\(\alpha_2\)-Antiplasmin Enschede: Dysfunctional \(\alpha_2\)-Antiplasmin Molecule Associated with an Autosomal Recessive Hemorrhagic Disorder

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Abstract

\(\alpha_2\)-Antiplasmin (\(\alpha_2\)-AP) is a major fibrinolysis inhibitor, whose complete, congenital absence has been found to be associated with a distinct hemorrhagic diathesis.

We studied a 15-yr-old male with a hemorrhagic diathesis after trauma from early childhood on. This bleeding tendency was associated with a minimal \(\alpha_2\)-AP level recorded functionally in the immediate plasmin inhibition test: \(\leq\) 4% of normal. However, a normal plasma concentration of \(\alpha_2\)-AP antigen (83%) was found. His sister (5 yr old) showed similar results (2 and 92%). In their family, eight heterozygotes could be identified by half-normal activity results and normal antigen concentrations. The inheritance pattern is autosomal recessive.

On analysis, the \(\alpha_2\)-AP of the propositus was homogeneous in all respects tested, suggesting a homozygous defect. We designated the abnormal \(\alpha_2\)-AP as \(\alpha_2\)-AP Enschede.

\(\alpha_2\)-AP Enschede showed the following characteristics: (a) complete immunological identity with normal \(\alpha_2\)-AP; (b) normal molecular weight (sodium dodecyl sulfate–polyacrylamide gel electrophoresis); (c) normal \(\alpha\)-electrophoretic mobility; (d) presence in plasma of both molecular forms excluding an excessive conversion to the less reactive non–plasminogen-binding form; (e) quantitatively normal binding to lys-plasminogen and to immobilized plasminogen kringle 1-3; and (f) normal Factor XII–mediated binding to fibrin. Functional abnormalities were found in: (i) no inhibition of amidolytic activities of plasmin and trypsin, even on prolonged incubation; (ii) no formation of plasmin–antiplasmin complexes in plasma with plasmin added in excess; and (iii) no inhibition of fibrinolysis by fibrin-bound \(\alpha_2\)-AP. In the heterozygotes, the presence of abnormal \(\alpha_2\)-AP did not interfere with several functions of the residual normal \(\alpha_2\)-AP. One-dimensional peptide mapping showed an abnormal pattern of papain digestion.

We conclude that in this family, abnormal antiplasmin molecules, defective in plasmin inhibition but with normal plasminogen-binding properties, have been inherited. The residual plasminogen-binding properties do not protect against a hemorrhagic diathesis.

Introduction

The relevance of \(\alpha_2\)-antiplasmin (\(\alpha_2\)-AP), 1 or \(\alpha_2\)-plasmin inhibitor, as a major regulatory inhibitor in fibrinolysis, has been made clear since the discovery of congenital deficiencies in 1979 (1). The homozygous-deficient cases discovered so far show a distinct hemorrhagic diathesis. Bleeding symptoms have also been observed (1–6) in some heterozygotes with approximately half-normal plasma concentrations of \(\alpha_2\)-AP.

\(\alpha_2\)-AP is a 67,000-mol-wt glycoprotein synthesized in the liver (7) and present in plasma at a concentration of \(\sim\) 1 \(\mu\)M (8). In the circulation, the inhibitor is present in two molecular forms that have a distinct difference in affinity for plasminogen: one form has affinity for plasminogen (plasminogen-binding [PB] form); the other does not (non–plasminogen-binding [NPB] form) (9–11). The two forms circulate in a ratio of PB/1NPB = 2.2; thus, PB = 0.67 \(\mu\)M and NPB = 0.30 \(\mu\)M (12). There is evidence that the NPB form is formed out of the PB form in the circulation (13, 14).

The PB–\(\alpha_2\)-AP molecule has three functional sites that determine an intimate interplay of this inhibitor in fibrinolysis.

(i) The first site is the reactive site for proteases, which can be cleaved by plasmin, trypsin, and some other proteases, and can result in a 1:1 covalent complex, possibly stabilized by an ester bond (15).

(ii) The second, lysine-donor site(s) interact(s) reversibly with lysine-binding site(s) of plasminogen and plasmin. The participation of this second site in the case of plasmin determines the unique rapid inactivation rate (\(k_t = 2-4 \times 10^7\) M\(^{-1}\) s\(^{-1}\)) observed for this protease (16). The inactivation rates are much slower (10 to 60 times) for other proteases, e.g., trypsin (16), and also for a modified (mini) plasmin that lacks lysine-binding sites (17).

(iii) The third functional site of \(\alpha_2\)-AP is located at the NH\(_2\)-terminal end of the molecule, presenting a site for the transglutaminase Factor XIII which cross-links \(\alpha_2\)-AP to fibrin during coagulation (18). About 20% of the total plasma \(\alpha_2\)-AP becomes covalently linked to fibrin clots and renders them more resistant to lysis after in vitro coagulation (19).

The difference between the PB and NPB forms of antiplasmin on the functional level appears to be that the NPB form only retains its reactive site for proteases (site 1), but has no other functional sites, thus lacking plasminogen and also fibrin.

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1. Abbreviations used in this paper: \(\alpha_2\)-AP, \(\alpha_2\)-antiplasmin; BAU, blood activator units; FDP, fibrinogen) degradation products; IPIT, immediate plasmin inhibition test; KIU, kallikrein inactivator units; MCE, modified crossed immunoelectrophoresis; NPB, nonplasminogen binding; PB, plasminogen binding; t-PA, tissue-type plasminogen activator.

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binding (20). The inactivation rate of plasmin is strongly reduced (11).

In this paper we describe a patient with a bleeding tendency associated with a minimal plasma antiplasmin functional activity and a normal α2-AP antigen level. Functional and structural abnormalities and residual functions of the defective α2-AP are described.

Methods

Materials

Unless otherwise specified, reagents were of analytical grade and were obtained from E. Merck, Darmstadt, FRG. Microbiological grade gelatin was from N. K. Merk. Agarose for electrophoresis (lot 33006), sodium dodecyl sulfate (SDS), and ethylene-diamine-tetraacetic acid disodium salt (EDTA) were obtained from BDH Chemicals Ltd., Poole, England. Carboxaw 6000 was from Fluka AG, Buchs, Switzerland. Trasylol (5,880 kalilkrein inactivator units [KIU]/mg) was a gift from Bayer AG, Leverkusen, FRG, through the courtesy of Dr. E. Philipp. Activated partial thromboplastin time reagent, reptilase reagent, FM test, A23187 and chromozym TRY (benzoyl-Val-Gly-Arg-p-nitroanilide) were obtained from Boehringer Mannheim GmbH, Mannheim, FRG. Thromboplastin-C reagent, ADP, and epihemin were from Dade Diagnostics Inc., Aqua, PR. Coagulation-deficient plasmas for Factors V, VII, IX, X, XI, prekallikrein, and high molecular weight kininogen were obtained from George King Bio-Medical Inc., Overland Park, KS. Factor VIII–deficient plasma, Factor XII–deficient plasma, and α2-AP–deficient plasma (see reference 2) were obtained from congenitally deficient patients. Platelet-poor citrated human plasma and pooled plasma were prepared as previously described (21). Dextran sulfate, sodium salt (500,000 mol wt) was from Pharmacia Fine Chemicals, Uppsala, Sweden. Coomassie Brilliant Blue R-250 was from Serva Feinbiochemica GmbH & Co., Heidelberg, FRG. Tissue-type plasminogen activator (t-PA) was a partially purified preparation (step 3, material [22]) from human uterus and obtained by extraction with 0.3 M potassium acetate buffer, pH 4.2, ammonium sulfate precipitation, and zinc chelate–agarose chromatography (22). Papain (type III) was from Sigma Chemical Co., St. Louis, MO.

Plasminogen-rich bovine fibrinogen was prepared according to Brakman (23). EDTA buffer (μ = 0.15) consisted of 0.05 M sodium diethylbarbiturate, 0.1% (wt/vol) gelatin, and 2.7 mM EDTA adjusted to pH 7.8 with an HCI solution. The synthetic substrates S-2238, S-2222, S-2444, S-2302, and S-2251 were from Kabi Vitrum AB, Stockholm, Sweden; collagen, Hormon-Chemie, Munich, FRG; arachidonic acid, Bio Data Corp. (Hatboro, PA); and reticocitin, from H. Lüöndek & Co. A/S, Copenhagen, Denmark. Antiserum against α1-antitrypsin, α2-macroglobulin, C1-inactivator, antithrombin III, histidine-rich glycoprotein, and Factor XIII, subunit A, were from Behringwerke AG Diagnostica, Marburg, FRG. Antiserum directed against fibrinogen was raised in goats and antiserum to Von Willebrand factor in rabbits. Bovine thrombin (EC 3.4.21.5) was from Leo Pharmaceutical, Ballerup, Denmark, or from Roche, Basel, Switzerland. Plasmin (EC 3.4.21.7) was prepared as previously described (24) and the concentration determined by active site titration with p-nitrophenyl-p'-guanidinobenzoate, according to Chase and Shaw (25). Trypsin (EC 3.4.21.4) from bovine pancreas was obtained from Boehringer Mannheim GmbH. Protein A–purified IgG of a rabbit antiserum against high molecular weight urokinase (M0, of 54,000) from urine was prepared as specified in reference 26. Rabbit anti–goat IgG antibody conjugated with alkaline phosphatase was from Sigma Chemical Co. Rabbit antiserum against α2-AP were obtained from (a) Nordic Immunological Laboratories, Tilburg, The Netherlands; (b) as a gift from Dr. D. Collen, Center for Thrombosis and Vascular Research, University of Leuven, Belgium (batch DC 2); (c) as a gift from Dr. I. Clemmensen, Statens Serum Institute at Hvidovre Hospital, Copenhagen, Denmark; (d) as a gift from Behringwerke AG Diagnos-}


tics; and (e) as a gift from Servo, Asnières, France. Goat IgG and antiserum was obtained from BioRad AB, Umed, Sweden, and Nordic Immunological Laboratories, respectively. Lys-plasminogen was prepared from human Cohn fraction III by affinity chromatography on lysine–agarose followed by gel filtration on Sephadex G-150.

Methods

Platelet function. The bleeding time was performed according to Mielke (27) using a Simplate II device (General Diagnostics, Div. of Warner-Lambert Co., Morris Plains, NJ). Platelet aggregation studies were performed in a dual channel aggregation module (Payton Assoc., Inc., Buffalo, NY) at 37°C with ADP (final concentrations, 2.5 and 5.0 μM), collagen (1.0 and 4.0 μg/ml), epinephrine (1.0 and 5.0 μM), arachidonic acid (1.5 mM), and A23187 (5.0 and 10.0 μM). The platelet number, as measured with the platelet analyzer 810 (Baker Diagnostics Ltd., Bethlehem, PA), was adjusted to 250,000/μl by dilution with autologous platelet-poor plasma. Total ATP and ADP were measured using the firefly luciferase technique described by Holmsen et al. (28). Serotonin was assayed according to Rao et al. (29).

Coagulation tests. Prothrombin times and activated partial thromboplastin times were performed by standard methods using thromboplastin-C and activated partial thromboplastin time reagent, respectively. Factors VIII, IX, XI, XII, prekallikrein, and high molecular weight kininogen were determined in a one-stage assay using congenitally deficient plasma as substrate. The urea solubility test was done by standard techniques. Clottable fibrinogen was measured according to Claus (30). Soluble fibrin–monomer complexes were determined by the ethanol gelation test and the FM test (Boehringer Mannheim GmbH). Antithrombin III, α1-antitrypsin, α2-macroglobulin, and C1-inactivator were assayed with the chromogenic substrates S-2238, S-2222, and S-2444, respectively.

Fibrinolysis techniques. The normal euglobulin fractions of plasmas were prepared at pH 5.9 with a plasma dilution of 1:10 as described previously (21). Precipitates were redissolved in EDTA buffer (21). Activities were assayed on plasminogen-rich bovine fibrin plates (31) and results expressed in diameters of lysed zones in the plates after 18 h incubation at 37°C. Total plasminogen activator plus proactivator level in plasma was assayed on fibrin plates with the blood activator inventory test (26). The activity of the dextran sulfate euglobulin fraction was expressed in arbitrary blood activator units (BAU) (BAU·ml⁻¹) and the contribution of the plasma urokinase–related activity was determined as the amount of activity quenched by excess of α2-AP IgG of an antiserum raised against urinary urokinase (26). For whole blood clot lysis, spontaneously clotted blood held at 37°C was observed for lysis.

The dilute blood clot lysis time method was performed as described by Chohan et al. (32) recording the lysis time of 10% blood.

The plasma activity of t-PA was assayed by a spectrophotometric assay (33). The activity was expressed in (milli) international units of urokinase, and established with a clot lysis time method as described by Rijken et al. (22). Inhibition of t-PA by plasma (Table II) was assayed as recently described (34) and expressed in percent of pooled normal plasma. Plasminogen was determined using the streptokinase activation procedure of Fribert et al. (35). Inhibition of the fibrinolytic activity of plasmin or human t-PA (Fig. 3) was carried out by a fibrin clot lysis method with plasma dilutions added. The fibrin clot was formed by mixing 0.1 ml enzyme, 0.1 ml plasma dilution, 0.05 ml human plasminogen (3 mg/ml), 0.05 ml thrombin (40 NIH U/ml), and 0.5 ml plasminogen-containing human fibrinogen (2.4 mg/ml) at 0°C, followed by incubation at 37°C. The time between clotting and lysis was determined and used for calculation of the residual activity of the enzyme. Results of individual plasmas were compared with those of the normal plasma pool and, using a standard curve obtained with a series of normal plasma dilution, expressed as percent inhibition (2).
the corresponding antisera. FDPs were also measured with the Thrombo-WellcoTest (Wellcome Diagnostics, Beckenham, England). Histidine-rich glycoprotein was determined by the method according to Maskarinec et al. (37). I-PA antigen was determined with an enzyme immunoassay, as recently described (38).

SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotting. SDS-PAGE was carried out according to Laemmli (39) or Weber and Osborn (40), as indicated. Immunoblotting was performed by incubating the blots successively with 10 μg/ml goat anti-α2-AP IgG (Biopool AB) or 1,000-fold diluted goat anti-plasminogen antisemur (Nordic Immunological Laboratories) and 1,000-fold diluted rabbit anti-goat IgG antibody conjugated with alkaline phosphatase. Staining was performed according to Blake et al. (41). Control blots were incubated with 1,000-fold diluted normal goat serum or buffer, as indicated. To eliminate nonspecific staining, in some experiments (Fig. 8) α2-AP-related antigen in plasma was extracted by incubating plasma samples with rabbit anti-α2-AP IgG Sepharose. This procedure was followed by washing the gel and elution with SDS sample buffer (60 min at 60°C).

Papain digestion. Papain was activated by incubating a solution of 1 mg/ml in phosphate-buffered saline that contained 5 mM cysteine and 1 mM EDTA for 30 min at room temperature. Plasma samples were incubated for 1 h at 37°C with a previously selected concentration of papain (18.5 μg/ml). The reaction was stopped by addition of iodoacetamide (1 mM, final concentration).

α2-AP functions. The functional assay of α2-AP with synthetic substrate, the immediate plasmin inhibition test (IPIT), was performed as described in detail elsewhere (2). The IPIT has been shown to record 1.00 × PB + 0.14 × NbP (12). Titration of a fixed amount of plasma with increasing plasmin concentrations was performed, also using the IPIT setup (Fig. 1) (42).

In such an experiment (Fig. 1), the activity of free plasmin that did not react with α2-AP is measured as a function of the added plasmin concentration. Plasmin activity and plasmin concentration are correlated to a buffer control without plasma (Fig. 1, top left, filled circles), and the concentrations of plasmin [PL] bound to α2-AP (PLb) and of plasmin that did not bind (P[PL]) are obtained from the titration (Fig. 1, top left, open circles) as indicated. Since

\[ [PL]b = \frac{[α2-AP]}{1 + \frac{1}{K_{app}}} \]

double reciprocal plot of bound plasmin ([PL]b) against free plasmin ([P[PL]]) gives a linear curve (Fig. 1) with intercept on the abscissa of 1/K_{app} (inhibition constant, apparent); and intercept on the ordinate of 1/[α2-AP]. For normal plasmin this value represents the PB-α2-AP = 0.67 μM, while K_{app} = 1.33 nM, well in accordance with literature data of the plasmin-α2-AP reactions in purified systems (16).

Modified crossed immunoelectrophoresis (MCIE). MCIE with added plasminogen was carried out as described in detail elsewhere (10). In brief: the 1% agarose gel for the first dimension in 0.03 M buffer, pH 8.6, contained 1,000 KIU/ml trastylol and 0.04 mg lys-plasminogen/ml added to the agarose solution just before casting the gel. Before electrophoresis, 5 μl of plasma, 2 μl of lys-plasminogen solution (2 mg/ml), and 1 μl 10,000 KIU/ml trastylol were sequentially introduced into the punched well. The gel for the second dimension comprised antiserum against α2-AP. The antisemur (c) used for this technique has a comparable titer for both forms of α2-AP as checked by assay of mixtures of the PB and NPB forms (12).

The affinity of α2-AP to lys-plasminogen was assessed by the MCIE using varying plasminogen concentrations in the agarose gel. The retardation of the PB form was recorded relative to the position of the NPB form (see Figs. 3 and 6). The lys-plasminogen concentration giving half-optimal retardation was used to represent the dissociation constant of lys-plasminogen PB-antiplasmin.

The binding of α2-AP to fibrin was studied by clotting 180 μl citrated plasma with a 120 μl calcium chloride (37.5 mM), thrombin (4 NIH U·ml⁻¹), NaCl (37.5 mM) mixture for 1 h at 37°C. In the clot supernatant and in a plasma sample incubated with 120 μl 0.15 M NaCl, α2-AP antigen concentration or activity was assayed. The difference represented the amount of α2-AP bound to fibrin.

The function of fibrin-bound α2-AP was assessed as follows: 0.5 ml citrated plasma, 5 μl purified t-PA, and 30 μl 0.15 M NaCl or IgG prepared from Factor XIII subunit A antiserum were incubated for 15 min at 0°C. Subsequently, a sample of 0.375 ml of the mixture was mixed with 70 μl calcium chloride (0.025 M) and thrombin (10 NIH U/ml) mixture and incubated for 30 min at 37°C. The formed clot was condensed by mechanical manipulation and centrifugation. The clot was washed with 0.15 M NaCl, blotted on filter paper, and placed on a plasminogen-rich bovine fibrin plate. Lyzed zones were recorded after 18 h incubation at 37°C. The IgG of Factor XIII A antiserum that was used completely prevented α- and γ-chain cross-linking of fibrin, as checked by SDS-PAGE.

Tryptsin and plasmin inhibition by purified α2-AP. PB-α2-AP from normal plasma and plasma of the propositus were purified on a plasminogen kriple 1-3 column essentially as described by Wiman (43). α2-AP preparations were obtained by elution with a buffer containing 3% albumin and 0.01% Tween 80. The preparations were found to contain only PB-α2-AP as shown by MCIE. The concentration of normal α2-AP was determined by titration with active site titrated plasmin; the concentration of abnormal α2-AP was established by Laurell immunoelectrophoresis.

In inhibition experiments trypsin, plasmin, or buffer was incubated at 37°C for various lengths of time with the α2-AP preparations in 160 μl buffer (0.05 M Tris/HCl, 0.11 M NaCl, pH 7.4, containing 1.4 mg/ml Carbowaq 6000; 0.017% (vol/vol) Tween 80) in a polystyrene test tube. Activity was assayed spectrophotometrically (405 nm) after addition of 40 μl chromogenic substrate to a final concentration of 0.7 mM and transferred to a semi-microcuvette. For trypsin, the chromogenic substrate Chromozym TRY was used and a solution of 1 mg/ml trypsin was found to neutralize 6.8 nM normal α2-AP (cf. Fig. 9).

Results

(a) Case history

The patient, a 15-yr-old white male, was referred for evaluation of easy bruising from early childhood on. He had no umbilical bleeding at birth. After minor trauma subcutaneous hematomas easily developed. There was no prolonged hemorrhage from small cuts but sometimes bleeding started again after 24 h. At the age of four, he began to bleed 12 h after tonsilectomy. Bleeding persisted for 2 d and ceased after blood transfusion. At age eight, there was a bleeding some hours after tooth extraction. The hemorrhage stopped 3 d later after transfusion. At age 14, he suffered again from prolonged bleeding after dental extraction. Epistaxis, spontaneous gingival bleeding, and muscle or joint bleedings did not occur. His 5-yr-old sister bruised easily, but had not had any operable procedures or injuries. Both siblings had normal growth and development. There were no signs suggestive of liver disease and routine liver function tests were normal. Besides a mild bleeding after tooth extraction, the father of the siblings had no bleeding tendency. The other members of the pedigree (see Fig. 2) did not have any signs of a hemorrhagic diathesis.

(b) Identification of the defect

Assessment of coagulation and platelet functions of the propositus in general tests showed no defects in either system (Table 1). Factor concentrations were within normal ranges for fibrinogen (clottable, Laurell), Factors II, V, VII, VIII:C, IX,
X, XI, XII, XIII subunit A, prekallikrein, and high molecular weight kininogen. With regard to platelet function, aggregation profiles with ADP, collagen, epinephrine, arachidonic acid, and A23187 were normal, as was the platelet content of ATP, ADP, and serotonin. Von Willebrand’s disease was ruled out by normal Factor VIII:C and VIII R:AG level and by a normal ristocetin cofactor activity.

In fibrinolysis, as evident from Tables I and II, all profibrinolytic and antifibrinolytic factors were found to be normal except for the activity of α2-AP which is ≤ 4% for both the propositus and his sister. This latter finding was confirmed for several plasma samples from the propositus that were obtained over a period of one year. On the other hand, the concentrations of α2-AP determined immunochemically (Laurell) were found to be 83 and 92%, respectively, in the plasmas of the propositus and his sister, which is within the normal range of 65–145% (n = 61). The siblings also had a reduced dilute blood clot lysis time (sister, 108 min) (Table I). The propositus’ plasma exhibited spontaneous lysis in fibrin plates. There were no signs of active processes (fibrin monomer, FDP).

The activity assay of α2-AP by plasmin inhibition in whole plasma containing other plasmin inhibitors is a kinetic assay with its specificity based upon the unique rapid reaction between plasmin and α2-AP.

The results of ≤ 4% in this test for the propositus and his sister indicated the absence of the rapid α2-AP activity. Also, in titration experiments using increasing plasmin concentrations (Fig. 1), no α2-AP activity could be detected. In normal plasma, analysis in such an experiment results in a Kᵢ (apparent) of 1.33 nM and an apparent antiplasmin concentration of 0.667 μM (Fig. 1, top). The propositus’ plasma (Fig. 1, bottom), however, showed inhibition of plasmin by an inhibitor with an apparent Kᵢ > 0.3 μM and a concentration in plasma which is at least one order of magnitude higher than that of the normal α2-AP.

Additionally, it was observed that the propositus’ plasma did not interfere with the α2-AP activity assay in normal plasma up to addition of a 16-fold excess of the propositus α2-AP (immunochemical amount). In a clot lysis inhibition test using the natural fibrinolysis substrate fibrin, the plasmas of the propositus and his sister showed a reduced inhibition of plasmin, as well as of t-PA-induced lysis. These data are comparable with results found previously for a plasma of a congenitally α2-AP–deficient case (type I deficiency) (refer to Fig. 3) (1–4). Fibrin-bound α2-AP of the propositus also did not inhibit plasmin (Table III).

It can be concluded that the propositus and his sister have an α2-AP deficiency, with a complete absence of the main rapid plasmin inhibition function of the molecule.

(c) Family study
Analysis of α2-AP levels in the family of the propositus (see pedigree, Fig. 2) revealed normal values with immunochemical methods in all members (Fig. 3). However, activity methods revealed another case with very low activity (2%): the sister of the propositus already discussed. Eight members of the family showed approximately half-normal α2-AP activity.

Table I. Results of Hemostasis Tests

<table>
<thead>
<tr>
<th>Assay</th>
<th>Propositus</th>
<th>Normal range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prothrombin time (s)</td>
<td>12.0</td>
<td>10.3–12.3</td>
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<tr>
<td>Activated partial thromboplastin time (s)</td>
<td>40</td>
<td>32–42</td>
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<tr>
<td>Thrombin time (s)</td>
<td>22.7</td>
<td>19.5–25.5</td>
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<tr>
<td>Reptilase time (s)</td>
<td>22.3</td>
<td>19.3–22.3</td>
</tr>
<tr>
<td>Fibrin monomer test</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>Ureum test</td>
<td>Normal</td>
<td></td>
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<tr>
<td>Antithrombin III activity (%)</td>
<td>130</td>
<td>80–120</td>
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<tr>
<td>Protein C antigen (%)</td>
<td>76</td>
<td>67–140</td>
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<tr>
<td>Bleeding time (min)</td>
<td>5.15</td>
<td>3–8</td>
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<tr>
<td>Platelet count (μ/l⁻¹)</td>
<td>271,000</td>
<td>150,000–320,000</td>
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<tr>
<td>Platelet aggregation studies</td>
<td>Normal</td>
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<tr>
<td>Whole blood clot lysis (h)</td>
<td>&gt;36</td>
<td>&gt;36</td>
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<tr>
<td>Dilute blood clot lysis, 10% (min)</td>
<td>87</td>
<td>&gt;162</td>
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<td>Plasma on fibrin plates, 30 μl (mm)</td>
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<td>Euglobulin activity on fibrin plate (mm)</td>
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<td>FDP (Wellco test) (μg/ml)</td>
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Table II. Results of Fibrinolysis Assays

<table>
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<th>Propositus’ sister</th>
<th>Normal range</th>
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<tr>
<td>Plasminogen (%)</td>
<td>80</td>
<td>88</td>
<td>75–125</td>
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<tr>
<td>Histidine-rich glycoprotein (%)</td>
<td>84</td>
<td>85</td>
<td>60–140</td>
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<tr>
<td>t-PA activity (mU/ml)</td>
<td>118</td>
<td>5</td>
<td>0–250</td>
</tr>
<tr>
<td>t-PA antigen (ng/ml)</td>
<td>11.8</td>
<td>16.1</td>
<td>10–30</td>
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<tr>
<td>Fast-acting t-PA inhibition (%)</td>
<td>64</td>
<td>133</td>
<td>20–350</td>
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<tr>
<td>Plasmin activity (BAU/ml)</td>
<td>51</td>
<td>53</td>
<td>35–60</td>
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<tr>
<td>Factor XIII–dependent activator activity (BAU/ml)</td>
<td>49</td>
<td>47</td>
<td>35–60</td>
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<tr>
<td>Cl–inactivator activity (%)</td>
<td>78</td>
<td>125</td>
<td>80–120</td>
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<tr>
<td>α2-Macroglobulin activity (%)</td>
<td>225</td>
<td>250</td>
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<tr>
<td>α2-AP activity (%)</td>
<td>4</td>
<td>2</td>
<td>85–140</td>
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Table III. Lysis Induced by Cross-Linked and Non-cross-Linked Washed Plasma Clots

<table>
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<tr>
<th>Type of plasma</th>
<th>Lysis zone (mm) in fibrin plates by clots</th>
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<tr>
<td></td>
<td>Cross-linked</td>
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<tr>
<td>Pooled normal</td>
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<tr>
<td>Propositus’ father</td>
<td>27.0</td>
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<tr>
<td>Propositus</td>
<td>35.9</td>
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<tr>
<td>Factor XIII–deficient</td>
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<tr>
<td>Pooled normal</td>
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</tbody>
</table>

Washed plasma clots prepared by coagulation of citrated plasma enriched in t-PA with thrombin/CaCl₂ were placed on fibrin plates. Lysis zones after 18 h of 37°C incubation are recorded. Added amount of t-PA is slightly different for the two last plasmas. Cross-linking is prevented for the “non-cross-linked” clots by addition of IgG of an antiserum directed towards Factor XIII subunit A. The crosslinking and its absence (γ-γ dimers absent) was confirmed by SDS-PAGE in each case.
levels in plasma. The classification of these members as heterozygotes was supported by the ratio of activity/immunochemical level of α2-AP (Fig. 3) and the inheritance pattern (Fig. 2), which apparently is autosomal recessive.

The half-normal values of inhibition of plasmin and t-PA by plasma in a clot lysis assay (Fig. 3) confirmed the absence of part of the α2-AP function in the heterozygotes. None of the inhibition parameters presented in Fig. 3 showed a significant difference between heterozygotes from the paternal or maternal family. Both families have lived for generations in the eastern part of The Netherlands. The family history has been obtained by interview and examination of the official registration dating to ∼1780, but no (official) consanguinity was found in six generations.

(d) Further characterization of the dysfunctional α2-AP
On Ouchterlony analysis with five different antisera against α2-AP (see Materials), complete identity between α2-AP in normal pooled plasma and in the propositus’ plasma was found (not shown). In crossed immunoelectrophoresis for α2-AP, a normal single peak pattern at α-mobility in the propositus’ plasma and that of his parents was obtained (cf. Fig. 7). SDS-PAGE and immunoblotting of normal pooled plasma and the plasma of the propositus showed that the apparent molecular weights of normal and dysfunctional α2-AP are very similar (Fig. 4).

Plasminogen binding
The propositus (Fig. 5) and his sister showed, qualitatively speaking, a normal pattern in MCE. In this method, plasminogen was incorporated in the agarose of the first-dimension electrophoresis to retard the PB form of α2-AP to β-mobility. This demonstrates that the PB form of α2-AP is present, thus excluding the possibility of a deficiency in this form. The ratios (PB/NPB) between the two forms for the propositus and his sister were 1.58 and 1.42, respectively, which are outside the normal range, 2.3±0.3 SD, range 1.86 to 3.00, in 29 normal individuals. This was confirmed in other plasma samples, and indicates a quantitative defect in the relation between the two forms.

The affinity of PB antiplasmin for plasminogen was assessed by varying the concentration of lys-plasminogen in the agarose gel, and showed a 50% retardation of PB-α2-AP at 0.4 μM for normal plasma (Fig. 6). The propositus’ plasma showed similar results (Fig. 6), indicating a normal binding to lys-plasminogen of the PB form.

The normal binding to plasminogen was endorsed by the observations during purification of PB-α2-AP by chromatography on immobilized kringle 1-3 from plasminogen. No difference in behavior between normal and propositus’ α2-AP was observed in binding to the column and the elution was at similar aminohexanoic acid concentrations. The NPB form of the propositus did not bind.
tus showed some $\alpha_2$-AP-related antigen with a faster electrophoretic mobility. This material probably represents proteolytically degraded $\alpha_2$-AP, which is sometimes observed in larger quantities after purification.

**Fibrin binding**

Fibrin binding of $\alpha_2$-AP mediated by Factor XIII was assessed by clotting of plasma samples with thrombin/CaCl$_2$ and immunochromatography analysis. A normal amount of 16% of $\alpha_2$-AP (normal range, 18±9% [SD]), $n = 12$) became bound to fibrin in the propositus' plasma. As in normal plasma, the PB form was predominantly bound, as revealed by comparison of the MCIE patterns of the plasma and the serum (not shown).

In the heterozygotes, the binding of $\alpha_2$-AP to fibrin mediated by Factor XIII was half of normal: 18.5±5% (SD), $n = 8$, as compared with 36±8% (SD), $n = 11$, in the normal family members. The binding was assessed by “activity” assay of $\alpha_2$-AP, which only reveals binding of the normal $\alpha_2$-AP in the heterozygotes. The results are similar to the results of heterozygotes in the previously described Dutch family (type I deficiency) (2), indicating the absence of interference of the dysfunctional $\alpha_2$-AP with the binding to fibrin of the normal $\alpha_2$-AP.

**Protease interaction**

As shown in Fig. 7, the addition of excess plasmin to plasma and incubation at 37°C results in normal pooled plasma in the formation of irreversible plasmin–$\alpha_2$-AP complexes appearing at $\beta$-mobility. In the propositus, no such complexes are formed, and in a heterozygote of his family an intermediate situation occurs.

After addition of plasmin to plasma and incubation for 45 min at 37°C, $\alpha_2$-AP-related antigen was isolated by immuno-

![Figure 5](image_url)  
**Figure 5.** MCIE of $\alpha_2$-AP in normal plasma and plasma of the propositus. The plasminogen incorporated in the agarose gel during first-dimension electrophoresis retards the PB form of $\alpha_2$-AP to $\beta$-mobility, while the NPB form retains its $\alpha$-mobility.

adsorption chromatography and analyzed by SDS-PAGE. As shown in Fig. 8, immunoblotting with anti-plasminogen IgG in normal plasma showed a band apparently corresponding to a complex of 140,000 plasmin–$\alpha_2$-AP and some dissociated plasmin. No complex was found in the propositus' plasma in a similar experiment, further substantiating the absence of plasin–$\alpha_2$-AP complex formation.

The PB–$\alpha_2$-AP of the propositus' plasma was purified on immobilized kringle 1-3 from plasminogen. This preparation was used to study possible slow type of inhibition of plasmin and inhibition of trypsin. As shown in Fig. 9, neither plasmin nor trypsin were inhibited to any extent by an excess of the PB–$\alpha_2$-AP of the propositus in 120 min. This is in clear contrast to the results with normal PB–$\alpha_2$-AP.

**Papain digestion**

One-dimensional peptide maps of $\alpha_2$-AP in plasma of the family members of the propositus (son) are shown in Fig. 10. Plasma samples were digested with papain. In the propositus and his sister, the smallest polypeptide, between 14.4 and 20.1

![Figure 4](image_url)  
**Figure 4.** SDS-PAGE and immunoblotting of the propositus' plasma (lanes 1-3, 7 and 8) and pooled normal plasma (lanes 4-6, 9 and 10). 40-µl amounts of 1,600-fold (lanes 1 and 4), 800-fold (lanes 2, 5, 7, and 9), and 400-fold (lanes 3, 6, 8, and 10) diluted plasma were electrophoresed on a 7.5% polyacrylamide gel (Laemmli system). After blotting, the nitrocellulose sheet was cut into two pieces. The left part (lanes 1-6) was stained after incubation with goat anti-$\alpha_2$-AP IgG, the right part (lanes 7-10) after incubation with buffer as a control. Lanes 1-6 show that the apparent molecular weight of normal and propositus' $\alpha_2$-AP are very similar. Staining in the upper half of lanes 1-6 is due to nonspecific interactions (the bands close to the origins are not visible in the control, but are visualized when normal goat serum is used instead of buffer). The plasma of the propositus' shows increased amounts of a faster migrating species of $\alpha_2$-AP.

![Figure 6](image_url)  
**Figure 6.** Changes in electrophoretic migration of PB–$\alpha_2$-AP by lys-plasminogen incorporated in the agarose gel in various concentrations. The migration of PB–$\alpha_2$-AP in a fixed electrophoresis time is expressed relative to the mobility of NPB–$\alpha_2$-AP. The half-maximal retardation of PB–$\alpha_2$-AP is at 0.4 µmol/liter lys-plasminogen. (c) Normal plasma; (●) propositus plasma.
activity (≤ 4% of normal) of the fibrinolysis inhibitor α₂-AP in plasma.

The importance of α₂-AP in the inhibition of fibrinolysis and the relation between unrestrained fibrinolysis and bleeding complications has been extensively dealt with (1–4) in relation to previously described cases of congenital homozygous α₂-AP deficiency. Also, some of the heterozygotes in the reported deficient families show a moderate hemorrhagic diathesis (2, 3, 5, 6). The father of the propositus in the present family was the only heterozygote with a mild bleeding tendency. The other seven heterozygotes did not have spontaneous hemorrhages and did not bleed after the many operative procedures in this group (tonsillectomy, hysterectomy, dental extractions, hip operations).

Study of the propositus and his sister revealed the presence in plasma of normal amounts of α₂-AP, detected immunochemically, despite the low activity values. This indicates an abnormal α₂-AP molecule, and represents the first reported type II deficiency of α₂-AP. We designated the dysfunctional molecule as “α₂-AP Enschede” (the city of birth of the propositus).

The propositus and his sister present with a homozygous expression of the deficiency with the residual activity of ≤ 4% similar to reported type I deficiencies. In all aspects (inhibitory activity, fibrin binding, PB, electrophoretic mobility, immunochemical reaction, molecular weight, and peptide mapping) of the dysfunctional α₂-AP studied in the “homozygotes” and the heterozygotes, the defects/results are homogeneous. No differences between heterozygotes from paternal and maternal origin were recorded.

No evidence for consanguinity was obtained from the family history; however, the homogeneity of the defects (e.g., peptide mapping) and the very specific functional defect in the molecule strongly suggest the existence of a true homozygous state, whether or not by consanguinity. Detailed analysis of the genomic DNA of the defective molecule might provide a definite answer.

The defective α₂-AP molecule lacks its function of very rapid plasmin inhibition. Such a defect could theoretically be due to excessive conversion of the PB form to the NPB form. This option has been excluded because, firstly, the PB form can be demonstrated in plasma of the propositus and has been purified based on its affinity for plasminogen. Secondly, the occurrence of heterozygotes with ≈ 50% residual activity argues against a hyperactive conversion process but points to a defect in the α₂-AP molecule. Thirdly, unlike even normal NPB–α₂-AP, the purified α₂-AP Enschede completely lacks slow inhibitory action towards plasmin as well as trypsin. Note that the PB/NPB ratio in both the homozygotes is lower than normal, indicating a slightly increased conversion of PB to NPB–α₂-AP for the α₂-AP Enschede.

The primary defect appears to be the complete inability of PB–α₂-AP Enschede to inhibit plasmin and trypsin. No inhibition of plasmin and trypsin activity on prolonged incubation (120 min) with purified PB–α₂-AP Enschede was observed, and no complexes with plasmin could be obtained (Figs. 7 and 8). The reaction with plasmin has been shown to involve the protease bait region of the molecule and secondary binding site(s) interacting with lysine-binding site(s) of the plasmin (16, 17). The reaction with trypsin does not involve the latter site (17), thus indicating a defect in the protease bait region of the molecule. Furthermore, the said secondary binding site is also
considered to be involved in binding to plasminogen and this property is shown to be retained in the α₂-AP Enschede (Figs. 5 and 6).

Other functions of the inactive PB–α₂-AP Enschede, such as PB and fibrin binding, are found qualitatively and quantitatively intact. The fibrin binding involves residues at the NH₂-terminal end of the molecule (18). The PB has been recently reported to reside most likely in the COOH-terminal part (26 amino acids) of the molecule (44). These areas do not comprise the protease-bait region and the findings are compatible with a hypothesis of a rather localized defect in the latter region that does not affect the other functions. The occurrence of a small defect is supported by the results of peptide mapping showing a tiny difference (< 1,000 d) in the smallest peptide generated by papain digestion. It suggests the occurrence of a small substitution or deletion in the gene for the abnormal molecule resulting in a change in papain cleavage site or generated peptide. The abnormality and residual functions of the PB–α₂-AP Enschede are summarized in Fig. 11 and its legend.

Note that the dysfunctional α₂-AP, by virtue of its residual actions (fibrin and PB) in the fibrinolytic process, does not interfere with the action of the normal α₂-AP in the heterozygotes. In the in vitro tests the dysfunctional molecule is not found to interfere with the immediate plasmin inhibition and other functions of the normal α₂-AP.

The residual interactions of the α₂-AP Enschede (fibrin and plasminogen binding) are not sufficient to prevent a hemorrhagic diathesis. In view of the age of the two homozygous patients, it is premature to discuss the severity of the bleeding tendency that appears, so far, to be less than in the reported type I-deficient cases. On the other hand, a very mild hemorrhagic diathesis was noted in the heterozygote father.

Specifically, the PB of α₂-AP, capable of retarding fibrinolysis by means of reduction of the binding of plasminogen to fibrin, may be of significance (45). The significance of this aspect has thus far been difficult to evaluate separately because

![Figure 9](image-url) **Figure 9.** Inhibition of plasmin and trypsin by purified PB–α₂-AP. (A) Normal α₂-AP (AP normal) inhibits trypsin (1 mg/liter) dose dependently (equivalence at 6.8 nmol/liter final concentration, abscissa) in contrast to α₂-AP of the propositus (AP propositus). Enzyme and inhibitor were incubated for 10 min at 37°C. (B and C) Plasmin (11 nmol/liter) and trypsin (6.8 nmol/liter) were incubated with α₂-AP (excess, 25 nM) or buffer for varying periods of time at 37°C (abscissa), and the residual activities on the synthetic substrates S 2251 and chromozym TRY, respectively, are recorded (ordinate).

![Figure 10](image-url) **Figure 10.** SDS-PAGE and immunoblotting of papain-digested plasma samples of the members of the family of the propositus. SDS-PAGE (15% gel) was carried out according to Laemmli. The blots were stained after incubation with goat anti-α₂-AP IgG (lanes 1–5) or with normal goat serum (lanes 6–9). See Methods for experimental details. Lane 1: homozygous daughter; lanes 2 and 6: heterozygous mother; lanes 3 and 7: heterozygous father; lanes 4 and 8: homozygous son (propositus); lanes 5 and 9: pooled normal plasma. The arrow indicates the position of the abnormal peptide parents. Numbers on the left represent molecular weights (×10³).

![Figure 11](image-url) **Figure 11.** Schematic representation of plasmin and PB–α₂-AP. In the α₂-AP, three functional sites are distinguished, from which site 1 has been found to be defective in α₂-AP Enschede. Site 1 in α₂-AP Enschede: no inhibition of plasmin and trypsin, no formation of plasmin–antiplasmin complexes, no inhibition of fibrinolysis by fibrin-bound antiplasmin. Site 2: normal binding to lys-plasminogen, and to immobilized kringle 1-3. Site 3: normal Factor XIII-mediated binding to fibrin.
of the inhibitory effect of $\alpha_2$-AP on plasmin (45). The reported family presents a clinical evaluation of the separate significance of the said PB. Apparently, a hemorrhagic diathesis could not be prevented, and points to a primary importance of plasmin inhibition.

It is therefore likely that the main function of $\alpha_2$-AP concerns its plasmin inhibition, and that the other functions, such as fibrin and plasminogen binding, support this function and concern the targeting of this action.

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Note added in proof: Sequence analysis of cloned genomic DNA fragments by Holmes et al. (46) recently demonstrated the presence of an alaniine insertion near the active site region of $\alpha_2$-AP Enschede.

References

35. Friberger, P., P. Knös, S. Gustavsson, L. Aurell, and G. Cla-


