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PROTHROMBIN *and* OTHER VITAMIN K PROTEINS

Volume II

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CRC PRESS

Prothrombin and Other Vitamin K Proteins

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CRC Press, Inc.
Boca Raton, Florida

Library of Congress Cataloging in Publication Data
Main entry under title:

Prothrombin and other vitamin K proteins.
Includes bibliographies and index.
1. Prothrombin. 2. Vitamin K. 3.Blood coagulation factors. I. Seegers, Walter H. (Walter Henry), 1910- II. Walz, David A. [DNLM: 1. Prothrombin—metabolism. 2. Vitamin K--metabolism. WH 310 P9667]
QP93.5.P767 1986 615'.718 85-7869
ISBN 0-8493-6308-X (v. 1)
ISBN 0-8493-6309-8 (v. 2)

Direct all inquiries to CRC Press, Inc., 2000 Corporate Blvd., N.W., Boca Raton, Florida, 33431.

International Standard Book Number 0-8493-6308-X (v. 1)
International Standard Book Number 0-8493-6309-8 (v. 2)

Library of Congress Card Number 85-7869
Printed in the United States

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Walter Seegers contributed research on nutrition and reproductive biology, and to the isolation of enzymes from blood. He achieved the industrial production of thrombin as an hemostatic agent, and contributed to understanding biochemical abnormalities in blood coagulation, participated in the discovery of blood coagulation factors, and conducted research on blood platelets. He collaborated with University Professor John M. Dorsey, M.D. in authoring the book *Living Consciously: The Science of Self.*

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Dr. Walz's research interests include protein structure, particularly of those proteins involved in blood coagulation, platelet secreted proteins, and the chromatographic separation of proteins and peptides.

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Chapter 1

ANTITHROMBIN III: PROPERTIES AND FUNCTION

L. Róka and Th. Eckhardt

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I. INTRODUCTION

Blood is able to inactivate thrombin. This phenomenon was first demonstrated by Morawitz. Shortly after spontaneous clotting of blood, in vitro thrombin activity is no longer detectable in the serum.[1] The thrombin inactivating capacity of plasma is due to different components which Seegers has characterized and designated antithrombin I to VI (AT-I to AT-VI) and which are complied in Table 1.[2] It was established that AT-II and AT-III are identical, representing one single molecule exerting its inhibitory effect under different experimental conditions.[8] This unique molecule, which has kept the term AT-III, is the most important of all thrombin inhibitors in plasma. It should be pointed out, however, that various other serine proteases besides thrombin are irreversibly inactivated by AT-III (Table 2). On the other hand, eight components differing in substrate specificity are known to inactivate plasma serine proteases (Table 3).

II. MOLECULAR STRUCTURE

AT-III is synthesized in the liver. The human gene for AT-III synthesis has been isolated.[19] Analysis of the gene structure reveals that a proantithrombin molecule with a signal peptide consisting of 32 amino acid residues residing in the N terminus is synthesized. After the signal peptide is split off, AT-III is a single-chain glycoprotein with a molecular weight of 65,000 daltons.

The AT-III molecule consists of 432 amino acid residues and four oligosaccharide side chains which are attached to the protein core via asparagine residues 96, 135, 155, and 192, respectively (Figure 1).[20] Secondary structure is established by three distinct disulfide bridges (Cys 8-Cys 128, Cys 21-Cys 95, Cys 247-Cys 430). Tertiary structure reveals 31% α-helix, 16% β-sheet, 9% β-turns, and 44% random coil (Figure 2).[21] AT-III shares significant homology in primary structure with α_1-antiprotease, ovalbumin, angiotensinogen,[22] and possibly heparin cofactor II, as well.

III. BIOLOGICAL FUNCTION

A. Mechanism of Action

The biological function of AT-III is the irreversible inactivation and binding of various serine proteases. One molecule of a serine protease cleaves the peptide bond Arg 393-Ser 394 of AT-III (see Figure 3), which is located close to the C terminus within the disulfide ring Cys 247-Cys 430. Cleavage of this peptide bond induces formation of a permanent acyl binding between AT-III via the carboxy group of Arg 393 and the hydroxyl group of Ser at the active site of the protease. This implies that the serine residue at the active site of the protease is blocked and that the cleaved AT-III molecule remains bound within this complex, both molecules being unavailable for further reactions.[23] The cleaved C-terminal peptide of AT-III remains attached to the parent molecule even after cleavage of the disulfide bond Cys 247-430. During the reaction between AT-III and protease, complex formation prevails. It could be demonstrated however that an AT-III derivative, in which peptide bond Arg 393-Ser 394 is cleaved, is formed as well, resulting from hydrolytic cleavage of the acyl bond previously formed.[24] After the acyl binding has been hydrolyzed the modified AT-III molecule does not regain inhibitory properties, whereas the protease is liberated in its active form. This secondary reaction was pointed out by Seegers in 1960.[25] AT-III is susceptible to proteolytic attack by PMN elastase, which cleaves the peptide bond Arg 393-Ser 394 within the AT-III molecule without losing its own proteolytic activity.[26] Certain snake venom proteases act in a similar fashion.

Table 1
DIFFERENT ANTITHROMBIN (AT)-ACTIVITIES

		Ref.
AT-I	Fibrin clot adsorbs thrombin	3
AT-II	Heparin cofactor	4
AT-III	Progressive antithrombin	5
AT-IV	Inhibitor activity derived from prothrombin activation	2
AT-V	Immunologic inhibition	6
AT-VI	Fibrin split products	7

Table 2
SERINE PROTEASES INACTIVATED AND NOT INACTIVATED BY AT-III

Serine Protease	Ref.
Inactivated by AT-III	
Thrombin	9
F Xa	10
F IXa	11
F XIa	12
F XIIa	13
Plasmin	8
Kallikrein	14
Urokinase	15
Trypsin	8
m β-Acrosin	16
Cls	17
Not inactivated by AT-III	
F VIIa	18

Table 3
PLASMA PROTEASE INHIBITORS

Name	Concentration $\times 10^{-6}$ M/ℓ	Inhibitor activity IU/mℓ (25°C)	Inhibitor reactive site
α-1-Protease inhibitor	25		Met-Ser
α-1-Antichymotrypsin	6		Leu-Ser
α-2-Macroglobulin	3.5		Arg-Val
Antithrombin III	2.3—3.1	11.4 ± 1.6	Arg-Ser
Inter-α-trypsin inhibitor	3.0		—
C-1 inhibitor	1.8		—
α-2-Plasmin inhibitor	0.9		Leu-Met
Heparin cofactor II	0.4—0.6	3.8 ± 0.6	—

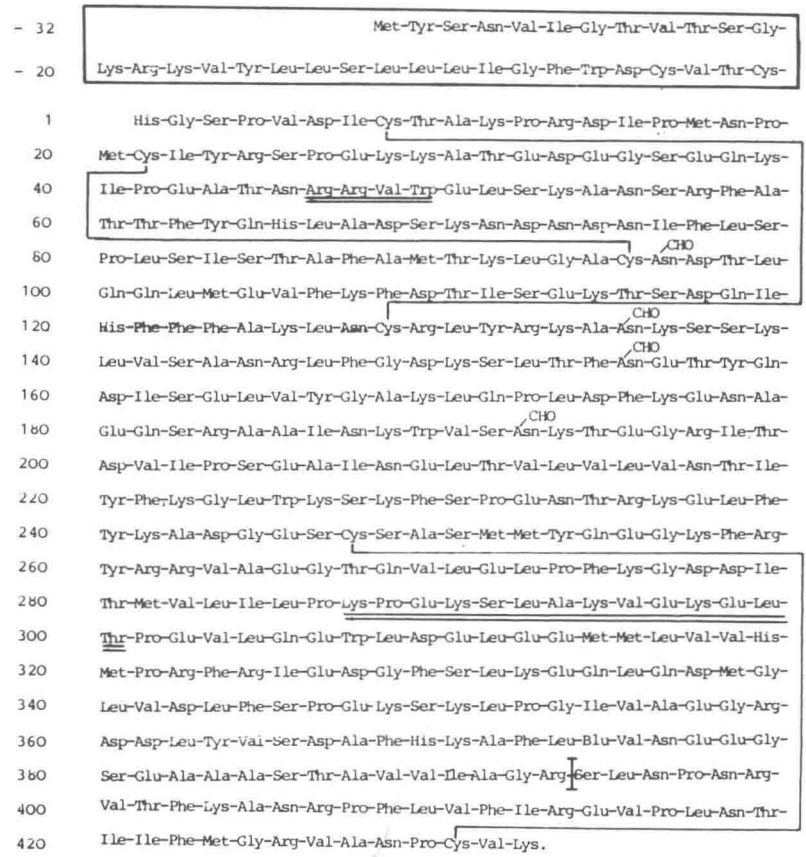

FIGURE 1. Amino acid sequence of AT-III with signal peptide −32 to −1. Disulfide bridges: Cys-Cys. Polysaccharide side chains: CHO. Bond split by serine proteases at Arg 393-Ser 394. Binding domains for heparin double underlined.

B. Interaction with Heparin

The reaction between AT-III and various serine proteases is greatly enhanced by heparin. This is due to equimolar reversible binding of heparin to AT-III, which induces a crucial conformational change within the AT-III structure.[27] Two distinct domains within the AT-III molecule which have been shown to bind heparin are located within the N- and the C-terminal region, respectively. The heparin binding site at the carboxyterminus involves residues 287-300.[21] This sequence is part of an α-helix structure which unfolds during denaturation or by cleaving the disulfide ring Cys 247-Cys 430. In its unfolded configuration this region no longer binds to heparin. Heparin binds to AT-III by means of an octasaccharide structure (Figure 4). Binding is mediated by four sulfate groups of glucosamine residues in positions 2, 4, 6, and 8 of the octasaccharide.

The N-terminal heparin binding site consists of amino acid residues Arg 47-Trp 49.[28] This is inferred from data on AT-III "Toyama" which is unable to bind heparin. Amino acid sequence analysis of this AT-III variant revealed that Arg 47 is replaced by Cys 47.[29] This conceivably alters the sterical exposure of Trp 49 which was shown to be crucial for heparin binding.[28] Heparin binding is lost by cleavage of disulfide bonds.[30] Whereas the heparin-antithrombin III complex is able to accelerate the inhibition of Factor Xa, plasmin, and presumably also other proteases, this does not apply for thrombin. In this case accelerated inhibition affords a thrombin-heparin complex.[31,32] At physiologic pH the acceleration of the reaction between AT-III and thrombin requires only catalytic amounts of heparin, because

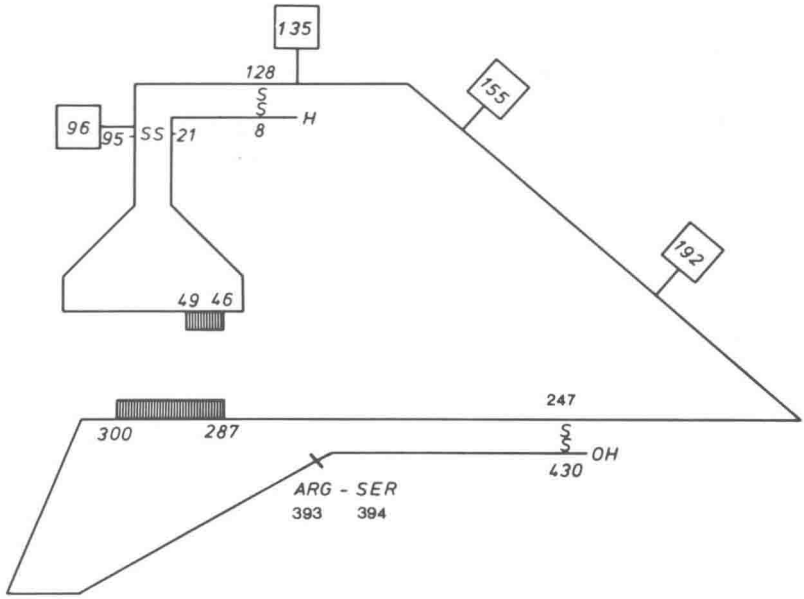

FIGURE 2. Schematic presentation of the AT-III molecule with three disulfide bridges (−SS−), and four oligosaccharide residues at Asn 96, 135, 155, and 192. Peptide bond cleaved by proteases at Arg 393-Ser 394 and two heparin binding domains 46—49 and 287—300.

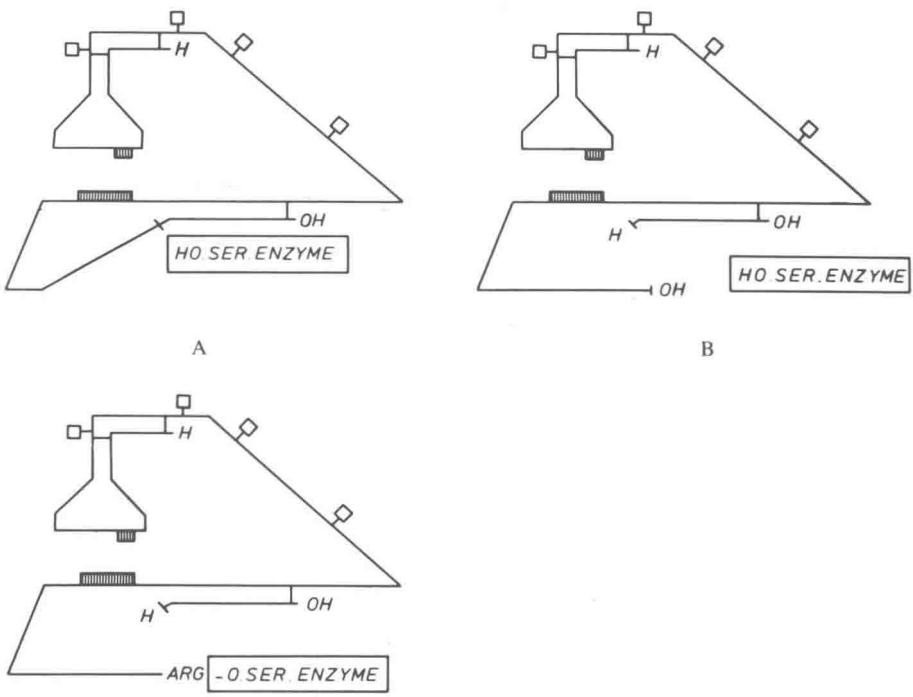

FIGURE 3. Reaction between protease and AT-III. (A) Protease cleaves peptide bond Arg 393-Ser 394; (B) AT-III-protease complex is established by acyl binding in which both partners are inactive; and (C) protease is reactivated by cleavage of acyl bond, whereas the two-chain derivative of AT-III stays inactive.

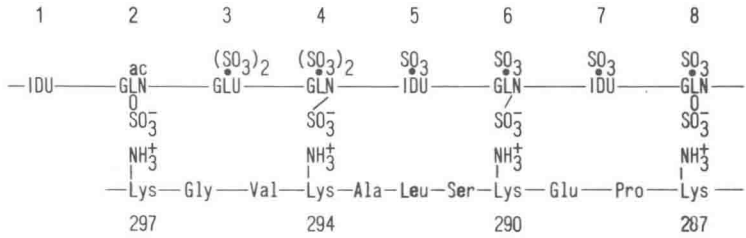

FIGURE 4. Octasaccharide (1-8)-fragment of heparin, which binds to the C-terminal binding domain (Lys 287-Lys 297) of AT-III. Position 1: α-L-iduronic acid; Position 2: *N*-acetyl-D-glucosamine-6-sulfate; Position 3: β-D-glucuronic acid; Position 4: *N*-sulfate-D-glucosamine-3,6-sulfate; Position 5: α-L-iduronic-acid-2-sulfate; Position 6: *N*-sulfate-D-glucosamine-6-sulfate; Position 7: α-L-iduronic-acid-2-sulfate; Position 8: *N*-sulfate-D-glucosamine-6-sulfate.

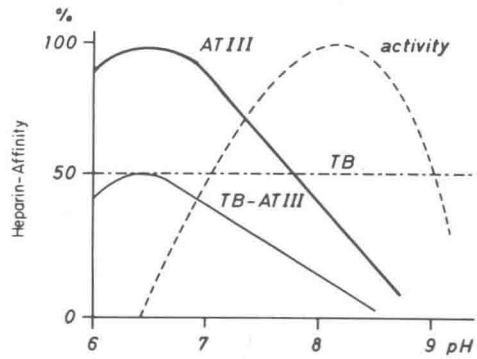

FIGURE 5. pH-Dependent heparin affinity of free antithrombin III (AT-III), free thrombin (TB), and thrombin-antithrombin complex (TB-AT-III); pH-dependent reaction rate (activity) of the inactivation of thrombin by antithrombin III in the presence of a constant amount of heparin.

heparin binds to thrombin and AT-III with considerably higher affinity than with the thrombin-AT-III complex. At acid pH (pH 6.4) stoichiometric amounts of heparin have to be present because heparin remains firmly attached to the AT-III protease complex and is not available for binding with thrombin (Figure 5).[33] In the absence of heparin millimolar concentrations of calcium ions facilitate the reaction between AT-III and Factor Xa, whereas the reaction between AT-III and thrombin is unaffected.[34]

Estradiol binds to AT-III thereby inhibiting the reaction between AT-III and thrombin in the absence of heparin. This also applies to other steroids which, however, bind with lower affinity.[35]

C. Biological Data

Normal plasma concentration of AT-III is 150 to 200 mg/ℓ (2 to 3 μ*M*).[36,37] Half-life as determined after administration of ^{125}I-labeled AT-III was shown to be 2.8 days in patients with normal AT-III levels.[38] Shorter times for elimination have also been published. Values ranging from 8.4 to 13.6 hr reported by Marciniak and Gockermann[9] are comparable with the data communicated by Schmidt et al. (7.5 to 10 hr) measured in a 4-month-old male infant suffering from DIC.[40]

The distribution volume of AT-III between the intra- and extravascular compartments is analogous to that of albumin with an intravascular fraction of 0.45.[38] Concentration of AT-

III in cerebrospinal fluid is only 80 to 400 n*M*. Functionally intact AT-III is not detectable in urine under physiological conditions. The amount of AT-III excreted in the urine, measured by immunological techniques, is about 30 to 50 μg/day.[41] AT-III was also demonstrated in platelets. Platelet AT-III was shown to be immunologically identical with plasma AT-III and to be released after platelet stimulation with ADP and collagen.[42] The existence of AT-III within platelets has not been confirmed by others. Bleyl found the platelet AT-III concentration to be maximal 1.5 p*M*, which is equivalent to 5000 molecules of AT-III per platelet, a concentration 10^6 less than in plasma.[41] Whether AT-III is an essential constituent of platelets is an open question.

AT-III is not an acute phase reactant. Its basal concentration provides an inhibitory potential adequate for neutralizing physiologically occurring serine proteases.

AT-III has been localized by immunohistological technique in different organs including microvasculature and walls of larger vessels in human lung, kidney, liver, and spleen,[43] vascular endothelial basement membrane of human skin,[44] and in human breast, colon, and anal tissue as well as in malignant tissue from stomach and ileum.[45] Hepatic synthesis of AT-III is easily hampered by tissue hypoxia[46] whereas endotoxin increases AT-III synthesis.[47] In stored human blood, human plasma, human serum, bovine blood, bovine plasma, and bovine serum, AT-III retains its functional activity for about 4 weeks at 5°C.[48] Antithrombin replacement does not require fresh blood or fresh frozen plasma.

D. Physiological Implications

AT-III is an essential component of the circulating blood. It allows appropriate limitation of the extent to which the hemostatic mechanism is activated in a particular situation. If a vessel injury requires the hemostatic mechanism to be initiated, activated proteases are quickly formed. The process however remains strictly localized because proteases are activated after being attached to the phospholipid layer, which is exposed only at the site of injury. Phospholipid- or platelet-bound active proteases are not available for AT-III attack.[49,50] If, however, proteases enter the fluid phase, complex formation with AT-III occurs, thereby preventing generalization of a localized coagulation process. As thrombin does not bind to phospholipids, AT-III is crucial to provide efficient thrombin binding. This implies that free AT-III as well as AT-III protease-complexes are demonstrable in the circulating blood. Complexed AT-III leaves the circulation with a t $^1/_2$ of 7 to 15 min.[51,52] Due to complex formation of AT-III with proteases during spontaneous clotting in vitro, AT-III activity in serum is decreased by 43.5%.[53] It is not known whether and to what extent the physiologic action of AT-III involves endogenous heparin. AT-III inactivates "anticoagulant" thrombin bound to the endothelial cell membrane receptor thrombomodulin. This reaction is not enhanced by heparin.[54]

E. Methodology

Antithrombin III is purified from plasma by affinity chromatography on heparin Sepharose using an increasing salt gradient for elution;[55] this procedure can be improved by introducing a pH-gradient as well.[56]

AT-III can be measured as an immunologically reactive protein or as a functionally active protease inhibitor. Functional activity is assessed by three distinct features; namely, progressive inhibition, heparin cofactor activity, and heparin affinity. Each of these properties can be impaired in various forms of AT-III-deficiency (see later).

Antisera raised against human AT-III react with free AT-III, heparin-antithrombin complex as well as with the protease AT-III complex. The use of the two-dimensional immunoelectrophoresis with heparin containing buffer in the second dimension allows one to discriminate between free AT-III and AT-III protease-complex. Due to its higher heparin affinity free AT-III moves faster than does the complex.[57]

AT-III binds an equimolar amount of thrombin or Factor Xa in the presence of heparin in vitro. Therefore the decline of thrombin or Xa activity after addition of AT-III containing sample in a test system containing heparin is proportional to the amount of AT-III in the sample. Thrombin and Factor Xa activity are easily measured using specific chromogenic substrates. The reaction between thrombin and AT-III is a molecular second-order reaction.[58]

Measuring thrombin inhibition with the chromogen Bz-Gly-Pro-Arg-pNA in the plasma of healthy adults the antithrombin III-inhibitor activity was found to be 11.4 ± 1.6 IU/mℓ (25°C).[59] Newborns have approximately 50% of the activity and concentration of adults.[60,61]

IV. HEPARIN COFACTOR II

A second thrombin inhibitor which has been designated heparin cofactor II and antithrombin BM, respectively, has been discovered.[62,63] It differs from AT-III by its lower heparin affinity and its thrombin specificity.

Heparin cofactor II has a molecular weight of 70,000 and amino acid composition related to that of AT-III. The only region of the molecule sequenced so far is the N terminus which does not reveal homologies with AT-III.[64]

There is evidence that heparin cofactor II is synthesized in the liver. Physiologic and pathologic changes in AT-III and heparin cofactor II levels parallel each other.[64]

V. CLINICAL RELEVANCE

A. Current Perspective

AT-III has received considerable attention in clinical practice. Chromogenic assays for AT-III activity were introduced into the clinical laboratory and helped to gain a better insight into the pathophysiology of thromboembolic disease. In some important clinical entities relevance of secondary AT-III deficiency is clearly established, whereas it is subject to controversy in others.[65,66]

There is a causal relationship between congenital AT-III deficiency and a markedly increased thrombotic tendency (thrombophilia) in afflicted patients. A single molecular defect causing a thromboembolic syndrome has so far been clearly defined in only very few other clinical syndromes such as protein C, protein S, and plasminogen deficiences, as well as in certain cases of dysfibrinogenemia.

Clinical conditions with AT-III deficiency as an important etiologic factor in the development of thromboembolism will now be discussed.

B. Congenital AT-III Deficiency

Congenital AT-III deficiency is either a quantitative (type I) or a qualitative (type II) defect. Until 1980 about 50 families with hereditary AT-III deficiency had been described in the literature.[65] Presumably many more cases have been detected in the meantime, because the amidolytic assay for AT-III activity has been widely employed. It is likely, however, that most newly discovered cases are not published. According to Norwegian and American statistics the frequency of congenital AT-III deficiency in the general population is estimated to be between 1:2000 and 1:5000, respectively.[67,68] AT-III deficiency is therefore more often to be encountered than hemophilia A. It has been estimated that this defect is to be expected in 2 to 3% of all patients hospitalized for thromboembolic disease.[69]

Inheritance is autosomal-dominant, although variable patterns of inheritance have been reported.[70] Both sexes are equally affected.

The first case to be presented in the literature was characterized as having a quantitative reduction of AT-III synthesis by about 50% (type I).[71] It is a striking phenomenon that this concentration of AT-III does not protect against thrombosis, whereas only minute activities

of coagulation proteases are required for hemostasis to be effective. Most patients with AT-III deficiency thus far described share the following features with the original case of classical, quantitative AT-III deficiency. In heterozygotes AT-III level is reduced to about half normal, even though great variations within one family and among families occur. The functions of AT-III, measurable as progressive AT-III activity, heparin cofactor activity and heparin affinity are normal.[65,72,73] A variant of type I deficiency with decreased heparin affinity has been described.[70]

Single cases of qualitative AT-III variants (type II) have been discovered. In such patients the circulating AT-III pool consists of one normal and one functionally abnormal AT-III species. Normal AT-III levels are measured by immunological techniques. Functional defects of these rare AT-III variants are heterogenous. Different functional qualities of the AT-III molecule may be impaired.

In AT-III Budapest all functional properties of the molecule (progressive AT-III-activity, heparin-cofactor activity, and heparin-affinity) are impaired. The change in molecular conformation (which has not been biochemically defined) apparently involves both thrombin- and heparin-binding sites.[74,75]

The functional defect of AT-III Aalborg is confined to thrombin binding to AT-III. Impaired progressive AT-III activity is associated with decreased heparin cofactor activity. Heparin affinity however is normal.[76]

Antithrombins Basel, Paris, and Toyama lack the ability to bind heparin (heparin-cofactor activity and heparin affinity are abnormal, progressive AT-III activity is normal).[77,78] In AT-III Toyama the defect was shown to reside in a single gene mutation introducing into residue position 47 cysteine instead of arginine thus impairing heparin binding at AT-III.[29]

AT-III Chicago is similar to AT-III Budapest in that both thrombin and heparin binding are altered.[79] Interestingly, while heparin affinity is increased, thrombin-binding and consequently heparin cofactor activity are impaired.

These qualitative AT-III variants, all of which are defective in heparin cofactor activity, cause a high incidence of thromboembolism.

AT-III variants with impaired heparin cofactor activity but lacking clinical evidence of thrombophilia have also been described (antithrombins Padua, Ann Arbor, and Padua II).[80-82] In the Ann Arbor family an abnormally fast reacting progressive AT-III was suspected because complex formation with thrombin could be demonstrated.

AT-III Vincenza belongs to type II deficiency.[83,84] It is peculiar, however, because the migration patterns in two-dimensional immunoelectrophoresis in the presence of heparin differ markedly if performed in plasma and serum. It was hypothesized that this is due to enhanced proteolytic cleavage of AT-III by thrombin in the presence of heparin. In one family thromboembolic disease was associated with a reduced platelet AT-III content.[85]

Congenital AT-III deficiency is associated with a high risk for venous thrombosis and pulmonary thromboembolism. At least one thromboembolic episode occurs in 55% of all patients. Thrombosis occurs before the age of 15 in less than 10% of cases. There is evidence of thrombosis in 85% of all patients by the age of 50. Thromboembolic disease usually becomes clinically apparent between the age of 10 and 35. In only 58% of cases the first thromboembolic episode coincides with a clinical event known to predispose for thrombosis, such as trauma, major surgery, delivery, etc.[65]

Thromboses are most commonly localized in the deep veins of the lower extremities and the pelvis. In more than 60% of cases thrombosis is recurrent. Rare manifestations, which are however quite characteristic of congenital AT-III deficiency, are mesenterial vein thrombosis and venous thrombosis in early pregnancy.[86-88]

The thrombotic tendency varies widely within one family as well as among families and is not strictly correlated with the level of AT-III activity measured. Some patients require life-long oral anticoagulation, whereas others suffer from an initial thromboembolic episode