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# Non-Covalent Interaction of α2-Antiplasmin with Fibrin(ogen): Localization of α2-Antiplasmin Binding Sites

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## Abstract

Covalent incorporation (cross-linking) of plasmin inhibitor α2-antiplasmin (α2-AP) into fibrin clots increases their resistance to fibrinolysis. We hypothesized that α2-AP may also interact non-covalently with fibrin prior to its covalent cross-linking. To test this hypothesis, we studied binding of α2-AP to fibrin(ogen) and its fragments by ELISA and Surface Plasmon Resonance. The experiments revealed that α2-AP binds to polymeric fibrin and surface-adsorbed fibrin(ogen) while no binding was observed with fibrinogen in solution. To localize the α2-AP-binding sites, we studied the interaction of α2-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 sub-domains, which together encompass practically the whole fibrin(ogen) molecule. In ELISA, α2-AP bound to immobilized $D_1$, D-D, αC region, αC-domain and its C-terminal sub-domain. The binding was Lys-independent and was not inhibited by plasminogen or tPA. Furthermore, the affinity of α2-AP to 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 sub-domain of the αC-domain contain high affinity α2-AP-binding sites that are cryptic in fibrinogen and exposed in fibrin or adsorbed fibrinogen, and the presence of plasminogen facilitates interaction of α2-AP with the D regions. The discovered non-covalent interaction of α2-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 α2-AP to the fibrin clot.

The fibrinolytic system including 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 between plasminogen, tissue-type plasminogen activator (tPA[1]), and fibrin, and results in generation of active fibrinolytic enzyme plasmin (4,5). Plasmin activity is controlled by a number of inhibitors; the major physiological inhibitor of plasmin is α2-antiplasmin (α2-AP). The importance of such a control is highlighted by the fact that congenital deficiency of α2-AP results in a severe hemorrhagic disorder due to increased susceptibility to fibrinolysis (5–7).

Plasmin inhibitor α2-AP is a single chain glycoprotein consisting of 464 amino acid residues with $NH_2$-terminal Met residue, Met-α2-AP (3,8,9). In the circulation, it undergoes proteolytic cleavage between Pro12 and Asn13 by an antiplasmin-cleaving enzyme resulting

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in a 452-residue version with $NH_2$-terminal Asn residue, Asn-$\alpha_2$-AP, which accounts for approximately 70% of circulating $\alpha_2$-AP (10–13). $\alpha_2$-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, $\alpha_2$-AP has a COOH-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 close proximity to the reactive loop (15), binds to Lys-binding kringles of plasmin increasing the inhibitory efficiency of $\alpha_2$-AP (16,17). Thus, $\alpha_2$-AP efficiently inhibits free plasmin in the circulation thereby preventing fibrinogenolysis. Upon blood coagulation, $\alpha_2$-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-$\alpha_2$-AP or Asn-$\alpha_2$-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 $\alpha_2$-AP in solution is well established, that by $\alpha_2$-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 non-identical polypeptide chains denoted A$\alpha$, B$\beta$, and $\gamma$ (21). These chains are folded into a number of structural domains that compose several regions (22). The central region E is formed by the disulfide-linked $NH_2$-terminal portions of all six chains converging from both subunits. The COOH-terminal regions of the B$\beta$ and $\gamma$ chains and a portion of the A$\alpha$ chain form the terminal D region, one in each subunit, while the remaining COOH-terminal portion of the two A$\alpha$ chains (residues A$\alpha$221–610) form two $\alpha$C regions. Each $\alpha$C region is composed of the flexible $\alpha$C-connector (residues A$\alpha$221–391) and compact $\alpha$C-domain (residues A$\alpha$392–610) (23). Thus, the structure of fibrinogen can be presented as consisting of three linearly arranged regions, D-E-D, with the $\alpha$C-domains attached to the D regions via the $\alpha$C-connectors (Fig. 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 (Fig. 1B) (24). The $\alpha$C regions are susceptible to proteolysis and degraded into smaller fragments (21); they, as well as their constituents $\alpha$C-connector and $\alpha$C-domain, can be prepared by recombinant techniques (25) (Fig. 1C).

Fibrinogen is rather inert in the circulation, however, it becomes highly reactive towards 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 $NH_2$-terminal portions of the A$\alpha$ and B$\beta$ 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 (Fig. 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 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 $\alpha$C-domains (reviewed in Ref: 4). Whether fibrin(ogen) contains binding sites for plasminogen inhibitor, $\alpha_2$-AP, remains to be established.

Factor XIIIa can incorporate $\alpha_2$-AP to both fibrinogen and fibrin (28). The incorporation occurs by covalent cross-linking of $\alpha_2$-AP to a single Lys residue (Lys303) located in the COOH-terminal portion of the fibrin(ogen) A$\alpha$ chain (29,30), namely in its $\alpha$C-connector. While covalent cross-linking of $\alpha_2$-AP to fibrin(ogen) is well established, its molecular

mechanism is not well understood. Particularly, it is not known whether the efficient cross-linking requires spatial arrangement of the cross-linking sites. In this connection, it was shown that factor XIIIa-mediated γ-γ cross-linking is markedly enhanced in the presence of a fibrin fragment E template that bring 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 proper orientation of the cross-linking sites (32). Thus, we hypothesized that $\alpha_2$-AP may interact non-covalently with fibrin prior to its covalent cross-linking. The major goal of the present study was to test this hypothesis and to further clarify the mechanism of incorporation of $\alpha_2$-AP into a fibrin clot that plays a critical role in controlling fibrinolysis.

## EXPERIMENTAL PROCEDURES

### Proteins, Enzymes, and Antibodies

Human $\alpha_2$-antiplasmin, namely, its Asn-$\alpha_2$-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, human Glu-plasminogen was from an Enzyme Research Laboratories. Bovine α-thrombin, aprotinin, and carboxypeptidase B were from Sigma. The rabbit anti-plasminogen polyclonal antibodies were purchased from Chemicon, the anti-tPA monoclonal antibody mAb GMA-043 was from Calbiochem. The alkaline phosphatase conjugated ExtrAvidin was from Sigma.

### Preparation of Fibrin(ogen) Fragments

Fibrinogen $D_1$ and $E_3$ fragments (Fig. 1B) were prepared by plasmin digestion of human fibrinogen by the procedures described in (34). Their NH$_2$-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 earlier (35). The fibrin-derived D-D fragment and D-D:E complex (Fig. 1D) were prepared by plasmin digestion of cross-linked fibrin as previously described in (26).

The recombinant Aα221–610, Aα221–391, and Aα392–610 fragments corresponding to the human fibrinogen αC region, αC-connector, and αC-domain, respectively, (Fig. 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 sub-domains of the αC-domain, respectively, (36) were expressed in *Escherichia 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, code for 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 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 earlier (25). The purified fragments were refolded at 4 °C by slow dialysis from urea using the protocol described in (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: $E_{280, 1\%} = (5{,}690W + 1{,}280Y + 120S\text{-}S)/(0.1\ M)$, 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 based on their amino acid composition. The following values of molecular masses and $E_{280, 1\%}$ were obtained: 12,394 Da and 5.74 for Aα392–503, and 11,841 Da and 8.42 for Aα504–610. Note that these values take into account the $NH_2$-terminal fMet residue (see above) while the numbering of these fragments does not. The molecular masses and $E_{280, 1\%}$ 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 (39) with some modifications. Microtiter Immulon 2HB plate wells (Thermo) were coated overnight with 100 μL/well of fibrinogen or fibrin(ogen) fragments, 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, it was incubated with 100 μL of a mixture of thrombin (1 NIH u/mL) and aprotinin (400 u/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 COOH-terminal Lys residues. The wells were then blocked with 2% bovine serum albumin in TBS-Ca containing 0.01% Tween-20. Followed by washing with TBS-Ca and 0.01% Tween-20, the indicated concentrations of the proteins used as ligands were added to the wells and incubated for 1 h at 37 °C. $α_2$-AP was labeled with biotin using 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 were detected by reactions with specific polyclonal or monoclonal antibodies and the peroxidase-conjugated anti-rabbit or anti-mouse polyclonal antibodies as described earlier (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 Equation (1): $A = A_{max} /(1 + K_d/[L])$, where $A$ represents 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 1,000 RU, respectively, using the amine coupling kit (BIAcore AB, Uppsala, Sweden) according

to the manufacturer 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 10 μL/min flow rate. 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 adding 2 M NaCl in binding buffer for 30 sec followed by re-equilibration with binding buffer. Experimental data were analyzed using BIAevaluation 4.1 software 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 10,000 RU using the amine coupling kit (BIAcore AB, Uppsala, Sweden) according to the manufacturer instructions. Conversion of immobilized fibrinogen to fibrin was done by treatment with a mixture of thrombin (1 NIH u/mL) and aprotinin (400 u/mL) for 30 min. To prepare oligomers/polymers on the surface of the chip, 1 mg/mL fibrinogen was applied to immobilized fibrin for 20 min followed by a mixture of 3 NIH u/mL thrombin and 5 μg/mL factor XIII for 30 min; non-bound proteins were then washed out with HBS-Ca. This application was repeated three times to achieve immobilization of additional fibrin (~600 RU).

## RESULTS

### Interaction of α₂-Antiplasmin with Fibrin(ogen)

Interaction of α₂-AP with fibrinogen and fibrin was tested by two methods, ELISA and Surface Plasmon resonance (SPR). In ELISA, when microplate wells were coated with fibrinogen and then increasing concentrations of α₂-AP were added, fibrinogen exhibited a dose-dependent binding with $K_d$ = 179 nM (Fig. 2 and Table 1). In contrast, when microplate wells were coated with α₂-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 α₂-AP on the surface of microplate wells could result in the loss of its binding activity. To select between these alternatives we first performed sandwich ELISA, in which microplate wells were coated with streptavidin that captured biotin-labeled α₂-AP. When fibrinogen was added to α₂-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 α₂-AP (up to 2 μM) were added, no binding was observed (not shown). In another SPR experiment, when fibrinogen was added to immobilized α₂-AP, again no binding was observed (not shown). Thus, these experiments indicate that fibrinogen interacts with α₂-AP only when it is adsorbed onto a surface.

To test interaction of α₂-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 ELISA, α₂-AP exhibited a dose-dependent binding to surface-adsorbed fibrin with $K_d$ = 102 nM (Fig. 2 and Table 1) while in SPR no binding to immobilized fibrin was observed even at 2 μM α₂-AP (not shown). Although one

cannot exclude that the luck 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/immobilized at comparatively low concentrations, the resulting fibrin was most probably monomeric. Since the conformational changes and exposure of cryptic binding sites occur mainly upon fibrin polymerization (26,27), this may explain why such monomeric fibrin exhibited fibrinogen-like binding property towards $\alpha_2$-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 ELISA, as expected, while no binding to immobilized fibrin was observed in SPR (not shown). These results suggest that monomeric fibrin does not interact with $\alpha_2$-AP.

To test whether fibrin polymer may interact with $\alpha_2$-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 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/polymers of fibrin. In contrast to immobilized fibrin monomers described above, such immobilized oligomers/polymers exhibited in control experiments significant interaction with plasminogen (not shown) suggesting that their fibrin-specific binding sites were exposed. When $\alpha_2$-AP at increasing concentrations was added, it also exhibited a dose-dependent binding (Fig. 3). The $K_d$ value determined by the kinetic analysis of association/dissociation data (see Experimental Procedures) was found to be $45 \pm 8$ nM, that determined by the equilibrium analysis was close, $68 \pm 12$ nM. These experiments indicate that $\alpha_2$-AP binds to polymeric fibrin with high affinity.

## Localization of $\alpha_2$-AP Binding Sites in Fibrin(ogen)

To localize the $\alpha_2$-AP binding site(s) in fibrin(ogen), we studied the interaction of $\alpha_2$-AP with the $D_1$ or D-D fragments and $E_3$ fragment corresponding to the fibrin(ogen) D and E regions, respectively, and the recombinant $\alpha$C region ($A\alpha221$–610), which together encompass practically the whole fibrin(ogen) molecule (Fig. 1B–D). In ELISA, when these fragments were adsorbed on the surface of microplate wells and increasing concentrations of $\alpha_2$-AP were added, a prominent binding was observed with the D-D and $A\alpha221$–610 fragments while $E_3$ exhibited no binding (Fig. 4). The binding was dose-dependent and the $K_d$ values determined for D-D and $A\alpha221$–610 were 94 and 131 nM, respectively (Table 1). In another experiment, $\alpha_2$-AP also exhibited binding to the surface-adsorbed fibrinogen-derived $D_1$ fragment (Table 1). In reverse ELISA, when the D-D, $D_1$, $E_3$, and $A\alpha221$–610 fragments were added to immobilized $\alpha_2$-AP, no binding was detected (not shown). In agreement, in SPR experiments none of these fragments exhibited binding to immobilized $\alpha_2$-AP (not shown). These results suggest that the D and $\alpha$C regions contain $\alpha_2$-AP binding sites that are cryptic in fibrinogen and the $D_1$, D-D, and $A\alpha221$–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/immobilized $\alpha_2$-AP suggests that in solution these fragments have fibrinogen-specific conformation and their $\alpha_2$-AP binding sites are cryptic. Indeed, X-ray studies of fibrinogen-derived D and fibrin-derived D-D 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 (Fig. 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 $\alpha_2$-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 $\alpha_2$-AP, it exhibited a dose-dependent binding (Fig. 5). The $K_d$ value determined by the kinetic analysis of the data was found to be $1.4 \pm 0.4$ $\mu$M, that determined by the equilibrium analysis was practically the same, $1.4 \pm 0.2$ $\mu$M. These results clearly indicate that the $\alpha_2$-AP-binding sites in the D-D:E complex are accessible for $\alpha_2$-AP. They further reinforce the above suggestion that the $\alpha_2$-AP-binding sites of the D regions are cryptic in fibrinogen and become exposed in fibrin.

## Localization of $\alpha_2$-AP-Binding Site in the $\alpha$C Region

To localize the $\alpha_2$-AP-binding site in the $\alpha$C region, we studied the interaction of $\alpha_2$-AP with the recombinant A$\alpha$221–391 and A$\alpha$392–610 fragments, which correspond to the $\alpha$C-connector and $\alpha$C-domain, respectively, and compose the entire $\alpha$C region (Fig. 1C). In ELISA, $\alpha_2$-AP exhibited binding only to the surface-adsorbed A$\alpha$392–610 fragment while no binding was observed to A$\alpha$221–391 (Fig. 6); the affinity of the binding ($K_d$ = 150 nM) was similar to that determined for the $\alpha$C region (Table 1). This indicates that only the $\alpha$C-domain of the $\alpha$C region is involved in the interaction with $\alpha_2$-AP, i. e. this domain contains $\alpha_2$-AP-binding site.

We have shown recently that the $\alpha$C-domain consists of two independently folded sub-domains, N-terminal and C-terminal (36). To further localize the $\alpha_2$-AP binding site in this domain, we constructed and expressed A$\alpha$392–503 and A$\alpha$504–610 fragments corresponding to its N- and C-terminal subdomains, respectively (see Experimental Procedures). In ELISA, when increasing concentrations of $\alpha_2$-AP were added to both fragments immobilized on microtiter plate wells, only A$\alpha$504–610 exhibited binding (Fig. 6). The $K_d$ value of 123 nM determined for this binding was similar to those determined for the interaction of $\alpha_2$-AP with the $\alpha$C region or $\alpha$C-domain (Table 1). Thus, these results indicate that the $\alpha_2$-AP-binding site is located in the C-terminal sub-domain of the $\alpha$C-domain.

## Further Characterization of the Interaction Between $\alpha_2$-AP and Fibrin(ogen)

Since plasminogen, tPA, and $\alpha_2$-AP are the major players in initiation and regulation of fibrinolysis, we next tested whether the former two interfere in the interaction of $\alpha_2$-AP with fibrin(ogen). It is well established that plasminogen and tPA both interact with fibrin(ogen) D regions and $\alpha$C-domains in a Lys-dependent manner, although a Lys-independent binding of tPA to the D region was also observed (4). To test if $\alpha_2$-AP-fibrin(ogen) interaction involves Lys-binding sites, we studied binding of $\alpha_2$-AP to several fibrin(ogen) fragments that exhibited $\alpha_2$-AP-binding properties in the presence or absence of Lys-binding inhibitor, $\varepsilon$-aminocaproic acid ($\varepsilon$-ACA). In ELISA, when microplate wells were coated with these fragments and then increasing concentrations of $\alpha_2$-AP were added in the presence of 100 mM $\varepsilon$-ACA, all fragments exhibited a dose-dependent binding with $K_d$ values very similar to those determined in the absence of $\varepsilon$-ACA (Table 1). These results indicate that the binding of $\alpha_2$-AP to fibrin(ogen) is Lys-independent. Since the binding of plasminogen and tPA to fibrin(ogen) is mostly Lys-dependent, these results also suggest that $\alpha_2$-AP-binding site are different from those for plasminogen and tPA.

To test the above suggestion, we first studied simultaneous binding of all three species, $\alpha_2$-AP, tPA, and plasminogen, to surface-adsorbed fibrin in the presence or absence of $\varepsilon$-ACA. In ELISA, when a mixture of $\alpha_2$-AP, tPA, and plasminogen, each at saturating concentrations, 0.5 $\mu$M, 2.5 $\mu$M, and 2.5 $\mu$M, respectively, was added to immobilized fibrin, the binding of each species was detected (Fig. 7, filled bars). The addition of 100 mM $\varepsilon$-

ACA did not affect the binding of $\alpha_2$-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 (Fig. 7, empty bars). In another ELISA experiments, when fibrin, D-D and A$\alpha$392–610 fragments were adsorbed onto the surface of microplate wells and increasing concentrations of $\alpha_2$-AP were added in the presence of saturating concentrations of tPA (0.5 μM for A$\alpha$392–610 and 2.5 μM for fibrin and D-D), the binding curves and $K_d$ values for all three species were very similar to those obtained in the absence of tPA (Fig. 8A and Table 1). These results indicate that there was no competition between $\alpha_2$-AP and tPA for the binding sites on fibrin or the D-D and A$\alpha$392–610 fragments, i.e. the $\alpha_2$-AP and tPA binding sites do not overlap.

Next, we performed similar binding experiments with $\alpha_2$-AP in the presence of saturating concentrations of plasminogen (0.5 μM for A$\alpha$392–610, and 2.5 μM for fibrin and D-D). In the case of the surface-adsorbed A$\alpha$392–610 fragment the binding of $\alpha_2$-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 (Fig. 8B and Table 1). In contrast, binding of $\alpha_2$-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 D-D were found to be 25 and 5.5 nM, respectively. These results confirm that plasminogen does not compete with $\alpha_2$-AP for its binding site on fibrin(ogen). They also indicate that in the presence of plasminogen the binding of $\alpha_2$-AP to the surface-adsorbed D-D fragment and fibrin is significantly increased.

## DISCUSSION

The major physiological inhibitor of plasmin, $\alpha_2$-AP, plays an important role in the regulation of fibrinolysis. This occurs through direct inhibition of plasmin activity by $\alpha_2$-AP, either in the circulation or on the surface of a fibrin clot. In the circulation, $\alpha_2$-AP rapidly inhibits free plasmin thus preventing fibrinogenolysis (8,41). $\alpha_2$-AP also inhibits fibrin-bound plasmin, although much less efficiently (12). Meanwhile, incorporation of $\alpha_2$-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 $\alpha_2$-AP to fibrin(ogen) with factor XIIIa (6). In the present study, we found that $\alpha_2$-AP can also be incorporated into fibrin or adsorbed fibrin(ogen) through a non-covalent binding to its D regions and αC-domains.

The non-covalent interaction of $\alpha_2$-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 $\alpha_2$-AP (1 μM). This suggests that *in vivo* $\alpha_2$-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 allows the following speculation. Lys303 residue to which factor XIIIa cross-links $\alpha_2$-AP is located in the fibrin(ogen) αC-connector while one of the $\alpha_2$-AP-binding sites localized in the present 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 non-covalent 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 non-covalent binding of $\alpha_2$-AP to the αC-domain may provide its proper orientation towards the cross-linking site (Lys303) to enhance the covalent stage of the interaction (cross-linking). This is presented schematically in Fig. 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 thereby initiating fibrinolysis in places of fibrin deposition (4). In the present study, we localized the $\alpha_2$-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 $\alpha_2$-AP are Lys-independent. These findings suggest that fibrin can accommodate all three proteins simultaneously in close proximity to 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 $\alpha_2$-AP. Thus, non-covalent binding of $\alpha_2$-AP to the D regions and $\alpha$C-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, D-D:E complex, interacted with $\alpha_2$-AP while neither fibrinogen nor fibrin monomer exhibited such interaction. In ELISA, soluble fibrinogen did not interact with immobilized $\alpha_2$-AP while the latter exhibited strong interaction with surface adsorbed fibrin(ogen) and its fragments. These indicate that $\alpha_2$-AP-binding sites are cryptic in fibrinogen and become exposed in fibrin upon polymer formation or in adsorbed fibrinogen. This finding is not unexpected since 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 $\alpha_2$-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 $\alpha_2$-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 $\alpha_2$-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 $\alpha_2$-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 $\alpha_2$-AP, the latter may also be cross-linked by factor XIIIa to the former, as revealed in the recent study (28). However, since such cross-linking is not preceded by the non-covalent interaction, it should be less efficient than that to fibrin.

The present study also revealed that the presence of plasminogen significantly facilitates the non-covalent binding of $\alpha_2$-AP to the fibrin(ogen) D regions. When $\alpha_2$-AP was added to the immobilized D-D fragment or fibrin in the presence of plasminogen, the affinity of the binding to both species was dramatically increased while no change was observed in the presence of tPA (Fig. 8 and Table 1). No change in the affinity was also observed when $\alpha_2$-AP was added to the immobilized $\alpha$C-domain fragment in the presence of plasminogen or tPA. The reason for such increased affinity of $\alpha_2$-AP to 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 that this binding may elicit conformational changes in the D regions resulting in further exposure of the $\alpha_2$-AP-binding sites and increased affinity to $\alpha_2$-AP. Alternatively, the increased affinity may be connected with the reported ability of $\alpha_2$-AP to interact with plasminogen (6,8). In this case simultaneous binding of $\alpha_2$-AP to fibrin and fibrin-bound plasminogen could increase the overall affinity. This alternative may seem to be less probable since the interaction of $\alpha_2$-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 $\alpha_2$-AP and plasminogen for binding to immobilized fibrinogen or D-D (Fig. 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 towards

fibrin and $\alpha_2$-AP. Indeed, it was proposed that $\alpha_2$-AP interacts with plasminogen through 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 plasminogen-induced increased affinity of $\alpha_2$-AP to fibrin(ogen) D regions and its possible role in regulation of fibrinolysis.

In summary, the present study revealed the non-covalent interaction between $\alpha_2$-AP and fibrin or surface adsorbed fibrin(ogen), localized $\alpha_2$-AP-binding sites in the fibrin(ogen) D region and the $\alpha$C-domains, namely, its C-terminal sub-domain, and further clarified the mechanism of incorporation of $\alpha_2$-AP into fibrin clot. This mechanism includes the exposure of cryptic $\alpha_2$-AP-binding sites upon conversion of fibrinogen into fibrin polymer and non-covalent binding of $\alpha_2$-AP to the same regions of fibrin which bind tPA and plasminogen. Such exposure may serve to localize the activity of $\alpha_2$-AP to places of fibrin deposition. The non-covalent binding prior to the cross-linking may provide proper orientation of the cross-linking site and enhance the covalent cross-linking of $\alpha_2$-AP to the fibrin clot. The non-covalent binding may also serve to bring $\alpha_2$-AP to the clot for efficient inhibition of plasmin formed upon initiation of fibrinolysis while subsequent covalent cross-linking keeps $\alpha_2$-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 $\alpha_2$-AP.

## Acknowledgments

## Abbreviations

| | |
|---|---|
| **tPA** | tissue-type plasminogen activator |
| **$\alpha_2$-AP** | $\alpha_2$-antiplasmin |
| **$\varepsilon$-ACA** | $\varepsilon$-aminocaproic acid |
| **ELISA** | enzyme-link 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) |

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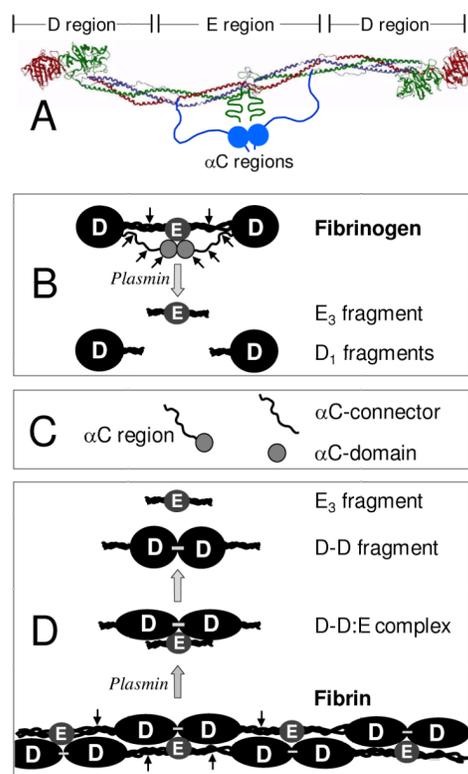
**Fig. 1.**
Schematic representation of fibrinogen, fibrin, and their fragments prepared for this study. *Panel A*: Ribbon diagram of fibrinogen based on its crystal structure (47); the individual fibrinogen chains, Aα, Bβ, and γ, are colored blue green, and red, respectively, the vertical lines denote approximate boundaries between the D and E regions. The αC regions, whose structure have 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. *Panel B*: Schematic representation of the fibrinogen molecule and its products of plasminolysis, $D_1$ and $E_3$ fragments. *Panel C*: Recombinant αC region (Aα221–610 fragment), αC-connector (Aα221–391 fragment), and αC-domain (Aα392–610 fragment). *Panel 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 non-covalent 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.
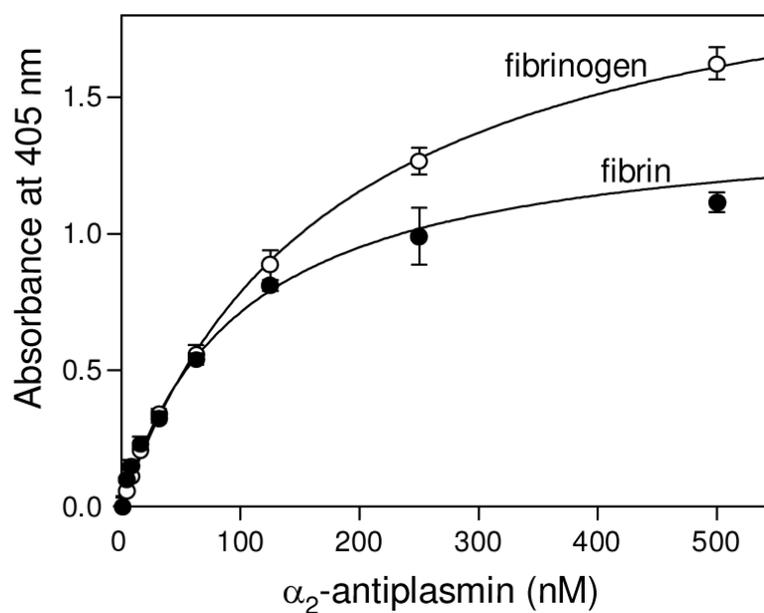
**Fig. 2.**
Analysis of the interaction of $\alpha_2$-antiplasmin with fibrinogen and fibrin by ELISA. Increasing concentrations of biotinylated $\alpha_2$-AP were added to surface-adsorbed fibrinogen (empty circles) or fibrin (filled circles). Bound biotinylated $\alpha_2$-AP was detected with avidin 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.
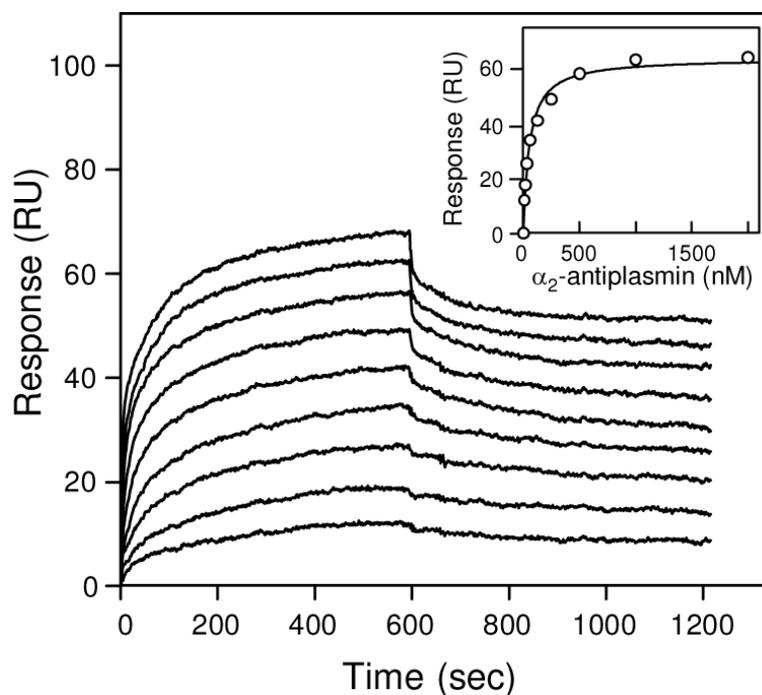
**Fig. 3.**
Analysis of the interaction of α2-antiplasmin with polymeric fibrin by surface plasmon resonance. α2-AP at increasing concentrations, 8, 16, 32, 63, 125, 250, 500, 1000 and 2000 nM, was added to immobilized fibrin polymers (see Experimental Procedures) and its association/dissociation was monitored in real time. The inset shows the results of the equilibrium analysis; the amount of bound a2-antiplasmin at equilibrium for each concentration is presented by circles and the best fit is presented by solid curve.
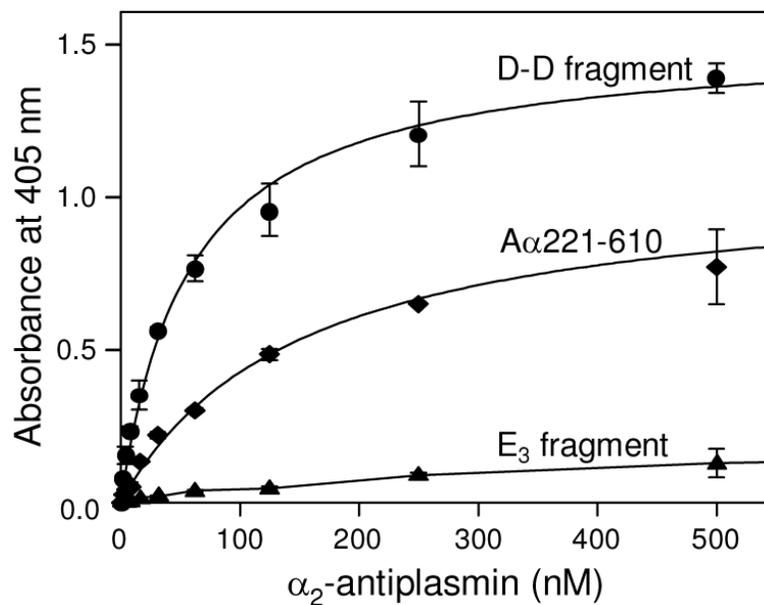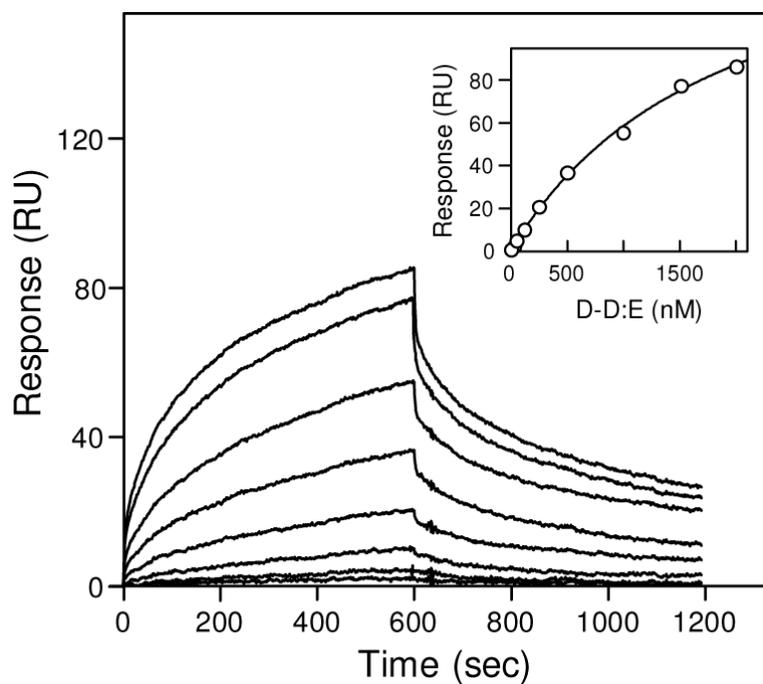
**Fig. 4.**
ELISA-detected binding of $\alpha_2$-antiplasmin to immobilized fibrin(ogen) fragments.
Increasing concentrations of biotinylated $\alpha_2$-AP were added to the immobilized D-D
(circles), A$\alpha$221–610 (diamonds), and E$_3$ (triangles) fragments. Bound biotinylated $\alpha_2$-AP
was detected 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.

**Fig. 5.**
Analysis of the interaction between $\alpha_2$-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 $\alpha_2$-AP and its association/dissociation was monitored in real time. The inset shows the results of the equilibrium analysis; the amount of bound D-D:E for each concentration is presented by circles and the best fit is presented by solid curve.
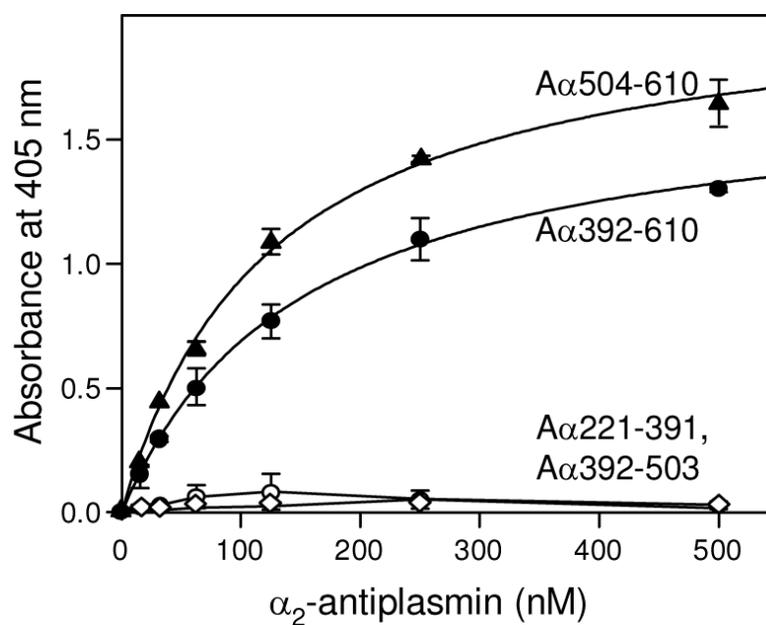
**Fig. 6.**
ELISA-detected binding of $\alpha_2$-antiplasmin to immobilized recombinant $\alpha$C-fragments. Increasing concentrations of biotinylated $\alpha_2$-AP were added to the immobilized A$\alpha$221–391 (empty circles), A$\alpha$392–610 (filled circles), A$\alpha$392–503 (empty diamonds), and A$\alpha$504–610 (filled triangles) fragments. Bound biotinylated $\alpha_2$-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.
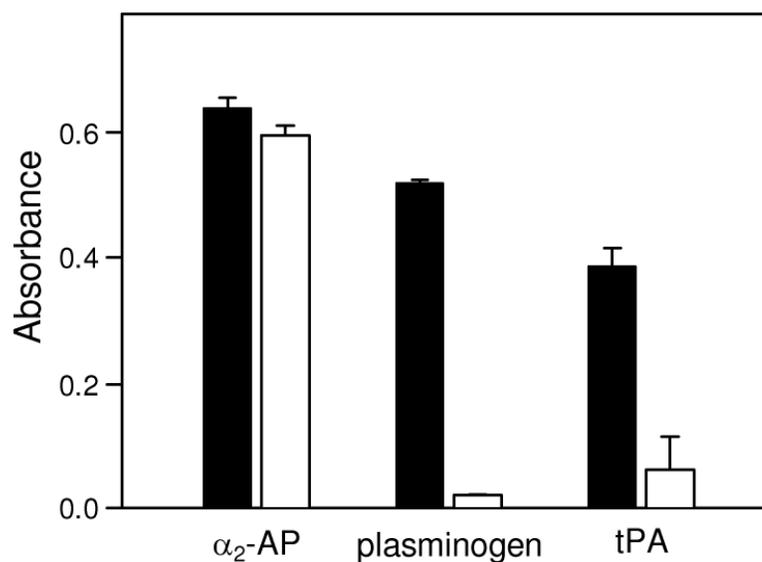
**Fig. 7.**
Simultaneous binding of α$_2$-antiplasmin, tPA, and plasminogen to immobilized fibrin detected by ELISA. A mixture of 0.5 μM biotinylated α$_2$-AP, 2.5 μM tPA, and 2.5 μM plasminogen was added to surface-adsorbed fibrin in the absence (filled bars) or presence (empty bars) of 100 mM ε-ACA. Bound biotinylated α$_2$-AP was detected spectrophotometrically at 405 nm using avidin conjugated to alkaline phosphatase, 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.
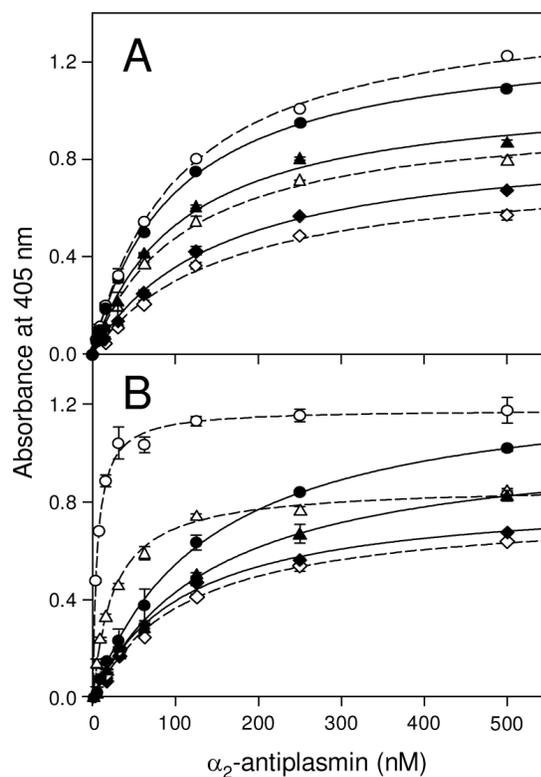
**Fig. 8.**
The effect of tPA or plasminogen on the binding of $\alpha_2$-antiplasmin to immobilized fibrin and its fragments detected by ELISA. *Panel A*: Increasing concentrations of biotinylated $\alpha_2$-AP were added to surface-adsorbed fibrin (triangles), the D-D (circles), or A$\alpha$392–610 fragments (diamonds) in the absence (filled symbols, solid lines) or presence (empty symbols, broken lines) of 2.5 $\mu$M tPA. *Panel B*: Increasing concentrations of biotinylated $\alpha_2$-AP were added to surface-adsorbed fibrin (triangles), the D-D (circles), or A$\alpha$392–610 fragments (diamonds) in the absence (filled symbols, broken lines) or presence (empty symbols, broken lines) of 2.5 $\mu$M plasminogen. Bound biotinylated $\alpha_2$-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.
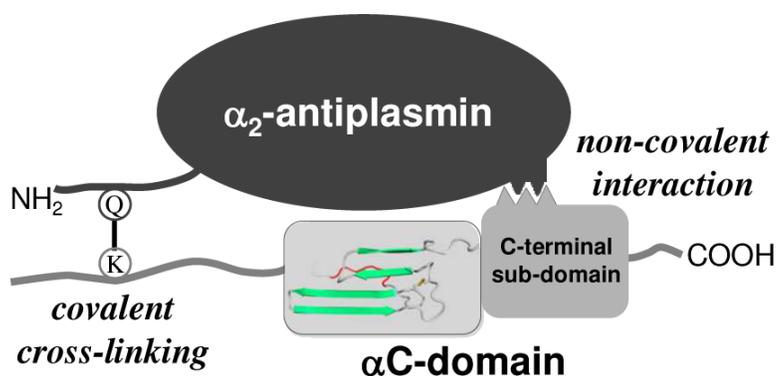
**Fig. 9.**
Schematic representation of the non-covalent and covalent interactions between $\alpha_2$-antiplasmin and fibrin(ogen) αC-domain. $\alpha_2$-AP with flexible $NH_2$-termial portion containing reactive Gln14 (Q) is presented on the top. The αC region containing the flexible αC-connector with reactive Lys303 (K) and compact αC-domain consisting of two sub-domains is presented on the bottom; the structure of the αC-domain is adapted from (36). The non-covalent interaction is shown on the right, the covalent cross-linking (Q–K) between reactive Gln14 and Lys303 is shown on the left.

**Table 1**

Dissociation constants ($K_d$) for the interaction of $\alpha_2$-antiplasmin with surface-adsorbed fibrinogen, fibrin, and their fragments obtained by ELISA.

| Proteins/Fragments | $K_d$ (nM)[a] | | | |
|---|---|---|---|---|
| | | + εACA | + tPA | + plasminogen |
| fibrinogen | 179 ± 19 | 102 ± 4 | - | - |
| fibrin | 102 ± 4 | 101 ± 12 | 109 ± 9 | 25 ± 2 |
| E₃ fragment | n. b.[b] | - | - | - |
| D₁ 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 | n. b. | - | - | - |
| Aα392–503 fragment | n. 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.