Volume 300, number 3, 241-246 FEBS 10890 April 1992

Antithrombin Budapest 3

An antithrombin variant with reduced heparin affinity resulting from the substitution L99F

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Received 11 February 1992

The molecular basis and functional properties of a variant antithrombin (AT) protein, AT Budapest 3, were studied. A single base substitution was identified in codon 99, CTC→TTC, altering the normal leucine to phenylalanine. The proband presented with a history of venous thrombotic disease and was found to be homozygous for the mutation. The variant protein demonstrated reduced heparin affinity and reduced antiproteinase activity in the presence of either unfractionated heparin or the AT-binding heparin pentasaccharide, when compared to normal AT. A small change in the isoelectric point was also identified. The substituted amino acid residue of AT Budapest 3 is located near to the proposed AT heparin binding site, and it is suggested that reduced heparin affinity of the variant protein may result from substitution-induced distortion of positive charge geometry in the binding site and/or changes in its position relative to the rest of the inhibitor molecule.

Antithrombin; Heparin binding; Homozygous mutation; Hereditary thrombosis

1. INTRODUCTION

AT is the main physiological inhibitor of the activated serine proteinases of the coagulation system. Formation of complexes between thrombin and AT is accelerated at least two-thousand-fold in the presence of heparin and a similar enhancement is seen for inhibition of factors Xa and IXa (reviewed in [1]). There is evidence that in vivo heparin sulphate, located on the endothelial cell surface, fulfills the same role as extrinsic heparin [2]. The mechanism by which heparin accelerates the antiproteinase activity of AT is broadly understood but several aspects remain to be clarified. In this respect the identification of the heparin binding domain of the AT protein is of crucial importance. One approach is the study of naturally occurring variants with impaired ability to interact with this glycosaminoglycan. We report here the analysis of AT Budapest 3 and show that the reduced heparin affinity of the variant is associated with an amino acid substitution at position 99.

2. MATERIALS AND METHODS

2.1. *Case history*

We studied the presenting individual from a previously identified kindred suspected of having an AT variant with impaired heparin affinity, AT Budapest 3 [3]. The proband is the only family member to have had clinical thrombotic disease. Heparin cofactor and immunological AT assays and crossed immunoelectrophoresis performed in the presence of heparin demonstrated an abnormal AT protein with impaired heparin binding in the plasma of the presenting female, with no trace of normal AT. Both her parents, her two sisters and maternal grandmother were found to have the abnormal AT in addition to a normal component.

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2.2. *AT protein analysis*

Blood was collected into citrate anticoagulant from the proband, the plasma separated from the cells and stored at −70°C. Control normal plasma was obtained from healthy volunteers. AT antigen levels were assayed using Behring NorPartigen plates. The affinity of plasma AT for heparin was measured by elution from a heparin-Sepharose (Pharmacia) column with a NaCl gradient as described [4]. The ability of unfractionated heparin and the specific AT-binding pentasaccharide to accelerate the antiproteinase activity of the variant was studied using plasma from the proband and normal individuals in a heparin cofactor assay, based upon the anti-factor Xa (Coatest, KabiVitrum) assay. For this, the conditions were essentially identical to those suggested by the manufacturer, except the concentration of added heparin (Leo Laboratories, Princes Risborough, UK) and pentasaccharide (a kind gift from Dr. J. Choay, Sanofi/Choay, Paris) was varied, as indicated.

SDS-PAGE was carried out using the Phast system (Pharmacia). Isoelectric focusing also was carried out on the Phast system, using premade 4–6.5 IEF gels. For this, plasma was subject to focusing and the protein was transferred (Phast Transfer, Pharmacia) onto Immoblin membranes, incubated with anti-AT antibody (Dakopatts, High Wycombe, UK) and then peroxidase coupled swine anti-rabbit IgG (Dakopatts, High Wycombe).

2.3. *AT gene sequence analysis*

DNA was extracted from blood leucocytes by standard methods. The coding regions and flanking intron sequences of the AT gene were amplified by the polymerase chain reaction (PCR) and directly se-

quenced using oligonucleotide primers and conditions as reported previously [5,6]. For several segments of the gene, biotinylated oligonucleotide primers were used in the amplification reaction, allowing subsequent preparation of single DNA strands by the Dynabead system (Dynal UK Ltd.) for sequencing [7].

AT gene sequence polymorphisms were identified by DNA sequencing or by examination of the amplified DNA fragments on agarose gels in the case of the length polymorphism in the 5'-region of the gene [8].

AT alleles from a normal population were examined for the presence of the single base substitution identified in the AT Budapest 3 allele by allele-specific priming of the PCR [5,9]. An oligonucleotide primer complementary to the noncoding mutant DNA strand (5'-ACCTCCATCAGTTGCTCGAA-3'; mutation underlined) was used in conjunction with an upstream primer (5'-GTTGCAGCC-TAGCTTAACTTGGCA-3'); under stringent conditions specific amplification of a 409 bp DNA fragment was observed only when the mutation was present. Efficiency of amplification was monitored by an independent set of primers specific for an independent (δ globin) gene segment [5]. The PCR was performed in a 25 μl volume composed of 100–200 ng DNA, 5 pmol of each primer, 2.5 mM $MgCl_2$, 50 mM KCl, 100 mM Tris-HCl, pH 8.3, 100 μM of each dNTP and 0.5 U *Taq* polymerase. Thermal cycling conditions were 27 cycles of 94°C for 1 min, 58°C for 1 min and 72°C for 2 min, with the last extension phase prolonged to 10 min.

3. RESULTS

AT assays showed a reduction in both heparin cofactor activity (Fig. 1) and AT immunological quantitation (77%; normal 80–120%). A reduction in heparin affinity was observed by heparin-Sepharose chromatography. Whereas normal AT from control plasma eluted from the column at 0.88 M NaCl, AT from the plasma of the proband was recovered at 0.48 M NaCl, as a single peak, consistent with the presence of AT protein with reduced heparin affinity in her plasma and no normal AT component. Both unfractionated heparin and the AT-binding pentasaccharide were found to have diminished ability to accelerate the antiproteinase activity of the Budapest 3 AT, when compared to normal AT, in a plasma based assay (Fig. 1). At high concentrations of the pentasaccharide, however, maximal antiproteinase activity could be induced in the Budapest 3 AT, consistent with a mutation that alters the heparin binding domain, but not the reactive site of the inhibitor. AT Budapest 3 purified by heparin-Sepharose chromatography showed equal mobility to normal AT on SDS-PAGE under nonreducing conditions (Fig. 2).

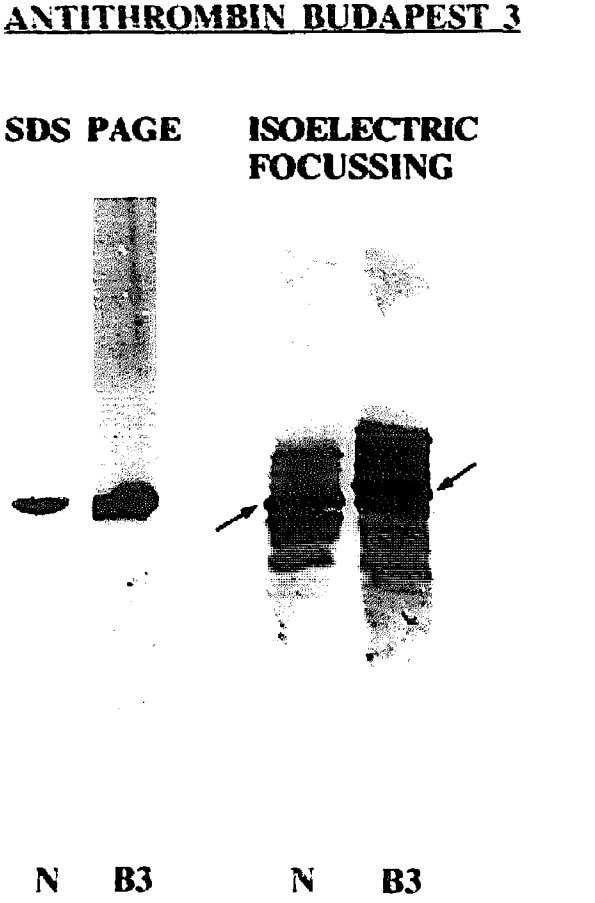

Fig. 2. Nonreducing SDS polyacrylamide gel and isoelectric focusing gel, each with normal (N) AT in left lane and AT Budapest 3 (B3) in the right lane. The major normal AT band (arrowed) migrates with pI 5.50, while that of AT Budapest 3 (arrowed) migrates with pI 5.75.

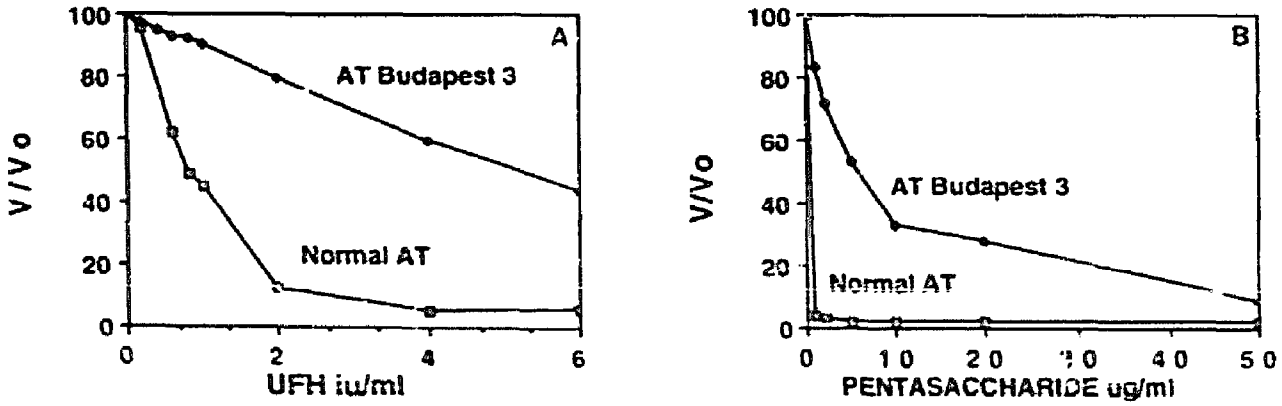

Fig. 1. Inhibition of factor Xa by either normal plasma or plasma from the proband with AT Budapest 3, in the presence of either (A) unfractionated heparin (UFH) or (B) the specific AT-binding pentasaccharide.

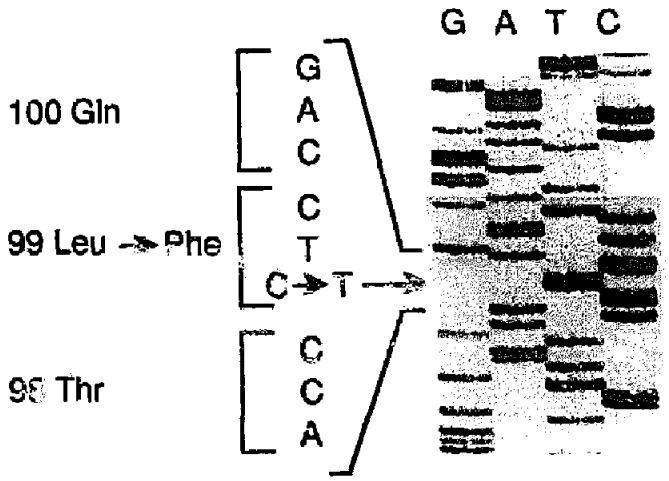

Fig. 3. DNA sequence analysis of the AT genes of the proband in the region of codon 99. The sequencing template is amplified DNA so both alleles are sequenced together. A single T (arrowed) is seen in the first position of codon 99, instead of the normal C, indicating a homozygous C→T substitution.

Isoelectric focusing revealed a shift in the isoelectric point of the major component of the variant AT (Fig. 2) from 5.50 to 5.75.

DNA sequence analysis revealed a single mutation, the substitution of T for C in the first position of codon 99 (Fig. 3) in both AT alleles of the proband. This predicts the replacement of the normal leucine by phenylalanine. The proband is clearly homozygous for the mutation, compatible with the inheritance of a mutant allele from each of her parents, who as protein phenotypic data had already suggested were both heterozygous for a heparin binding AT variant. The total coding region of the AT alleles of the proband and the flanking intron segments were sequenced and no further mutations were observed. Furthermore, the proband was homozygous for the previously described AT gene sequence polymorphisms; the Budapest 3 allele was associated with the short length polymorphism 5′ to the gene [8], the absence of the *Pst*I cutting site within exon 4 [10], the GTA sequence within codon 295 [11] and the presence of the *Dde*I cutting site at position 60 within intron 5 [12]. The C→T substitution within codon 99 was not found in 142 AT alleles from normal unrelated individuals, screened by allele-specific priming of the PCR, confirming that the change was not a sequence polymorphism but unique to AT Budapest 3.

4. DISCUSSION

We have identified the mutation L99F (CTC→TTC) as the basis of the AT variant Budapest 3. The proband from the Budapest 3 kindred is homozygous for the mutant allele and has suffered recurrent venous thromboembolic disease. Since the proband was also homozygous for sequence polymorphisms within the AT gene, it is likely that the mutant AT alleles are of a single origin. This would be consistent with previous laboratory evidence that both parents were heterozygous for this AT variant and would suggest consanguinity within the family. The absence of clinical thrombotic disease in the parents and other family members who appear to be heterozygous on laboratory testing [3] is consistent with the previous demonstration that thrombosis in individuals heterozygous for heparin binding variants is very uncommon [13].

The proband represents the fifth reported individual homozygous for an AT variant whose primary abnormality is decreased heparin affinity. It is apparent that homozygosity for L99F has a milder phenotype both in terms of laboratory assays and clinical disease. In three of the other four cases the mutation was identified as R47C [14–16] while the molecular basis of AT Fontainebleau [17] has not been reported. In contrast to AT Budapest 3, the other four cases all had minimal affinity for heparin and this was reflected by the absence of heparin cofactor activity. Whereas the quantity of AT Budapest 3 in the plasma of the proband was just below the lower limit of the normal range, plasma AT in the other homozygous individuals was actually raised. Clinically the proband studied here has had only venous thrombotic disease, while all four of the other patients had episodes, sometimes recurrent, of arterial thrombosis. It is tempting to suggest that the difference in clinical course is a reflection of the severity of impairment of anticoagulant activity of the variant antithrombins.

Table I

Residues implicated in AT heparin binding

AT residue	Substitution or modification	Equivalent residue of α_1-antitrypsin*	Reference
I7	17N, AT Rouen III	none	[23]
R24	R24C, AT Rouen IV	none	[24]
P41	P41L, AT Basel	H16	[25]
R47	R47C, AT Toyama	T22	[15]
	K47T, Rouen I		[26]
	R47S, AT Rouen II		[19]
W49	HNB modification	N24	[27]
L99	AT Budapest 3	H73	this report
K107	S-DABITC modification	N81	[28]
K114	TNBS modification	I87	[29]
K125	TNBS modification	E98	[29]
	S-DABITC modification		[28]
	P5P modification		[30]
R129	R129Q, AT Geneva	T102	[31]
	HPG modification		[32]
N135	AT-β	S108	[33]
K136	S-DABITC modification	Q109	[28]
R145	HPG modification	G117	[32]

*see Fig. 4a alignment. HNB, hydroxynitrobenzyl; HPG (*p*-hydroxyphenyl)glyoxal; P5P, pyridoxal 5′-phosphate; S-DABITC, 4-*N*,*N*-dimethylaminoazobenzene-4′-isothiocyano-2′-sulfonic acid; TNBS, trinitrobenzene sulfonic acid.

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       1         10        20        30        40
AT     HGSPVDICTAKPRDIPMNPMCIYRSPEKKATEDEGSEQKIPE

A1AT                            EDPQGDAAQKTDTSHHD
                                1        10

            50        60        70        80
AT     ATNRRVWELSKANSRFATTFYQHLADSKNDNDNIFLSPLSIS
         [          hA              ]    s6B [  hB
A1AT   QDHPTFNKITPNLAEFAFSLYRQLAHQSNS-TNIFFSPVSIA
        20        30        40        50

          90        100       110       120
AT     TAFAMTKLGACNDTLQQLMEVFKFDTISEKTSDQIHFFFAKL
                ][    hC     ] [  hC1 ][        hD
A1AT   TAFAMLSLGTKADTHDEILEGLNFN-LTEIPEAQIHEGFQEL
       60        70        80        90

        130       140       150       160
AT     NCRLYRKANKSSKLVSANRLFGDKSLTFNETYQDISELVYGK
             ]  [     s2A      ]    [    hE     ]
A1AT   LRTLNQPDSQ-LQLTTDGGLFLSEGLKLVDKFLEDVKKLYHS
       100       110       120       130
(A)
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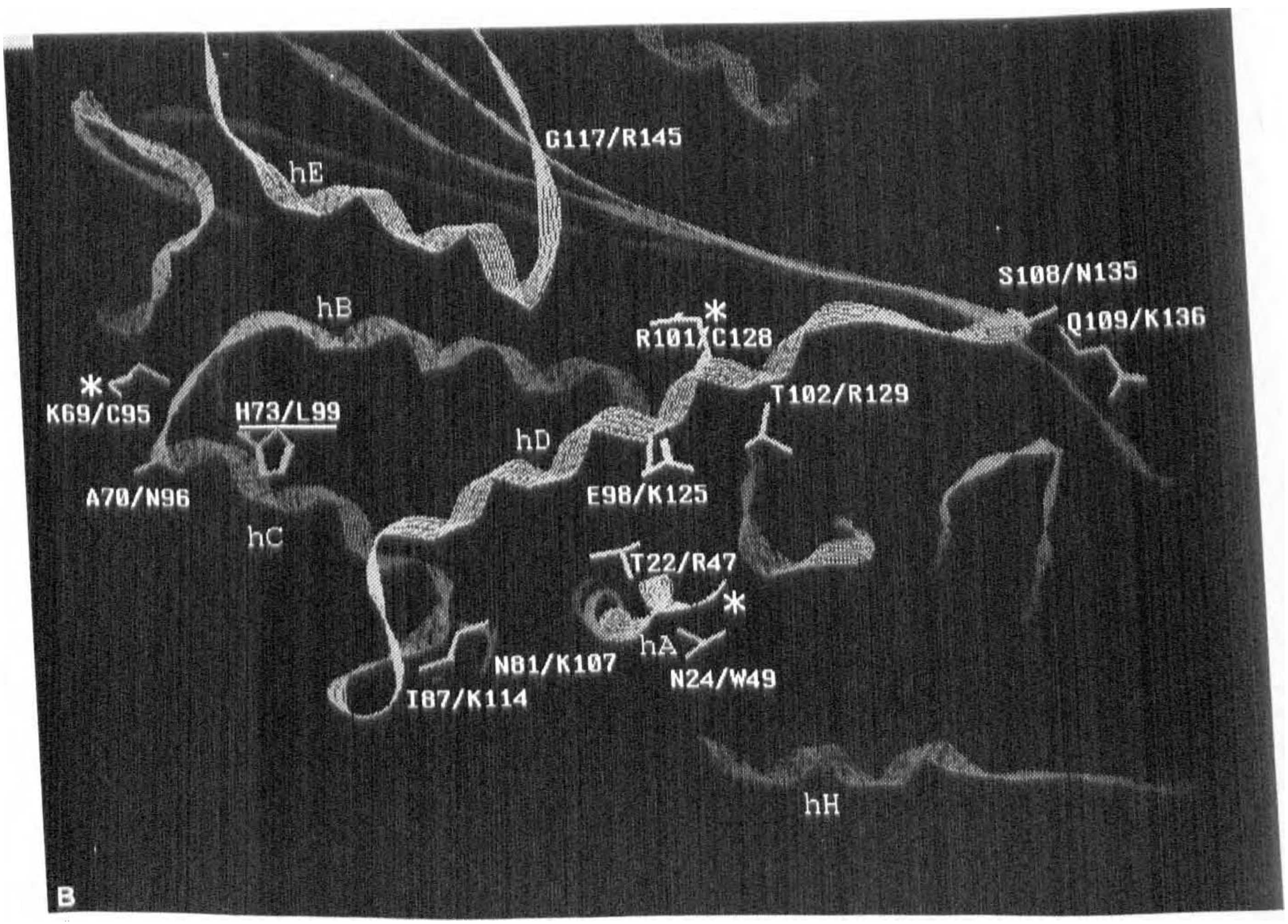

The AT Budapest 3 variant demonstrates reduced heparin affinity by heparin-Sepharose chromatography, and a diminished rate of antiproteinase activity compared to normal AT, in the presence of either unfractionated heparin or the AT-binding pentasaccharide. Reduced heparin affinity and heparin cofactor activity are consistent with the identification of a L99F substitution and current concepts of the AT heparin binding domain. The AT residues listed in Table I appear to participate directly in heparin binding or lie in close proximity to the heparin binding site. These residues were identified by studies of antithrombin genetic variants and the glycosylation isoform, AT-β, and through chemical modification experiments (references in Table I). Crystallographic studies of cleaved AT [18] and modelling studies using the structurally homologous serpin α_1-antitrypsin [19,20] show that the residues implicated in AT heparin binding map to two spatially contiguous elements on the surface of the inhibitor molecule. These are: (i) the amino-terminal polypeptide of AT, which is not present in other members of the serpin family, and (ii) the helix D region, which contains a high density of positively charged amino acids in AT relative to other serpins. Basic residues of the AT heparin binding site are believed to interact with negatively charged sulphate groups on heparin.

Figure 4 shows the location of the AT Budapest 3 substitution in a model of the AT heparin binding site based in the crystal structure of the prototypical serpin α_1-antitrypsin which shares a common three-dimensional template with AT [18,20]. The illustration in Fig. 4b was generated by aligning the sequences of AT and α_1-antistrypsin (Fig. 4a), and marking the positions of amino acids corresponding to AT residues implicated in heparin binding (Table I) onto the structure for postcomplex (cleaved) α_1-antitrypsin [21]. In this model, the heparin binding domain is present as a region of high positive charge density, composed of basic residues from the A and D helices and probably also the amino terminal polypeptide. A model of the AT heparin binding site based on the structure of native ovalbumin [22] leads to the same conclusion (data not shown).

L99, the substituted residue of AT Budapest 3, is located in helix C, which lies underneath the heparin binding domain. In this location, the L99F substitution could perturb the geometry of the positively charged surface and/or alter its position relative to the rest of the inhibitor molecule. This could occur in several ways. For example, L99 is located near to N96, a glycosylation site of AT; alteration in the degree of glycosylation at this site could affect heparin interaction with its binding site on AT through steric blockage or increased exposure of charged amino acids. Alternatively, the L99F substitution may displace the N-terminal element of the heparin binding domain, which is fixed to helix C by a disulphide bond between the nearby C95 and C21. Finally, substitution of L99 by a bulkier phenylalanine residue may distort interaction between helix C and the amino terminal end of helix D above it. Distortions produced through any of these mechanisms could alter the relative geometry of positive charges in the binding site so that heparin is bound less efficiently, and might additionally interfere with protein conformational changes proposed as the basis for AT heparin cofactor activity.

Fig. 4. (A) Partial sequences of human AT and human α_1-antitrypsin, aligned as in Huber and Carrell [20]. Residues implicated in AT heparin binding are boxed (see Table I). (−) indicates gaps in the aligned sequences. Invariant and highly conserved serpin residues are underlined. (^) marks residues located in alpha helices and (*) marks residues in β-sheets of cleaved α_1-antitrypsin [21]. hX designates helix X; sXY, strand X in sheet Y. (B) Location of the AT Budapest 3 mutation in a model of the AT heparin binding site based on the structure of postcomplex α_1-antitrypsin [21]. Ribbon diagram shows the helix D-containing surface of Brookhaven Protein Structure Database entry 5API onto which the side chains for α_1-antitrypsin residues equivalent to those implicated in AT heparin binding (see Table I and Fig. 4a alignment) have been drawn. Notation aaX/aaY indicates that amino acid X of α_1-antitrypsin corresponds to amino acid Y of AT. Location of the non-homologous N-terminal polypeptide of AT can be inferred from the disulphide bond pattern (C8-C128, C21-C95) to extend from the asterisk marking the position of AT C128, through that marking C95, to that marking the amino terminal of helix A.

Acknowledgements: The support of the Wellcome Trust, USPHS Grant HL30712 and AHA Established Investigatorship 88-1202 is acknowledged. We thank Professor D.J. Weatherall for encouragement and support.

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