Antithrombin III (AT) is a major plasma serine protease inhibitor and a member of the serpin family of proteins. We have characterized the molecular and genetic basis of AT Budapest, an inherited variant of AT that is associated with thrombotic disease in affected family members. A single amino acid substitution, 429Pro to Leu, was identified, occurring in a region of the molecule that is highly conserved in members of the serpin family. Two forms of variant protein were present in approximately equal amounts in the plasma of the propositus, who is homozygous for the mutation. One form, which had apparently normal $K_m$ bound heparin strongly and retained some residual thrombin inhibitory activity. The other form had only weak heparin affinity and no antiproteinase activity, and had slightly decreased mobility on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under nonreducing conditions; this normalized in the presence of a reducing agent, suggesting it was caused by a change in conformation. Additional support for a difference in conformation of the two forms of variant was provided by the finding that the fraction that bound heparin-Sepharose was recognized by a monoclonal antibody raised against normal AT, whereas the weak-affinity fraction was not.

AT Budapest was the first type 2a AT variant described. The propositus and several members of the kindred have had thrombembolic episodes, and examination of the family tree shows the parents of the propositus are consanguinous. In this report, we show that the AT Budapest allele, for which the propositus is homozygous, contains a single nucleotide substitution leading to the substitution of proline for codon 429. The presence of a proline at the equivalent position is highly conserved across the whole of the serpin family of proteins.

MATERIALS AND METHODS

Patients. The history and family tree have been described previously. Plasma was obtained from the propositus, and genomic DNA was extracted from blood leukocytes of the propositus, a sister with clinical and laboratory evidence of the abnormal AT, and the two children of the propositus, who have been clinically unaffected, but have abnormal laboratory AT assays.

Isolation of AT protein. Normal pooled plasma was prepared by centrifugation of citrated plasma from laboratory staff. Citrated plasma containing AT Budapest was prepared from the propositus, who (as shown) is homozygous for the abnormal AT. AT was isolated from normal plasma by dextran sulfate precipitation and stepwise elution from heparin-Sepharose (Pharmacia, Uppsala, Sweden). The plasma was applied in 0.1 mol/L Tris-HCl, pH 7.4, and normal AT eluted exclusively between 0.4 and 1.5 mol/L NaCl. To maximize the yield of the AT fractions from the propositus, plasma was applied to the heparin-Sepharose in 0.4 mol/L NaCl; under these conditions, one major AT pool (designated AT Budapest “variant”) was collected with the washthrough, while the second AT pool (designated AT Budapest “normal”) was collected after stepwise elution of the column with 1.5 mol/L NaCl. AT Budapest variant was further purified by chromatography on
immobilized polyclonal antibody to AT. The bound AT Budapest variant was eluted with MgCl₂ after prior washing with 0.5 mol/L NaCl, as described previously. The normal AT and both AT Budapest fractions were subjected to a final ion-exchange chromatography step before study. The AT preparations were applied to a Mono Q column (Pharmacia) in 0.02 mol/L triethylammonium, pH 7.3, and eluted with an NaCl gradient using a fast protein liquid chromatography (Pharmacia) delivery system.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. SDS-PAGE was performed under reducing conditions (with dithiothreitol) and nonreducing conditions, using the Phast System (Pharmacia) and 10% to 15% gradient gels. Immunoblotting was performed as described previously. The concentrations of all AT preparations were determined using a commercial radial immunodiffusion method employing a polyclonal antibody to normal AT (Hoechst, Hounslow, England).

Measurement of thrombin inhibitory activity. The reaction of AT with thrombin was studied at 37°C in the absence of heparin. Human thrombin (20 nmol/L) was incubated with varying concentrations of AT (as indicated in the figure legends) in 0.15 mol/L NaCl, 0.05 mol/L Tris-HCl, pH 7.4, containing 1% polyethylene glycol 6000. At different times, the incubation mixtures were subsampled into cuvettes containing the chromogenic substrate S2238 (KabiVitrum, Stockholm, Sweden) to determine residual thrombin. Relative velocities of substrate hydrolysis, V/V₀, were plotted against time in a semilog plot. The second-order rate constant was determined from a reciprocal plot of the pseudo first-order rate constant against AT concentration.

Monoclonal antibody (MoAb) affinity. The ability of three different murine MoAbs, ESAH 1, ESAH 5, and ESAH 6, raised against a covalent heparin-AT complex (J. Dawes, manuscript in preparation) to recognize AT Budapest normal and AT Budapest variant was tested using a radioimmunoassay with "%AT." The labeling procedure resulted in the retention of normal biological activities by the AT. Serial dilutions of AT Budapest normal and AT Budapest variant (50 μL) were incubated overnight at room temperature with 50 μL ¹²⁵I-labeled normal AT (10 ng/mL), 50 μL MoAb (0.125 to 10.0 μg/mL), and 50 μL 0.05 mol/L phosphate buffer, pH 7.5, containing 0.15 mol/L NaCl, 0.25% gelatin, and 1% Tween 20. The buffer used minimized nonspecific binding, which was measured in tubes containing only radiolabeled antigen and buffer. Antibody-antigen complexes were separated from unbound antigen using Sepharose 4B-coupled sheep anti-mouse immunoglobulin antisum.

DNA sequence analysis. The polymerase chain reaction was used to amplify each of the seven AT exons, with flanking intron sequences, as previously described. Amplified DNA fragments were purified from agarose gel by electroelution and then directly sequenced. Sequences were confirmed by analysis of both coding and noncoding strands, and the region containing the identified mutation was sequenced in at least three independently prepared templates.

Allele-specific oligonucleotide hybridization. Allele-specific oligonucleotide (ASO) probe hybridization was used to confirm the observed mutation and to assure that it was not a sequence polymorphism. Exon 6 of the AT gene from 35 randomly selected individuals from a variety of ethnic groups was enzymatically amplified, electrophoresed in 1% agarose, and blotted onto nitrocellulose membrane (Hybond C extra; Amersham, UK). The membrane was then sequentially hybridized to ³²P-end-labeled ASOs (normal allele 5'-GTAGCCAAACCCGTTGTGGTTAA-3', coding strand sequence; mutant allele 5'-TTAACCAGAGGTGGCC-TAC-3', noncoding strand sequence). Hybridization was performed at 40°C for 18 hours in 6 X SET (6 X SET = 1 mol/L NaCl, 6 mmol/L EDTA, 0.1 mol/L Tris-HCl, pH 7.5, 0.1% SDS, and 100 ng/mL sheared salmon sperm DNA. The membrane was washed in 6 X SSC (20 X SSC = 3 mol/L NaCl, 0.3 mol/L Na citrate); the stringent wash for the mutant probe was 5 minutes at 50°C, and for the wild-type probe 5 minutes at 55°C. Autoradiographs were exposed overnight at room temperature.

RESULTS

AT heparin affinity. Preliminary experiments indicated that AT in the plasma of the propositus was totally retained on heparin-Sepharose when the column was equilibrated in 0.1 mol/L Tris-HCl, pH 7.4. By application of a NaCl gradient to the column, we confirmed the prior observation that the AT of the propositus consisted of two fractions of approximately equal quantity with different heparin binding affinities. To maximize yield, the two fractions were separated by applying the plasma to the heparin-Sepharose column in 0.4 mol/L NaCl. One fraction, AT Budapest variant, demonstrated reduced heparin affinity and eluted in the washthrough, while the other antithrombin component, AT Budapest normal, eluted at the position of normal AT (0.8 to 1.0 mol/L NaCl).

SDS-PAGE mobility. Normal AT migrated as a single band on SDS-PAGE, under reducing and nonreducing conditions, with a Mr, approximately 60,000. AT Budapest normal had seemingly identical mobility to that of normal AT both under reducing and nonreducing conditions (Fig 1). However, AT Budapest variant had slightly decreased mobility under nonreducing conditions, which normalized on reduction. All of these AT bands reacted with polyclonal antibodies to AT on immunoblotting, in parallel experiments conducted on separate gels.

Reactivity of AT with MoAbs. AT Budapest normal and AT Budapest variant differed in their interaction with MoAbs. Binding of all three MoAbs, ESAH 1, ESAH 5, and ESAH 6, to normal ¹²⁵I-AT was competitively inhibited by AT Budapest normal, although inhibition was incomplete (20% for ESAH 1, 40% with ESAH 5 and 50% with ESAH 6) at a concentration at which native AT inhibited by greater than 90%. These quantitative differences probably reflect reduced affinities of the MoAbs for the variant protein. While normal ¹²⁵I-AT binding to ESAH 5 and ESAH 6 was quantitatively inhibited by AT Budapest variant and AT Budapest normal to a similar extent, ESAH 1 apparently failed to recognize AT Budapest variant (Fig 2).

Thrombin inhibition studies. Increasing concentrations of normal AT progressively inactivated thrombin (Fig 3A). AT Budapest normal was also able to inactivate thrombin (Fig 3B); however, derivation of the second-order rate constants from the replotted results of Fig 3 indicated that the rate constant for AT Budapest normal was 36% of that of normal AT. AT Budapest variant was completely unable to inactivate thrombin (Fig 3C).

AT Budapest gene sequence. The sequences of each of the seven exons, with flanking intron regions, of the AT genes of the propositus were determined and the mutation was identified as the substitution of a T for C in the second position of codon 429 (Fig 4). This mutation predicts the presence of leucine (CTT) instead of the normal proline
AT BUDAPEST

non reduced

reduced

N BN BV

N BN BV

(CCT) at this position. The propositus was homozygous for this allele, while sequence analysis of amplified exon 6 DNA from his sister and his two children showed each of them to be heterozygous for the substitution (Fig 5). ASO probe hybridization studies confirmed that the propositus was homozygous for the 429 CCT to CTT mutation, and that his three relatives tested were each heterozygous. Additionally, screening 70 alleles from randomly selected individuals did not identify further instances of the substitution. The propositus was also homozygous for each of the three described DNA polymorphisms within the AT locus.16-20 We have identified another (unpublished) sequence polymorphism within exon 4 of the AT gene, codon 295 GTG/GTA, which does not alter the normally encoded valine, and the propositus was also homozygous for this polymorphism. In addition, when compared with the most complete published AT DNA sequence,21 derived from cloned AT genes, four intronic sequence variations were identified. Using the base numbering of Bock et al,21 these were (1) intron 2 position 549, A to G; (2) intron 2 position 561, C deletion; (3) intron 4 position 62, C insertion; and (4) intron 5 position 194, C insertion. The patient was homozygous for all of these changes, and we have observed these sequences in all other AT alleles we have sequenced, suggesting they represent cloning-induced sequence artifacts in the original report.

Fig 1. SDS-PAGE of AT Budapest. Electrophoresis was performed on a 10% to 15% gel, under nonreducing and reducing conditions. N, normal; BN, AT Budapest normal; BV, AT Budapest variant.

DISCUSSION

AT Budapest was the first variant AT to be recognized and is associated with significant thromboembolic episodes in affected members of the kindred.2,17 Previous family studies have shown that the propositus and affected relatives have normal immunological AT levels, but that both heparin affinity and serine protease inhibitory activity were impaired.1,8,17,22 Furthermore, the AT functional abnormalities were more pronounced in the propositus than his relatives, findings reflected at the phenotypic level where he has suffered more frequent clinical thromboembolic episodes than other affected family members.

We have further investigated the properties of the AT isolated from the plasma of the propositus and have identified the mutation in the AT Budapest allele. A single-base substitution was found in codon 429, CCT to CTT, which leads to the replacement of the normal proline by leucine. Direct DNA sequence analysis and ASO hybridization showed that the propositus was homozygous, while his sister and his two children were heterozygous for the mutation. Presence of two copies of the mutant allele in the propositus is consistent with the consanguinous marriage of his parents and would also explain the more pronounced abnormal AT functional studies. It is likely that the mutation is of a single origin, since the propositus is homozygous for each of the four DNA sequence polymorphisms.

Two forms of the variant AT protein, of approximately equal concentration, were identified within the plasma of the propositus, confirming previous observations.17,22 The two AT fractions demonstrated different affinities for

ATIII Budapest (ug/ml)

Fig 2. Inhibition of binding of 125I-AT to MoAb ESAH 1 by AT Budapest normal (○) and AT Budapest variant (●). Incubations were performed as described in Materials and Methods, using ESAH 1 at 5 μg/mL.
Fig 3. Inactivation of thrombin (20 nmol/L) by AT, at the concentrations indicated. Thrombin and AT were incubated in 0.15 mol/L NaCl, 0.05 mol/L Tris-HCl, pH 7.4, containing 1% polyethylene glycol. Aliquots were subsampled into cuvettes containing the chromogenic substrate S2238, to determine residual thrombin activity. (A) normal AT. (B) AT Budapest normal, (C) AT Budapest variant.
heparin-Sepharose; one fraction, which we called AT Budapest variant, had low heparin affinity, while the other component, AT Budapest normal, had heparin affinity similar to normal AT. This property allowed the isolation and further purification of the two components for further comparison with normal AT. Whereas the AT Budapest normal component demonstrated a reduced thrombin inhibitory function, the AT Budapest variant fraction was found to have no thrombin inhibitory activity when measured at a range of concentrations.

The basis for the impaired functional activity of the two AT Budapest protein fractions was not identified in the current study and remains a matter for speculation. However, the evidence suggests that the substitution of 429Pro to Leu is associated with a conformational change in the protein. Proline in position 429 of AT is highly conserved in the equivalent position of the other serpins, whether or not they have heparin-binding or protease inhibitory actions (Table 1). Although the crystal structure of AT has not been solved, the tertiary structure has been modeled on α1-antitrypsin, another serpin with which AT displays significant sequence homology. On this basis, 429Pro of AT (equivalent to 391Pro of α1-antitrypsin) is predicted to lie within a hydrophobic pocket along with two other highly conserved residues, 239Phe (208Phe of α1-antitrypsin) and 408Phe (370Phe of α1-antitrypsin). The highly conserved nature of this group of residues suggests that they may play an important role in maintaining the tertiary structure of the serpin group of proteins. The substitution of 429Pro by leucine in AT Budapest may disrupt the integrity of this association and it is likely that similar mutations in other serpins would also alter their functional activities.

Altered folding of the mutant AT is supported by the reduced affinities of the MoAbs observed in this study. In addition, the failure of the MoAb ESAH1 to recognize the Budapest variant fraction, while maintaining some affinity for AT Budapest normal, is consistent with a further conformational difference between the two protein fractions. A more extensive change in the folding of the variant component, as compared with Budapest normal, would explain the more severe disturbance in functional activity, with a loss of antiprotease action and a reduction in heparin affinity. This proposition is also supported by the observed behavior of the variant component on SDS-PAGE.

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Fig 4. Autoradiograph of the noncoding strand sequencing gel of amplified exon 6 from a normal subject and the AT Budapest propositus. The complementary coding strand sequence and deduced amino acids are also shown. The propositus is homozygous for the replacement (arrowed) of the normal C in the second position of codon 429 by T.

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Fig 5. Noncoding strand sequencing gel of amplified AT exon 6 from the sister of the propositus. The deduced sequence of the coding strand is shown on the left. The sequence is heterozygous for C (normal) and T (mutant) in the second position of codon 429. The two children of the propositus showed sequences identical to the sister.
ANTITHROMBIN III BUDAPEST

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Consensus: F G R V N P

Alignment of carboxy terminal amino acids of serpins' numbered according to AT.

Abbreviations: HCII, hepafin cofactor II; PCI, protein C inhibitor; PNI, protease nexin; α1AT, α1-antitrypsin; α2ACT, α2-antichymotrypsin; C1inh, C1 inhibitor; α2AP, α2-antiplasmin; pPAI, placental plasminogen activator inhibitor; pPAI, placental plasminogen activator inhibitor; AGTH, angiotensinogen; TBG, thyroxine-binding globulin; Ctpsn, contrapsin; Oval, ovalbumin; BPZ, barley protein Z; CPV38, cowpox virus 38-Kd protein; Gene Y, chicken ovalbumin-related gene Y protein.

The AT Budapest protein identified in the proposal can adopt two different conformations of the same primary sequence is unknown. We suggest that one form of protein, represented by AT Budapest normal, displays only localized disturbance of the structure manifested by some decrease of thrombin inhibitory activity. The other form of protein, represented by AT Budapest variant, has a more global conformational change, abolishing thrombin inhibited, decreasing heparin affinity, and resulting in the loss of the recognition site for the MoAb ESAH 1. Further work will be required to resolve the basis for these differences.

ACKNOWLEDGMENT

Professor D.J. Weatherall is thanked for his support.

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Antithrombin III Budapest: a single amino acid substitution (429Pro to Leu) in a region highly conserved in the serpin family

RJ Olds, DA Lane, R Caso, M Panico, HR Morris, G Sas, J Dawes and SL Thein