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New carbohydrate site in mutant antithrombin (7 Ile→Asn) with decreased heparin affinity

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A mutant antithrombin was isolated from the plasma of a patient with pulmonary embolism. The new protein, which accounted for 55% of the antithrombin, had decreased heparin affinity and contained two components when analysed on the basis of either charge or molecular mass. Sialidase and endo-β-*N*-acetylglucosaminidase F treatment suggested that this heterogeneity was due to a partial glycosylation occurring at a new carbohydrate attachment sequence. Peptide mapping by reverse-phase HPLC showed that the abnormality involved the N-terminal tryptic peptide. Sequence analysis demonstrated that the underlying mutation was 7 Ile→Asn which introduces a new Asn-Cys-Thr glycosylation sequence. This new oligosaccharide attachment site occupies the base of the proposed heparin-binding site, and the finding explains the consequent decrease in heparin affinity.

Antithrombin mutant; Thromboembolism; Carbohydrate attachment; Heparin binding

1. INTRODUCTION

Antithrombin is the principal protease inhibitor of the coagulation cascade and requires activation by the sulphated oligosaccharide heparin [1]. The major circulatory form of the protein has a molecular mass of 58 kDa and contains four asparagine-linked oligosaccharide side chains [2]. The tertiary structure of antithrombin has been inferred from the known three-dimensional structure of α_1-antitrypsin with which it shares a 30% identity of sequence [3,4].

We describe here the characterisation of a new, low-heparin-affinity variant, antithrombin Rouen-III (7 Ile→Asn), from a patient with pulmonary embolism. This finding supports the previous conclusions, based on other functional variants and protein homologies, that heparin binds to a positively charged surface extending from Arg 47 in the A helix and along the outer face of the D helix to Lys 133 [5].

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Abbreviations: antithrombin, antithrombin III; endo F, endo-β-*N*-acetylglucosaminidase F

2. MATERIALS AND METHODS

2.1. *Materials*

Sialidase type VI *(C. perfringens)* was obtained from Sigma. Endo-β-*N*-acetylglucosaminidase F (Endo F, *F. meningosepticum*) was obtained from New England Nuclear.

2.2. *Antithrombin measurements in plasma*

Antithrombin concentration was determined by Laurell electroimmunoassay [5]. Functional studies were carried out using synthetic chromogenic substrates of thrombin (IIa) and factor Xa. Progressive anti-IIa activity was assayed with S2232 (Kabi, Flow-Puteaux, France) and human IIa [7], while progressive anti-Xa activity was evaluated using CBS 3139 (Serbio-Asnieres, France) [8]. Antithrombin activity in the presence of heparin or

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purified heparan sulphate was evaluated using bovine thrombin and substrate CBS 3447 as detailed in [5]. Anti-Xa activity was evaluated in the presence of both heparin and synthetic pentasaccharide (IC 851589, Institut Choay, Paris) using bovine factor Xa and substrate CBS 3139 as described [5]. Results are expressed as a percentage of a pool of control plasma from 20 normal individuals.

2.3. *Fractionation of antithrombin*

Typically, 1 ml of 5 mM $CaCl_2$ and 1 ml of 10% dextran sulphate were added to 100 ml plasma. The supernatant was applied to a 1.5 × 25 cm column of heparin-Sepharose equilibrated in 50 mM Tris-HCl, 10 mM citrate, pH 7.4 [5]. The column was eluted with a linear gradient to 1.3 M NaCl in the same buffer.

2.4. *Peptide mapping*

Antithrombin was carboxyamidomethylated in 8 M urea [9] and digested with trypsin [10]. Reverse-phase HPLC mapping was carried out using a Waters 8 mm inner diameter radial compression cartridge with a 10 μm C-18 packing. The initial solvent (A) was 49 mM KH_2PO_4 adjusted to pH 2.9 with orthophosphoric acid, while solvent B consisted of a 50:50 mixture of A in acetonitrile [10]. The flow rate was 1.5 ml/min and a linear gradient was run from 3 to 67% B over 46 min and then to 100% B over the next 8 min. Usually, 0.1 mg digest (1.7 nmol) was applied per run. Peaks were collected, dried, and the insoluble phosphate residue was extracted with 20 μl of 60% ethanol to recover peptide material [11].

2.5. *Structural studies*

Peptides were hydrolysed (110°C for 16 h) under vacuum, using vapour from 6 M HCl/1% phenol [12]. Phenylthiocarbamyl derivatives of the amino acids were quantified by reverse-phase HPLC using a Waters Nova pac column [13]. Protein sequences were determined using an Applied Biosystems 475 A sequencer; the PTH amino acids were identified by HPLC.

3. RESULTS

3.1. *Case history*

The propositus was a 60-year-old women who developed a massive pulmonary embolism 10 days after total hip replacement. The embolism occurred during an episode of severe heparin-induced thrombocytopenia. A variant antithrombin was diagnosed several weeks later and was also found in the woman's daughter (table 1). Both mother and daughter had normal antigenic levels of antithrombin, and normal progressive anti-IIa and anti-Xa activity. However, there was impairment of activation of antithrombin by heparin, heparan sulphate and by the synthetic pentasaccharide (table 1). These data suggested the (heterozygous) presence of a variant form of antithrombin with a defect in the heparin-binding domain.

Table 1

Antithrombin levels in the propositus's and daughter's plasma

	Propositus	Daughter	Normal range
Antithrombin antigen	105	130	80–120
Progressive anti-IIa activity	115	105	80–120
Progressive anti-Xa activity	93	93	78–114
Heparin cofactor activity (anti-IIa)	66	65	82–118
Heparan sulphate cofactor activity (anti-IIa)	70	60	84–120
Heparin cofactor activity (anti-Xa)	70	75	82–116
Pentasaccharide cofactor activity (anti-Xa)	60	68	84–116

Average of three separate plasma samples expressed as % of control plasma

3.2. *Isolation and characterisation*

Chromatography of 100 ml plasma on heparin-Sepharose gave two well-resolved peaks of antithrombin. One eluted at 0.48 M NaCl, the other eluting in the position of normal antithrombin at 0.85 M NaCl. The low-heparin-affinity antithrombin was present in a slight excess over the product of the normal gene when measured antigenically and gravimetrically (table 2). The purified abnormal antithrombin retained normal progressive anti-IIa activity but showed negligible activation by either heparin or the synthetic pentasaccharide (table 2).

The low-heparin-affinity antihrombin migrated as two bands on agarose gel electrophoresis, both bands reacting with antisera against antithrombin (fig.1, lanes 3,9). The position of the more anodal band indicated an increase of two net negative

Table 2

Antithrombin levels in normal and abnormal fractions separated by heparin-Sepharose chromatography

	Abnormal fraction	Normal fraction
Yield from 100 ml plasma (mg)	6.6	5.9
Antithrombin antigen[a]	60	40
Progressive anti-IIa activity[a]	50	35
Heparin cofactor activity (anti-IIa)[a]	5	45
Pentasaccharide cofactor activity (anti-Xa)[a]	< 5	42

[a] Results expressed as % of control plasma

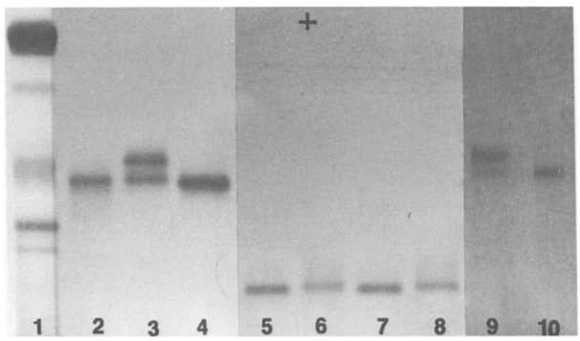

Fig.1. Agarose gel electrophoresis, pH 8.6. (1) Serum control. (2) Purified antithrombin from pooled normal plasma. (3) Purified low-heparin-affinity fraction from propositus. (4) Normal antithrombin from propositus. (5,7) Normal fraction after 1 and 20 h incubation, respectively, with sialidase. (6,8) Low-heparin-affinity fraction after 1 and 20 h incubation with sialidase. Antithrombin (1.5 mg/ml) was incubated (20°C) with 5% (w/w) sialidase in 50 mM acetate buffer, pH 5.5/5 mM $CaCl_2$. (9,10) Immunofixation of 3 and 4, respectively, with anti-antithrombin antisera.

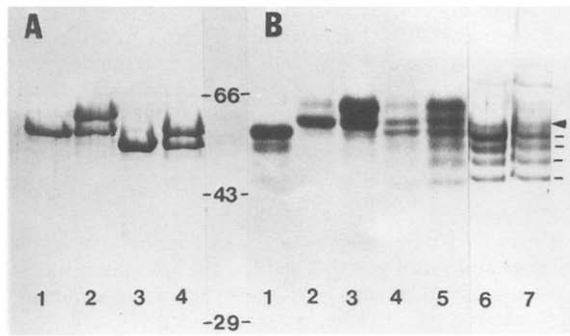

Fig.2. SDS-polyacrylamide gel (10%) electrophoresis in the presence of β-mercaptoethanol. (A) Effect of sialidase. (1,3) Normal antithrombin component before and after 1 h incubation with sialidase, respectively. (2,4) Low-heparin-affinity antithrombin before and after sialidase treatment. (B) Effect of endo F. (1) Marker of antithrombin β which lacks one of the four oligosaccharide side chains [14]. (2) Normal antithrombin from propositus. (3) Low-heparin-affinity antithrombin. (4,6) Normal antithrombin after 20 and 48 h incubation with endo F. (5,7) Low-heparin affinity antithrombin after 20 and 48 h incubation with endo F. Arrow shows position of fully glycosylated antithrombin while (−) indicates successive removal of four oligosaccharide side chains. 1 μl endo F was added to 25 μg antithrombin in 25 μl of 0.1 M phosphate buffer, pH 6.1/50 mM EDTA/1% NP-40/0.1% SDS and incubated for 24 h at 20°C.

charges, while the cathodal band had no formal increase in charge, running only marginally but consistently ahead of normal antithrombin. The proportion of the two low-heparin-affinity components varied from preparation to preparation and, indeed, the fast component disappeared on storage of the purified solution for more than 24 h. The low-heparin-affinity material was heterogeneous with respect to molecular mass as well as charge. On SDS-polyacrylamide gel electrophoresis in both the presence and absence of β-mercaptoethanol, this material ran as two bands, one comigrating with normal antithrombin at 58 kDa, and the other at 2.5 kDa higher (fig.2). The proportion of these components did not alter, and solutions could be stored for several days without change in the pattern.

Sialidase treatment would be expected to remove eight negatively charged sialic acid residues from antithrombin and in the process, reduce its molecular mass by approx. 2.5 kDa. On digestion with sialidase, both components of the low-heparin-affinity antithrombin lost negative charge to run as a single band with a mobility similar to that of asialo-antithrombin A on agarose gel electrophoresis (fig.1). However, the molecular mass difference between the two low-heparin-affinity components was retained on this treatment with sialidase (fig.2A). after extensive digestion with endo F, which cleaves the oligosaccharide component to give a single asparagine-linked *N*-acetylglucosamine residue, the low, and normal-affinity antithrombins behaved identically on SDS-polyacrylamide gel electrophoresis (fig.2B, lanes 6,7).

Evaluation of the data suggested that the underlying medical condition was explicable in terms of a point mutation, which did not involve a change in charge, but introduced a new partially utilised Asn-X-Thr/Ser carbohydrate attachment sequence.

3.3. *Peptide mapping*

Repeated SDS-polyacrylamide gel electrophoresis of CNBr fragments [14] failed to show any differences between the low-heparin-affinity and normal antithrombin fractions, despite the fact that control samples incubated in 70% formic acid alone still retained the 60.5 kDa as well as the 58 kDa band (not shown). This served to exlude CNBr fragments with molecular masses greater than 7 kDa as possible new carbohydrate sites and implied that the mutation was in a smaller fragment, i.e.

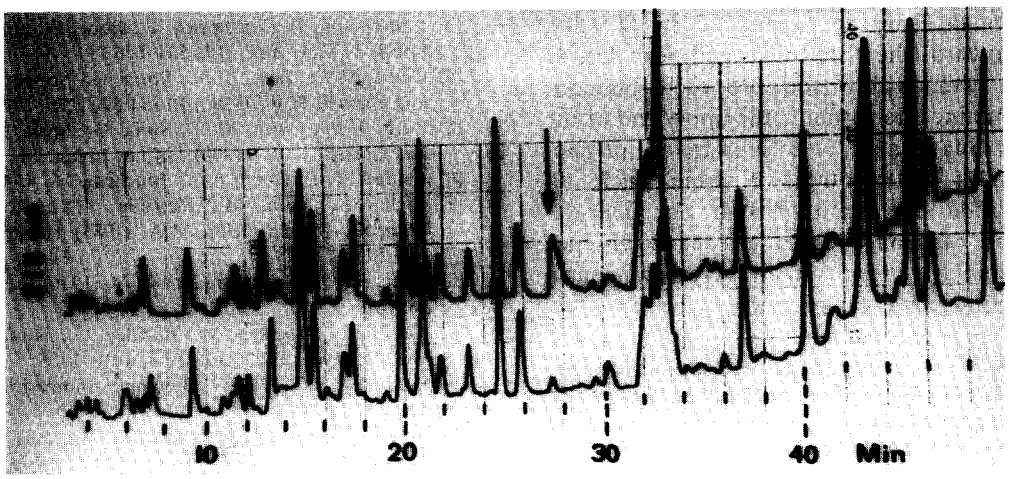

Fig.3. Reverse-phase tryptic peptide maps of carboxyamidomethylated antithrombin from the propositus. (Lower trace) low-heparin-affinity fraction; (upper trace) normal fraction. The peak at 27.5 min was present in all normal antithrombins analysed but missing from digests of antithrombin Rouen-III.

between residues 1–20, 90–103, 253–338 (four peptides generated from this region) or 424–432. Similarly, but not conclusively, partial cleavage at cysteine residues with nitrothiocyanobenzoic acid [15] implied that the mutation occurred before the second cysteine at position 22.

Tryptic peptide mapping by reverse-phase HPLC gave the first definitive clue as to the location of the mutation site. Maps of the normal antithrombin fraction were essentially identical to maps of the low-heparin-affinity fraction except that the peak at 27.5 min was not present in the abnormal antithrombin (fig.3). Amino acid analysis of this peak (His 1.0, Gly 1.3, Ser 0.9, Pro 2.0, Val 1.0, Asx 0.8, Ile 1.0, CMCys 0.7, Thr 0.9, Ala 1.2, Lys 0.8, Arg 0.9) showed that it represented the expected 13-residue N-terminal tryptic fragment. As no new aberrant peak was clearly apparent in digests of the abnormal fraction, the native carboxyamidomethylated protein was subjected to direct gas-phase sequence analysis. This yielded an initial sequence of His-Gly-Ser-Pro-Val-Asp-Asn-Cys-Thr-Ala-Lys-Pro-Arg-Asp-Ile-Pro-Met-Asn-Pro-Met-Cys-Ile-. The expected isoleucine at position 7 was absent and replaced instead by asparagine. The yield of Asn 7, however, was about half that expected considering the yield of Asn 14. This finding of a reduced yield of asparagine is consistent with partial glycosylation occurring at the new Asn-Cys-Thr sequence.

4. DISCUSSION

The pulmonary embolism suffered by the propositus during a period of heparin treatment is understandable in the light of the subsequent finding of a genetic variant of antithrombin with impaired heparin activation. The identification of the primary mutation in antithrombin Rouen-III as 7 Ile→Asn explains its charge and molecular mass heterogeneity. There is no inherent alteration in charge or molecular mass with this substitution. However, the new oligosaccharide attachment sequence of Asn-Cys-Thr allows for the observed heterogeneity of the new antithrombin.

The decreased heparin affinity of the variant is in accord with the currently accepted model of the heparin-binding domain of antithrombin. The evidence from functional genetic mutants and protein homologies indicates that the negatively charged heparin binds through ionic bonds to Arg 47 of the A helix and along the outer surface of, and parallel to, the D helix (Lys 125, Arg 129, Arg 132, Lys 133) [4,5]. Unfortunately, there is no precise information on the position of residue 7 of antithrombin, modelled on the three-dimensional structure of α_1-antitrypsin. Position 7 has no equivalent in antitrypsin because antithrombin has a sequence of 25 additional amino acids at its N-terminus. The Cys at position 8, however, is disulfide bonded to Cys 128 of the D helix. This

would place Ile 7 in the vicinity of the D helix, probably towards the N-terminal end near Arg 47 [4,5], and well within the proposed heparin-binding domain. Interestingly, the upper end of the heparin-binding domain is defined by the physiological variant, antithrombin B, which lacks an oligosaccharide side chain on Asn 135. The decreased steric hindrance resulting from the absence of the carbohydrate results in an increased heparin affinity in this particular instance.

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