The mechanism of action of heparin in purified systems and in plasma

Citation for published version (APA):

Pieters, J. J. S. M. (1989). *The mechanism of action of heparin in purified systems and in plasma*. [Doctoral Thesis, Maastricht University]. Rijksuniversiteit Limburg. <https://doi.org/10.26481/dis.19890224jp>

Document status and date:

Published: 01/01/1989

DOI:

[10.26481/dis.19890224jp](https://doi.org/10.26481/dis.19890224jp)

Document Version:

Publisher's PDF, also known as Version of record

Please check the document version of this publication:

- A submitted manuscript is the version of the article upon submission and before peer-review. There can be important differences between the submitted version and the official published version of record. People interested in the research are advised to contact the author for the final version of the publication, or visit the DOI to the publisher's website.
- The final author version and the galley proof are versions of the publication after peer review.
- The final published version features the final layout of the paper including the volume, issue and page numbers.

[Link to publication](#)

General rights

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal.

If the publication is distributed under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license above, please follow below link for the End User Agreement:

www.umlib.nl/taverne-license

Take down policy

If you believe that this document breaches copyright please contact us at:

repository@maastrichtuniversity.nl

providing details and we will investigate your claim.

THE MECHANISM OF ACTION OF HEPARIN
IN PURIFIED SYSTEMS AND IN PLASMA

Druk: Wibro Dissertatiedrukkerij, Helmond
Omslag: Janine Voncken

The Mechanism of Action of Heparin in Purified Systems and in Plasma

Proefschrift

ter verkrijging van de graad van doctor
aan de Rijksuniversiteit Limburg te Maastricht,
op gezag van Rector Magnificus, Prof. Dr. F.I.M. Bonke,
volgens het besluit van het College van Dekanen,
in het openbaar te verdedigen op vrijdag 24 februari 1989
om 16.00 uur

door

Jean Joseph Servais Marie Pieters

geboren te Heerlen

Promotor:

Prof. Dr. H.C. Hemker

Co-promotor:

Dr. T. Lindhout

Beoordelingscommissie:

Prof. Dr. H.A.J. Struyker Boudier, Rijksuniversiteit Limburg, Maastricht (voorzitter)

Prof. Dr. U. Abildgaard, Universiteit van Oslo, Noorwegen

Prof. Dr. F. Duckert, Universiteit van Basel, Zwitserland

Prof. Dr. P.B. Soeters, Rijksuniversiteit Limburg, Maastricht

Prof. Dr. M.A. Allesie, Rijksuniversiteit Limburg, Maastricht

"In de wetenschap gelijken wij op kinderen, die aan de oever der kennis
hier en daar een steentje oprapen, terwijl de wijde oceaan van het
onbekende zich voor onze ogen uitstrekt."

John Newton

*aan Chantal
aan mijn ouders*

Contents

Chapter I	Introduction.	9
Chapter II	Neutralization of heparin by prothrombin activation products. [Thromb Res 45:573, 1987]	41
Chapter III	Inhibition of factor IX _a and factor X _a by antithrombin III/heparin during factor X activation. [J Biol Chem 263:15313, 1988]	51
Chapter IV	The limited importance of factor X _a inhibition to the anticoagulant property of heparin in thromboplastin-activated plasma. [Blood, in press]	71
Chapter V	Factor XI _a catalyzed generation of factor IX _a in human plasma. Regulation by plasma proteinase inhibitors. [Submitted]	91
Chapter VI	In situ generated thrombin is the only enzyme that effectively activates factor VIII:C and factor V in plasma. [Submitted]	107
Chapter VII	General discussion.	117
Summary		125
Samenvatting		127
Dankwoord		129
Curriculum Vitae		131

ABBREVIATIONS

AT III	antithrombin III
DEAE	diethylaminoethyl
EDTA	ethylenediaminetetraacetic acid
FPLC	fast protein liquid chromatography
GLA	γ -carboxyglutamic acid
HA-heparin	high affinity heparin
MW	molecular weight
PC	phosphatidylcholine
PS	phosphatidylserine
PPACK	Phe-Pro-Arg-chloromethylketone
RVV	Russell's viper venom
SDS	sodium dodecyl sulfate
SDS-PAGE	polyacrylamide gel electrophoresis with SDS
STI	soybean trypsin inhibitor
S2238	D-Phe-(Pipicolyl)-Arg-p-nitroanilide
S2337	N-Benzoyl-Ile-Glu-(Piperidyl)-Gly-Arg-p-nitroanilide
Tris	tris(hydroxymethyl)aminomethane
UFH	unfractionated heparin

Chapter I

Introduction

In this chapter we will provide some background information on the process of blood coagulation, its initiation and its regulation. First of all, we will briefly discuss the historical events which lead to our present understanding of blood coagulation. After presenting a pathway for the initiation of coagulation, we will discuss the regulatory/inhibitory reactions occurring in plasma, with the emphasis on the antithrombin III/heparin system. In a last section we introduce the subsequent chapters of this thesis.

I. The Early Years

More than 300 years ago, in 1666, Malpighi (1) isolated white fibrous strands by washing clotted blood. This event represents the first written evidence of interest in the mysteries of blood coagulation (2).

About 100 years later, Hewson (3) localized the source of these fibrous strands to the plasma, rather than to the blood corpuscles, and by the middle-nineteenth century the formation of this fibrous material, named fibrin, became subject to investigations, which led to the beginning of the study of blood coagulation.

Figure 1. The "four factor" theory of blood clotting (taken from reference 23).

In 1845, Buchanan (5) showed the ability of a serum component to induce blood coagulation. This component was initially named thrombin by Schmidt (6), but he later postulated that it was derived during clotting from a precursor in plasma, prothrombin.

It is now well established (7) that the discovery of prothrombin was due to the work of Pekelharing (8).

The next important observation was made by de Blainville (9): he saw that intravenous injection of a suspension of brain tissue led to immediate death, caused by total occlusion of the blood vessels by clots. Buchanan (5) confirmed that a variety of tissue's could induce clot formation, and initially it was thought that bodily tissue's contained thrombin. However, Schmidt (10), and later Morawitz (11) found that these tissue extracts did not clot fibrinogen directly, but rather by converting prothrombin into thrombin with the necessary presence of calcium ions. Therefore these clot-promoting substances were called tissue thromboplastin.

These results led to the so-called classical theory, also named the "four-factor" theory of blood clotting, which is schematically depicted in Figure 1. In this theory, tissue thromboplastin, in the presence of calcium ions first converts prothrombin into thrombin, which then in turn converts fibrinogen into fibrin. This theory held strong for almost half a century and persisted in the textbooks even longer.

Figure 2. The enzyme cascade in the blood coagulation mechanism as published by MacFarlane in 1964. This reaction sequence originally represented what is now known the intrinsic pathway of coagulation. These reactions proceed upon exposure of blood to foreign surfaces (modified from reference 45)

In the late thirties several experiments suggested that the conversion of prothrombin into thrombin required participation of additional factors (12-14), and three additional factors were postulated.

In 1947, work from Owren (16) on a family with a congenital bleeding tendency gave the first clear indication of the existence of an additional clotting factor. He named it factor V, being the fifth clotting factor discovered, but renamed it later in "proaccelerin" (17).

Several investigators suggested that there existed a serum component important for the conversion of prothrombin into thrombin (18,19,20). Koller et. al. finally performed experiments on a familiar bleeding tendency from which they inferred the existence of a procoagulant plasma protein which they called factor VII, and that appeared to be identical with the serum component (23)

In 1956, studies of two patients with an abnormal one stage prothrombin time that was corrected by serum, revealed that the factor VII preparations then available contained a second procoagulant factor, as predicted earlier by Duckert (24,25). This factor was named Stuart-Prower factor (factor X) (26,27), after the surnames of the affected individuals.

However, at the time of Morawitz classical theory, studies of Nolf (33) and Bordet and Gengou (34) laid the basis for the understanding of what is now known as the intrinsic pathway of coagulation. They demonstrated that blood depleted of cells clotted in glass tubes. Originally it was thought that such plasma contained thromboplastic substances (35). This was believed until Conley's classic experiment, that clearly demonstrated plasma to clot upon exposure to glass in the absence of tissue thromboplastin (42).

At that time, mostly by means of the occurrence of people with a bleeding disorder, and who appeared to be deficient in a clotting factor, factor XII (37), factor XI (38) and the antihaemophilic factor (factor VIII) (39,40) were discovered.

In 1947, Pavlovsky's observation of a second type of haemophilia (41) led to the discovery of factor IX (42, 43,44).

This completed the view on blood coagulation in the early sixties, and resulted in publications of the so-called "cascade" (45) or "waterfall" (46) hypothesis of the blood coagulation system (see Figure 2).

The interaction of components of the blood clotting system with phospholipid vesicles was first demonstrated by Hanahan and Papahadjopoulos in 1964 (30). In 1967, Hemker et al performed experiments on the conversion of prothrombin into thrombin in a purified system, and proposed a kinetic model in which phospholipid provided a surface on to which coagulant proteins bind, in order to form a complex (31). It was also shown that for the activation of factor X into factor X_a a complex of phospholipid, calcium, activated factor IX and VIII:C was responsible (32).

II. A Modern View

Nowadays, 25 years after the cascade or waterfall hypothesis was presented, this reaction sequence still forms the backbone of most reviews on blood coagulation. As was described in the previous section, this model was based on two historical findings, first, the observation of de Blainville (9), leading to the evidence that tissue factor promotes blood coagulation, and second, the experiments of Conley (36), showing the clotting of plasma upon exposure to glass. This division in extrinsic (tissue factor) and intrinsic (glass contact) pathways for coagulation was of considerable practical significance for the clinic in testing the integrity of

the blood coagulation pathway: The one-stage prothrombin time (PT) is used to screen the extrinsic pathway (15), while the partial thromboplastin time (PTT) screens the intrinsic pathway (14,47).

However, in this section we would like to formulate an alternative scheme for the blood coagulation cascade. To do this, we will first have to briefly recapitulate the most important features of the separate blood coagulation factors who will form part of the scheme; after discussing tissue factor we will mention the vitamin-K dependent clotting factors (i.e. factor VII, factor IX, factor X and prothrombin), and then we will present some information on the non-enzymatic protein cofactors factor VIII and factor V. Finally, a pathway for the initiation of coagulation as we think it proceeds in plasma under physiological circumstances is proposed.

A. Components of the Blood Coagulation Scheme

1. Tissue Factor

TF is a protein present on the plasma membranes of many cells. Especially brain, lung and placenta tissue display a high activity in TF. It is a 53,000 dalton glycoprotein, rich in carbohydrates (56,57). Purification of TF requires removal of phospholipids by extraction with organic solvents (58). The resultant purified TF is devoid of proteolytic activity and has an absolute requirement for phospholipids to express its coagulant activity (58,59). Recently, an inhibitor of the TF:factor VII(a) complex was identified (59,60; see the section on factor VII). Also it was suggested that the carbohydrate moiety of TF provides a regulatory mechanism, as it was reported that concanavalin A binds to and inhibits TF (58,61,62).

No deficiencies of TF are known, indicating the possibility that absence of TF is incompatible with life (62).

2. The Vitamin-K Dependent Coagulation Factors

In 1935, the necessity of vitamin-K for normal hemostasis was recognised by Dam (63). Many years later it was shown that vitamin-K was necessary for the biological activity of prothrombin and factors VII, IX and X (64-66). It is now known that vitamin-K provides a post-translational carboxylation of glutamic

acid residues to yield the γ -carboxyglutamic acid residues (Gla's) (67). These Gla's are thought to bind calcium ions, thereby promoting the association of these proteins to lipid membranes. Calcium ions act as "bridges" in this model, by interacting with negatively charged sites at the phospholipid on the one hand and the Gla residues on the protein on the other hand (68).

However, the role of Gla-residues may extend beyond Ca^{2+} -mediated, protein-lipid association. Factor VII for example, binds via calcium ions to a very hydrophilic domain in TF that contains no Gla's (69). Also the activation of factor X by Russel's viper venom occurs in the absence of phospholipid, but in the presence of calcium, where intact factor X is activated much faster than factor X from which the Gla-domain has been removed (70). Another example of a calcium dependent, non-phospholipid dependent reaction is the activation of factor IX by activated factor XI (71). Moreover, for the activation of factor X by factor VII_a/TF in the presence of phospholipid, it was demonstrated that the concentration of factor X in solution controls the reaction velocity, rather than the concentration of phospholipid-bound factor X (72).

Figure 3. The four vitamin-K dependent coagulation factors. The shadow shaded part represents the amino terminal Gla-containing region. the active site serine on the carboxyterminal part of the zymogen is symbolized with an asterisk. Activated peptides released upon activation of the zymogens are shown by solid bars.

Thus, as there apparently is no a priori reason to invoke protein-phospholipid binding to explain Ca^{2+} - and Gla-mediated acceleration of reactions in which vitamin-K dependent coagulation factors are involved, it is speculated that a Gla-mediated, Ca^{2+} -induced conformational change may cause the observed acceleration of these reactions (48).

Which of these possibilities, calcium induced "bridge" formation, calcium induced conformational change, or both, causes the biological activity of these proteins, remains to be established. The Gla-containing, vitamin-K dependent proteins are schematically depicted in Figure 3.

Factor VII

Factor VII is a single chain glycoprotein (MW = 48,000 dalton), present in plasma at the very low concentration of 10 nM (73). Factor VII is unique among the coagulation enzymes in that the zymogen factor VII has intrinsic activity (74). Nevertheless, factor VII clotting activity is some 100-fold increased by proteolytic cleavage into its two chain derivative, factor VII_a. This is catalyzed most rapidly by factor X_a and at a slow rate by factor IX_a (75,76). Factor XII_a has also been reported to activate factor VII (77,78).

Until recently, no inhibitors of factor VII(a) were described. In 1987, Rao et al found that an inhibitor, requiring the presence of TF, factor VII(a) and factor X_a could inhibit the TF:factor VII(a) activity, probably by the formation of an inert TF:VII(a):X_a:inhibitor complex in the presence of calcium (59,60). Indeed we have shown that when plasma is triggered with a relatively high concentration of thromboplastin, the activated factor X is able to back-inactivate the activating TF:VII_a complex, thus providing a regulation of its own generation (79).

Deficiencies in factor VII(a) activity have been described, some patients having serious bleeding difficulties, others quite moderate or mild (80).

Factor IX

Factor IX (Christmas factor) is a single chain glycoprotein with a molecular weight of 57,000 dalton (81,82). Its plasma concentration is about 80 nM. Factor IX is activated into two chain factor IX_a by TF:VII(a), as mentioned above, and by factor XI_a, as we shall discuss in a later section (83,84).

The inhibition of factor IX_a is accomplished by antithrombin III (84,85), and this is stimulated by heparin (see later). In a purified system, this stimulation is quite moderate (86), however, we and others showed that the action of heparin on the antithrombin III/factor IX_a reaction in plasma is tremendous (87-89).

Deficiency of factor IX results in hemophilia B, a sex linked, recessive bleeding disorder (90). About 10% of the patients have normal plasma levels of immunologically detectable factor IX (CRM⁺), and the remaining 90% can be derived into two groups: those with reduced levels of factor IX (CRM^{red}) and

those with no detectable levels of factor IX (CRM) (90-92). Hemophilia B occurs in a severe, moderate or mild form according to the level of factor IX activity in plasma; Patients with severe hemophilia B have less than 1% factor IX; the patients possessing 5-25% of normal factor IX activity have only mild symptoms.

Factor X

Factor X (Stuart-Prower factor) is a two chain glycoprotein of 55,000 dalton MW, circulating in plasma at a concentration of about 160 nM (93,94). Factor X is converted to α -factor X_a by the complex of factor IX_a , factor $VIII_a$, Ca^{2+} and phospholipids, or by the TF:VII(a) complex. This product can then autocatalytically be converted to a lower molecular weight form, β -factor X_a . Factor X_a is also able to convert factor X autocatalytically into β -factor X and β -factor X_a (95).

Factor X_a is inactivated by antithrombin III, α_2 -macroglobulin and α_1 -antitrypsin (84,96,97).

To our present knowledge, no patients with a severe factor X deficiency were described. Because activated factor X is the only known physiological activator of prothrombin, factor X deficient patients possibly die in utero at the first signs of microtraumatation (98).

Prothrombin

Prothrombin (factor II) is a single chain glycoprotein of a molecular weight of 72,000 dalton, circulating in plasma at concentration of about 1.5 μ M (99). Upon activation by factor X_a into thrombin, the Gla-containing activation peptide (fragment 1.2) is released (100) (see Figure 3). The activation peptide of prothrombin is almost half of the molecule, which suggests that it might be more than a mere activation peptide. Its function has been subject to many investigations. It was found to play a role in Ca^{2+} mediated binding to membranes, thus enhancing the factor X_a -induced activation (101,102). It also seems to be involved in the binding of the non-enzymatic protein cofactor of prothrombin activation, factor V_a (see later) (50,102). The activation fragment has been found to regulate the activation of prothrombin (103) and to neutralize heparin (103), although the in vivo importance of these last two findings remain to be established.

The thrombin formed is inhibited by antithrombin III, α_2 -macroglobulin, α_2 -antiplasmin and heparin cofactor II (105-108).

No patients lacking factor II were described, indicating the importance of this factor for normal hemostasis.

3. Factor VIII and Factor V

The non-enzymatic protein cofactors VIII and V have a lot in common. Both of these cofactors accelerate the activation of a Gla-containing substrate (factor X or prothrombin) by a Gla-containing enzyme (factor IX_a or factor X_a). Both require calcium ions and a lipid surface for optimal activity (109). Another similarity is that both factor VIII and factor V activity is regulated by limited proteolysis; both cofactors circulate as inactive proteins and are activated by serine proteases such as thrombin or factor X_a, and are inactivated by another Gla-containing serine protease, namely activated protein C (110). These inactivation reactions also require a Gla-containing cofactor (protein S), Ca²⁺ and a lipid surface. Deficiency in factor VIII or factor V result in respectively hemophilia A (111) or parahemophilia (112) which represent mild to severe bleeding disorders. Also a combined deficiency of factors VIII and V was described (113).

Factor VIII

Factor VIII clotting activity occurs principally in plasma as a complex with von Willebrand factor (114). Factor VIII procoagulant protein is indicated as factor VIII:C, to distinguish from von Willebrand factor. Due to its very low concentration (about 1-1.5 nM) in plasma (115) and the fact that in plasma it circulates as an inactive cofactor tightly bound to von Willebrand factor, it was extremely difficult to purify. However, the isolation of full length cDNA's coding for human factor VIII:C, and its expression in mammalian cells (116-119), led to an explosion of knowledge about the structure and function of factor VIII:C. This cDNA codes for a peptide with a MW of 265,000.

As already mentioned, factor VIII:C must be activated by thrombin (51-53,120), factor X_a (121) or factor IX_a (122), although thrombin must be considered to be the physiologic activator in plasma.

The inactivation of factor VIII:C is, as already mentioned, accomplished by the protein C/protein S pathway, which is further discussed in a later section.

Deficiency of factor VIII:C (hemophilia A) results in hemorrhage and hematomas. As for hemophilia B, the patients can be divided in severe (1%), moderate (1-5%) or mild (5-25%), according to the relative amount of factor VIII:C activity in plasma (109)

Factor V

Factor V is a plasma glycoprotein that circulates in plasma at a concentration of 30 nM in a single chain form (123). It was isolated and purified from human plasma in 1980 (124), whereas platelet factor V has also been isolated. Bovine aortic endothelial cells were also found to contain factor V activity (125,126).

Factor V is activated by thrombin and factor X_a (127,128). Activated factor V changes the reaction pathway and the intermediate generated during thrombin formation. In the absence of factor V_a , the intermediate prethrombin 2 is generated, whereas in its presence meizothrombin is the principal intermediate (129,130). Both intermediates are then converted to the same thrombin species.

The inactivation of factor V_a proceeds also through the protein C/protein S mechanism (131), as will be described later.

Deficiency in factor V results in parahemophilia, either caused by low levels of factor V or dysfunctional forms of factor V (132, 133). The bleeding of these patients is usually milder and more easily controlled than the bleeding associated with hemophilia A. (112).

4. Factor XI

Human factor XI (plasma thromboplastin antecedent, PTA) circulates in plasma non-covalently associated with high molecular weight kininogen (HMWK) (134). It is present at a concentration of approximately 30 nM (135) and occurs as two identical disulphide-linked polypeptide chains of 80,000 dalton MW each (136). Factor XI is activated by proteolysis due to factor XII_a . Factor XI_a is composed of two heavy chains and two light chains held together by disulphide bonds (137).

Factor XI_a is inhibited by α_1 -antitrypsin, antithrombin III, C1-inhibitor and α_2 -antiplasmin. It is not clear whether α_1 -antitrypsin, or the combined action of α_1 -antitrypsin, antithrombin III and α_2 -antiplasmin are responsible for the rapid inactivation of factor XI_a in plasma (89,138,139).

Factor XI deficiencies are sometimes associated with a bleeding disorder (38), suggesting a possible physiological role for factor XI in normal hemostasis.

B. Initiation of Blood Coagulation

Now we would like to discuss in more detail the modern views on blood coagulation. Especially the initiation of blood coagulation was a subject of debate during the recent years. Some investigators stress the importance of the contact activation for the initiation of blood coagulation, others deny it. We would like to defend the position in the following part that indeed, the contact factors are not needed to explain normal and abnormal haemostasis.

Due to the intrinsic activity of factor VII and the fact that TF requires no proteolytical processing for its activity, the initial step in coagulation could be accomplished by the mere physical complexation of tissue factor with factor

VII(a) (48). (As factor VII zymogen is thought to have activity, factor VII(a) is used when both enzyme and zymogen is referred to (48)). It is interesting to note that none of the usual inhibitory proteins is active against factor VII(a). Recently, an inhibitor of the factor VII(a):TF complex was found. However, this so-called extrinsic pathway inhibitor (EPI, 60), becomes active by its association with factor X_a , i.e. after coagulation already has started.

Once the active TF:factor VII is formed, this complex activates factor X, giving rise to small amounts of activated factor X. This factor X_a then converts the zymogen factor VII into its activated form, which possesses a 100-fold increased activity. This enzymatic complex of TF:factor VII_a activates enough factor X to produce the amount of factor X_a necessary to convert some prothrombin to yield trace amounts of thrombin. These traces of thrombin can trigger the platelets to expose their procoagulant activity and activate factor V. Only then sufficient amounts of thrombin are generated to ensure proper coagulation (54,55).

However, in this course of events the antihemophilic factors, factor IX and factor VIII:C, seem not to be involved, although they must play a significant role in normal hemostasis. To explain their action, we have to go back to 1965, the year in which Josso showed that factor VIII:C and factor IX are essential for thrombin formation induced by diluted thromboplastin (140).

Figure 4. The Jossso-loop. A: Factor VII(a) activates both factor IX and factor X, after which the generated activated factor X converts prothrombin into thrombin. B: The completed Jossso-loop. Her, also the non enzymatic cofactors and phospholipid surface are indicated (taken from reference 98).

Because usually, all experiments in which the TF:factor VII initiation was involved, were carried out with extremely high concentrations of thromboplastin, it was not until the late seventies that this alternative pathway via factor IX got enough attention. By that time Osterud and Rapaport demonstrated clearly that besides factor X, also factor IX is activated in considerable amounts by TF:factor VII(a) (49,141). The generation of factor IX_a activity may become important if clotting is started by diluted thromboplastin, then providing a reinforcement loop in the generation of factor X_a. This reinforcement loop, which was proposed to be named the "Josso loop", after its discoverer (98), is depicted in Figure 4a.

Thus, besides producing directly factor X_a, the TF:factor VII(a) complex also provides an indirect activation of factor X by activating factor IX. As mentioned above, the first traces of thrombin activate factor VIII:C, leading to a view on thrombin formation as depicted in Figure 4b. The reinforcement loop via the antihemophilic factors, the Josso loop, will become more important the less thromboplastin is present. This might explain the fact that hemophiliacs tend to bleed in thromboplastin poor organs. In general, it is very likely that the *in vivo*

Figure 5. A modern view on blood coagulation. Upon the exposure of tissue factor, the complex of factor VII(a):TF activates as well as factor IX as factor X, leading to the generation of factor X_a. this activated factor X then converts prothrombin into thrombin, leading to the generation of fibrin (modified from reference 48).

coagulation starts upon exposure of very small amounts of tissue factor, equivalent to highly diluted thromboplastin. Indeed, already in 1834 de Blainville demonstrated that high concentrations of thromboplastin completely knocked out the hemostatic balance: the animals he injected with tissue extracts died through total occlusion of the blood vessels with clots (9)!

A modern view on blood coagulation as explained above is schematically presented in Figure 5.

Now, what about the role of the contact factors in hemostasis? It is significant that people deficient in a contact factor are entirely asymptomatic (142). Also, the contact activation reactions are linked to several other proteolytic plasma systems (62,143). Finally, the initiation of blood coagulation by negatively charged surfaces such as kaolin, sulfatides or glass must be considered extremely rare in a physiological situation. Instead of regarding contact activation as an alternative pathway for initiating blood coagulation, one could rather suspect the use of glass material in the early years of coagulation research to be a historical error!

However, one problem remains to be solved. In the above presented view on coagulation, no role for factor XI was considered, although factor XI deficient patients clearly show bleeding problems in a certain (unknown) percentage of the cases. Now there are 2 possibilities: Either the bleeding problems observed in those patients are not the consequence of the decreased factor XI levels (alone), or factor XI does play a role in normal hemostasis. Because the activation of factor IX by factor XI_a does not require a cofactor or a surface for optimal activity, the factor XI_a catalyzed activation of factor IX could possibly play a role in those situations in which no tissue factor is available.

III. The Regulation of the Coagulation Cascade

Uninhibited thrombin formation would lead to an uncontrolled explosive thrombin generation that would cause lethal thrombosis at the slightest provocation. As already mentioned throughout the previous section, the reaction sequence leading to the generation of thrombin is controlled at various levels by several inhibitory mechanisms. In this section we will discuss a number of these inhibitory mechanisms, who can be divided into four groups.

A. The Protein C/Protein S Pathway

The protein C/protein S pathway provides an inhibitory mechanism for the activities of factor VIII:C_a (120) and factor V_a (144). Protein C is a vitamin-K dependent glycoprotein with a molecular weight of about 62,000 dalton and occurring in plasma at a concentration of 63 nM (62,145). Upon proteolysis by thrombin, protein C is activated at a very slow rate (146) and this activation process is further inhibited by physiological calcium concentrations (147). However, the endothelial cell was found to possess cofactor activity, now known to be caused by the presence of thrombomodulin at its surface (148), which forms a 1:1 complex with thrombin. This complex then rapidly activates protein C in a Ca²⁺ dependent reaction (149). In 1983 it was found that the factor V_a molecule also possesses cofactor activity for the activation of protein C by thrombin (150,151).

Activated protein C requires the presence of phospholipid, calcium ions and protein S, a vitamin-K dependent protein that functions as a cofactor for optimal inhibitory activity towards activated factors V and VIII:C (152).

Activated protein C is inhibited by activated protein C inhibitor (APC-inhibitor (153,154)).

The importance of the protein C/protein S pathway in the regulation of blood coagulation might be indicated by the fact that deficiencies of protein C/protein S are associated with thrombotic disorders (155). It is significant that for thrombomodulin to be present so that the protein C/protein S mechanism can get started, intact endothelium is required.

B. Inhibitors of Surface Binding

This group of anticoagulants comprises a variety of compounds, all interfering with the blood coagulation factor-surface binding. This group can be divided into several subgroups (156), of which a first group is formed by the phospholipases, who bind to a phospholipid surface bilayers and hydrolyses various of its components. Thus, they occupy the surface and change its properties (157).

The second group consists of the lupus anticoagulant, a heterogenous group of acquired immunoglobulins which bind to the procoagulant surface (158). The lupus anticoagulant has been found to act as an inhibitor of *in vitro* clotting tests (159), and is associated with thrombosis (159), although the relationship between these phenomena remains to be established.

In a third group we mention the Gla-containing activation fragments of prothrombin and factor X, who inhibit in a competitive manner the tissue factor dependent activation of factor X (160) and the factor X_a-catalyzed prothrombin activation (103). Also a Gla-containing peptide derived from factor X was found to inhibit surface mediated reactions (161).

As a last member of this group we name the vascular anticoagulant (VAC) discovered by Reutelingsperger in 1985 (162). This protein, devoid of Gla-residues, binds in a calcium-dependent way with high affinity to negatively charged phospholipids, thus interfering with the coagulant factor phospholipid interaction.

C. The Extrinsic Pathway Inhibitor

In 1985, Sanders et al observed an inhibition of the factor VII_a/TF activity during coagulation in plasma (163). This inhibitor which they called the extrinsic pathway inhibitor (EPI), apparently required the presence of the TF:VII_a complex and factor X_a, thus providing a control mechanism in the initial steps of coagulation, triggered by the exposure of TF.

D. Serpins

The last group of naturally occurring inhibitors comprises the serpins, the serine protease inhibitors. This includes antiplasmin, α_1 -antitrypsin, heparin cofactor II and antithrombin III (164). These are structurally homologous plasma proteins, while their functional diversity is determined by their reactive site amino acids. The molecular weight of these proteins ranges from 40,000 to 100,000 dalton. These serpins function as suicidal proteins in that they form a tight 1:1 complex with their target protease, after which these complexes are removed from the circulation and catabolized.

As the major part of this thesis deals with the effect of antithrombin III and heparin (a mucopolysaccharide greatly enhancing the activity of antithrombin III) on several activated blood coagulation factors, we dedicate a separate section to this protein inhibitor.

The Antithrombin III-Heparin System

Antithrombin III

Antithrombin III is one of the most important physiological inhibitors of blood coagulation. Its inhibitory action is directed against thrombin and factor X_a, whereas factor IX_a, factor XI_a and factor XII_a were also shown to be liable to in-

hibition (for a recent review see reference 165). The inhibition of factor XI_a and factor XII_a, however, seems to be of no importance in plasma. The activity towards factor X_a and factor IX_a will be discussed further in this paragraph.

Antithrombin III, purified from plasma for the first time in 1968 (166), showed to be a single chain glycoprotein with a molecular weight of 55,000 dalton (167). Its plasma concentration is about 2.5 μ M (168,169). Two forms of antithrombin III were found in plasma, due to different carbohydrate composition (170,171). The inhibitory activity of antithrombin III is greatly enhanced in the presence of heparin, as we shall discuss in a later section.

The interaction of antithrombin III with thrombin is extensively studied. Antithrombin III reacts with thrombin to form a 1:1 complex (172). In serum this complex was found to possess a molecular weight in the 180,000 - 200,000 dalton range instead of the 90,000 dalton expected (173,174). Marciniak et al, however, showed that this was due to aggregation (175). The formation of the antithrombin III thrombin complex involves the cleavage of a peptide bond in antithrombin III. The formed complex dissociates under physiological conditions spontaneously but very slowly (176), thus liberating active thrombin and proteolytically modified inactive antithrombin III (177). This modified two-chain antithrombin III is also formed by direct activation by thrombin, without complex formation (178).

As already mentioned, antithrombin III also inactivates factor X_a and factor IX_a. The inactivation of factor X_a by antithrombin III is analogous to the inhibition of thrombin. However, factor X_a seems to be protected from inactivation by antithrombin III by the presence of Ca²⁺/phospholipid or Ca²⁺/V_a/phospholipid (179,180,86). The inhibition of factor IX_a by antithrombin III was found to be very slow in a purified system, and negligible in plasma. The addition of heparin caused a dramatic increase in inactivation rate of factor IX_a (86,89).

Deficiencies in antithrombin III have been described, with antithrombin III levels varying from 40 - 70 % of normal (165). These deficiencies are a result of either reduced antithrombin III levels (type I deficiency), or of abnormal variants (type II deficiency). Among type II deficiency, several different variants were identified, e.g. antithrombin III molecules with abnormal thrombin and factor X_a inhibition and abnormal heparin binding (AT III "Budapest", AT III "Tokyo"); abnormal thrombin and factor X_a inhibition but normal heparin binding (AT III "Vicenza", AT III "Denver"), and normal thrombin and factor X_a inhibition but abnormal heparin binding (AT III "Paris", AT III "Basel"). The occurrence of this last variant with thrombosis shows that the binding of antithrombin III to heparin also in vivo probably plays a significant role. It has been suggested that the binding of antithrombin III to the heparin-like molecules (at the endothelial cell surface) in vivo must be important for its optimal activity as a coagulation inhibitor (165).

Heparin.

Heparin is a heterogenous, polydisperse glycosaminoglycan, with a molecular weight range from 5,000 to 30,000 dalton giving a mean molecular weight of 15,000 dalton. In mammalian species, this complex carbohydrate is widely distributed throughout a variety of organs, including liver, lungs, heart, kidney and intestine (181). Heparin is composed of individual mucopolysaccharide chains composed of a multitude of possible arrangements of uronic acid and glucosamine residues (165).

The antithrombin III binding sequence is an oligosaccharide segment that contains D-glucuronic acid and a unique trisulphated D-glucosamine residue, whereas the actual binding site for antithrombin III appears to be a pentasaccharide (182). The structure of this pentasaccharide is shown in Figure 6, and was reproduced by total synthesis by Choay et al (183). The chemical groups, most probably responsible for the binding to antithrombin III, are the 3-O-sulphate group and the 6-O-sulphate group on the N-acetylated glucosamine unit.

However, only one third of the chains of unfractionated heparin contain this unique pentasaccharide sequence. The remaining two-third are essentially inactive as an anticoagulant, although not necessarily as an antithrombotic agent (165).

Figure 6. Structure of the antithrombin III binding pentasaccharide sequence. The 3-O-sulphate group of unit 3 (marked by an asteriks) is unique to the antithrombin III binding region of heparin, while each one of the sulphate groups indicated by (e) is essential to the high affinity binding of antithrombin III (modified from reference 183).

Because the administration of heparin, besides the desired antithrombotic effect, also causes non-desired side effects such as bleeding (184,197) and platelet aggregation (185), one of the aims in heparin research over the past years was to isolate or synthesize heparin preparations that maximize the sequences with desirable activities, and minimize those sequences who probably cause the undesirable side effects (186). The preparation of heparin fractions is achieved either by fractionation, chemical modification, or synthesis.

Fractionation of heparin chains is done in various ways, including affinity chromatography with antithrombin III (187), gelfiltration (188) and precipitation with alcohols. Affinity chromatography with antithrombin III results in high affinity heparin fractions, containing the antithrombin III-binding sequence, and in low affinity heparin fractions. Gel filtration results in heparin fractions with different molecular weight. Because sulphated species are less soluble in alcohol than the less sulphated species, fractional precipitation with alcohol results in fractions with a different degree of sulphation.

Chemical modification is achieved mostly by removal of sulphate groups or controlled depolymerization, which produces fragments of the size of 8 - 30 monosaccharide residues, ranging in molecular weight from 2,500 - 9,000 dalton. Fragments of 8 - 16 monosaccharides that contain the antithrombin III binding pentasaccharide show very high anti- X_a activities but are devoid of anti-thrombin activity.

The above mentioned discrimination in anti- X_a and anti-thrombin activity of heparin was made because it was found that for the inhibition of thrombin, the binding of heparin to the protease is an absolute requirement (189,190). In contrast, for the inactivation of factor X_a , the presence of the pentasaccharide sequence is sufficient to obtain a high inhibition of factor X_a by antithrombin III (191,192). Therefore a difference was made for heparins which exert high anti- X_a activity (i.e. low molecular weight heparins containing the pentasaccharide sequence) and heparins which exerted high anti-thrombin activity as well as anti-factor X_a activity (i.e. heparins containing at least 14 - 18 saccharides (193,194).

For the inhibition of factor IX_a and factor XI_a the same molecular weight dependence as for thrombin was shown by Holmer et al (189). However, we provided clear evidence that in plasma the inactivation of factor IX_a is stimulated by pentasaccharide (see Chapter 5).

In the clinic, heparin is used for the prophylaxis of venous thrombosis, for the treatment of overt thrombosis, for the prophylaxis of recurrent thrombosis and for maintaining the fluidity of the blood in extracorporeal circulations, such as in heart-lung machines and renal dialysis (195).

The prevention and treatment of these thrombotic events with classical unfractionated heparin is often accompanied with hemorrhage and sometimes with undesirable platelet interactions (196). Therefore, a number of years ago it was

proposed that heparin preparations of low molecular weight would have advantages in anticoagulant therapy. Experiments in animal models suggested that with heparin fractions of about 5,000 dalton molecular weight, equal antithrombotic efficacy was obtained with less bleeding tendency as compared to classical unfractionated heparin (197,198).

Also it was found that heparins with a mean molecular weight of between 4,000 and 6,000 dalton had relatively little effect on tests measuring overall clotting such as APTT, but maintained their potency as measured by anti-X_a assay's (199,200). Thus, it was suggested that heparins with high anti-X_a/APTT ratios (i.e. high anti-X_a/anti-thrombin ratios) would maintain the efficacy of unfractionated heparin by exerting an antithrombotic effect through their preserved anti-X_a function, and cause less bleeding because of their reduced action on overall clotting (201, 202).

However, preparations of heparin in which the molecule is large enough to retain some thrombin blocking action appeared to be more effective as an antithrombotic agent (203). Nor have the clinical studies published so far convincingly demonstrated that low molecular weight heparins are safer and cause less bleeding than unfractionated heparin (204).

Another aspect is that although most clinical studies make use of the anti-X_a activity of heparin/low molecular weight heparins to monitor their antithrombotic potency, the relationship between antithrombotic efficacy and anti-X_a activity may severely be questioned (see chapter 4 and reference 205).

Some properties of the low molecular weight heparin, however, clearly overcome disadvantages of the classical unfractionated heparin preparations. First, low molecular weight heparins are eliminated from the blood more slowly than unfractionated heparin; its biological half life is about twice as long. Second, low molecular weight heparins apparently do not cross the placenta.

IV. The Present Investigation.

This thesis describes a number of studies which deal with the regulatory (inhibitory) action of the heparin/antithrombin III system at several levels of the blood coagulation cascade. The reactions of the blood coagulation cascade and the interaction of heparin/antithrombin III on these reactions, are schematically drawn in Figure 7. As is shown, antithrombin III and heparin act as well on factors XI_a and IX_a, as on factor X_a and thrombin.

However, because the reactions presented in Figure 7 are the result of studies mostly performed with isolated coagulation factors, the relative importance for the regulation of the *in vivo* haemostasis may severely be questioned. First of all, until now no information was available about the amounts of activated coagulation factors that were necessary for normal haemostasis. Secondly, the importance of a number of feed-back activation and inactivation reactions were not always recognized. To illustrate the first point: what good would it be for example to inhibit 95 % of the generated factor IX_a or factor X_a, if the remaining 5 % could result in as adequate haemostatic processes as would 100 % would? Also, coagulation factors which provide an amplification of the whole haemostatic mechanism by means of a number of feed-back reactions, of course are more important targets for regulation as are coagulation factors providing a single activation reaction.

With respect to these feed-back reactions, the role of thrombin can be considered as extremely important in blood coagulation. In Figure 8 the role of thrombin in a number of reactions leading to clot formation is depicted. Of course, thrombin is the enzyme that converts fibrinogen into fibrin, but, moreover, also is responsible for the activation of factor VIII:C and factor V, the essential protein cofactors for respectively factor X and prothrombin activation. Also the exposure of the platelet-procoagulant surface is the result of their activation by thrombin. Therefore, one can easily imagine the impact of thrombin inhibition on the overall process of blood coagulation.

An other aspect of the limited importance of results obtained from *in vitro* studies on purified factors to *in vivo* haemostasis is the fact that these results can

Figure 7. Interaction of the antithrombin III/heparin system with activated clotting factors XI_a, IX_a, X_a and thrombin.

Figure 7. Interaction of the antithrombin III/heparin system with activated clotting factors XI_a, IX_a, X_a and thrombin. mostly not be extrapolated to the *in vivo* situation. For example, the reaction between factor X_a and antithrombin III in buffer has been described to be totally different from the reaction in which phospholipids and calcium are present. One can thus imagine what the effect of hundreds of plasma constituents are on such a reaction!

Therefore, in this thesis we gradually approach a situation which can be considered very near to the physiological one. The first two studies (chapters II and III) were performed in purified systems, whereas chapters IV, V and VI describe studies performed in whole plasma.

Chapter II describes studies performed with several activation products of prothrombin. We showed that meizothrombin, a major product during prothrombin activation did not bind to heparin, whereas thrombin does. Also, we showed that the activation peptides, which are released from prothrombin upon its activation, are able to neutralize heparin. These results might indicate an important role for

Figure 8. The central role of thrombin in the blood coagulation system. Thrombin is responsible for acceleration of its own generation by means of activation of the non enzymatic protein cofactors factor VIII:C and factor V, and by means of providing a procoagulant surface by activating the platelets. thrombin is responsible for inhibition of its generation by activating protein C, an important inhibitor of factor VIII:C_a and factor V_a.

fragment 1.2 formation in the regulation of blood coagulation by competing with antithrombin III for the available heparin.

The following chapter (chapter III) deals with the action of antithrombin III/heparin on the activated coagulation factors during enzymatic conversion of factor X into factor X_a by activated factor IX in the presence of phospholipids and calcium. We clearly demonstrated that under these more physiologic conditions, the kinetics of inactivation differ considerably from the kinetics of inhibition found for isolated, purified coagulation factors. For example, we found that factor X bound to phospholipid vesicles was able to protect factor X_a from inhibition by antithrombin III, which may be an important mechanism for maintaining coagulation in vivo. Also, we unexpectedly found that calcium ions stimulated the heparin accelerated inhibition of factor IX_a by antithrombin III.

Next we moved to whole plasma. To be able to measure specific activated coagulation factors in plasma without interference by other coagulation factors, we developed extremely sensitive bio-assay's, for factor X_a , factor IX_a , factor V_a and factor VIII:C_a. In chapter IV we demonstrated that for the anticoagulant effect of heparin in plasma, the inactivation of factor X_a (and thus the anti- X_a activity of heparin) is of no importance. Rather the ability of heparin to inhibit factor V_a generation (via the inhibition of thrombin) causes the anticoagulant effect.

In chapter V we investigated the kinetics of factor IX_a catalyzed factor IX activation in plasma. Furthermore, we studied the effect of heparin and the synthetic pentasaccharide on both the inactivation of factor IX_a and factor XI_a in plasma. Our results showed that the kinetics of factor XI_a catalyzed factor IX activation were identical to those found in purified systems. Furthermore, in contrast to what was expected, heparin and pentasaccharide did have significant effects on factor IX_a generation in plasma.

In chapter VI finally we studied the activation of the non enzymatic protein cofactors factor V and factor VIII:C in thromboplastin-activated plasma. The enzymes in this system which possibly activate these cofactors are factor X_a or thrombin. However, our results show that thrombin is the only enzyme capable of activating factor V and factor VIII:C under our conditions.

References

1. Malpighi M: De Polype Cordis. In Opera Omnia, London, 1686. Quoted from a reprint published by Almqvist and Wiksells, Stockholm, 1953, pp 123-139

2. Ratnoff OD: Blood clotting mechanisms: An overview, in Ogston D, Bennet B (eds): Haemostasis: Biochemistry, Physiology, and Pathology. John Wiley & Sons, 1977, pp 1-24
3. Hewson W: An experimental inquiry into the properties of blood, with some remarks on its morbid appearances. Reprinted in The Works of William Hewson, F.R.S., Sydenham Society. London, 1846, p 40
4. Chaptal HA: Annales de Chimie 21:284, 1797
5. Buchanan A: on the coagulation of the blood and other fibrineferous liquids. London Med Gaz 1:617, 1845
6. Schmidt A: Zur blutlehre. F.C.W. Vogel Verlag, Leipzig, 1892. Quoted by Morawitz: Die chemie der blutgerinnung. Ergebn Physiol 4:307, 1905
7. Hemker CW: De ontdekking van het prothrombine. PhD Thesis, Rijksuniversiteit Limburg, Maastricht, The Netherlands, 1981, p 76
8. Pekelharing CA: Over den aard van het fibrineferment. Onderz v h Phys Lab te Utrecht, 4e reeks, 1:77, 1891
9. de Blainville H. In: Gaz Med Paris, Ser 2, 2:524, 1834
10. Schmidt A: Ueber den faserstoff und die ursache seiner gerinnung. Arch Anat Physiol Wissensch Medizin, 28:545, 1861
11. Morawitz P: Beitrage zur kenntnis der blutgerinnung. Deutsche Archiv Klin Med, 79:1, 1903
12. Warner ED, Brinkhous KM, Smith HP: The prothrombin conversionrate in various species Proc Soc Exp Biol Med 40:197, 1939
13. Quick AJ: On the constitution of prothrombin. Amer J Physiol 140:212, 1943
14. Warner ED, Brinkhous KM, Smith HP: A quantitative study on blood clotting. Prothrombin fluctuates under experimental conditions. Amer J Physiol 114:667, 1936
15. Quick AJ: The nature of the bleeding in jaundice. Journ A M A, 110:1658, 1938
16. Owren PA: The coagulation of blood, investigations on a new clotting factor. I. Demonstration of a new factor in the coagulation process. Acta Med Scand, Suppl 194:1, 1947
17. Owren PA: The prothrombin activating complex and its clinical significance. Report of the 3rd International Congress of Haematology, Grune and Stratton, New York, 1950, pp 379-390
18. Owen CA, Bollman JL: Prothrombin conversion factor of dicoumarol plasma. Proc Soc Exp Biol NY, 67:231, 1948
19. De Vries A, Alexander B, Goldstein R: A factor in serum which accelerates the conversion of prothrombin to thrombin. I. Its determination and some physiologic and biochemical properties. Blood 4:247, 1949
20. Alexander B, De Vries A, Goldstein R: A factor in serum which accelerates the conversion of prothrombin to thrombin. II. Its evolution with special reference to the influence of conditions which affect blood coagulation. Blood 4:739, 1949
21. Koller F, Loeliger A, Duckert F: Experiments on a newclotting factor (factor VII). Acta Haematologica 6:1, 1951
22. Koller F, Loeliger A, Duckert F: Le facteur VII. Rev Hemat 7:156, 1952
23. MacFarlane RG: The theory of blood coagulation, in Biggs R (ed): Human Blood Coagulation, Haemostasis and Thrombosis. Blackwell Scientific Publishers, 1976, pp 1-31
24. Duckert F, Fluckinger P, Koller F: Le role du facteur X dans la formation de la thromboplastin sanguine. Rev Hemat 9:489, 1954
25. Duckert F, Fluckiger P, Matter M, Koller F: Clotting factor X. Physiologic and physicochemical properties. Proc Soc Exp Biol NY, 90:17, 1955
26. Hougie C, Barrow EM, Graham JB: Stuart clotting defect. I. Segregation of an hereditary hemorrhagic state from the heterogeneous group heretofore called "stable factor" (SPCA, proconvertin, factor VII) deficiency. J Clin Invest, 36:485, 1957

27. Telfer TP, Denson KW, Wright DR: A "new" coagulation defect. *Brit J Haemat* 2:308, 1956
28. Nemerson Y: The reaction between bovine brain tissue factor and factors VII and X. *Biochemistry* 5:601, 1966
29. Williams WJ, Norris DG: Purification of a bovine plasma protein (factor VII) which is required for the activity of lung microsomes in blood coagulation. *J Biol Chem* 241:1847, 1966
30. Hanahan DJ, Papahadjopoulos D: Interactions of phospholipids with coagulation factors. *Thromb Diath Haemorrh suppl* 17:71, 1965
31. Hemker HC, Esnouf MP, Hemker PW, Swart ACW, MacFarlane RG: Formation of prothrombin converting activity. *Nature* 215:248, 1967
32. Varadi K, Hemker HC: Kinetics of the formation of the factor X activating enzyme of the blood coagulation system. *Thromb Res* 8:303, 1976
33. Nolf P: The coagulation of the blood. *Medicine* 17:381, 1938
34. Bordet J, Gengou O: Recherches sur la coagulation du sang et les serums anticoagulants. *Ann Inst Pasteur* 15:129, 1901
35. Morawitz P: Die chemie der blutgerinnung. *Ergeb Physiol* 4:307, 1905
36. Conley CL, Hartmann RC, Morse WI: The clotting behaviour of human "platelet-free" plasma: evidence for the existence of a "plasma thromboplastin". *J Clin Invest* 28:340, 1949
37. Ratnoff OD, Colopy JE: A familial hemorrhagic trait associated with a deficiency of a clot promoting fraction in plasma. *J Clin Invest* 34:602, 1955
38. Rosenthal RL, Dreskin OH, Rosenthal N: New hemophilia-like disease caused by deficiency of a third plasma thromboplastin factor. *Proc Soc Exp Biol N Y* 82:171, 1953
39. Patek AJ, Stetson RP: Haemophilia. I. The abnormal coagulation of the blood and its relation to the blood platelets. *J Clin Invest* 15:531, 1936
40. Shanberge JN, Gore I: Studies on the immunologic and physiologic activities of anti-haemophilic factor (AHF). *J Lab Clin Med* 50:954, 1957
41. Pavlovsky A: Contribution to the pathogenesis of hemophilia. *Blood* 2:185, 1947
42. Aggeler PM, White SG, Glendening MB, Page EW, Leake TB, Bates G: Plasma thromboplastin component (PTC) deficiency: a new disease resembling hemophilia. *Proc Soc Exp Biol N Y* 79:692, 1952
43. Biggs R, Douglas AS, Macfarlane RG, Dacie JV, Pitney WR, Merskey C, O'Brien JR: Christmas disease: a condition previously mistaken for haemophilia. *Brit Med J* 2:1378, 1952
44. Schulman I, Smith CH: Hemorrhagic disease in an infant due to deficiency of a previously undescribed clotting factor. *Blood* 7:794, 1952
45. Macfarlane RG: An enzyme cascade in the blood clotting mechanism, and its function as a biochemical amplifier. *Nature* 202:498, 1964
46. Davie EW, Ratnoff OD: Waterfall sequence for intrinsic blood clotting. *Science* 145:1310, 1964
47. Bennet B: Coagulation pathways: Interrelationships and control mechanisms. *Seminars in Hematology* 14:301, 1977
48. Nemerson Y: Tissue factor and hemostasis. *Blood* 71:1, 1988
49. Osterud B, Rapaport SI: Activation of factor IX by the reaction product of tissue factor and factor VII: Additional pathway for initiating blood coagulation. *Proc Natl Acad Sci USA* 74:5260, 1977
50. Jobin F, Esnouf MP: Studies on the formation on the prothrombin converting complex. *Biochem J* 102:666, 1967
51. Rapaport SI, Schiffman S, Patch MJ, Ames SB: The importance of activation of anti-hemophilic globulin and proaccelerin by traces of thrombin in the generation of intrinsic prothrombinase activity. *Blood* 21:221, 1963

52. Biggs R, Macfarlane RG, Denson KWE, Ash BJ: Thrombin and the interaction of factors VIII and IX. *Brit J Haemat* 11:276, 1965
53. Osterud B, Rapaport SI, Schiffman S, Chang MMY: Formation of intrinsic factor X-activator activity, with special reference to the role of thrombin. *Br J Haematol* 21:643, 1971
54. Bailey K, Bettelheim FR, Lorand L, Middelbrook WR: Action of thrombin in the clotting of fibrinogen. *Nature* 167:233, 1951
55. Shuman MA, Majerus PW: The measurement of thrombin in clotting blood by radioimmunoassay. *J Clin Invest* 58:1249, 1976
56. Broze GJ, Leykam JE, Schwartz BD, Miletich JP: Purification of human tissue factor. *J Biol Chem* 260:10917, 1985
57. Guha A, Bach R, Konigsberg W, Nemerson Y: Affinity purification of human tissue factor: Interaction of factor VII and tissue factor in detergent micelles. *Proc Natl Acad Sci USA* 83:299, 1986
58. Zur M, Nemerson Y: Tissue factor pathway of coagulation. in Bloom, Thomas (eds). *Haemostasis and Thrombosis*. Churchill Livingstone, 1981, pp 124-139
60. Rao LVM, Rapaport SI: Studies of a mechanism inhibiting the initiation of the extrinsic pathway of coagulation. *Blood* 69:645, 1987
61. Pitlich FA: Concanavalin A inhibits tissue factor coagulant activity. *J Clin Invest* 55:175, 1975
62. Lämmle B, Griffin JH: Formation of the fibrin clot: the balance of procoagulant and inhibitory factors. *Clinics in Haematology* 14:281, 1985
63. Dam H: The antihemorrhagic vitamin of the chick. *Biochem J* 29:1273, 1935
64. Hemker HC, Velkamp JJ, Hensen A, Loeliger EA: Nature of prothrombin biosynthesis: preprothrombinaemia in vitamin K-deficiency. *Nature* 200:589, 1963
65. Josso F, Lavergne JM, Gouault M, Prou-Wartelle O, Soulier JP: Différents états moléculaires du facteur II (prothrombine). Leur étude à l'aide de la staphylocoagulase et d'anticorps anti-facteur II. *Thromb Diath Haemorrh* 20:88, 1968
66. Stenflo J: A new vitamin K-dependent protein. Purification from bovine plasma and preliminary characterization. *J Biol Chem* 251:355, 1976
67. Suttie JW: Mechanism of action of vitamin K: Synthesis of -carboxyglutamic acid. *CRC Crit Rev Biochem* 8(2):189, 1980
68. Nelsestuen GL: Role of -carboxyglutamic acid. An unusual protein transition required for the calcium-dependent binding of prothrombin to phospholipid. *J Biol Chem* 251:5-648, 1976
69. Bach R, Nemerson Y, Konigsberg W: Purification of bovine tissue factor. *J Biol Chem* 256:8324, 1981
70. Morita T, in Suttie (ed): Vitamin-K metabolism and vitamin-K dependent proteins. (University Park Press, Baltimore) 1979, pp 124-128
71. van der Graaf F, Greengard JS, Bouma BN, Kerbiriou DM, Griffin JH: Isolation and functional characterisation of the active light chain of activated human blood coagulation factor XI. *J Biol Chem* 258:9669, 1983
72. Forman SD, Nemerson Y: Membrane-coagulation reaction is dependent of the concentration of phospholipid-bound substrate: Fluid phase factor X regulates the extrinsic system. *Proc Natl Acad Sci USA* 83:4675, 1986
73. Bajaj SP, Rapaport SI, Brown SF: Isolation and characterisation of human factor VII. Activation of factor VII by factor X_a. *J Biol Chem* 256:253, 1981
74. Zur M, Nemerson Y: The esterase activity of coagulation factor VII. *J Biol Chem* 253:2203, 1978
75. Rao LVM, Rapaport SI, Bajaj SP: Activation of human factor VII in the initiation of tissue factor-dependent blood coagulation. *Blood* 68:685, 1986
76. Radcliffe R, Nemerson Y: Mechanism of activation of bovine factor VII. Products of cleavage by factor X_a. *J Biol Chem* 251:4797, 1976

77. Radcliffe R, Bagdasarian, Colman R, Nemerson Y: Activation of bovine factor VII by Hageman factor fragments. *Blood* 50:611, 1977
78. Seligson J, Osterud B, Brown SF, Griffin JH, Rapaport SI: Activation of human factor VII in plasma and in purified systems. Roles of activated factor IX, kallikrein, and activated factor XII. *Clin Invest* 64:1056, 1979
79. Pieters J, Lindhout T: The limited importance of the anti-X_a activity of heparin in thromboplastin activated plasma. *Blood*, in press
80. Giles AR, Tinlin S, Brosseau L, Hoogendoorn H: In vivo studies of the role of factor VII in Hemostasis. *Blood* 65: 1197, 1985
81. DiScipio RG, Kurachi K, Davie EW: Activation of human factor IX (Christmas Factor). *J Clin Invest* 61:1528, 1978
82. Osterud B, Bouma BN, Griffin JH: Human blood coagulation factor IX. Purification, properties, and mechanism of activation by activated factor XI. *J Biol Chem* 253:5946, 1978
83. Ratnoff OD, Davie EW, Mallett DC: Studies on the action of Hageman Factor: Evidence that activated Hageman Factor in turn activates plasma thromboplastin antecedent. *J Clin Invest* 40:803, 1961
84. Rosenberg JS, McKenna PW, Rosenberg RD: Inhibition of human factor IX_a by human antithrombin. *J Biol Chem* 250:8883, 1975
85. Kurachi K, Fujikawa K, Schmer G, Davie EW: Inhibition of factor IX_a and factor X_a by antithrombin III. *Biochemistry* 15:373, 1976
86. Pieters J, Willems G, Hemker HC, Lindhout T: Inactivation of factor IX_a and factor X_a during factor X activation by antithrombin III/heparin. *J Biol Chem* 263:15313, 1988
87. Fuchs J, Trapp HG, Griffith MJ, Roberts HR, Pizzo SV: Regulation of factor IX_a in vitro in human and mouse plasma and in vivo in the mouse. *J Clin Invest* 73:1696, 1984
88. McNeely T, Griffith MJ: The anticoagulant mechanism of action of heparin in contact-activated plasma: Inhibition of factor X activation. *Blood* 65:1226, 1985
89. Pieters J, Soons H, Willems G, Franssen J, Hemker HC, Lindhout T: Factor XI_a catalyzed activation of factor IX in human plasma; Effect of heparin. Submitted for publication
90. Hedner U, Davie EW: Factor IX. in: Colman RW (ed): Hemostasis and thrombosis. Philadelphia JB Lippincott, 1982, pp 29
91. Roberts HR, Gross GP, Webster WP, Dejanov II, Penick GD: Acquired inhibitors of plasma factor IX. A study of their induction properties and neutralisation. *Am J Med Sci* 251:43, 1966
92. Thompson J: Factor IX antigen by radioimmunoassay. Abnormal factor IX protein in patients on warfarin therapy and with hemophilia B. *J Clin Invest* 59:900, 1977
93. Fujikawa K, Legaz ME, Davie EW: Bovine factor XI (Stuart Factor). Mechanism of activation by a protein from Russell's viper venom. *Biochemistry* 11:4882, 1972
94. Fair J, Plow EF, Edgington TS: Combined functional and immunochemical analysis of normal and abnormal factor X. *J Clin Invest* 64:884, 1979
95. Jesty J, Spencer AK, Nemerson Y: The mechanism of activation of factor X. Kinetic control of alternative pathways leading to the formation of activated factor X. *J Biol Chem* 249: 5614, 1974
96. Yin ET, Wessler S, Stoll PJ: Rabbit plasma inhibitor of the activated species of blood coagulation factor X. *J Biol Chem* 246:3694, 1971
97. Fuchs J, Pizzo SV: Regulation of factor X_a in vitro in human and mouse plasma and in vivo in the mouse. Role of the endothelium and plasma proteinase inhibitors. *J Clin Invest* 72:2041, 1983
98. Hemker HC: In memory of François Josso. Why do hemophiliacs bleed? *Scand J Haematol (Suppl 40)* 33:11, 1984
99. Suttie JW, Jackson CM: Prothrombin structure, activation and biosynthesis. *Physiol Rev* 57:1, 1977

100. Stenn KS, Blout ER: Mechanism of bovine prothrombin activation by an insoluble preparation of bovine factor X_a (thrombokinase). *Biochemistry* 11:4502, 1972
101. Esnouf MP, Jobin F: Lipids in prothrombin conversion. *Thromb Diath Haemorrh* 17:103, 1965
102. Esmon CT, Owen WG, Jackso CM: A plausible mechanism for prothrombin activation by factor X_a, factor V_a, phospholipids, and calcium ions. *J Biol Chem* 249:8045, 1974
103. Govers-Riemslog JWP, Speijer H, Zwaal RFA, Rosing J: The effects of prothrombin fragment 1 and fragment 1.2 on prothrombin activation. *Thromb Res* 38:375, 1985
104. Pieters J, Franssen J, Visch C, Lindhout T: Neutralization of heparin by prothrombin activation products. *Thromb Res* 45:573, 1987
105. Abilgaard In The physiological inhibitors of blood coagulation and fibrinolysis. Collen et al. (eds), Amsterdam, Elsevier/ North-Holland, 1979, pp 31-33
106. Steinbach Rev Française d'études cliniques et biologiques 13:179, 1968
107. Saito H, Goldsmith GH, Moroi M, Aoki N: Inhibition spectrum of α -2-plasmin inhibitor. *Proc Natl Acad Sci Usa* 76:2013, 1979
108. Tollefsen DM, Majerus DW, Blank MK: Heparin cofactor II. Purification and properties of a heparin-dependent inhibitor of thrombin in human plasma. *J Biol Chem* 257:2162, 1982
109. Kane WH, Davie EW: Blood coagulation V and VIII: Structural and functional similarities and their relationship to hemorrhagic and thrombotic disorders. *Blood* 71:539, 1988
110. Esmon CT: The regulation of natural anticoagulant pathways. *Science* 235:1348, 1987
111. Sadler JE, Davie EW: Hemophilia A, hemophilia B and von Willebrand's disease, in Stamatoyannopoulos G, Nienhuis AW, Leder P, Majerus PW (eds): The molecular basis of blood diseases. Philadelphia, Saunders, 1987, p 575
112. Seeler RA: Parahemophilia. Factor V deficiency. *Med Clin North Am* 56:119, 1972
113. Soff GA, Levin J: Familial multiple coagulation factor deficiencies. *Semin Thromb Hemost* 7:112, 1981
114. Zimmerman TS, Fulcher CA: Factor VIII procoagulant protein. *Clinics in haematology* 14:343, 1985
115. Fulcher CA, Zimmerman TS: Characterization of the human factor VIII procoagulant protein with a heterologous precipitating antibody. *Proc Natl Acad Sci Usa* 79:1648, 1982
116. Toole JJ, Knopf JL, Wozney JM, Sultzman LA, Buecker JL, Pittman DD, Kaufman RJ, Brown, E, Shoemaker C, Orr EC, Amphlett GW, Foster WB, Coe ML, Knutson GJ, Fass DN, Hewick RM: Molecular cloning of a cDNA encoding human antihemophilic factor. *Nature* 312:342, 1984
117. Gitscher J, Wood WI, Goralka TM, Wion KL, Chen EY, Eaton DH, Vehar GA, Capon DJ, Lawn RM: Characterization of the human factor VIII gene. *Nature* 312:326, 1984
118. Wood WI, Capon DJ, Simonsen CC, Eaton DL, Gitscher J, Keyt B, Seebyrg PH, Smith DH, Hollingshead P, Wion KL, Delwart E, Tuddenham EGD, Vehar GA, Lawn RM: Expression of active human factor VIII from recombinant DNA clones. *Nature* 312:330, 1984
119. Vehar GA, Keyt B, Eaton D, Rodriguez H, O'Brien DP, Rotblat F, Oppermann H, Keck R, Wood WI, Harkins RN, Tuddenham EGD, 312:337, 1984
120. Vehar GA, Davie EW: Preparation and properties of bovine factor VIII (Antihemophilic Factor). *Biochemistry* 19:401, 1980
121. Eaton DL, Wood WI, Eaton D, Hass PI, Hollingshead P, Wion K, Mather J, Lawn RM, Vehar GA, Gorman C: Construction and characterization of an active factor VIII variant lacking the central one-third of the molecule. *Biochemistry* 25:505, 1986
122. Rick ME: Activation of factor VIII by factor IX_a. *Blood* 60:74, 1982
123. Tracy PB, Ride LL, Bowie EJW, Mann KG: Radioimmunoassay of factor V in human plasma and platelets. *Blood* 60:95, 1982

124. Dahlbec B: Human coagulation factor V purification and thrombin-catalyzed activation. *J Clin Invest* 66:583, 1980
125. Viskupp RW, Tracy PB, Mann KG: The isolation of human platelet factor V. *Blood* 69:1188, 1987
126. Cervený TJ, Fass DN, Mann KG: Synthesis of coagulation factor V by cultured aortic endothelium. *Blood* 63:1467, 1984
127. Suzuki K, Dahlbeck B, Stenflo J: Thrombin-catalyzed activation of human coagulation factor V. *J Biol Chem* 257:6556, 1982
128. Foster WB, Nesheim ME, Mann KG: The factor X_a-catalyzed activation of factor V. *J Biol Chem* 258:13970, 1983
129. Krisnaswamy S, Mann KG, Nesheim ME: The prothrombinase-catalyzed activation of prothrombin proceeds through the intermediate meizothrombin in an ordered, sequential reaction. *J Biol Chem* 261:8977, 1986
130. Rosing J, Zwaal RFA, Tans G: Formation of meizothrombin as intermediate in factor X_a-catalyzed prothrombin activation. *J Biol Chem* 261:4224, 1986
131. Walker FJ, Sexton PW, Esmon CT: The inhibition of blood coagulation by activated protein C through the selective inactivation of activated factor V. *Biochim Biophys Acta* 571:333, 1979
132. Tracy PB, Mann KG: Abnormal formation of the prothrombinase complex: Factor V deficiency and related disorders. *Hum Pathol* 18:162, 1987
133. Chin HC, Whitaker E, Colman RW: Heterogeneity of human factor V deficiency. Evidence for the existence of antigen-positive variants. *J Clin Invest* 72:493, 1983
134. Thompson RE, Mandle R, Kaplan AP: Association of factor XI and high molecular weight kininogen in human plasma. *J Clin Invest* 60:1376, 1977
135. Saito H, Goldsmith GH Jr: Plasma Thromboplastin Antecedent (PTA), Factor XI: A specific and sensitive radioimmunoassay. *Blood* 50:377, 1977
136. Bouma BN, Griffin JH: Human blood coagulation factor XI. Purification, properties, and mechanism of activation by activated factor XII. *J Biol Chem* 252:6432, 1977
137. Kurachi K, Davie EW: Activation of human factor XI (Plasma Thromboplastin Antecedent) by factor XII_a (Activated Hageman Factor). *Biochemistry* 16:5831, 1977
138. Scott CF, Schapira M, James HL, Cohen AB, Colman RW: Inactivation of factor XI_a by plasma protease inhibitors. Predominant role of α_1 -protease inhibitor and protective effect of high molecular weight kininogen. *J Clin Invest* 69:844, 1982
139. Meijers JCM: Regulation of the blood coagulation mechanism by plasma proteinase inhibitors. Thesis at the University of Utrecht, Utrecht, the Netherlands, 1988
140. Josso F, Prou-Wartelle O: Interaction of tissue factor and factor VII at the earliest phase of coagulation. *Thromb Diathes Haemorrh* 14(Suppl 17):35, 1965
141. Osterud B, Papaport SI: Activation of 125I-factor IX and 125I-factor X: Effect of tissue factor and factor VII, factor X_a and thrombin. *Scand J Haematol* 24:213, 1980
142. Ratnoff OD, Saito H: Surface-mediated reactions. Current topics in hematology 2:1, 1979
143. Kaplan AP, Silverberg M: The coagulation-kinin pathway of human plasma. *Blood* 70:1, 1987
144. Kiesel W, Canfield WM, Ericsson LH, Davie EW: Anticoagulant properties of bovine plasma protein C following activation by thrombin. *Biochemistry* 16:5824, 1977
145. Griffin JH, Mosher DF, Zimmerman TS, Kleiss AJ: Protein C, an antithrombotic protein, is reduced in hospitalized patients with intravascular coagulation. *Blood* 60:261, 1982
146. Kiesel W: Human plasma protein C. Isolation, characterization and mechanism of activation by human α -thrombin. *J Clin Invest* 64:761, 1979
147. Esmon CT: Protein C: Biochemistry, physiology, and clinical implications. *Blood* 62:1155, 1983

148. Esmon NL, Owen WG, Esmon CT: Isolation of a membrane-bound cofactor for thrombin-catalyzed activation of protein C. *J Biol Chem* 257, 859, 1982
149. Esmon N, DeBault L, Esmon C: Proteolytic formation and properties of γ -carboxyglutamic acid-domainless protein C. *J Biol Chem* 258:5548, 1983
150. Salem HH, Broze GJ, Miletich JP, Majerus PW: Human coagulation factor V_a is a cofactor for the activation of Protein C. *Proc Natl Acad Sci USA* 80:1584, 1983
151. Salem HH, Broze GJ, Miletich JP, Majerus PW: The light chain of factor V_a contains the activity of factor V_a that accelerates protein C activation by thrombin. *J Biol Chem* 258:8531, 1983
152. Walker F: Regulation of activated protein C by a new protein: A possible function for protein S. *J Biol Chem* 255:5521, 1980
153. Marlar RA, Griffin JH: Deficiency of protein C inhibitor in combined factor V/VIII deficiency disease. *J Clin Invest* 66:1186, 1980
154. Suzuki K, Nishioka J, Hashimoto S: Protein C inhibitor. Purification from human plasma and characterization. *J Biol Chem* 258:163, 1983
155. Bertina RM, Broekmans AW, van der Linden IK, Mertens K: Protein C deficiency in a Dutch family with thrombotic disease. *Thromb Haemost* 48:1, 1982
156. Reutelingsperger CPM: Vascular anticoagulant, a new physiological anticoagulant mechanism. Thesis, University of Limburg, Maastricht, the Netherlands, 1987
157. Verhey HM, Boffa M-C, Rothen C, Brycaert M-L, Verger R, de Haas GH: Correlation of enzymatic activity and anticoagulant properties of phospholipase A2. *Eur J Biochem* 112:25, 1980
158. Thiagarajan P, Shapire SS, De Marco L: Monoclonal Immunoglobulin M1 coagulation inhibitor with phospholipid specificity. *J Clin Invest* 66:397, 1980
159. Feinstein Prog Hemostasis Thromb 1:75, 1972
160. Forman S, Bach R, Nemerson Y: *Fed Proc* 43:1946, 1984
161. Nawroth PP, Kiesel W, Stern DM: Anticoagulant and antithrombotic properties of a γ -carboxyglutamic acid-rich peptide derived from the light chain of blood coagulation factor X. *Thromb Res* 44:625, 1986
162. Reutelingsperger CPM, Hornstra G, Hemker HC: Isolation and partial purification of a novel anticoagulant from arteries of human umbilical cord. *Eur J Biochem* 151:625, 1985
163. Sanders NL, Bajaj SP, Zivelin A, Rapaport SI: Inhibition of tissue factor/factor VII_a activity in plasma requires factor X and an additional plasma component. *Blood* 66:204, 1985
164. Carrel RW, Christey PB, Boswell DR: Serpins: antithrombin and other inhibitors of coagulation and fibrinolysis. Evidence from amino acid sequences. In Verstraete M, Vermeylen J, Lijnen R, Arnout J (eds): *Thrombosis and Haemostasis 1987*, Leuven University Press, Leuven, pp 1
165. Barrowcliffe TW, Thomas DP: Antithrombin III and heparin, in Bloom AL, Thomas DP (eds): *Haemostasis and Thrombosis*, Second edition, Churchill Livingstone, 1987, pp 849
166. Abildgaard U: Highly purified antithrombin III with heparin cofactor activity prepared by disc electrophoresis. *Scand J Clin Lab Invest* 21:89, 1968
167. Kurachi K, Schmer G, Hermodson MA, Teller DC, Davie EW: Characterization of human, bovine, and horse antithrombin III. *Biochemistry* 15:368, 1976
168. Murano G, Williams L, Miller-Andersson M, Aronson DL, King C: Some properties of antithrombin III and its concentration in human plasma. *Thromb Res* 18:259, 1980
169. Conard J, Brosstad F, Lie Larsen M, Samama M, Abildgaard U: Molar antithrombin concentration in normal human plasma. *Haemostasis* 13:363, 1983
170. Peterson CB, Blackburn MN: Isolation and characterization of an antithrombin III variant with reduced carbohydrate content and enhanced heparin binding. *J Biol Chem* 260:610, 1985

171. Milner AE, Burnett D, Rutter J, Bradwell AR: Detection of antithrombin III micro-heterogeneity. *Thromb Res* 37:127, 1985
172. Abildgaard U: Binding of thrombin to antithrombin III. *Scand J Clin Lab Invest* 24:23, 1969
173. Binder B: On the complex formatio of antithrombin III with thrombin. Gel filtration studies on human plasma and serum. *Thromb Diath Haemorrh* 30:280, 1973
174. Pepper DS, Bânhegyi D, Cash JD: The different forms of AT III in serum. *Thromb Haemost* 38:494, 1977
175. Marciniac E, Gora-Maslak G: High molecular weight forms of antithrombin III complexes in blood. *Thromb Haemost* 49:32, 1983
176. Jesty J: Dissociation of complexes and their derivates formed during inhibition of bovine thrombin and activated factor X by antithrombin III. *J Biol Chem* 254:1044, 1979
177. Danielsson A, Björk I: Slow, spontaneous dissociation of the antithrombin thrombin complex produces a proteolytically modified form of the inhibitor. *FEBS Letters* 119:2-41, 1980
178. Björk I, Fish WW: Production in vitro and properties of a modified form of antithrombin, cleaved at the active site by thrombin. *J Biol Chem* 257:9487, 1982
179. Marciniac E, Tsukamura S: Two progressive inhibitors of factor X_a in blood. *Br J Haematol* 22:341, 1972
180. Lindhout T, Baruch D, Schoen P, Franssen J, Hemker HC: Thrombin gneration and in-activation in the presence of antithrombin III and heparin. *Biochemistry* 25:5962, 1986
181. Rosenberg RD: Heparin antithrombin III system. In: Colman RW (ed): Hemostasis and thrombosis. Philadelphia JB Lippincott, 1982, pp 962
182. Björk I, Lindahl U: Mechanism of the anticoagulant action of heparin. *Molecular and Cellular Biochemistry* 48:161, 1982
183. Choay J, Petitou M, Lormeau JC, Sinay P, Casu B, Gatti G: Structure activity relationship in heparin: A synthetic pentasaccharide with high affinity for antithrombin III and eliciting high anti-factor X_a activity. *Biochem Biophys Res Comm* 116: 492, 1983
184. Esquivel CO, Bergqvist D, Blörck C-G, Nilson B: Comparisom between comercial heparin, low molecular weight heparin and pentosan polysulfate on hemostasis and platelets in vivo. *Thromb Res* 28:389, 1982
185. Ellison N, Edmunds LH, Colman RW: Platelet aggregation following heparin and protamine administration. *Anesthesiology* 48:65, 1978
186. Casu B: Structure of heparins and their fragments. *Nouvelle Revue Française d'Hematologie* 26:211, 1984
187. Lam LH, Silbert JE, Rosenberg RD: The separation of active and inactive forms of heparin. *Biochim Biophys Res Comm* 69:570, 1976
188. Rosenberg RD, Jordan RE, Favreau LV, Lam LH: Highly active heparin species with multiple binding sites for antithrombin. *Biochim Biophys Res Comm* 86:1319, 1979
189. Holmer E, Kurachi K, Söderström G: The molecular weight dependence of the rate enhancing effect of heparin on the inhibition of thrombin, factor X_a, factor IX_a, factor XI_a, factor XII_a and kallikrein by antithrombin. *Biochem J* 193:295, 1981
190. Griffith MJ: Kinetics of the heparin-enhanced antithrombin III.thrombin reaction. Evidence for a template model for the mechanism of action of heparin. *J Biol Chem* 157:7360, 1982
191. Lane DA, MacGregor IR, Michalski R, Kakkar VV: Anticoagulant activities of four unfractionated and fractionated heparins. *Thromb Res* 12:257, 1978
192. Petitou M: synthetic heparin fragments: new and efficient tools for the studie of heparin and its interactions. *Nouv Rev Franç d'Hematol* 26:221, 1984
193. Oosta GM, Gardner WT, Beeler DL, Rosenberg RD: Multiple functional domains of the heparin molecule. *Proc Natl Acad Sci USA* 78:829, 1981

194. Lane DA, Denton J, Flynn AM, Thunberg L, Lindahl U: Anticoagulant activities of heparin oligosaccharides and their neutralization by platelet factor 4. *Biochem J* 218:725, 1984
195. Ireland H, Lane DA, Curtis JR: Objective assesment of heparin requirements for haemodialysis in humans. *J Lab Clin Med* 103:643, 1984
196. Salzman EW: Low-molecular-weight heparin. Is small beautiful? *New Eng J Med* 315:957, 1986
197. Carter CJ, Kelton JG, Hirsh J, Cerskus A, Santos AV, Gent M: The relationship between the hemorrhagic and antithrombotic properties of low molecular weight heparin in rabbits. *Blood* 59:1239, 1982
198. Holmer E, Mattson C, Nilsson S: Anticoagulant and antithrombotic effects of heparin and low molecular weight heparin fragments in rabbits. *Thromb Res* 25:475, 1982
199. Barrowcliffe TW, Johnson EA, Eggleton CA, Kembell-Cook G, Thomas DP: Anticoagulant activities of high and low molecular weight heparin fractions. *Br J Haematol* 41:573, 1979
200. Andersson L-O: Anticoagulant properties of heparin fractionated by affinity chromatography on matrix-bound antithrombin III and by gel filtration. *Thromb Res* 9:575, 1976
201. Thomas DP: Current status of low molecular weight heparin. *Thromb Haemost* 56(3):241, 1986
202. Thomas DP: Heparin in prophylaxis and treatment of venous thromboembolism. *Sem Haematol* 15:1, 1978
203. Ofosu FA, Blajchman MA, Modi GJ, Smith LM, Buchanan MR, Hirsh J: The importance of thrombin inhibition for the expression of the anticoagulant activities of heparin, dermatan sulphate, low molecular weight heparin and pentosan polysulphate. *Br J Haematol* 60:695, 1985
204. Berqvist D, Burmark US, Frisell J: Low molecular weight heparin once daily compared with conventional low dose heparin twice daily. A prospective double blind multicentre trial on prevention of postoperative thrombosis. *Br J Surg* 73:204, 1986
205. Hemker HC: The mode of action of heparin in plasma, in Verstraete M, Vermeylen J, Lijnen R, Arnout J (eds): *Thrombosis and Haemostasis 1987*. Leuven University Press, Leuven

Chapter II

Neutralization of Heparin by Prothrombin Activation Products^o

Abstract

The neutralization of heparin by active site blocked meizothrombin and thrombin, prothrombin fragment 1.2, fragment 1 and fragment 2 was probed by the heparin-dependent factor X_a inactivation by antithrombin III (AT III). Meizothrombin had no effect on the inactivation of factor X_a , whereas thrombin had an inhibitory effect ($IC_{50} = 700$ nM). After factor X_a catalyzed cleavage of meizothrombin, the resulting products, prothrombin fragment 1.2 plus thrombin, did not show any heparin neutralizing properties. However, after isolation of the reaction products, both thrombin and prothrombin fragment 1.2 exhibited heparin neutralizing properties in the factor X_a inactivation reaction. The IC_{50} -values were 700 nM and 100 nM, respectively. Prothrombin fragment 1, when present at 125 nM, caused a 50% reduction of the heparin-dependent rate of inactivation of factor X_a and prothrombin fragment 2 had no effect at all. From this we conclude that, in addition to the thrombin part of the prothrombin molecule, the fragment 1 region also exhibits a rather high affinity for heparin.

^o Based on: Pieters J, Franssen J, Visch C, Lindhout T: *Thromb Res* 45:573, 1987

Introduction

During the activation of prothrombin by factor X_a various activation products are formed. Dependent on the order of cleavage, prethrombin 2 or meizothrombin occur as intermediates, which finally give rise to formation of thrombin and the activation peptides fragment 1.2, fragment 1 and fragment 2 (1,2). As the activation peptide of prothrombin that is released during activation is almost half the prothrombin molecule, it may be related to important biological functions, as suggested by Walker and Esmon (3). Especially, the activation peptides could play a regulatory role in the formation of thrombin at the site of its generation. The persistence of thrombin away from the site of its generation is prevented by several natural inhibitors of serine proteases, of which AT III is the main one (4,5).

Heparin, a highly sulfated mucopolysaccharide, has been shown to greatly enhance the inhibition of thrombin and other serine proteases by AT III. The mechanism is thought to be by binding to AT III and inducing a conformational change which facilitates AT III binding to activated serine proteases (6,7) and/or by direct interaction with these plasma proteins rendering them more susceptible to neutralization by AT III (8,9). The smallest saccharide with an intact AT III binding domain is a pentasaccharide (10).

Recently, Rosing et al. (2) showed the formation of considerable amounts of meizothrombin during prothrombin activation. Their evidence was partly based on the different reactivities of meizothrombin and thrombin towards the heparin-enhanced inactivation by AT III. In fact, heparin appeared to be devoid of any catalytic activity in the meizothrombin/AT III reaction (11). A possible explanation for the latter phenomenon could be the absence of heparin binding sites on the meizothrombin molecule. By making use of the heparin-dependent inactivation of factor X_a by AT III, we were able to show that meizothrombin does not neutralize heparin, whereas thrombin does. Furthermore, this report demonstrates that the prothrombin activation products fragment 1.2 and fragment 1 are inhibitors of the heparin-enhanced factor X_a inactivation by AT III, and that this effect is due to their heparin neutralizing properties.

Materials and Methods

Materials- Bz-Ile-Glu-(Piperidyl)-Gly-Arg-p-nitro-anilide (S-2337) and D-Phe-(Pipicolyl)-Arg-p-nitro-anilide (S-2238) were purchased from AB Kabi Diagnostica, Stockholm. Phe-Pro-Arg-chloromethylketone (PPACK) was obtained from Calbiochem, La Jolla, USA. Human serum albumin, fatty free (HSA), soybean trypsin inhibitor (STI) and Echis carinatus venom were obtained from Sigma. Antithrombin III high affinity heparin (HA-heparin) was obtained by chromatography on an AT III affinity column and was a kind gift from Pharmuka Laboratories, France. The synthetic pentasaccharide (IC 831423) was a product of Laboratoire Choay, France. All reagents used were of the highest grade commercially available.

Proteins- Bovine factor X_a, factor V_a, prothrombin, thrombin, prothrombin fragment 1 and prothrombin fragment 2 were purified as described before (12-14). Bovine AT III was purified according to Thaler and Schmer (15). The AT III was subsequently freed of heparin contamination by ionic exchange chromatography (16). Prothrombin fragment 1.2 was prepared as an activation product of PPACK-meizothrombin by incubation of PPACK-meizothrombin with factor X_a (10 nM), factor V_a (50 nM), phospholipids (0.1 mM) and CaCl₂ (10 mM) in 20 mM Tris-HCl, 100 mM NaCl (pH 7.5) at 37° C. After 30 minutes the reaction was stopped by the addition of EDTA to a final concentration of 20 mM and the reaction mixture was chromatographed on a soybean trypsin inhibitor-Sepharose column (bed volume 2 mL) directly followed by a QAE-Sephadex A-50 column (bed volume 94 mL) at 4° C with a flow rate of 15 mL/h. The column was eluted with a linear gradient (2 x 250 mL) of 0.10 to 0.60 M NaCl in 20 mM Tris-HCl (pH 7.5).

Protein Concentrations- Concentrations of thrombin, factor X_a, factor V_a and prothrombin were determined as reported earlier (12,17). The molar concentrations of bovine AT III, prothrombin fragment 1.2, fragment 1 and fragment 2 were calculated using E₂₈₀ of 6.0, 12.3, 10.1, 12.5 and MW of 56,600, 35,000, 22,500, 12,500 respectively (3,13,15).

Preparation of Active Site Blocked-Meizothrombin and Thrombin- Active site blocked meizothrombin was prepared by activation of purified prothrombin (10 μM) by Echis carinatus venom (5 μg/mL) in the presence of 60 μM PPACK at 37° C in 50 mM Tris-HCl, 175 mM NaCl (pH 7.9). The solution was extensively dialyzed against the aforementioned buffer. PPACK-thrombin was prepared by incubating purified thrombin (11.5 μM) with PPACK (60 μM) at 37° C in the same buffer until virtually no amidolytic activity could be detected and finally dialyzed against the same buffer.

Neutralization of Factor X_a activity- Purified factor X_a (10 nM) was added to AT III (200 nM) and HA-heparin (30 ng/mL), in the presence of various concentrations of PPACK-thrombin, PPACK-meizothrombin, prothrombin fragment 1.2, fragment 1 or fragment 2 at 37° C in 50 mM Tris-HCl, 175 mM NaCl and 0.5 mg HSA/mL (pH 7.9). At timed intervals after the addition of factor X_a , 20 μ L aliquots were transferred into 330 μ L of substrate solution [containing 50 mM Tris-HCl, 175 mM NaCl, 20 mM EDTA, 0.5 mg HSA/mL and 0.22 mM S2337 (pH 7.9)] at 37° C. After 6 minutes, 150 μ L of 50% acetic acid (v/v) was added and the absorbance of the mixture was measured at 405 nm using an Aminco DW-2 spectrophotometer. Pseudo-first-order rate constants of inhibition of factor X_a were calculated from a plot of the logarithm of residual factor X_a concentrations versus time.

Results

Effects of PPACK-Thrombin and PPACK-Meizothrombin on the Heparin-Catalyzed AT III/Factor X_a Reaction- Figure 1A depicts the pseudo-first-order plots of inhibition of Factor X_a by AT III at varying HA-heparin concentrations. It is shown that under these conditions the pseudo-first-order rate constant increased

Figure 1: Pseudo-first-order plots of inhibition of factor X_a (10 nM) by AT III (200 nM) in the presence of 10 ng/mL (●), 20 ng/mL (■) or 30 ng/mL (▲) HA-heparin (panel A), and replot of pseudo-first-order rate constant versus the HA-heparin concentration (panel B).

proportionally with the HA-heparin concentration (Figure 1B). Thus, the addition of a compound which neutralizes heparin will decrease the apparent pseudo-first-order rate constant as a result of competition of the added compound with AT III for the heparin.

We next examined the effects of thrombin and meizothrombin on the heparin-catalyzed AT III/factor X_a reaction. In order to avoid depletion of AT III we utilized active site blocked proteases (PPACK-thrombin and PPACK-meizothrombin). It has been reported that PPACK-thrombin has the same affinity for heparin when compared to thrombin, but does not react with AT III (18). Thus a decrease of the pseudo-first-order rate constant will be the result of the neutralization of heparin to the added compound which removes it from the AT III/factor X_a reaction.

Figure 2 depicts the apparent pseudo-first-order rate constants of inhibition of factor X_a , as a function of the PPACK-thrombin and PPACK-meizothrombin concentration. It is clearly demonstrated that PPACK-meizothrombin does not influence the inhibition of factor X_a , whereas the addition of PPACK-thrombin reduces the rate constant in a dose-dependent manner. The rate constants are corrected for the heparin-independent reaction ($k = 0.1 \text{ min}^{-1}$). The concentration of PPACK-thrombin required to obtain half the maximal rate constant of the heparin-dependent factor X_a inhibition was 700 nM. In order to establish that solely the heparin neutralizing capacity of PPACK-thrombin caused the lower rate constant, we utilized the synthetic pentasaccharide IC 831423. Our results demonstrate that the pentasaccharide (15 ng/mL) is a highly effective catalyst of the AT III/factor X_a reaction, but its action is not influenced by the addition of PPACK-thrombin (Figure 2). This result can readily be explained by the

Figure 2: Effect of PPACK-thrombin (●) and PPACK-meizothrombin (■) on the apparent pseudo first order rate constant of the HA-heparin-dependent inhibition of factor X_a . The effect of PPACK-thrombin on the pseudo first order rate constant of the pentasaccharide-dependent inhibition of factor X_a (▲).

absence of any anti-thrombin activity and thus thrombin-binding capacity of this compound (10). Taking these results together, it is our conclusion that PPACK-meizothrombin is unable to interact with heparin. This observation, therefore, might explain our previously lylylreported finding that the AT III/meizothrombin reaction is not catalyzed by heparin (11).

Conversion of PPACK-Meizothrombin into Prothrombin Fragment 1.2 and PPACK-Thrombin and the Neutralization of Heparin by the Reaction Products- We initially assumed that the presence of covalently-bound prothrombin fragment 1.2 in the meizothrombin molecule caused the difference between thrombin and meizothrombin in their heparin-neutralizing properties. Thus, we set out to demonstrate that after cleavage of the PPACK-meizothrombin molecule the obtained prothrombin fragment 1.2 and PPACK-thrombin would show the same properties as the purified proteins. To this end, PPACK-meizothrombin ($4\text{ }\mu\text{M}$) was incubated with factor X_a (6 nM), factor V_a (30 nM), phospholipid composed of 20% phosphatidylserine/80% phosphatidylcholine ($60\text{ }\mu\text{M}$) and CaCl_2 (6 mM) in 50 mM Tris-HCl, 175 mM NaCl (pH 7.9) at 37°C . The reaction, when monitored by SDS-PAGE, was completed after 30 minutes and gave rise to prothrombin fragment 1.2 and PPACK-thrombin (data not shown). Increasing amounts of this reaction mixture, up to $2\text{ }\mu\text{M}$ of converted PPACK-meizothrombin, were unable to slow down the HA-heparin-catalyzed AT III/factor X_a reaction, indicating that cleaved meizothrombin is unable to neutralize heparin. It is important to note that under these conditions non-covalent association of prothrombin fragment 1.2 and (PPACK-)thrombin might be occurring (19) which can form a product with properties indistinguishable from meizothrombin.

To further investigate the heparin neutralizing properties of prothrombin fragment 1.2 and PPACK-thrombin, we analyzed the reaction mixture by FPLC on a mono-Q column equilibrated with 50 mM Tris, 175 mM NaCl (pH 7.5), de-

Figure 3: FPLC-analysis of factor X_a cleaved PPACK-meizothrombin on a mono-Q column.

veloped with a linear gradient of 175 to 600 mM NaCl at room temperature and with a flow rate of 1 mL/min. PPACK-thrombin eluted with the equilibration buffer containing 175 mM NaCl and prothrombin fragment 1.2 eluted at 350 mM NaCl (Figure 3A). The protein peaks, indicated by the solid bars, were dialyzed against 50 mM Tris-HCl, 175 mM NaCl (pH 7.9) and then added to the HA-heparin containing AT III-factor X_a reaction mixture to a final concentration of 100 nM of PPACK-thrombin and prothrombin fragment 1.2, respectively. Both PPACK-thrombin and prothrombin fragment 1.2 decreased the pseudo-first-order rate constant of factor X_a inhibition from 0.5 min^{-1} to 0.4 and 0.1 min^{-1} , respectively (Figure 3B). Whereas the effect of PPACK-thrombin was expected, fragment 1.2 was to our surprise even more effective in neutralizing heparin.

Effect of Prothrombin Fragment 1.2 on the Heparin-Catalyzed ATIII/Factor X_a Reaction- - Figure 4 depicts the effect of various concentrations of prothrombin fragment 1.2 on the pseudo-first-order rate constant of the HA-heparin-dependent inhibition of factor X_a by AT III. An IC_{50} value of 100 nM was found.

We next examined which region of the prothrombin fragment 1.2 molecule contains the site of interaction with heparin. To this end, prothrombin fragment

Figure 4: Effect of prothrombin fragment 1.2 (Δ), fragment 1 ($\blacksquare$) and fragment 2 ($\bullet$) on the apparent pseudo first order rate constant of the HA-heparin dependent factor X_a inhibition by AT III.

1.2 (5 μ M) was converted into fragment 1 and fragment 2 by incubation with thrombin (50 nM). Analysis by SDS-PAGE revealed that the reaction was completed after 30 min. When samples were taken during the reaction and tested at 100 nM concentrations for their heparin-neutralizing effect, a 50% inhibition of the factor X_a inactivation reaction was observed, independent on the extend of the prothrombin fragment 1.2 cleavage reaction. It was tested that the amount of thrombin carried-over did not interfere with the assay.

Purified fragment 1 and fragment 2 at various concentrations were then added to factor X_a inactivation mixtures. Their effects on the pseudo-first-order rate constants are shown in Figure 4. Fragment 1 showed the highest affinity for heparin with an IC_{50} -value of 125 nM which is somewhat higher than that for prothrombin fragment 1.2 (IC_{50} = 100 nM). However, fragment 2 had an IC_{50} -value far above 1500 nM. Our results indicate that also prothrombin fragment 1 contains a domain which interacts with heparin and at even a higher affinity when compared to the one present in the thrombin region of the prothrombin molecule.

Discussion

Using the heparin-dependent factor X_a inactivation by AT III, we were able to show that meizothrombin, an intermediate in prothrombin activation (2) is not able to neutralize heparin, whereas thrombin does. When active site blocked meizothrombin (PPACK-meizothrombin) was titrated into a factor X_a inactivation mixture, containing 10 nM factor X_a , 200 nM AT III and 30 ng/mL HA-heparin, there was no effect at all on the pseudo-first-order rate constant of the heparin-dependent factor X_a inactivation reaction. Performing the same experiment with active site blocked thrombin (PPACK-thrombin) we obtained a 50% inhibition of the inactivation reaction at a concentration of 700 nM PPACK-thrombin. Although our method measures the neutralization of heparin, it seems reasonable to explain this phenomenon in terms of binding affinity between negatively charged heparin and basic amino acids in the neutralizing proteins. Thus, our observations show that PPACK-meizothrombin is not able to bind to heparin, while PPACK-thrombin does contain heparin-binding sites and therefore explains the absence of a stimulatory effect of heparin on the AT III/meizothrombin reaction (11). As meizothrombin is formed by cleavage of the Arg and the Ile in the prothrombin molecule, it still contains the intact fragment 1.2 region. The intriguing question, therefore, is whether covalently-bound fragment 1.2 prevents the exposure of the heparin-binding site(s) present at the thrombin part of the molecule.

After cleavage of PPACK-meizothrombin by factor X_a , however, the converted PPACK-meizothrombin was not able to inhibit the HA-heparin-catalyzed AT III-factor X_a reaction up to a concentration of $2 \mu\text{M}$, although electrophoretic analysis (SDS-PAGE) revealed that after 30 min PPACK-meizothrombin was completely converted to prothrombin fragment 1.2 and PPACK-thrombin. However, after separating the reaction products by FPLC, PPACK-thrombin and, quite surprisingly, prothrombin fragment 1.2 were able to decrease the pseudo-first-order rate constant of factor X_a inhibition. These results suggest that after cleavage of PPACK-meizothrombin, prothrombin fragment 1.2 and PPACK-thrombin remain non-covalently associated and thus exhibit, like the PPACK-meizothrombin molecule, no heparin-binding properties.

To investigate the binding properties of the separate prothrombin fragment region, we purified fragment 1 and fragment 2. Our data show that the sites responsible for the heparin affinity are located on fragment 1. Fragment 2 hardly shows any affinity for heparin. Aronson et al. (20) demonstrated that during the clotting of blood and plasma in systems in which thrombin is rapidly inactivated, fragment 1.2 can account for at least 90% of the prothrombin originally present. Together with our results, that indicate strong binding properties of fragment 1.2 towards heparin, one could assume that fragment 1.2 formation indeed plays an important role in the regulation of blood coagulation by competing with AT III for the available heparin.

References

1. Suttie JW, Jackson CM: Prothrombin structure, activation and biosynthesis. *Physiol Rev* 57:1, 1977
2. Rosing J, Zwaal RFA, Tans G: Formation of meizothrombin as intermediate in factor X_a -catalyzed prothrombin activation. *J Biol Chem* 261:4224, 1986
3. Walker FJ, Esmon CT: The effect of prothrombin fragment 2 on the inhibition of thrombin by antithrombin III. *J Biol Chem* 254:5618, 1979
4. Rosenberg RD, Damus PS: The purification and mechanism of action of human antithrombin-heparin cofactor. *J Biol Chem* 248:6490, 1973
5. Hemker HC, Willems G, Beguin S: A computer assisted method to obtain the prothrombin activation velocity in whole plasma independent of thrombin decay processes. *Thrombos Haemostas* 56:9, 1986
6. Einarson R, Andersson LO: Binding of heparin to human antithrombin III as studied by measurements of tryptophan fluorescence. *Biochim Biophys Acta* 490:104, 1977
7. Villanueva GB, Danishefsky I: Evidence for a heparin-induced conformational change of AT III. *Biochem Biophys Res Comm* 79:803, 1977
8. Smith GF: The heparin-thrombin complex in the mechanism of thrombin inactivation by heparin. *Biochem Biophys Res Comm* 77:111, 1977

9. Machovich R, Staub M, Pattby L: Decreased heparin sensitivity of cyclohexanedione-modified thrombin. *Eur J Biochem* 83:473, 1978
10. Choay J, Petitou M, Lormeau JC, Sinay P, Casu P, Gatti G: Structure activity relationships in heparin: a synthetic pentasaccharide with high affinity for antithrombin III and eliciting high anti-factor X_a activity. *Biochem Biophys Res Comm* 116:492, 1983
11. Lindhout T, Baruch D, Schoen P, Franssen J, Hemker HC: Thrombin generation and inactivation in the presence of antithrombin III and heparin. *Biochemistry* 25:5962, 1986
12. Lindhout T, Govers-Riemslog JWP, van de Waard P, Hemker HC Rosing J: Factor V_a-Factor X_a interaction. Effect of phospholipid vesicles of varying composition. *Biochemistry* 21:5494, 1982
13. Owen WG, Esmon CT, Jackson CM: The conversion of prothrombin to thrombin. I. Characterization of the reaction products formed during the activation of bovine prothrombin. *J Biol Chem* 249:594, 1974
14. Govers-Riemslog JWP, Speijer H, Zwaal RFA, Rosing J: The effects of bovine prothrombin fragment 1 and fragment 1.2 on prothrombin activation. *Thromb Res* 38:375, 1985
15. Thaler EJ, Schmer G: A simple two-step isolation procedure for human and bovine antithrombin II/III (heparin cofactor): A comparison of two methods. *Br J Haematol* 31:233, 1975
16. Miller-Andersson M, Borg H, Andersson LO: Purification of antithrombin III by affinity chromatography. *Thromb Res* 5:439, 1977
17. van Dieijen G, Tans G, Rosing J, Hemker HC: The role of phospholipid and factor VIII_a in the activation of bovine factor X. *J Biol Chem* 256:3433, 1981
18. Griffith HJ, Kingdon HS, Lundblad RL: Inhibition of the heparin-antithrombin III/thrombin reaction by active site blocked thrombin. *Biochem Biophys Res Comm* 87:686, 1979
19. Nesheim ME: The correlation between the thrombin-catalyzed cleavage of prothrombin fragment 1.2 and release of thrombin from prothrombinase. *Thrombos Haemostas* 54:148, 1985
20. Aronson DL, Stevan L, Ball AB, Franca BR jr, Finlayson JS: Generation of the combined prothrombin activation peptide (F 1.2) during the clotting of blood and plasma. *J Clin Invest* 60:1410, 1977

Chapter III

Inhibition of Factor IX_a and Factor X_a by Antithrombin III/Heparin During Factor X Activation^o

Abstract

We investigated the kinetics of the inhibitory action of antithrombin III and antithrombin III plus heparin during the activation of factor X by factor IX_a. Generation and inactivation curves were fitted to a 3-parameter 2-exponential model to determine the pseudo first order rate constants of inhibition of factor IX_a and factor X_a by antithrombin III/heparin. In the absence of heparin, the second order rate constant of inhibition of factor X_a generated by factor IX_a was 2.5-fold lower than the rate constant of inhibition of exogenous factor X_a. It appeared that phospholipid-bound factor X protected factor X_a from inactivation by antithrombin III. It is, as yet, unclear whether an active-site or a nonactive-site interaction between factor X_a and factor X at the phospholipid surface is involved. The inactivation of factor IX_a by antithrombin III was found to be very slow and was not affected by phospholipid, calcium and/or factor X. With unfractionated heparin (UFH) above 40 ng/mL and antithrombin III at 200 nM, the apparent second order rate constant of inhibition of exogenous and generated fac-

^o Based on: Pieters J, Willems G, Hemker HC, Lindhout T: J Biol Chem 263 (30):15313, 1988

tor X_a were the same. Thus, in this case phospholipid-bound factor X did not protect factor X_a from inhibition. In the presence of the synthetic pentasaccharide heparin, however, phospholipid-bound factor X reduced the rate constant about 5-fold. Pentasaccharide had no effect on the factor IX_a /antithrombin III reaction. UFH (1 $\mu\text{g/mL}$) stimulated the antithrombin III-dependent inhibition of factor IX_a during factor X activation 400-fold. In the absence of reaction components this stimulation was 65-fold. We established that calcium stimulated the heparin-dependent inhibition of factor IX_a .

Introduction

Antithrombin III (AT III) is a plasma protein that plays an important role in the regulation of a series of activation reactions leading to clot formation (1). From studies with purified serine proteases and inhibitor, it is well known that the main target of AT III is thrombin whereas the activity towards activated factor X and activated factor IX is less pronounced (2,3).

Heparin accelerates the rate at which AT III inactivates the serine proteases of the blood coagulation system. Dependent on the molecular weight of the heparin preparation used, the rate enhancing effect of heparin on the inhibition of factor IX_a and factor X_a seems to be different (4). Inactivation of factor IX_a was found to decrease when heparins were used with decreasing molecular weight, whereas the inhibition of factor X_a was potentiated by low molecular weight heparin fractions who have virtually no effect on the inactivation of factor IX_a .

As factor X_a is the only known physiological activator of prothrombin, it plays a key role in the process of coagulation. Its formation has been postulated to be an important target for the control mechanism of thrombin formation (5,6). Furthermore, factor X_a may play a role in the activation of factors V, VIII and VII (7-9).

Among the control mechanism which regulate the generation of factor X_a , the inhibition by antithrombin III is one of the most important ones, especially when heparin is present. Therefore the effect of antithrombin III and antithrombin III plus heparin on isolated factor X_a and factor IX_a has been studied (10-13). However, little is known about the kinetics of inactivation of factor IX_a and factor X_a during factor X activation, which presents a more physiological situation. Previous studies reported the effect of AT III on factor X_a generated by simple activators of factor X, such as the factor X_a -activating protein from Russell's Viper venom (14). Recently, Jesty (15) described the inhibition of in situ generated

factor X_a by AT III. When using factor IX_a as an activator of factor X, he could not accurately analyze the overall factor X_a generation and inactivation, however. Also, this author did not present kinetic parameters on the inactivation of factor X_a, generated by factor IX_a.

In this paper we present a method which allows the simultaneous analysis of the kinetics of both factor IX_a and factor X_a inactivation by AT III or AT III plus heparin during factor X activation. By this approach we were able to assess the relative importance of factor IX_a inhibition and factor X_a inhibition to the overall effect of unfractionated heparin (UFH) and a synthetic pentasaccharide heparin on factor X_a generation. We report some marked differences between the kinetics of the inhibition reactions during factor X activation and those observed in the absence of factor X, phospholipid and/or calcium.

Materials and Methods

Materials- The synthetic peptide substrates Bz-Ile-Glu-(Piperidyl)-Gly-Arg-p-nitro-anilide hydrochloride (S-2337) and H-D-Phenylalanyl-L-pipecolyl-L-arginine-p-nitro-anilide dihydrochloride (S-2238) were purchased from AB Kabi Diagnostica, Stockholm. Fatty acid-free ovalbumin was a product of Sigma Chemical Co., St. Louis. All reagents used were of the highest grade commercially available.

Unfractionated heparin (168 units/mg) was from Pharmuka Laboratories, France, and the synthetic pentasaccharide heparin (800 anti-factor X_a units/mg and no anti-thrombin activity) was a kind gift from Institut Choay, France.

Proteins- Bovine AT III was purified by the method of Thaler and Schmer (16). The AT III was subsequently freed of heparin contamination by ion-exchange chromatography (17). All other bovine clotting factors were prepared and quantitated as previously described (18-20).

Phospholipid Vesicles- Phospholipid vesicles were made from a mixture of phosphatidylserine (PS) and phosphatidylcholine (PC) as previously described (20). Vesicles composed of 25 mole% PS and 75 mole% PC were used throughout the experiments unless stated otherwise.

Determination of Inhibition Rate of Purified Protease- The inactivation of factor X_a by AT III was investigated under pseudo-first order conditions essentially as previously described (3). The inactivation of factor IX_a was followed by incuba-

tions of factor IX_a with AT III/heparin in the presence or absence of accessory components and at several time points residual factor IX_a activity was assayed as described previously (21) with the following modifications. Factor IX_a containing samples were diluted 1:1000 into buffer containing 50 mM Tris-HCl (pH 7.9), 175 mM NaCl, 0.5 mg ovalbumin/mL, 5 mM CaCl₂, 20 μ M phospholipid and 100 nM thrombin, prewarmed at 37° C for 4 min. Factor VIII:C (10 U/mL) was added and allowed to be activated for 15 seconds, after which the reaction was started by the addition of factor X (0.5 μ M). The rate of factor X activation was determined by measuring the amount of factor X_a formed after 30 and 90 seconds using the chromogenic substrate S-2337 as described earlier (3). Factor X_a formation was linear in time for at least 2 minutes. From the observed rate of factor X activation the amount of factor IX_a present in the sample was calculated using a calibration curve made with known amounts of active site titrated factor IX_a.

Inactivation of Factor IX_a and Factor X_a by AT III/Heparin During Factor X Activation- Factor X (1 μ M) was activated by 80 nM factor IX_a, in the presence of 100 μ M phospholipid, 10 mM CaCl₂ and heparin when indicated in 50 mM Tris-HCl, 175 mM NaCl and 0.5 mg/mL ovalbumin, pH 7.9 at 37° C. At timed intervals after the addition of factor IX_a, aliquots (7 μ L) were removed and assayed for factor X_a activity. Factor X_a formation was linear in time for at least 30 min. After 10 min an aliquot (200 μ L) was taken from the reaction mixture and added to a solution (14 μ L) containing AT III and rabbit anti-factor IX IgG. At the same time another aliquot (200 μ L) was taken from the reaction mixture and added to a solution (14 μ L) containing AT III alone. Addition of anti-factor IX resulted in instantaneous and complete inhibition of factor X_a formation, but had no effect on factor X_a activity or on the AT III/factor X_a reaction. After the addition of AT III or AT III plus anti-factor IX, timed samples (7 μ L) were taken and assayed for factor X_a activity.

Because the rabbit anti-factor IX IgG neutralized the unfractionated heparin, anti-factor IX IgG could not be used to block further factor X_a formation to measure the effects of UFH on factor X_a inhibition. Therefore, we modified the experimental set-up for the determination of the effect of UFH on the inactivation of factor IX_a and factor X_a during factor X activation. Factor X was activated in the presence of UFH under the same conditions as described before. Factor X_a formation was monitored by removing aliquots (7 μ L) and assayed for factor X_a activity. After 10 minutes, 200 μ L was removed and added to a solution of AT III (14 μ L). From this reaction mixture, timed samples (10 μ L) were taken, and assayed for factor IX_a activity as already described. From the same mixture, aliquots (7 μ L) were taken and assayed for factor X_a activity. The pseudo first order rate constants of inhibition of factor X_a and factor IX_a were calculated as described in the following section.

Kinetic Analysis of the Inhibition of Factor IX_a and Factor X_a During Factor X Activation- Under the experimental conditions of our studies the factor X_a formation was linear during the time-period of the experiment when AT III was absent. This was obtained by using factor X concentrations well above the K_m for factor X and less than 20% of factor X consumed during the experiment. Secondly, the AT III concentrations were in excess over the factor X_a and factor IX_a concentrations, resulting in pseudo-first order kinetics of inhibition. When AT III or AT III plus heparin was present during the activation of factor X by factor IX_a we assumed that the amount of amidolytic active factor X_a present at time t is a function of the rate of inactivation of factor IX_a and the rate of inactivation of factor X_a. In formula this reads:

$$d/dt [X_a] = \nu e^{-k_{IX_a} t} - k_{X_a} [X_a] \quad (1)$$

where ν is the rate of factor X_a formation in the absence of AT III or AT III plus heparin, k_{IX_a} and k_{X_a} are the pseudo first order rate constants of inhibition of factor IX_a and factor X_a, respectively.

Integration of Equation 1 by Laplace transformation gives the following equation for the generation of factor X_a concomitant with its decay.

$$[X_a]_t = (\nu / (k_{X_a} - k_{IX_a})) (e^{-k_{IX_a} t} - e^{-k_{X_a} t}) \quad (2)$$

the parameters, ν , k_{IX_a} and k_{X_a} could be determined by fitting equation 2 to the experimental data points. However, we preferred an analysis of our data which also allows independent determinations of the parameters ν and k_{X_a} . To this end, the activation of factor X in the absence of AT III was performed for t_0 min, followed by the addition of AT III or AT III plus anti-factor IX IgG.

When AT III is added at time t_0 , the factor X_a concentration then obtained

$$[X_a]_{t=t_0} = \nu t_0 \quad (3)$$

has to be included into Equation 2:

$$[X_a]_t = (\nu / (k_{IXa} - k_{IXa})) (e^{-k_{IXa}(t-t_0)} - e^{-k_{Xa}(t-t_0)}) + [X_a]_{t=t_0} e^{-k_{Xa}(t-t_0)} \quad (4)$$

When AT III plus anti-factor IX IgG are added at time t_0 , factor X_a formation is completely blocked but not the decay of factor X_a and reduces Equation 4 to

$$[X_a]_t = [X_a]_{t=t_0} e^{-k_{Xa}(t-t_0)} \quad (5)$$

If $t \leq t_0$ then

$$[X_a]_t = \nu t \quad (6)$$

Equations 4-6 were simultaneously fitted to experimental data points, obtained in the absence or presence of AT III/heparin or in the presence of AT III/heparin plus anti-factor IX IgG. The fit procedure, based upon a nonlinear least squares regression method which uses a re-iterative Gauss-Newton algorithm (22), produced ν , k_{IXa} and k_{Xa} . In addition, k_{Xa} and ν were separately cal-

culated using Equations 5 and 6, respectively, to estimate the validity of the parameter values as calculated by the fit procedure. The reported errors represent the 95% confidence interval, about twice the S.D. of the estimate.

Results

Determination of Factor IX_a and Factor X_a Inactivation by Antithrombin III During Factor X Activation- Factor X was activated by factor IX_a in the presence of phospholipid and calcium. After 10 min to a part of the reaction mixture a solution of AT III and rabbit anti-factor IX IgG was added, and at the same time AT III alone was added to another part of the reaction mixture. The result of

Figure 1: Factor X_a generation in the presence of AT III. Factor X (1 μ M) was activated by 80 nM factor IX_a in the presence of 100 μ M phospholipid and 10 mM calcium (curve A). At 10 minutes to 200 μ L of the reaction mixture, 1 μ M AT III was added (curve B). At the same time, to another 200 μ L of the reaction mixture 1 μ M AT III and anti-factor IX IgG was added (curve C). Factor X_a activity was assayed as described under Materials and Methods. The solid lines represent the factor X_a generation and inactivation as calculated by the fit procedure.

Inset: semi-logarithmic replot of curve C.

such a typical experiment is shown in Figure 1. Curve A shows a linear factor X_a generation and addition of both anti-factor IX IgG and AT III at $t = 10$ min resulted in the exponential decay of the formed factor X_a (curve C), whereas addition of AT III alone resulted in significant higher residual factor X_a activities because of ongoing factor X_a generation (curve B).

Equations 4-6 (see Materials and Methods) were fitted to the experimental data and the factor X_a generation and inactivation curves, indicated by the solid lines, are the results of the best fit to the data points. The pseudo first order rate constants of inactivation of factor X_a and factor IX $_a$ by AT III as well as the velocity of factor X_a formation in the absence of AT III were obtained by the computer fit-procedure. In this typical experiment the velocity of factor X_a formation (v) was found to be $2.1 \pm 0.1 \text{ nM}^{-1}\text{min}^{-1}$, whereas the pseudo first order rate constants (k) of inactivation of factor X_a by AT III ($1 \mu\text{M}$) was $0.21 \pm 0.01 \text{ min}^{-1}$. However, the pseudo first order rate constant of inactivation of factor IX $_a$ by AT III was too small to measure accurately ($\leq 0.01 \text{ min}^{-1}$).

The calculated parameters v and k can also be determined directly from part A and part C of the curves, respectively. The rate of factor X_a formation was 2.1 nM/min (part A). The pseudo first order rate constant of inactivation of factor X_a by AT III was determined from the semilogarithmic plot of part C of the curve versus time, $k_{X_a} = 0.21 \text{ min}^{-1}$ (Figure 1, inset). Thus, the independently determined values for the rate of factor X activation and the rate constant of factor X_a inactivation were the very same as the values from the fit-procedure, indicating the validity of our mathematical model.

This procedure was repeated in the presence of various AT III concentrations (0 – $1.5 \mu\text{M}$). A linear relationship between the pseudo first order rate constants of inhibition of factor X_a and the AT III concentration was found. The second order rate constant was $2.1 \pm 0.1 \times 10^5 \text{ M}^{-1}\text{min}^{-1}$. The inactivation of factor IX $_a$ during factor X activation at the AT III concentrations used was too small ($k_{IX_a} \leq 0.010 \text{ min}^{-1}$) to be measured accurately. Higher AT III concentrations, however, could not be used in this experimental setup, because then the factor X_a generated by factor IX $_a$ was too fast inactivated and resulted in very low residual factor X_a activities.

In order to determine the inactivation of factor IX $_a$ at higher AT III concentrations, we made use of a factor IX $_a$ -assay in which functional factor IX $_a$ activity was determined via the factor VIII:C $_a$ enhanced activation of factor X (see Materials and Methods). The pseudo first order rate constants of factor IX $_a$ inactivation by AT III in the presence of factor X, phospholipid and calcium were plotted versus the AT III concentration (0 – $10 \mu\text{M}$). A second order rate constant of $8.2 \times 10^3 \text{ M}^{-1}\text{min}^{-1}$ was calculated. In the absence of the reaction components

(factor X, phospholipid and Ca^{2+}) the second order rate constant of factor IX₃ inactivation was found to be the very same as in their presence ($k_2 = 8.2 \times 10^3 \text{ M}^{-1}\text{min}^{-1}$).

Effect of Accessory Components on Factor X_a Inactivation by Antithrombin III- As several investigators reported contradictory effects of phospholipid or calcium (23-27) on the inactivation of factor X_a by AT III, we examined whether our decay constants, determined by the fit-procedure during factor X activation, differed from the decay constants of factor X_a in free solution.

To this end, we incubated purified factor X_a (10 nM) with AT III (0-1.5 μM), either in the absence or presence of phospholipid (100 μM), calcium (10 mM) or both. Figure 2 shows the results, revealing a second order rate constant of inhibition of factor X_a by AT III in free solution was $5.0 \times 10^5 \text{ M}^{-1}\text{min}^{-1}$. It is clear that neither the addition of phospholipid or calcium or phospholipid plus calcium protected factor X_a from being inactivated by AT III. Under all conditions the rate constants were $5.0 \pm 0.1 \times 10^5 \text{ M}^{-1}\text{min}^{-1}$. However, these rates of inactivation of factor X_a appeared to be still about 2.5-fold faster when compared to the rates of inactivation under the conditions of factor X activation (see previous section). In order to find an explanation for this discrepancy we added besides

Figure 2: Pseudo first order rate constant of inactivation of factor X_a (10 nM) as a function of the antithrombin III concentration in the presence of phospholipid (100 μM) and 10 mM calcium (●); Factor X (1 μM) and 10 mM calcium (△); Factor X (1 μM), phospholipid (100 μM) and 10 mM calcium (▲); or alone (○).

phospholipid and calcium, also factor X ($1\ \mu\text{M}$) to the inactivation mixture. The second order rate constant of $2.1 \times 10^5\ \text{M}^{-1}\text{min}^{-1}$ for factor X_a inactivation by AT III thus obtained, was the very same as determined during factor X activation (Figure 2; closed triangles).

These results suggest that the decrease of the second order rate constant of the factor X_a - AT III reaction during factor X activation is due to the presence of phospholipid-bound factor X. Indeed, the pseudo first order rate constant of inhibition of factor X_a in the presence of a fixed phospholipid concentration decreased with increasing factor X concentration (Figure 3A). Also an increase of the phospholipid concentration at a fixed factor X concentration resulted in a decrease of the rate constant (Figure 3B). In both cases the rate constant progressively decreased to an apparent end value of $0.2\ \text{min}^{-1}$.

One of the explanations for this phenomenon could be the autocatalytical activation of factor X by factor X_a . Indeed, it has been reported that bovine factor X can be converted by factor X_a into β -factor X, factor X_a and/or β -factor X_a (28-30).

Figure 3: Effect of factor X and phospholipid on the inactivation of factor X_a by AT III. Panel A, factor X_a ($10\ \text{nM}$) was incubated with AT III ($1\ \mu\text{M}$) in the presence of varying amounts of factor X, Ca^{2+} ($10\ \text{mM}$) and $100\ \mu\text{M}$ phospholipid ($\bullet$) or in the absence of phospholipid ($\circ$). Panel B, the same incubation but in the presence of varying amounts of phospholipid, Ca^{2+} ($10\ \text{mM}$) and $1\ \mu\text{M}$ factor X ($\bullet$) or in the absence of factor X ($\circ$). The pseudo first order rate constants of inactivation of factor X_a by AT III were determined as described under Materials and Methods.

In the presence of phospholipid the major reaction product appeared to be β -factor X (30). An exponential increase in factor X_a generation as a result of the autocatalytic activation of factor X by factor X_a might explain the apparent lower pseudo first order rate constant of inactivation of factor X_a in the presence of phospholipid-bound factor X. However, under none of our experimental conditions, an increase in amidolytic factor X_a activity could be detected. In addition, sodium dodecylsulfate polyacrylamide gel electrophoresis of samples taken from a mixture containing 10 nM factor X_a, 200 μ M phospholipid, 10 mM Ca²⁺ and 5 μ M of factor X revealed the formation of β -factor X at a very slow rate.

Under the same conditions but in the absence of phospholipid, no cleavage of factor X was observed over an 1-hour period.

In order to explore the effect of phospholipid-bound factor X in more detail, the following experiment was performed. Pseudo first order rate constants of inactivation of factor X_a (10 nM) by AT III (1 μ M) were determined in the presence of factor X (10 μ M), Ca²⁺ (10 mM) and varying concentrations of

Figure 4: Effect of factor X and phospholipids of different compositions on the inactivation of factor X_a by antithrombin III. Factor X_a (10 nM) was incubated with antithrombin III (1 μ M) in the presence of factor X (10 μ M), calcium (10 mM) and varying concentrations of phospholipid vesicles composed of 10 mol% PS/90 mol% PC (▲), or 25 mol% PS/75 mol% PC (●).

phospholipid composed of 10 mol% PS/90 mol% PC or 25 mol% PS/75 mol% PC. The rate constants progressively decreased until apparent end levels of 0.1 min^{-1} and 0.3 min^{-1} in the presence of vesicles containing 25 mole %PS or 10 mole % PS, respectively (Figure 4). From reported factor X-phospholipid binding parameters (31,32) it was calculated that at each phospholipid concentration used, virtually all available phospholipid binding sites were saturated with factor X, i.e. $12.3 \text{ } \mu\text{mol}$ of factor X bound/mmol of 25% PS - 75% PC and $6.7 \text{ } \mu\text{mol}$ factor X bound/mmol of 10% PS - 90% PC. Thus, the fraction of factor X bound to phospholipid linearly increased with the phospholipid concentration. Although factor X and factor X_a interact competitively with the same phospholipid binding sites, factor X, by virtue of its affinity for phospholipid and factor X_a , promotes the binding of factor X_a at the surface. Hence, an apparent inhibition of the inactivation reaction might be a consequence of a decreased fluid phase factor X_a concentration concomitant with an increase in the concentration of phospholipid-bound factor X.

Additional support for our notion that the factor X_a -factor X association rate at the surface might be the controlling element was obtained from an identical set of experiments but using a non-saturating factor X concentration ($0.2 \text{ } \mu\text{M}$). The observed rate constant of the inactivation reaction went through a minimum of 0.4 min^{-1} at $60 \text{ } \mu\text{M}$ phospholipid (25% PS - 75% PC) and then increased with further increase in the concentration of the phospholipid until an almost non-inhibited value (0.55 min^{-1}) at $400 \text{ } \mu\text{M}$ of phospholipid.

Effect of Unfractionated Heparin and Pentasaccharide on Factor IX_a and Factor X_a Inactivation by Antithrombin III during Factor X Activation- For the determination of the pseudo first order reaction rate constants of factor IX_a and factor X_a inactivation during factor X activation by AT III (200 nM) in the presence of UFH (0-120 ng/mL) or pentasaccharide (0-12 ng/mL), the same fit-procedure as already described was used. However, as the rabbit antifactor IX IgG neutralized the UFH, but not pentasaccharide, we modified the experimental set-up for the investigation of the effect of UFH. Instead of blocking the factor IX_a activity by the addition of anti-factor IX IgG, only AT III was added and factor IX_a inactivation was determined separately as described under Materials and Methods. In order to obtain the decay constant of factor X_a generated by factor IX_a , the Equations 1-4 were fitted to the experimental data with the parameters V_{X_a} and k_{IX_a} set at values calculated from the direct measurements according to Equation 6 and to $[IX_a]_t = [IX_a]_0 \exp(-k_{IX_a} t)$, respectively. Our mathematical model for analysis of the data depends on heparin-dependent factor X_a and factor IX_a inactivation being a pseudo first order reaction. This approach will only work if the protease concentrations remain far below saturation levels (1). Therefore, pseudo first order rate constants had to be determined as a function of the protease concentration under the same experimental conditions. To this end, factor IX_a at different concentrations (40 and 80 nM) was incubated with AT III (200 nM) and heparin (300 ng/mL) in 50 mM Tris-HCl (pH 7.9), 175 mM NaCl, 5

Figure 5: Pseudo first order rate constants of inactivation of factor X_a by AT III as a function of the UFH (panel A) and the pentasaccharide (panel B) concentration. (●), factor X_a (10 nM) plus AT III (200 nM) and (▲), factor X_a during factor X activation by [factor IX_a-phospholipid-Ca²⁺] in the presence of AT III (200 nM).

mM CaCl₂ and 0.5 mg ovalbumin/mL. Linear first order rate plots were obtained for at least 70% of the reaction. Rate constants SE were calculated from a linear least-squares fit using 6 time points in triplicate to be $0.203 \pm 0.017 \text{ min}^{-1}$ (40 nM factor IX_a) and $0.213 \pm 0.011 \text{ min}^{-1}$ (80 nM factor IX_a). We concluded that the apparent pseudo first order rate constant of inhibition of factor IX_a under our conditions and within the range used, was independent of the factor IX_a concentration. This finding also holds for the inactivation of factor X_a by AT III (200 nM) in the presence of heparin (80 ng/mL). The rate constants were $0.756 \pm 0.033 \text{ min}^{-1}$ (10 nM factor X_a) and $0.759 \pm 0.039 \text{ min}^{-1}$ (20 nM factor X_a).

Figure 5 shows the effect of UFH and pentasaccharide on the inactivation of factor X_a by AT III, both for free solution and for factor X_a during factor X activation. The stimulating effect of UFH on the inactivation of purified factor X_a by AT III (Figure 5A) was the same either in the presence or in the absence of phospholipids and/or calcium, with a rate constant of $0.012 \text{ min}^{-1}(\text{ng/mL})^{-1}$. However, during factor X activation the inhibition of factor X_a generated by factor IX_a was markedly reduced at heparin concentrations up to 40 ng/mL. At higher UFH concentrations the stimulating effect was the same as observed with purified factor X_a in free solution. Because this phenomenon was also observed

Figure 6: Pseudo first order rate constants of inactivation of factor X_a (10 nM) as a function of the factor X concentration in the presence of 100 μM phospholipid, 10 mM calcium, 200 nM anti-thrombin III and 40 ng/mL UFH.

in the presence of factor X alone, it might indicate that factor X and AT III compete with each other for UFH. Indeed, in Figure 6 it is shown that when increasing amounts of factor X were added to a mixture containing factor X_a (10 nM), AT III (200 nM) and 40 ng/mL UFH, the rate constant of the inactivation reaction progressively decreased with increasing factor X until the value of the heparin-independent AT III- X_a reaction. The factor X concentration that neutralized half the amount of available UFH was 0.2 μM .

The stimulating effect of pentasaccharide on the inactivation of factor X_a by AT III was 5-fold lower during factor X activation as compared to factor X_a inactivation in free solution (Figure 5B). The rate constants were $0.014 \text{ min}^{-1}(\text{ng/mL})^{-1}$ and $0.071 \text{ min}^{-1}(\text{ng/mL})^{-1}$, respectively. Factor X alone did not have an effect on the pentasaccharide-dependent inactivation of factor X_a . Thus, the lower rate constant during factor X activation was again the result of the protective effect of phospholipid-bound factor X on the inactivation of factor X_a .

Pentasaccharide was unable to enhance the inactivation of factor IX_a , either in the absence or presence of calcium, phospholipid and/or factor X. Figure 7 shows that UFH had a very small effect on the inactivation of factor IX_a in free solution, $k = 0.11 \text{ min}^{-1}(\mu\text{g/mL})^{-1}$. However, during factor X activation a 6-fold higher rate constant of inactivation of factor IX_a was found, a $k = 0.68 \text{ min}^{-1}(\mu\text{g/mL})^{-1}$. In order to establish the cause of this discrepancy, we examined the

Figure 7: Pseudo first order rate constants of inactivation of factor IX_a (80 nM) by AT III (200 nM) as a function of the UFH concentration. ($\bullet$) factor IX_a alone, ($\circ$) factor IX_a during factor X activation and ($\triangle$) factor IX_a in the presence of calcium (10 mM).

effect of UFH on factor IX_a inactivation in the presence of the individual components present in the factor X activation mixture. From Figure 7 it is clear that the enhanced inactivation rate of factor IX_a by antithrombin III and heparin is solely due to the presence of calcium.

Discussion

Several investigators have studied the inactivation of activated coagulation factors IX and X by AT III (10-13). However, the majority of these studies dealt with the effect of AT III on purified factor IX_a and factor X_a in free solution.

In the present paper, we describe a method for the determination of decay constants under more physiological conditions by modelling the inactivation of factor IX_a and factor X_a during factor X activation. The validity of this model was confirmed by the close resemblance between the data computed by the fit-procedure and the experimentally obtained data points (Figure 1). Furthermore, our experimental approach provided independent control of the velocity of fac-

tor X_a formation and rate of inactivation of generated factor X_a . These values were in good agreement with the values as determined by the fit-procedure. The second order rate constant for factor X_a inactivation by AT III thus obtained was $2.1 \times 10^5 \text{ M}^{-1}\text{min}^{-1}$. Because of the much lower rate of inhibition of factor IX_a under these conditions when compared with factor X_a , we were unable to calculate the rate constant of inhibition of factor IX_a by AT III. However, by making use of a factor IX_a assay and high AT III concentrations, we found that the reaction of factor IX_a and AT III proceeded with a second order rate constant of $8.2 \times 10^3 \text{ M}^{-1}\text{min}^{-1}$, thus about 25-fold slower when compared with the factor X_a /AT III reaction.

As several authors reported that human factor X_a (25) and bovine factor X_a (26) are protected from inactivation by AT III in the presence of phospholipids, we compared the decay constant of factor X_a determined during factor X activation with the decay constant of free factor X_a . Indeed, we found that the second order rate constant of factor X_a inactivation by AT III was $5.0 \times 10^5 \text{ M}^{-1}\text{min}^{-1}$, compared to a second order rate constant of $2.1 \times 10^5 \text{ M}^{-1}\text{min}^{-1}$ during factor X activation. However, in contrast to Ellis et al. (24) and Walker and Esmon (26), but in accordance with Marciniak (27) and Jesty (15), we did not observe that factor X_a was protected from inhibition by phospholipids. Using previously reported binding data of the factor $X_{(a)}$ -phospholipid interaction (31,32), it can be calculated that in the presence of $100 \mu\text{M}$ of phospholipid (25 mole% phosphatidylserine/75 mole% phosphatidylcholine) 90% of total factor X_a (10 nM) is bound to the phospholipid surface and yet, the pseudo first order rate constant of inhibition of factor X_a is the very same as that in the absence of phospholipid (Figures 2 and 3). Therefore, the presence of phospholipid do not explain the lower decay constant of factor X_a during factor X activation. Surprisingly, factor X appeared to reduce the rate of the factor X_a /AT III reaction. This phenomenon was only observed when both calcium and phospholipid were present. The similar effects of either increasing amounts of phospholipid at a fixed factor X concentration or increasing factor X at a fixed phospholipid concentration on the decay constant of factor X_a , strongly suggest that phospholipid-bound factor X protected factor X_a from inhibition (Figure 3).

In view of a factor X_a -catalyzed conversion of phospholipid-bound factor X to β -factor X (28-30) it seems reasonable to rationalize the observed inhibition of the inactivation reaction in terms of a competitive enzyme-substrate interaction. Thus, the rate constant of the AT III-factor X_a reaction will be attenuated by a factor of $1/(1 + [S]/K_m)$ and should approach zero at saturating substrate levels (33). However, a substantial increase in surface-bound factor X did not result in a further decrease of the apparent rate constant when factor X_a was quantitatively bound to the surface (Figure 4). Moreover, the level to which the inactivation reaction could be inhibited appeared to be a function of the phosphatidyl-

serine content of the phospholipid vesicle. Because the binding capacity of vesicles increases with increasing phosphatidylserine content (31) it seems that the local factor X concentration at the surface, rather than the bulk phospholipid-bound factor X concentration, is the effective substrate concentration for the competitive reaction.

At present, an alternative mechanism, in which the active site of factor X_a is not involved in the factor X_a-factor X interaction at the surface, can not be ruled out. That is, the decrease in the rate of inactivation of factor X_a could also reflect a reduced reactivity of factor X_a in the ternary complex of factor X-phospholipid-factor X_a towards AT III.

The inactivation of factor IX_a was not affected by the accessory components (phospholipid, calcium) and factor X. Regardless of the presence of factor X, phospholipids and/or calcium, the second order rate constant for factor IX_a inactivation by AT III was $8.2 \times 10^3 \text{ M}^{-1} \text{ min}^{-1}$. In view of the protection of factor X_a by phospholipid-bound factor X, it is quite striking that the interaction between factor IX_a and factor X at the surface does not compete with the AT III-factor IX_a reaction. However, because the phospholipid-factor IX_a interaction would be one of the affecting elements, it is interesting to note that the binding constant of this interaction is substantially larger than the one reported for the factor X_a-phospholipid interaction (34).

We next examined the effects of two types of heparin, unfractionated heparin (UFH) and synthetic pentasaccharide heparin, on the inhibition of factor IX_a and factor X_a during factor X activation. Pentasaccharide was included in our study because of reported effects of the non-AT III binding region of heparin molecules on factor X_a (25) and factor IX_a inhibition (13). Comparing the inhibition of factor X_a during factor X activation in the presence of UFH and pentasaccharide, it is evident that a polysaccharide sequence in addition to the AT III binding sequence alters the kinetics of factor X_a inhibition in such a way that it diminishes the protection of factor X_a by phospholipid-bound factor X. It is feasible that the competitive interaction between factor X_a and phospholipid-bound factor X is counterbalanced more strongly by the interaction between factor X_a and UFH-AT III because of the higher affinity of factor X_a for UFH-AT III than for pentasaccharide-AT III.

Pentasaccharide could not enhance the inhibition of factor IX_a by AT III. Apparently, factor IX_a binding regions on the heparin molecule are absolutely required for its catalytic function. The decay constant of factor IX_a increased linearly with the concentration of UFH, $k = 0.65 \text{ min}^{-1} \text{ per } \mu\text{g UFH/mL}$. To our surprise, the stimulatory effect of UFH on the inactivation of factor IX_a was about 6-fold higher during factor X activation as in free solution. It appeared that this stimu-

lation was due to the presence of calcium (Figure 7). The same phenomenon has been reported for human factor X_a (25). Calcium, because of its ability to induce structural changes in factor IX_a (35,36) may affect the heparin-catalyzed reaction either by enhancing ternary complex formation or by increasing the intrinsic rate constant of the AT III-factor IX_a reaction at the heparin surface.

Acknowledgment. We wish to thank Dr. Hans Soons for valuable discussions.

References

1. Griffith MJ, in Zwaal RFA, Hemker HC (eds): Blood Coagulation. Elsevier, Amsterdam, 1986, pp 239-283
2. Jordan RE, Oosta GM, Garder WT, Rosenberg RD: The kinetics of hemostatic enzyme-antithrombin III interactions in the presence of low molecular weight heparin. *J Biol Chem* 255:10081, 1980
3. Lindhout T, Baruch D, Schoen P, Franssen J, Hemker HC: Thrombin generation and inactivation in the presence of antithrombin III and heparin. *Biochemistry* 25:5962, 1986
4. Holmer E, Kurachi K, Soderstrom G: The molecular weight dependence of heparin on the rate-enhancing effect of heparin on the inhibition of thrombin, factor X_a , factor IX_a , factor XI_a , factor XII_a and kallikrein by antithrombin III. *Biochem J* 193:395, 1981
5. Gitel SN, Stephenson RC, Wessler S: In vitro and in vivo correlation of clotting protease activity: Effect of heparin. *Proc Natl Acad Sci USA* 74:3028, 1977
6. McNeely TB, Griffith MJ: The anticoagulant mechanism of action of heparin in contact-activated plasma: Inhibition of factor X activation. *Blood* 65:1226, 1985
7. Foster WB, Nesheim ME, Mann KG: The factor X_a -catalyzed activation of factor V. *J Biol Chem* 258:13970, 1983
8. Mertens K, Bertina RM: Activation of human coagulation factor VIII by activated factor X, the common product of the intrinsic and the extrinsic pathway of blood coagulation. *Thromb Haemostas* 47:96, 1982
9. Rao LVM, Rapaport SI, Bajaj SP: Activation of human factor VII in the initiation of tissue factor-dependent coagulation. *Blood* 68:685, 1986
10. Osterud B, Miller-Andersson M, Abildgaard U, Prydz H: The effect of antithrombin III on the activity of the coagulation factors VII, IX and X. *Thromb Haemostas* 35:295, 1976
11. Jesty J: The inhibition of activated bovine coagulation factors X and VII by antithrombin III. *Arch Biochem Biophys* 185:165, 1978
12. Jesty J: Dissociation of complexes and their derivatives formed during inhibition of bovine thrombin and activated factor X by antithrombin III. *J Biol Chem* 254:1044, 1979
13. Kurachi K, Fujikawa K, Schermer G, Davie EW: Inhibition of bovine factor IX_a and factor X_a by antithrombin III. *Biochemistry* 15:373, 1976
14. Jesty J, Morrison SA, Harpel PC: Measurement of human activated factor X_a -antithrombin III complex by an enzyme-linked differential-antibody immunosorbent assay. *Anal Biochem* 139:158, 1984
15. Jesty J: analysis of the generation and inhibition of activated coagulation factor X in pure systems and in human plasma. *J Biol Chem* 261:8695, 1986

16. Thaler E, Schmer G: A simple two-step isolation procedure for human and bovine antithrombin II/antithrombin III (heparin cofactor): a comparison of two methods. *Br J Haematol* 31:233, 1975
17. Miller-Andersson M, Borg H, Andersson L-O: Purification of antithrombin III by affinity chromatography. *Thromb Res* 5:439, 1974
18. Van Dieijen G, Tans G, Rosing J, Hemker HC: Role of phospholipid and factor VIII_a in the activation of bovine factor X. *J Biol Chem* 256:3433, 1981
19. Rosing J, van Rijn JLML, van Dieijen G, Comfurius P, Zwaal RFA: The role of activated human platelets in prothrombin and factor X activation. *Blood* 65:319, 1980
20. Lindhout T, Govers-Riemsag JWP, van de Waart P, Hemker HC Rosing J: Factor V_a-factor X_a interaction. Effects of phospholipid vesicles of varying composition. *Biochemistry* 21:5494, 1982
21. Soons H, Janssen-Claessen T, Hemker HC, Tans G: The effect of platelets in the activation of human blood coagulation factor IX by factor XI_a. *Blood* 68:140, 1986
22. Gill PE, Murray W:Siam J Num Anal 15:977, 1978
23. Ellis V, Scully M, Kakkar V: The effect of divalent metal cations on the inhibition of human coagulation factor X_a by plasma proteinase inhibitors. *Biochim Biophys Acta* 747:123, 1983
24. Ellis V, Scully MF, Kakkar V: Inhibition of prothrombinase complex by plasma proteinase inhibitors. *Biochemistry* 23:5882, 1984
25. Barrowcliffe TW, Havercroft SJ, Kembell-Cook G, Lindahl U: The effect of Ca⁺⁺, phospholipid and factor V on the anti-(factor X_a) activity of heparin and its high-affinity oligosaccharides. *Biochem J* 243:31
26. Walker FJ, Esmon CT: The effects of phospholipids and factor Va on the inhibition of factor X_a by antithrombin III. *Biochem Biophys Res Comm* 90:641, 1979
27. Marciniak E: Factor X_a inactivation by antithrombin III: evidence for biological stabilization of factor X_a by factor V-phospholipid complex. *Br J Haematol* 24:391, 1973
28. Jesty J, Spencer AK, Nemerson Y: The mechanism of activation of factor X. Kinetic control of alternative pathways leading to the formation of activated factor X. *J Biol Chem* 249:5614, 1974
29. Jesty J, Spencer AK, Nakashima Y, Nemerson Y, Konigsberg W: The activation of coagulation factor X. Identity of cleavage sites in the alternative activation pathways and characterization of the COOK-terminal peptide. *J Biol Chem* 250:4497, 1975
30. Link RP, Castellino FJ: The activation of bovine factor X by bovine factor X_a. *Arch Biochem Biophys* 215:215, 1982
31. Nelstue GL, Broderius M: Interaction of prothrombin and blood-clotting factor X with membranes of varying composition. *Biochemistry* 16:4172, 1977
32. van de Waart P, Bruls H, Hemker HC, Lindhout T: Interaction of bovine blood clotting factor V_a and its subunits with phospholipid vesicles. *Biochemistry* 22:2427, 1983
33. Griffith MJ: Measurement of the heparin enhanced antithrombin III thrombin reaction rate in the presence of synthetic substrate. *Thromb Res* 25:245, 1982
34. Beals JM, Castellino FJ: The interaction of bovine factor IX, its activation intermediate, factor IX_a, and its activation products, factor IX_a and factor IX_a, with acidic phospholipid vesicles of various compositions. *Biochem J* 236:861, 1986
35. Chuang TF, Sargeant RB, Hougie C: The effect of calcium ions on the properties of factor IX and its activated form. *Br J Haematol* 27:281, 1974
36. Amphlett GW, Byrne R, Castellino FJ: The binding of calcium to the activation products of bovine factor IX. *J Biol Chem* 254:6333, 1979

Chapter IV

The Limited Importance of Factor X_a Inhibition to the Anticoagulant Property of Heparin in Thromboplastin-Activated Plasma^o

Abstract

The anti-factor X_a activities of heparin fractions are widely used as an *ex vivo* index of their antithrombotic efficacy. Its clinical meaning, however, remains speculative. In this study we measured the effects of standard heparin, a synthetic pentasaccharide heparin (anti-factor X_a activity only), and a low molecular weight heparin (LMWH) on factor X_a, factor V_a and thrombin generation in thromboplastin-activated plasma. We clearly demonstrated that the anti-factor X_a activity of heparin contributed little to its anticoagulant activity. The inhibition of factor V_a generation, dependent of the heparin anti-thrombin activity only, is of prime importance to the inhibition of thrombin generation in plasma. The inhibition of thrombin generation by LMWH was comparable with that of standard heparin on basis of their respective anti-thrombin specific activities but not on basis of their anti-factor X_a activities.

^o Based on: Pieters J, Lindhout T: Blood, in press

Introduction

Until now, the antithrombotic activity of heparin is very poorly correlated with any laboratory parameter. Especially the recently developed low molecular weight heparins have made this problem quite clear. To reduce the bleeding risk associated with classical unfractionated heparin, a lot of clinical trials have been necessary to find an acceptable dosage scheme that optimizes the antithrombotic properties of heparin and minimizes its bleeding risk. For the recently developed low molecular weight heparins (LMWH) the approach was not to perform so many clinical trials until the right dosage was found, but rather to use laboratory tests to compare the new heparin types with the classical heparins. However, the main problem that arose with this approach was that it was not very clear what laboratory properties of heparin indicated its antithrombotic or hemorrhagic activities. Thus, the search for the proper laboratory correlate to *in vivo* antithrombotic action has become a central theme in heparin research.

Experiments in animal models suggested that these low molecular weight heparins might be as good an antithrombotic agent as unfractionated heparin, while at the same time reducing the bleeding risk (1-4). Then, one started to look for a property that was different in both types of heparin, and which was found in the relative anti-factor X_a potency. Low molecular weight heparins namely, which have a molecular weight between 4,000 and 9,000 daltons, loose their anti-thrombin action but retain their anti-factor X_a activity (5). It was therefore thought that the anti-factor X_a activity correlated with the antithrombotic properties, and the anti-thrombin action of heparin with its bleeding risk (6-8). Consequently, the anti-factor X_a specific activity became the *in vitro* index of the *in vivo* antithrombotic properties of heparin.

However, it is becoming increasingly apparent that the anti- X_a activity of heparin is not closely correlated with its clinical antithrombotic potency (9). Buchanan et al (10) reported that better antithrombotic effects were achieved by heparin or heparinoid capable of influencing the inactivation and/or the generation of thrombin. Hemker et al (11) developed a method which allowed the determination of the prothrombinase activity in the absence and presence of heparin, and they thus found that inhibition of thrombin is of major importance for the anticoagulant activity of heparin in plasma. The low molecular weight heparins they studied using this approach, could be divided into two classes: heparins that only inhibited thrombin (S-type heparins, after the similarity of these heparins with standard heparin), and heparins that do not act on thrombin but do inhibit prothrombinase (11,12).

In the present study, we studied the effect of heparin in a situation very close to the physiological one, namely in plasma that is activated with thromboplastin. To this end, we first had to develop very sensitive assays that were able to measure picomolar amounts of activated coagulation factors. The measurement of these activated factors directly with the available chromogenic substrates is not possible in plasma, because of the interference of various other plasma proteins, e.g. thrombin, antithrombin III, etc.. Therefore, we made use of the ability of factor X_a to activate (bovine) prothrombin in the presence of (bovine) factor V_a, phospholipids and calcium ions. In a similar way the plasma factor V_a concentration was determined, namely by the ability of factor V_a to accelerate prothrombin activation by factor X_a, phospholipids and calcium ions. In that way, we could very specifically determine the concentrations of factor X_a and factor V_a. Because of the large amounts that are usually generated, thrombin activity could be measured straightforward using a chromogenic substrate.

Using these assays, we investigated the effect of heparin and heparin analogues on the generation of thrombin, factor X_a and factor V_a in thromboplastin-activated plasma. For these studies we made use of three different heparin preparations; first of all we used the 4th international standard heparin, which possesses both high anti-thrombin and anti-factor X_a activity, second we made use of the synthetic pentasaccharide which possesses only anti-factor X_a activity, and as a third heparin preparation we used the low molecular weight heparin CY 216 which exerts a high anti-factor X_a activity and a low anti-thrombin activity. Our results indicate that factor V_a generation is the rate limiting step in the formation of the prothrombin converting complex, that consists of factor X_a, factor V_a and phospholipid. We hypothesized that the effect of heparin on thrombin generation is related mainly to its ability to influence factor V_a formation and thus to its anti-thrombin activity rather than to its anti-factor X_a activity.

Materials and Methods

Materials- The synthetic peptide substrates Bz-Ile-Glu-(Piperidyl)-Gly-Arg-p-nitro-anilide hydrochloride (S-2337) and H-D-Phenylalanyl-L-pipecolyl-L-arginine-p-nitro-anilide dihydrochloride (S-2238) were purchased from AB Kabi Diagnostica, Stockholm. Fatty acid-free ovalbumin was a product of Sigma Chemical Co., St. Louis. All reagents used were of the highest grade commercially available.

The 4th International Standard for Heparin (195 U/mg) was supplied by the National Institute for Biological Standards and Control (NIBSC). A synthetic pentasaccharide heparin (manufacturers specific activity of 0 USP U/mg and

4,000 anti-factor X_a U/mg) and a low molecular weight heparin CY 216 (manufacturers specific activity of 10-20 anti-thrombin U/mg and 200 anti-factor X_a U/mg) were a kind gift from Choay Institute (Paris).

Human brain thromboplastin was prepared by the method of Owren and Aas (13). It was homogenated in a Potter Elvehjem homogenizer for 3 minutes, centrifugated at $2,000 \times g$ for 15 minutes and stored at -80°C . Its concentration was adjusted with Tris-saline to give a prothrombin time of 13 s.

Proteins- All bovine clotting factors were prepared and quantitated as previously described (14-16).

Phospholipid Vesicles- Phospholipid vesicles were made from a mixture of phosphatidylserine (PS) and phosphatidylcholine (PC) as previously described (16). Vesicles composed of 25 mole% PS and 75 mole% PC were used throughout the experiments.

Preparation of Human Normal Plasma- Blood from healthy donors was collected on 0.13 M trisodium citrate; nine parts blood to one part of citrate solution. The blood was then centrifugated twice at room temperature for 15 minutes at $3,000 \times g$. A third centrifugation was done at 4°C for one hour at $23,000 \times g$. The obtained platelet free plasma was stored at -80°C .

Thromboplastin-Induced Factor X_a , Factor V_a and Thrombin Generation in Platelet Free Plasma- To 0.13 mL of citrated human plasma, 9 μL of a Tris-buffer (50 mM Tris, 180 mM NaCl, 0.5 mg ovalbumin/mL, pH 7.9) with or without heparin(fraction) was added and incubated for 4 min in a flat-bottom plastic tube while stirring. Clotting was initiated by the addition of 11 μL of the Tris-buffer containing 0.27 mol/L CaCl_2 and human brain thromboplastin. Timed samples (10 μL) were removed from incubations and assayed for either factor V_a , factor X_a or thrombin activity.

Factor V_a Assay- Factor V_a was assayed in plasma samples, diluted 1:200 in Tris-buffer containing 5 mM CaCl_2 and 0.5 μg UFH/mL. Ten μL of the diluted sample was added to a cuvette containing 13 pmol/L factor X_a , 5 mM CaCl_2 and 0.05 mM phospholipid (25 mol% phosphatidylserine and 75 mol% phosphatidylcholine) in Tris-buffer. After 4 min thrombin generation was initiated by the addition of prothrombin (0.2 μM final concentration in 125 μL). The reaction was stopped after 2 min by the addition of 325 μL Tris-buffer containing 20 mM EDTA. The amount of thrombin was measured in a dual-wavelength spectrophotometer using S-2238 as described previously (15).

Factor X_a Assay- Factor X_a was assayed in plasma samples diluted 1:200 in Tris-buffer. Ten μL of the diluted sample was added to a cuvette containing 0.6 nmol/L factor V_a, 0.05 mM phospholipid and 5 mM CaCl_2 in Tris-buffer. After 4 min prothrombin (1.5 μM final concentration in 50 μL) was added and the assay was proceeded as described for factor V_a.

Thrombin Assay- Undiluted plasma samples (5 μL) were added to a cuvette containing 495 μL Tris-buffer, 20 mM EDTA and 0.25 mM S-2238. The thrombin activity was calculated from the change in absorbance as described previously (17).

Results

Measurement of Activated Coagulation Factors in Plasma- In order to be able to measure very specific the generation of factor X_a and factor V_a in plasma, we developed extremely sensitive assays. These determinations are based on the activity of these factors on their natural substrates.

Plasma factor X_a was assayed by its ability to activate (bovine) prothrombin in the presence of calcium, phospholipids and (bovine) factor V_a; the sample taken from activated plasma was diluted a thousand-fold into the factor X_a assay mixture, and the assay was performed as described under Materials and Methods. Figure 1A shows a calibration curve made with a human factor X_a preparation of a known concentration. Factor X_a concentrations down to 0.1 pM could be detected, corresponding to an amount of 0.1 nM in plasma. Thrombin was determined by diluting the plasma directly a hundred-fold in EDTA-buffer containing the chromogenic substrate for thrombin.

The determination of activated factor V proceeded in a similar way. We made use of the ability of human factor V_a to accelerate prothrombin activation by factor X_a, phospholipid and calcium tremendously. An over thousand-fold diluted plasma sample was added to the factor V_a assay mixture, and the factor V_a present in the sample was measured via the prothrombin activation (see Materials and Methods). Factor V did not show any activity in this assay. However, one problem aroused with this factor V_a determination, which was the carry-over of factor X_a in this assay. This factor X_a, present in the diluted sample, also accelerated the prothrombin activation. By adding heparin to the first dilution buffer in a concentration of 0.5 $\mu\text{g/mL}$, the present antithrombin III inactivated this factor X_a within 15 minutes.

Figure 1. Calibration curve for the factor X_a and factor V_a assays. The rate of (bovine) prothrombin activation was determined in the presence of A: Phospholipid, calcium and (bovine) factor V_a, and B: Phospholipid, calcium and (bovine) factor X_a.

Figure 1B shows the calibration curve made with known amounts of (bovine) factor V_a (control experiments revealed that bovine and human factor X_a were comparable with respect to their activity in the prothrombinase complex).

Generation of Activated Coagulation Factors in Thromboplastin-Activated Human Plasma- To study the activation of prothrombin, factor X_a and factor V_a generation in normal human plasma, citrated human plasma was activated as described under Materials and Methods, using a thromboplastin concentration of 1/30 (vol/vol). With such a high thromboplastin concentration, the activation of factor X_a generation in normal plasma is similar to factor X_a generation in factor IX deficient plasma, indicating that in this case intrinsic factor X activation is not relevant (data not shown).

Figure 2A shows the obtained thrombin generation curve. After a very short lag-phase, explosive thrombin formation is followed by a very rapid decay of the formed thrombin. A maximum of about 350 nM of thrombin was formed. The inactivation, which occurs after the peak at 1 minute, is mainly due to the action of antithrombin III and α_2 -macroglobulin (19). The complex of thrombin and α_2 -macroglobulin still has amidolytic activity towards the chromogenic substrate S-2238, thus resulting in an apparent end level of thrombin activity (11).

Figure 2. Generation of activated coagulation factors in plasma. Plasma was activated with 1/30 (vol./vol.) thromboplastin and the generation of thrombin (panel A), factor X_a (panel B) and factor V_a (panel C) was determined. Panel B, inset: semi-logarithmic plot of the decay part of the factor X_a generation curve in the absence of heparin. Panel C: the generation of factor V_a in protein C depleted plasma is also shown (Δ).

In Figure 2B the factor X_a generation curve is depicted. No lag phase is present, and the maximal amount of factor X_a generated is 12 nM. This is only about 10 % of the total amount of factor X present in plasma. Surprisingly, as is depicted in the inset, the decay phase, which starts after 2 minutes, can be modelled according to pseudo-first order reaction rate kinetics. This means that after 2

minutes, no factor X is further activated, and only factor X_a inactivation is observed. The calculated pseudo first order rate constant of inhibition of in situ formed factor X_a thus, 0.30 min^{-1} , is in accordance with the value reported by Jesty (18). The fact that factor X activation stops after 2 minutes, might be caused by the extrinsic pathway inhibitor (EPI) in combination with factor X_a , recently described by Rao et al (19, see also scheme I). Indeed, addition of an extra 1/30 (vol/vol) amount of thromboplastin resulted in a similar factor X_a generation as depicted in figure 2B (results not shown).

The factor V activation is shown in Figure 2C by the closed circles. As for thrombin generation, also here a short lag phase is observed, followed by explosive factor V_a generation, and a rapid decay. The inhibition of factor V_a appeared to be due to activated protein C, as in protein C deficient plasma (a kind gift of Dr. Bertina), no inactivation of the generated factor V_a was observed. Also the addition of EDTA immediately stopped the factor V_a inactivation reaction. Factor V_a generation in protein C depleted plasma is shown in Figure 2C by the open triangles.

Effect of Standard Heparin on the Generation of Activated Coagulation Factors in Plasma- To assess the effect of standard heparin on the generation of activated coagulation factors in plasma, we added standard heparin in several concentrations to the plasma. The plasma was subsequently activated with 1/30 (vol/vol) thromboplastin and the generation of thrombin, factor X_a and factor V_a was determined. Figure 3 depicts the effect of the addition of standard heparin at a concentration of 1 and 2 $\mu\text{g/mL}$ on the thrombin, factor X_a and factor V_a generation curves.

From Figure 3A it is clear that standard heparin induces a prolonged lag time, and also the total amount of thrombin formed is greatly reduced. The factor X_a generation curves, as depicted in Figure 3B, do not show a lag phase, but instead the effect of heparin is to greatly reduce the factor X_a yield. Figure 3C shows the effect of standard heparin on the generation of factor V_a . As for thrombin generation, also factor V_a is generated only after a lag period, which length is dependent on the heparin concentration added to the plasma.

From Figure 3 it is already clear, that the lag-phase observed for thrombin generation is not correlated with the inhibition of factor X_a generation, because no lag phase was induced in factor X_a generation upon the addition of standard heparin.

Figure 3. Effect of standard heparin on the generation of thrombin (panel A), factor X_a (panel B) and factor V_a (panel C) in thromboplastin activated plasma. Concentrations used are 0 $\mu\text{g/mL}$ (●), 1 $\mu\text{g/mL}$ (▲) and 2 $\mu\text{g/mL}$ (△).

Effect of Pentasaccharide on the Generation of Activated Coagulation Factors in Plasma- Next, we investigated the effect of the synthetic pentasaccharide on the generation of thrombin, factor X_a and factor V_a in thromboplastin-activated plasma. As pentasaccharide possesses only anti-factor X_a activity, the addition of pentasaccharide would show the effect of the anti- X_a activity to the inhibition of thrombin generation and thus clotting in plasma. Figure 4 shows the effect of adding the pentasaccharide in a concentration of 0.5 and 1 $\mu\text{g/mL}$ to the plasma.

From Figure 4A it is clear that pentasaccharide has practically no effect on thrombin generation. The peak amount of formed thrombin remains at 1 minute, and only the total amount of the thrombin formed is reduced. As expected, the generation of factor X_a was significantly reduced (panel B). It is noteworthy that, although factor X_a levels in plasma containing $1\ \mu\text{g/mL}$ are very low (panel B), thrombin generation is only slightly reduced (panel A). Panel C shows that, as for the generation of thrombin, factor V_a generation is not significantly influenced.

Figure 4. Effect of the synthetic pentasaccharide on the generation of thrombin (panel A), factor X_a (panel B) and factor V_a (panel C) in thromboplastin activated plasma. Concentrations used are $0\ \mu\text{g/mL}$ ($\bullet$), $0.5\ \mu\text{g/mL}$ ($\blacktriangle$) and $1\ \mu\text{g/mL}$ ($\triangle$).

Figure 5. Effect of the low molecular weight heparin CY 216 on the generation of thrombin (panel A), factor X_a (panel B) and factor V_a (panel C) in thromboplastin activated plasma. Concentrations used are 0 µg/mL (●), 10 µg/mL (▲) and 20 µg/mL (△).

Effect of the Low Molecular Weight Heparin CY 216 on the Generation of Activated Coagulation Factors in Plasma- As a third heparin preparation we used CY 216, a low molecular weight heparin with a high anti-X_a over anti-thrombin ratio. The obtained results are depicted in Figure 5. We added CY 216 in a concentration of 10 and 20 µg/mL to the plasma. As can be seen in panel A, the addition of 20 µg/mL CY 216 results in a similar inhibition of the thrombin generation

curve as was the case with 1 $\mu\text{g/mL}$ standard heparin. Indeed, this parallels their anti-thrombin activities: standard heparin possessing 195 U/mL, and CY 216 10-20 U/mL. In panel B the effect of CY 216 on the generation of factor X_a is depicted. CY 216 exerts the same concentration dependence as standard heparin for the inhibition of factor X_a generation, and indeed, the two heparin preparations possess the same specific anti-factor X_a activities. The factor V_a generation curves again parallel the thrombin generation, as already was seen for standard heparin and pentasaccharide (see panel C).

Table I summarizes the effects of the three used heparins and heparin analogues in thromboplastin activated plasma. The effects of these heparins on thrombin generation and factor X_a generation is expressed as percentages of the peak values. Also the effect on the clotting time is given. As can be seen in Table I, pentasaccharide and CY 216, when compared with standard heparin are much less effective than was expected from their given specific anti-factor X_a activities. Also, Table I shows that reducing the factor X_a yield, does not cause a similar reduction in thrombin yield; a reduction of the factor X_a yield to 5 % for example, causes a reduction of the thrombin generation peak value of 14 % (standard heparin), 25 % (CY 216) or 71 % (pentasaccharide). This shows that the reduction in thrombin yield parallels rather the anti-thrombin activity of the heparins, than their anti-factor X_a activity. The prolongation of the clotting time also parallels the anti-thrombin activity, rather than the anti-factor X_a activity.

Table 1. Thromboplastin-dependent plasma factor X_a and thrombin generation as a function of the heparin concentration.

Heparin Type	Concentration			Peak Values (%)		Clotting Time (sec)
	($\mu\text{g/mL}$)	(a-IIa U/mL)	(a- X_a U/mL)	IIa	X_a	
4 th ISH	0	0	0	100	100	45
	0.5	0.1	0.1	50	45	60
	1.0	0.2	0.2	30	17	85
	2.5	0.5	0.5	14	6	510
CY 216	2.5	0.025 - 0.05	0.5	60	49	48
	10.0	0.1 - 0.2	2.0	38	19	70
	20.0	0.2 - 0.4	4.0	25	6	90
	50.0	0.5 - 1.0	10.0	5	nd	630
Pentasaccharide	0.5	0	2.0	90	29	50
	2.5	0	10.0	71	4	62
	10.0	0	40.0	60	nd	75
	40.0	0	160	35	nd	90

Experimental conditions were the same as in Figures 3-5. nd: not detectable

Effect of the Inhibition of Factor X_a on the Anticoagulant Effect of Heparin in Plasma- As already is clear from Figures 3-5 and Table I, the inhibition of the factor X_a generation did not in all cases reduce the thrombin generation in plasma. Therefore, in order to investigate the contribution of factor X_a inhibition to thrombin generation, we used a weight amount of each heparin that caused similar factor X_a generation curves in which the factor X_a peak values were 5% of the control, as is depicted in Figure 6, i.e. 2.5 µg/mL of standard heparin (panel B), 20 µg/mL of CY 216 (panel C), and 2.5 µg/mL of pentasaccharide (panel D).

Figure 6. Effects of heparin and heparin fractions on the generation of thrombin (●), factor X_a (▲) and factor V_a (○) in thromboplastin activated plasma. (A) no heparin, (B) 2.5 µg/mL of standard heparin, (C) 20 µg/mL of CY 216 and (D) 2.5 µg/mL of the synthetic pentasaccharide. The amounts are expressed as a percentage of the maximum amounts in the absence of heparin, i.e. 1 U/mL (25 nmol/L) of factor V_a, 320 nmol/L of thrombin and 12 nmol/L of factor X_a.

Thus, the only variable in these experiments was the anti-thrombin activity, 0.5 U/mL (Standard heparin), 0.2 - 0.4 U/mL (CY 216) and 0 U/mL for pentasaccharide, respectively. Figure 6 shows that despite similar factor X_a generation curves, thrombin generation differed markedly with the heparin used. Our results indicate that indeed thrombin generation is not directly associated with factor X_a generation. That is, the prolongation of the lag phase observed in thrombin generation in the presence of standard heparin is not solely the result of factor X_a inhibition, because the same, quantitative, factor X_a inhibition in the presence of pentasaccharide had a minor effect on the lag phase and slightly reduced the thrombin yield. In the presence of standard heparin (2.5 μ g/mL) the maximum thrombin activity is about 10% of the control, but it should be recognized that the virtual thrombin concentration is much higher because of the rapid decay of the thrombin activity once it is formed. Thus, despite a greatly reduced factor X_a yield, even below our detection limit of 0.1 nmol/L in plasma, significant amounts of thrombin can be produced (Figure 6B).

The explanation for the relatively weak anticoagulant activities of pentasaccharide and CY 216 can be found in their effects on factor V_a generation in plasma. Factor V_a is an essential cofactor of factor X_a and is generated from the pro-cofactor V by thrombin. As also shown in Figure 6, factor V_a generation is greatly delayed in plasma containing a relatively high anti-thrombin activity (standard heparin) but hardly in plasma containing pentasaccharide, devoid of anti-thrombin activity. Also, the inhibition of the formed factor V_a appeared to be dependent on the amount of thrombin present, probably because protein C activation is thrombin dependent.

In support of our notion that factor V_a generation by thrombin is the rate-limiting step in prothrombin activation, the addition of factor V_a to heparinized plasma at different time intervals after thromboplastin addition, immediately caused an extremely rapid thrombin generation, followed by a rapid decay as the result of prothrombin depletion and inactivation of thrombin (Figure 7). Clot formations were observed within 10 s after the additions of factor V_a . The thrombin peak activities thus obtained appeared to parallel the amount of residual factor X_a activity at that time present. Factor X_a generation was not influenced by the addition of factor V_a and maximum plasma factor V_a levels observed were about twice the value found when no factor V_a was added. These results indicate that the presence of factor V_a makes extremely low levels of factor X_a sufficient to produce thrombin at a high rate.

Figure 7. Factor V_a stimulated thrombin generation in heparinized plasma. Thrombin was measured in mixtures containing human plasma, thromboplastin (1/30 of total volume) and 2.5 $\mu\text{g/mL}$ of the standard heparin ($\square$). In separate experiments purified factor V_a (final concentration of 25 nmol/L) was added 1 min ($\circ$), 2 min ($\bullet$), 4 min ($\triangle$) or 6 min ($\blacktriangle$) after the addition of thromboplastin.

Discussion

The process of blood coagulation comprises a complicated series of protein interactions that functions in a series of interlinked positive and negative feedback loops. It is because of this complexity that at present no definite information is available to assess the relative importance of the anti-thrombin and anti-factor X_a activities of heparin to the overall reaction rate of the coagulation sequence *ex vivo*. In addition, the activated clotting factors generated in clotting plasma were not directly available for quantitation because of the lack of sensitive and specific bioassays. In the present paper we presented a method by which factor V_a and factor X_a generation could be monitored in clotting plasma. The essential feature of these assays is that the natural substrate, prothrombin, is used to measure the enzyme and cofactor activities. Because of the fact that for the prothrombin activation only very small amounts of factor X_a or factor V_a are needed, the plasma could be diluted a thousand-fold before measurement, thus eliminating all factors that could interfere with our factor X_a or factor V_a determination.

The aim of our study was to estimate the relative contributions of the heparin-dependent inhibition of factor X_a and thrombin to the anticoagulant effect of heparin in plasma. Thereto, the action of heparin on the following processes have to be considered: i) formation of the prothrombinase complex (inhibition of factor X_a and factor V_a generation), ii) inhibition of prothrombinase activity and iii) inhibition of thrombin.

The generation of both factor V_a and factor X_a are controlled by positive feedback reactions. In particular, the so-called intrinsic activation of factor X is a complicated process in which activation of factor VIII:C by thrombin and probably also by factor X_a is of crucial importance (20). As a first attempt we, therefore, restricted ourselves to an investigation of the so-called extrinsic pathway of blood coagulation. To this end, human brain thromboplastin was used at a concentration that produced factor X_a with a rate indistinguishable from that in factor IX deficient plasma. The activation and inactivation reactions that are then important are depicted in Scheme 1. The TF:factor VII_a complex activates factor X into factor X_a , which then converts some prothrombin into thrombin. These first traces of thrombin are sufficient to activate factor V into factor V_a , to form together with factor X_a , phospholipids and calcium the so-called prothrombinase complex. Only then sufficient amounts of thrombin are produced to form a fibrin-clot.

The inhibitory pathways are also depicted in Scheme 1. First of all there is the extrinsic pathway inhibitor, inhibiting the activity of tissue factor when factor X_a and calcium are present (19). Second, activated protein C inhibits the activated factor V, and finally antithrombin III is a very important inhibitor of thrombin and factor X_a , which inhibition is dramatically accelerated when heparin is present.

Scheme 1. Activation (—) and inactivation (---) reactions upon triggering plasma with a high concentration of thromboplastin.

First of all we determined how the activated coagulation factors were generated in plasma, when triggered with a high concentration of thromboplastin. The fact that no lag phase was observed in factor X_a generation, whereas factor V_a generation did show a lag phase, already indicates that factor V_a formation might be the rate limiting step in the formation of the prothrombinase complex and thus in the generation of sufficient amounts of thrombin (about 10 nM) to produce the critical fibrin concentration that results in clot formation.

We determined the effects of three different types of heparin on the generation of thrombin, factor X_a and factor V_a in plasma. These three differed from each other in their anti-thrombin and anti-factor X_a activities. The most important conclusions from these experiments are that apparently, the generation of factor X_a does not correlate with the generation of thrombin. Rather the generation of factor V_a activity is correlated to thrombin generation.

In order to investigate further whether the inhibition of factor X_a generation is really important for the inhibition of thrombin generation, we added the three heparins in a concentration which caused the factor X_a generation to be only 5 % of normal.

When solely the generation of factor X_a is inhibited (Figure 6D) the rather low thrombin concentrations required for factor V_a formation are readily produced as indicated by a minor increase in the lag phase of factor V_a formation. The slight decrease of the maximum thrombin activity suggest a minor reduction of prothrombinase activity, either caused by the lower levels of factor X_a and/or by inhibition of factor X_a in the complex. At higher pentasaccharide concentrations, where factor X_a generation was diminished below our detection limit, and only sub-nanomolar amounts of prothrombinase might be present, still significant amounts of thrombin were generated. All together, these findings strongly suggest that the anti-factor X_a activity is of minor importance to the overall anticoagulant activity of heparin. In contrast, the heparin-dependent inhibition of thrombin during the initial stage of thromboplastin-triggered coagulation, when factor V is converted into factor V_a by thrombin (Figure 6B), appeared to be of prime importance to the anticoagulant activity of heparin.

Indeed, the anticoagulant effect of heparin could be reversed upon the addition of purified factor V_a to thromboplastin-activated heparinized plasma during the period where still no substantial thrombin generation was observed and where factor X_a activity was already greatly reduced (Figure 7).

Barrowcliffe et al. (21) have demonstrated in a purified system that when molar activities are compared unfractionated heparin is a more efficient inhibitor of the amidolytic factor X_a activity in the prothrombinase complex than pentasaccharide. In the experiments shown in Figure 6, the molar activities of stand-

ard heparin (MW = 15,000, 40% of the molecules with high affinity for AT III) and pentasaccharide (MW = 1714) were 0.07 μ M and 1.5 μ M, respectively. Thus, it is unlikely that the dramatic differences observed in the thrombin generation curves in the presence of heparin and pentasaccharide are the result of a higher anti-prothrombinase activity in case of standard heparin. In addition, it has been pointed out by Béguin et al. (22) that no direct action of heparin on prothrombinase needs to be assumed if one takes into account the effect of thrombin inhibition on thrombin-mediated feedback reactions.

It is tempting to speculate that the rather insignificant contribution of the heparin anti-factor X_a activity in the anticoagulant activity of heparin, as demonstrated here, might be one of the reasons why the heparin anti-factor X_a activity does not parallel antithrombotic efficacy. However, we have to emphasize that if factor X_a generation via the intrinsic pathway is more relevant to the *in vivo* coagulation and if activation of factor VIII:C by factor X_a contributes significantly to the positive feedback reaction of factor X_a generation in plasma, then the anti-factor X_a activity has to be taken into account as well. In a purified system, however, thrombin-catalyzed activation of factor VIII:C appeared to be about 15-fold more efficient in the factor IX $_a$ -catalyzed factor X activation than the activation of factor VIII:C by factor X_a (20). Currently, the initiation of feedback activation of factor VIII:C in plasma is poorly understood and requires further studies.

References

1. Choay J, Petitou M, Lormeau JC, Sinay P, Casu B, Gatti G: Structure-activity relationship in heparin: a synthetic pentasaccharide with high affinity for antithrombin III and eliciting high anti-factor X_a activity. *Biochim Biophys Res Comm* 116: 492, 1983
2. Esquivel CO, Bergqvist D, Björk CG, Nilsson B: Comparison between commercial heparin, low molecular weight heparin and pentosan polysulfate on hemostasis and platelets *in vivo*. *Thromb Res* 28: 389, 1982
3. Holmer E, Mattson C, Nilsson S: Anticoagulant and antithrombotic effects of low molecular weight heparin fragments in rabbits. *Thromb Res* 25: 475, 1982
4. Diness Y, Nielson JJ, Pedersen PC, Wolffbrandt KH, Ostergaard PB: A comparison of the antithrombotic and haemorrhagic effects of a low molecular weight heparin (LHN-1) and conventional heparin. *Thromb Haemost* 55: 410, 1986
5. Carter CJ, Kelton JG, Hirsh J, Cerskus A, Santos AV, Gent M: The relationship between the hemorrhagic and antithrombotic properties of low molecular weight heparin in rabbits. *Blood* 59: 1239, 1982
6. Fussi F, Girolami A, Cella G: Low molecular weight heparins and their effect *in vitro*, in experimental models and in humans, in Lundblat RL, Brown WV, Mann KG, Roberts HR (eds): *Chemistry and biology of heparin*. Elsevier/North Holland, New York, 1981, p 535

7. Thomas DP, Merton RE, Lewis WE, Barrowcliffe TW: Studies in man and experimental animals of a low molecular weight heparin fraction. *Thromb Haemost* 45:218, 1981
8. Holmer E, Mattson C, Nilsson S: Anticoagulant and antithrombotic effects of heparin and low molecular weight heparin fragments in rabbits. *Thromb Res* 25:475, 1982
9. Thomas DP: Current status of low molecular weight heparin. *Thromb Haemost* 56:241, 1986 (Letter)
10. Buchanan MR, Boneu B, Ofosu F, Hirsh J: The relative importance of thrombin inhibition and factor X_a inhibition to antithrombotic effects of heparin. *Blood* 65: 198, 1985
11. Hemker HC, Willems GM, Béguin S: A computer assisted method to obtain the prothrombin activation velocity in whole plasma independent of thrombin decay processes. *Thromb Haemost* 56(1):9, 1986
12. Hemker HC: The mode of action of heparin in plasma, in Verstraete M, Vermeylen J, Lijnen HR, Arnout J (eds): *Thrombosis and haemostasis 1987*, ISTH, Leuven, 1987, p17
13. Owren PA, Aas K: The control of dicoumarol therapy and the quantitative determination of prothrombin and proconvertin. *Scand J Clin Lab Invest* 3:201, 1951
14. Van Dieijen G, Tans G, Rosing J, Hemker HC: Role of phospholipid and factor VIII_a in the activation of bovine factor X. *J Biol Chem* 256:3433, 1981
15. Rosing J, van Rijn JLML, van Dieijen G, Comfurius P, Zwaal RFA: The role of activated human platelets in prothrombin and factor X activation. *Blood* 65:319, 1980
16. Lindhout T, Govers-Riemslog JWP, van de Waart P, Hemker HC, Rosing J: Factor Va-factor X_a interaction. Effects of phospholipid vesicles of varying composition. *Biochemistry* 21:5494, 1982
17. Lindhout T, Baruch D, Schoen P, Franssen J, Hemker HC: Thrombin generation and inactivation in the presence of antithrombin III and heparin. *Biochemistry* 25: 5962, 1986
18. Jesty J: Analysis of the generation and inhibition of activated coagulation factor X in pure systems and in human plasma. *J Biol Chem* 261: 8695, 1986
19. Rao LVM, Rapaport SI: Studies of a mechanism inhibiting the initiation of the extrinsic pathway of coagulation. *Blood* 69: 645, 1987
20. Lollar P, Knutson GJ, Fass DN: Activation of porcine factor VIII:C by thrombin and factor X_a. *Biochemistry* 24: 8056, 1985
21. Barrowcliffe TW, Havercroft SJ, Kembell-Cook G, Lindahl U: The effect of Ca²⁺, phospholipid and factor V on the anti- (factor X_a) activity of heparin and its high-affinity oligosaccharides. *Biochem J* 243: 31, 1987
22. Béguin S, Lindhout T, Hemker HC: The mode of action of heparin in plasma. *Thromb Haemost* (in press)

Chapter V

Factor XI_a Catalyzed Generation of Factor IX_a in Human Plasma.

Regulation by Plasma Proteinase Inhibitors and Heparin^o

Abstract

The generation and inhibition of factor IX_a have been studied in factor XI_a-activated plasma to determine the kinetics of inhibition of factor XI_a and factor IX_a during factor IX activation. Interference of other (activated) factors with the factor IX_a activity measurements could be avoided by using a highly specific and sensitive bioassay. Factor IX_a generation curves were analyzed according to a 3-parameter model to determine the k_{cat}/K_m ratio for factor IX activation by factor XI_a. Simultaneously, apparent first order rate constants of inhibition of factor XI_a and *in situ* generated factor IX_a were determined in plasma containing free Ca²⁺ levels of 4 mM and phospholipids. The fitted value for the k_{cat}/K_m ratio, 320 $\mu\text{M}^{-1}\text{min}^{-1}$, was in good agreement with the reported ratio. In the absence of heparin no decay of factor IX_a activity was observed. Maximal levels of factor IX_a varied with the factor XI_a concentration as a result of inhibition of factor XI_a by plasma proteinase inhibitors at a rate constant of 0.35 min^{-1} . In the presence of unfractionated heparin the apparent second order rate constant of inhibition of factor IX_a was 6.16 $\text{min}^{-1}/\mu\text{g heparin/mL}$. The rate constant in the presence of

^o Based on: Pieters J, Soons H, Willems G, Franssen J, Hemker HC, Lindhout T, submitted

a synthetic pentasaccharide heparin was $0.70 \text{ min}^{-1}/\mu\text{g}$ pentasaccharide/mL. Ca^{2+} ions (4 mM) were required to obtain heparin-dependent inhibition of factor IX_a . Clot formation but not thrombin formation abolished the heparin stimulated inhibition of factor IX_a . Factor XI_a inhibition was not affected by heparin in the range studied.

Introduction

Factor IX can be activated via both the extrinsic and intrinsic coagulation pathway. It thus forms a link between these pathways that is thought to be of crucial importance for the initiation phase of blood coagulation (for recent reviews, see references 1 and 2)

The majority of studies on the regulation of factor IX_a by plasma proteinase inhibitors have been done with purified enzyme either in plasma or in buffer (3-5). It has been shown that bovine and human factor IX_a react slowly with antithrombin III but not with α_2 -macroglobulin and α_1 -proteinase inhibitor (4,6). The reaction with antithrombin III is greatly stimulated by the presence of heparin. Like in case of thrombin, the simultaneous binding of both the inhibitor and enzyme to the same heparin molecule seems to be required for maximal rates of inactivation of factor IX_a (7). Indeed, for bovine factor IX_a it was found that pentasaccharide did not stimulate its inactivation by antithrombin III (5). Whereas Ca^{2+} ions reduce the rate of inactivation of thrombin by antithrombin III/heparin (8), they greatly enhance the reaction between bovine factor IX_a and antithrombin III/heparin (5).

In addition to these studies, two groups have reported on the effects of heparin on the progress of activation of radiolabelled factor IX in contact-activated plasma (3) and in thromboplastin-activated plasma (9). In the latter case, 5 U/mL of heparin almost completely blocked factor IX activation. During contact activation, heparin did not significantly affect the steps preceding factor X activation. That is, in the absence or presence of 0.5 U/mL heparin, the extent of radiolabelled factor IX cleavages at 30 minutes were essentially identical. Factor IX_a activity levels were reduced in the presence of heparin but still to be found significantly higher than was required for factor X activation to occur in the absence of heparin.

However, a straightforward analysis of the generation and inhibition of factor IX_a activity in heparinized plasma on basis of a model that describes the time course of factor IX_a generation and decay is currently not available. One of the reasons might be the problem of measuring factor IX_a activity generated *in situ* without interference by other activated coagulation factors formed.

The present studies were undertaken to examine the effects of two types of heparin, i.e. unfractionated heparin and ultra low molecular weight heparin, on factor XI_a-catalyzed factor IX_a generation in plasma and inactivation of *in situ* generated factor IX_a. It was possible by using a highly specific and sensitive bioassay to monitor the generation and inhibition of factor IX_a in factor XI_a-activated plasma. Factor IX_a generation curves were fitted to a 3-parameter model to assess quantitatively the effects of unfractionated heparin and a synthetic pentasaccharide heparin on factor IX_a and factor XI_a inhibition by antithrombin III.

Materials and Methods

Materials- The synthetic peptide substrate Bz-Ile-Glu(Piperidyl)-Gly-Arg-p-nitro-anilide hydrochloride (S-2337) was purchased from AB Kabi Diagnostica, Stockholm, Sweden.

Factor IX deficient plasma was obtained from congenitally deficient patients and was purchased from DiaMed AG, Switzerland. Ovalbumin was a product of Sigma Chemical Co., St. Louis. All reagents used were of the highest grade commercially available.

The 4th International Standard for Heparin (ISH; 195 units/mg) was obtained from the National Institute for Biological Standards and Control, London. The synthetic pentasaccharide (IC 83.1423; 800 anti-factor X_a units/mg) was a kind gift from Institut Choay, France.

Proteins- Human factor IX and human factor XI were purified as described earlier (10). All other bovine clotting factors were prepared and quantitated as previously described (11-13).

Phospholipid Vesicles- Phospholipid vesicles were made from a mixture of phosphatidylserine (PS) and phosphatidylcholine (PC) as previously described (13). Vesicles composed of 25 mole% PS and 75 mole % PC were used throughout the experiments.

Preparation of Human Normal Plasma- Blood from healthy donors was collected on 0.13 M trisodium citrate; nine parts blood to one part of citrate solution. The blood was then centrifuged twice at room temperature for 15 minutes at 3,000 x g. A third centrifugation was done at 4° C for one hour at 23,000 x g. The obtained platelet free plasma was stored at -80° C.

Determination of Free Ca^{2+} Concentration in plasma- For the determination of the amount of free Ca^{2+} ions in recalcified plasma, a reference curve was made using a calomel electrode from 0 - 100 mM Ca^{2+} in buffer containing 50 mM Tris-HCl (pH 7.9), 175 mM NaCl. The free calcium ion concentration thus measured in citrated plasma reconstituted with 20 mM Ca^{2+} was 4 mM.

Factor IX_a Determination- To follow the activation of factor IX by factor XI_a either in plasma or in a purified system, an assay was developed in which factor IX_a was determined via its ability to activate bovine factor X in the presence of CaCl_2 , phospholipid and activated factor VIII:C. Factor IX_a containing samples were diluted 1:1000 into buffer containing 50 mM Tris-HCl (pH 7.9), 175 mM NaCl, 0.5 mg ovalbumin/mL, 5 mM CaCl_2 , 20 μM phospholipid 10 U/mL factor VIII:C and 0.5 μM factor X. Factor X activation was started by the addition of 100 nM thrombin and the reaction was allowed to proceed for 90 seconds, after which 20 mM EDTA was added to stop the reaction. Subsequently, the chromogenic substrate S-2337 was added in order to determine the amount of factor X_a formed as described previously (5). From the observed rate of factor X_a formation, the amount of factor IX_a present in the sample was calculated using a calibration curve made with known amounts of human factor IX_a .

Factor IX_a Generation in Plasma- To 160 μL of citrated human plasma, 23 μL of a Tris-buffer (50 mmol/L Tris, 180 mmol/L NaCl, 0.5 mg ovalbumin/mL, pH 7.9) with or without heparin(fraction) was added and incubated for 4 min in a flat-bottom plastic tube while stirring. Phospholipids were also added to yield a final concentration of 20 μM . The plasma was activated by the addition of factor XI_a and calcium ions at the concentrations indicated. Timed samples (5 μL) were removed from incubations and assayed for factor IX_a activity.

Analysis- The analysis of the factor IX_a generation curves in the presence of proteinase inhibitors was based on the following assumptions: 1. Factor IX activation by factor XI_a followed Michaelis-Menten kinetics; 2. Inactivation of factor IX_a generated and factor XI_a can both be described as simple pseudo first order reactions.

If we assume that the actual concentration of factor IX at time t equals

$$[\text{IX}]_t = [\text{IX}]_0 - [\text{IX}_a] - [\text{IX}_a\text{-I}] \quad (1)$$

where $[IX]_0$ is the factor IX concentration at $t = 0$, $[IX_a]$ is the concentration of free factor IX_a at $t=t$ and $[IX_a.I]$ is the concentration of factor IX_a -inhibitor complex at $t=t$, then the factor IX_a generation curves can be described by the following set of differential equations:

$$d/dt [XI_a] = -k_{XIa}[XI_a] \quad (2)$$

$$d/dt [IX_a.I] = k_{IXa}[IX_a] \quad (3)$$

and

$$d/dt [IX_a] = ((k_{cat}/K_m) [XI_a] [IX]) - k_{IXa}[IX_a] \quad (4)$$

where $[IX] = [IX]_0 - [IX_a] - [IX_a.I]$, and k_{IXa} and k_{XIa} are the pseudo first order rate constants of inhibition of factor IX_a and factor XI_a , respectively.

The initial conditions are:

1. $[XI_a]$ at $t=0$ is the amount of factor XI_a added to the plasma;
2. $[IX_a] = 0$ at $t=0$;
3. $[IX_a.I] = 0$ at $t=0$.

The values for K_m/k_{cat} , k_{IXa} and k_{XIa} were estimated by a least squares fit of the solution $IX_a(t)$ of Equation 4 to the experimentally determined factor IX_a generation curves which uses a iterative Gauss-Newton algorithm (14).

The differential equations for $[IX_a]$,

$$\delta [IX_a]/\delta k_{IXa} \qquad \text{and} \qquad \delta [XI_a]/\delta k_{XIa}$$

were then solved numerically by the fourth order Runge-Kutta method (15) with stepsize $dt=1$ s. Accuracy estimated by halving the stepsize was better than 0.1%. The approximate 95% confidence intervals of the parameters were estimated by linearization of the non-linear equations at the obtained parameter values (16).

Results

Factor IX_a Bioassay- We developed an assay for activated factor IX based on the formation of a highly efficient factor X converting complex that consists of factor IX_a , phospholipids, Ca^{2+} and activated factor VIII:C. Under the assay conditions used, the rate of factor X activation is linear in time and factor IX is the only rate limiting factor. Thus, the amount of factor X_a generated, measured as amidolytic activity towards S-2337, is proportional with the amount of factor IX_a present in the assay. A reference curve, made with known amounts of purified, human factor IX_a is shown in Figure 1. Plasma could be highly diluted because of the high sensitivity of the assay, thus abolishing interference of other activated clotting factors. A 200-fold dilution of an activated plasma sample was sufficient to stop further factor IX_a generation and inhibition. Typical factor IX_a generation curves are shown in Figure 2. Purified human factor IX (70 nM) was added to congenital factor IX deficient plasma (less then 1% factor IX) after which the plasma was activated with purified human factor XI_a (2.5 nM) in the presence of 4 mM free calcium and 20 μ M phospholipid. Although phospholipids are not required for the factor XI_a catalyzed factor IX activation reaction (17), it was included to let the process of blood coagulation to proceed via factor X_a and thrombin generation into fibrin formation. The plasma clotting time was 2.5 minutes. As shown in Figure 2 virtually all factor IX added to the plasma could be recovered as activated factor IX. Interestingly, inhibition of activated factor IX was not observed. The same experiment but performed with purified factor XI_a and factor IX in a buffer system revealed the very same factor IX_a generation curve. As expected, no factor IX_a activity could be demonstrated in

Figure 1: Calibration of the factor IX_a assay

factor IX deficient plasma. To check that our factor IX_a assay could not be influenced by the generation of other activated factors, e.g. factor X_a or thrombin, factor IX deficient plasma was activated with thromboplastin. Although the plasma clotted at 45 seconds and peak values for factor X_a and thrombin were 10 nM and 360 nM respectively, no amidolytic activity could be detected with the actor IX_a bioassay (data not shown).

Figure 2: Factor IX_a a generation upon the addition of 70 nM factor IX_a and 2.5 nM factor XI_a to factor IX deficient plasma (▲), or buffer (○). (Δ): control, factor IX deficient plasma without factor IX reconstitution.

Figure 3: Factor IX_a generation curves in plasma by 2.5 nM factor XI_a at a final concentration of 70 nM factor IX (●), 250 nM factor IX (○), 360 nM factor IX (▲) or 650 nM factor IX (□).

Analysis of Factor IX_a Generation Curves in Plasma- In order to be able to evaluate the kinetics of factor IX_a and factor XI_a inhibition in plasma, we first had to determine the k_{cat}/K_m value for factor XI_a catalyzed factor IX activation in plasma. This ratio was determined from a set of factor IX_a generation curves as is depicted in Figure 3. Factor IX was added to normal plasma, in order to get a final concentration ranging from 70 nM up to 650 nM.

Factor IX activation was started by the addition of 2.5 nM factor XI_a, 4 mM free Ca²⁺ and 20 μM phospholipid to the plasma. The lines drawn are the result of the best fit of the mathematical model (described in Materials and Methods) to the data points. The by the fit procedure estimated k_{cat}/K_m ratio was 320 μM⁻¹min⁻¹. The estimated values for the inhibition of factor XI_a and factor IX_a was 0.35 ± 0.05 min⁻¹ and 0, respectively.

Effect of Heparin on Factor IX_a Generation and Inhibition in Plasma- The effects of the 4th International Standard for Heparin (ISH) and the synthetic pentasaccharide heparin on factor IX_a generation and inhibition were studied in normal plasma using factor XI_a (1.25 nM), phospholipid (20 μM) and Ca²⁺ (free concentration of 4 mM) to start coagulation.

Whereas fitting of factor IX_a activity data from activated plasma in the absence of heparin to our model appears to be excellent, it was not in the presence of heparin. Large deviation between the computed and the experimental gene-

Figure 4: Factor IX_a decay by antithrombin III/heparin in plasma with (●) or without (○) the addition of hirudin. Plasma was activated by factor XI_a as described, and after 4 minutes heparin (0.175 $\mu\text{g/mL}$) was added and the decay was determined.

ration curves were seen at the decay phase following the plasma clotting time. Obviously, our assumption that the decay of factor IX_a activity in the presence of heparin could be described as a simple pseudo first order reaction during the entire generation curve was not correct.

In an attempt to examine whether the heparin-dependent inhibition of *in situ* generated factor IX_a was different prior to and after fibrin formation the following experiments were performed. Hirudin (250 nM) was added to plasma to prolong the clotting time from 2.5 to over 6 minutes. In order to be able to measure factor IX_a decay, plasma clotting had to be prolonged, because at the clotting time (2.5 min.) factor IX activation was still going on. Standard heparin (0.035 U/mL) was added at 4 minutes to plasma containing hirudin or not. Although in both experiments the very same factor IX_a levels were obtained, with or without the addition of hirudin, the decay curves, as is shown in Figure 4 were quite different. Whereas heparin-dependent inhibition of *in situ* generated factor IX_a followed simple pseudo first order kinetics when the plasma was not clotted (in the case of hirudin addition), it seems that the heparin-dependent factor IX_a decay once the plasma clots behaves very different (Figure 4).

As yet, we have no explanation for this unexpected phenomenon. The very same observation was made in factor VIII:C deficient plasma prior to and after clotting was initiated by adding factor X_a (10 nM) to factor XI_a activated plasma. From this we concluded that protection of factor IX_a from inhibition by antithrombin III/heparin as a result of complex formation between factor IX_a, phospholipid and factor VIII:C_a could be ruled out.

As the factor IX_a inhibition in heparinized plasma did not follow first order kinetics because of clot formation, all experiments were performed in the presence of 0.5 μM hirudin. This amount of hirudin was sufficient to prolong the clotting time from 2.5 to more than 10 minutes, and was found not to influence the factor XI_a-catalyzed factor IX_a generation in plasma (Pieters and Lindhout, unpublished results).

Figure 5 shows factor IX_a generation curves in the presence of varying amounts of standard heparin. The drawn lines represent the enzyme generation curves as calculated by the fit-procedure. To reduce the number of variable parameters in our mathematical model, the $k_{\text{cat}}/K_{\text{m}}$ ratio was set at the fixed value of $320 \text{ min}^{-1} \mu\text{M}^{-1}$, as determined earlier (Figure 3). The by the fit-procedure calculated parameter values k_{IXa} and k_{XIa} are depicted in Table I.

Whereas the decay constants of inhibition of *in situ* generated factor IX_a increased linear with the heparin concentration ($6.16 \text{ min}^{-1}/(\mu\text{g/mL})$), the decay constant of inhibition of factor XI_a remained the same whether or not heparin was present (see Table I).

The effect of pentasaccharide on factor IX_a generation and inhibition were analyzed in the very same way. Figure 6 shows the factor IX_a generation curves in the presence of varying amounts of pentasaccharide. The drawn lines are the

Figure 5: Effect of 0.05 $\mu\text{g/mL}$ (Δ), 0.10 $\mu\text{g/mL}$ ($\blacktriangle$) and 0.175 $\mu\text{g/mL}$ ($\circ$) standard heparin on the generation of factor IX_a in plasma. ($\bullet$): control, no heparin.

best fit of the model to the data points. The obtained decay constants of inhibition of factor IX_a and factor XI_a are listed in Table I. Inhibition of factor XI_a is not affected by pentasaccharide. Factor IX_a inhibition, however, is stimulated by pentasaccharide, and the second order rate constant for *in situ* factor IX_a inhibition normalized for the pentasaccharide concentration was 0.70 min⁻¹ per μg/mL, thus about 9-fold slower than for standard heparin (on a weight basis; see Table I).

Effect of Calcium Ions on Heparin-Dependent Inhibition in Situ Generated Factor IX_a— We recently reported that calcium ions stimulate the inhibition of bovine factor IX_a by antithrombin III/heparin (5). To see whether calcium ions have an effect on the heparin-catalyzed inhibition of human factor IX_a by antithrombin III in plasma, we performed an experiment as was shown in Figure 5. Before heparin was added to the activated plasma, EDTA was added at a final concentration of 10 mM. At the heparin concentrations used, up to 0.175 μg/mL standard heparin and 5 μg/mL pentasaccharide no inhibition of factor IX_a could be detected. Thus, the drastically increased rate of inhibition of *in situ* generated factor IX_a caused by either standard heparin or pentasaccharide was completely abolished when free calcium ions were not present. However, when calcium was added to the plasma to retain a free calcium concentration of 4 mM, factor IX_a was inactivated at about the same rate constants as found by analysis of the factor IX_a generation curves in heparinized plasma. Because these independently

Figure 6: Effect of 5 μg/mL (○), 2.5 μg/mL (▲) and 1 μg/mL (△) pentasaccharide on the generation of factor IX_a in plasma. (●): control, no pentasaccharide.

Table I. Pseudo first order rate constants for the inhibition of factor XI_a and factor IX_a in plasma, containing heparin or pentasaccharide as indicated.

[Standard Heparin], $\mu\text{g/mL}$	$k_{\text{XIa}}, \text{min}^{-1}$	$k_{\text{IXa}}, \text{min}^{-1}$
0	0.35	0
0.05	0.39	0.30
0.10	0.30	0.64
0.175	0.30	1.21
[Pentasaccharide], $\mu\text{g/mL}$		
1.0	0.34	0.70
2.5	0.39	1.51
5.0	0.38	3.49

determined decay constants of factor IX_a are in good agreement with those obtained from the fit-procedure, we conclude that plasma factor IX_a generation curves can be acceptably described by the mathematical model.

Discussion

The purpose of this study was to determine the kinetics of factor XI_a-catalyzed factor IX activation in whole plasma and to investigate the effect of heparin on factor XI_a and factor IX_a during factor IX activation.

We preferred the use of a factor IX_a activity bioassay, rather than a radiometric assay because it was aimed to examine both the generation and inactivation reactions. Therefore we made use of an assay that measures sensitively and specifically functional factor IX_a via its ability to activate factor X in the presence of phospholipid, calcium and activated factor VIII:C.

Our approach to analyze the factor IX_a generation curves in plasma involved simple Michaelis-Menten kinetics to describe the activation phase of the reaction system and simple first order decay of both factor XI_a and factor IX_a. From Figure 3 where factor IX_a generation curves at different factor IX concentrations are depicted, it is clear that fitting our mathematical model to the obtained factor IX_a generation curves is a valid approach for the determination of the catalytic efficiency, k_{cat}/K_m , which was calculated to be $320 \text{ min}^{-1} \mu\text{M}^{-1}$, and the pseudo first order rate constant of inhibition of factor XI_a ($0.35 \pm 0.05 \text{ min}^{-1}$).

Due to the plasma concentration of factor IX, we were not able to determine an independent value for k_{cat} and K_m . However, the k_{cat}/K_m ratio of $320 \text{ min}^{-1} \mu\text{M}^{-1}$ is in good agreement with the values reported by Walsh et al. (19) and from our laboratory (18) for factor XI_a-catalyzed factor IX activation in purified systems (471 and $227 \text{ min}^{-1} \mu\text{M}^{-1}$, respectively).

We tried to determine independent values for k_{cat} and K_m , but even at a factor IX concentration as high as 650 nM , the initial rate of factor IX activation by factor XI_a linearly increased with the factor IX concentration. Therefore, as the K_m for factor IX activation by factor XI_a in plasma appeared to be in the μmolar range, the k_{cat}/K_m ratio was used instead of the separate kinetic parameters in order to analyze the factor IX_a generation curves in the presence of heparin or pentasaccharide.

The value of $0.35 \pm 0.05 \text{ min}^{-1}$ for factor XI_a inhibition in plasma is in agreement with the value determined by Scott et al (20) for factor XI_a inhibition by α_1 -antitrypsin in plasma. However, Meijers et al (21) showed that in plasma factor XI_a is mainly inactivated by the concerted action of α_1 -antitrypsin (24%), α_2 -antiplasmin (25%) and C1-inhibitor (31%).

The determination of the effect of heparin on factor IX_a generation in normal plasma was not possible because clotting of the plasma abolished the heparin-stimulated inactivation of factor IX_a. We established that this effect was not due to protection of factor IX_a by activated factor VIII:C from inhibition by antithrombin III. This neutralization of heparin activity is related to clot formation, but not to thrombin formation. Whether the generated fibrinogen-degradation products cause the altered inhibition kinetics of factor IX_a by antithrombin III, remains to be established.

In order to prolong the clotting time to over 10 minutes, hirudin was added at a concentration of 500 nM to the plasma. Standard heparin was found to stimulate the inhibition of factor IX_a with a rate constant normalized for the heparin

concentration of $6.16 \text{ min}^{-1}/\mu\text{g heparin/mL}$ (Table I). The fact that heparin greatly stimulates factor IX_a inhibition in plasma is in agreement with previous studies (4).

The effect of pentasaccharide on factor IX_a generation was quite unexpected. Table I shows that pentasaccharide is capable of stimulating the inhibition of factor IX_a with $0.70 \text{ min}^{-1}/\mu\text{g pentasaccharide/mL}$. Although little is known about the interaction of factor IX_a-heparin-antithrombin III, it is generally assumed that the mechanism of factor IX_a inhibition resembles that of thrombin inhibition. That is, the inhibition of factor IX_a by heparin/antithrombin III proceeds via the binding of heparin to factor IX_a. However, pentasaccharide (22) represents the antithrombin III binding sequence of heparin, and thus probably exerts its anti-protease effect not via the binding to the enzyme, but solely via binding to antithrombin III. And indeed, a previous study of the effect of pentasaccharide on the inactivation of (bovine) factor IX_a in a pure system showed that pentasaccharide, added in high concentrations had no effect at all.

The inhibition of factor IX_a by antithrombin III/heparin in plasma only occurred in the presence of free calcium ions (4 mM). The addition of EDTA completely abolished the heparin-dependent inhibition of factor IX_a. This is in agreement with the results obtained from studies with bovine factor IX_a in a purified system (5), but in contrast to the effect of calcium ions in the heparin-stimulated inhibition of e.g. thrombin, for which it was found that Ca^{2+} inhibits the heparin-dependent antithrombin III inhibition (8).

Acknowledgement. The authors wish to thank Drs. Wagevoord and Hendrix for help with the protein purification.

References

1. Nemerson Y: Tissue factor and hemostasis. *Blood* 71:1, 1989
2. Thompson AR: Structure, function and molecular defects of factor IX. *Blood* 67:565, 1986
3. McNeely T, Griffith MJ: The anticoagulant mechanism of action of heparin in contact-activated plasma: inhibition of factor X activation. *Blood* 65:1226, 1985a
4. Fuchs HE, Trapp HG, Griffith MJ, Roberts HR, Pizzo SV: Regulation of factor IX_a in vitro in human and in mouse plasma and in vivo in the mouse. Role of Endothelium and the plasma proteinase inhibitors. *J Clin Invest* 73:1696, 1984
5. Pieters J, Willems G, Hemker HC, Lindhout T: Inhibition of factor IX_a and factor X_a by antithrombin III/heparin during factor X activation. *J Biol Chem*, 263(30):15313, 1988

6. Rosenberg JS, McKenna PW, Rosenberg RD: Inhibition of human factor IX_a by human antithrombin III. *J Biol Chem* 250:8883, 1975
7. Holmer E, Kurachi K, Söderström G: The molecular-weight dependence of the rate-enhancing effect of heparin on the inhibition of thrombin, factor X_a, factor IX_a, factor XI_a, factor XII_a, and kallikrein by antithrombin III. *Biochem J* 193:395, 1981
8. Speight MO, Griffith MJ: Calcium inhibits the heparin-catalyzed antithrombin III/thrombin reaction by decreasing the apparent binding affinity of heparin for thrombin. *Arch Biochem Biophys* 225:958, 1983
9. Morrison SA, Jesty J: Tissue factor-dependent activation of tritium-labeled factor IX and factor X in plasma. *Blood* 63:1338, 1984
10. Lindhout T, Baruch T, Schoen P, Franssen J, Hemker HC: Thrombin generation and inactivation in the presence of antithrombin and heparin. *Biochemistry* 25:5962, 1986
11. Van Dieijen G, Tans G, Rosing J, Hemker HC: The role of phospholipid and factor VIII_a in the activation of bovine factor X. *J Biol Chem* 256:3433, 1981
12. Rosing J, van Rijn JLML, van Dieijen G, Comfurius P, Zwaal RFA: The role of activated platelets in prothrombin and factor X activation. *Blood* 65:319, 1985
13. Lindhout T, Govers-Riemslog JWP, van de Waart P, Hemker HC, Rosing J: Factor Va-factor X_a interaction. Effects of phospholipid vesicles of varying composition. *Biochemistry* 21:5494, 1982
14. Gill PE, Murray W: *SIAM J Num Anal* 15:977, 1978
15. Press WH, Flannery BP, Teukolski SA, Vetterling WT in: *Numerical Recipes*, Cambridge University Press, pp 547, 1986
16. Beck JV, Arnold KJ: *Parameter estimation in engineering and science*, John Wiley & Sons, New York, pp 334, 1977
17. Van der Graaf F, Greengard JS, Bouma BN, Kerbirou DM, Griffin JH, *J Biol Chem* 258:9669, 1983
18. Soons H: Studies on the inhibition of blood coagulation factor XI_a. Thesis at the University of Limburg, Maastricht, the Netherlands, 1987
19. Walsh PN, Bradford H, Sinha D, Piperno JR, Tuszyński GP: Kinetics of the factor XI_a catalyzed activation of human blood coagulation factor IX. *J Clin Invest* 73:1392, 1984
20. Scott CF, Schapira M, James HL, Cohen AB, Colman RW: Inactivation of factor XI_a by plasma protease inhibitors: Predominant role of α_1 -protease inhibitor and protective effect of high molecular weight kininogen. *J Clin Invest* 68:844, 1982
21. Meijers JCM: Regulation of the blood coagulation mechanism by plasma proteinase inhibitors. Thesis at the University of Utrecht, the Netherlands
22. Choay J, Petitou M, Lormeau JC, Sinay P, Casu P, Gatti G: Structure activity relationships in heparin: a synthetic pentasaccharide with high affinity for antithrombin III and eliciting high anti-factor X_a activity. *Biochem Biophys Res Comm* 116:492, 1983

Chapter VI

In Situ Generated Thrombin is the Only Enzyme that Effectively Activates Factor VIII:C and Factor V in Plasma^o

Abstract

We investigated the activation of the non-enzymatic protein cofactors factor VIII:C and factor V in plasma when coagulation was initiated by thromboplastin. Using sensitive bio-assays we were able to measure specifically the generation of factor VIII:C_a and factor V_a in plasma.

Our results showed that when plasma was triggered with a relative high concentration of thromboplastin, factor VIII:C and factor V were completely activated at the clotting time of plasma. However, when the generation of thrombin, but not that of factor X_a was delayed by adding hirudin to the plasma, factor V_a was only generated at the time thrombin generation overcame the hirudin inhibition. Also the generation of factor VIII:C_a correlated with the thrombin generation, and not with factor X_a generation. Furthermore, addition of high amounts of factor X_a to hirudinized plasma did not show any factor VIII:C or factor V activation.

^o Based on: Pieters J, Hemker HC, Lindhout T, submitted

We concluded that in plasma, activated with thromboplastin, the enzyme responsible for the activation of factor V and factor VIII:C is thrombin, and not factor X_a .

Introduction

Clotting of plasma is the result of a series of enzymatic reactions acting in a staged cascade system (1). Starting either from the intrinsic or extrinsic coagulation pathway, the coagulation system comprises a number of feed-back activation reactions. For example, the generation of thrombin via the intrinsic pathway proceeds only at a considerable rate after the non enzymatic protein cofactors factor V and factor VIII:C have been activated (2,3). Activated factor VIII:C serves as an essential cofactor for the activation of factor X by factor IX_a , while factor V_a plays an important role in the factor X_a -catalyzed conversion of prothrombin into thrombin.

Conversion of factor VIII:C into its activated form is thought to proceed principally through the proteolytic activity of thrombin (4), although factor X_a is also considered to be a possible important activator of factor VIII:C (4,5). The activation of factor VIII:C by factor IX_a has been demonstrated in a purified system. However, the factor IX_a concentrations used in this study exceeded the plasma factor IX concentration, making it unlikely that factor IX_a could be physiologically important for this activation (6).

Activators of factor V include thrombin and factor X_a (7,8). However, the thrombin-catalyzed activation of factor V appeared to be a 100-fold faster than factor X_a on a molar base (8). Proteases present in platelets and endothelial cells have also been shown to activate factor V, although it is not clear if these activation reactions occur under physiologic conditions (9,10, see reference 11 for a review on factor V and factor VIII:C).

The relative importance of the above mentioned activators for the *in vivo* normal haemostasis is not clear. Although a number of enzymes have been described to activate factor VIII:C and factor V in purified systems, the relative catalytic efficiencies of these enzymes in plasma are unknown.

Therefore, we investigated the activation of factor V and factor VIII:C under more physiological conditions, namely in whole, platelet free, plasma activated with thromboplastin. With the use of very specific bio-assays we were able to follow the generation of factor V_a and factor VIII:C_a during coagulation in plasma. With the use of hirudin we separated the thrombin generation from the factor X_a generation, and clearly demonstrated that in this system, thrombin is the only enzyme responsible for the activation of both factor V and factor VIII:C.

Materials and methods

Materials- The synthetic peptide substrates Bz-Ile-Glu-(Piperidyl)-Gly-Arg-p-nitro-anilide hydrochloride (S-2337) and H-D-Phenylalanyl-L-pipecolyl-L-arginine-p-nitro-anilide dihydrochloride (S-2238) were purchased from AB Kabi Diagnostica, Stockholm. Ovalbumin was a product of Sigma Chemical Co., St. Louis. All reagents used were of the highest grade commercially available.

Human brain thromboplastin was prepared by the method of Owren and Aas (12). It was homogenated in a Potter Elvehjem homogenizer for 3 minutes, centrifugated at $2,000 \times g$ for 15 minutes and stored at -80°C . Its concentration was adjusted with Tris-saline to give a prothrombin time of 13 s.

Proteins- Recombinant desulphato-hirudin variant 1 (CGP 39393) was kindly provided by Dr. Wallis, Ciba-Geigy, Horsham, United Kingdom. All bovine clotting factors were prepared and quantitated as previously described (2,13,14).

Phospholipid- Phospholipid vesicles were made from a mixture of phosphatidylserine (PS) and phosphatidylcholine (PC) as previously described (14). Vesicles composed of 25 mole% PS and 75 mole% PC were used throughout the experiments.

Preparation of Human Normal Plasma- Blood from healthy donors was collected on 0.13 mol/L trisodium citrate; nine parts blood to one part of citrate solution. The blood was then centrifugated twice at room temperature for 15 minutes at $3,000 \times g$. A third centrifugation was done at 4°C for one hour at $23,000 \times g$. The obtained platelet free plasma was stored at -80°C .

Thromboplastin-Induced Factor X_a , Factor V_a , Factor VIII:C_a and Thrombin Generation in Platelet Free Plasma- To 0.13 mL of citrated human plasma, 9 μL of a Tris-buffer (50 mM Tris, 180 mM NaCl, 0.5 mg ovalbumin/mL, pH 7.9) with or without hirudin was added and incubated for 4 min in a flat-bottom plastic tube while stirring. Clotting was initiated by the addition of 11 μL of the Tris-buffer containing 0.27 mol/L CaCl_2 and human brain thromboplastin. Timed samples (10 μL) were removed from incubations and assayed for either factor V_a , factor X_a or thrombin activity.

Factor V_a Assay- Factor V_a was assayed in plasma samples, diluted 1:200 in Tris-buffer (pH 7.9) containing 5 mM CaCl_2 and 0.5 μg unfractionated heparin/mL. Ten μL of the diluted sample was added to a cuvette containing 13 pM factor X_a , 5 mM CaCl_2 and 50 μM phospholipid in Tris-buffer. After 4 min thrombin generation was initiated by the addition of prothrombin (0.2 μM final

concentration in 125 μL). The reaction was stopped after 2 min by the addition of 325 μL Tris-buffer containing 20 mM EDTA. The amount of thrombin was measured in a dual-wavelength spectrophotometer using S-2238 as described previously (15).

Factor VIII:C_a assay- Factor VIII:C_a was assayed in plasma samples diluted 1:40 directly into the assay-mixture containing 100 nM bovine factor IX_a, 20 μM phospholipids and 5 mM CaCl₂ in Tris-buffer (pH 7.9) containing 0.5 mg/mL ovalbumin. The reaction was started after 15 seconds upon the addition of bovine factor X (final concentration 0.5 μM). At 30 and 90 seconds 50 μL was taken and assayed for factor X_a activity using the chromogenic substrate S-2337. From these two time points the rate of factor X activation was determined and was linear with the added amounts of factor VIII:C_a.

Factor X_a Assay- Factor X_a was assayed in plasma samples diluted 1:200 in Tris-buffer (pH 7.9). Ten μL of the diluted sample was added to a cuvette containing 0.6 nM factor V_a, 50 μM phospholipid and 5 mM CaCl₂ in Tris-buffer. After 4 min prothrombin (1.5 μM final concentration in 50 μL) was added and the assay was proceeded as described for factor V_a.

Thrombin Assay- Undiluted plasma samples (5 μL) were added to a cuvette containing 495 μL Tris-buffer, 20 mM EDTA and 0.25 mM S-2238. The thrombin activity was calculated from the change in absorbance as described previously (15).

Results

Factor VIII:C Bioassay- In order to be able to determine activated factor VIII:C in plasma, a highly sensitive and specific bio-assay was developed making use of the activity of factor VIII:C_a as a cofactor in the activation of factor X by factor IX_a in the presence of phospholipids and calcium. By judicious choice of the concentrations of the reaction components, we were able to measure factor VIII:C_a without interference of non-activated factor VIII:C. Figure 1 shows the calibration curve constructed from a series of plasma dilutions which were activated with thrombin (50 nM) (Figure 1, closed circles). Assuming that the plasma concentration of factor VIII:C is about 1 nM (11,16), Figure 1 shows that this assay is linear up to 30 pM activated factor VIII:C and concentrations as low as 5 pM can be determined. The intersection at the ordinate represents the activity of

Figure 1: Calibration curve for factor VIII:C_a (●). Human normal plasma was diluted to obtain the concentrations factor VIII:C indicated, assuming that the concentration of plasma factor VIII:C is 1 nM. The (diluted) plasma samples were activated with 50 nM thrombin, and the activity was determined as described under Materials and Methods. The activity of plasma that was not activated with thrombin was also determined (▲).

the assay without factor VIII:C_a). The closed triangles in Figure 1 represent the activity of plasma which was not activated with thrombin. Control experiments in factor VIII:C deficient plasma showed the blanc activity on the assay.

Effect of Hirudin on Thrombin and Factor X_a Generation- In order to investigate the relative importance of thrombin and factor X_a to the activation of factor V and factor VIII:C, we first had to separate the thrombin generation and factor X_a generation, as these two are the possible activators of the cofactors V and VIII:C in our plasma system. To this end, we incubated plasma in the presence of hirudin and activated it using thromboplastin at a final concentration of 1/30 (vol/vol). Thrombin generation and factor X_a generation were determined as described under Materials and Methods. Figure 2 shows the effect of the addition of different amounts of hirudin on thrombin and factor X_a generation. The thrombin generation curve without hirudin is characterized by a very short lag period followed by explosive thrombin formation. The fast inactivation of the formed thrombin, which occurs after the peak at 1 minute, is mainly due to the

Figure 2: Effect of hirudin on thrombin generation (Panel A) and factor X_a generation (Panel B) in thromboplastin-activated plasma. The concentrations hirudin used were 0 (●), 0.5 (▲) and 0.75 (△) μ M.

action of antithrombin III and 2-macroglobulin. From Figure 2A it is clear that the more hirudin is present, the more the thrombin generation is delayed, and also the maximal amount of thrombin formed is decreased. The factor X_a generation, however, is not at all influenced by hirudin.

Effect of Hirudin on Factor V_a and Factor VIII: C_a Generation- Figure 3 shows factor V_a generation curves as they were obtained when plasma was activated with thromboplastin in the presence of varying amounts of hirudin. Apparently, factor V_a generation is closely correlated with thrombin generation but not at all with factor X_a generation. As in the case of thrombin generation, increasing

Figure 3: Effect of hirudin on factor V_a generation in thromboplastin-activated plasma. The concentrations hirudin used were 0 (●), 0.5 (▲) and 0.75 (△) μ M.

Figure 4: Effect of hirudin on factor $VIII:C_a$ generation in thromboplastin-activated plasma. The concentrations hirudin used were 0 (●), 0.5 (▲) and 0.75 (△) μ M.

amounts of hirudin caused a prolongation of the lag-phase of factor V_a generation. It should be noted that despite a drastic decrease of non-complexed thrombin with increasing hirudin, maximal factor V_a activities could be generated. Like the factor V_a generation curves, the factor VIII:C_a generation curves (Figure 4) showed a very rapid activation phase followed by a decay of factor VIII:C_a activity. Again, factor VIII:C_a generation is closely correlated with thrombin generation but not with factor X_a generation.

Exogenous Factor X_a - In an attempt to see whether or not factor V and factor VIII:C could be activated by factor X_a in plasma at a concentration approaching the amount of factor X present in plasma, 100 nM of purified human factor X_a was added to plasma incubated for 8 minutes with thromboplastin (1/30 vol/vol), Ca^{2+} (20 mM) and hirudin (2 μ M). Prior to the addition of factor X_a , no factor V_a , nor factor VIII:C_a or thrombin activities could be detected. Factor X_a level was about 2 nM. After the addition of factor X_a , samples were taken and assayed for factor V_a and factor VIII:C_a. Up to 5 minutes following the addition of factor X_a , no factor V_a nor factor VIII:C_a activity could be detected, neither thrombin activity.

Discussion

A number of investigators have explained their findings on the effects of the anticoagulant drug heparin on the intrinsic pathway of blood coagulation by assuming that trace amounts of factor X_a are able to activate factor VIII:C and factor V (17). Although thrombin might be the most efficient activator of both cofactors (11), thrombin is also the end product of the coagulation process. Therefore, a (partial) activation of factor V or factor VIII:C by factor X_a earlier in coagulation could amplify thrombin generation by inducing small amounts of thrombin which than in turn by a positive feedback mechanism activate factor VIII:C and factor V. However, that indeed factor VIII:C and factor V activation by factor X_a is essential to attain "physiological" rates of factor X_a and thrombin generation in plasma has, to our knowledge, never been demonstrated.

In this study we determined the relative contribution of factor X_a and thrombin to the in situ activation of the non-enzymatic protein cofactors factor VIII:C and factor V in plasma. The plasma was triggered with a high concentration of thromboplastin, thus limiting the activation reactions to the extrinsic pathway (18). Preliminary studies on the intrinsic pathway of blood coagulation revealed that factor X_a generation was strongly controlled by specific thrombin inhibitors

like hirudin. As in such a system thrombin and factor X_a generation cannot be separated, it is extremely difficult to study the relative contribution of thrombin and factor X_a generation to the activation of factor V and factor VIII:C (Pieters J, Lindhout T, unpublished results). Therefore, thromboplastin-activated plasma was used rather than contact-activated plasma.

We developed a very specific assay to monitor the generation of factor VIII:C_a in plasma, making use of the fact that (human) factor VIII:C_a is able to catalyze (bovine) factor X activation by (bovine) factor IX_a in the presence of phospholipids and calcium. A sensitive assay for the determination of factor V_a generation in plasma was developed during earlier studies (18,19).

The generation of factor VIII:C_a in thromboplastin-activated plasma follows exactly the generation of thrombin, and shows a maximum around 1 minute. However, at that time point also factor X_a is generated, so that it is not clear what the relative contribution of thrombin and factor X_a is in this system to the activation of factor VIII:C. In order to obtain a thrombin generation, independent from factor X_a generation, we added hirudin to the plasma in several concentrations. The thrombin generation curve obtained was indeed dependent on the hirudin concentration used, while the factor X_a generation curve was not at all influenced by hirudin. When we determined the generation of factor VIII:C_a in this hirudinized plasma, it appeared that this generation paralleled the generation of thrombin but not that of factor X_a . Moreover, addition of a high amount of factor X_a to thromboplastin-activated plasma containing 2 μ M hirudin, did not cause any activation of factor VIII:C.

The activation of factor V was investigated in a similar way; thrombin generation and factor X_a were separated by means of hirudin addition, and factor V_a generation was determined. Also in this case, generation of factor V_a entirely parallels thrombin generation, and addition of a high amount of factor X_a did not induce factor V_a generation.

The results of this study using plasma as a physiological environment for coagulation, and thromboplastin as a clotting trigger, show that under these conditions thrombin is the only activator of the cofactors factor VIII:C and factor V. The mechanism of plasma clotting evolving from our results is one in which via the TF:factor VII_a complex factor X is activated. This initially present factor X_a is able to convert at a low rate prothrombin into thrombin, which then is able to activate factor VIII:C and factor V. Only then sufficient amounts of thrombin are generated to ensure proper clotting.

The conclusions derived from this study once again emphasizes the central role of thrombin in the overall process of blood coagulation.

References

1. Jackson CM, Nemerson Y: Blood coagulation. *Ann Rev Biochem* 49:765, 1980
2. Rosing J, Tans G, Govers-Riemslog JWP, Zwaal RFA, Hemker HC: The role of phospholipids and factor V_a in the prothrombinase complex. *J Biol Chem* 255:274, 1980
3. van Dieijen G, Tans G, Rosing J, Hemker HC: The role of phospholipid and factor VIIIa in the activation of bovine factor X. *J Biol Chem* 256:3433, 1981
4. Vehar GA, Davie EW: Preparation and properties of bovine factor VIII (antihemophilic factor). *Biochemistry*, 19:401, 1980
5. Lollar P, Knutson GJ, Fass DN: Activation of porcine factor VIII:C by thrombin and factor X_a. *Biochemistry* 24:8056, 1985
6. Rick M: Activation of factor VIII by factor IX_a. *Blood* 60:744, 1982
7. Suzuki K, Dahlbäck B, Stenflo J: Thrombin-catalyzed activation of human blood coagulation factor V. *J Biol Chem* 257: 6556, 1982
8. Foster WB, Nesheim ME, Mann KG: The factor X_a-catalyzed activation of factor V. *J Biol Chem* 258:13970, 1983
9. Kane WH, Mruk JS, Majerus PW: Activation of coagulation factor V by a platelet protease. *J Clin Invest* 70:1092, 1982
10. Rodgers GM, Kane WH: Activation of endogenous factor V by a homocysteine-induced vascular endothelial cell activator. *J Clin Invest* 77:1909, 1986
11. Kane WH, Davie EW: Blood coagulation factors V and VIII: structural and functional similarities and their relationship to hemorrhagic and thrombotic disorders. *Blood* 71:539, 1988
12. Owren PA, Aas K: The control of dicoumarol therapy and the quantitative determination of prothrombin and proconvertin. *Scand J Clin Lab Invest* 3:201, 1951
13. Rosing J, van Rijn JLML, van Dieijen G, Comfurius P, Zwaal RFA: The role of activated human platelets in prothrombin and factor X activation. *Blood* 65:319, 1980
14. Lindhout T, Govers-Riemslog JWP, van de Waart P, Hemker HC, Rosing J: Factor V_a-factor X_a interaction. Effects of phospholipid vesicles of varying composition. *Biochemistry* 21:5494, 1982
15. Lindhout T, Baruch D, Schoen P, Franssen J, Hemker HC: Thrombin generation and inactivation in the presence of antithrombin III and heparin. *Biochemistry* 25:5962, 1986
16. Hoyer LW: The factor VIII complex. Structure and function. *Blood* 58:1, 1981
17. McNeely TB, Griffith MJ: The anticoagulant mechanism of action of contact-activated plasma: Inhibition of factor X activation. *Blood* 65:1226, 1985
18. Pieters J, Lindhout T: The relative importance of factor X_a inhibition to the anticoagulant effect of heparin in thromboplastin-activated plasma. *Blood*, in press
19. Lindhout T, Pieters J, Béguin S, Hemker HC: The mode of action of CY 216 in plasma, in Breddin K, Fareed J, Samama M (eds): *Proceedings of the First International Symposium on Fraxiparine*, Schattauer Verlag, Stuttgart-New York, 1988, in press

Chapter VII

General Discussion

Introduction

Over the past decades, a number of proteins were discovered, all forming part of the overall scheme of the blood coagulation system. By means of several purification techniques these factors could be purified to homogeneity, and once they were available in a purified form, they could be intensively studied. In that way we gained a great deal of knowledge about structure, function and interactions with other proteins of these coagulation factors (for recent reviews on structures and functions of blood coagulation factors see references 1-3).

Amino acid sequences led to more information about protein structures, and based on these sequences, oligonucleotide probes could be constructed to isolate cDNA clones, coding for these factors (4). Expression of especially those proteins with limited availability (e.g. factor VIII:C) in mammalian cells provided them in large quantities (5,6). Furthermore, the ability to manipulate DNA structures *in vitro*, thereby creating mutant promotor elements, provides a powerful strategy to define the functionally important regions (7).

However, it became more and more apparent that the interactions between clotting factors, inhibitors and other plasma constituents should be studied under more complex circumstances than only in isolated form. Only then we will get more information about what reactions are really important in the process of coagulation. Also with regard to anticoagulation, the target reactions for the inhibition of clotting should be better determined in plasma. Antithrombotic therapy can then be directed against the relevant reactions in plasma.

Therefore we initiated the studies described in this thesis, starting with two studies with purified proteins (Chapters II and III), but approaching the physiological situation by reconstituting several proteins in a buffer system. Then we turned to whole plasma in which we were able to measure specifically generation of activated coagulation factors (Chapter IV - VI).

Our goal was to come to a better insight into the interactions of activated blood coagulation factors with antithrombin III and heparin in more complex systems and in plasma.

Antithrombin III is a very important inhibitor of activated clotting factors in plasma, and heparin greatly stimulates this inhibition. Two main topics are treated in the several chapters of this thesis. The first topic deals with the inhibition of activated coagulation factors by antithrombin III/heparin, and the effect of other (plasma) constituents (substrates, cofactors, phospholipids, etc.) on this inhibition. The second topic concerns the determination of the crucial stages in plasma leading to the formation of a fibrin-clot, with the idea in mind that, if those crucial reactions can be controlled by regulatory mechanisms, like the heparin stimulated inhibition by antithrombin III, we might have found a firmer basis for a better antithrombotic therapy. The remaining part of this chapter will discuss the obtained results with respect to the above mentioned topics.

Effect of Heparin/Antithrombin III in Complex Reaction Systems

Prothrombin activating complex

In chapter II we describe the effect of prothrombin activation products on the heparin catalyzed factor X_a inhibition by antithrombin III.

It was known that the antithrombin III dependent inactivation of meizothrombin, a possible major activation product after factor X_a catalyzed prothrombin activation, was not stimulated by heparin (9), whereas the inactivation of thrombin is. We showed that (active site blocked) meizothrombin indeed is not able to bind heparin, explaining the absence of a stimulatory effect of heparin on the antithrombin III/meizothrombin reaction (8). Fragment 1.2 and thrombin, however, very clearly showed heparin binding properties. Also after factor X_a catalyzed activation of (active site blocked) meizothrombin to give (active site blocked) thrombin and fragment 1.2 these components were not able to neutralize heparin. Only after separation they regained their heparin binding properties.

These results show very clearly that the presence of additional constituents can have important consequences; generation of fragment 1.2 at the sites of prothrombin activation may play an important role in the regulation of blood coagulation by competing with AT III for the available heparin.

Factor X Activating Complex

Next we started to investigate what the effect of antithrombin III/heparin on the factor IX_a catalyzed factor X activation was in the presence of calcium and phospholipids (Chapter III).

The inactivation of factor X_a and factor IX_a by antithrombin III/heparin was already extensively studied (10-12). However, these studies dealt with the inhibition of factor IX_a and factor X_a in isolated forms. We were able to analyze simultaneously the kinetics of factor X_a and factor IX_a inactivation during factor X activation by factor IX_a. Generation and inactivation curves were fitted to a 3-parameter 2-exponential model to determine the pseudo first order rate constants of inhibition of factor IX_a and factor X_a by antithrombin III/heparin. We showed that the inhibition of factor X_a by antithrombin III, formed through activation by factor IX_a was 2.5-fold reduced when compared with factor X_a inhibition by antithrombin III in free solution. Phospholipid alone did not show any effect of the inhibition of factor X_a, and this is in contrast to earlier findings (13,14). Further investigations on this phenomenon showed that the protective effect of this so-called "tenase-complex" was due to the presence of phospholipid-bound factor X. From the observed effects on factor X_a inhibition antithrombin III, two possible explanations evolved from these observations, first, β -factor X formation at the phospholipid surface (15) could explain the observed inhibition in terms of a competitive enzyme substrate interaction. A second explanation could not be ruled out, i.e. a reduced activity of factor X_a in the ternary factor X-phospholipid-factor X_a complex towards antithrombin III, without involvement of the factor X_a active site. The inactivation of factor IX_a, however was not at all affected by the presence of additional components.

To investigate the effect of heparin on the inhibition of (generated) factor X_a and factor IX_a, we used two different heparin preparations. First, we used an unfractionated heparin preparation with both high anti-factor X_a and antithrombin activity. As a second heparin preparation we made use of the synthetic pentasaccharide (16), which represents the antithrombin III binding sequence (see Chapter I) in order to explore the reported effects of the non-antithrombin III- binding region of heparin molecules on factor X_a and factor IX_a (17,18). When we compared the effect of pentasaccharide with that of unfractionated heparin, it was clear that an additional sequence to the antithrombin III binding one leads to different inactivation kinetics of factor X_a by antithrombin III, probably because unfractionated heparin and antithrombin III show a greater affinity for factor X_a than the pentasaccharide-antithrombin III complex.

Unexpectedly, the heparin catalyzed inhibition of factor IX_a by antithrombin III was stimulated about 6-fold during factor X activation as compared to factor IX_a inhibition in free solution. It appeared that this effect was due to the presence of calcium, probably by inducing structural changes in factor IX_a (19,20).

Effect of Heparin/Antithrombin III in Plasma

From the two first studies described in this thesis (Chapters II and III), it followed that activated coagulation factors behave quite different in more complex systems than was predicted from studies performed in isolated situations, with respect to their inhibition by antithrombin III/heparin. Therefore we decided to continue our research in whole plasma systems.

Importance of Factor X_a Inhibition in Plasma

As already mentioned in Chapter I, the importance of the anti-factor X_a effect of heparin to the anticoagulant effect of heparin was not at all clear (21,22). It was found in animal models that heparins with a high anti-factor X_a over antithrombin ratio were a better antithrombotic than unfractionated heparin, while producing less bleeding tendency (23). Although the so-called low molecular weight heparins clearly show the advantage of a better pharmacokinetic compared with classical heparin, the fact that they give rise to less bleeding problems, while being an as good antithrombotic as unfractionated heparin remained unclear (23, 24). Therefore, as described in Chapter IV, we investigated the contribution of factor X_a inhibition to the overall anticoagulant potency of heparin in plasma. And our results showed, that not factor X_a inhibition, but inhibition of the (thrombin-induced) factor V_a generation is of prime importance to inhibit coagulation in thromboplastin-activated plasma. Thus it is our conclusion that the inhibition of thrombin, and not of factor X_a is crucial to anticoagulate (thromboplastin-triggered) plasma.

Inhibition of Factor IX_a Generation in Plasma

The role of factor XI_a in the process of blood coagulation is as yet unclear. Factor XI_a is the product of contact activation, and, under physiological circumstances, it is hard to believe that this contact activation plays an important role (see Chapter I). However, a certain percentage people deficient in factor XI clearly show bleeding problems (in contrast to those deficient in a contact factor).

Because factor XI_a is a possible important activator of factor IX in plasma (25), we decided to study the kinetics of inactivation (by antithrombin III/heparin) of the factor XI_a -catalyzed factor IX activation in plasma.

For the analysis of the factor IX_a generation curves in the presence of heparin or pentasaccharide in plasma, we used a 3-parameter model, based on the Michaelis-Menten equation and first order kinetics of inactivation to determine k_{cat}/K_m , and inactivation constants for factor IX_a and factor XI_a .

The inactivation of factor XI_a appeared to be fast in plasma, with a pseudo first order rate constant of 0.35 min^{-1} . This inhibition constant was not dependent on the addition of heparin or pentasaccharide, and therefore the inactivation of factor XI_a is probably not the result of the action of antithrombin III. This inhibition can be explained by the data of Scott et al (26), who found a pseudo first order rate constant for factor XI_a of 0.59 min^{-1} by α_1 -antitrypsin. However, the data of Meijers et al (27) indicate that the inhibition of factor XI_a in plasma is mainly accomplished by the combined action of α_1 -antitrypsin, α_2 -antiplasmin and C1-inhibitor.

Inhibition of the generated factor IX_a was not observed in normal plasma. However, the addition of heparin caused a dramatic effect on the factor IX_a inactivation curve ($6.16 \text{ min}^{-1}(\mu\text{g/mL})^{-1}$). Moreover, even pentasaccharide, which does not show an effect on factor IX_a in a purified system, did have a very clear effect on factor IX_a inactivation, and caused an inactivation of $0.70 \text{ min}^{-1}(\mu\text{g/mL})^{-1}$.

The Central Role of Thrombin in Blood Coagulation

The general conclusion from Chapter IV, namely the importance of thrombin in the activation of factor V to ensure proper coagulation, already indicates that thrombin plays a key role in the formation of a fibrin-clot. The central role of thrombin in the clotting scheme is also emphasized in Chapter I. From the study described in Chapter IV, the idea developed that in general, thrombin is responsible for the activation of the non enzymatic protein cofactors factor VIII:C and factor V in order to accelerate its own formation. The literature, however, describes studies in which also factor X_a or factor IX_a were found to activate factor VIII:C (28,29). Also factor V has been described to be activated by other proteases than thrombin (30,31).

Unfortunately, all these studies deal with investigations done in purified systems. Because we were able to measure all the relevant activated coagulation factors in plasma, we investigated the activation of factor VIII:C and factor V in plasma. Chapter VI describes this study, and we found that in thromboplastin-ac-

tivated plasma, *in situ* generated thrombin, but not factor X_a catalyzed activation factors VIII:C and V. Even addition of high amounts of factor X_a did not result in factor VIII:C_a or factor V_a formation.

We hope that the investigations presented in this thesis throw some more light on the process of blood coagulation as it nowadays, after more than 300 years of investigations, is thought to proceed. We think that we succeeded at least in pointing out that this process should be investigated *ex vivo*, or in any case in a physiological more relevant environment as mere buffer. We presented some interesting data on how activated clotting factors behave under more complex conditions (first topic), and we tried to trace out the factor(s) which play a key role in plasma clot-formation (second topic). Our results showed that thrombin is the enzyme that plays a key role in plasma coagulation, and should be the prime target in anticoagulation.

References

1. Lämle B, Griffin JH: Formation of the fibrin clot: the balance of procoagulant and inhibitory factors. *Clinics in Haematology* 14:281, 1985
2. Thompson AR: Structure, function and molecular defects of factor IX. *Blood* 67:565, 1988
3. Barrowcliffe TW, Thomas DP: Antithrombin III and heparin, in Bloom AL, Thomas DP (eds): *Haemostasis and Thrombosis*, Second edition, Churchill Livingstone, 1987, pp 849
4. Rosenberg RD: Regulation of the hemostatic mechanism, in Stamatoyannopoulos G, Nienhuis AW, Leder P, Majerus PW (eds): *The molecular basis of blood diseases*. Philadelphia, Saunders, 1987, p 534
5. Toole JJ, Knopf JL, Wozney JM, Sultzman LA, Buecker JL, Pittman DD, Kaufman RJ, Brown, E, Shoemaker C, Orr EC, Amphlett GW, Foster WB, Coe ML, Knutson GJ, Fass DN, Hewick RM: Molecular cloning of a cDNA encoding human antihemophilic factor, *Nature* 312:342, 1984
6. Vehar GA, Keyt B, Eaton D, Rodriguez H, O'Brien DP, Rotblat F, Oppermann H, Keck R, Wood WI, Harkins RN, Tuddenham EGD, Lawn RM, Capon DJ: Structure of human factor VIII. *Nature* 312:337, 1984
7. Nienhuis AW, Maniatis T: Structure and expression of globin genes in erythroid cells, in Stamatoyannopoulos G, Nienhuis AW, Leder P, Majerus PW (eds): *The molecular basis of blood diseases*. Philadelphia, Saunders, 1987, p 28
8. Lindhout T, Baruch D, Schoen P, Franssen J, Hemker HC: Thrombin generation and inactivation in the presence of antithrombin III and heparin. *Biochemistry* 25:5962, 1986
9. Rosing J, Zwaal RFA, Tans G: Formation of meizothrombin as intermediate in factor X_a-catalyzed prothrombin activation. *J Biol Chem* 261:4224, 1986

10. Jordan RE, Oosta GM, Gardner WT, Rosenberg RD: The kinetics of hemostatic enzyme-antithrombin interactions in the presence of low molecular weight heparin. *J Biol Chem* 255:10081, 1980
11. Holmer E, Kurachi K, Söderström G: The molecular - weight dependence of the rate - enhancing effect of heparin on the inhibition of thrombin, factor X_a, factor IX_a, factor XI_a, factor XII_a and kallikrein by antithrombin. *Biochem J* 193:295, 1981
12. Jesty J: The inhibition of activated bovine coagulation factors X and VII by antithrombin III. *Arch Biochem Biophys* 185:165, 1978
13. Ellis V, Scully MF, Kakkar V: Inhibition of prothrombinase complex by plasma proteinase inhibitors. *Biochemistry* 23:5882, 1984
14. Walker FJ, Esmon CT: The effects of phospholipids and factor V_a on the inhibition of factor X_a by antithrombin III. *Biochem Biophys Res Comm* 90:641, 1979
15. Link RP, Castellino FJ: The activation of bovine factor X by bovine factor X_a. *Arch Biochem Biophys* 215:215, 1982
16. Choay J, Petitou M, Lormeau JC, Sinay P, Casu B, Gatti G: Structure - activity relationship in heparin: A synthetic pentasaccharide with high affinity for antithrombin III and eliciting high anti-factor X_a activity. *Biochem Biophys Res Comm* 116:492, 1983
17. Barrowcliffe TW, Havercroft SJ, Kemball-Cook G, Lindahl U: The effect of Ca²⁺, phospholipid and factor V on the anti-(factor X_a) activity of heparin and its high-affinity oligosaccharides. *Biochem J* 243:31, 1987
18. Kurachi K, Fujikawa K, Schmer G, Davie EW: Inhibition of bovine factor IX_a and factor X_a by antithrombin III. *Biochemistry* 15:373, 1976
19. Chuang TF, Sargeant RB, Hougie C: The effect of calcium ions on the properties of factor IX and its activated form. *Brit J Haematol* 27:281, 1974
20. Amphlett GW, Byrne R, Castellino FJ: The binding of calcium to the activation products of bovine factor IX. *J Biol Chem* 254:6333, 1979
21. Thomas DP: heparin in prophylaxis and treatment of venous thromboembolism. *Sem Haematol* 15:1, 1978
22. Ofosu FA, Blajchman MA, Modi GJ, Smith LM, Buchanan MR, Hirsh J: The importance of thrombin inhibition for the expression of the anticoagulant activities of heparin, dermatan sulphate, low molecular weight heparin and pentosan polysulphate. *Br J Haematol* 60:695, 1985
23. Carter CJ, Kelton JG, Hirsh J, Cerskus A, Santos AV, Gent M: The relationship between the hemorrhagic and antithrombotic properties of low molecular weight heparin in rabbits. *Blood* 59:1239, 1982
24. Thomas DP: Current status of low molecular weight heparin. *Thromb Haemost* 56(3):241, 1986
25. Warn-Cramer BJ, Bajaj SP: Intrinsic versus extrinsic coagulation. Kinetic considerations. *Biochem J* 239:757, 1986
26. Scott CF, Schapira M, James HL, Cohen AB, Colman RW: Inactivation of factor XI_a by plasma proteinase inhibitors. Predominant role of α_1 -protease inhibitor and protective effect of high molecular weight kininogen. *J Clin Invest* 69:844, 1982
27. Meijers JCM: Regulation of the blood coagulation mechanism by plasma proteinase inhibitors. Thesis at the State University of Utrecht, Utrecht, The Netherlands, 1988
28. Lollar P, Knutson GJ, Fass DN: Activation of porcine factor VIII:C by thrombin and factor X_a. *Biochemistry* 24:8056, 1985
29. Rick M: Activation of factor VIII by factor IX_a. *Blood* 60:744, 1982
30. Suzuki K, Dahlbäck B, Stenflo J: Thrombin-catalyzed activation of human blood coagulation factor V. *J Biol Chem* 257: 6556, 1982
31. Foster WB, Nesheim ME, Mann KG: The factor X_a-catalyzed activation of factor V. *J Biol Chem* 258:13970, 1983
32. Soons H: Studies on the inhibition of blood coagulation factor XI_a. Thesis at the University of Limburg, Maastricht, the Netherlands, 1987

Summary

The work presented in this thesis comprise a number of studies concerning the action of the antithrombin III/heparin system at several levels of the coagulation cascade. Our goal was to gain a better insight into the interactions of activated coagulation factors with the plasma inhibitor antithrombin III and the modulation of this action by heparin.

Heparin is a mucopolysaccharide that greatly accelerates the reaction between antithrombin III and activated coagulation factors. In the clinic, heparin is used in a variety of ways to treat or prevent thrombosis. Its mechanism of action in the patient, however, is not very well understood, because little is known about the effect of heparin on the complex reaction systems that govern blood coagulation *in vivo*.

Therefore it was our aim to obtain more information on the mechanism of action of heparin in (complex) purified systems and in plasma.

After a general introduction concerning the coagulation process, its initiation and inhibition, it is demonstrated in Chapter II that the prothrombin activation peptides, that are released during the generation of thrombin, are able to neutralize heparin. These results show a possible role for these fragments in the regulation of blood coagulation.

Chapter III describes the effect of antithrombin III and heparin during the enzymatic conversion of factor X into factor X_a by factor IX_a in the presence of phospholipids and calcium. The situation is a complicated one because both the enzyme (factor IX_a) and the generated product (factor X_a) are inactivated by antithrombin III/heparin. Under these circumstances, that start to approach the complexities of plasma, important differences become apparent with the effect of antithrombin III and heparin on isolated activated coagulation factors.

Chapters II and III clearly show that in more complex systems the activated coagulation factors behave quite different from what would be predicted from studies performed on isolated factors. In order to approach the *in vivo* situation still further, the investigations were continued in whole (platelet free) plasma.

To assess the effect of heparin and heparin-analogues on activated coagulation factors in plasma, sensitive and specific bio-assays were developed that can monitor the generation of activated coagulation factors in clotting plasma.

In Chapter IV it is demonstrated that not factor X_a inhibition, but rather the inhibition of (thrombin-induced) factor V_a generation by heparin explains the inhibition of coagulation in thromboplastin-activated plasma.

Using an assay for factor IX_a , the kinetics of inactivation (by antithrombin III/heparin) of the factor XI_a -catalyzed factor IX_a generation in plasma were studied (Chapter V). It was found that the inactivation of factor XI_a in plasma was fast, and independent of the presence of heparin or heparin analogues. Factor IX_a , generated in plasma by factor XI_a , was not significantly inactivated. However, heparin and pentasaccharide clearly caused inactivation of this factor IX_a .

In Chapter VI, the activation of the non enzymatic protein cofactors VIII:C and V was studied. It was demonstrated that in thromboplastin-triggered plasma, *in situ* generated thrombin, but not factor X_a is responsible for the activation of factors VIII:C and V.

From these studies, it can be concluded that (1) inhibition of activated coagulation factors by antithrombin III/heparin in complex reaction systems is quite different from inhibition of these factors in isolated systems, and (2) thrombin is the enzyme that plays the key role in plasma coagulation. It therefore should be the prime target of antithrombotic therapy by anticoagulation.

Samenvatting

In dit proefschrift worden een aantal studies beschreven met betrekking tot de werking van antitrombine III en heparine op verschillende niveaus van de stollcascade. Ons doel was een beter inzicht te verkrijgen in de interacties van geactiveerde stoffactoren met de plasma remmer antitrombine III en heparine.

Heparine is een mucopolysaccharide dat in staat is de reactie tussen antitrombine III en geactiveerde stoffactoren drastisch te versnellen. In de kliniek wordt heparine gebruikt ter behandeling of voorkoming van trombose. Het werkingsmechanisme van heparine in de patiënt is echter slecht begrepen, omdat weinig bekend is over het effect van heparine op de meer complexe reactie systemen die deel uitmaken van de bloedstolling *in vivo*.

Vandaar dat wij ons ten doel gesteld hebben meer informatie te verkrijgen over het werkingsmechanisme van heparine in (complexe) gezuiverde systemen en in plasma.

Na een algemene inleiding betreffende het bloedstollingsproces, de initiatie en de remming van dit proces, wordt in hoofdstuk II aangetoond dat de protrombine activeringspeptiden die gevormd worden tijdens de trombine generatie, in staat zijn om heparine te neutraliseren. Deze resultaten tonen een mogelijke rol aan voor deze fragmenten in de regulatie van de bloedstolling.

Hoofdstuk III beschrijft het effect van antitrombine III en heparine gedurende de enzymatische omzetting van factor X in factor X_a door factor IX_a in aanwezigheid van fosfolipiden en calcium. Deze situatie is gecompliceerd omdat zowel het enzym (factor IX_a) als het gevormd product (factor X_a) geïnactiveerd worden door antitrombine III/heparine. Onder deze omstandigheden, die de complexiteit van het plasma benaderen, worden belangrijke verschillen duidelijk met het effect van antitrombine III en heparine op geïsoleerde stoffactoren.

De hoofdstukken II en III laten zien dat in complexe systemen geactiveerde stoffactoren zich geheel anders gedragen als voorspeld zou worden op basis van studies uitgevoerd met geïsoleerde factoren.

Om de *in vivo* situatie nog meer te benaderen, werden de studies voortgezet in vol (plaatjesvrij) plasma.

Om het effect van heparine en heparine analoga op geactiveerde stoffactoren in plasma te bestuderen, werden gevoelige en specifieke bepalingen ontwikkeld om de generatie van geactiveerde stoffactoren in plasma te kunnen volgen.

In hoofdstuk IV wordt aangetoond dat niet de remming van factor X_a , maar veeleer de remming van de (trombine-afhankelijke) factor V_a generatie door heparine de remming van de stolling in tromboplastine-geactiveerd plasma verklaard.

Met behulp van een factor IX_a bepaling, werd de kinetiek van de remming (door antitrombine III/heparine) van de factor XI_a gecatalyseerde factor IX_a generatie in plasma bestudeerd (hoofdstuk V). De resultaten laten zien, dat factor XI_a in plasma snel wordt geremd, en dat deze remming onafhankelijk is van de toevoeging van heparine of heparine analoga. Factor IX_a , gegenereerd in plasma door factor XI_a , werd niet significant geïnactiveerd. Toevoeging van heparine of pentasaccharide veroorzaakte echter een duidelijke remming van factor IX_a .

In hoofdstuk VI werd de activering van de niet enzymatische eiwitcofactoren VIII:C en V bestudeerd. De resultaten tonen aan, dat in tromboplastine-geactiveerd plasma, *in situ* gegenereerd trombine, en niet factor X_a , verantwoordelijk is voor de activering van factor VIII:C en V.

Uit de studies die beschreven worden in dit proefschrift, kan geconcludeerd worden dat (1) inactivatie van geactiveerde stoffactoren door antitrombine III en heparine in complexe reactie systemen grote verschillen vertoont met de remming van deze factoren in geïsoleerde systemen, en (2) dat trombine het enzym is dat een sleutelrol speelt in de stolling van plasma. Vandaar dat trombine de belangrijkste rol zou kunnen spelen als doelwit enzym bij antistolling.

Dankwoord

Dit proefschrift is tot stand gekomen dankzij een intensieve samenwerking met vele anderen. Iedereen die hieraan heeft bijgedragen, wil ik graag bedanken.

Mijn promotor, professor H.C. Hemker wil ik bedanken voor de gelegenheid die hij mij geboden heeft om onder zijn leiding dit promotie-onderzoek te verrichten.

Mijn co-promotor en dagelijkse begeleider Dr. T. Lindhout, ben ik veel dank verschuldigd. Ik heb onze samenwerking gedurende de afgelopen jaren steeds als bijzonder positief ervaren. Voor zijn enorme energie, inzet en deskundigheid heb ik grote bewondering.

Hans, Truus, Jo en Pieter bedank ik voor de hulp en steun tijdens de lab-uren.

George Willems wil ik bedanken voor zijn bijdrage aan de wiskundige analyses van onze resultaten.

Rob Wagenvoord en Hans Hendrix stonden altijd klaar om hulp te bieden bij de factor VIII zuiveringen.

Alle medewerkers van de vakgroep Biochemie bedank ik voor de goede werksfeer op het lab gedurende de afgelopen jaren.

Mijn ouders, die mij altijd gestimuleerd hebben en voortdurend interesse toonden, ben ik daarvoor zeer dankbaar.

Tenslotte gaat mijn dank uit naar Chantal, voor haar begrip en steun in de afgelopen tijd.

Curriculum Vitae

De schrijver van dit proefschrift werd geboren te Heerlen op 5 januari 1962. Hij behaalde in 1980 het diploma Gymnasium-B aan het Stedelijk Lyceum te Maastricht. Vanaf oktober 1980 studeerde hij aan de faculteit Landbouwwetenschappen van de Katholieke Universiteit Leuven (België), richting scheikunde. Gedurende het laatste studiejaar verrichtte hij onderzoek op het laboratorium virologie van het Rega Instituut te Leuven. In 1985 behaalde hij het ingenieursdiploma in de afstudeerrichting biochemie en microbiologie. Vanaf oktober 1985 is hij werkzaam als wetenschappelijk assistent bij de vakgroep Biochemie van de Rijksuniversiteit Limburg, alwaar hij onder leiding van Prof. Dr. H.C. Hemker en Dr. T. Lindhout het onderzoek verrichtte dat heeft geleid tot dit proefschrift.