An hereditary abnormal antithrombin III (ATIII Geneva) with defective heparin cofactor activity was characterized by DNA single strand amplification and subsequent direct sequencing. ATIII Geneva was found to have a G to A transition in Exon IIIa leading to an Arg-129 to Gln mutation. This amino acid is part of the ATIII region comprising residues 114–154, which contains the highest proportion of basic residues (Arg or Lys), and is known from chemical modification studies to be involved in heparin binding. The variant protein did not bind heparin-Sepharose and was isolated from the propositus plasma by immunoaffinity chromatography. High affinity (for ATIII) heparin had only a minimal effect on thrombin and activated factor X inhibition by the purified abnormal ATIII. Taken together, these results demonstrate an important role for Arg-129 in the binding and interaction of ATIII with heparin of high affinity. We propose that a cooperation between Lys-125, Arg-129, Lys-136, and Arg-47 exposed at the surface of the inhibitor allows the binding of the essential pentasaccharide domain of heparin which is specific for the ATIII interaction.

Antithrombin III (ATIII) is a 132-amino acid serine proteinase inhibitor (Serpin) which plays a key role in the natural antithrombotic mechanism. It forms stoichiometric inactive complexes with thrombin, activated factor X (FXa), and other clotting proteases, the rates of inhibition of the proteases being greatly enhanced in the presence of heparin (for review see Ref. 1). The gene contains seven exons and six introns (1990 by The American Society for Biochemistry and Molecular Biology, Inc.)

**EXPERIMENTAL PROCEDURES**

**DNA Studies**

*Preparation of DNA—Peripheral blood samples from the patient were collected on EDTA. DNA was extracted from the leukocytes as described in a previous study (11).*

*Enzymatic Amplification of Specific Genomic DNA Sequences—Amplification of exon II of the ATIII gene was performed using the *Thermus aquaticus* (Taq) polymerase (Perkin Elmer-Cetus Instruments) according to Saiki et al. (12) and as previously described (11). Deoxynucleotides, deoxyadenosine triphosphate (dATP), deoxythymidine triphosphate (dCTP), deoxythymidine triphosphate (dTTP), and deoxyguanosine triphosphate (dGTP) were purchased from Pharmacia.*

*Exons IIIa and VI were amplified by asymmetric chain reaction (13) using two sets of oligonucleotides as extension primers, PS17C and PS18B for exon IIIa amplification, and PS11C and PS12B for exon VI amplification. Oligonucleotides were synthesized on a gene assembler (Pharmacia Fine Chemicals) using the 3'-cyanolylphosphorothioate internucleotide linkages. The putative heparin-binding domain in ATIII is thought to consist of two regions encompassing amino acids 35–50 (2) and 114–154 (3). The latter is rich in basic amino acids including Lys-125 and Lys-136 which are known to be critical for heparin binding and the conformation change that is subsequently induced (4). Three-dimensional modeling based upon the results of x-ray crystallography suggested that these two regions are adjacent (5, 6).*

*Natural mutations of Pro-41 (7) and Arg 47 (6, 8), encoded by exon II, have been described in variants of ATIII with reduced heparin-enhanced protease inhibition, and these have confirmed that this part of the molecule is involved in its interaction with the polysaccharide.*

*We present here the first case of a natural mutation detected within the 114–154 domain, which is encoded by exon IIIa. A variant ATIII, first described as ATIII Geneva (10) has an Arg-129 to Gln mutation. The genomic DNA abnormality in codon 129 (CGA to CAA) was identified using an asymmetric polymerase chain reaction (PCR) amplification of the third exon of the ATIII gene and direct sequencing of the single strand amplified DNA.*

*The variant ATIII in the patient's plasma did not bind to heparin-Sepharose. Furthermore, accelerated thrombin inhibition by the purified variant was minimal in the presence of heparin, showing that replacement of the basic amino acid Arg by Gln in position 129 strongly impairs the interaction of heparin with ATIII.*

*These results confirm that the region encompassing Arg-129 in ATIII is important for heparin binding and subsequent activation of the inhibitor.*
and 1.5 M NaCl. AT111 Geneva did not bind to heparin-Sepharose in using a mini system (miniprotean I, Bio-Rad).

TABLE I

<table>
<thead>
<tr>
<th>Primers used for amplification of exon IIIa and exon VI of the ATIII gene</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Location of these primers in intervening sequences (IVS) or coding sequences.</strong></td>
</tr>
<tr>
<td><strong>EXON IIIa</strong></td>
</tr>
<tr>
<td><strong>Coding strand</strong></td>
</tr>
<tr>
<td>PS17C</td>
</tr>
<tr>
<td>Non-coding strand</td>
</tr>
<tr>
<td>175 176 177</td>
</tr>
<tr>
<td>PS18B</td>
</tr>
<tr>
<td><strong>EXON VI</strong></td>
</tr>
<tr>
<td><strong>Coding strand</strong></td>
</tr>
<tr>
<td>IVS 5</td>
</tr>
<tr>
<td>IVS 3a</td>
</tr>
<tr>
<td>PS11C</td>
</tr>
<tr>
<td>Non-coding strand</td>
</tr>
<tr>
<td>3' flanking region</td>
</tr>
<tr>
<td>PS12B</td>
</tr>
</tbody>
</table>

phoramidine method and purified on polyacrylamide gel. Their sequences are summarized in Table I. As shown in Fig. 1, PS17C was located in intervening sequence 2 (IVS 2) and PS18B at the end of exon III and beginning of IVS 5. The resulting 270-base pair fragment contained the sequence encoding for amino acids 105-174. PS11C overlapped the ends of IVS 5 and the beginning of exon VI, and PS12B was located in the 3'-flanking region. The amplification with PS11C and PS12B gave a 210-base pair fragment sequence encompassing amino acids 379-432.

Each PCR contained 50 pmol of one primer (PS18B or PS12B) and 0.5 or 1 pmol of the other (respectively PS17C or PS11C), 200 μM of each dNTP, 0.5 μg of genomic DNA, 1 X PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.01% (w/v) gelatin) and 2.5 units of Taq polymerase in a final volume of 100 μl. The reactions were performed in 1.0 ml microcentrifuge tubes (Sarstedt) with a Hybaid thermal cycler.

This asymmetric polymerase chain reaction led to a preferential amplification and enrichment of the noncoding strands of exons IIIa and VI.

Specificity of this PCR was checked on a 6% polyacrylamide gel using a mini system (miniprotein I, Bio Rad).

Exons IIIa and VI PCR products were first desalted and the excess of dNTPs removed by spin dialysis on a Centrinon 100 unit (Amicon). The single-stranded templates were sequenced using the sequencing kit from United States Biochemical Corporation and with PS17C and PS11C as sequencing primer and α-[35S]dATP from Amersham.

**Variant Protein Purification and Analysis**

Normal pooled plasma was prepared by centrifugation of citrated blood from healthy laboratory staff. Citrated plasma containing the variant ATIII Geneva was prepared from the propositus, who was heterozygous for the abnormality. ATIII was isolated from normal plasma by dextran sulfate precipitation and stepwise elution from heparin-Sepharose (Pharmacia) (14). The plasma was applied in 0.1 M Tris-Cl, pH 7.4, and normal ATIII eluted exclusively between 0.4 and 1.5 M NaCl. ATIII Geneva did not bind to heparin-Sepharose in 0.4 M NaCl (the normal ATIII in the propositus’ plasma was, however, retained on the heparin-Sepharose) and was purified by further chromatography on immobilized polyclonal antibody to ATIII (16). The bound variant ATIII (Geneva) was eluted with MgCl₂ after prior washing with 0.5 M NaCl, as described (16). For the kinetic studies, an additional chromatography step was performed to remove any contaminating heparin. The ATIII preparations were applied to a Mono Q column in 0.02 M triethanolamine, pH 7.3, and eluted with a NaCl gradient using a fast protein liquid chromatography (Pharmacia) delivery system (17).

The reaction of ATIII with thrombin was studied at 37 °C in the absence and presence of high affinity (for ATIII heparin (this was a generous gift from Professor Ulf Lindahl, Biomedical Center, Upsala, Sweden).

20 nm human thrombin (a kind gift from Dr. J.-M. Freyssinet, INSERM U 311, Strasbourg, France) was incubated with varying concentrations of ATIII (as indicated in the figures) in 0.15 M NaCl, 0.05 M Tris-HCl, pH 7.4, containing 1% polyethylene glycol 6000. At different times, the incubation mixtures were subsampled into cuvettes containing the chromogenic substrate S 2238 (KabiVitrum) to determine the residual thrombin. Relative velocities of substrate hydrolysis V/V₀, where V is the velocity at a given time and V₀ is the initial velocity, were plotted against time. Replotting the reciprocal pseudo first-order rate constant against reciprocal ATIII concentration allowed the second-order rate constant to be determined (18). Similar experiments were conducted using FXa rather than thrombin. Bovine FXa was purchased from Diagen (Thame, United Kingdom) and the chromogenic substrate S 2222 was purchased from KabiVitrum.

**RESULTS**

The two regions of the ATIII gene suggested previously as coding for the heparin-binding domain were sequenced.

The first, with exon II, coding for amino acids 15-78, was analyzed after subcloning the amplified DNA as described in a previous study (11) and was found normal.

In the present study, a large part of exon IIIa, coding for amino acids 105-172, was sequenced directly using a single strand DNA obtained by asymmetric PCR. The patient was found to have both G and A in the codon corresponding to amino acid 129, leading to allele codons CGA and CAA. The patient is thus heterozygous for an Arg-129 to Gln mutation (Fig. 2). This result was confirmed after subcloning (data not shown).

As the purified variant showed decreased antithrombin activity (see further), we also sequenced exon VI corresponding to the amino acids 381-432 which encompasses the reactive site. Using the same techniques (asymmetric PCR and direct sequencing), we found a normal sequence, excluding another mutation in this part of the gene.

A small amount of the Arg-129 to Gln (ATIII Geneva) variant could be purified, as described under “Experimental Procedures.” Normal and variant ATIII preparations migrated as single bands on 10-15% gradient polyacrylamide gel electrophoresis under non-reducing and reducing conditions (Fig. 3). These bands reacted with polyclonal antibodies to normal ATIII following immunoblotting (17); results not illustrated.

Thrombin was inactivated by different concentrations of...
A Novel Mutation in the ATIII Heparin-binding Site

normal ATIII (Fig. 4a), such that reploting the reciprocal of the observed pseudo first-order rate constant against the reciprocal antithrombin concentration yielded a zero ordinate intercept and a second-order rate constant of inhibition of $2.86 \times 10^{5} \text{M}^{-1} \text{min}^{-1}$ (Fig. 4c). Antithrombin Geneva was less well able to inactivate thrombin (Fig. 4b), and a rate constant of inhibition $1.06 \times 10^{5} \text{M}^{-1} \text{min}^{-1}$ was calculated (Fig. 4c). Whether the reduced rate constant of inhibition was due directly to the structural defect or to partial inactivation of the variant inhibitor during purification (perhaps due to its decreased stability) could not be ascertained.

Preliminary studies were performed to investigate the effect of heparin on the ATIII-thrombin reaction. When 0.5 μg of high affinity heparin was added to a reaction mixture containing 20 nM thrombin and 0.023 μM normal ATIII, a large increase in rate of inactivation of thrombin was observed (as might be expected), as shown by Fig. 5a. The same amount of high affinity heparin produced a much smaller change on the inactivation rate of thrombin by ATIII Geneva (0.065 μM), see Fig. 5b. Indeed, increasing the amount of heparin 4-fold only produced minimal additional acceleration of inhibition of thrombin (Fig. 5b). These results provide, therefore, additional evidence that the interaction of ATIII Geneva with heparin is impaired but also indicate that the interaction is not completely abolished by the molecular defect.

Similar results have been obtained in a parallel study of inactivation of FXa by normal and variant ATIII preparations (results not shown). The variant inhibitor inactivates FXa and this inactivation is only weakly accelerated by high affinity heparin.

DISCUSSION

The identification of novel mutations in ATIII is of interest for several reasons. It may allow the evaluation of the physiological role of the inhibitor from a consideration of the clinical consequences of the mutation; it provides a unique means of understanding the molecular mechanisms involved in its interaction with clotting proteases and heparin; and it highlights the membership of ATIII in the Serpin superfamily.

We describe here the first natural mutation at Arg-129, a residue located in a region of ATIII which has been shown to be of importance for heparin binding by enzymatic fragmentation and chemical modification of the normal protein (3-5). The mutation was identified by detection of the base change G → A in the sense strand of the codon CGA, leading to the replacement of Arg by Gln. This probably results from a C → T transition in the antisense strand and further
carried out in the absence or the presence of high affinity heparin, at the concentration indicated: normal ATIII (a) and ATIII Geneva, were incubated as described in the legend of Fig. 4. Experiments were carried out in the absence or the presence of high affinity heparin (21). This suggests that if the activation of ATIII molecules by heparin-like glycosaminoglycans on the vascular endothelium (22) is important, then only 50% of functionally intact ATIII needs to be present for its expression.

The purified variant presented a diminished rate of thrombin and FXa inhibition even in the absence of heparin. In the patient’s plasma, these activities appeared to be normal (10). The part of exon VI coding for all the amino acid known to be important for protease binding was found to have a normal nucleotide sequence which reduced the possibility of the existence of a second mutation. Either the mutation impairs the function of the reactive site once the protein is removed from its physiological milieu, or it renders the protein more susceptible to denaturation during the purification process.

At physiological ionic strength and pH the Arg-129 to Gln variant could not bind to heparin-Sepharose (10). The replacement of Arg-47 by Cys observed in ATIII Alger, Paris I, Paris II, and Tours (11, 23, 24) also dramatically reduced the binding affinity for heparin (25-28). Recent observations made with ATIII Barcelona, another Arg-47 Cys mutation showed that the additional thiol group does not form a disulfide bond with other proteins (29). Pro-41 to Leu (30-32), Arg-47 to Ser (5) and Arg-47 to His mutations (9, 33) did not abolish completely the binding to heparin-Sepharose, which enabled the purification of the variant after elution by lower salt concentration than those required for normal ATIII. The binding affinity of ATIII for heparin is thus more strongly impaired by Arg-129 to Gin and Arg-47 to Cys than by other Arg-47 mutations, Pro-41 to Leu and Ile-7 to Asn mutations. The latter mutation is reported to create an additional glycosylation site and so the oligosaccharide may partly occupy the heparin-binding site (34).

Surprisingly, the purified variant ATIII had antithrombin and anti-FXa activities which were weakly stimulated by high affinity heparin. Thus, the Arg-129 to Gln mutation strongly modifies the heparin inhibitor interaction but does not completely abolish the enhancing effect, as might have been suspected by the absence of binding on heparin-Sepharose.

The heparin-binding site involves two distinct regions in the secondary structure of ATIII, respectively, encompassing Arg-47 and Arg-129. A composite heparin-binding domain involving arginines 47, 129, and 132 and lysines 125 and 136 has been suggested from projection in a three-dimensional model derived from that of α1-antitrypsin (6). Recent studies which used a chromogenic reagent labeling the Lys residues located at the surface of the molecule have confirmed the essential role of Lys 135 and Lys 136 of ATIII (4, 35). These 2 Lys residues and Arg-129 may be adjacent at the surface of an α-helix, assuming 3.6 amino acids/twist of the helix. The model proposed by Church et al. (36) suggests that the other basic residues, including Arg-132, Lys-133, and Lys-139, are present on the same side of a helical structure encompassing amino acids 124–141. We propose that within this domain, putative conformation of which is represented in Fig. 6, Lys-125, Arg-129, and Lys-136 play a crucial role in heparin binding.

Heparin also binds and activates another Serpin, heparin cofactor II (HCII), which has some conserved basic amino acid 125, Arg-129, and Lys-136 play a crucial role in heparin binding.
A Novel Mutation in the ATIII Heparin-binding Site

19001

acid residues when its sequence is aligned with that of ATIII. Arg-129 in ATIII corresponds to Arg-189 in HCII and similar correspondences were found for Arg-47, Lys-114, Lys-125, Arg-132, and Lys-133 (37). The mutation of Arg-189 to His in a congenital mutant of HCII, HCII Oslo, resulted in its decreased affinity for dermatan sulfate but, apparently, did not impair heparin binding (38). The ATIII interaction with heparin requires a specific pentasaccharide sequence with a unique S-O sulfate group (39). The conformation of the heparin binding site of ATIII is thus unique, which implies more stringent ligand specificity and affinity than does the corresponding domain in HCII. The presence of a disulfide bond in position 128 may place Arg-129 in a favorable conformation in ATIII that could facilitate its cooperation with Arg-47. The replacement of Arg-47 by Cys may also change the respective positions of the 2 Arg: this could explain the difference in heparin binding and subsequent activation observed with the Arg-129 to Gln mutation in ATIII Geneva gives the first conclusive evidence for the involvement of this amino acid in the interaction between the inhibitor and high affinity heparin.

Although some details regarding the interaction of heparin with ATIII remain to be clarified, the dramatic reduction of heparin binding and subsequent activation observed with the Arg-129 to Gln mutation in ATIII Geneva gives the first conclusive evidence for the involvement of this amino acid in the interaction between the inhibitor and high affinity heparin.

REFERENCES

Important role of arginine 129 in heparin-binding site of antithrombin III. Identification of a novel mutation arginine 129 to glutamine.
S Gandrille, M Aiach, D A Lane, D Vidaud, P Molho-Sabatier, R Caso, P de Moerloose, J N Fiessinger and E Clauser


Access the most updated version of this article at http://www.jbc.org/content/265/31/18997

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/265/31/18997.full.html#ref-list-1