α_2-Antiplasmin Enschede: Alanine Insertion and Abolition of Plasmin Inhibitory Activity

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An abnormal α_2-antiplasmin that is associated with a serious bleeding tendency has been found in a Dutch family and is referred to as α_2-antiplasmin Enschede. This abnormal α_2-antiplasmin is converted from an inhibitor of plasmin to a substrate. The molecular defect of α_2-antiplasmin Enschede, as revealed by sequencing of cloned genomic DNA fragments, consists of an alanine insertion near the active site region of the molecule. Substitution of this fragment into complementary DNA for a wild-type α_2-antiplasmin yields a translation product with physical and functional properties typical of the abnormal α_2-antiplasmin Enschede. The naturally occurring mutant may serve as a model for investigating the structures that determine the properties of an inhibitor versus those of a substrate in serine protease inhibitors.

HUMAN α_2-ANTIPLASMIN (α_2-AP), the primary physiological inhibitor of plasmin in plasma (*1*) belongs to the serpin superfamily of serine protease inhibitors (*2*), which share a significant degree of amino acid homology (*3*) and a common inhibitory mechanism (*4*). A model of the crystal structure of the serpin α_1-antitrypsin (α_1-AT), cleaved at its reactive site (a substrate-like region) (*5*), indicates that the reactive site sequence is contained in and exposed in a strained loop that could allow direct binding of an attacking serine protease. Enzyme inhibition is the consequence of an irreversible 1:1 stoichiometric complex formation by reaction of the inhibitors' reactive site P1-P'1 peptide bond (*4*) and the active site serine residue of the protease. A tetrahedral intermediate is formed and subsequently breaks down with the concomitant release of an amine from the COOH-terminal portion of the inhibitor. The remaining complex between the modified inhibitor and the protease is very stable, but the exact nature of the uniting forces is not fully understood. Certainly, other amino acids are involved in stabilizing the complex and directing inhibitor specificity. Sequence divergence within the serine protease contact area (P1 and surrounding residues) is a characteristic of probably all serine protease inhibitors, including members of the serpin superfamily (*3, 4, 6*). This variability is considered to be an evolutionary tool used to generate different inhibitory spectra. Recent amino acid sequencing of avian ovomucoid third domains has shown strongly hypervariable enzyme-inhibitor contact residues (*7*), thus bringing to question the belief that orthologous proteins of related species maintain a conservation of these functional positions. Another question that remains unresolved is, What makes a serine protease inhibitor an inhibitor rather than simply a substrate?

The rapid inhibition of plasmin by α_2-AP [the second-order rate constant $k_1 = 2 \times 10^7$ to $4 \times 10^7 M^{-1}\text{sec}^{-1}$] (*8*) is dependent on two functional areas of α_2-AP. The first is the reactive-site P1-P'1 peptide bond, which in α_2-AP is Arg-364–Met-365 (*2, 9*). Cleavage of this bond releases a peptide of relative molecular size (M_r) ~8000 (*10*). The second is a 26–amino acid COOH-terminal region (*11*) that allows reversible binding between α_2-AP and the lysine-binding sites (LBS) of plasmin or plasminogen. In normal plasma both plasminogen-binding and non-plasminogen-binding forms of α_2-AP exist (*12, 13*). Miniplasmin, a plasmin derivative lacking the LBS, but possessing a fully intact active site, is neutralized ~60 times more slowly by α_2-AP (*14*), demonstrating the relevance of the interaction with the LBS.

Congenital deficiencies of α_2-AP may result in bleeding disorders (*15*). An abnormal α_2-AP, referred to as α_2-AP Enschede, was found in two siblings living in Enschede, the Netherlands. These individuals have 3% of normal functional activity and 100% of normal antigen levels (*16*). Their apparently heterozygous parents have 50% functional activity and 100% antigen levels. Analysis of the variant protein showed that its ability to reversibly bind plasmin or plasminogen has not been affected. SDS–polyacrylamide gel electrophoresis (SDS-PAGE), however, showed no complex formation with plasmin but did show release of a small fragment. Analysis of papain digests revealed a fragment from α_2-AP Enschede migrating with a slightly different M_r (*17*) from the corresponding fragment of normal α_2-AP. It was suggested that α_2-AP Enschede has been converted from an inhibitor to a substrate of plasmin by a small deletion or point mutation (*17*).

Genomic DNA fragments from the Enschede family were cloned and their nucleotide sequences determined (*18*) to investigate the lesion responsible for the phenotype of α_2-AP Enschede and to gain a better understanding of what makes the normal molecule an inhibitor. Hybridization with an α_2-AP complementary DNA (cDNA) probe to genomic Enschede DNA revealed a restriction fragment length polymorphism (RFLP) within the DNA encoding the reactive site region (Fig. 1). Two alleles are evident in each confirmed heterozygous parent—one corresponding in size to the single slightly larger and more intense band in both children and one corresponding in size to the smaller 174-bp wild-type alleles of a normal individual.

Pst I–Hind III genomic DNA fragments, containing either the mutant or the wild-type sequence from each family member and the normal individual, were cloned and sequenced. Figure 2 presents the partial nucleotide and deduced amino acid sequence of both alleles from the father. One allele contains the normal sequence and the other reveals a GCG in-frame insertion. Identical results were obtained with DNA from the mother. All clones from both the son and daughter contain the GCG insertion. This rare type of mutation results in the insertion of an alanine somewhere between amino acid residues 353 and 357, seven to ten positions on the NH_2-terminal side of the P1 residue (Arg-364) in the reactive site of α_2-AP.

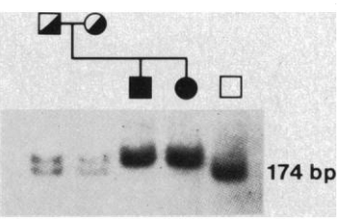

Fig. 1. Identification of a genetic lesion in α_2-AP Enschede. Demonstration of RFLP in Sst I–digested genomic DNA of Enschede family members by Southern analysis; (□) normal male control. Sst I–digested genomic DNA (20 μg) from each individual was separated on a 6% polyacrylamide gel, and electroblotted to a Zeta-probe membrane (Bio-Rad). Hybridization was with the Pst I–Hind III fragment of α_2-AP cDNA (nucleotides 1061 to 1393 containing the Sst I fragment and encoding amino acids 318 to 429, which encompass the reactive site) (*2*), nick-translated to a specific activity of 6×10^8 cpm/μg.

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Because the cloned 332- to 335-bp Pst I–Hind III fragments contain no untranslated DNA, the genomic structure of α_2-AP must be different from those of some other serpins. Indeed, both α_1-AT and rat angiotensinogen have an intron in this region (*19*), whereas chicken ovalbumin (*20*) does not. This raises the question of whether α_2-AP has followed the evolutionary course of α_1-AT and angiotensinogen but lost an intron or whether it has arisen from the ovalbumin side of the family.

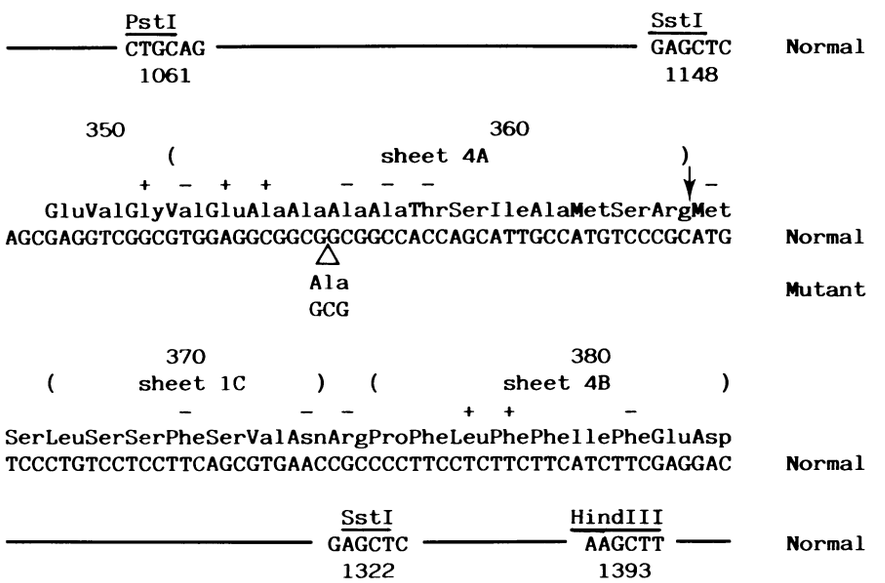

Fig. 2. Partial nucleotide and deduced amino acid sequence of normal and mutant alleles of α_2-AP. Nucleotides 1061 to 1393 [α_2-AP numbering system (*2*)] are shown. Amino acids are numbered above the sequence. The reactive site is indicated with an arrow. Secondary structural elements (β-pleated sheet strands) are indicated as determined by amino acid homology with α_1-antitrypsin cleaved at the reactive site (*3, 5*). Symbols above the sequence indicate varying degrees of amino acid sequence homology among 11 serpins; (+) eight or more homologous amino acids; (–) six or more homologous amino acids. Genomic DNA clones were obtained by digesting 40 μg of Pst I–Hind III DNAs from each individual and separating them on 5% polyacrylamide gels. This was followed by fractionation, electroelution of the ~320- to 370-bp fragments, ligation in pUC18 (*23*), transformation of *Escherichia coli* HB101, and screening of lysed colonies on nitrocellulose membranes by hybridization with the same nick-translated Pst I–Hind III fragment as was used for identification of the RFLP. Pst I–Hind III fragments of plasmid DNA from colonies indicating positive hybridization were subcloned in M13mp18 or M13mp19 (*23*) for dideoxynucleotide sequencing (*24*).

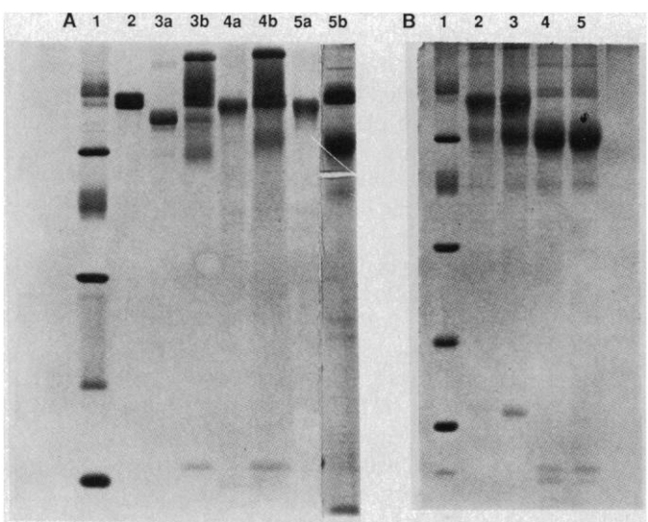

Fig. 3. SDS-PAGE of α_2-AP moieties. (**A**) SDS-PAGE (15% gel) under nonreducing conditions of α_2-AP moieties before (a) and after (b) reaction with plasmin. (Lane 1) Protein calibration mixture (M_r's 94,000, 67,000, 43,000, 30,000, 20,000, and 14,450); (lane 2) plasmin; (lane 3) nα_2-AP; (lane 4) rα_2-AP; and (lane 5) rα_2-AP-∇Ala. (**B**) SDS-PAGE (15% gel) under nonreducing conditions of rα_2-AP-∇Ala after reaction with plasmin for (lane 2) 0 minutes; (lane 3) 1 minute; (lane 4) 10 minutes; and (lane 5) 20 minutes. The protein standards (lane 1) were as in (A). nα_2-AP and rα_2-AP were purified as described (*21*). rα_2-AP-∇Ala was purified from the serum-free conditioned media of pPAAP-∇Ala-transfected CHO cells as described (*21*) with addition of aprotinin (10 kallikrein inhibitor units per milliliter) to the buffers used for chromatography on LBSI-Sepharose. Protein concentration was measured by enzyme-linked immunosorbent assay (ELISA) (*21*) or by amino acid analysis. (A) Plasmin (10 μ*M*) was incubated with 10 μ*M* nα_2-AP, rα_2-AP, or rα_2-AP-∇Ala for 1 minute at 37°C; the synthetic plasmin inhibitor D-Val-Phe-Lys-CH_2Cl was then added to attain a concentration of 2×10^{-4} *M* prior to gel loading. (B) Plasmin (0.5 μ*M*) was incubated with 10 μ*M* rα_2-AP-∇Ala for 0, 1, 10, and 20 minutes at 37°C, and D-Val-Phe-Lys-CH_2Cl was added prior to gel loading.

The question, whether the alanine insertion is indeed responsible for the inability of α_2-AP Enschede to inhibit plasmin, was examined as follows. A Pst I–Hind III fragment containing the insertion was exchanged with the normal sequence in α_2-AP within a eukaryotic expression plasmid, pPAAP, based on the SV40 early promoter (*21*), to yield a plasmid encoding the Ala insert (pPAAP-∇Ala). The plasmid containing the insert was then delivered to Chinese hamster ovary (CHO) cells by cotransfection with pDHFR5 (*22*), a plasmid containing the selectable marker dihydrofolate reductase, for secretion of recombinant α_2-AP-∇Ala (rα_2-AP-∇Ala). The recombinant α_2-AP-∇Ala was then purified from serum-free conditioned media and characterized.

SDS-PAGE shows a nearly homogeneous rα_2-AP-∇Ala migrating slightly more slowly than the plasminogen-binding form of natural α_2-AP (nα_2-AP) but in the same relative position as rα_2-AP (Fig. 3). Amino acid compositions of rα_2-AP-∇Ala, rα_2-AP, and nα_2-AP are indistinguishable. After preincubation with plasmin, both nα_2-AP and rα_2-AP form stable complexes with plasmin, as shown by SDS-PAGE, and complex formation is associated with the release of a peptide with a low M_r (Fig. 3). In these gels, this peptide migrates with an apparent $M_r >$ 14,000, but amino acid sequencing has shown that its true M_r is ~8000 (*10*). When treated in the same way, rα_2-AP-∇Ala shows no stable complex formation but does show release of a peptide with a slightly faster migration than that of nα_2-AP or rα_2-AP.

A time-course analysis of the reaction between plasmin and rα_2-AP-∇Ala (molar ratio 1:20) reveals the initial release of a peptide with a M_r similar to that of the peptide released from nα_2-AP and rα_2-AP when they are treated with equal molar amounts of plasmin; the peptide is then further degraded to two Coomassie blue-staining fragments (Fig. 3). These results suggest that the first cleavage may occur at the displaced reactive site Arg-365–Met-366 and that at least one other secondary plasmin cleavage site exists in the COOH-terminal peptide.

Crossed immunoelectrophoresis (CIE) (Fig. 4) shows that nα_2-AP in plasma and purified rα_2-AP-∇Ala migrate with similar mobility. After preincubation with plasmin (molar ratio of α_2-AP to plasmin, 2:1) (Fig. 4), purified rα_2-AP displays two peaks, the peak with slower mobility representing plasmin–α_2-AP complex. In this system, rα_2-

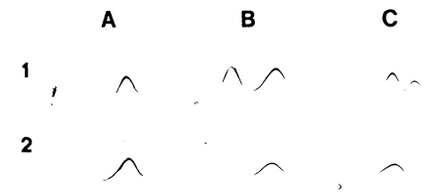

Fig. 4. Crossed immunoelectrophoresis of α_2-AP moieties without (**A** and **B**) or with (**C**) the addition of Lys-plasminogen in the first dimension. (A1) nα_2-AP in plasma; (A2) purified rα-AP-∇Ala; (B1) purified rα_2-AP preincubated with plasmin; (B2) purified rα_2-AP-∇Ala preincubated with plasmin; (C1) nα_2-AP in plasma; and (C2) purified rα_2-AP-∇Ala. Crossed immunoelectrophoresis with rabbit antiserum to α_2-AP with or without the addition of purified Lys-plasminogen (140 μg/ml) was performed as described (*13*). Preincubations of 10 μ*M* rα_2-AP-∇Ala with 5 μ*M* plasmin were for 1 minute at 37°C, after which $2 \times 10^{-4}M$ D-Val-Phe-Lys-CH_2Cl was added.

AP-∇Ala shows no complex formation, in agreement with the SDS-PAGE results. That rα_2-AP-∇Ala maintains the plasminogen-binding function associated with the 26 amino acids at the COOH-terminal of nα_2-AP (*11*) is shown by modified CIE (*13*), in which Lys-plasminogen, a partially degraded form of native Glu-plasminogen, is added to the gel matrix. In this case nα_2-AP in plasma reveals two peaks of plasminogen-binding (slower mobility) and non-plasminogen-binding (faster mobility) material, whereas nearly all of the rα_2-AP-∇Ala, which was purified with LBSI-Sepharose, is in the plasminogen-binding form, as evidenced by its retarded migration (Fig. 4). In CIE, the behavior of rα_2-AP-∇Ala is similar to that of nα_2-AP-∇Ala.

Although rα_2-AP-∇Ala reacts normally with the LBS of plasminogen, it does not inhibit plasmin irreversibly. Whereas under second-order conditions, plasmin is inhibited by nα_2-AP or rα_2-AP with a k_1 of 2×10^7 to $4 \times 10^7 M^{-1}$ sec^{-1} (*8*), no inhibition is observed with rα_2-AP-∇Ala. Even when plasmin is incubated with up to a 20-fold molar excess of rα_2-AP-∇Ala, no irreversible plasmin inhibition is observed.

In conclusion, a three–base pair (GCG) in-frame insertion within the structural gene of α_2-AP has been identified in two true heterozygotes and in their two homozygous children. This alanine insertion, in an area homologous to the 4A β-pleated sheet of cleaved α_1-AT (immediately adjacent to the reactive site), must have resulted in some structural perturbation that has abolished the plasmin inhibitory activity of α_2-AP Enschede and converted the protein to a plasmin substrate. To our knowledge, α_2-AP Enschede constitutes the only instance in which a mutation within the reactive-site region has inactivated a serpin, yet it has converted the inhibitor to a substrate. This naturally occurring mutant may serve as a model for further investigation of the structure-function relationships in α_2-AP, or in serpins in general, that determine the relative properties of an inhibitor as opposed to those of a substrate.

REFERENCES AND NOTES

1. D. Collen, *Eur. J. Biochem.* **69**, 209 (1976); M. Moroi and N. Aoki, *J. Biol. Chem.* **251**, 5956 (1976); S. Müllertz and I. Clemmensen, *Biochem. J.* **159**, 545 (1976).
2. W. E. Holmes, L. Nelles, H. R. Lijnen, D. Collen, *J. Biol. Chem.* **262**, 1659 (1987).
3. R. W. Carrell and D. R. Boswell, in *Proteinase Inhibitors*, vol. 12 of *Research Monographs in Cell and Tissue Physiology*, A. J. Barrett and G. Salvesen, Eds. (Elsevier, Amsterdam, 1986), pp. 404–420.
4. J. Travis and G. S. Salvesen, *Annu. Rev. Biochem.* **52**, 655 (1983).
5. H. Loebermann, R. Tokuoka, J. Deisenhofer, R. Huber, *J. Mol. Biol.* **177**, 531 (1984).
6. M. Laskowski, Jr., and I. Kato, *Annu. Rev. Biochem.* **49**, 593 (1980).
7. M. Laskowski, Jr., *et al.*, *Biochemistry* **26**, 202 (1987).
8. B. Wiman and D. Collen, *Eur. J. Biochem.* **84**, 573 (1978).
9. B. H. Shieh and J. Travis, *J. Biol. Chem.* **262**, 6055 (1987).
10. B. Wiman and D. Collen, *ibid.* **254**, 9291 (1979).
11. T. Sasaki, T. Morita, S. Iwanaga, *J. Biochem. (Tokyo)* **99**, 1699 (1986).
12. I. Clemmensen, S. Thorsen, S. Müllertz, L. C. Petersen, *Eur. J. Biochem.* **120**, 105 (1981).
13. C. Kluft and N. Los, *Thromb. Res.* **21**, 65 (1981).
14. B. Wiman, L. Boman, D. Collen, *Eur. J. Biochem.* **87**, 143 (1978).
15. K. Koie, T. Kamiga, K. Ogata, J. Takamatsu, *Lancet* **1978-II**, 1334 (1978); N. Aoki *et al.*, *J. Clin. Invest.* **63**, 877 (1979); L. A. Miles *et al.*, *Blood* **59**, 1246 (1982); A. Yoshioka *et al.*, *Haemostasis* **11**, 179 (1982); H. Stormorken, G. O. Gogstad, F. Brosstad, *Thromb. Res.* **31**, 647 (1983); L. Kordich, L. Feldman, P. Porterie, O. Lago, *ibid.* **40**, 645 (1985); P. Kettle and E. G. Maynee, *J. Clin. Pathol.* **38**, 428 (1985); C. Kluft, E. Vellenga, E. J. P. Brommer, G. Wijngaards, *Blood* **59**, 1169 (1982); E. A. R. Knot, J. W. ten Cate, R. J. Lamping, G. L. Kian, *Thromb. Haemostasis* **55**, 375 (1986).
16. H. K. Nieuwenhuis *et al.*, *Thromb. Haemostasis* **50**, 170 (abstr.) (1983); C. Kluft *et al.*, *J. Clin. Invest.*, in press.
17. D. C. Rijken *et al.*, *Fibrinolysis* (suppl. 1) (abstr. 55), 1986.
18. Blood from both parents and two children of the Enschede family was collected with informed consent by H.K.N. and peripheral blood leukocyte DNA was prepared in the laboratory of D.C.R. and C.K.
19. T. Tanaka, H. Ohkubo, S. Nakanishi, *J. Biol. Chem.* **259**, 8063 (1984).
20. M. Leicht *et al.*, *Nature (London)* **297**, 655 (1982).
21. W. E. Holmes *et al.*, *Biochemistry* **26**, 5133 (1987).
22. S. Subramani, R. Mulligan, P. Berg, *Mol. Cell. Biol.* **1**, 854 (1981).
23. C. Yannisch-Perron, J. Vieira, J. Messing, *Gene (Amsterdam)* **33**, 103 (1985).
24. F. Sanger, S. Nicklen, A. R. Coulson, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463 (1977).

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