BIOCHEMISTRY
including biophysical chemistry & molecular biology
Article

Biochemistry **2010,** *49,* 7643–7651 7643
DOI: 10.1021/bi1010317

Noncovalent Interaction of α_2-Antiplasmin with Fibrin(ogen): Localization of α_2-Antiplasmin-Binding Sites[†]

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Received June 28, 2010; Revised Manuscript Received August 4, 2010

ABSTRACT: Covalent incorporation (cross-linking) of plasmin inhibitor α_2-antiplasmin (α_2-AP) into fibrin clots increases their resistance to fibrinolysis. We hypothesized that α_2-AP may also interact noncovalently with fibrin prior to its covalent cross-linking. To test this hypothesis, we studied binding of α_2-AP to fibrin(ogen) and its fragments by an enzyme-linked immunosorbent assay (ELISA) and surface plasmon resonance. The experiments revealed that α_2-AP binds to polymeric fibrin and surface-adsorbed fibrin(ogen), while no binding was observed with fibrinogen in solution. To localize the α_2-AP-binding sites, we studied the interaction of α_2-AP with the fibrin(ogen)-derived D_1, D-D, and E_3 fragments, and the recombinant αC region and its constituents, αC connector and αC domain and its subdomains, which together encompass practically the whole fibrin(ogen) molecule. In the ELISA, α_2-AP bound to immobilized D_1, D-D, αC region, αC domain, and its C-terminal subdomain. The binding was Lys-independent and was not inhibited by plasminogen or tPA. Furthermore, the affinity of α_2-AP for D-D was significantly increased in the presence of plasminogen, while that to the αC domain remained unaffected. Altogether, these results indicate that the fibrin(ogen) D region and the C-terminal subdomain of the αC domain contain high-affinity α_2-AP-binding sites that are cryptic in fibrinogen and exposed in fibrin or adsorbed fibrinogen, and the presence of plasminogen facilitates interaction of α_2-AP with the D regions. The discovered noncovalent interaction of α_2-AP with fibrin may contribute to regulation of the initial stage of fibrinolysis and provide proper orientation of the cross-linking sites to facilitate covalent cross-linking of α_2-AP to the fibrin clot.

The fibrinolytic system, including the fibrinolytic proenzyme plasminogen and its activators, plays an important role in the dissolution of blood clots and vascular remodeling (*1*−*3*). Formation of a blood clot triggers plasminogen activation, which occurs through a number of orchestrated interactions among plasminogen, tissue-type plasminogen activator (tPA)[1], and fibrin, and results in generation of the active fibrinolytic enzyme plasmin (*4*, *5*). Plasmin activity is controlled by a number of inhibitors; the major physiological inhibitor of plasmin is α_2-antiplasmin (α_2-AP). The importance of such a control is highlighted by the fact that congenital deficiency of α_2-AP results in a severe hemorrhagic disorder because of increased susceptibility to fibrinolysis (*5*−*7*).

Plasmin inhibitor α_2-AP is a single-chain glycoprotein consisting of 464 amino acid residues with an N-terminal Met residue, Met-α_2-AP (*3*, *8*, *9*). In the circulation, it undergoes proteolytic cleavage between Pro12 and Asn13 by an antiplasmin-cleaving enzyme, resulting in a 452-residue version with an N-terminal Asn residue, Asn-α_2-AP, which accounts for approximately 70% of the circulating α_2-AP (*10*−*13*). α_2-AP is a member of the serpin (serine protease inhibitor) family whose inhibitory mechanism includes formation of a covalent complex with target proteases and inhibition of the latter. However, in contrast to the other family members, α_2-AP has a C-terminal extension (approximately 50 residues long) that contains a number of Lys residues (*14*). This extension, which according to the X-ray structure is located in the proximity of the reactive loop (*15*), binds to Lys-binding kringles of plasmin, increasing the inhibitory efficiency of α_2-AP (*16*, *17*). Thus, α_2-AP efficiently inhibits free plasmin in the circulation, thereby preventing fibrinogenolysis. Upon blood coagulation, α_2-AP is covalently cross-linked to forming fibrin by activated factor XIII (factor XIIIa) and becomes an effective inhibitor of fibrinolysis. The cross-linking occurs through Gln14 or Gln2 in Met-α_2-AP or Asn-α_2-AP, respectively; however, the latter is cross-linked to fibrin much faster than the former (*18*−*20*). While the molecular mechanism of plasmin inhibition by α_2-AP in solution is well-established, that by α_2-AP cross-linked to fibrin needs to be further clarified.

Fibrinogen consists of two identical disulfide-linked subunits, each of which is formed by three nonidentical polypeptide chains denoted Aα, Bβ, and γ (*21*). These chains are folded into a number of structural domains that compose several regions (*22*). Central region E is formed by the disulfide-linked N-terminal portions of all six chains converging from both subunits. The C-terminal regions of the Bβ and γ chains and a portion of the Aα

[†] This work was supported by National Institutes of Health Grant HL-56051 (L.M.) and by National Institutes of Health Grant HL-072995 and the Warren Medical Research Center (P.M.).

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[1] Abbreviations: tPA, tissue-type plasminogen activator; α_2-AP, α_2-antiplasmin; ε-ACA, ε-aminocaproic acid; ELISA, enzyme-linked immunosorbent assay; SPR, surface plasmon resonance; HBS, HEPES buffer saline [20 mM HEPES (pH 7.4) containing 150 mM NaCl]; TBS, Tris buffer saline [20 mM Tris (pH 7.4) containing 150 mM NaCl].

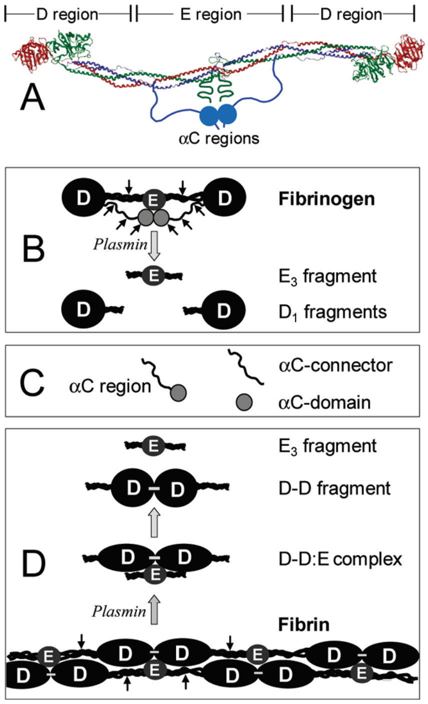

FIGURE 1: Schematic representation of fibrinogen, fibrin, and their fragments prepared for this study. (A) Ribbon diagram of fibrinogen based on its crystal structure (*47*). The individual fibrinogen chains, Aα, Bβ, and γ, are colored blue, green, and red, respectively, and the vertical lines denote approximate boundaries between the D and E regions. The αC regions, whose structure has not been identified, are shown schematically as two blue spheres representing αC domains, each attached to the bulk of the molecule with the flexible αC connector. (B) Schematic representation of the fibrinogen molecule and its products of plasminolysis, D_1 and E_3 fragments. (C) Recombinant αC region (residues Aα221−610), αC connector (residues Aα221−391), and αC domain (residues Aα392−610). (D) Schematic representation of fibrin and its products of fibrinolysis, the D-D:E complex, and the D-D and E_3 fragments. For the sake of simplicity, only two strands of fibrin molecules without the αC regions are shown; the molecules are linked through the noncovalent DD−E interactions and covalent γ−γ cross-linking between the D regions (shown by small horizontal bars). Small arrows in panels B and D indicate plasmin cleavage resulting in fibrin(ogen) fragments.

chain form the terminal D region, one in each subunit, while the remaining C-terminal portions of the two Aα chains (residues Aα221−610) form two αC regions. Each αC region is composed of the flexible αC connector (residues Aα221−391) and compact αC domain (residues Aα392−610) (*23*). Thus, the structure of fibrinogen can be presented as consisting of three linearly arranged regions, D−E−D, with the αC domains attached to the D regions via the αC connectors (Figure 1A). The D and E regions correspond to the D and E fragments, respectively, which can be prepared by proteolytic digestion of fibrinogen with plasmin (Figure 1B) (*24*). The αC regions are susceptible to proteolysis and degraded into smaller fragments (*21*); they, as well as their constituents αC connector and αC domain, can be prepared by recombinant techniques (*25*) (Figure 1C).

Fibrinogen is rather inert in the circulation; however, it becomes highly reactive toward different proteins and cell types after thrombin-mediated conversion into fibrin. Thrombin cleaves two pairs of fibrinopeptides, fibrinopeptide A (FpA) and fibrinopeptide B (FpB), from the N-terminal portions of the Aα and Bβ chains, respectively, to expose polymerization sites or “knobs” “A” and “B” in the central E region. Monomeric fibrin molecules interact with each other through these sites and complementary “a” and “b” sites, also called “holes”, which are located in the D regions and are always reactive. Such interaction through D and E regions, also called DD−E interaction, results in formation of fibrin polymers (Figure 1D). Fibrin polymerization is accompanied by the conformational changes and exposure of multiple binding sites in the polymer (*26*, *27*). Among them are tPA- and plasminogen-binding sites that play a major role in the initiation of fibrinolysis (*4*). Namely, as soon as these sites are exposed, binding of tPA and plasminogen to them brings these two proteins together and provides efficient activation of plasminogen into active plasmin by tPA. Numerous studies localized tPA- and plasminogen-binding sites in the D regions and the αC domains (reviewed in ref *4*). Whether fibrin(ogen) contains binding sites for the plasminogen inhibitor, α_2-AP, remains to be established.

Factor XIIIa can incorporate α_2-AP into both fibrinogen and fibrin (*28*). The incorporation occurs by covalent cross-linking of α_2-AP to a single Lys residue (Lys303) located in the C-terminal portion of the fibrin(ogen) Aα chain (*29*, *30*), namely in its αC connector. While covalent cross-linking of α_2-AP to fibrin(ogen) is well established, its molecular mechanism is not well understood. In particular, it is not known whether the efficient cross-linking requires the spatial arrangement of the cross-linking sites. In this manner, it was shown that factor XIIIa-mediated γ−γ cross-linking is markedly enhanced in the presence of a fibrin fragment E template that brings together its two D regions through the DD−E interaction (*31*). Further, our previous studies revealed that fibronectin, another plasma protein that is cross-linked to fibrin by factor XIIIa, binds with high affinity to fibrin to provide the proper orientation of the cross-linking sites (*32*). Thus, we hypothesized that α_2-AP may interact noncovalently with fibrin prior to its covalent cross-linking. The major goal of this study was to test this hypothesis and to further clarify the mechanism of incorporation of α_2-AP into a fibrin clot that plays a critical role in controlling fibrinolysis.

EXPERIMENTAL PROCEDURES

Proteins, Enzymes, and Antibodies. Human α_2-antiplasmin, namely, its Asn-α_2-AP form, was prepared as previously described in detail (*33*). Plasminogen-depleted human fibrinogen and bovine serum albumin were purchased from Calbiochem. Recombinant single-chain tPA was a Genentech product, and human Glu-plasminogen was from Enzyme Research Laboratories. Bovine α-thrombin, aprotinin, and carboxypeptidase B were from Sigma. The rabbit anti-plasminogen polyclonal antibodies were purchased from Chemicon, and anti-tPA monoclonal antibody mAb GMA-043 was from Calbiochem. Alkaline phosphatase-conjugated ExtrAvidin was from Sigma.

Preparation of Fibrin(ogen) Fragments. Fibrinogen D_1 and E_3 fragments (Figure 1B) were prepared by plasmin digestion of human fibrinogen by the procedures described in ref *34*. Their N-terminal residues determined by direct sequencing for 10 cycles using a Hewlett-Packard model G 1000S sequenator were essentially the same as those we reported previously (*35*). The fibrin-derived D-D fragment and D-D:E complex (Figure 1D) were prepared by plasmin digestion of cross-linked fibrin as previously described in ref *26*.

The recombinant Aα221−610, Aα221−391, and Aα392−610 fragments corresponding to the human fibrinogen αC region, αC connector, and αC domain, respectively (Figure 1C), were expressed in *Escherichia coli* and subsequently purified and refolded by the procedures described previously (*23*). Recombinant Aα392−503 and Aα504−610 fragments corresponding to N- and C-terminal subdomains of the αC domain, respectively (*36*), were expressed in *E. coli* using the pET-20b expression vector (Novagen Inc.). The cDNAs encoding these fragments were amplified by polymerase chain reaction using a plasmid carrying the full-length human αC region sequence (*23, 37*). The following oligonucleotides were used as primers: 5′-AGAGACATATGGGCACATTTGAAGAGG-3′ (forward) and 5′-AGAGAAAGCTTTTACCAAGTGTCGAAGAAGGCAGC-3′ (reverse) for the Aα392−503 fragment and 5′-AGAGACATATGGCCTCAACTGGAAAAACA-3′ (forward) and 5′-AGAGAAAGCTTTTAGACAGGGCGAGATTTAG-3′ (reverse) for the Aα504−610 fragment. The forward primers incorporated the *Nde*I restriction site immediately before the coding region; the final three bases of the *Nde*I site, ATG, encode the fMet residue that initiates translation. The reverse primers included a TAA stop codon immediately after the coding segment, followed by a *Hin*dIII site. The amplified cDNA fragments were purified by electrophoresis in an agarose gel, digested with *Nde*I and *Hin*dIII restriction enzymes, and ligated into the pET-20b expression vector. The resulting plasmids were used for transformation of DH5α and then B834(DE3) pLysS *E. coli* host cells. Both cDNA fragments were sequenced in both directions to confirm the integrity of the coding sequences. The expressed Aα392−503 and Aα504−610 fragments were found in inclusion bodies, from which they were purified by the procedure described previously (*25*). The purified fragments were refolded at 4 °C by slow dialysis from urea using the protocol described in ref *23* and then additionally purified by size-exclusion chromatography on a Superdex S-75 column equilibrated with TBS [20 mM Tris buffer (pH 7.4) containing 150 mM NaCl] and 0.2 mM PMSF. The fragments were concentrated to 1−2 mg/mL with a Centriprep 10 concentrator (Millipore), filtered through 0.2 μm filter unit, and stored at 4 °C.

Protein Concentration Determination. Concentrations of the recombinant Aα392−503 and Aα504−610 fragments were determined spectrophotometrically using extinction coefficients ($E_{280,1\%}$) calculated from the amino acid composition by the following equation: $E_{280,1\%} = (5690W + 1280Y + 120\text{S-S})/(0.1M)$, where W, Y, and S-S represent the number of Trp and Tyr residues and disulfide bonds, respectively, and M represents the molecular mass (*38*). Molecular masses of these fragments were calculated on the basis of their amino acid composition. The following values of molecular masses and $E_{280,1\%}$ were obtained: 12394 Da and 5.74 for the Aα392−503 fragment and 11841 Da and 8.42 for the Aα504−610 fragment, respectively. Note that these values take into account the N-terminal fMet residue (see above) while the numbering of these fragments does not. The molecular masses and $E_{280,1\%}$ values for the recombinant Aα221−610, Aα221−391, and Aα392−610 fragments were determined previously (*23*).

Solid-Phase Binding Assay. Solid-phase binding was performed in plastic microtiter plates using an enzyme-linked immunosorbent assay (ELISA) as described in ref *39* with some modifications. Microtiter Immulon 2HB plate wells (Thermo) were coated overnight with 100 μL of fibrinogen or fibrin(ogen) fragments per well, all at 10 μg/mL in TBS with 1 mM Ca^{2+} (TBS-Ca), followed by washing with the same buffer. To convert adsorbed fibrinogen into fibrin, we incubated the sample with 100 μL of a mixture of thrombin (1 NIH unit/mL) and aprotinin (400 units/mL) in TBS-Ca for 30 min at 37 °C, followed by washing with the same buffer. Adsorbed fibrin was treated with 5 μg/mL carboxypeptidase B for 30 min at room temperature to remove possible C-terminal Lys residues. The wells were then blocked with 2% bovine serum albumin in TBS-Ca containing 0.01% Tween 20. Following washing with TBS-Ca and 0.01% Tween 20, the proteins used as ligands at the indicated concentrations were added to the wells and incubated for 1 h at 37 °C. α_2-AP was labeled with biotin using the EZ-Link Sulfo-NHS-Biotinylation Kit (Pierce), and bound α_2-AP was detected by reaction with the alkaline phosphatase-conjugated avidin. A PPNP Microwell Alkaline Phosphatase Substrate (Kirkegaard & Perry Laboratories Inc.) was added to the wells, and the amount of bound ligand was measured spectrophotometrically at 405 nm. Bound plasminogen or tPA was detected by reactions with specific polyclonal or monoclonal antibodies and the peroxidase-conjugated anti-rabbit or anti-mouse polyclonal antibodies as described previously (*35*). A TMB Microwell Peroxide Substrate (Kirkegaard & Perry Laboratories Inc.) was added to the wells, and the amount of bound ligand was measured spectrophotometrically at 450 nm. Data were analyzed by nonlinear regression analysis using the equation

$$A = A_{\max}/(1 + K_d/[L]) \qquad (1)$$

where A represents the absorbance of the oxidized substrate, which is assumed to be proportional to the amount of ligand bound, $A_{\max}$ is the absorption at saturation, [L] is a molar concentration of the ligand, and K_d is the dissociation constant.

Biosensor Assay. The interaction of fibrin(ogen) and its fragments with α_2-AP was also studied by surface plasmon resonance (SPR) using the BIAcore 3000 biosensor (Biacore AB, Uppsala, Sweden), which measures association and dissociation of proteins in real time. α_2-AP at 5 μg/mL or fibrinogen at 10 μg/mL was immobilized to the CM5 sensor chip at 250 or 1000 RU, respectively, using the amine coupling kit (BIAcore AB) according to the manufacturer's instructions. Binding experiments were performed in 20 mM HEPES (pH 7.4) buffer with 150 mM NaCl, 1 mM Ca^{2+}, and 0.01% Tween 20 (HBS-Ca) at a flow rate of 10 μL/min. The association between the immobilized proteins and added ligands was monitored as the change in the SPR response; the dissociation was measured upon replacement of the ligand solution for the buffer without ligand. To regenerate the chip surface, complete dissociation of the complex was achieved by addition of 2 M NaCl in binding buffer for 30 s followed by re-equilibration with binding buffer. Experimental data were analyzed using BIAevaluation version 4.1 supplied with the instrument. The dissociation equilibrium constant, K_d, was calculated as $K_d = k_{diss}/k_{ass}$, where k_{ass} and k_{diss} represent kinetic constants that were estimated by global analysis of the association−dissociation data using the 1:1 Langmurian interaction model (kinetic analysis). To confirm the kinetic analysis, K_d was also estimated by analysis of the association data using the steady-state affinity model provided by the same software (equilibrium analysis).

To prepare a fibrin surface for SPR experiments, fibrinogen was additionally purified on Superdex 200 and covalently immobilized to the CM5 sensor chip at 10000 RU using the amine coupling kit (BIAcore AB) according to the manufacturer's instructions. Conversion of immobilized fibrinogen to fibrin was achieved via treatment with a mixture of thrombin (1 NIH unit/mL) and aprotinin (400 units/mL) for 30 min. To prepare oligomers and polymers on the surface of the chip, 1 mg/mL

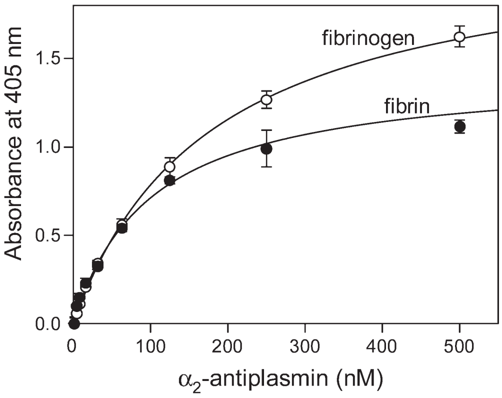

FIGURE 2: Analysis of the interaction of α_2-antiplasmin with fibrinogen and fibrin by an ELISA. Increasing concentrations of biotinylated α_2-AP were added to surface-adsorbed fibrinogen (○) or fibrin (●). Bound biotinylated α_2-AP was detected with avidin conjugated to alkaline phosphatase as described in Experimental Procedures. The curves represent the best fit of the data to eq 1. All results are means ± the standard deviation of duplicate determinations.

fibrinogen was applied to immobilized fibrin for 20 min followed by a mixture of 3 NIH units/mL thrombin and 5 μg/mL factor XIII for 30 min; nonbound proteins were then washed out with HBS-Ca. This application was repeated three times to achieve immobilization of additional fibrin (~600 response units).

RESULTS

Interaction of α_2*-Antiplasmin with Fibrin*(*ogen*). Interaction of α_2-AP with fibrinogen and fibrin was tested by two methods, ELISA and surface plasmon resonance (SPR). In the ELISA, when microplate wells were coated with fibrinogen and then increasing concentrations of α_2-AP were added, fibrinogen exhibited a dose-dependent binding with a K_d of 179 nM (Figure 2 and Table 1). In contrast, when microplate wells were coated with α_2-AP and then increasing concentrations of fibrinogen (up to 2 μM) were added, no binding was observed (not shown). Such a discrepancy may be explained by a well-established fact that adsorption of fibrinogen to various surfaces results in conformational changes and exposure of its fibrin-specific binding sites (reviewed in ref *27*). Alternatively, adsorption of α_2-AP on the surface of microplate wells could result in the loss of its binding activity. To select between these alternatives, we first performed a sandwich ELISA, in which microplate wells were coated with streptavidin that captured biotin-labeled α_2-AP. When fibrinogen was added to α_2-AP immobilized in such a manner, no binding was observed (not shown). Then we used SPR, in which proteins are immobilized by covalent cross-linking to the spacer on the surface of a sensor chip and, therefore, may preserve their original conformation. When fibrinogen was immobilized in such a manner and then increasing concentrations of α_2-AP (up to 2 μM) were added, no binding was observed (not shown). In another SPR experiment, when fibrinogen was added to immobilized α_2-AP, again no binding was observed (not shown). Thus, these experiments indicate that fibrinogen interacts with α_2-AP only when it is adsorbed onto a surface.

To test the interaction of α_2-AP with fibrin, we converted fibrinogen adsorbed on microplate wells or immobilized on the surface of a sensor chip into fibrin by treatment with thrombin, as described in Experimental Procedures. In the ELISA, α_2-AP exhibited a dose-dependent binding to surface-adsorbed fibrin with a K_d of 102 nM (Figure 2 and Table 1) while in SPR no binding to immobilized fibrin was observed even at 2 μM α_2-AP (not shown). Although one cannot exclude the possibility that the lack of binding in SPR was connected with the immobilization procedure, another explanation seems to be more reasonable. Namely, because in these experiments fibrinogen was adsorbed or immobilized at comparatively low concentrations, the resulting fibrin was most probably monomeric. Because the conformational changes and exposure of cryptic binding sites occur mainly upon fibrin polymerization (*26*, *27*), this may explain why such monomeric fibrin exhibited a fibrinogen-like binding property toward α_2-AP, i.e., interacted with the latter only when adsorbed to the surface. In agreement, in control experiments, plasminogen, which is known to interact only with adsorbed fibrin(ogen) or polymeric fibrin, exhibited binding to adsorbed fibrin only in the ELISA, as expected, while no binding to immobilized fibrin was observed via SPR (not shown). These results suggest that monomeric fibrin does not interact with α_2-AP.

To test whether fibrin polymer may interact with α_2-AP, we prepared a fibrin surface on which fibrin was presumably in polymeric form (see Experimental Procedures). Briefly, fibrinogen was immobilized onto the surface of a sensor chip at a high density to increase the probability of intermolecular contacts and then treated with thrombin to convert it into fibrin and enable the DD−E interactions. Next, additional fibrinogen was added, which specifically bound to immobilized fibrin through the DD−E interactions, and then such fibrin−fibrinogen complexes were treated with factor XIIIa, which reinforced the complexes by covalent cross-linking; thrombin was added to convert the additionally immobilized fibrinogen into fibrin. Such addition of fibrinogen and subsequent treatment with factor XIIIa and thrombin was repeated three times to immobilize additional molecules that presumably formed oligomers or polymers of fibrin. In contrast to immobilized fibrin monomers described above, such immobilized oligomers or polymers exhibited in control experiments significant interaction with plasminogen (not shown), suggesting that their fibrin-specific binding sites were exposed. When α_2-AP at increasing concentrations was added, it also exhibited dose-dependent binding (Figure 3). The K_d value determined by the kinetic analysis of association−dissociation data (see Experimental Procedures) was found to be 45 ± 8 nM, and that determined by the equilibrium analysis

Table 1: Dissociation Constants (K_d) for the Interaction of α_2-Antiplasmin with Surface-Adsorbed Fibrinogen, Fibrin, and Their Fragments Obtained by an ELISA

	K_d (nM)[a]			
protein or fragment	no addition	with εACA	with tPA	with plasminogen
fibrinogen	179 ± 19	102 ± 4	–	–
fibrin	102 ± 4	101 ± 12	109 ± 9	25 ± 2
E_3 fragment	nb[b]	–	–	–
D_1 fragment	197 ± 33	165 ± 11	–	–
D-D fragment	94 ± 4	98 ± 10	105 ± 4	5.5 ± 0.4
Aα221−610 fragment	131 ± 25	–	–	–
Aα392−610 fragment	150 ± 5	141 ± 14	157 ± 21	111 ± 14
Aα221−391 fragment	nb[b]	–	–	–
Aα392−503 fragment	nb[b]	–	–	–
Aα504−610 fragment	123 ± 10	130 ± 15	–	–

[a] Values are means ± the standard deviation of at least three independent experiments. [b] No binding was observed.

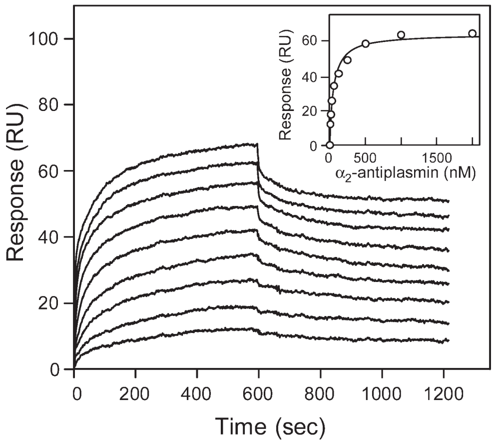

FIGURE 3: Analysis of the interaction of α_2-antiplasmin with polymeric fibrin by surface plasmon resonance. α_2-AP at increasing concentrations (8, 16, 32, 63, 125, 250, 500, 1000, and 2000 nM) was added to immobilized fibrin polymers (see Experimental Procedures), and its association and dissociation were monitored in real time. The inset shows the results of the equilibrium analysis; the amount of bound α_2-antiplasmin at equilibrium for each concentration is presented as circles, and the best fit is presented as a solid curve.

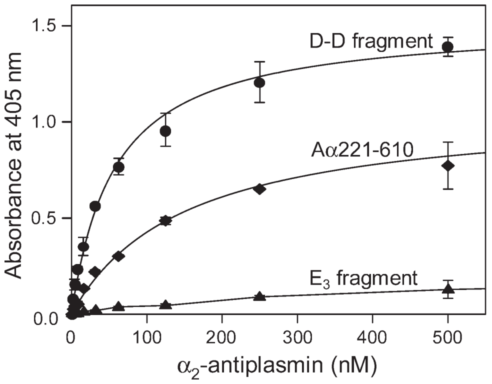

FIGURE 4: ELISA-detected binding of α_2-antiplasmin to immobilized fibrin(ogen) fragments. Increasing concentrations of biotinylated α_2-AP were added to the immobilized D-D (●), Aα221−610 (◆), and E_3 (▲) fragments. Bound biotinylated α_2-AP was detected with avidin conjugated to alkaline phosphatase. The curves represent the best fits of the data to eq 1. All results are means ± the standard deviation of duplicate determinations.

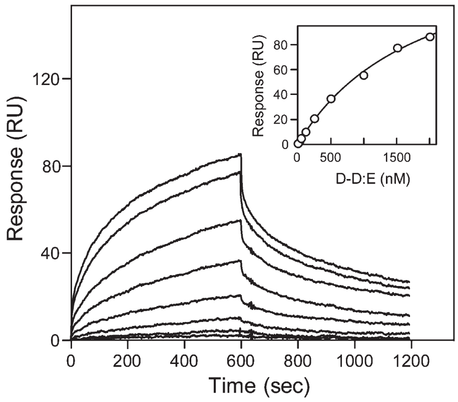

FIGURE 5: Analysis of the interaction between α_2-antiplasmin and the D-D:E complex by surface plasmon resonance. The D-D:E complex at increasing concentrations (32, 63, 125, 250, 500, 1000, 1500, and 2000 nM) was added to immobilized α_2-AP, and its association and dissociation were monitored in real time. The inset shows the results of the equilibrium analysis; the amount of bound D-D:E complex for each concentration is presented as circles, and the best fit is presented as a solid curve.

was close, 68 ± 12 nM. These experiments indicate that α_2-AP binds to polymeric fibrin with high affinity.

Localization of α_2-AP-Binding Sites in Fibrin(ogen). To localize the α_2-AP-binding site(s) in fibrin(ogen), we studied the interaction of α_2-AP with the D_1 or D-D fragments and the E_3 fragment corresponding to the fibrin(ogen) D and E regions, respectively, and the recombinant αC region (Aα221−610), which together encompass practically the whole fibrin(ogen) molecule (Figure 1B−D). In the ELISA, when these fragments were adsorbed on the surface of microplate wells and increasing concentrations of α_2-AP were added, a prominent binding was observed with the D-D and Aα221−610 fragments while E_3 exhibited no binding (Figure 4). The binding was dose-dependent, and the K_d values determined for the D-D and Aα221−610 fragments were 94 and 131 nM, respectively (Table 1). In another experiment, α_2-AP also exhibited binding to the surface-adsorbed fibrinogen-derived D_1 fragment (Table 1). In a reverse ELISA, when the D-D, D_1, E_3, and Aα221−610 fragments were added to immobilized α_2-AP, no binding was detected (not shown). In agreement, in SPR experiments none of these fragments exhibited binding to immobilized α_2-AP (not shown). These results suggest that the D and αC regions contain α_2-AP-binding sites that are cryptic in fibrinogen and the D_1, D-D, and Aα221−610 fragments and become exposed upon their adsorption to a surface.

Our finding that D_1 and D-D fragments did not interact with surface-adsorbed or immobilized α_2-AP suggests that in solution these fragments have a fibrinogen-specific conformation and their α_2-AP-binding sites are cryptic. Indeed, X-ray studies of the fibrinogen-derived D fragment and the fibrin-derived D-D fragment revealed that the overall fold of the D fragment in the monomer and the dimer is very similar (*40*). At the same time, it was shown that the fibrin-derived D-D:E complex (Figure 1D) preserves its fibrin-specific conformation and that its fibrin-specific binding sites, including those for tPA and plasminogen, are accessible (*26*). To test if the α_2-AP-binding sites are also accessible in this complex, we used SPR. When the D-D:E complex at increasing concentrations was added to immobilized α_2-AP, it exhibited a dose-dependent binding (Figure 5). The K_d value determined by the kinetic analysis of the data was found to be 1.4 ± 0.4 μM, and that determined by the equilibrium analysis was practically the same, 1.4 ± 0.2 μM. These results clearly indicate that the α_2-AP-binding sites in the D-D:E complex are accessible for α_2-AP. They further reinforce the suggestion made above that the α_2-AP-binding sites of the D regions are cryptic in fibrinogen and become exposed in fibrin.

Localization of the α_2-AP-Binding Site in the αC Region. To localize the α_2-AP-binding site in the αC region, we studied the interaction of α_2-AP with the recombinant Aα221−391 and Aα392−610 fragments, which correspond to the αC connector and αC domain, respectively, and compose the entire αC region (Figure 1C). In the ELISA, α_2-AP exhibited binding only to the surface-adsorbed Aα392−610 fragment while no binding to the Aα221−391 fragment was observed (Figure 6); the affinity of the binding (K_d = 150 nM) was similar to that determined for the αC region (Table 1). This indicates that only the αC domain of the αC region is involved in the interaction with α_2-AP; i.e., this domain contains the α_2-AP-binding site.

We have shown recently that the αC domain consists of two independently folded subdomains, N-terminal and C-terminal (*36*).

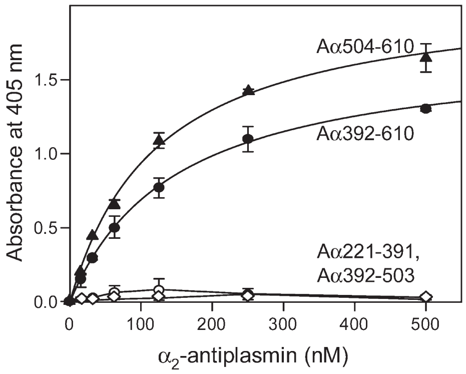

FIGURE 6: ELISA-detected binding of α_2-antiplasmin to immobilized recombinant αC fragments. Increasing concentrations of biotinylated α_2-AP were added to the immobilized Aα221−391 (○), Aα392−610 (●), Aα392−503 (◇), and Aα504−610 (▲) fragments. Bound biotinylated α_2-AP was detected with avidin conjugated to alkaline phosphatase. The curves represent the best fits of the data to eq 1. All results are means ± the standard deviation of duplicate determinations.

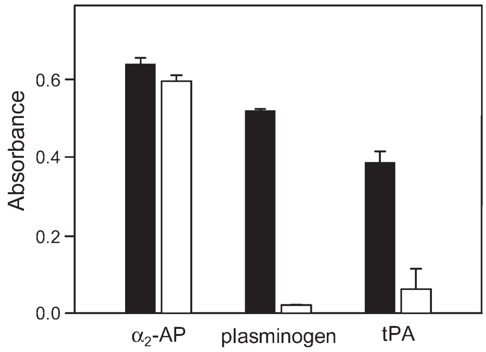

FIGURE 7: Simultaneous binding of α_2-antiplasmin, tPA, and plasminogen to immobilized fibrin detected by an ELISA. A mixture of 0.5 μM biotinylated α_2-AP, 2.5 μM tPA, and 2.5 μM plasminogen was added to surface-adsorbed fibrin in the absence (black bars) or presence (white bars) of 100 mM ε-ACA. Bound biotinylated α_2-AP was detected spectrophotometrically at 405 nm using avidin conjugated to alkaline phosphatase, and bound tPA and plasminogen were detected spectrophotometrically at 450 nm using the anti-tPA monoclonal antibody and specific polyclonal antibodies, respectively, as described in Experimental Procedures. All results are means ± the standard deviation of two independent experiments, each performed in duplicate.

To further localize the α_2-AP-binding site in this domain, we constructed and expressed Aα392−503 and Aα504−610 fragments corresponding to its N- and C-terminal subdomains, respectively (see Experimental Procedures). In the ELISA, when increasing concentrations of α_2-AP were added to both fragments immobilized on microtiter plate wells, only Aα504−610 exhibited binding (Figure 6). The K_d value of 123 nM determined for this binding was similar to those determined for the interaction of α_2-AP with the αC region or αC domain (Table 1). Thus, these results indicate that the α_2-AP-binding site is located in the C-terminal subdomain of the αC domain.

Further Characterization of the Interaction between α_2-AP and Fibrin(*ogen*). Because plasminogen, tPA, and α_2-AP are the major players in the initiation and regulation of fibrinolysis, we next tested whether the former two interfere in the interaction of α_2-AP with fibrin(ogen). It is well established that plasminogen and tPA both interact with fibrin(ogen) D regions and αC domains in a Lys-dependent manner, although a Lys-independent binding of tPA to the D region was also observed (*4*). To test if α_2-AP-fibrin(ogen) interaction involves Lys-binding sites, we studied binding of α_2-AP to several fibrin(ogen) fragments that exhibited α_2-AP binding properties in the presence or absence of the Lys-binding inhibitor, ε-aminocaproic acid (ε-ACA). In the ELISA, when microplate wells were coated with these fragments and then increasing concentrations of α_2-AP were added in the presence of 100 mM ε-ACA, all fragments exhibited a dose-dependent binding with K_d values very similar to those determined in the absence of ε-ACA (Table 1). These results indicate that the binding of α_2-AP to fibrin(ogen) is Lys-independent. Because the binding of plasminogen and tPA to fibrin(ogen) is mostly Lys-dependent, these results also suggest that α_2-AP-binding sites are different from those for plasminogen and tPA.

To test the suggestion made above, we first studied simultaneous binding of all three species, α_2-AP, tPA, and plasminogen, to surface-adsorbed fibrin in the presence or absence of ε-ACA. In the ELISA, when a mixture of α_2-AP, tPA, and plasminogen, each at saturating concentrations, 0.5, 2.5, and 2.5 μM, respectively, was added to immobilized fibrin, the binding of each species was detected (Figure 7, black bars). The addition of 100 mM ε-ACA did not affect the binding of α_2-AP, while the binding of plasminogen and tPA was abolished, as expected; there was some residual binding of tPA, most probably due to its Lys-independent site (Figure 7, white bars). In other ELISA experiments, when fibrin, D-D, and Aα392−610 fragments were adsorbed onto the surface of microplate wells and increasing concentrations of α_2-AP were added in the presence of saturating concentrations of tPA (0.5 μM for the Aα392−610 fragment and 2.5 μM for fibrin and D-D fragments), the binding curves and K_d values for all three species were very similar to those obtained in the absence of tPA (Figure 8A and Table 1). These results indicate that there was no competition between α_2-AP and tPA for the binding sites on fibrin or the D-D and Aα392−610 fragments; i.e., the α_2-AP- and tPA-binding sites do not overlap.

Next, we performed similar binding experiments with α_2-AP in the presence of saturating concentrations of plasminogen (0.5 μM for the Aα392−610 fragment and 2.5 μM for fibrin and the D-D fragment). In the case of the surface-adsorbed Aα392−610 fragment, the binding of α_2-AP was not affected by the presence of plasminogen and the determined K_d value of 111 nM was very close to that determined in the absence of plasminogen (Figure 8B and Table 1). In contrast, binding of α_2-AP to surface-adsorbed fibrin or the D-D fragment was much stronger in the presence of plasminogen; the K_d values for fibrin and the D-D fragment were found to be 25 and 5.5 nM, respectively. These results confirm that plasminogen does not compete with α_2-AP for its binding site on fibrin(ogen). They also indicate that in the presence of plasminogen the binding of α_2-AP to the surface-adsorbed D-D fragment and fibrin is significantly strengthened.

DISCUSSION

The major physiological inhibitor of plasmin, α_2-AP, plays an important role in the regulation of fibrinolysis. This occurs through direct inhibition of plasmin activity by α_2-AP, either in the circulation or on the surface of a fibrin clot. In the circulation, α_2-AP rapidly inhibits free plasmin, thus preventing fibrinogenolysis (*8*, *41*). α_2-AP also inhibits fibrin-bound plasmin, although much less efficiently (*12*). Meanwhile, incorporation of

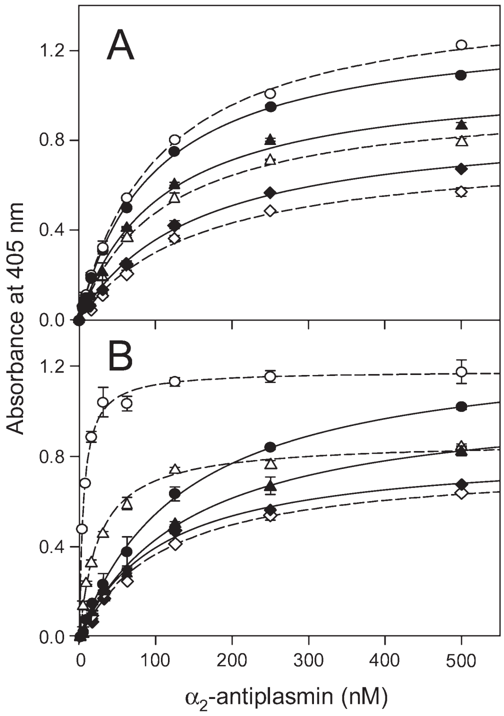

FIGURE 8: Effect of tPA or plasminogen on the binding of α_2-antiplasmin to immobilized fibrin and its fragments detected by an ELISA. (A) Increasing concentrations of biotinylated α_2-AP were added to surface-adsorbed fibrin (triangles), the D-D fragment (circles), or the Aα392−610 fragment (diamonds) in the absence (black symbols, solid lines) or presence (white symbols, dashed lines) of 2.5 μM tPA. (B) Increasing concentrations of biotinylated α_2-AP were added to surface-adsorbed fibrin (triangles), the D-D fragment (circles), or the Aα392−610 fragment (diamonds) in the absence (black symbols, solid lines) or presence (white symbols, dashed lines) of 2.5 μM plasminogen. Bound biotinylated α_2-AP was detected with avidin conjugated to alkaline phosphatase. The curves represent the best fit of the data to eq 1. All results are means ± the standard deviation of duplicate determinations.

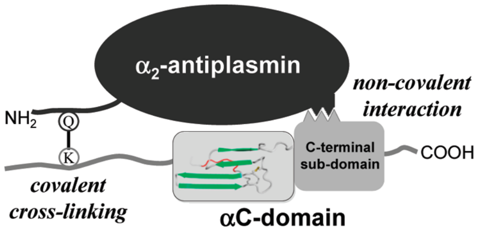

FIGURE 9: Schematic representation of the noncovalent and covalent interactions between α_2-antiplasmin and the fibrin(ogen) αC domain. α_2-AP with a flexible N-termial portion containing reactive Gln14 (Q) is presented at the top. The αC region containing the flexible αC connector with reactive Lys303 (K) and the compact αC domain consisting of two subdomains are presented at the bottom; the structure of the αC domain is adapted from ref *36*. The noncovalent interaction is shown at the right, and the covalent cross-linking (Q-K) between reactive Gln14 and Lys303 is shown at the left.

α_2-AP into fibrin endows the fibrin clot with resistance to fibrinolysis (*18*, *19*). According to the current view, this incorporation occurs by covalent cross-linking of α_2-AP to fibrin(ogen) with factor XIIIa (*6*). In this study, we found that α_2-AP can also be incorporated into fibrin or adsorbed fibrin(ogen) through a noncovalent binding to its D regions and αC domains.

The noncovalent interaction of α_2-AP with polymeric fibrin occurs with high affinity; the equilibrium dissociation constant determined by SPR (K_d = 45−68 nM) is far below the physiological concentration of α_2-AP (1 μM). This suggests that in vivo α_2-AP should efficiently bind to fibrin clots, thereby contributing to the regulation of fibrinolysis. Although the exact physiological role of this binding is yet to be established, the accumulated data allow the following speculation. The Lys303 residue to which factor XIIIa cross-links α_2-AP is located in the fibrin(ogen) αC connector, while one of the α_2-AP-binding sites localized in this study is in the fibrin(ogen) αC domain; i.e., the binding and cross-linking sites are located in different portions of the αC region. A similar situation was observed with fibronectin, in which binding and cross-linking sites are also located in different portions of the αC region and whose noncovalent binding to the αC connector promotes its cross-linking by factor XIIIa to the αC domain (*32*). Thus, as in the case with fibrin−fibronectin interaction, the noncovalent binding of α_2-AP to the αC domain may provide its proper orientation toward the cross-linking site (Lys303) to enhance the covalent stage of the interaction (cross-linking). This is presented schematically in Figure 9.

Numerous previous studies localized the tPA- and plasminogen-binding sites in the fibrin(ogen) D and αC regions (αC domains) (reviewed in ref *4*). Binding of tPA and plasminogen to these sites brings them together, enabling efficient activation of the latter by the former and thereby initiating fibrinolysis in places of fibrin deposition (*4*). In this study, we localized the α_2-AP-binding sites in the same regions. We also found that these binding sites do not overlap and have a different nature; while tPA- and plasminogen-binding sites are mainly Lys-dependent, those for α_2-AP are Lys-independent. These findings suggest that fibrin can accommodate all three proteins simultaneously in the proximity of each other. Such spatial arrangement may be necessary for efficient activation of plasminogen by tPA upon initiation of fibrinolysis and subsequent rapid inhibition of newly formed plasmin by bound α_2-AP. Thus, noncovalent binding of α_2-AP to the D regions and αC domains may play a role in controlling the initiation of fibrinolysis on fibrin clots.

Our SPR experiments revealed that polymeric fibrin or its soluble model, the D-D:E complex, interacted with α_2-AP while neither fibrinogen nor fibrin monomer exhibited such interaction. In the ELISA, soluble fibrinogen did not interact with immobilized α_2-AP while the latter exhibited strong interaction with surface-adsorbed fibrin(ogen) and its fragments. These indicate that α_2-AP-binding sites are cryptic in fibrinogen and become exposed in fibrin upon polymer formation or in adsorbed fibrinogen. This finding is not unexpected because previous studies revealed that conformational changes upon conversion of fibrinogen into fibrin or upon its adsorption result in the exposure of its numerous cryptic binding sites (*27*). For example, a similar situation was previously observed with tPA, plasminogen, apolipoprotein(a), fibronectin, and Mac-1 receptor, which exhibited binding only to fibrin or adsorbed fibrin(ogen) and its fragments (*25*, *26*, *32*, *35*, *42*, *43*). Thus, the interaction of α_2-AP with fibrin(ogen) represents another example of the conformation-dependent exposure of cryptic sites. Such exposure in vivo may serve to localize the activity of α_2-AP to the surface of a fibrin clot where the initiation and propagation of fibrinolysis by tPA and plasmin(ogen) take place. One can also expect that binding of α_2-AP to fibrinogen deposited on blood-contacting foreign surfaces, for example, on implanted biomaterials, may contribute to the regulation of plasminogen activation on such surfaces and may promote cross-linking of α_2-AP to adsorbed fibrinogen, thereby rendering the latter more resistant to plasmin digestion. It should be noted that although fibrinogen in solution does not interact with α_2-AP, the latter may also be cross-linked by factor XIIIa to the former, as revealed in a recent study (*28*). However, because such cross-linking is not preceded by the noncovalent interaction, it should be less efficient than that to fibrin.

This study also reveals that the presence of plasminogen significantly facilitates the noncovalent binding of α_2-AP to the fibrin(ogen) D regions. When α_2-AP was added to the immobilized D-D fragment or fibrin in the presence of plasminogen, the affinity of binding to both species was dramatically increased while no change was observed in the presence of tPA (Figure 8 and Table 1). No change in the affinity was also observed when α_2-AP was added to the immobilized αC domain fragment in the presence of plasminogen or tPA. The reason for such increased affinity of α_2-AP for the D region is not clear. It is known that plasminogen binds to fibrin or immobilized fibrinogen and its D-containing fragments (*4*). Thus, one cannot exclude the possibility that this binding may elicit conformational changes in the D regions resulting in further exposure of the α_2-AP-binding sites and increased affinity for α_2-AP. Alternatively, the increased affinity may be connected with the reported ability of α_2-AP to interact with plasminogen (*6*, *8*). In this case, simultaneous binding of α_2-AP to fibrin and fibrin-bound plasminogen could increase the overall affinity. This alternative may seem to be less probable because the interaction of α_2-AP with plasminogen was shown to occur through its Lys-binding sites (*44*) and to competitively inhibit binding of plasminogen to fibrin (*6*, *45*). However, the experiments performed in this study revealed no competition between α_2-AP and plasminogen for binding to immobilized fibrinogen or the D-D fragment (Figures 7 and 8). Such a discrepancy may be connected with the presence in plasminogen of five kringle domains, at least three of which contain Lys-binding sites with different selectivity toward fibrin and α_2-AP. Indeed, it was proposed that α_2-AP interacts with plasminogen through the Lys-binding site of kringle-1 while the primary interaction of fibrin with plasminogen may occur through kringle-5 (reviewed in ref *46*). Further experiments are required to test this speculation and to clarify the reasons for the plasminogen-induced increased affinity of α_2-AP for fibrin(ogen) D regions and its possible role in the regulation of fibrinolysis.

In summary, this study reveals the noncovalent interaction between α_2-AP and fibrin or surface-adsorbed fibrin(ogen), localized α_2-AP-binding sites in the fibrin(ogen) D region and the αC domains, namely, its C-terminal subdomain, and further clarified the mechanism of incorporation of α_2-AP into the fibrin clot. This mechanism includes the exposure of cryptic α_2-AP-binding sites upon conversion of fibrinogen into fibrin polymer and noncovalent binding of α_2-AP to the same regions of fibrin that bind tPA and plasminogen. Such exposure may serve to localize the activity of α_2-AP to places of fibrin deposition. The noncovalent binding prior to the cross-linking may provide the proper orientation of the cross-linking site and enhance the covalent cross-linking of α_2-AP to the fibrin clot. The noncovalent binding may also serve to bring α_2-AP to the clot for efficient inhibition of plasmin formed upon initiation of fibrinolysis, while subsequent covalent cross-linking keeps α_2-AP bound to the clot, thereby increasing its resistance to fibrinolysis upon its propagation. This mechanism can also be applied to fibrinogen deposited on surfaces of implanted biomaterials, which is expected to be resistant to plasmin digestion due to the binding and cross-linking of α_2-AP.

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