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Summary – Antithrombin III is a plasma glycoprotein responsible for thrombin inhibition in the blood coagulation cascade. The X-ray structure of its cleaved form has been determined and refined to 3.2 Å resolution. The overall topology is similar to that of α_1-antitrypsin, another member of the serpin (serine protease inhibitor) superfamily. The biological activity of antithrombin III is mediated by a polysaccharide, heparin. The binding site of this effector is described. A possible structural transition from the native to the cleaved structure is discussed.

antithrombin III / X-ray structure / heparin / serpin superfamily

Introduction

Antithrombin III (ATIII) is a single chain glycoprotein involved in the coagulation process. It is synthesized primarily in the liver with a signal peptide of 32 amino acids necessary for its intracellular transport through the endoplasmatic reticulum; the peptide is then cleaved prior to secretion [1]. Antithrombin III has a normal plasma concentration of $\approx 2\ \mu M$ [2].

ATIII inhibits serine proteases of the intrinsic coagulation pathway such as Factor IXa, Xa and XIa and thrombin [3] which is its principal physiological target considering the relative rates of inactivation [4]. Thus, functional deficiency of antithrombin III results in a predisposition towards thrombosis and pulmonary embolism. Inhibition of thrombin by ATIII occurs through the formation of a stable equimolar complex between inhibitor and protease [3, 5] involving the formation of a possible covalent bond between 2 residues. This tight complex can only be dissociated by nucleophilic agents or at alkaline pH and the released antithrombin III is cleaved at the reactive center site Arg393-Ser394 (sequence numbering of human antithrombin III unless otherwise stated) [6]. This modified inhibitor has lost all or most of its ability to inhibit thrombin and is made up of 2 polypeptide fragments held together by a disulfide bridge [7].

The rate of thrombin inactivation is enhanced by catalytic amounts of a polysaccharide, heparin, without affecting the stoichiometry of the enzyme–inhibitor reaction [8, 9]. A unique pentasaccharide sequence of heparin is required for the specific interaction with antithrombin III [10]. Furthermore, this pentasaccharide, which presents the same activity as heparin for the inhibition of Factor Xa, has been chemically synthetized [11, 12]. However, the size of the oligosaccharide that can enhance thrombin inhibition by ATIII is at least 18 monosaccharide units long [13].

The primary structure of human ATIII (MW 58000 Da) was elucidated both by protein and cDNA sequencing [14, 15]. Based on the close similarity between their primary structures, several proteins have been classified in the so-called serpin (serine protease inhibitor) superfamily [16]. This includes inhibitors of serine proteases like α_1-antitrypsin, as well as, for example, ovalbumin, of no known anti-protease activity [17] and some hormone carriers. Sequence alignment within this family led to the hypothesis that all its members had evolved from a common ancestor [18]. The crystal structure of the cleaved α_1-antitrypsin was determined at 3.0 Å resolution [19].

The binding site of heparin in ATIII is located in the N-terminal domain of the protein. Some residues which are essential for heparin binding to ATIII were identified using chemical affinity labeling techniques, others through genetic studies of natural variants unable to bind heparin. Many antithrombin III variants with functional deficiency are known and have been classified [20].

The reaction of heparin with antithrombin is of clear clinical importance. The X-ray structure determination of bovine ATIII was undertaken in order to study such

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Abbreviations: ATIII, antithrombin III; α_1-AT, α_1-antitrypsin

interaction at the molecular level, and to provide structural information for the design of chemical analogs of heparin. On the other hand, knowledge of the thrombin binding site would allow rational site directed mutagenesis experiments to be carried out. ATIII is thus an exemplary system for drug design and genetic engineering. We describe the preliminary refinement of the structure as determined by X-ray, and review issues concerning the structure–function relationships of ATIII.

Crystallization and data collection

Bovine antithrombin III (MW 56 kDa) was purified from plasma by affinity chromatography on heparin–sepharose [21]. Activity assays of the purified protein against thrombin were normal. ATIII crystals were obtained by the microdialysis method at 4°C with ammonium sulphate as a precipitating agent, and in the presence of divalent cations [22]. The crystalline material was characterized by SDS–polyacrylamide gel electrophoresis and N-terminal amino acid sequencing. The protein in the crystal appeared to be cleaved at residue Leu395, one amino acid away from the known reactive site (Arg393-Ser394).

The large unit cell necessitated the use of X-ray synchrotron radiation (fig 1). Data were collected on oscillation photographs at Lure (Orsay, France) and Chess (Cornell University, Ithaca, NY, USA). The diffraction limit was 3.0 Å. One platinum derivative isomorphous to 4.5 Å resolution to the native data was prepared. The crystal and intensity data are presented in table IA and IB.

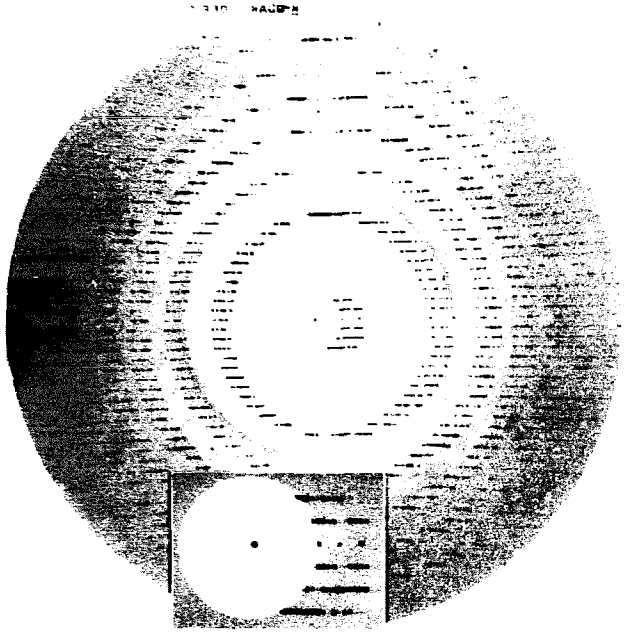

Fig 1. 2° Oscillation photograph of native crystal of ATIII. The crystal was oriented with the long axis (c axis) perpendicular to the incident beam. An enlargment of the central zone is shown.

Table IA. Crystal data. The diffraction limit is 3.0 Å.

Space group $P4_32_12$	
a = 91.3 (Å)	α = 90°
b = 91.3 (Å)	β = 90°
c = 383.1 (Å)	γ = 90°
2 molecules / asymmetric unit	
V = 3.193 10^6 (Å^3)	

Table IB. Intensity data statistics.

Derivative	*Native*	*PtCl*[a]
Resolution (Å)	3.2	3.5
Independent reflections	23500	14500
Independent reflections at 3.5 Å	20300	14500
Ratio of measured to possible reflections	0.94	0.70
R	0.092	0.076

[a]Native crystals soaked with a 2 mM solution of $PtCl_4^{2-}$ for 3 days.

Structure determination

From the 33% amino acid sequence homology between ATIII and α_1-AT, the assumption was made that the core of these 2 serpins might have a homologous 3-dimensional structure. The molecular replacement method using α_1-AT structure as a model turned out to be successful [23]. Briefly, the rotation function was determined for each of the 2 independent molecules of the asymmetric unit using the fast rotation function [24] with a wide range of conditions tested. The Crowther and Blow translation function [25] proved to be very discriminative for the different possible orientations. The correct solution was refined by rigid body using the Corels program [26]. The 2 molecules were related by a screw axis (κ = 123°, t = 42.2 Å). The phases calculated from this model were used to compute a Fourier difference map of the platinum derivative (PTCL in table IB).

This revealed 3 platinum sites which satisfied the Patterson function. The heavy atom positions were located near cysteine residues at the surface of the protein. Phasing was improved by solvent flattening and non crystallographic symmetry averaging.

Further real space density modification combined with phase extension is now under investigation to provide a map which is free of any bias introduced by the initial model.

Model building and crystallographic refinement

The starting model for crystallographic refinement was the CORELS solution with the α_1-AT molecular fold [19]. The bovine protein sequence was under determination at that time, but very high sequence homology was suggested between human and bovine ATIII [6, 27]. We thus used the human ATIII sequence without taking into account insertions–deletions, which were all located in loop regions. Forty-four residues in the N-terminal part of ATIII were model-built, taking advantage of the presence of 2 disulfide bridges in that region and taking into account secondary structure prediction, distance constraints and molecular compactness. X-Ray refinement using energy minimization and 1 ps molecular dynamics simulation with the GROMOS program [28] led to an R-factor of 32% at 3.6 Å resolution [23]. This model was inspected on an Evans and Sutherland PS390 graphics system with the FRODO program [29] using an OMIT Fourier map [30]. At that time, most of the amino acid sequence of bovine ATIII (90% complete) was introduced*. Further crystallographic refinement using GROMOS was pursued on the VP200 at CIRCE (Paris, France), which led to an R-factor of 28% between 8–3.2 Å (table II and fig 2). A detailed description of the refinement will be published later.

Table II. Results of energy minimization and molecular dynamics in crystallographic refinement of bovine ATIII.

Step	*Resolution (Å)*	*t (ps)*	*R-factor (%)*
EMX[a]	20–3.6	–	start 52 final 42
MDX[b]	20–3.6	1	32
Construction with 90% of the bovine sequence:			
EMX	8–3.6		start 48 final 35
MDX	8–3.6	2.5	27
MDX	8–3.2	4.0	28

[a]EMX, energy minimization; MDX, molecular dynamics simulation (both using a crystallographic potential). [b]The time step for all molecular dynamic rounds was 0.002 ps.

Antithrombin III and the serpin superfamily

The sequence of bovine ATIII displays very high homology (≈ 90%) with the human protein. Thus, the structure description of the former can be applied to the human protein. The secreted human ATIII contains 432 amino acids, 6 of which are cysteines that form 3 disulfide bridges which are conserved in the bovine protein: Cys8-Cys128, Cys21-Cys95, Cys247-Cys430.

Sequence alignments of human ATIII with 3 other members of the serpin family are shown in table III. Human or bovine ATIII and α_1-AT can be aligned with few insertions–deletions: 14 insertions and 1 deletion have to be made when comparing α_1-AT to ATIII. Almost all of them occur in connecting loops between secondary structural elements, except for the insertions of Arg399, Val400 and Thr401 which occur inside strand 1C. The overall topology of ATIII (fig 3) resembles that of α_1-antitrypsin with helices, mainly located in the N-terminal domain (1-200), and 3 β sheets. The ATIII specific N-terminal extension (44 residues) contains helices and loops. Both structures represent cleaved molecules. α_1-AT was intentionally modified for X-ray structure determination at the reactive site peptide bond Met358-Ser359 (α_1-AT numbering) while ATIII appeared to be cleaved after characterization of the crystals [22] one residue away (Leu395) from the known reactive site. Nevertheless, and as in the structure of α_1-AT, the 2 residues (*ie* Arg394 and Leu395) are separated by ≈ 60Å and are at opposite ends of the molecule. After the cleavage site, the C-terminal part Leu395-Lys432 of ATIII is linked to the core of the molecule by a disulfide bridge.

Two other proteins are shown in table III: ovalbumin and corticosteroid binding globulin. Ovalbumin, which has no known anti-protease activity, has 28% sequence homology to ATIII. The X-ray structure determination of its native form is currently being investigated [31]. In the crystal structure of plakalbumin (the nicked form of ovalbumin), a folding similar to that of α_1-AT is found, except for the topology of the central β sheet, where strand 4A is lost and 2 adjacent strands are displaced [32]. A 3-dimensional model of corticosteroid binding globulin (26% sequence homology to ATIII),

*Reinbolt J, Medjoub A (1990) personal communication

Table III. Alignment of amino acid sequences of 4 members of the serpin family after Huber and Carrell [62]. *Abbreviations:* hATIII, human antithrombin III; hA1PI, human α_1-proteinase inhibitor (α_1-antitrypsin); cOVAL, chicken ovalbumin; hCBG, human corticosteroid binding globulin. Numbering is that of human antithrombin III and the secondary structural elements of α_1-antitrypsin [19] are indicated in the bottom line. On the top line: * ATIII variant with functional abnormality limited to the heparin binding site; ¤ ATIII variant with functional abnormality limited to the reactive site or a variant that affects the integrity of the overall structure; # residue which has been mapped by chemical affinity labeling techniques or predicted to be located in the heparin binding site. The reactive centre P1 residue is also indicated.

```
                                                    1        10         20
                                                             *
hATIII   1.  mysnvigtvtsgkrkvyllsllligfwdcvtcHGSPVDICTAKPRDIPMNPMCIY
hA1PI    2.                                          mpssvswgilllaglcclvpvs
cOVAL    3.
hCBG     4.                                                   mplllytcllw
sec. struc.

                    30         40         50         60         70
                               *      * #
hATIII   1.  RSPEKKATEDEGSEQKIPEATNRRVWELSKANSRFATTFYQHLADSKNDNDNIFL
hA1PI    2.  laEDPQGDAAQKTDTSHHDQDHPTFNKITPNLAEFAFSLYRQLAHQSNS-TNIFF
cOVAL    3.                              MGSIGAASMEFCFDVFKELKVHHAN-ENIFY
hCBG     4.  lptsglwtvqaMDPNAAYVNMSNHHRGLASANVDFAFSLYKHLVALSPK-KNIFI
sec. struc.                        <-----------hA----------->     <s6B>

               80         90        100        110                120
                                               #                  #
hATIII   1.  SPLSISTAFAMTKLGACNDTLQQLMEVFKFDTIS---------EKTSDQIHFFFA
hA1PI    2.  SPVSIATAFAMLSLGTKADTHDEILEGLNFN-LT---------EIPEAQIHEGFQ
cOVAL    3.  CPIAIMSALAMVYLGAKDSTRTQINKVVRFDKLPGFGDSIEAQCGTSVNVHSSLR
hCBG     4.  SPVSISMALAMLSLGTCGHTRAQLLQGLGFN-LT---------ERSETEIHQGFQ
sec. struc. <-----hB----->   <----hC---->               <------hD---

                  130        140        150        160        170
                 #          #
hATIII   1.  KLNCRLYRKANKSSKLVSANRLFGDKSLTFNETYQDISELVYGAKLQPLDFKENA
hA1PI    2.  ELLRTLNQPDS-QLQLTTDGGLFLSEGLKLVDKFLEDVKKLYHSEAFTVNFGDT-
cOVAL    3.  DILNQITKPND-VYSFSLASRLYAEERYPILPEYLQCVKELYRGGLEPINFQTAA
hCBG     4.  HLHQLFAKSDT-SLEMTMGNALFLDGSLELLESFSADIKHYYESEVLAMNFQDW-
sec. struc. -------->     <----s2A--->     <-----hE----><-s1A->    <

            180        190        200        210        220        230

hATIII   1.  EQSRAAINKWVSNKTEGRITDVIPSEAINELTVLVLVNTIYFKGLWKSKFSPENT
hA1PI    2.  EEAKKQINDYVEKGTQGKIVDLV--KELDRDTVFALVNYIFFKGKWERPFEVKDT
cOVAL    3.  DQARELINSWVESQTNGIIRNVLQPSSVDSQTAMVLVNAIVFKGLWEKAFKDEDT
hCBG     4.  ATASRQINSYVKNKTQGKIVDLF--SGLDSPAILVLVNYIFFKGTWTQPFDLAST
sec. struc. -------hF------>                  <-----s3A---->

                  240        250        260        270        280

hATIII   1.  RKELFYKADGESCSASMMYQEGKFRYRRVAE-GTQVLELPFKGDDITMVLILPKP
hA1PI    2.  EEEDFHVDQVTTVKVPMMKRLGMFNIQHCKKLSSWVLLMKYLG-NATAIFFLPD-
cOVAL    3.  QAMPFRVTEQESKPVQMMYQIGLFRVASMASEKMKILELPFASGTMSMLVLLPDE
hCBG     4.  REENFYVDETTVVKVPMMLQSSTISYLHDSELPCQLVQMNYVG-NGTVFFILPD-
sec. struc.            <----s3C---> <-s1B->  <--s2B-->  <--s3B-->
```

```
                290        300        310        320        330        340
                  #    #  #
hATIII    1.  EKSLAKVEKELTPEVLQEWLDEL--EEMMLVVHMPRFRIEDGFSLKEQLQDMGLV
hA1PI     2.  EGKLQHLENELTHDIITKFLENE--DRRSASLHLPKLSITGTYDLKSVLGQLGIT
cOVAL     3.  VSGLEQLESIINFEKLTEWTSSNVMEERKIKVYLPRMKMEEKYNLTSVLMAMGIT
hCBG      4.  KGKMNTVIAALSRDTINRWSAGL--TSSQVDLYIPKVTISGVYDLGDVLEEMGIA
sec. struc.    <-hG->    <---hH-->          <--s2C--><-s6A-><--hI-->

                     350        360        370        380        390 P1
                                                          ¤          ¤¤
hATIII    1.  DLFSPEKSKLPGIVAEGRDDLYVSDAFHKAFLEVNEEGSEAAASTAVVIAGRSLN
hA1PI     2.  KVFSNG-ADLSGVTEEA--PLKLSKAVHKAVLTIDEKGTEAAGAMFLEAIPMSIP
cOVAL     3.  DVFSSS-ANLSGISSAE--SLKISQAVHAAHAEINEAGREVVGSAEAGVDAASVS
hCBG      4.  DLFTNQ-ANFSRITQDA--QLKSSKVVHKAVLQLNEEGVDTAGSTGVTLNLTSKP
sec. struc.                          <-----s5A-----><-----s4A---->

                400        410        420        430
                           ¤
hATIII    1.  PNRVTFKANRPFLVFIREVPLNTIIFMGRVANPCVK
hA1PI     2.  PE---VKFNKPFVFLMIEQNTKSPLFMGKVVNPTQK
cOVAL     3.  EE---FRADHPFLFCIKHIATNAVLFFGRCVSP
hCBG      4.  II---LRFNQPFIIMIFDHFTWSSLFLARVMNPV
sec. struc. <--s1C--> <--s4B--> <---s5B--->
```

a plasma corticosteroid carrier protein, has been proposed on the basis of the known α_1-AT structure [33].

Carbohydrate

There are 4 glycosylation sites in human ATIII. Three of them, Asn96, 155 and 192, have been identified in the bovine ATIII sequence. They are located at the surface of the protein, their side-chain pointing towards the solvent. Asn96 is located in a loop between 2 helices. Asn155 lies in the N-terminal part of a helix and Asn192 is located at the C-terminal part of another helix. The fourth (Asn135) belongs to a region of the polypeptide chain which has not yet been sequenced. In 2 cases (Asn155 and Asn192), there is electron density at the expected locations that might account for the carbohydrate chains (fig 4).

The location of the carbohydrate chains are of clear importance concerning the structure–function relationships in antithrombin III. There is experimental evidence concerning ATIII variants of increased or decreased heparin affinity: one of them, antithrombin β, is deprived of its carbohydrate side chain at position Asn135 and has been shown to display a higher affinity to heparin [34]. On the contrary, the mutation at position 7 from Ile to Asn, which is then subject to glycosylation, leads to a decreased affinity for heparin [35]. It is clear that these carbohydrate chains are in the neighborhood of the heparin binding site in antithrombin III. The description of their conformations and interactions will be of interest with respect to functional and crystal packing effects. We observed during the crystallization assays that the results were dependent upon the protein batches and the microheterogeneity of the carbohydrate contents.

Antithrombin pathology

Three goals might be aimed at in the design of site-directed mutagenesis experiments on ATIII: i) to stabilize the native structure; ii) identify the residues involved in thrombin and heparin recognition and binding; iii) characterize the essential functional amino acids. Important information is already provided by natural mutants characterized by protein or cDNA sequencing. They all show single amino acid substitution.

ATIII deficiency can be classified as follows into 2 types and 5 subtypes [1, 20]:

Type I: low functional and immunological antithrombin
— subtype Ia: reduced synthesis and / or increased turnover of a normal molecule;

— subtype Ia: reduced synthesis and/or increased turnover of a normal molecule;
— subtype Ib: reduced synthesis and/or turnover of antithrombin with abnormal heparin binding properties.

Type II: low functional, but normal immunological antithrombin
— subtype IIa: functional abnormalities affecting both the reactive site and the heparin binding site;
— subtype IIb: functional abnormalities limited to the reactive site;
— subtype IIc: functional abnormalities limited to the heparin binding site.

Thrombin binding deficiency

The following mutations, resulting in ATIII deprived of thrombin inhibition, have been characterized:

— antithrombin Northwick Park, Arg393 to Cys [36];
— antithrombin Glasgow, Arg393 to His [37];
— antithrombin Denver, Ser394 to Leu [38];
— antithrombin Utah, Pro407 to Leu [39];
— antithrombin Hamilton, Ala382 to Thr [40].

Three of these mutations occur at the P1 and P1′ positions (P1, P1′ define the ATIII reactive site Arg393-Ser394). Interestingly, it was shown for α_1-AT that the protease selectivity could be changed by a single mutation: a genetically engineered α_1-AT, where the Met to Arg mutation at P1 position was made, transformed the anti-elastase inhibitor to an anti-thrombin inhibitor [41].

Heparin binding deficiency

The rate of association of ATIII with thrombin and factor Xa is increased by 3–4 orders of magnitude

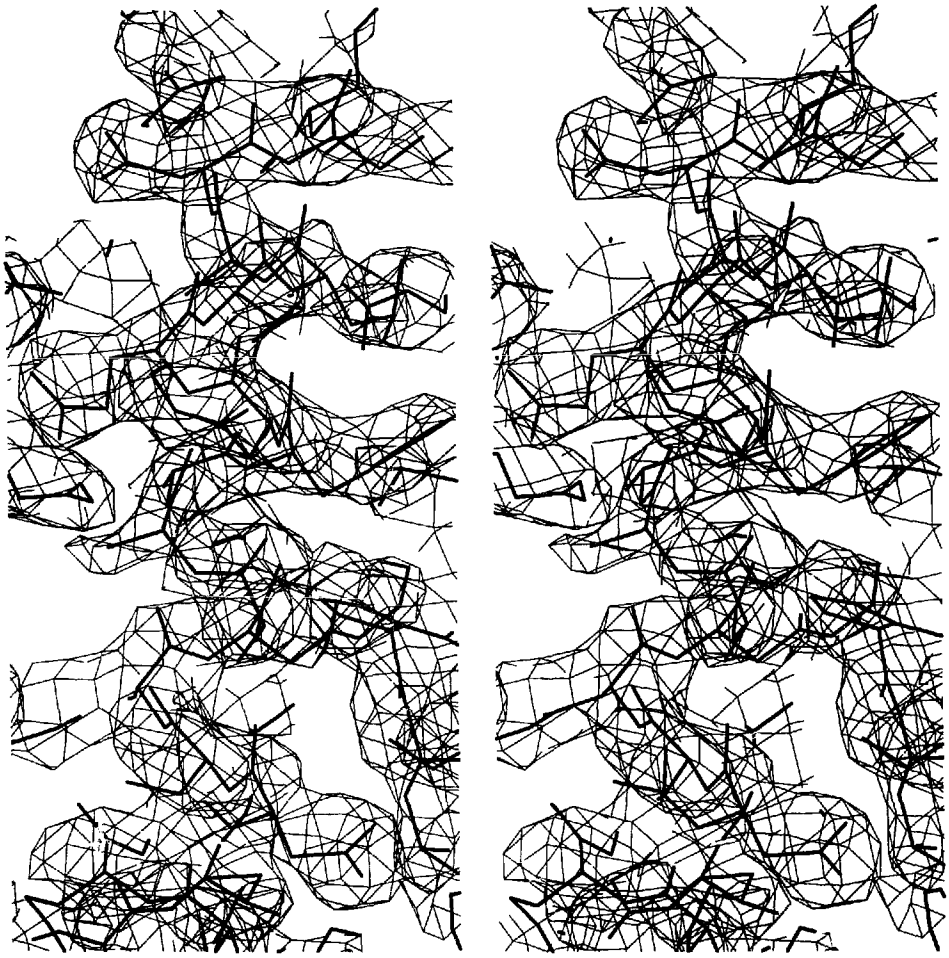

Fig 2. Electron density (OMIT map) of a helical portion of bovine ATIII. The model results from molecular dynamics refinement at 3.2 Å (R-factor = 28%).

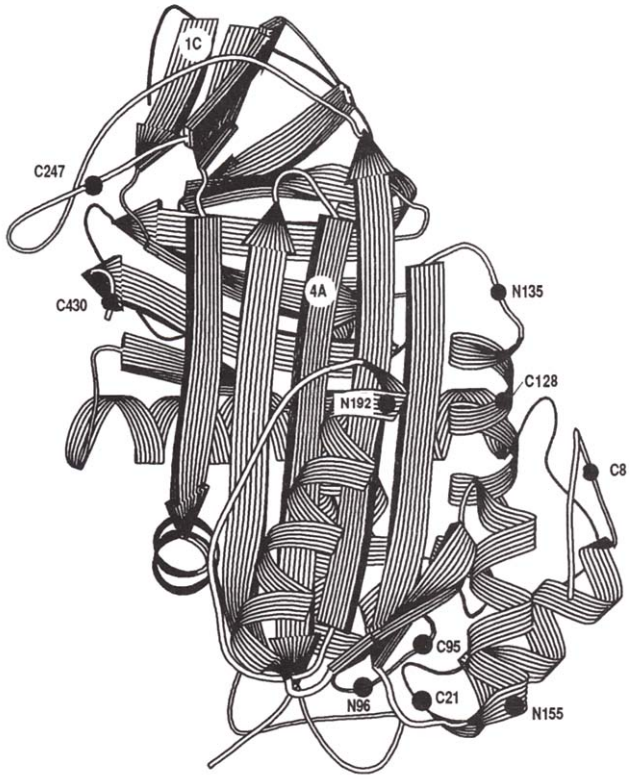

Fig 3. Schematic representation of ATIII using the RIBBON program [64]. Residues 394 and 395 (the cleavage site) are respectively found at the C-terminal end of strand 4A and at the N-terminal part of strand 1C. Strands 4A and 1C are labelled according to the structural element classification of α_1-AT. The 3 disulfide bridges, C8-C128, C21-C95 and C247-C430, and the 4 glycosylation sites (N96, N135, N155 and N192) are visible in this orientation.

upon binding of heparin [8, 42]. The single pentasaccharide sequence responsible for the specific heparin binding to ATIII was determined [10, 11]. Some ATIII amino acids involved in the binding site site of heparin were identified in congenital variants of ATIII with decreased heparin affinity (subtype IIc; after Finazzi *et al* [20]):

— antithrombin Toyama, Arg47 to cys [43];
— antithrombin Rouen I, Arg47 to His [44];
— antithrombin Rouen II, Arg47 to Ser [45];
— antithrombin Basel, Pro41 to Leu [46];
— antithrombin Rouen III, Ile7 to Asn [35].

The binding of heparin to ATIII is mediated through the interaction of some specific sulphate groups of heparin with basic amino acids of ATIII. Indeed, several of these residues were identified as essential for heparin binding: Lys107 [47], Lys114 [48], Lys125 [49] and Lys136 [47]. Three other lysine residues, Lys290, 294 and 297, were predicted as also being close to the heparin binding site [50]. It has been shown by chemical modification studies that tryptophan 49 is also involved [51]. These essential residues for heparin binding are displayed on the 3-dimensional structure of ATIII in figure 5. They form a large contact area, wrapped around the molecule from Ile7 to Lys297.

Kinetically, heparin binding to ATIII occurs as a 2-step reaction with a dissociation constant of $4.3\ 10^{-5}$ M for the initial binding step. Complexed ATIII then undergoes a conformational change which leads to a 300-fold increase in affinity of heparin for ATIII [52]. Furthermore, it has been shown that the binding constant for the low affinity state is the same as that for cleaved antithrombin [53]. Thus, it is postulated that the cleaved molecule cannot undergo the conformational change which leads to the high affinity state. This conformational change of ATIII might also modify the reactive site. Another explanation for the heparin accelerating effect of ATIII-thrombin formation arises from the hypothesis that a ternary complex thrombin–heparin–ATIII is formed [54], in which heparin behaves as a template in order to bring the active site of the protease into close contact to the reactive site of ATIII [55]. However, such a mechanism cannot account for factor Xa activation, which is known to be due to a conformational change.

Discussion and Conclusion

The structure–function relationships in antithrombin III mainly concern 2 aspects: the double specificity of the molecule; and its relationships to the serpin family.

Obviously, the full molecular recognition process between ATIII and heparin requires knowledge of the native structure of the inhibitor. Thus 2 questions to be answered are: what does the native structure look like; and how can the drastic conformational change that occurs upon cleavage be explained?

It has been shown from denaturation experiments that some members of the serpin superfamily (*eg* ATIII and α_1-AT) undergo an increase in heat stability upon cleavage [18, 56–58], while others such as ovalbumin and angiotensinogen do not [59]. Further experiments on the intact and proteolytically modified forms of these inhibitors have been performed using circular dichroism and fluorescence spectra [60] and ^{1}H NMR spectroscopy [61]. When heat stability of the cleaved form has been observed, the hypothesis of a native stressed (S) and cleaved relaxed (R) structures has been postulated [18, 56, 57]. The corresponding structural hypothesis was that in their native form, these

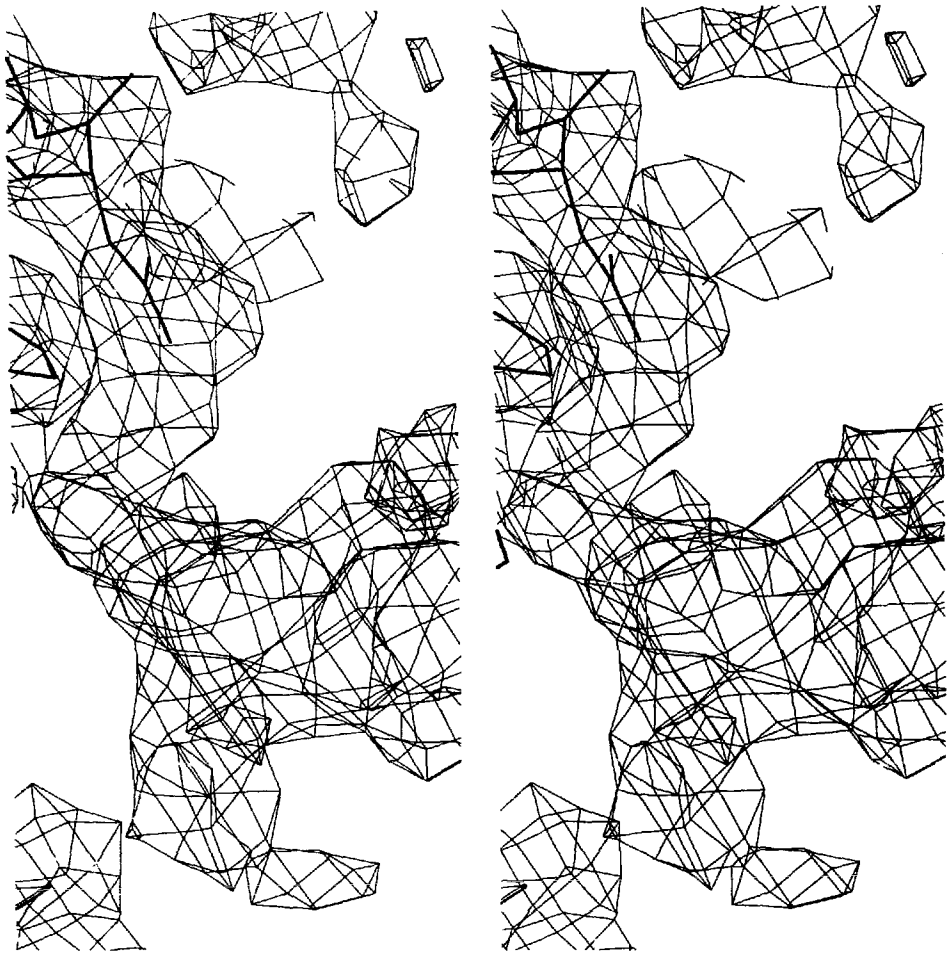

Fig 4. Electron density in the vicinity of Asn192 side chain. The density which clearly extends towards the solvent corresponds to the carbohydrate chain.

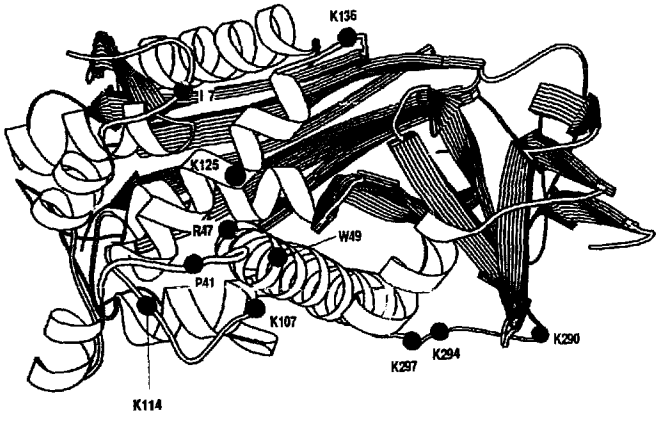

Fig 5. Ribbon schematic representation of ATIII. The amino acids involved in heparin binding are shown (ATIII sequence numbering and one letter code amino acid symbols).

proteins possess an accessible loop built up after extraction of strand 4A from the main pleated sheet [19, 62] (see fig 2). This loop would fold in sheet A in the (R) structure. However, the nature of the structural transition proposed and the fact that it cannot explain some punctual mutations (*eg* Ala382 to Thr, Pro407 to Leu) justify the fact that alternative models should not be underestimated. Toma *et al* [63] proposed a model of the native structure of protein C inhibitor, a member of the serpin family which inhibits activated protein C. The reconstruction, based on the cleaved α_1-AT structure, involves a movement of serine 359 together with strand 1C towards Met358 (α_1-AT numbering). An approach based on small modifications of the cleaved structure of ATIII was performed and led to a model of native ATIII which does not alter the overall topology of the molecule and allows explanation of biochemical data and mutants with functional deficiency (unpublished results).

In conclusion, we believe that the structural nature of the native state of ATIII and other members of the serpin family remains an open question.

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