Proposed Heparin Binding Site in Antithrombin Based on Arginine 47
A New Variant Rouen-II, 47 Arg to Ser

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Abstract

Antithrombin Rouen-II, a new inherited variant of antithrombin-III, was found in two members of a family with no definite history of thrombosis. The subjects had normal antigenic concentrations of antithrombin and normal progressive inhibitory activity. However, the variant had defective heparin and heparan sulfate cofactor activities, and was not activated by a synthetic pentasaccharide representing the minimum heparin sequence.

The abnormal antithrombin was isolated using heparin-Sepharose chromatography, and on electrophoresis at pH 8.6 migrated more anodally than normal. Two-dimensional peptide mapping of tryptic and Staphylococcus aureus V8 protease digests was performed and the abnormal peptide was located by tryptophan staining. Amino acid sequence studies demonstrated a substitution of arginine at residue 47 by a serine.

Evidence strongly suggests that arginine 47 is a prime heparin binding site in antithrombin and that it forms part of a proposed positively charged linear site (to which heparin binds) that stretches across the surface of the molecule from the A to the D helix.

Introduction

Antithrombin (antithrombin-III) a member of the serpin superfamily (1), is the major inhibitor of thrombin and several other coagulation serine proteases. It plays an important physiological role in controlling the coagulation cascade. Inherited quantitative deficiencies of antithrombin result in an increased risk of thromboembolic disease (2).

The formation of the inactive protease-antithrombin complexes is greatly accelerated by commercial heparins or by endothelial, heparin-like substances (3) which are most likely to be one of the heparan sulfates (4).

The mechanism by which heparin activates the protease inhibitory activity of antithrombin remains puzzling, but some illuminating data have recently emerged. The antithrombin binding sequence in heparin is a unique specific pentasaccharide (5) bearing at least four sulfate groups that play an essential role in this interaction (6). On the other hand, the localization of the heparin binding site on antithrombin remains uncertain. The first clues have come either from chemically modified antithrombins or from molecular characterization of natural mutant inhibitors. In vitro alteration of the tryptophan 49 (7) or of lysine residues (8) prevent the heparin acceleration of the inhibitory activity without affecting the progressive protease inhibitor interaction when the mucopolysaccharide is absent. Only five variants of antithrombin with impaired heparin binding ability have been extensively studied. In four of them, arginine at residue 47 is replaced either by a cysteine in antithrombin Toyama (9), Tours (10), and Alger (11), or by a histidine in the variant Rouen-I we recently reported (12). The fifth one, antithrombin Basel, has a Pro to Leu substitution at residue 41 (13).

We describe in this paper the qualitative investigations and the molecular characterization of a new inherited antithrombin mutant, Rouen-II. Rouen-II has normal progressive inhibitory activity but defective heparin binding and low heparin and heparan sulfate cofactor activities which occur as a result of the replacement of arginine 47 by serine. This variant provides new evidence that arginine 47 is a prime site in antithrombin for the binding of heparin and endothelial mucopolysaccharides.

Methods

Case report. The propositus, a 40-yr-old man suffering from hypertriglyceridemia, was admitted to hospital because of a sudden myocardial infarction that lacked extensive coronary artery disease (this was found out through angiography). As a result of standard investigations he was found to have an abnormal antithrombin. There was no previous familial history of thrombosis, but his 13-yr-old daughter displayed the same antithrombin abnormality.

Antithrombin measurements in plasma. Antithrombin concentrations were quantified by Laurell electroimmunoassay (14). Qualitative determinations were carried out by amidolytic methods using synthetic chromogenic substrates of thrombin (IIa) or Factor Xa. Progressive anti-IIa activity was assayed with S2238 from Kabi (Flow, Puteaux, France) and human IIa, as proposed by Aiach (15), progressive anti-Xa activity was evaluated with CBS 3139 (Serbio, Asnières, France) according to Odegard (16). Antithrombin activity was evaluated in the presence of heparin using the reagent kit of Diagnosticla Stago (Asnières, France) on a discrete analyzer as recommended by Aiach (17): 5 μl of plasma were diluted in 500 μl of pH 8.7, Tris buffer

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containing an excess of bovine thrombin and 3 IU/ml heparin. After a 20-

s incubation time, 100 μl of chromo-thrombin was added and the

increase in absorbance recorded during 40 s. Antithrombin activity

was also measured in the presence of purified heparan sulfate (HS Ila),

obtained from B. Casu (18), using an end-point method: 200 μl of

d plasma (diluted 1:10 in Tris-EDTA buffer, pH 8.4, containing 2.25 US
Pharmacopeia U/ml heparan sulfate) were incubated with 200 μl bo-

vine thrombin during 30 s; 200 μl of CBS 3447 was added and the

reaction was stopped after 60 s. Anti–Factor Xa activity was evaluated

in the presence of 3 IU/ml heparin or 1 I.C. anti-Xa U/ml synthetic
pentasaccharide (IC 851589; a gift from Institut Choay, Paris, France)

with the same conditions as for heparan sulfate cofactor activity, but in

the presence of bovine Factor Xa and CBS 3139 (from Stachrom
heparin reagent kit, Diagnostica Stago). Results are expressed in per-

centage of control plasma, which comprised a pool of 20 normal

plasmas.

Crossed immunoelectrophoresis of antithrombin. This was

achieved according to Sas’ method (19), with or without the addition in

the first dimension of 25 IU/ml unfractionated heparin or of 25 anti-

Xa I.C. U/ml low molecular weight heparin (CY 222; Institut Choay,

mean molecular weight, 2.500).

Purification and characterization of antithrombin. Antithrombin

was isolated on heparin-Sepharose as previously described (12). Elec-

trophoresis on agarose and SDS polyacrylamide, 14C-carboxymethyla-

tion and proteolytic digestion with trypsin and S. aureus V8 protease,

thin layer peptide mapping, HPLC peptide separations, and peptide

sequencing were performed as previously described (12).

Antithrombin activation studies. These were performed using puri-

fied normal and propositus antithrombins, adjusted to equal antigenic

concentration by varying both the heparin concentration (0.1, 0.5, 5.0

IU) and the ionic strength of the phosphate buffer, pH 7.4 (isotonic, 2×
isotonic, 3× isotonic). 0.2 μg of purified antithrombin was added to

600 μl of buffer, pH 7.4, which contained heparin. Excess thrombin

(10 μl of Parke Davis, Detroit, topical bovine thrombin diluted 15 μl to

1 ml) was added and, after 20 min, 25 μl of Chromozym-TH (5 mg/ml)

was added and the increase in absorbance was monitored at 405 nm.

Results

The propositus and his daughter both demonstrated the same

antithrombin abnormality in their plasmas (Table I). Immuno-

reactive levels and progressive activities of antithrombin were

within the normal range, whereas heparin cofactor activi-

ties measured by antithrombin or anti–Factor Xa assays were
decreased to 64–70% of the control, and heparan sulfate co-

Table I. Antithrombin Levels* in the Propositus

and Daughter’s Plasma

<table>
<thead>
<tr>
<th></th>
<th>Propositus</th>
<th>Daughter</th>
<th>Normal range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antithrombin antigen</td>
<td>102</td>
<td>115</td>
<td>80–120</td>
</tr>
<tr>
<td>Progressive anti-Xa activity</td>
<td>100</td>
<td>122</td>
<td>80–120</td>
</tr>
<tr>
<td>Progressive anti-Xa activity</td>
<td>120</td>
<td>100</td>
<td>78–114</td>
</tr>
<tr>
<td>Heparin cofactor activity (anti-Xa)</td>
<td>64</td>
<td>65</td>
<td>82–118</td>
</tr>
<tr>
<td>Heparan sulfate cofactor activity (anti-Ila)</td>
<td>60</td>
<td>68</td>
<td>84–120</td>
</tr>
<tr>
<td>Heparan sulfate cofactor activity (anti-Xa)</td>
<td>64</td>
<td>70</td>
<td>82–116</td>
</tr>
<tr>
<td>Pentasaccharide cofactor activity</td>
<td>60</td>
<td>66</td>
<td>84–116</td>
</tr>
</tbody>
</table>

* Values expressed as a percentage of that obtained from pooled

plasma (20 normal samples).

factor activity evaluated by an anti–IIa measurement was

equally low at 60 and 68%, respectively, of the control. In

the same way, the pentasaccharide only partially potentiated ini-

hbitation of Factor Xa in the patient’s plasma.

Normal patterns were obtained by crossed immunoelectropho-

resis in the absence of heparin. But in the presence of

unfractionated heparin or low molecular weight heparin, nor-

mal antithrombin migrated as a major, more anodal peak,

while both abnormal plasmas displayed a double-peak pattern;

one peak had the same fast mobility as the control, the other

had the same slower migration as antithrombin in the absence

of heparin (gels not shown).

The results of heparin-Sepharose chromatography of the

patient’s plasma at pH 7.4 show heterozygosity for an ant-

ithrombin of decreased heparin binding affinity (Fig. 1). The

variant antithrombin eluted with 0.45 mol/liter NaCl com-

pared with the normal antithrombin which eluted at 0.95

mol/liter. Quantitation by electroimmunoassay confirmed the

presence of an excess of variant compared with the normal

component (Table II). The activities of the two separate frac-

tions were also compared (as seen on Table II) using the exper-

imental conditions described for the plasma measurements.

The abnormal fraction had normal progressive inhibition ac-

tivity but defective heparin or pentasaccharide cofactor activi-

ties.

The thrombin inhibitory activity of purified Rouen-II and

normal antithrombin were compared in PBS that was two or

time its isotonic strength with varying amounts of hepa-

rin (Fig. 2). This showed that the variant antithrombin was

incompletely activated in the presence of low levels of heparin

when there was sufficient competition from the sodium and

chloride ions for the charged groups on heparin and ant-

ithrombin. At the concentrations of heparin used in the

experiment there was no significant difference between Rouen-

II and normal antithrombin III under isotonic conditions. How-

ever, at 2× isotonic concentration Rouen-II showed <50% activity

at the low heparin level. The difference in activity between nor-

mal antithrombin and Rouen-II is even more dra-

matic in 3× isotonic strength buffer. Here Rouen-II with 5.0 U

of heparin had less activity than normal antithrombin with 0.5

U of heparin.

Electrophoresis in agarose at pH 8.6 showed that the ab-

normal antithrombin migrated faster (more anodally) than

normal, implying that the variant had an increased negative

charge. SDS-PAGE showed the molecular weight of normal and

abnormal antithrombins to be identical at 56,000.

A series of peptide maps were prepared of both the normal

and abnormal antithrombins. These were identical apart from

a consistent finding in both tryptic and V8 protease maps of a

shifted tryptophan-containing peptide. Normal tryptic maps

Figure 1. Elution pro-

file of heparin-Sepha-

rose chromatography,

showing the resolution of

antithrombin Rouen-

II from normal an-

tithrombin at pH 7.4.

The proteins were

eluted with an increas-

ing linear NaCl gradient.
Table II. Antithrombin Levels* in Antithrombin Rouen-II Normal and Abnormal Fractions Separated by Heparin-Sepharose Chromatography

<table>
<thead>
<tr>
<th></th>
<th>Abnormal fraction</th>
<th>Normal fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antithrombin antigen</td>
<td>60</td>
<td>40</td>
</tr>
<tr>
<td>Progressive anti-IIa activity</td>
<td>50</td>
<td>35</td>
</tr>
<tr>
<td>Heparin cofactor activity (anti-IIa)</td>
<td>&lt;5</td>
<td>45</td>
</tr>
<tr>
<td>Pentasaccharide cofactor activity (anti-Xa)</td>
<td>&lt;5</td>
<td>35</td>
</tr>
</tbody>
</table>

* Values expressed as a percentage of that obtained from pooled plasma (20 normal samples).

showed three tryptophan-staining peptides whereas Rouen-II had only two. This suggested that the abnormality had occurred within residues 47–53. The V8 maps gave two tryptophan peptides from both normal and abnormal antithrombins, with the peptide corresponding to residues 43–50 being shifted. The amino acid composition of the implicated peptides showed an arginine to be missing in the variant, but the identity of the new amino acid could not be unequivocally determined from the peptide maps. The tryptic peptide, residues 47–53, was isolated from both normal and Rouen-II digests by reversed phase HPLC using 49-mmol/liter phosphate buffer, pH 2.9, and an increasing concentration gradient of acetonitrile. The peptide was rerun on reversed phase HPLC in 0.1% trifluoroacetic acid with increasing acetonitrile. The peptides were monitored at 215 nm, and to detect the tryptophan-peptides, also at 280 nm. Amino acid sequence studies were performed on these peptides. These showed that arginine, which was at position 1 in the normal tryptic peptide, had been replaced by a serine. This corresponds to position 47 in the intact protein.

Discussion

Rouen-II is a new genetic variant of antithrombin with normal progressive inhibitory activity, but decreased cofactor activity, with both unfraccionated or synthetic pentasaccharide heparins. As demonstrated by crossed immunoelectrophoresis with both types of heparin, and also by heparin-Sepharose chromatography, this dysfunction is due to impaired heparin binding as has been previously demonstrated in other analogous variants (9–13, 20–23). The biochemical defect in Rouen-II was demonstrated by repeated peptide maps of both tryptic and V8 protease digests on thin layer plates, and by HPLC. These showed a single consistent difference in peptides overlapping residue 47. Sequence analysis showed this difference to be due to the replacement of the arginine normally present at position 47 by a serine. This mutation incidentally creates an unused oligosaccharide attachment site at position 45, i.e., Asn-Arg-Ser.

The identification of the mutation in antithrombin Rouen-II as 47 arginine to serine, is not a surprise. Among the seven other genetic variants so far identified (9–13, 24, 25) four with impaired heparin binding involve mutations of arginine 47: to cysteine in the independently observed variants Toyama (9), Tours (10), and Alger (11), and to histidine in Rouen-I (12). One reason for this recurrence of mutations at position 47 may be the susceptibility of the DNA codon for this arginine, CGT, to base substitutions; in particular, the replacement of arginine 47 by a cysteine is due to a thymine for cytosine substitution, a particularly frequent DNA mutation (26). Recurrent mutations in hemophilia A also provide evidence that CG dinucleotides are “hotspots” for mutations in man (27). However, the occurrence of three different mutations at the one site (to cysteine, to histidine, and to serine) all provide evidence of the critical role played by arginine 47 in the binding of heparin by antithrombin.

The results shown in Fig. 2 support the deduction that heparin binds to antithrombin by ionic bonding (salt-bridges) between the negatively charged sulfates of the heparin and positively charged basic amino acids (arginines and lysines) of antithrombin. Increasing salt concentrations compete for these bonds and diminish the heparin affinity particularly in the absence of arginine 47 in Rouen-II.

Several recent reports provide evidence that antithrombin can be activated on vascular heparin-like substances. This pathway could be responsible, in part, for the nonthrombogenic properties of endothelium (28). Among these substances, heparan sulfate proteoglycans bind with high affinity via sulfated saccharides similar to those of the unique pentasaccharide sequence of commercial heparin (29). The activation of Rouen-II by anticoagulantly active heparan sulfate is defective and from this we deduce that arginine 47 is also a prime site for binding to endothelial proteoglycans. As we previously noticed (30), the incidence of thrombosis in heterozygous antithrombin variants with defective heparin binding is very low compared with that observed in quantitative defects or other types of variants. In the Rouen-II family, there is no definite history of thrombosis; the variant antithrombin retains its progressive activity, and as shown in Fig. 2, also has the potential to be activated by heparin though less readily than normal antithrombin. The observation of a greater concentration of the variant vs. the normal antithrombin (Table II) suggests increased consumption of the normal component and hence decreased functional activity of the variant. It is possible that this decreased in vivo functional activity of antithrombin Rouen-II was a factor that contributed to the coronary thrombosis of the propositus. Indeed, the homozygous patient for antithrombin Alger, with complete loss of heparan sulfate binding, does suffer from thromboembolic disease (22).
The results shown in Fig. 2 demonstrate that the defect in the Rouen-II variant is one of heparin (or heparan) binding and not of heparin activation. The identification of Rouen-II together with other variants of arginine 47, led us (with P. C. Christey and D. R. Boswell) to look at other basic residues that are uniquely conserved in the two heparin-activated serpins, antithrombin and heparin cofactor-II, and which might therefore be involved in the binding of heparin (31). Using computer alignments of the serpin family it is possible to determine homologous residues and project them (1) on the known crystallographic structure of the archetype of the family, alpha-1-antitrypsin (32). Seven such basic residues occur and five of them, including arginine 47, are plotted in Fig. 3 to give a proposed binding site for heparin. Further evidence for this binding site is given by the identification of asparagine 135 as the site of the oligosaccharide chain whose absence gives the increased natural affinity variant, beta-antithrombin (33), and also by the independent evidence of Blackburn that lysine 125 is directly involved in heparin binding (34). The proposed binding site, as shown in Fig. 3, primarily involves the A and D helices, and is projected on the cleaved, postcomplex structure of alpha-1-antitrypsin (32). Reconstruction of the native structure in both antitrypsin and antithrombin involves a change in conformation but observations based on space-filling models make it unlikely that this transition is accompanied by gross alterations of the A and D helices (31).

Altogether then there is good evidence that heparin (and inferentially heparan) bind to a positively charged linear site extending across the molecule from arginine 47, as shown in Fig. 3. There is as yet no direct evidence as to how the binding of heparin activates the inhibitory function of the molecule; presumably there is an accompanying conformational change. Evidence for the events involved in this activation will come from the study of variants that bind heparin but do not activate it, and ultimately from crystallographic studies of antithrombin that show its three-dimensional conformation in the presence of heparin.

Acknowledgments

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References


