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Evidence For Covalent Linkage Between Some Plasma Α2-Antiplasmin Molecules and Aα Chains of Circulating Fibrinogen

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Plasma alpha2-antiplasmin (α2AP) is a single-chain serine protease inhibitor acting mainly through the fibrinolytic system1-3. Its physiological importance is underscored by the observation that homozygous α2AP deficiency results in a severe hemorrhagic disorder due to rapid fibrin clot lysis (hyperfibrinolysis)4-6.

Some plasma α2AP molecules are tightly bound to purified plasma fibrinogen7, 8, but it is not known whether the binding force is covalent. From an historical perspective, a plasmin/trypsin inhibitory activity was identified many years ago in purified fibrinogen preparations9. Years later, evidence from electroimmuno-diffusion and ELISA studies indicated that inhibitory activity bound to plasma fibrinogen was due to binding of α2AP (‘fibrinogen-bound α2AP’)7, 8. However, these analyses could not directly address the question of whether the binding observed could be accounted for by covalent linkage between plasma fibrinogen and α2AP.

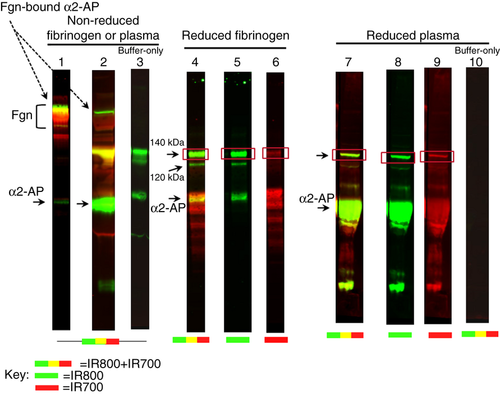
It is established that α2AP can be covalently linked to fibrin or fibrinogen by *thrombin-activated* factor XIII (FXIIIa). This occurs through formation of a covalent ε-(γ-glutamyl) lysine isopeptide bond between α2AP and lysine 303 of the fibrin(ogen) Aα chain10, 11. More recently, we demonstrated that α2AP could readily be covalently incorporated into the Aα chains of fibrinogen by *non-thrombin activated* factor XIII (FXIII)8.

In this present study we addressed the question of the covalent nature of fibrinogen-α2AP binding by conducting immuno-electrophoretic blotting (Western blotting) experiments on: (i) purified plasma fibrinogens (three) prepared from plasma by standard fractionation procedures12; (ii) citrated plasma from two healthy single donors as well as from a freeze-dried pooled plasma specimen obtained from Enzyme Research Laboratories (South Bend, IN, USA); and (iii) ‘serum’ prepared from plasma by thrombin defibrination.

Samples for electrophoresis were first heated for 5 min at 95 °C in a Tris-buffered (0.1 M, pH 6.8), SDS (5%), urea (4 M) solution, with or without added β-mercaptoethanol (5%). They were then loaded onto 4–12% ‘Bis-Tris’ gradient gels (Criterion XT, Bio Rad Laboratories) and subjected to electrophoresis using a ‘MOPS’ running buffer, as suggested by the manufacturer. Material in the gels was subsequently electrophoretically transferred to PVDF membranes.

PVDF-bound protein in the membrane was reacted with a polyclonal rabbit anti-human fibrinogen Aα chain IgG (‘H-300’, Santa Cruz Biochemicals) and/or an affinity-purified polyclonal goat anti-human α2AP IgG (EB08777, Everest Biotechnology, Oxfordshire, UK). EB08777 had been raised against an internal α2AP peptide, KDFLQSLKGPRGDK. These procedures were followed by reacting the membrane with fluorescently-labeled secondary antibodies (LI-COR Biosciences, Lincoln, NE, USA) against goat IgG [donkey anti-goat IgG tagged with a near-infrared green fluorescent dye (IRdye800)] or against rabbit IgG [donkey anti-rabbit IgG tagged with a red fluorescent dye (IRdye700)] according to a LI-COR protocol. Fluorescense was detected in a LI-COR Odyssey Infrared Imaging system by scanning at one or both available wavelength channels (680- and 800 nm). The results could be displayed at either or both wavelengths. Images that were displayed simultaneously at both wavelengths frequently appeared yellow. Unambiguous delineation of the location of material in any given region or band was obtained by displaying 680 nm or 800 nm scans separately.

Non-reduced purified fibrinogen (Fig. 1, lane 1) or plasma (lane 2) showed a prominent red-staining fibrinogen band (‘Fgn’, approximately 340 kDa). The cathodal region also possessed a prominent yellow/green-staining band indicating the presence of α2AP covalently linked to fibrinogen (‘Fgn-bound α2AP’). As expected, a 340 kDa fibrinogen band was absent from serum (data not shown). In addition to the specimen of intermediate solubility shown in lane 1, we examined lower solubility (fraction I-1) and higher solubility fibrinogens (fraction I-9). Every specimen showed the green-staining Fgn-bound α2AP band (data not shown).

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**Figure 1** Lanes selected from scanned PVDF membranes were from two experiments (lanes 1, 4, 5, 6 and lanes 2, 3, 7–10, respectively). The wavelengths used are indicated beneath the lanes. Non-reduced fibrinogen (‘Fgn’) is shown in lane 1, non-reduced single-donor plasma in lane 2. The dashed arrows indicate the location of fibrinogen (‘Fgn’)-bound α2AP. The ‘buffer-only’ plasma control in lane 3 had been reacted with buffer followed by secondary antibodies. A reduced fibrinogen specimen is displayed in lanes 4–6 at either or both wavelengths. Reduced single-donor plasma is displayed at either or both wavelengths in lanes 7–9. A rectangle imageencloses the α2AP-fibrinogen Aα chain 140 kDa heterodimer band. A faint doubly fluorescent heterodimer band at approximately 120 kDa is visible in lanes 4–9.

The non-reduced ‘buffer-only’ plasma control (lane 3) showed green staining material at approximately 150 kDa and at approximately 80 kDa, of uncertain nature. This ‘non-specific’ staining pattern did not overlap the fibrinogen-bound α2AP position or the ‘monomolecular’ α2AP position (approximately 70 kDa). The same non-specific IRdye 800 staining pattern also appeared in the plasma specimen (lane 2), and overlapped IRdye 700-staining material migrating between the fibrinogen and α2AP bands. These red bands probably represent fibrinogen fragments of fibrinogen-bearing Aα chain epitopes.

The 340 kDa fibrinogen band was absent from disulfide-bridge reduced plasma and from purified fibrinogen specimens. In its place there was a prominent approximately 140 kDa IRdye 800/IR dye 700-staining band reflecting covalently-linked α2AP-Aα chain heterodimers (lanes 4–6 and 7–9). There also was a relatively faint IRdye 800/IRdye 700-staining band at approximately 120 kDa in reduced fibrinogen and plasma, probably representing a heterodimeric fragment containing a smaller-sized Aα chain derivative. There was no staining in the ‘buffer-only’ control (lane 10).

In reduced fibrinogen-containing specimens (lanes 4–9) there was an IRdye800-stained band corresponding to monomeric α2AP that was situated amidst red-staining Aα chains and smaller Aα chain derivatives. The α2AP band was intense in the plasma samples, no doubt reflecting its relatively high plasma concentration. A fainter monomeric α2AP band was also visualized in non-reduced fibrinogen (e.g. lane 1). As monomeric α2AP had carried through all fibrinogen purification procedures, we infer that it, like the covalently bound α2AP, was tightly ‘bound’ to plasma fibrinogen. As expected, there was an intense α2AP monomer band in non-reduced plasma (lane 2).

Finally, in reduced plasma there was a prominent IRdye800 positive band at approximately 25 kDa (lanes 7, 8) of unknown identity. Material in this band was possibly derived from the non-reduced green-staining region shown in lane 3. The nature of the IRdye700 band at approximately 25 kDa in lane 9 is unknown.

Our immune-blotting experiments demonstrate that in plasma purified fibrinogen and in plasma fibrinogen there is a covalent linkage between α2AP and fibrinogen Aα chains that forms heterodimers. This structure accounts for most of the previously observed tight binding between plasma fibrinogen and α2AP7-9. Our experiments also indicate that the process accounting for heterodimer formation takes place *in vivo*, and we infer that this process involves the action of plasma FXIII.

There appears to be a correlation between reduced fibrinolytic potential and an increased tendency for intravascular thrombosis. Lisman *et al*.13 reported that reduced plasma fibrinolytic potential is a risk factor for venous thrombosis. Guimares *et al*.14 subsequently found that hypofibrinolysis was a risk factor for arterial thrombosis at a young age. More recently, Hoekstra *et al*.15 determined that impaired fibrinolysis was a risk factor in the Budd-Chiari syndrome (spontaneous thrombosis in hepatic veins or the inferior vena cava). Taken together these findings imply that hypofibrinolysis contributes importantly to the functional burden imposed by venous or arterial thrombosis. The role of fibrinogen-bound α2AP in regulating that process remains to be determined.

# Addendum

M. W. Mosesson designed and carried out most experiments and wrote the manuscript. T. Holyst, I. Hernandez and K. R. Siebenlist helped design and carry out experiments and helped edit the manuscript.

# Disclosure of Conflicts of Interests

The authors state that they have no conflict of interests.

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