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Research Article

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: less than or equal to 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 XIII-mediated binding to fibrin. Functional abnormalities were found in: (i) no inhibition of amidolytic activities of plasmin and trypsin, […]

α_2-Antiplasmin Enschede: Dysfunctional α_2-Antiplasmin Molecule Associated with an Autosomal Recessive Hemorrhagic Disorder

C. Kluft,* H. K. Nieuwenhuis,‡ D. C. Rijken,* E. Groeneveld,* G. Wijngaards,* W. van Berkel,§ G. Dooijewaard,* and J. J. Sixma‡
*Gaubius Institute TNO, 2313 AD Leiden, The Netherlands; ‡Department of Haematology, University Hospital, Utrecht, The Netherlands; and §Ziekenzorg Hospital, Enschede, The Netherlands

Abstract

α_2-Antiplasmin (α_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 α_2-AP level recorded functionally in the immediate plasmin inhibition test: ≤ 4% of normal. However, a normal plasma concentration of α_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 α_2-AP of the propositus was homogeneous in all respects tested, suggesting a homozygous defect. We designated the abnormal α_2-AP as α_2-AP Enschede.

α_2-AP Enschede showed the following characteristics: (*a*) complete immunological identity with normal α_2-AP; (*b*) normal molecular weight (sodium dodecyl sulfate–polyacrylamide gel electrophoresis); (*c*) normal α-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 XIII–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 α_2-AP. In the heterozygotes, the presence of abnormal α_2-AP did not interfere with several functions of the residual normal α_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.

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Address correspondence to Dr. Kluft, Gaubius Institute TNO, Herenstraat 5d, 2313 AD Leiden, The Netherlands.

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Introduction

The relevance of α_2-antiplasmin (α_2-AP),[1] or α_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 α_2-AP.

α_2-AP is a 67,000-mol-wt glycoprotein synthesized in the liver (7) and present in plasma at a concentration of ~ 1 μM (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/NPB = 2.2; thus, PB = 0.67 μM and NPB = 0.30 μM (12). There is evidence that the NPB form is formed out of the PB form in the circulation (13, 14).

The PB-α_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 ($k_1 = 2\text{–}4 \times 10^7$ 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 α_2-AP is located at the NH_2-terminal end of the molecule, presenting a site for the transglutaminase Factor XIII which cross-links α_2-AP to fibrin during coagulation (18). About 20% of the total plasma α_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

1. *Abbreviations used in this paper:* α_2-AP, α_2-antiplasmin; BAU, blood activator units; FDP, fibrin(ogen) degradation products; IPIT, immediate plasmin inhibition test; KIU, kallikrein inactivator units; MCIE, modified crossed immunoelectrophoresis; NPB, nonplasminogen binding; PB, plasminogen binding; t-PA, tissue-type plasminogen activator.

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 α_2-AP antigen level. Functional and structural abnormalities and residual functions of the defective α_2-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 E. Merck.

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. Carbowax 6000 was from Fluka AG, Buchs, Switzerland. Trasylol (5,880 kallikrein 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 epinephrine were from Dade Diagnostics Inc., Aquada, 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 α_2-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 (μm = 0.15) consisted of 0.05 M sodium diethylbarbiturate, 0.10 M NaCl, 0.25% (wt/vol) gelatin, and 2.7 mM EDTA adjusted to pH 7.8 with an HCl 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 ristocetin, from H. Lündbeck & Co. A/S, Copenhagen, Denmark. Antisera against α_1-antitrypsin, α_2-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 Pharmaceuticals, 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 (M_r 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 antisera against α_2-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 Diagnostica; and (*e*) as a gift from Serbio, Asniéres, France. Goat IgG and antiserum was obtained from Biopool AB, Umeå, 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). Serotinin 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 Clauss (30). Soluble fibrin–monomer complexes were determined by the ethanol gelation test and the FM test (Boehringer Mannheim GmbH). Antithrombin III, α_1-antitrypsin, α_2-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 added 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 Friberger 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).

Immunological techniques. Fibrinogen, fibrin(ogen) degradation products (FDP), Factor VIIIR:Ag, protein C (kindly performed by Dr. R. Bertina, Academic Hospital, Leiden, The Netherlands), and Factor XIII subunit A were determined by the Laurell technique (36) using

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 Mancini et al. (37). t-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-α_2-AP IgG (Biopool AB) or 1,000-fold diluted goat anti-plasminogen antiserum (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) α_2-AP–related antigen in plasma was extracted by incubating plasma samples with rabbit anti-α_2-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).

α_2-AP functions. The functional assay of α_2-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 $1.00 \times PB + 0.14 \times NPB$ (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 α_2-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 α_2-AP ($[PL]_b$) and of plasmin that did not bind ($[PL]_f$) are obtained from the titration (Fig. 1, *top left, open circles*) as indicated. Since

$$[PL]_b = \frac{[\alpha_2 - AP]}{1 + \frac{K_{i,app}}{[PL]_f}},$$

a double reciprocal plot of bound plasmin ($[PL]_b$) against free plasmin ($[PL]_f$) gives a linear curve (Fig. 1) with intercept on the abscissa of $1/K_{i,app}$ (inhibition constant, apparent); and intercept on the ordinate of $1/[\alpha_2\text{-AP}]$. For normal plasmin this value represents the PB-α_2-AP = 0.67 μM, while $K_{i,app}$ = 1.33 nM, well in accordance with literature data of the plasmin–α_2-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 trasylol 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 trasylol were sequentially rapidly introduced into the punched wall. The gel for the second dimension contained antiserum against α_2-AP. The immunoprecipitation peak surface at β-mobility represents the concentration of the PB form of α_2-AP; idem at α-mobility, that of the NPB form. The antiserum (c) used for this technique has a comparable titer for both forms of α_2-AP as checked by assay of mixtures of the PB and NPB forms (12).

The affinity of α_2-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. 5 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 α_2-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, α_2-AP antigen concentration or activity was assayed. The difference represented the amount of α_2-AP bound to fibrin.

The function of fibrin-bound α_2-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. Lysed 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.

Trypsin and plasmin inhibition by purified α_2-AP. PB-α_2-AP from normal plasma and plasma of the propositus were purified on a plasminogen kringle 1-3 column essentially as described by Wiman (43). α_2-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-α_2-AP as shown by MCIE. The concentration of normal α_2-AP was determined by titration with active site titrated plasmin; the concentration of abnormal α_2-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 α_2-AP preparations in 160 μl buffer (0.05 M Tris/HCl, 0.11 M NaCl, pH 7.4, containing 1.4 mg/ml Carbowax 6000; 0.017% (vol/vol) Tween 80) in a polystyrene 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/l trypsin was found to neutralize 6.8 nM normal α_2-AP (cf. Fig. 9).

Results

(a) Case history

The patient, a 15-yr-old white male, was referred for evaluation of easy bruisability 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 tonsillectomy. 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 operative 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 I). Factor concentrations were within normal ranges for fibrinogen (clottable, Laurell), Factors II, V, VII, VIII:C, IX,

Table I. Results of Hemostasis Tests

Assay	Propositus	Normal range
Prothrombin time (*s*)	12.0	10.3–12.3
Activated partial thromboplastin time (*s*)	40	32–42
Thrombin time (*s*)	22.7	19.5–25.5
Reptilase time (*s*)	22.3	19.3–22.3
Fibrin monomer test	Negative	Negative
Ureum test	Normal	
Antithrombin III activity (%)	130	80–120
Protein C antigen (%)	76	67–140
Bleeding time (*min*)	5.15	3–8
Platelet count (μl^{-1})	271,000	150,000–320,000
Platelet aggregation studies	Normal	
Whole blood clot lysis (*h*)	>36	>36
Dilute blood clot lysis, 10% (*min*)	87	>162
Plasma on fibrin plates, 30 μl (*mm*)	6.7	0
Euglobulin activity on fibrin plate (*mm*)	15.0	9–15
FDP (Wellco test) ($\mu g/ml$)	<10	<10

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 α_2-AP which is $\leq 4\%$ 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 α_2-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).

Table II. Results of Fibrinolysis Assays

Assay	Propositus	Propositus' sister	Normal range
Plasminogen (%)	80	88	75–125
Histidine-rich glycoprotein (%)	84	85	60–140
t-PA activity (*mU/ml*)	118	5	0–250
t-PA antigen (*ng/ml*)	11.8	16.1	10–30
Fast-acting t-PA inhibition (%)	64	133	20–350
Plasma urokinase activity (*BAU/ml*)	51	53	35–60
Factor XII–dependent activator activity (*BAU/ml*)	49	47	35–60
C1-inactivator activity (%)	78	125	80–120
α_2-Macroglobulin activity (%)	225	250	80–120
α_2-AP activity (%)	4	2	85–140

The activity assay of α_2-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 α_2-AP.

The results of $\leq 4\%$ in this test for the propositus and his sister indicated the absence of the rapid α_2-AP activity. Also, in titration experiments using increasing plasmin concentrations (Fig. 1), no α_2-AP activity could be detected. In normal plasma, analysis in such an experiment results in a K_i (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 $K_i > 0.3$ μM and a concentration in plasma which is at least one order of magnitude higher than that of the normal α_2-AP.

Additionally, it was observed that the propositus' plasma did not interfere with the α_2-AP activity assay in normal plasma up to addition of a 16-fold excess of the propositus α_2-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 α_2-AP–deficient case (type I deficiency) (refer to Fig. 3) (1–4). Fibrin-bound α_2-AP of the propositus also did not inhibit plasmin (Table III).

It can be concluded that the propositus and his sister have an α_2-AP deficiency, with a complete absence of the main rapid plasmin inhibition function of the molecule.

(c) Family study

Analysis of α_2-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 α_2-AP activity

Table III. Lysis Induced by Cross-Linked and Non-cross-Linked Washed Plasma Clots

	Lysis zone (*mm*) in fibrin plates by clots	
Type of plasma	Cross-linked	Non-cross-linked
Pooled normal	0	34.0
Propositus' father	27.0	36.1
Propositus	35.9	35.5
Factor XIII–deficient	—	32.7
Pooled normal	0	33.0

Washed plasma clots prepared by coagulation of citrated plasma enriched in t-PA with thrombin/$CaCl_2$ 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.

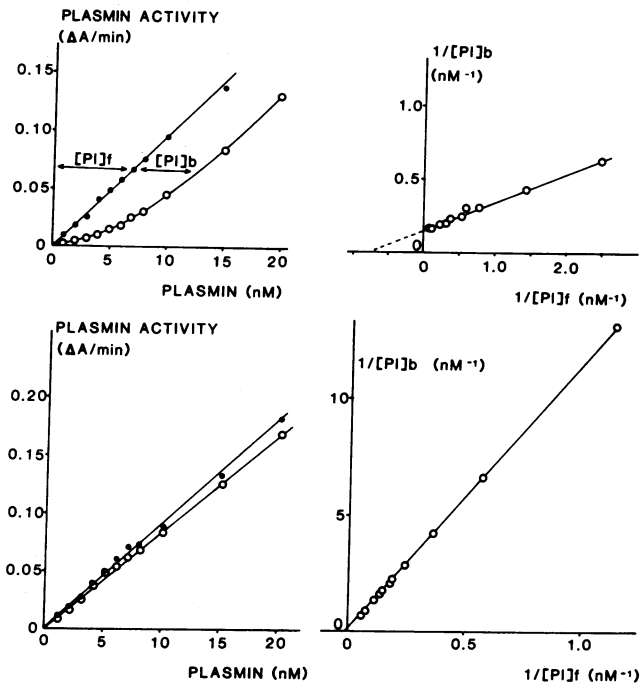

Figure 1. Titration of α_2-AP in plasma with increasing concentrations of plasmin. The activity of the plasmin that did not react with α_2-AP was assayed in the setup of the immediate plasmin inhibition test. The concentration of the plasmin was determined by active site titration. (*Top*) Normal plasma. (*Bottom*) Plasma from the propositus. (*Top left*) How, from the plasma titration curve (○), and the control in buffer (●), $[PL]_f$ (equals free plasmin concentration) and $[PL]_b$ (equals bound plasmin) are obtained. In the reciprocal plots (*top and bottom right*) the linear relation (curve fitting) between $1/[PL]_b$ and $1/[PL]_f$ provides values for $1/[\alpha_2\text{-AP}]$ and $1/K_i$ apparent, respectively, at the intercepts on the ordinate and the abscissa (see Methods).

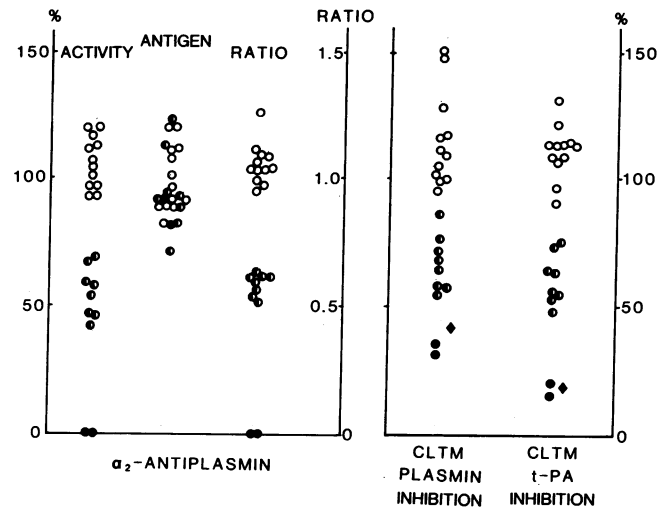

Figure 3. α_2-AP in the family with a dysfunctional α_2-AP molecule. Members are classified as with homozygous expression (●); heterozygotes (◐); normals (○). (♦) Homozygous type I case of α_2-AP deficiency described previously (2). α_2-AP is assayed functionally with the IPIT and immunochemically according to Laurell (36). The ratio of these data is calculated (*third column from left*). Plasmin and t-PA inhibition are also recorded in a clot lysis time assay (CLTM) (see Methods). All percentage values are expressed relative to pooled normal plasma.

levels in plasma. The classification of these members as heterozygotes was supported by the ratio of activity/immunochemical level of α_2-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 α_2-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 α_2-AP

On Ouchterlony analysis with five different antisera against α_2-AP (see Materials), complete identity between α_2-AP in normal pooled plasma and in the propositus' plasma was found (not shown). In crossed immunoelectrophoresis for α_2-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 α_2-AP are very similar (Fig. 4).

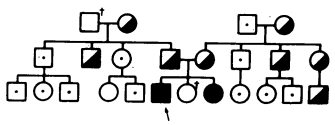

Figure 2. Pedigree of a family with a dysfunctional α_2-AP molecule (α_2-AP Enschede). (□) Male normal; (○) female normal; (◪) heterozygote; (■) homozygote; (▣) not tested; (†) deceased; (↖) propositus.

Plasminogen binding

The propositus (Fig. 5) and his sister showed, qualitatively speaking, a normal pattern in MCIE. In this method, plasminogen was incorporated in the agarose of the first-dimension electrophoresis to retard the PB form of α_2-AP to β-mobility. This demonstrates that the PB form of α_2-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–α_2-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–α_2-AP by chromatography on immobilized kringle 1-3 from plasminogen. No difference in behavior between normal and propositus' α_2-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.

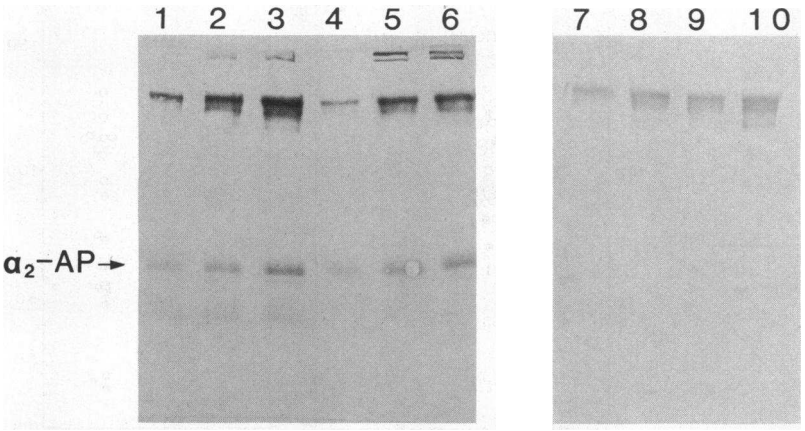

Figure 4. SDS-PAGE and immunoblotting of the propositus' plasma (lanes *1–3, 7* and *8*) and pooled normal plasma (lanes *4–6, 9* and *10*). 40-μl amounts of 1,600-fold (lanes *1* and *4*), 800-fold (lanes *2, 5, 7,* and *9*), and 400-fold (lanes *3, 6, 8,* and *10*) diluted plasma were electrophoresed 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-α_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' α_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 showed some α_2-AP–related antigen with a faster electrophoretic mobility. This material probably represents proteolytically degraded α_2-AP, which is sometimes observed in larger quantities after purification.

Fibrin binding

Fibrin binding of α_2-AP mediated by Factor XIII was assessed by clotting of plasma samples with thrombin/$CaCl_2$ and immunochemical analysis. A normal amount of 16% of α_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 α_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 α_2-AP, which only reveals binding of the normal α_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 α_2-AP with the binding to fibrin of the normal α_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–α_2-AP complexes appearing at β-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, α_2-AP–related antigen was isolated by immunoadsorption 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–α_2-AP and some dissociated plasmin. No complex was found in the propositus' plasma in a similar experiment, further substantiating the absence of plasmin–α_2-AP complex formation.

The PB–α_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–α_2-AP of the propositus in 120 min. This is in clear contrast to the results with normal PB–α_2-AP.

Papain digestion

One-dimensional peptide maps of α_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

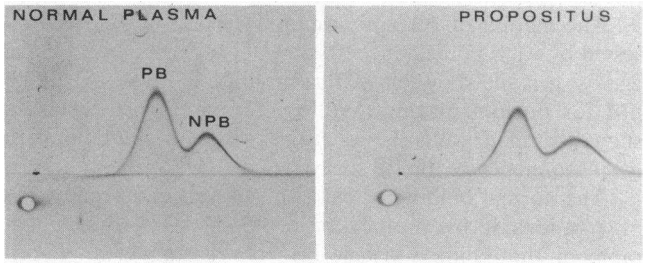

Figure 5. MCIE of α_2-AP in normal plasma and plasma of the propositus. The plasminogen incorporated in the agarose gel during first-dimension electrophoresis retards the PB form of α_2-AP to β-mobility, while the NPB form retains its α-mobility.

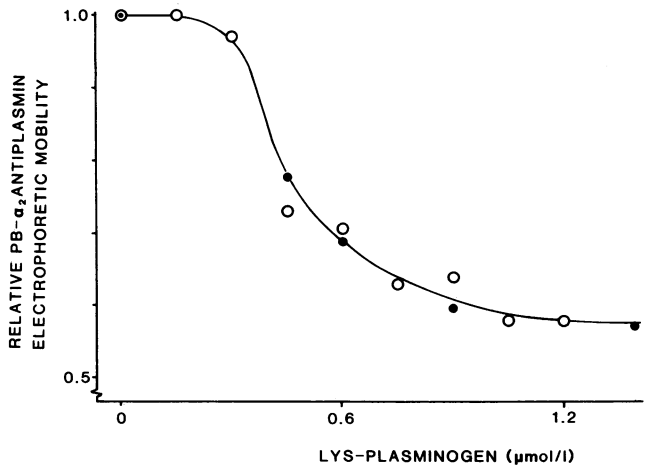

Figure 6. Changes in electrophoretic migration of PB–α_2-AP by lys-plasminogen incorporated in the agarose gel in various concentrations. The migration of PB–α_2-AP in a fixed electrophoresis time is expressed relative to the mobility of NPB–α_2-AP. The half-maximal retardation of PB–α_2-AP is at 0.4 μmol/liter lys-plasminogen. (○) Normal plasma; (●) propositus plasma.

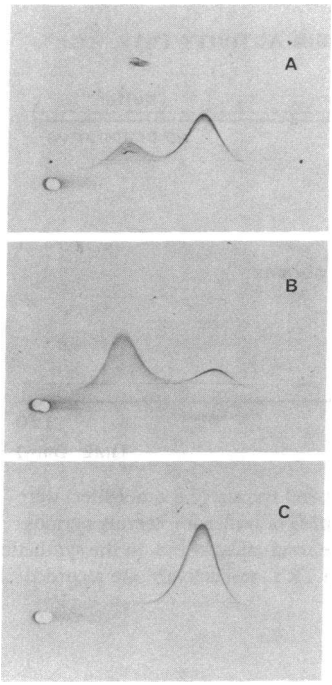

Figure 7. Crossed immunoelectrophoresis of α_2-AP after addition of plasmin to plasma. *B* shows the result for normal plasma with a large amount of plasmin–α_2-AP complexes at β-mobility. Excess plasmin was added to plasma and electrophoresis was carried out after 2 to 30 min incubation at 37°C. The agarose gels contained trasylol. *A* and *C* concern the father (heterozygote) and the propositus, respectively.

kD (see *arrow* in Fig. 10), showed a tiny difference in electrophoretic mobility, with a difference in apparent molecular weight of < 1,000. Both the heterozygous mother and father showed a doublet consisting of a normal and abnormal band, indicating that the two parents have the same or a similar defect. This suggests a small substitution or deletion in the gene for the abnormal molecule.

Discussion

The propositus and his sister manifest themselves with easy bruising and bleeding after operative procedures or injury. The crucial defect (which was found after extensive analysis of coagulation, platelet, and fibrinolysis parameters) was a very low activity (≤ 4% of normal) of the fibrinolysis inhibitor α_2-AP in plasma.

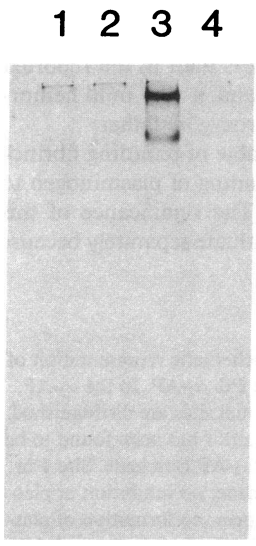

Figure 8. Demonstration of plasmin–α_2-AP complexes in plasma by SDS-PAGE and immunoblotting. Plasma samples were incubated with and without 1 μM plasmin for 45 min at 37°C. α_2-AP-related antigen was isolated by immunoadsorption, electrophoresed on a 10% polyacrylamide gel (Weber and Osborn system); and immunoblotted using goat anti-plasminogen IgG. Plasmin–α_2-AP complexes (and some dissociated plasmin) are only visible in lane *3*. Lane *1:* propositus' plasma plus plasmin; lane *2:* propositus' plasma; lane *3:* pooled normal plasma plus plasmin; lane *4:* pooled normal plasma. Numbers on the right represent molecular weight ($\times 10^3$).

The importance of α_2-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 α_2-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 α_2-AP, detected immunochemically, despite the low activity values. This indicates an abnormal α_2-AP molecule, and represents the first reported type II deficiency of α_2-AP. We designated the dysfunctional molecule as "α_2-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 α_2-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 α_2-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 α_2-AP molecule. Thirdly, unlike even normal NPB–α_2-AP, the purified α_2-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–α_2-AP for the α_2-AP Enschede.

The primary defect appears to be the complete inability of PB–α_2-AP Enschede to inhibit plasmin and trypsin. No inhibition of plasmin and trypsin activity on prolonged incubation (120 min) with purified PB–α_2-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

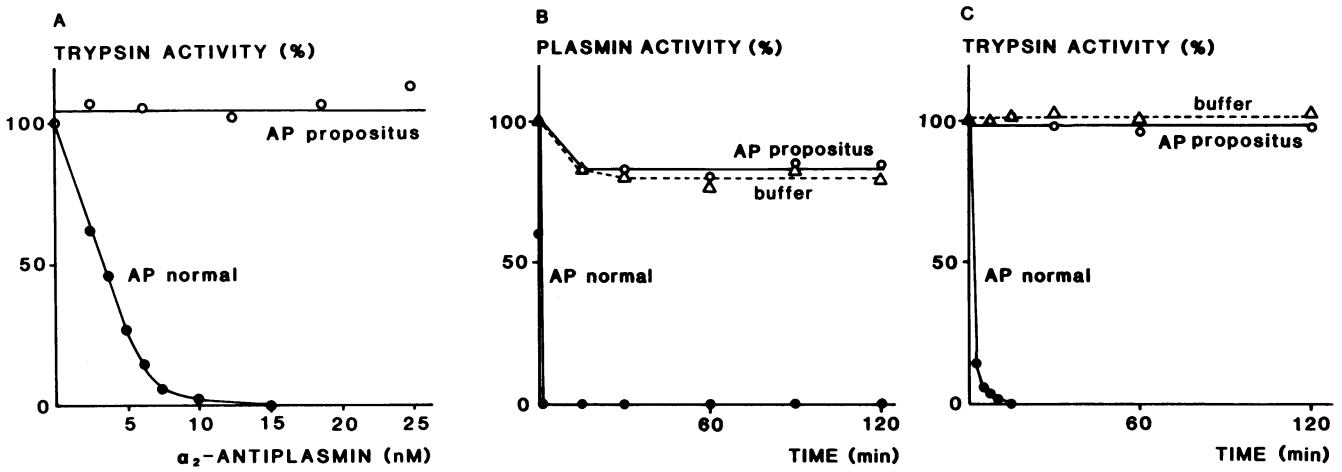

Figure 9. Inhibition of plasmin and trypsin by purified PB–α_2-AP. (*A*) Normal α_2-AP (AP normal) inhibits trypsin (1 mg/liter) dose dependently (equivalence at 6.8 nmol/liter final concentration, *abscissa*) in contrast to α_2-AP of the propositus (AP propositus). Enzyme and inhibitor were incubated for 10 min at 37°C. (*B* and *C*) Plasmin (11 nmol/liter) and trypsin (6.8 nmol/liter) were incubated with α_2-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*).

considered to be involved in binding to plasminogen and this property is shown to be retained in the α_2-AP Enschede (Figs. 5 and 6).

Other functions of the inactive PB–α_2-AP Enschede, such as PB and fibrin binding, are found qualitatively and quantitatively intact. The fibrin binding involves residues at the NH_2-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–α_2-AP Enschede are summarized in Fig. 11 and its legend.

Note that the dysfunctional α_2-AP, by virtue of its residual actions (fibrin and PB) in the fibrinolytic process, does not interfere with the action of the normal α_2-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 α_2-AP.

The residual interactions of the α_2-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 α_2-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

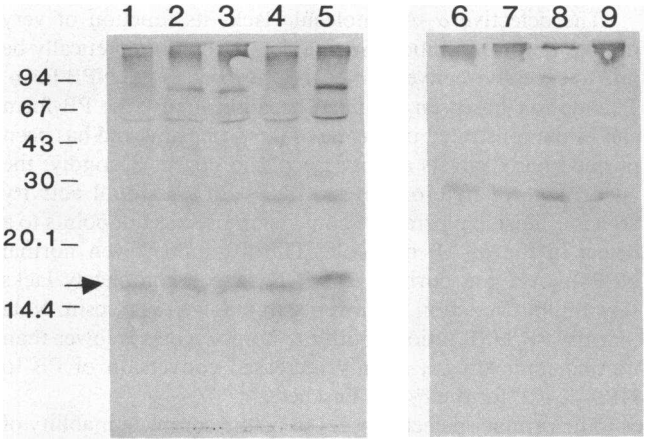

Figure 10. SDS-PAGE and immunoblotting of papain-digested plasma samples of the members of the family of the propositus. SDS-PAGE (15% gel) was carried out according to Laemmli. The blots were stained after incubation with goat anti-α_2-AP IgG (lanes *1–5*) or with normal goat serum (lanes *6–9*). See 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 ($\times 10^3$).

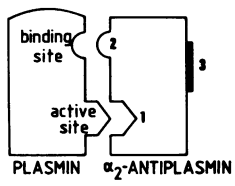

Figure 11. Schematic representation of plasmin and PB–α_2-AP. In the α_2-AP, three functional sites are distinguished, from which site 1 has been found to be defective in α_2-AP Enschede. Site 1 in α_2-AP Enschede: no inhibition of plasmin and trypsin, no formation of plasmin–antiplasmin complexes, no 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 α_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 α_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 alanine insertion near the active site region of α_2-AP Enschede.

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