Studies on the influence of heparin and heparin derivatives on the generation of thrombin

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STUDIES ON THE INFLUENCE OF HEPARIN AND HEPARIN
DERIVATIVES ON THE GENERATION OF THROMBIN

PROEFSCHRIFT

ter verkrijging van de graad van doctor in de geneeskunde
aan de Rijksuniversiteit Limburg te Maastricht, op gezag
van de Rector Magnificus, Prof. Dr. F.I.M. Bonke, volgens
het besluit van het College van Dekanen, in het openbaar
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CONTENTS

Abbreviations		5
Chapter I	INTRODUCTION	
	The influence of heparin on thrombin formation	7
Chapter II	KINETICS OF THROMBIN-INDUCED RELEASE AND ACTIVATION OF PLATELET FACTOR V	37
	based on:	
	Baruch, D., Hemker, H.C., and Lindhout, T. Eur.J.Biochem. 154, 213-218 (1986)	
Chapter III	THROMBIN-INDUCED PLATELET FACTOR Va FORMATION IN PATIENTS WITH A GRAY PLATELET SYNDROME	53
	based on:	
	Baruch, D., Lindhout, T., Dupuy, E., and Caen, J.P. Submitted for publication.	
Chapter IV	INHIBITION OF THROMBIN-CATALYZED REACTIONS IN BLOOD COAGULATION AND PLATELET ACTIVATION	
	Effect of heparin and low molecular weight heparins in the absence of antithrombin III	65
	based on:	
	Baruch, D., Franssen, J., Hemker, H.C., and Lindhout, T. Thromb. Res. 38, 447-458 (1985)	
	Baruch, D., Lindhout, T., Wagenvoord, R., and Hemker, H.C. Haemostasis (1986) in press	

Chapter V	THE ROLE OF HEPARIN CHARGE DENSITY IN THE ANTITHROMBIN III DEPENDENT AND INDEPENDENT INACTIVATION OF THROMBIN	87
	based on:	
	Baruch, D., Franssen, J., Hemker, H.C., and Lindhout, T. Submitted for publication.	
Chapter VI	THROMBIN GENERATION AND INACTIVATION IN THE PRESENCE OF ANTITHROMBIN III AND HEPARIN	103
	based on:	
	Lindhout, T., Baruch, D., Schoen, P., Franssen, J., and Hemker, H.C. Submitted for publication	
Chapter VII	GENERAL DISCUSSION	127
SUMMARY		135
RESUME		138
SAMENVATTING		141
ACKNOWLEDGEMENTS		145
CURRICULUM VITAE		

ABBREVIATIONS

DEAE	Diethylaminoethyl
EDTA	Ethylenediamine tetraacetic acid
Hepes	4-2-(hydroxyethyl)-1-piperazine-ethanesulfonic acid dimethyl-sulfoxide
HSA	Human serum albumin
p-NPGB	p-nitrophenyl-p-guanidinobenzoate hydrochloride
PC	Phosphatidylcholine
PS	Phosphatidylserine
PPCA	Platelet prothrombin converting activity
S2337	N-benzoyl-L-isoleucyl-L-glutamyl-piperidyl-L-glycyl-L-arginine-paranitroanilide hydrochloride
S 2238	HD-phenylalanyl-L-pipecolyl- L-arginine- paranitroanilide hydrochloride
SDS	Sodium-dodecyl-sulfate
Tris	Tris (hydroxymethyl) aminomethane
USP	United States Pharmacopeia

CHAPTER I

THE INFLUENCE OF HEPARIN ON THROMBIN FORMATION

* Thrombin formation and its regulation.

- Thrombin formation
- The regulation of thrombin formation by thrombin dependent positive feedback reactions.
 1. Factor VIII:C activation
 2. Factor V activation
 3. Platelet factor V release and activation
 4. Procoagulant activity of platelets in factor X and prothrombin activation.

* The function of heparin in the regulation of thrombin formation.

- Introduction
- The inactivation of thrombin by natural inhibitors.
 1. Antithrombin III
 2. Heparin cofactor II
- Biosynthesis and structure of heparin
- Heparin-antithrombin III interaction
- Heparin-protease interaction
- Kinetics of the heparin-catalyzed antithrombin III/protease reactions
- Kinetics of the heparin-catalyzed antithrombin III/protease reactions in prothrombin activation by the prothrombinase complex
- The antithrombin III-independent effect of heparin
- Heparin-platelet interaction

* The present study.

THROMBIN FORMATION AND ITS REGULATION

Thrombin formation

The process that governs the generation of thrombin is a series of proteolytic proenzyme activations that occur at a phospholipid-solute interface. In addition, the limited proteolysis of non-enzymatic proteins provides specific cofactors for some of the enzymes of blood coagulation. The sequence of the proteolytic activation reactions can be simplified to a main activation triangle with three reinforcement loops (1). The activation of factor X occurs by two different pathways: activated factor VII (factor VII_a) activates factor X or factor VII_a activates first factor IX which in turn activates factor X. The intrinsic pathway consists of factor IX activation by the contact factors (factor XI, which is activated by factor XII and prekallikrein). Reinforcement loops are formed by positive feed back reactions such as activation of factor VII by factor X_a or factor IX_a (2), the mutual activation of prekallikrein and factor XII and the activation of factor VII by factor XII (3) [Figure 1 taken from reference (1)]. In addition thrombin mediates other positive feedback reactions as will be discussed in the next section.

Figure 1: The proteases of blood coagulation. The roman numerals indicate the respective coagulation factors. PK = prekallikrein. The arrows indicate activation by limited proteolysis. For example, $X \rightarrow II$ means that activated factor X exerts its proteolytic action upon factor II so as to activate it (taken from reference 1).

Figure 2: The overall coagulation process. In addition to figure 1 the non-enzymatic cofactors and the surfaces at which the reaction takes place are indicated. NS = negatively-charged surface. HMWK = High Molecular Weight Kininogen; TF = tissue factor; PL = phospholipid.

Each of the activation reactions has its specific non-enzymatic protein cofactor: factor VII_a which activates factor X and factor IX needs the protein component of tissue factor. Factor IX_a which activates factor X needs activated factor VIII. Prothrombin activation by factor X_a requires activated factor V. Contact activation reactions require high molecular weight kininogen. Thus each stage in the process of blood coagulation can be viewed as a dissociable three component enzyme (enzyme-cofactor-surface) acting on a substrate bound to the same surface. Whereas the physiological surface involved in the so-called contact activation (activation of factor XII, factor XI and prekallikrein) has yet to be established, wounded cells and/or activated platelets provide the procoagulant surfaces for the activation of factor IX, factor X, factor VII and prothrombin. The overall scheme of blood coagulation is depicted in Figure 2.

The regulation of thrombin formation by thrombin-dependent positive feedback reactions

Thrombin is responsible for positive feedback reactions that lead to an amplification of its own formation. Four specific reactions are catalyzed by thrombin.

1. Factor VIII:C activation. Activated factor VIII:C ($VIII_a$) is the cofactor of the factor-X-activating complex. Factor $VIII_a$ forms a stoichiometric complex with factor IX_a and promotes factor IX_a binding to phospholipid by lowering the dissociation constant (4,5). Non-activated human factor VIII consists of a single polypeptide chain, $M_r=100,000$ to $210,000$, this heterogeneity is presumably due to proteolysis (6). Thrombin-catalyzed factor VIII activation results in the formation of a heavy chain of $M_r=92,000$ and a light chain of $M_r=71,000 - 72,000$ (7). This is analogous to the active forms shown for human and bovine factor V (8,9). There is evidence that thrombin activated factor VIII is labile and that stabilization is achieved by performing the activation in the presence of phospholipid and factor IX_a (10). Whether thrombin is responsible for the inactivation of factor $VIII_a$ (6) or whether factor VIII undergoes both thrombin dependent activation and thrombin independent inactivation (11) is still unknown.

2. Factor V activation. Factor V from bovine or human origin is a single chain glycoprotein with $M_r=330,000$. The activation process of either molecule by thrombin yields factor V_a , a two-subunit protein. The two subunits together represent about one-half of the mass of factor V, and the remaining mass represents activation fragments. Bovine factor V_a consists of a light chain of $M_r=80,000$ and a heavy chain of $M_r=94,000$. Both chains are non-covalently linked by Ca^{++} ions (9,12). Factor V_a increases the maximal rate of thrombin formation about 2000-fold (13). With factor X_a it forms a stoichiometric complex with a K_d of $3 \cdot 10^{-9}$ M. Phospholipids promote factor X_a -factor V_a complex formation by decreasing the K_d from 10^{-9} M to 10^{-11} M. The K_d of the factor X_a -factor V_a complex for phospholipids is dependent on the phosphatidylserine content of the latter (9). The phospholipid binding site of factor V_a is located on the factor V_a light chain. The interaction is primarily electrostatic and does not require Ca^{++} ions (14) although hydrophobic interactions cannot be ruled out. Factor X_a and prothrombin bind to intact factor V_a . That is, binding occurs when the factor V_a light chain and heavy chain are associated via Ca^{++} ions (15).

3. Platelet factor V release and activation. Factor V localization in platelets was first shown by Breederveld (16), using immunofluorescence techniques. Immunological and functional studies, as well as studies of partial granule deficiencies in platelet, have established that factor V is stored in α -granules (17-23). Osterud et al. (18) reported that the protein was stored in its activated form because no increase in factor V_a activity was observed when frozen-thawed platelet suspensions were incubated with thrombin. However, it is generally accepted now that factor V is stored in its non-activated form and can be released by a variety of agents (thrombin, collagen, ADP, ionophore A 23187). The findings of Osterud et al. (18) can be explained by the presence of a platelet factor V activator (19). Several reports suggested that collagen induced the incomplete release of factor V present in platelets (20-22). In contrast with these studies, Kane et al. (23) showed that virtually all factor V present in platelets could be released by the action of thrombin. This discrepancy, as well as the one existing for the assessment of platelet factor V concentration relies on the absence of a well-defined factor V bioassay. Based on the radioimmunoassay described by Tracy et al. (24) human platelet factor V represents 10-20% of human plasma factor V (25 nM).
- The importance of platelet factor V in maintaining normal hemostasis has been suggested by Miletich et al. (25) who demonstrated that among patients with a factor V deficiency the severity of bleeding correlated with the extent of platelet factor V deficiency. Tracy et al. (26) reported a severe familial bleeding disorder related to the almost complete absence of functionally active platelet factor V with a ratio activity/antigen of 2%. This compares with a ratio of 70% in plasma. This abnormality was not caused by an impaired release reaction, nor by a defect in the assembly or functioning of the prothrombinase complex in the presence of exogenous factor V_a .
4. Procoagulant activity of platelets in factor X and prothrombin activation. Extensive studies from our laboratory have demonstrated the need for the combined action of collagen and thrombin in the generation of platelet procoagulant activity in factor X and prothrombin

activation (27-29). This activity results from the exposure at the surface of activated platelets, of the negatively-charged phospholipids, phosphatidylserine and phosphatidylethanolamine. The physiological importance of platelet procoagulant activity was confirmed by the study of a patient with a bleeding disorder ascribed to an isolated deficiency of platelet procoagulant activity in prothrombin activation (29) and presumed to be due to the selective lack of a membrane protein (receptor) required for factor V_a binding (30). However, the results of a recent study showed an equally impaired ability of the patient's platelets to promote prothrombin and factor X activation after stimulation of platelets by collagen and thrombin. The decreased stimulatory effect of the patient's platelets was accompanied by a reduced exposure of phosphatidylserine as assessed by treatment of the membranes with phospholipases (29).

THE FUNCTION OF HEPARIN IN THE REGULATION OF THROMBIN FORMATION

Introduction

Heparin is one of the most important drugs for the treatment and prevention of venous thrombosis. However it still raises many puzzling questions on different levels for clinicians and clinical chemists, biologists and biochemists. Clinicians and clinical chemists know that the major drawback to the therapeutic use of heparin is the absence of any reliable laboratory test to keep the patient in the narrow range that ensures an efficient antithrombotic action but without causing a hemorrhagic tendency. Research over the last few years has led to the preparation of heparin fractions which allowed considerable progress in understanding the structure function relationships. The main *in vitro* and *ex vivo* anticoagulant function of heparin occurs by accelerating the inactivation of serine proteases by antithrombin III. However the properties of heparin to catalyze factor X_a or thrombin inactivation by antithrombin III *in vitro* do not necessarily reflect the complexity of heparin actions in blood coagulation reactions *ex vivo*, and are far from being sufficient to explain the *in vivo* effects.

The inactivation of thrombin by natural inhibitors

1. Antithrombin III. Antithrombin III is the main inhibitor of thrombin. It is present in human plasma at a concentration of $2.5 \mu\text{M}$ (31). The correlation between thrombotic disorders and plasma antithrombin III deficiency suggests that antithrombin III is essential in the regulation of hemostasis.

Antithrombin III from human or bovine plasma is a single-chain α_2 -glycoprotein with $M_r=56,000-57,000$ and $M_r=56,600$ as determined by SDS-gelelectrophoresis and equilibrium sedimentation techniques, respectively (32). It contains about 10% carbohydrates. The complete primary structure of human antithrombin III has been reported and showed a pronounced sequence homology with α_1 -antitrypsin (33), histidine-rich glycoprotein (34) and ovalbumin (35).

Antithrombin III inhibits proteases by means of the formation of a stable 1 : 1 molar complex between the active site of the enzyme and the reactive site of antithrombin III (36). This reactive site, the antithrombin III (Arg 385 - Ser 386) peptide bond, appears to be the same for thrombin (37), factor IX_a and factor X_a (38).

The mechanism of thrombin inhibition by antithrombin III has been extensively studied. It resembles the mechanism described for the formation of a stable bond between a protease and a substrate and is shown in Figure 3. The first step of the reaction involves the formation of a non covalent enzyme-substrate (Michaelis) complex, which after internal bondshifts leads to the formation of a covalent tetrahedral complex. The third step of the reaction involves the breakdown of a peptide bond in the tetrahedral complex, leading to an acylenzyme intermediate and an amine product. The reverse reaction might occur, but very slowly. Water replaces the peptide leaving group in the reaction sequence (39). Evidence for the formation of an acylenzyme intermediate in the reaction between antithrombin III and proteases came from studies of the dissociation pattern of these complexes under the denaturing conditions of SDS polyacrylamide electrophoresis. Whereas Rosenberg and Damus (36) showed the formation

Figure 3: The reaction sequence for serine protease and substrate complex formation. The enzyme is depicted by E-OH, -OH being the reacting group of the serine residue in the active centre. The reverse of the three reactions shown here can take place with the exception that the acylenzyme reacts with a water molecule to form a tetrahedral complex.

of a stable antithrombin III-thrombin complex, Jesty (40) demonstrated that the stability of the complexes (antithrombin III-thrombin and antithrombin III-factor X_a) was pH dependent. They were dissociated at pH 9 and could be reassociated at pH 7.9. Upon dissociation of the complexes a modified form of antithrombin III was produced. In a recent study it was shown that the equimolar thrombin-antithrombin III complex is the only product of the reaction of thrombin with antithrombin III over a wide range of ionic strengths (0.01-0.5). The reactive site-cleaved antithrombin III released at a slow rate from the complexes in the absence of heparin is identical to the proteolytically modified antithrombin III produced in the presence of heparin (41). Although the exact nature of the bonds involved in the stable enzyme-antithrombin III complex is not known, kinetic studies have demonstrated the involvement of a rapid preequilibrium step with formation of a Michaelis complex. Studies by means of stopped-flow fluorimetry with thrombin-bound-p-aminobenzamidine as a probe showed that the antithrombin III-thrombin reaction followed saturation kinetics when the antithrombin III concentration exceeded 70 μM . The overall process of thrombin inactivation by antithrombin III was described as a two-step reaction, where the formation of an initial weak thrombin-antithrombin III complex with a K_d of 1.4×10^{-3} M is followed by the formation of a stable complex with a first order rate constant of 10.4 s^{-1} (42).

2. Heparin cofactor II. A second heparin-dependent inhibitor of thrombin, the heparin cofactor II has been demonstrated and purified from human plasma (43). The activity of this inhibitor is enhanced by high heparin concentration. The characteristics of the mucopolysaccharide structure required to enhance the heparin cofactor II-thrombin reaction have not been established. Subspecies of heparin other than the ones catalyzing the antithrombin III-protease reaction seem to be involved in this catalytic effect (see below, kinetics of the heparin-catalyzed antithrombin III-protease reaction) (44). Dermatan sulfate, which is a poor accelerator of the antithrombin III-thrombin reaction appears to be the main mucopolysaccharide able to catalyze the heparin cofactor II thrombin reaction (45). The mechanism by which dermatan sulfate catalyzes the heparin cofactor II-thrombin reaction seems essentially identical to the reaction mechanism described for the heparin-catalyzed antithrombin III-thrombin reaction (46). The physiological role of heparin cofactor II is not known. Its protease specificity seems to be restricted to thrombin, and in the presence of dermatan sulfate heparin cofactor II binds to thrombin as it is being generated in plasma (47). Rapid inhibition of thrombin by heparin cofactor II may occur in vivo only in the immediate vicinity of dermatan sulfate, which is the main proteoglycan of the intima and media of large arteries.

Biosynthesis and structure of heparin

Heparin is a highly sulfated mucopolysaccharide widely distributed throughout a variety of organs within mammalian species. The biosynthesis of heparin is initiated by the attachment of a carbohydrate-protein linkage region to the serine residues of a specific polypeptide chain (48). The polymer chain of the mucopolysaccharide is assembled by the alternate attachment of the N-acetylglucosamine and glucuronic acid (Figure 4). Thereafter this simple copolymer structure is modified by a multistep process that takes place to a varying extent, apparently at random places, within different segments of the mucopolysaccharide chain. These series of post-synthetic modifications are depicted in Figure 5. Each stage in the process is under the

Figure 4: Initiation of the synthesis of heparin. The serine residues of a specific polypeptide chain (heparin proteoglycan) bear carbohydrate-protein linkage regions. The polymer chain of the mucopolysaccharide is assembled by the alternate attachment of N-acetylglucosamine (NAG) and glucuronic acid (Glu).

control of a specific enzyme and is only initiated when the preceding transformation has been completed. Of particular interest for the development of the anticoagulant activity of heparin is the epimerization of glucuronic acid residues into iduronic residues at the C-2 position. Within the tissues a degradative process by different types of lysosomal enzymes takes place leading to single polysaccharide chains with molecular weights of approximately 3,000 to 35,000 daltons (49). They are composed of alternating residues of uronic acid and glucosamine, linked by alternating α -D and β -D1,4 bonds. The uronic acid groups may exist as iduronic acid-2 sulfate, glucuronic acid or non-sulfated iduronic acid. The glucosamine residues may be N-sulfated, N-acetylated or have free amino groups. They may have a free hydroxyl group at C-6 or bear an ester sulfate at this position (50). This mode of biosynthesis explains why commercial heparin preparations that are extracted from animal sources without further fractionation, exhibit a wide range of molecular weight, ratios of glucuronic acid over iduronic acid, degree of sulfation and N-acetylation (51).

Figure 5:

The biosynthesis of the mucopolysaccharide chains. Four major post-synthetic modifications of the simple copolymer structure (N-acetylglucosamine alternating with glucuronic acid) lead to the completed heparin molecule. They consist of:

- (1) deacetylation;
- (2) N-sulfation;
- (3) 5-epimerization of the D-glucuronosyl moieties;
- (4) O-sulfation at position 2 of the iduronosyl moieties and O-sulfation at position 6 of the glucosamine residues.

Heparin-antithrombin III Interactions

Heparin has been fractionated on the basis of its affinity for antithrombin III. Antithrombin III high affinity material represents only one third of unfractionated heparin (52,53). Preparation of heparin fragments by chemical or enzymatic degradation have led to the isolation of a polysaccharide sequence required for the heparin-antithrombin III interaction. The antithrombin III binding domain has been identified as a hexa-, penta-, or tetrasaccharide sequence (54,55,56). The pentasaccharide structure is shown in Figure 6.

Figure 6: The pentasaccharide sequence responsible for the binding to antithrombin III. $A_{8/S}$: N-acetyl-6-O-sulfate- α -D-glucosamine (A_a) or N-sulfate-6-O-sulfate- α -D-glucosamine (A_g); G: β -D-glucuronic acid; A_8^* : N-sulfate-3,6-di-O-sulfate- α -D-glucosamine; I_8 : 2-O-sulfate- α -L-iduronic acid; A_8 : N-sulfate-6-O-sulfate- α -D-glucosamine. Full circles indicate essential sulfate groups, the dotted circle indicates a sulfate group whose role has not been proved essential (taken from reference 55).

Rosenberg and Damus (36) demonstrated that the action of heparin in the antithrombin III thrombin-reaction could be removed by modification of the lysyl residues of the inhibitor. However, these alterations did not affect thrombin inhibition by antithrombin III in the absence of heparin. In addition, Longas et al. (57) demonstrated that disruption of a single disulfide bridge in antithrombin III leads to a reduced affinity for heparin and a corresponding loss of inhibitory activity in the presence, but not in the absence of heparin. This indicates that heparin and thrombin bind at different sites of the antithrombin III molecule.

Structural studies have provided evidence for two functional domains of the antithrombin III molecule. Upon exposure to guanidinium chloride, a biphasic denaturation behaviour was observed, suggesting a two-step folding mechanism of different parts of the molecule (58,59). However, correlation with the binding properties of antithrombin III differed in the two studies: whereas the irreversible loss of activity upon folding was accompanied by a parallel loss of heparin-binding properties and not of protease-binding in one study (58), opposite results were found in the other report concerning the simultaneous loss of binding properties, that were found to be strongly dependent on an aggregation of the thrombin-antithrombin III complexes (59). A hereditary abnormal antithrombin III characterized by a loss of heparin-enhanced activity, was biochemically identified as a single substitution of an arginine in the amino-terminal end (position 47) by a cysteine residue (60). The role of arginine residues in the heparin binding properties of antithrombin III has also been demonstrated in studies using chemical modifications of the arginine groups of the inhibitor (61,62). A loss of heparin binding was found independently from the loss of protease binding. Seven arginine residues are thought to be required for heparin binding, and a model was proposed where one or several arginine groups would accommodate the sulfate groups in the pentasaccharide structure of heparin, through positive charge clusters at the binding site of antithrombin III (62).

The involvement of tryptophan residues in the binding of heparin by antithrombin III has been shown by structural studies as well as with chemically modified antithrombin III. Using UV double difference spectral analysis, Einarsson (63) found that unfractionated heparin bound to antithrombin III with a molar stoichiometry of 2 : 1, and an average dissociation constant of 4.3×10^{-6} M, suggesting a strong and specific interaction. Fluorescence studies allowed determination of the respective dissociation constants for heparin fractions with high and low affinity for antithrombin III. High affinity heparin binds to antithrombin III with a K_d of 4.5×10^{-7} M (64), or 8×10^{-7} M (65) whereas low affinity heparin binds to the same site of antithrombin III with a K_d of 8×10^{-4} M (66). A general conformation change of the

antithrombin III molecule on its binding to heparin was suggested from these fluorescence studies. After binding to antithrombin III of the fluorophor probe 1-anilino-8-naphthalene sulfonate (ANS), a quenching of the antithrombin III emission fluorescence was observed, suggesting a hydrophobic interaction region in a near location of the tryptophanyl residues (66). Thus upon heparin binding, changes in the local micro-environment of buried tryptophan residues occurred (64). This was confirmed by circular dichroism spectral analysis in the presence of heparin (67). Chemical modification of the tryptophan residues of antithrombin III resulted in an impaired heparin enhancement of the rate of thrombin inactivation by antithrombin III (68). A recent study of the amino acid sequence of such modified antithrombin III led to the identification of a tryptophan residue in position 49 that is critical for heparin binding. The amino terminal sequence may constitute part of the heparin-binding domain that is widely separated from the protease-binding domain located on the carboxyl terminal end (69).

Heparin-protease interaction

The interaction between heparin and some activated blood coagulation factors has been established by direct binding techniques. However, most of the binding data were obtained from kinetic studies, as will be discussed later. It appears that the factors X_a , XII_a and kallikrein have little, if any, affinity for heparin, whereas thrombin, factor IX_a and XI_a bind strongly to heparin. The heparin-thrombin interaction is the best defined one. Besides the antithrombin III-binding site of heparin there appear to be additional structural properties of heparin that are related to its anticoagulant activity. Fractions with increasing molecular weight have been shown to exhibit increasing antithrombin activity (70-72). A minimum octadecasaccharide sequence is required for the action of heparin in the antithrombin III-thrombin reaction (73). Furthermore, heparin binding to thrombin in the heparin-catalyzed antithrombin III-thrombin reaction occurs via electrostatic interactions that increase with the degree of sulfation, i.e. the anionic charge density of heparin (74). Hurst et al. (75) found a

correlation between charge density and the anti-thrombin activity of either unfractionated heparin or antithrombin III high affinity fractions separated by a phase partition technique so that they systematically varied in anionic charge density. Thus the antithrombin III-binding domain of heparin appeared functionally independent of the protease-binding domain. However, no sequence has been identified for the latter and it seems that heparin binding to thrombin occurs along the whole dimension of the heparin chain.

Kinetics of the heparin-catalyzed antithrombin III/protease reactions

It has been shown that the kinetics of the heparin-catalyzed factor X_a inhibition by antithrombin III differ significantly from those of thrombin and factor IX_a inhibition. It is at present generally assumed that the apparent differences in the kinetics of the various antithrombin III/protease reactions are caused by the different affinities of the proteases for their binding to heparin.

Because heparin brings together the reactants and is released from the antithrombin III-protease complex once the reaction has occurred, heparin can be compared to an enzyme that catalyzes a two-substrate reaction. Either heparin binds simultaneously to both antithrombin III and the protease (random order bireactant model) or heparin binding results in a conformational change of antithrombin III that increases its affinity for the protease (ordered bireactant model).

The initial reaction velocities in each of the antithrombin III-protease reaction can be described by the general rate equation for a random order bireactant system (ref. 76 and references herein). In this model (the so-called template model) the reaction was first order with respect to the concentration of the ternary complex, with an apparent first order rate constant value of 13 s^{-1} , over a thousand-fold range of heparin concentrations (77). The heparin-catalyzed antithrombin III-thrombin reaction was shown to be saturable with respect to both antithrombin III ($K_d = 10^{-7} \text{ M}$) and thrombin ($K_d = 3.6 \times 10^{-8} \text{ M}$) (78). Hoylaerts et al. (79) were able to simplify the kinetic analysis by preparing covalent complex of heparin-antithrombin III.

Figure 7: A "sliding" model for heparin-antithrombin III-thrombin interaction according to Hoylaerts et al. (79). Antithrombin III binds to the pentasaccharide sequence of the heparin chain. Thrombin heparin interactions occur along the heparin chain length, allowing thrombin to diffuse towards the antithrombin III molecule.

The rate of thrombin inhibition was saturable with respect to the concentration of the covalent complex. Comparison of a high affinity heparin with $M_r=15,000$, and fragments of $M_r=4,300$ and $M_r=3,200$ revealed that the apparent dissociation constants for the interaction between thrombin and the covalent heparin-antithrombin III complexes were 7 nM, 100 nM and 6 μ M respectively. However, the apparent first order rate constant value, 2 s^{-1} , was independent of the heparin size. These findings are consistent with the random order model for the mechanism of heparin action. In addition, they showed that the binding of thrombin to heparin was of an electrostatic nature and was strongly dependent on the heparin chain length. Thus, the bound thrombin is able to diffuse along the heparin chain to interact with the bound antithrombin III as schematically represented in Figure 7.

Evidence has been presented that the heparin-catalyzed antithrombin III-protease reaction can also be described by the general rate equation for an ordered bireactant reaction mechanism. Fletcher and Nelsestuen (80) described a model in which the direct binding of

heparin to thrombin results in a decrease in the affinity of thrombin for the heparin-antithrombin III complex and thus in a decrease in the initial reaction velocity at high heparin concentrations. At low heparin concentrations, the rate-determining step was independent of thrombin but dependent on the antithrombin III concentration, while at high heparin concentrations, the reaction was dependent on both antithrombin III and thrombin. It was shown that fractionation of heparin by affinity for antithrombin III increases the maximum specific velocity of the reaction, not because the fractions had different K_m values for antithrombin III but due to the removal of polysaccharides with low affinity to antithrombin III (81). This model is essentially identical to the one proposed by Jordan et al. (82) where the interactions are analyzed as sequential events with firstly, direct binding of heparin to antithrombin III (allosteric effect) and secondly, interactions between the free enzyme and the heparin bound to antithrombin III (approximation effect). The second order rate constant for thrombin inactivation by the antithrombin III-heparin complex was $1.7 \times 10^9 \text{ M}^{-1} \cdot \text{min}^{-1}$, in contrast with a value in the absence of heparin of $4.25 \times 10^5 \text{ M}^{-1} \cdot \text{min}^{-1}$. Comparisons of the various kinetic constants indicated that the direct binding of heparin to antithrombin III is responsible for a 1000-fold acceleration of thrombin-antithrombin III complex formation whereas interaction between thrombin and heparin-antithrombin III provides an additional 4 to 15-fold increase in the rate of inactivation (83).

However, the heparin-antithrombin III-protease interaction does not seem to be restricted to either an ordered reaction sequence or a random reaction pathway, as underlined by Griffith (77) and Hoylaerts et al. (79). Upon saturation of thrombin with heparin, the formation of the ternary complex shifts from a random to a sequential pathway in which heparin-thrombin is inhibited by antithrombin III. Interestingly, when both thrombin and antithrombin III are saturated with heparin, the reaction rate minimizes to a rate which is only slightly slower than the rate of inhibition of factor X_a by antithrombin III-heparin. In addition, the rate of inhibition of factor X_a by antithrombin III-heparin is insensitive to the heparin chain length inasmuch as the antithrombin III-binding domain is present (46).

Kinetics of the heparin catalyzed antithrombin III/protease reactions in prothrombin activation by the prothrombinase complex.

In discussing the heparin-catalyzed antithrombin III-protease reaction, an important difference was found when factor X_a was studied in the presence of the accessory components (factor V_a and phospholipids) of the prothrombinase complex. It has already been known for several years that factor X_a bound to phospholipids is much less susceptible to the inactivation by antithrombin III-heparin than free factor X_a (84,85). Ellis et al. (86) reported that this effect was observed in the presence of the procoagulant surface of activated platelets or in the presence of phospholipid vesicles either in the absence or in the presence of factor V_a . However, Barrowcliffe et al. (87) reported that the protective effect of phospholipid and factor V_a could be eliminated at high concentrations of unfractionated heparin, whereas a fragment with $M_r=3,000$ was unable to overcome this protective effect. These authors suggested that in the presence of the components of the prothrombinase complex, an interaction between heparin and factor X_a occurred that required an additional sequence of the heparin molecule distinct from the saccharide sequence involved in the binding to antithrombin III.

In addition, an activation peptide from prothrombin (prothrombin fragment 2) was found to inhibit the antithrombin III inactivation of thrombin by altering the reversible binding of thrombin to antithrombin III. However, fragment 2 had no inhibitory effect on the formation of the stable antithrombin III-thrombin complex. This observation was interpreted as a potential protection mechanism against inactivation by antithrombin III of the formed thrombin (88).

The antithrombin III-independent effect of heparin.

Evidence for an antithrombin III-independent effect of heparin in reactions of blood coagulation and hemostasis is two-fold:

- Some heparin fractions with low affinity for antithrombin III and thus virtually devoid of any in vitro anti-thrombin or anti-factor X_a

activity significantly increased hemorrhage without influencing the in vivo antithrombotic effect in animal experimental models (89). Furthermore this antithrombin III low affinity heparin fraction potentiated the antithrombotic action of a high affinity heparin oligosaccharide of molecular weight lower than 3,500, which had an in vitro anti-factor X_a activity but no anti-thrombin activity (90).

- In a system consisting of purified coagulation factors and synthetic phospholipids, heparin can inhibit prothrombin and factor X activation. This effect was attributed to the displacement of the bound proteins from the phospholipid surface (91-93). However, these experimental results can alternatively be explained by an effect of heparin on the thrombin-catalyzed factor VIII activation. The effect of heparin in these experiments was practically eliminated if activated factor VIII was present. Furthermore, in an antithrombin III depleted plasma the inhibitory effect of heparin on factor X_a or thrombin generation is almost negligible (94). Comparison of crude heparin and low affinity heparin in prothrombin activation in antithrombin III depleted plasma showed that the inhibitory effect of both heparins was similar (95). However, these experiments do not exclude an effect due to other protease inhibitors.

Heparin-platelet interactions

The effect of heparin on platelet functions is rather unclear. Two main actions can be contrasted. Clinically, the most important one is the heparin-associated thrombocytopenia, that occurs either in isolation or with arterial thrombosis, but rarely leads to bleeding complications (for a review see reference 96). It represents the most serious adverse effect of heparin therapy for two reasons. It is encountered in 5% of patients receiving heparin therapy. Arterial thrombosis has a much lower frequency than isolated thrombocytopenia, but is a very severe complication with about 30% mortality being reported in patients (97,98). The onset of heparin-induced thrombocytopenia (plus arterial thrombosis) takes place between the 8th and 15th day of heparin

therapy, which is consistent with an anamnestic response. A heparin-dependent platelet aggregating factor has been isolated from the serum of the patients, and shown to be an antiplatelet antibody that caused increased platelet destruction (99). However, several arguments suggest that heparin-induced thrombocytopenia is not a typical drug-induced thrombocytopenia. Recovery of platelet count can occur despite the heparin type and dosage being maintained in the same patient. Patients with a history of heparin-induced thrombocytopenia can be rechallenged with heparin without recurrence of the thrombocytopenia (100) and heparin-dependent binding of the IgG from the patients' serum to normal platelets is not always convincingly demonstrated (101). The substitution of low molecular weight heparin preparations to treat the patients has been reported to be successful in some cases (102) and not in others (103). It is not yet known whether there are heparin preparations which have a negligible risk of thrombocytopenia.

The second type of heparin-platelet interaction is distinguished clinically by its immediate time of onset and does not seem to be an immunological process. It is not clear whether the different reactions are of clinical relevance. They include in vitro heparin-induced aggregation that can occur to a varying degree in almost every individual, and an impairment of the release reactions (104). The original observation of Salzman et al. (105) that high molecular weight heparin fractions irrespective of their affinity for antithrombin III, were more reactive on platelets than low molecular weight, antithrombin III high affinity fractions, suggested a correlation between the hemorrhagic effect of heparin and the anticoagulant properties in vitro. This was confirmed by in vivo animal studies, showing that the bleeding risk of heparin could be dissociated from its antithrombotic activity (106).

THE PRESENT STUDY

Although the anticoagulant action of heparin has been the subject of extensive investigations, two main problems emerge in the analysis of these studies. Firstly, the anti-thrombin activity has been almost exclusively studied in a simple in vitro bireactant system, where heparin catalyzes the interaction between thrombin and antithrombin III, whereas the situation in clotting plasma is much more complicated. Secondly, heparin is simply too heterogeneous to expect that the experimental results obtained in different laboratories, using different heparin preparations and fractions, would necessarily lead to a unique interpretation of data and identical conclusions regarding the mechanism of action. Our purpose was to focus on the interaction of heparin with reactions that can be considered as relevant for in vivo hemostasis and thrombosis, such as the thrombin-dependent feedback reactions. The interactions were analyzed by using purified proteins and washed platelets under well-defined conditions.

In chapter II we describe a kinetic study on platelet factor V_a formation from washed human platelets upon their activation by thrombin. The use of a prostacyclin analogue provides a way of analyzing the effect of thrombin on the release of factor V separately from its effect on the formation of activated factor V. This study raised the question of the relative importance of platelet factor V_a and plasma factor V_a formation in hemostasis. The study of patients with a moderate bleeding disorder ascribed to a Gray platelet syndrome, was undertaken in order to approach this question by determining whether the expected factor V storage defect would result in an impairment of platelet factor V_a formation (chapter III).

Platelet and plasma factor V_a formation are reactions involved in the feedback mechanism of thrombin formation, as are the generation of a negatively-charged surface from activated platelets and the activation of plasma factor VIII:C. The effect of heparin and heparin fractions in the absence of antithrombin III on these thrombin-dependent reactions is described in chapter IV. The interaction of heparin and thrombin can be quantitatively assessed as it results in an inhibition of the

catalytic action of thrombin towards its macromolecular substrates. The low molecular weight heparin fractions proved to be less effective in inhibiting the thrombin-dependent reactions than unfractionated heparin. Because heparin binding to thrombin is thought to be of an electrostatic nature, we have investigated whether and to what extent the antithrombin III-independent action of heparin on the inhibition of thrombin is a function of its charge density (chapter V).

Studying the antithrombin III-dependent inactivation of proteases, we were interested to assess this effect on thrombin formation when it occurs in the presence of prothombinase complexes of different compositions. Our aim was to simultaneously determine the rate of inactivation by antithrombin III and heparin of factor X_a bound to phospholipid and/or factor V_a and the rate of inactivation of thrombin formed under these different conditions. We compared these kinetics with those obtained for free enzyme inhibition by antithrombin III and heparin (Chapter VI).

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CHAPTER II

KINETICS OF THROMBIN-INDUCED RELEASE AND ACTIVATION OF PLATELET FACTOR V

SUMMARY

The kinetics of thrombin-induced platelet factor V activation were studied in suspension of washed human platelets. The effect of thrombin in stimulating the release reaction could be separated from its effect on factor V activation by use of a potent inhibitor of the release reaction, the prostacyclin analogue ZK 36374. When platelets were incubated with ZK 36374 prior to stimulation with thrombin, the amount of ZK 36374 required to inhibit 50% of factor V_a formation was 15 pM. ZK 36374 at a final concentration of 1 nM was found to block instantaneously and completely the release of factor V, whereas it has no effect neither on platelet factor V activation nor on the factor V_a assay. By varying the time interval between the addition of thrombin (0.5 nM) and ZK 36374 to suspensions of 4.6×10^6 platelets/ml the rate of factor V release was found to be 12 pM factor V/min. In the absence of ZK 36374, the total amount of factor V released was 8 pM, whereas Triton X-100-treated platelets gave 13 pM factor V. It appeared that the amount of factor V that could be released was dependent on the thrombin concentration. Maximum release was obtained at 1 nM thrombin. The rate of factor V release increased in proportion to the thrombin concentration. The rate of factor V activation was found to be proportional to the thrombin concentration as well as to the amount of released factor V. When 4.6×10^6 platelets/ml were activated by 0.5 nM thrombin, the rates of factor V activation were found to be 0.3 and 1.2 pM factor V_a /min at 20% and 90% completion of the release reaction. Therefore, the rate of factor V release was at least one order of magnitude faster than the rate of factor V activation. The kinetics of thrombin-induced platelet factor V activation were compared to those of plasma factor V activation in platelet-rich and platelet-free plasma. The results clearly demonstrate that platelets have no effect on the rate of factor V activation and that the kinetics of plasma factor V activation are identical to those of platelet factor V activation.

INTRODUCTION

Thrombin-activated platelets are essential to the normal hemostatic mechanism. Two types of procoagulant activity from activated platelets have been recognized. In the first place factor V is released and activated during stimulation by thrombin (1,2) and secondly the altered membrane of platelets activated by thrombin plus collagen provides a procoagulant surface (3,4). These two events enable factor X_a to function in a platelet bound complex of factor V_a and factor X_a , the so-called prothrombinase, thus causing a dramatic amplification of the thrombin-generating capacity of factor X_a (5,6). Factor V occurs as a plasma protein as well as a protein present in the alpha-granules of platelets.

The question of the physiological importance of platelet factor V over plasma factor V in affecting the efficiency of the hemostatic process has been recently addressed by Tracy et al. (7). These authors described an inherited factor V disorder and the association of a significant bleeding diathesis with a qualitative abnormality predominantly in platelet factor V. As yet the kinetics of thrombin-induced platelet factor V release and activation were difficult to study, because the release reaction induced by thrombin could not be separated from the factor V activation catalyzed by the same enzyme.

Here we report that a semi-synthetic prostacyclin analogue (ZK 36374) is able instantaneously to block thrombin-induced platelet factor V release during the time course of the reaction. This enabled us to study separately the kinetics of factor V release and thrombin-catalyzed activation of platelet factor V.

MATERIALS AND METHODS

Materials. S2238 was purchased from AB Kabi Diagnostica. Human serum albumin (fatty acid free) and 1,2-dioleoyl-sn-glycero-3-phosphocholine (PC) were obtained from Sigma. ZK 36374, a stable carbacyclin derivative of prostacyclin, was obtained from Schering AG (Berlin) as a 0.5 mg/ml solution of Tris-saline. All reagents used were of the highest grade commercially available.

Proteins. Human thrombin was prepared from prothrombin by the method of Fletcher and Nelsestuen (8). Bovine prothrombin, factor X_a and factor V_a were obtained as described by Lindhout et al. (9). The proteins were stored at -80°C in 50 mM Tris HCl, 175 mM NaCl, and 0.5 mg/ml human serum albumin, pH 7.9.

Protein concentrations. The molar concentration of thrombin was determined by active site titration with p-NPGb according to Chase and Shaw (10). Prothrombin concentration was determined by the same method after complete activation with E. carinatus venom. The concentration of factor V_a was determined by kinetic analysis as described by Lindhout et al. (9). Factor X_a concentrations were determined by active site titration according to Smith (11).

Phospholipid and phospholipid vesicle preparation. 1,2-Dioleoyl-sn-glycero-3-phosphoserine (PS) was prepared from 1,2-dioleoyl-sn-glycero-3-phosphocholine (PC) by enzymatic synthesis as described by Comfurius & Zwaal (12). Single-bilayer phospholipid vesicles were prepared according to the method of De Kruijff et al. (13). Phospholipid concentrations were determined by phosphate analysis as described by Böttcher et al. (14).

Isolation of human platelets. Blood was drawn by venepuncture from healthy volunteers directly into acid citrate-dextrose (0.18 M glucose-0.08 M sodium citrate-0.052 M citric acid), in the ratio of 5 volumes of whole blood to 1 volume of anticoagulant. Platelet-rich plasma was obtained by centrifugation at 220xg for 15 min. Platelets were isolated by centrifugation at 600xg for 20 min and the pellet was resuspended in 10 mM Hepes, 136 mM NaCl, 2.7 mM KCl, 2 mM MgCl_2 , 5 mM glucose and 4 mg human serum albumin (HSA)/ml, pH 6.7. The platelets were washed twice in this buffer by centrifugation at 350xg for 15 min. Before centrifugation one volume of acid citrate-dextrose was added to 14 volumes of the platelet suspension. Finally, the platelets were resuspended in Hepes buffer, 1 mg HSA/ml, pH 7.5. Platelet concentration was determined with a Coulter counter. Platelets were kept at room temperature and no loss of platelet functions was found for at least 10h.

Measurement of platelet factor V_a formation. Washed human platelets were suspended in 10 mM Hepes, 136 mM NaCl, 2.7 mM KCl, 2 mM MgCl_2 , 5mM glucose, 1 mg HSA/ml, pH 7.5. The platelet suspension ($292\ \mu\text{l}$, 4.6×10^6 platelets/ml) was incubated with 3 mM CaCl_2 and stirred (350 rev/min) for 4 min at 37°C . Subsequently, 5 μl of a thrombin solution was added, resulting in a

final volume of 0.35 ml. After different time intervals samples (20 μ l) were taken and transferred to cuvettes containing the factor V_a assay mixture. The assay mixture (205 μ l) contained factor X_a (13 pM), 20%PS -80%PC (10 μ M) and $CaCl_2$ (10 mM). The assay buffer was composed of 50 mM Tris, 175 mM NaCl, 0.5 mg HSA/ml, pH 7.9. Thrombin formation was initiated by the addition of 25 μ l of prothrombin (2 μ M). After 2 min, 0.65 ml buffer containing 50 mM Tris, 175 mM NaCl, 20 mM EDTA, 0.5 mg HSA/ml, pH 7.9 and 0.235 mM S2338 was added to the reaction mixture. The amount of thrombin formed was measured by the change in absorbance recorded on an Aminco DW-2 spectrophotometer operated in the dual wave-length mode ($\lambda_s = 405$ nm and $\lambda_r = 500$ nm). The procedure was performed at 37 $^{\circ}$ C. Standard curves were constructed by assaying dilutions of a bovine factor V_a preparation whose concentration was determined using active site titrated factor X_a (9). The assay had a functional range from 2×10^{-14} M to 5×10^{-12} M of factor V_a . Non-activated factor V had in this assay 1.2% of the activity of activated factor V.

Factor V release studies. At different time intervals, ZK 36374 was added as a small aliquot (10 μ l) to platelet suspensions incubated with thrombin. Factor V_a generation was monitored by the factor V_a assay until completion. The maximal amount of factor V_a formed was taken as the amount of factor V released at the time ZK 36374 was added to the platelet suspension.

Plasma factor V activation. Platelet-free plasma was obtained by centrifugation of platelet-rich plasma at 2000xg. Platelet-rich or platelet-free plasma was diluted in 10 mM Hepes, 136 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 5 mM glucose, 5 mM $CaCl_2$ 1mg HSA/ml, pH 7.5. The plasma dilution was stirred 4 min at 37 $^{\circ}$ C followed by addition of thrombin (0.5 nM), resulting in a final volume of 500 μ l. Subsequently, aliquots (20 μ l) were taken for factor V_a determination.

RESULTS AND DISCUSSION

Effect of ZK 36374 on thrombin-induced platelet factor V_a formation. When washed platelets (4.6×10^6 /ml) were preincubated with varying concentrations of ZK 36374 prior to stimulation with thrombin (0.5 nM) the maximal amount of factor V_a that could be formed was found to be dependent on the ZK 36374 concentration (Figure 1). At ZK 36374 concentrations of 1 nM platelet

Figure 1: Effect of ZK 36374 on thrombin-induced platelet factor V release. Platelets ($4.6 \times 10^6/\text{ml}$) were incubated with ZK 36374 (5-100 pM) for 1 minute before stimulation with thrombin (0.5 nM). Factor V_a was measured after 10 min (O) or 20 min (●). Inset: ratio of factor V_a generated in the absence of ZK 36374 over factor V_a generated in the presence of ZK 36374 as a function of the ZK 36374 concentration.

factor V_a generation was diminished to a baseline of about 2% of the control value in the absence of ZK 36374. In unstimulated platelets the baseline factor V_a activity was less than 1% of the control value. The ZK 36374 concentration required to diminish factor V_a generation to 50% of the control value, 15 pM, was determined from the intercept with the abscissa of a plot of ZK 36374 concentration versus the ratio of factor V_a formed in the absence of ZK 36374 over factor V_a formed in the presence of ZK 36374 (Figure 1, inset). In further release studies we used a ZK 36374 concentration of 1 nM.

Release of platelet factor V. Platelets ($4.6 \times 10^6/\text{ml}$) were stimulated with thrombin (0.5 nM). ZK 36374 was added in separate experiments prior to and at different time intervals after the addition of thrombin. The corresponding time courses of factor V_a generation are shown in Figure 2. It is demonstrated that the final level of factor V_a activity increased with the time interval between thrombin and ZK 36374 addition to the platelet suspension. Because ZK 36374 at this concentration (1 nM) was able to block the release reaction when preincubated with the platelet suspension, this result suggests that when ZK 36374 is added during the time course of thrombin-induced platelet factor V release, factor V release was instantaneously and completely inhibited. The increase in factor V_a formation after the addition of ZK 36374 therefore must be the result of activation of factor V that was

Figure 2: Effect of the time of ZK 36374 addition on the time course of factor V_a generation. Platelets ($4.6 \times 10^6/\text{ml}$) were stimulated with thrombin (0.5 nM) at 37°C . ZK 36374 was added 2 min prior to ($\square$) or at different time intervals after thrombin stimulation: ($\blacktriangle$) 5 s, ($\circ$) 15 s, ($\blacksquare$) 45 s, ($\triangle$) 2 min, ($\times$) 6 min ($\bullet$) no ZK 36374. Factor V_a was assayed as described under Materials and Methods.

Figure 3: Time course of thrombin-induced factor V release. Platelets were incubated at 37°C with thrombin (0.5 nM) at different time intervals followed by the addition of ZK 36374 (1 nM). Factor V was assayed as described in the text.

released, but not yet activated. Thus, the rate of factor V release can be estimated from a plot of the maximal amount of factor V_a , obtained 20 min after the addition of thrombin, versus the time of addition of ZK 36374 (Figure 3). The amount of released factor V showed a linear increase in time

during the first 30 s of the reaction. This enables us to estimate a true initial rate of factor V release of 12 pM factor V/min by 4.6×10^6 platelets/ml. It can be questioned whether ZK 36374 at a concentration of 1 nM affects platelet factor V activation and/or the factor V_a assay. To this end ZK 36374 was added at a final concentration of 100 nM to the factor V_a assay. We found that ZK 36374 had no effect on the determination of factor V_a activity. Because the rate of factor V_a generation, once the release reaction was completed, was identical whether or not ZK 36374 was present, we conclude that ZK 36374 had no effect on the platelet factor V activation reaction (Figure 2).

Obviously, our method to assess the amount of released factor V has clear advantages over other methods, where a separation is required between platelets and their supernatants. The method, however, is only applicable if there is no other effect of activated platelet constituents on our factor V_a assay, i.e. activated platelets provide a procoagulant surface by their ability to assemble the components of the prothrombinase complex. In order to avoid this effect phospholipid was present at a concentration that is sufficient to make the assay virtually insensitive for the procoagulant platelet surface. In addition, to assess the amount of platelet-bound factor V_a , platelets (4.6×10^6 /ml) were treated with 0.5 nM thrombin for 4 min and centrifuged for 1 min at 7000xg. Before and after the centrifugation, factor V_a activity was determined in the suspension and the supernatant respectively. We found that both the suspension and the supernatant contained virtually identical amounts of factor V_a (5 pM). This observation indicates that platelet-bound factor V_a must be a very small fraction of total factor V_a .

Whereas 50% of the platelet factor V is released within 20 s, half-maximal factor V_a formation was obtained only after a reaction time of 3 min (Figure 2). Apparently, the rate of thrombin-induced platelet factor V release is much faster than the rate of thrombin-catalyzed factor V activation.

Platelet factor V activation. Because the rate of factor V release is faster than the rate of factor V activation, the amount of non-activated factor V will increase rapidly during the time course of the release reaction. One can conceive that under our experimental conditions, i.e. high enzyme over substrate ratios, the rate of factor V activation is dependent on the factor V concentration. Thus, during the initial phase of the release reaction, the

rate of factor V activation will increase up to an optimal value when the release reaction is nearly completed.

Therefore, we studied the rate of factor V activation as a function of the factor V concentration. The different initial factor V concentrations were obtained by terminating the release reaction at different time intervals in thrombin-stimulated platelet suspensions of different platelet counts. When the prostacyclin analogue was added 10 or 20 s after thrombin (0.5 nM), a linear increase in factor V_a formation was observed during a subsequent time period, sufficient to release most of the platelet factor V when the release was not blocked. During that time interval, about 1 min for the different platelet concentrations, $20 \pm 5\%$ of the initial factor V was converted into factor V_a . Because the formation of factor V_a after addition of the prostacyclin analogue was linear in time, we have additional proof that the release reaction was instantaneously and completely blocked when ZK 36374 was added.

Figure 4A gives the amount of released, but yet not activated factor V as a function of the release time for the different platelet concentrations. These values were taken as the difference between total factor V_a formed (i.e. released factor V) and factor V_a formed during the release time. The rates of factor V activation at the corresponding factor V concentrations are depicted in Figure 4B. As was expected, the rates of factor V activation increased with the concentration of factor V (Figure 5). Thus, optimal rates were obtained when the release reaction reached completion. Independent of the platelet concentration we found that 10 s after initiating the reaction, the rate of factor V release was 20-fold higher than the rate of factor V activation. At the time the release reaction was nearly completed (90%) a 6-fold difference was observed.

Platelet factor V release and factor V_a generation as a function of thrombin concentration. Washed platelets (4.6×10^6 /ml) were incubated with varying concentrations of thrombin. ZK 36374 was added 2 min prior to thrombin or 3, 6, 10 and 15 s after thrombin. Initial rates of factor V release were estimated from plots of factor V_a concentration measured 20 min after the addition of thrombin, versus time of addition of ZK 36374. The initial rates of platelet factor V release as a function of thrombin concentration are given in Table I. It is apparent that the initial rate of release and rate of factor V activation are proportional to the thrombin concentration. When

Figure 4: Relationship between platelet factor V release and platelet factor V activation. Platelets ($3.8 - 25 \times 10^6/\text{ml}$) were stimulated with thrombin (0.5 nM) at 37°C .

A, the amount of released factor V after 10 s (▲), 20 s (○) and nearly completion (90%) of the release reaction (●).

B, rates of factor V_a formation after blocking the release reaction at 10 s (▲), 20 s (○) and at nearly completion (90%) of the release reaction (●).

C, rates of factor V release. Experimental details are described in the text.

Figure 5: Rate of factor V activation as a function of factor V concentration. The rates of factor V activation from figure 4B were plotted versus the corresponding initial factor V concentration from Figure 4A.

platelets were incubated with varying concentrations of thrombin in the absence of ZK 36374 we found that the final level of factor V_a increased proportionally to the thrombin concentration, reaching a maximum, 13 pM factor V_a , at about 1 nM thrombin (Table I).

When platelets (4.6×10^6 /ml) were exposed to 0.2% Triton X-100 followed by an incubation with 0.5 nM thrombin, 13 pM factor V_a was obtained. Thus at the optimal thrombin concentration (1 nM) all factor V present in the platelets was released.

It is apparent that at thrombin concentrations up to 0.6 nM a fraction of the total platelet factor V content can be released proportionally to the thrombin concentration.

Table I. Platelet factor V release and activation as a function of thrombin concentration

Platelets (4.6×10^6 /ml) were incubated with varying thrombin concentrations, at different time intervals before the addition of ZK 36374 (1nM), and in the absence of ZK 36374. The rates of factor V release, factor V activation and the amount of factor V released are measured as described in the text.

Thrombin	Rate of factor V release	Rate of factor V activation	Factor V released
nM	pM.min ⁻¹	pM.min ⁻¹	pM
0.2	2.5	0.7	4.2
0.4	8.0	1.2	8.5
0.6	16.5	3.0	12.0
0.8	27	4.0	12.5
1.0	35	5.0	13.0
1.5	58	8.0	13.2

Plasma factor V activation. An interesting question is whether the kinetics of plasma factor V activation, estimated under the conditions of platelet factor V activation, differ from those of platelet factor V activation. To this end different dilutions of platelet-free and platelet-rich plasma were incubated with 0.5 nM thrombin. At 30-s intervals up to 5 min, samples were removed and assayed for factor V_a activity. Factor V_a formation was found to be linear in time up to 2 min. The corresponding factor V concentrations in the dilutions were estimated from the factor V_a activities after complete activation. The results are depicted in Table II. Human plasma factor V concentration as determined by our factor V_a assay was found to be 6 nM. This value is 30-40% of that reported by Tracy et al. (15).

Table II. Rate of plasma factor V activation.

Platelet rich plasma (PRP) containing 2×10^8 platelets/ml and platelet free plasma (PFP) were diluted in 10 mM HEPES, 136 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 5 mM $CaCl_2$, 5 mM glucose and 1 mg HSA/ml, pH 7.5. Factor V activation was initiated by adding thrombin (0.5 nM).

	Factor V concentration		Rate of factor V activation		Rate. $[V]^{-1}$	
	(pM)		(pM. min ⁻¹)		(min ⁻¹)	
	PRP	PFP	PRP	PFP	PRP	PFP
Plasma dilution						
1 : 113	50	40	15.0	12.1	0.30	0.30
1 : 300	16	19	6.0	5.6	0.37	0.30
1 : 600	11	9	3.4	2.6	0.27	0.29
1 : 1200	5.4	4.5	1.9	1.5	0.35	0.33

It is also shown that the rate of factor V activation is proportional to the plasma factor V concentration. Apparently the rate of factor V activation in platelet-free plasma is identical to that in platelet-rich plasma. This

excludes a role of platelet constituents in factor V activation. Because of inter-individual variations we compared the kinetics of plasma factor V activation to those of platelet factor V activation using washed platelets from the same donor. The results show that the rates of platelet factor V activation, when normalized for the factor V concentration, were similar to those of plasma factor V activation (Table III). According to our bioassay, the amount of factor V that is provided by the platelets in platelet-rich plasma is about 10% of the total factor V concentration. This value is somewhat lower than that reported by Tracy et al. (15) and is in agreement with the findings of Chiu et al. (16).

Immunological identity between platelet and plasma factor V has been shown in human species (15,17,18), bovine species (19,20) and recently in guinea pig between plasma factor V and a precursor form synthesized in the megakaryocytes (16). Whereas in the latter case functional differences were found after exposure to thrombin, in other reports the same quotient of activation by thrombin was found between platelet and plasma factor V (1,2).

Table III. Rate of platelet factor V activation.

Washed platelets were prepared from the platelet rich plasma of Table 2. The rate of factor V activation by 0.5 nM thrombin was determined at the time the release reaction was nearly completed (90%). The factor V concentration at that time point was determined as described in the legends to Figure 4.

Platelet concentration	Factor V concentration	Rate of factor V activation	Rate. $[V]^{-1}$
10^{-6} x counts.ml $^{-1}$	pM	pM.min $^{-1}$	min $^{-1}$
31	45	10.0	0.22
12	16	5.0	0.30
6	10	3.0	0.31
3	6	2.3	0.38

CONCLUSION

In order to assess the time course of platelet factor V release by a bioassay it is important to separate the effect of thrombin on the release of platelet factor V from its effect on factor V activation because the rate limiting step in thrombin-induced platelet factor V_a formation is the thrombin-catalyzed activation of the released non-activated factor V. By utilizing the stable prostacyclin analogue ZK 36374 we could demonstrate that, in contrast to previous reports (1,2), the rate of platelet factor V release is as fast as the rate of release of another protein present in the platelet alpha-granules, i.e. beta-thromboglobulin (21). Our method is useful in the assessment of the kinetics of release of other proteins present in the alpha-granules and in the investigation of platelet disorders characterized by an impaired release process and/or storage defect.

Our findings on the kinetics of platelet factor V release and activation (platelet factor V_a generation) and plasma factor V_a formation raise questions as to the role of platelet factor V in the regulation of thrombin formation at the platelet surface. We found that the rate of activation of platelet factor V once it is released is identical to that of plasma factor V. Taking into account that 1) the rate of platelet factor V release and the amount that can be released depend on the thrombin concentration and 2) factor V present in the platelets is about 10% of that in plasma, it appears that factor V_a formation from plasma factor V is quantitatively far more important than factor V_a formation from platelet factor V.

However, one should bear in mind that at a site of vascular injury platelets do aggregate and as a result might provide locally high platelet factor $V_{(a)}$ concentrations. It has been reported that the absence of platelet factor V rather than plasma factor V activity appeared to be associated with a severe bleeding disorder (7,22). On the other hand, patients with a Gray platelet syndrome have a moderate bleeding disorder despite thrombocytopenia (23), a platelet factor V content that is about 20% of normal when expressed as factor V concentration per platelet count per ml and a severely impaired platelet factor V release reaction. However, those patients have an almost normal plasma factor V level (D. Baruch, T. Lindhout, E. Dupuy and J. P. Caen, unpublished results).

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CHAPTER III

THROMBIN-INDUCED PLATELET FACTOR V_a FORMATION IN PATIENTS WITH A GRAY PLATELET SYNDROME

SUMMARY

The contribution of human platelet factor V_a generation to the regulation of thrombin formation is not yet clear. We report a study on factor V_a formation in plasma and platelets of three patients with a mild bleeding disorder ascribed to a gray platelet syndrome. This inherited platelet disorder has been characterized by the absence of alpha-granules as observed by electron microscopy, and by specific deficiencies of alpha-granule proteins.

We found that the concentration of plasma factor V was slightly decreased (70% of normal values) but the rate of thrombin-catalyzed factor V activation was normal. In contrast, platelet factor V_a formation was severely impaired. Besides a much lower factor V content than in control platelets (6-20% of normal), the dependency of platelet factor V_a formation on thrombin concentration was altered. Increasing the thrombin concentration 4-fold compared to the concentration that results in optimal factor V_a concentration in normal platelets did not result in a maximal factor V_a formation in gray platelets. However, we have demonstrated that this factor V_a formation is the result of the thrombin-induced release reaction.

It is our conclusion that the almost normal plasma factor V_a formation in those patients compensates the markedly reduced functional platelet factor V_a formation and as such could account for the absence of a severe impairment of hemostasis.

INTRODUCTION

Factor V can be released from platelet alpha-granules after stimulation by a variety of agents like thrombin and collagen. In order to function as a cofactor in prothrombin activation, factor V requires to be activated by thrombin (1-3). The function of platelet factor V is not yet clearly established. We have recently developed a bioassay that allowed us to separately assess the kinetics of thrombin-induced platelet factor V release from those of platelet factor V activation (4). We have shown that all factor V stored in normal platelet suspensions (5×10^6 /ml) could be released by an optimal thrombin concentration (1 nM). Human platelet factor V represents 10 to 20% of plasma factor V (3-5). We found that the kinetics of thrombin-catalyzed platelet factor V activation were identical to those of plasma factor V activation (4). However, the crucial importance of platelet factor V in maintaining normal hemostasis was suggested by Miletich et al. (6), who showed a close relationship between the severity of bleeding and the extent of platelet factor V deficiency among a group of patients with a plasma factor V deficiency.

The gray platelet syndrome is a hereditary platelet disorder characterized by the almost complete absence of alpha-granules in the megakaryocytes and in the platelets (7,8). Cytochemical studies show that alpha-granules are present in the megakaryocytes at an immature state but are unable to mature. Thus, the compounds normally present in alpha-granules are synthesized but are discharged in distended demarcation membranes or vacuoles (9). Nurden et al. (10) demonstrated that up to 10% of the normal concentrations of the following proteins were present in the gray platelets: fibrinogen, fibronectin, thrombospondin, von Willebrand factor, platelet factor 4 and beta-thromboglobulin, whereas 50% of the normal albumin content was found.

The functional factor V concentration is not clearly established in gray platelets. Vicic et al. (3) showed in a patient with a combined deficiency of dense and alpha-granules, that platelet factor V activity was 50% of control values in frozen-thawed platelets as well as after stimulation by collagen. In a recent study, nearly normal amounts of factor V antigen were detected in lysed platelets of a patient with a gray platelet syndrome (11). However, the functional factor V concentration was not determined in that study.

The gray platelet syndrome is clinically characterized by a mild bleeding disorder, mainly of the type of a primary hemostasis abnormality of platelet adhesion or aggregation. However, two symptoms that have not been clearly described suggest a coagulation abnormality: a questionable recurrent hemarthrosis in one patient (7) and spontaneous hematoma in two others (12). This platelet disorder thus offers a possibility to further understand the contribution of platelet factor V in the efficiency of the hemostatic process.

In the present study we report on factor V_a formation in three patients with a gray platelet syndrome. We studied lyzed platelet suspensions, thrombin-activated washed platelet suspensions as well the kinetics of plasma factor V_a formation.

MATERIALS AND METHODS

S2238 was purchased from AB Kabi Diagnostica. Human serum albumin (fatty acid free) was from Sigma. ZK 36374, a stable carbacyclin derivative of prostacyclin was obtained from Schering (Berlin). Human proteins (prothrombin and factor X_a) were a kind gift from Dr. H. Soons. Purification procedures for human factor X and prothrombin were essentially the same as described earlier (13). Protein concentration determinations and phospholipid preparation were performed as previously reported (4).

Case history*. Three patients were investigated, two siblings H.B. and M.S., and a third unrelated patient B.O. The detailed case histories of the two siblings have been reported previously (12). Both suffered from epistaxis episodes and presented one severe hemorrhagic complication secondary to obstetrical or surgical interventions. The third patient, a woman aged 26 yr, had experienced mild bleeding episodes, essentially epistaxis that on one occasion required a blood transfusion. She had a miscarriage without any excessive bleeding. All patients had a thrombocytopenia at the time of the present study (circulating platelet counts 40,000/ μ l (M.S.), 90,000/ μ l (H.B.) and 60,000/ μ l (B.O.).

* This investigation was performed according to the Declaration of Helsinki and informed consent was obtained.

Isolation of Human Platelets. Blood was drawn by venepuncture directly into acid citrate-dextrose (0.18 M glucose-0.08 M sodium citrate-0.052 M citric acid), in the ratio of 5 volumes of whole blood to 1 volume of anticoagulant. PGE_1 was added (7 ng/ml). Normal platelet-rich plasma was obtained by centrifugation at 220xg for 15 min. Platelet-rich plasma from the patients was obtained by allowing whole blood to sediment for 1 hour at room temperature in tubes tilted at an angle of 45 degrees.

Platelets were isolated from platelet-rich plasma by centrifugation at 600xg for 20 min and the pellet was resuspended in 10 mM Hepes, 136 mM NaCl, 2.7 mM KCl, 2 mM MgCl_2 , 5 mM glucose and 1 mg human serum albumin (HSA)/ml, pH 6.7. The platelets were washed twice in this buffer by centrifugation at 350xg for 15 min. Before centrifugation one volume of acid citrate-dextrose was added to 14 volumes of the platelet suspension. Finally, the platelets were resuspended in the aforementioned Hepes buffer, 1 mg HSA/ml, pH 7.5. Platelet concentration was determined with a Coulter counter.

Measurement of Platelet Factor V_a Formation. Washed platelets (5×10^7 /ml) in Hepes buffer were incubated with thrombin at 37 °C. Factor V_a formation was determined by a factor V_a assay as previously described (4) with the modification that instead of bovine factors, human factor X_a and human prothrombin were used. Estimations of the factor V_a concentrations were obtained as follows. Maximal rate of thrombin formation, $v_{\max} = 48$ nM/min, is obtained when all factor X_a present, 16 pM, is saturated with factor V_a . At factor V_a concentrations far from saturation, the rate of thrombin formation, v , is proportional to the factor V_a concentration. Thus, the factor V_a concentration in the assay mixture can be calculated from $v/v_{\max}$ multiplied by the factor X_a concentration.

Measurement of plasma factor V_a formation. Platelet-free plasma was obtained by centrifugation of platelet-rich plasma at 2000xg. Serial dilutions (1:150, 1:300, 1:600) of platelet-free plasma were made in 10 mM Hepes, 136 mM NaCl, 2.7 mM KCl, 2 mM MgCl_2 , 5 mM glucose, 5 mM CaCl_2 , 1 mg HSA/ml, pH 7.5. The plasma dilutions were incubated at 37 °C for 4 min, followed by the addition of thrombin (1 nM). Aliquots were taken for factor V_a determination. The initial rate of factor V_a formation was determined during 2 min following the addition of thrombin. Final factor V_a concentration in plasma was determined after 16 min incubation of the

diluted plasma with thrombin, multiplied by the dilution factor. The factor V_a concentration in control plasma was obtained by assaying dilutions of platelet-free pooled plasma obtained from 50 human volunteers.

Factor V_a formation in lyzed platelets. Platelet suspensions (2×10^7 /ml) were incubated for 10 min with Triton X-100 (0.2%) in the presence of calcium (5 mM). Factor V_a formation was determined in Triton-treated platelet suspensions after the addition of thrombin (2 nM).

RESULTS

Plasma factor V activation. Factor V_a formation in several dilutions of platelet-free plasma of two patients was monitored after addition of 1 nM alpha-thrombin. Table I shows the final factor V_a concentrations measured for each dilution at complete activation of factor V by thrombin (16 min) and multiplied by the dilution factor. The variation between the different dilutions was within 10% and the results of 3 determinations were averaged. Factor V concentration in normal pooled plasma was found to be 5 nM. This value is in agreement with previously reported values of human factor V plasma concentration as determined with a bovine assay system (4).

Table I

<u>Plasma Factor V_a formation</u>		
Plasma	Final Factor V_a concentration (nM)	Initial rate/Factor V_a concentration (nM f V_a /min/nM fV)
Control	4.8	0.41
Patient H.B.	3.5	0.31
Patient B.O.	2.9	0.40

Plasma dilutions were assayed for factor V_a formation after activation by thrombin (1 nM). The initial rate of factor V_a formation and the factor V_a concentration were determined as described in Material and Methods.

Figure 1: Time course of thrombin induced platelet factor V_a formation. Washed platelets ($5 \times 10^7/\text{ml}$) were incubated at 37°C with thrombin (2 nM). Samples were taken in time and assayed for factor V_a activity. Control (●); patient H.B. (■); patient M.S. (▲); patient B.O. (Δ).

In the patients H.B. and B.O. plasma factor V concentrations were found to be 73% and 61% of control value, respectively. Furthermore, when the initial rate of factor V activation is normalized for the factor V concentration, it is apparent that this rate is the same (patient B.O.) or slightly lower (patient H.B.) than found in normal plasma (Table I). Platelet factor V_a formation. Washed platelet suspensions ($5 \times 10^7/\text{ml}$) were incubated with thrombin (2 nM). Figure 1 shows the time courses of factor V_a formation in the patients' and control platelets. Whereas in control platelets, the initial rate of factor V_a formation was 30 pM/min and the final factor V_a concentration was 75 pM, both values were markedly reduced

Figure 2: The dependency of factor V_a formation from gray platelets on the thrombin concentration. Platelet suspensions ($5 \times 10^7/\text{ml}$) were incubated with varying thrombin concentrations. Platelet factor V_a formation was determined after complete activation by thrombin (usually 15 min). Control (●); patient H.B. (■); patient M.S. (▲).

in suspensions of gray platelets. It is shown that in patients M.S. and B.O., factor V_a formation is almost negligible (1 pM in 30 min), whereas in patient H.B. 5 pM factor V_a was formed in 30 min.

Effect of thrombin concentrations on platelet factor V_a formation.

We determined platelet factor V_a formation in control, patients H.B. and M.S. as a function of the thrombin concentration. The results are shown in Figure 2. Whereas maximal factor V_a formation was obtained in control platelets at 2 nM thrombin, we found that factor V_a formation from gray platelets still increased at thrombin concentrations higher than 2 nM. Furthermore, with varying thrombin concentrations, the final amount of generated factor V_a was proportional to the initial rate of factor V_a formation in patient H.B.'s platelets (Figure 3).

Effect of the prostacyclin analogue ZK 36374 on platelet factor V_a formation.

We have previously demonstrated that the prostacyclin analogue ZK 36374 is a potent inhibitor of the thrombin-induced release of platelet factor V, but has no effect on the thrombin-catalyzed activation of factor V. When control platelets are incubated with ZK 36374 (1 nM) 3 min prior to the addition of thrombin, platelet factor V_a formation is reduced to 2% of the activity measured in the absence of ZK 36374 (4). Because of the low factor V_a activity that could be generated from the gray platelets, it can be questioned whether this amount is the result of the release reaction or

Figure 3: Initial rate of factor V_a formation and total factor V_a formation as a function of thrombin concentration. Platelet suspensions (5×10^7 /ml) from patient H.B. were incubated with increasing thrombin concentrations.

The initial rate of factor V_a formation (■) and the total factor V_a formation after 15 min (●) were determined as described in Material and Methods.

arose, for instance, from a contamination with plasma factor V. To this end, suspensions of gray platelets were incubated with ZK 36374 (1 nM) prior to the addition of thrombin (2 nM). Compared to the value measured in the absence of ZK 36374, we found a reduced factor V_a formation in the three patients' platelets. Because of the low factor V_a activities in the thrombin-treated platelet suspensions of patients B.O. and M.S., no firm conclusion can be drawn from those analyses. However, a 10-fold lower factor V_a activity was found in the ZK 36374 treated platelets of patient H.B. This finding indicates that factor V_a formation is the result of the thrombin-induced release reaction and subsequent activation of factor V.

Factor V_a formation in lyzed platelet suspensions. Total platelet factor V content was determined in 0.2% Triton X-100 lyzed platelet suspensions (2×10^7 /ml), after a further incubation with thrombin (2 nM). Factor V_a concentration in control platelets was 52 pM. Less than 10% of this value was obtained in patients H.B. (8%) and M.S (6%). However, 20% of the control value was found in patient B.O.

DISCUSSION

Platelet factor V_a formation was studied in suspensions of gray platelets in order to assess the amount of functional platelet factor V. It has been reported that most of the proteins normally present in alpha-granules are reduced 1 to 10% of the control values in gray platelets (10). Our findings on three gray platelet patients indicate that factor V is also reduced to 6%-20% of normal values as determined in Triton-treated platelet suspensions. In addition, we have demonstrated that the factor V_a formation can be induced by thrombin, but at least 4-fold higher thrombin concentration are required than for optimal release and activation of factor V from control platelets. Our observation that a prostacyclin analogue, a potent inhibitor of the platelet release reaction, was able to block the thrombin-induced gray platelet factor V_a formation, strongly suggests the presence of a factor V storage organelle rather than the localization of factor V in the cytoplasm of the gray platelet. In view of the low content in the proteins normally stored in the alpha-granules and the impaired release reaction, it is interesting to note that a small population of gray

platelets might contain immature (precursors of) alpha-granules (12). Our data on platelet factor V release are in good agreement with those of Levy-Toledano et al. (12) on the release of another alpha-granule protein, beta-thromboglobulin. As the same patients were described as in the present study, a direct comparison of the data is possible. In patients H.B. and M.S. respectively, 15% and 8% of the beta-thromboglobulin concentration of control platelets were obtained at a thrombin concentration of 1 NIH U/ml (10 nM) in patient H.B., and of 0.1 NIH U/ml (1 nM) in patient M.S. Unfortunately, the dependency of beta-thromboglobulin release on the thrombin concentration which might account for the different beta-thromboglobulin values was not reported. In addition, these authors demonstrated that platelet aggregation and serotonin release required higher concentrations of thrombin in the patients' than in control platelets (12). If one considers that the role of fibrinogen, and most probably of the platelet-fibrinogen, has been shown in thrombin-induced aggregation of normal platelets (14), it is possible to relate the platelet dysfunctions of gray platelet patients to an impaired release of proteins normally present in alpha-granules.

Our findings on the factor V content in gray platelets are in contrast with those reported by Tracy et al. (11). These authors claimed an almost normal content of factor V antigen. However, because the platelets were lyophilised, they were unable to assess the factor V content by a bioassay. One should bear in mind that a slight contamination with plasma factor V can account for this discrepancy.

Several studies have indicated that a severe bleeding disorder after trauma correlates with the absence of functional platelet factor V rather than with factor V levels in plasma (6,11). However, we believe that a straight forward conclusion as to the important role of platelet factor V over plasma factor V cannot be drawn from these studies. Firstly, a certain degree of dysfunctional plasma factor V (11) or reduced plasma factor V levels (6) was found concomitantly to the dysfunctional platelet factor V or reduced platelet factor V levels. Secondly, our results indicate that when platelet factor V_a formation is almost absent, and plasma factor V_a formation is nearly normal, the clinical symptoms can be characterized as a mild bleeding disorder. A particularly interesting observation is that when gray platelets are stimulated by thrombin and collagen, no abnormality was

found in their prothrombin-converting activity determined in the presence of exogenous factor V_a (15). That is, upon stimulation with thrombin and collagen, gray platelets appeared to generate a normal procoagulant surface. Because plasma factor V in these patients seems to account for an almost normal functional factor V_a activity, it seems unlikely that a quantitative platelet factor V disorder causes a severe impairment of hemostasis.

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CHAPTER IV

INHIBITION OF THROMBIN CATALYZED REACTIONS IN BLOOD COAGULATION AND PLATELET ACTIVATION. Effect of heparin and low molecular weight heparins in the absence of antithrombin III.

SUMMARY

The antithrombin III-independent effect of heparin was studied in the following thrombin-catalyzed reactions: activation of purified plasma factor V and partially purified plasma factor VIII:C, generation of factor V_a from the platelets and, in the presence of collagen, generation of platelet pro-coagulant activity. Three heparin fractions and a heparinoid were compared to crude heparin. The inhibitory effect of heparin (fractions) was quantitated by the concentration required to cause 50% inhibition of the initial rate of the thrombin-catalyzed reactions (IC₅₀).

Crude heparin was a more potent inhibitor of these reactions than the fractions or the heparinoid. The inhibitory action of the heparins was found to be the result of a direct effect on thrombin and not of an effect either on platelet activation functions or on the assembly or functioning of the prothrombinase complex. Probably this heparin inhibition is due to the masking of secondary macromolecular substrate binding sites on the thrombin molecule. We found no correlation between IC₅₀ values and the antithrombin III-dependent, anti-thrombin specific activities of the heparins. This supports the notion that besides its affinity for antithrombin III, heparin possesses additional functional properties that may contribute to its action in blood coagulation, as shown in the present study by the inhibition of the positive feedback reactions catalyzed by thrombin.

INTRODUCTION

Heparin is an effective antithrombotic agent, widely used in the treatment and prophylaxis of venous thrombosis and thromboembolism. However, its clinical use is limited by its major side effect, bleeding. For many years, it was generally assumed that both the antithrombotic and hemorrhagic

properties of heparin were related to its ability to augment the reactions between activated coagulation factors and antithrombin III. More recently, numerous comparative studies on the hemorrhagic, antithrombotic and anticoagulant effects of heparinoids and low molecular weight heparin fractions, prepared from crude heparin by a variety of methods, indicate that: 1) low molecular weight heparins with an anti-factor X_a to anti-thrombin ratio larger than that of crude heparin, have similar antithrombotic activities but produce less hemorrhage (1), 2) The anti-factor X_a activity of heparin is not related to its antithrombotic activity (2,3), 3) The excess low affinity material present in heparin with a low affinity to antithrombin III increases hemorrhage, without having either in vivo antithrombotic or ex vivo anticoagulant activity (4) and 4) Dermatan sulfate, known to potentiate only the activity of heparin cofactor II on thrombin, has an antithrombotic activity equal to that of crude heparin but leaves hemostasis largely unaffected (5).

These studies illustrate the difficulty in attempting to correlate in vitro assays and in vivo blood changes. It can be questioned whether one has to search for other, more specific markers of the anti-thrombin effect of heparin, that could be either an antithrombin III-dependent or independent effect. In this context it is important to note that coagulation and platelet reactions are strongly coupled by a positive feedback mechanism because thrombin-induced platelet activation generates procoagulant activities. This thrombin-dependent mechanism leads to an important amplification of thrombin generation and so of both hemostatic plug and thrombus formation.

The present study was undertaken to determine to what extent heparin (fractions) inhibit the thrombin-dependent feedback reactions in factor VIII and factor V activation, as well as in the formation of platelet procoagulant activities and to investigate whether heparin (fractions) exert such an anti-thrombin activity through a mechanism that is independent of antithrombin III.

MATERIAL AND METHODS

Materials. S 2238 (H-D-Phe-Pip-Arg-pNa) was purchased from AB Kabi Diagnostica, Stockholm, Sweden. Fatty acid free human serum albumin (HSA), Russell's Viper Venom and Echis Carinatus venom were obtained from Sigma.

Horse tendon collagen was obtained from Hormon-Chemie München, Germany. It was added in the non-fibrillar form dissolved in acidic dilution buffer obtained from Hormon-Chemie. Reaction tubes were 2 ml plastic flat-bottom tubes obtained from Sterilin Ltd (Teddington, England). Teflon-coated magnetic spinning bars 7x2 mm were from Bel-Art Products, USA. Phosphatidylcholine (18:1 cis/18:1 cis phosphatidylcholine) was from Sigma, USA. All reagents used were of the highest grade commercially available.

Preparation of Phospholipids and Phospholipid Vesicles. Phosphatidylserine (18:1 cis/18:1 cis) was prepared from phosphatidylcholine by enzymatic synthesis according to the method of Comfurius and Zwaal (6). Single bilayer vesicle solutions were prepared as previously described (7). Phospholipid concentrations were determined by phosphate analysis according to Böttcher et al. (8).

Proteins. Purification of factor VIII:C was performed by differential polyethyleneglycol precipitation. Bovine prothrombin, factor X, factor X_a , factor V and factor V_a were purified according to established procedures (9). Human and bovine α -thrombin were prepared as described by Pletcher et al. (10). The specific clotting activity was 2,800 NIH U/mg for human thrombin and 2,200 NIH U/mg for bovine thrombin. The protein preparations were stored at -70°C after dialysis against 50 mM Tris/HCl, 175 mM NaCl at pH 7.9.

Protein Concentrations. Concentrations of thrombin and factor X_a were determined by active site titration with p-nitrophenyl-p-guanidinobenzoate hydrochloride (11). Prothrombin concentrations were determined after complete activation with E.carinatus venom followed by active site titration. Similarly, factor X concentrations were obtained by active site titration after complete activation with factor X - converting protein from Russell's viper venom (11). Factor V_a concentrations were determined by kinetic analysis (9).

Heparin and Heparin Fractions. Crude porcine intestinal mucosal heparin (MW 1,500-30,000, mean MW 15,000, anti- X_a and anti-thrombin activity 175 U/mg) was obtained from Organon Laboratories (Oss, the Netherlands). The following low molecular weight compounds were prepared from standard heparin by Choay Institute (Paris): CY 216 was obtained by ethanol extraction (mean MW 4,500, USP activity 50 U/mg, anti- X_a activity 200 U/mg, anti-thrombin activity 10-20 U/mg) and CY 222 was obtained by nitrous acid depolymerization (mean MW 2,500, USP activity 25 U/mg, anti- X_a activity 250 U/mg, anti-thrombin activity 5-15 U/mg). Org 10172, a sulphated mucopoly-

saccharide was isolated by Organon Laboratories from porcine intestinal mucosa (mean MW 6,400, USP activity 2.94 U/mg, anti-X_a activity 8 U/mg, anti-thrombin activity 0.32 U/mg). We also used the fragment PK 10169 (Pharmuka laboratories) obtained by controlled depolymerization (mean MW 4,500, anti-X_a activity 120 U/mg, anti-thrombin activity 30 U/mg).

Isolation of Human Platelets. Blood was drawn by venepuncture from healthy volunteers who had not taken any medication for at least one week. As anticoagulant, one volume of acid citrate/dextrose (0.18 M glucose/0.08 M sodium citrate/0.052 M citric acid) was added to 5 volumes of whole blood. Platelet-rich plasma was obtained after centrifuging at 220xg for 15 min. Platelets were isolated by centrifuging at 600xg for 20 min and the pellet was resuspended in a Hepes buffer containing 136 mM NaCl, 2.68 mM KCl, 2 mM MgCl₂, 10 mM Hepes, 5 mM glucose and 0.4% HSA, pH 6.7. The platelets were washed twice in this buffer by centrifuging at 350xg, 15 min. Before centrifuging acid citrate/dextrose was added to the suspension (1 vol. to 14 vol.). Finally the platelets were resuspended in the Hepes buffer, pH 7.5 containing 0.1% HSA. The entire procedure for platelet preparation took about 2h and was carried out at room temperature (22 °C). Platelet concentration was determined with a Coulter counter. Platelets were kept at room temperature and no loss of platelet functions was found for at least 15h.

Partially purified factor VIII:C activation. Partially purified factor VIII:C was activated by bovine thrombin in the following way: factor VIII:C (20 µl, 27 U/ml) was incubated in 1 ml Michaelis buffer containing 29 mM sodium barbiturate, 29 mM sodium acetate, 116 mM NaCl pH 7.3. The reaction was started with the addition of thrombin (3 nM). Aliquots (10 µl) of factor VIII_a containing sample were added to the clotting assay containing: 60 µl factor VIII deficient plasma, 4 nM factor IX_a, 50 µM phospholipid [(25% phosphatidylserine (PS), 75% phosphatidylcholine (PC)] and 10 mM CaCl₂. The clotting time was recorded on an automatic coagulometer. A calibration curve was constructed with normal pool plasma activated with 9 nM thrombin, in order to determine the amount of factor VIII_a present in the sample.

Purified plasma factor V activation. Bovine factor V (100 nM) was incubated for 5 min at 37 °C in the presence of 3 mM CaCl₂. The reaction was started by the addition of thrombin (usually 0.5 nM). Aliquots were taken in time, diluted 1:1000 in buffer containing 50 mM Tris, 175 mM NaCl, 5 mM CaCl₂, 0.5 mg/ml HSA, pH 7.9. Factor V_a activity was measured by incubation of the aliquots (usually 20 µl) with factor X_a (1.3x10⁻¹¹ M), phospholipid

containing 20% phosphatidylserine and 80% phosphatidylcholine (1.0×10^{-5} M) and CaCl_2 (10 mM) in a final volume of 0.225 ml of the aforementioned buffer for 5 min at 37°C in a plastic cuvette. The reaction was initiated by the addition of 25 μl of prothrombin (2.0×10^{-6} M). After 2 min, 0.65 ml of the same buffer containing 20 mM EDTA and 0.24 μmol S 2238 was added to the reaction mixture. The amount of thrombin formed was measured by the change in absorbance recorded on an Aminco DW2 spectrophotometer operating in the dual wave-length mode ($\lambda_s=405$ nm and $\lambda_r=500$ nm) thermostated at 37°C . Standard curves were constructed by assaying dilutions of a bovine factor V_a preparation whose concentration was determined utilizing active site titrated factor X_a (9). The assay had a functional range from 2×10^{-14} M to 1×10^{-12} M of factor V_a .

Determination of Platelet Factor V Release and Activation. 292 μl of platelet suspension ($5.5 \times 10^6/\text{ml}$) were incubated with 13 μl of 75 mM CaCl_2 and stirred (350 revolutions/min) for 3 min at 37°C followed by the addition of thrombin, resulting in a final volume of 350 μl . Subsequently, aliquots were taken and assayed for factor V_a activity as described.

Platelet Beta-Thromboglobulin Release. To a reaction tube containing 292 μl of a platelet suspension ($5 \times 10^7/\text{ml}$), 13 μl of 75 mM CaCl_2 was added. The tube content was stirred for 5 min at 37°C and platelet activation was started by addition of a platelet activator (thrombin or collagen) resulting in a final volume of 350 μl . After a variable time periode at 37°C , the release reaction was stopped by adding to the platelet suspension 56 μl Hepes buffer containing 135 mM formaldehyde, 100 mM EDTA, and bringing it to a melting ice-bath (12). Platelets were centrifuged for 1 min in an Eppendorf centrifuge 5412, 200 μl of the supernatant was carefully removed, to which 50 μl of a 2 M Tris solution was added to neutralize the excess of formaldehyde. The sample was diluted 1:3 in Hepes buffer pH 7.5 containing 20 % of horse serum and the amount of β -thromboglobulin was measured using the radioimmunoassay kit from Amersham International Limited according to the instruction of the manufacturer.

Determination of Platelet Prothrombin Converting Activity (PPCA). 292 μl of platelet suspension ($5 \times 10^6/\text{ml}$) were incubated with 13 μl of 75 mM CaCl_2 and stirred for 3 min at 37°C . Platelet stimulation was started by adding thrombin or collagen plus thrombin resulting in a final volume of 325 μl and a CaCl_2 concentration of 3 mM. After a variable time of stirring at 37°C (i.e. platelet trigger time) the PPCA was determined as follows. 50 μl factor V_a (10 nM) and 50 μl factor X_a (5 nM) were added to the triggered

platelet suspension, to allow equilibration with the platelets. After 2 min period of preincubation, prothrombin activation was started with the addition of 75 μ l of a prepared mixture containing 6.6 μ M prothrombin and 27 mM CaCl_2 in 50 mM Tris, 175 mM NaCl and 0.5 mg/ml HSA, pH 7.9, to result in a final volume of 0.5 ml. The final concentrations of the reactants were: 0.5 nM factor X_a , 1 nM factor V_a , 1 μ M prothrombin and 6 mM CaCl_2 . After 30 and 60 s aliquots (usually 10 μ l) were taken from the reaction mixture to determine the amount of thrombin formed.

Analysis of the Data. We assume that thrombin forms a complex with heparin and that such a complex has no enzymatic activity. The reversible association between heparin and thrombin is expressed as:

$$K_d = [H][IIa]/[H.IIa] \quad (2)$$

where $[H]$ is the free heparin concentration and $[IIa]$ is the free thrombin concentration. Because heparin is present in excess over thrombin, $[H]$ can be equaled to the total heparin concentration. Under conditions where the initial rate of factor V_a generation or PPCA generation (v) are directly proportional to the free thrombin concentration:

$$v = k[IIa] = k([IIa]_0 - [H.IIa]) \quad (3)$$

where $[IIa]_0$ is the total thrombin concentration. From (2) and (3) we find:

$$v/(v_0 - v) = K_d/[H] \quad (4)$$

where $v_0 = k[IIa]_0$, is the initial rate of factor V_a or PPCA generation in the absence of heparin. A plot of $v/(v_0 - v)$ versus the reciprocal heparin concentration gives a straight line with slope K_d . Since K_d is the amount of heparin required to bind half the thrombin, the K_d value can also be expressed as IC_{50} , the amount of heparin causing 50 % inhibition.

RESULTS

Effect of heparin on protein-phospholipid interaction. First of all we had to exclude any effect of heparin carried over into the assay mixtures. Because an effect of heparin on protein-phospholipid interaction has been reported (13) we had to assess whether or not this effect of heparin occurred under our experimental conditions. To this end we used a protein-lipid equilibrium binding technique according to van de Waart et al. (14). Factor X_a (0.1 μM), factor V_a (0.1 μM), or prothrombin (0.05 μM) were incubated with large volume phospholipid vesicles (20 % PS - 80 % PC, 25 μM) in 50 mM Tris, 100 mM NaCl, pH 7.5 at 20 °C. After centrifugation for 30 min at 30,000 g at 20 °C the concentrations of the bound proteins were estimated by determination of the remaining activity in the supernatant. Both crude heparin and PK 10169 at a final concentrations of 0.5 mg/ml, could not displace factor V_a , factor X_a , or prothrombin from the phospholipid surface. The concentrations of bound protein in the presence and in the absence of heparin (fraction) were: 0.02 μM , 0.10 μM , 0.03 μM for factor X_a , factor V_a and prothrombin, respectively.

Effect of heparin on factor VIIIa assay. The effect of heparin on thrombin catalyzed factor VIII activation was measured in a clotting assay using a factor VIII deficient plasma, therefore heparin was carried over and indeed influenced the clotting times. This problem was circumvented by making calibration curve for factor VIII_a formation in the presence of the same amounts of heparin as those that were carried over. In that way heparin did not affect the relative changes occurring during the thrombin-catalyzed factor VIII activation.

Effect of heparin on prothrombinase activity. In all other studies we used a completely purified system, in which no effect of heparin carried over into the assay was observed. This was demonstrated as follows: at a time the reaction of factor V_a generation, or platelet prothrombin converting activity generation reached completion, heparin was added immediately before transferring a sample to the measuring system. No effect of heparin or heparin fractions at final concentrations of up to 0.5 mg/ml was observed after a 10 min incubation, showing that the amount of factor V_a activity or PPCA was independent of the presence of heparin in the activation mixture (Table I). This demonstrates that heparin did not interfere with the assembly or the functioning of the prothrombinase complex.

Table I The effect of heparin on prothrombinase activity

Compound		Rate of thrombin formation (nM/min)	PPCA (%)
None		24.0	100
Heparin	(0.64 mg/ml)	24.0	100
CY 216	(1 mg/ml)	22.4	92
CY 222	(1 mg/ml)	23.4	96
Org 10172	(1 mg/ml)	26.3	108

Demonstration of the absence of antithrombin III in the platelet suspensions. In the reactions where platelets are involved, we had to rule out the presence of antithrombin III in the washed platelet preparations (15). This was done by incubation of washed platelet suspension (10^7 /ml) with 5 nM thrombin in the presence of heparin (fractions) (10 μ g/ml). After 20 min, the amidolytic activity of thrombin appeared to be unaffected.

Effect of heparin (fractions) on thrombin-catalyzed plasma factor VIII activation. We used a semi-quantitative estimation of the effect of heparin on thrombin-catalyzed factor VIII activation. Activated factor VIII is the cofactor of factor IX_a in the intrinsic factor X activation reaction, whereas non-activated factor VIII has very little activity. In an assay where excess phospholipid is present, factor VIII_a activity is the rate limiting factor of the factor X_a formation. Thus the initial rate of factor VIII activation is proportional to the rate of factor X_a formation. In order to assess the effect of heparin on the thrombin-catalyzed factor VIII activation in a quantitative manner, it is important to establish the dependency of the reaction on the thrombin concentration. This relationship is shown in Figure 1. The rate of factor X_a formation and thus the rate of factor VIII_a formation appeared to be linear with the thrombin concentration up to 4 nM. From the data presented above we have chosen the following conditions to study the effect of heparin (fractions) on the thrombin-catalyzed factor VIII activation reaction. Thrombin (3 nM) was added to semi-purified factor VIII preincubated with varying amounts of heparin. At timed intervals, samples were taken and assayed for factor VIII_a as described in Materials and Methods.

Figure 1: Dependency of factor VIII activation on thrombin concentration. Normal plasma is incubated at 37 °C with varying bovine thrombin concentrations for 15 sec. A sample is brought to the factor X activation mixture containing phospholipide vesicles (25% PS/75% PC, 50 μ M), CaCl_2 (10 mM) and factor IX_a (4 nM). The rate of factor X activation is measured after 2 and 4 min. The results are expressed as rate of Factor X_a formation per min, per unit factor VIII_a present in 1 ml plasma.

Figure 2 shows the time course of factor VIII activation by thrombin (3 nM) in the absence and in the presence of varying amounts of unfractionated heparin. Heparin has an effect both on the initial rate of factor VIII activation, and on the rate of inactivation of factor VIII_a . It was observed that the level of factor VIII_a after 4 min was in all cases lower in the absence than in the presence of heparin.

We estimated the effect on the initial rate of activation, of unfractionated heparin, PK 10169, and Org 10172 (Fig. 3). The IC_{50} values were found to be 0.5 $\mu\text{g/ml}$, 1.75 $\mu\text{g/ml}$ and 25 $\mu\text{g/ml}$, respectively.

Effect of heparin (fractions) on thrombin-catalyzed plasma factor V activation. Factor V_a activity is determined by its potency to accelerate the rate of prothrombin activation. The non-activated factor V has little effect on the rate of thrombin activation. However, the thrombin formed during the assay is capable to activate factor V. We have to emphasize that even in the absence of factor V, prothrombin is slowly converted to thrombin by the factor X_a -prothrombin complex. Figure 4 shows the activation of factor V by thrombin formed during the assay. It is apparent that all factor V will be completely activated because the rate of thrombin formation becomes identical to the one observed in the presence of factor V_a . It also shows that the extent of factor V activation increases

Figure 2: Effect of unfractionated heparin on thrombin catalyzed factor VIII activation. Isolated factor VIII:C was incubated with thrombin (3 nM) and varying concentrations of heparin. (●), no heparin; (○) 0.14 $\mu\text{g/ml}$; (▲) 0.70 $\mu\text{g/ml}$; (Δ) 1.43 $\mu\text{g/ml}$; (■) 2.9 $\mu\text{g/ml}$; (□), 5.7 $\mu\text{g/ml}$. At timed intervals factor VIII_a containing samples were transferred to the assay. Factor VIII_a was determined by using a calibration curve established with normal plasma as described in Experimental Procedures.

Figure 3: Effect of heparin (fractions) on the initial rate of thrombin catalyzed factor VIII activation. Factor VIII:C was incubated with thrombin, in the presence of varying amounts of heparin. The initial rate of factor VIII activation was measured at 30 sec as described in the legend to Fig 2. Panel A, (●) unfractionated heparin; (○) PK 10169; Panel B, Org 10172.

Figure 4: Factor V (4 pM) was incubated with factor X_a (13 pM) phospholipid (10 μ M) in 50 mM Tris, 175 mM NaCl, 10 mM $CaCl_2$, 0.5 mg HSA/mL, pH 7.9 at 37 $^{\circ}$ C for 5 min. Prothrombin was added at a final concentration of 0.2 μ M. The results are depicted by the closed circles. Open circles represent the time course during the reaction of prothrombin activation in the presence of 4 pM V_a .

Figure 5: Dependency of factor V activation on thrombin concentration. Purified bovine factor V (100 nM) was incubated for 5 min at 37 $^{\circ}$ C in a buffer containing 50 mM Tris, 175 mM NaCl, 3 mM $CaCl_2$ 0.5 mg human serum albumin/mL in the presence of varying thrombin concentrations. Factor V_a activity was determined as described in Experimental Procedures.

exponentially with the time. Under the conditions of prothrombin activation that we used in further experiments, the reaction was stopped after 2 min. It appeared that the factor V activity is only 1.3% of factor V_a activity. Before studying the effect of heparin, we had to find conditions under which the rate of factor V_a formation is proportional to the thrombin concentration. Figure 5 shows that up to 0.6 nM thrombin these conditions are met. We also studied the time course of factor V_a formation and showed that up to 6 min the rate of factor V_a formation was linear in time.

From the data presented above we set the following experimental conditions to study the effect of heparin on the thrombin-catalyzed factor V activation. Bovine factor V (100 nM) was incubated at 37 °C with calcium (3 mM) and varying concentrations of heparin (fractions). The reaction was initiated by the addition of thrombin (0.3 nM). After 6 min diluted samples were assayed for factor V_a activity. The factor V_a concentration, expressed as a percentage of the factor V_a concentration found in the absence of heparin, was plotted as a function of heparin concentration (Fig. 6A). Determination of the IC_{50} values was performed by use of the double reciprocal plot as described in Material and Methods (Fig. 6B). It is clear that unfractionated heparin is a more efficient inhibitor ($IC_{50} = 10 \mu\text{g/ml}$) than the fractions CY 216 ($IC_{50} = 25 \mu\text{g/ml}$), CY 222 ($IC_{50} = 25 \mu\text{g/ml}$), PK 10169 (63 $\mu\text{g/ml}$) or the heparinoid Org 10172 ($IC_{50} = 125 \mu\text{g/ml}$). As straight lines were obtained, going through the origin, our assumption that the inhibition is caused by the formation of a heparin-thrombin complex, appears to be an acceptable minimal hypothesis.

Figure 6: Effect of heparin on the initial rate of thrombin-catalyzed factor V activation: Factor V (100 nM) was activated for 5 min by thrombin (0.3 nM) in the presence of CaCl_2 (3 mM) and varying concentrations of heparin. (○) crude heparin; (●) CY 216; (▲) CY 222; (□) PK 10169; (■) Org 10172. A, percentage of residual factor V_a activity as a function of heparin concentrations. B, replot of the data according to equation (4) described in the text.

Figure 7: Effect of varying thrombin concentrations on the time course of factor V_a generation. A, platelets (4.6×10^6 /ml) were incubated at 37°C with varying thrombin concentrations:

($\blacktriangle$) 0.125 nM; ($\square$) 0.25 nM;
 ($\blacksquare$) 0.5 nM; ($\triangle$) 0.75 nM;
 ($\circ$) 1 nM; ($\bullet$) 1.5 nM.

B, replot of the initial rate of factor V_a formation as a function of thrombin concentration.

In contrast with others (16), we found that heparin and heparin fractions up to 200 $\mu\text{g}/\text{ml}$ had no effect on the catalytic efficiency of thrombin on the chromogenic substrate S 2238 when the substrate concentration is above or near the K_m (data not shown). Thus, heparin alters the enzymatic activity of thrombin towards macromolecular substrates by interacting with accessory binding sites, whereas it does not affect the active site for small synthetic peptide substrates.

Inhibition of platelet factor V release and activation by heparin and heparin fractions. Factor V is released from the α -granules as a profactor and activated after the release reaction when thrombin is present (17,18). The initial rate of factor V_a generation, after triggering the platelets with thrombin, appeared to be proportional to the thrombin concentration up to 1.0 nM. The maximal amount of factor V_a that could be generated was about 8.5 pM/ 4.6×10^6 platelets (Figure 7A). This is in accordance with the amount of platelet factor V Tracy et al. (19) reported by a bioassay in a suspension of Triton-lyzed platelets.

Figure 8: Effect of heparin (fractions) on thrombin induced platelet factor V_a generation. Platelets ($4.6 \times 10^6/\text{ml}$) were stirred with 0.5 nM human thrombin for 3 min, in the presence of varying concentrations of heparin (●), CY 216 (○), CY 222 (▲), or Org 10172 (■). A, percentage of residual factor V_a activity as a function of heparin concentrations. B, replot of the data according to Equation 4 as described in Materials and Methods. 100% factor V_a activity in the absence of heparin was 3 pM factor V_a ($1 \text{ pM } V_a \cdot \text{min}^{-1}$).

To study the effects of heparin and heparin fractions on the initial rate of platelet factor V_a generation, 4.6×10^6 platelets/ml were stimulated with 0.5 nM thrombin and the amount of factor V_a formed was determined by assaying samples taken 3 min after the addition of thrombin. It is important to note that under these conditions the rate of factor V_a generation is proportional to the functional thrombin concentration (Figure 7B). The influence of varying amounts of crude heparin, CY 216, CY 222 and Org 10172 on the initial rate of factor V_a generation is shown in Figure 8A. It is apparent that crude heparin is the most potent inhibitor. When the data from Figure 8A were replotted according to Equation 4 (see Materials and Methods) straight lines were obtained going through the origin (Figure 8B). The IC_{50} values, as estimated from Figure 8B, are given in Table II. If human thrombin was replaced by bovine thrombin, the kinetics of bovine thrombin-induced platelet factor V_a generation were found to be identical to those reported herein for human thrombin (data not shown). However, the

IC₅₀ values for the different heparins are significantly lower than those for human thrombin (Table III). This is in accordance with the higher affinity of heparin for bovine thrombin (20) and supports the idea that the heparins interact primarily with thrombin.

Effect of heparin on platelet beta-thromboglobulin release. In order to investigate if any interaction between platelets and heparin might result in an impaired release reaction, we studied the release of another platelet α -granule protein, β -thromboglobulin. Platelets ($4.6 \times 10^7/\text{ml}$) were incubated with 10 μg collagen/ml or with 0.5 nM thrombin either in the absence or presence of crude heparin (0.1 mg/ml). When platelets were stimulated by collagen, heparin had no effect on the kinetics of β -thromboglobulin release, whereas heparin (0.1 mg/ml) diminished β -thromboglobulin release to less than 10% of the control value when thrombin was used to stimulate the platelets.

Effect of heparin and heparin fragments on the generation of platelet activity in prothrombin activation. Platelet prothrombin converting activity (PPCA) was measured under conditions where added factor V_a exceeded greatly the amount of factor V_a that is generated from platelets, so as to make the test independent of factor V release. By omitting phospholipids from our assay, the rate of thrombin formation becomes proportional to the amount of procoagulant surface that was generated upon platelet stimulation. It has been shown that platelets activated by a combination of collagen plus thrombin form a more suitable catalytic surface for the prothrombinase complex than platelets treated with thrombin alone (21). Therefore, we investigated the effect of heparins on human thrombin-plus-collagen-induced PPCA. Conditions had to be found where PPCA generation is

Figure 9: Time course of appearance of PPCA. Platelets ($4.6 \times 10^6/\text{ml}$) were incubated at 37°C with 0.3 nM human thrombin and 5 $\mu\text{g}/\text{ml}$ collagen. At different trigger times, coagulation factors X_a, V_a and prothrombin were added and thrombin formation was assayed as described in Material and Methods.

Figure 10: PPCA generation as a function of human thrombin concentrations. Platelets ($4.6 \times 10^6/\text{ml}$) were triggered at 37°C for 5 min with varying human thrombin concentrations in the absence (O) or in the presence (●) of $5 \mu\text{g}/\text{ml}$ collagen. Thrombin formation was assayed as described in Material and Methods.

Table II The effect of heparin fractions on human thrombin-induced factor V_a generation and human thrombin plus collagen-induced PPCA

Compound	Factor V_a generation IC_{50} ($\mu\text{g}/\text{ml}$)	PPCA IC_{50} ($\mu\text{g}/\text{ml}$)
Heparin	40	10
CY 216	130	100
CY 222	70	540
Org 10172	180	440

linear in time and proportional to the thrombin concentration. The generation of a procoagulant surface was followed in time for platelets ($4.6 \times 10^6/\text{ml}$) stimulated with 0.3 nM thrombin plus $5 \mu\text{g}$ collagen/ml (Figure 9). The dependency of platelet activation on the thrombin concentration is shown in Figure 10, either in the absence or presence of $5 \mu\text{g}$ collagen/ml. As a standard procedure to study the effect of heparin, platelets were stimulated for 3 min with 0.3 nM thrombin plus $5 \mu\text{g}$ collagen/ml. Although collagen had to be added in order to potentiate the PPCA generating efficiency of low thrombin concentrations, it has to be mentioned that under these conditions the rate of PPCA generation is proportional to the thrombin concentration (Figure 10).

Figure 11: Effect of heparin(fractions) on thrombin plus collagen-induced PPCA. Platelets ($4.6 \times 10^6/\text{ml}$) were triggered 3 min with 0.3 nM human thrombin and 5 μg collagen/ml in the presence of varying concentrations of heparin (●), CY 216 (○), CY 222 (▲), or Org 10172 (■).

A, percentage of residual PPCA as a function of heparin concentrations.

B, replot of the data as described in Materials and Methods. 100 % PPCA in the absence of heparin was 24 nM $\text{II}_\alpha/\text{min}$.

Figure 11 depicts the results of experiments where platelets ($4.6 \times 10^6/\text{ml}$) were incubated for 3 min at 37°C with varying concentrations of crude heparin, CY 216, CY 222 or Org 10172, followed by the stimulation of platelets during 3 min and measurement of platelet prothrombin converting activity. It is interesting to see that crude heparin is not able to block completely the generation of platelet prothrombin converting activity. Apparently, the remaining activity is equal to that generated by collagen in the absence of thrombin. Indeed, in a separate experiment we found that neither crude heparin nor heparin fragments were able to block the formation of collagen-induced PPCA. IC_{50} values were determined by analyzing the data as described in Materials and Methods. The replot of the data from Figure 11A according to Equation 4 is shown in Figure 11B. The intercept on the vertical axis represents the fraction of the PPCA activity generated by collagen alone. The IC_{50} values are listed in Table II.

Human thrombin plus collagen acting on human platelets requires more heparin or heparin fractions to attain 50% inhibition of PPCA than bovine thrombin does in the presence of collagen (Table III).

Table III The effect of heparin fractions on bovine thrombin-induced platelet factor V_a generation and bovine thrombin plus collagen-induced PPCA

Compound	Factor V_a generation	PPCA
	IC ₅₀ (μg/ml)	IC ₅₀ (μg/ml)
Heparin	0.5	3.4
CY 216	3.3	3.8
CY 222	1.8	90.0
Org 10172	15.0	125.0

DISCUSSION

The heparin preparations that are in general use in antithrombotic therapy are extremely complex mixtures with components that differ with respect to molecular weight, chemical composition, secondary structures as well as antithrombin III affinity and other biological properties. It is not surprising that, besides the well known antithrombin III-potentiating effect, many other inhibitory actions on the hemostatic system can be found. Crude heparin shows a marked anticoagulant and antithrombotic, but also a hemorrhage-inducing effect. It was generally assumed that these three effects were intimately linked. More recently it has been shown that these three biological functions of crude heparin can to a large extent be dissociated (1-5). This suggests that different fractions of crude heparin might have different effects as to anticoagulation, antithrombotic action and increasing of hemorrhagic tendencies. This is extremely interesting and of great practical interest because, where antithrombotic action is pursued and hemorrhage must be avoided, the therapeutic ratio between the two actions is always estimated on basis of anticoagulant action. However, the relationship between in vitro kinetics as assessed by clotting assays and the antithrombotic action of heparin is far from being

understood. Although binding to antithrombin III plays an important role in the antithrombotic action of heparin, antithrombin low affinity heparin seems to contribute to the antithrombotic action of heparin (22).

A major question is therefore whether the favourable therapeutic ratio of low molecular weight heparins is related to their specific properties in antithrombin III-independent anti-thrombin activity. The present study was undertaken to assess the effects of crude heparin, different heparin fractions and a heparinoid on the positive feedback reactions of thrombin, i.e. plasma factor VIII and factor V activation, and platelet activation, leading to the generation of platelet factor V_a and, in the presence of collagen, to the platelet prothrombin converting activity.

The effect of heparin on the activation of factor VIII results in lowering the initial rate of factor $VIII_a$ formation as well as slowing down the rate of disappearance of factor $VIII_a$. The inactivation of factor $VIII_a$ has been attributed either to a proteolytic action of thrombin (23) or a thrombin-independent inactivation (24). Therefore, the stabilizing effect of heparin on the factor $VIII_a$ could be due to an effect on thrombin and/or factor $VIII_a$.

It was recently shown in our laboratory that a highly sulphated polysaccharide, the pentosan polysulphate, exerts a strong inhibitory action on factor $VIII_a$ formation by increasing the amount of residual non-activated factor VIII rather than by inactivation of the formed factor $VIII_a$ (25). The exact nature of the heparin action on either the activation of factor VIII or inactivation of factor $VIII_a$ remains to be determined. Ofofu et al. (26) found that the inhibitory effect of heparin on factor X activation by the phospholipid factor IX_a -factor $VIII_{(a)}$ complexes, was markedly reduced when thrombin-activated factor VIII was used instead of non-activated factor VIII. Our findings, by a direct study of factor VIII activation, also suggest an effect of heparin on the thrombin-catalyzed activation of factor VIII.

Heparin IC_{50} values were determined under conditions where initial rates of either plasma factor V_a , or platelet factor V_a , or PPCA generation, were proportional to the thrombin concentration. The assumption was made that thrombin, when complexed with heparin, has no activity towards macromolecular substrates. Our experimental data appear to be consistent with that model (Figures 6B, 8B and 11B).

An interesting observation was the absence of effect of heparin (fractions) on the assembly and functioning of the prothrombinase complex at the outer

membrane of activated platelets (Table I). This seems to be in contrast with the findings of Walker et al. (13). They reported that heparin inhibited prothrombin activation by preventing the binding of activated clotting factors as well as prothrombin to a phospholipid surface. When we performed equilibrium binding studies according to Van de Waart et al. (14) we found that crude heparin (0.5 mg/ml) did not change the affinity of factor V_a , factor X_a and prothrombin for phospholipid as well as total phospholipid-binding capacity.

Thrombin-induced platelet factor V_a generation is the result of the release of non-activated factor V followed by its activation by thrombin. Because our factor V_a assay is virtually insensitive for factor V, the inhibitory action of heparin on the release reaction could not be separated from its action on thrombin-catalyzed factor V activation. However, we demonstrated that heparin did inhibit the thrombin-induced β -thromboglobulin release, whereas heparin did not inhibit the collagen-induced β -thromboglobulin release. This supports the notion that heparin exerts its effect through a heparin-thrombin rather than through a heparin-platelet interaction.

As to the IC_{50} values measured for the different heparins in the reactions catalyzed by thrombin, it is obvious that for crude heparin relatively low IC_{50} values were found, compared to CY 216, CY 222, PK 10169 and Org 10172. Therefore, on a weight basis ($\mu\text{g/ml}$) crude heparin is a more potent inhibitor of both factor V_a and PPCA generation, and of factor V and factor VIII activation. The inhibitory capacity of a heparin fraction on the thrombin-catalyzed reactions is not always in accordance with its antithrombin III-dependent anti-thrombin specific activity (anti-II $_a$ U/mg). Moreover when IC_{50} values are given in anti-II $_a$ U/ml, no correlation is found. This strongly suggests that, in studying the inhibitory action of heparin on thrombin, an antithrombin III-dependent effect can be distinguished from an independent one.

As yet, we can only speculate about the occurrence in different heparin molecular species, of such differential anti-thrombin actions. It would be interesting to establish whether or not antithrombin III low affinity heparins have anti-thrombin activities in blood coagulation or platelet reactions as demonstrated in the present report.

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CHAPTER V

THE ROLE OF HEPARIN CHARGE DENSITY IN THE ANTITHROMBIN III-DEPENDENT AND ANTITHROMBIN III-INDEPENDENT INACTIVATION OF THROMBIN

SUMMARY

We have previously demonstrated that, in the absence of antithrombin III, unfractionated heparin inhibits the activation of platelets and factor V by thrombin. Two low molecular weight heparin fractions and a heparinoid were less effective [Baruch et al. (1985) *Thromb. Res.* 38, 447-458]. It appeared that this inhibitory effect of heparin is the result of the formation of a heparin-thrombin complex.

In the present study we evaluated the thrombin-binding properties of heparin fractions with different charge density, obtained by ion-exchange chromatography on DEAE-Sephacel and a heparin fraction with very low affinity for antithrombin III obtained by affinity chromatography on antithrombin III-Sepharose. We found that the latter heparin fraction was not able to inhibit the thrombin-catalyzed platelet factor V_a formation. In addition, this fraction could not compete with unfractionated heparin for thrombin binding. The fractions with different charge density were able to bind to thrombin. This was shown by their inhibitory effect in the absence of antithrombin III on thrombin-catalyzed platelet factor V_a formation and by the ability of active-site-blocked thrombin to prevent the heparin (fraction)-dependent inhibition of thrombin by antithrombin III. The inhibitory effect of the fractions on factor V_a formation and the effect of active-site-blocked thrombin increased with the increase in charge density. However, these fractions not only differed in charge density but also in their content of antithrombin III binding species. That is, the increase in charge density was found to go along with an increase in antithrombin III high affinity material.

In addition, we established that when the heparin fraction with the lowest charge density and very low in antithrombin III binding material was chemically modified so as to achieve a higher charge density, it obtained a

higher affinity for thrombin but could not compete with the native fraction for binding to thrombin in the presence of antithrombin III. It is apparent from our study that the antithrombin III-independent inhibition of thrombin by heparin is the result of a heparin-thrombin interaction and depends on the charge density of the polysaccharide. But this particular property of heparin is found together with the presence of a specific antithrombin III-binding domain. This indicates that unfractionated heparin does not contain a fraction that has an antithrombin III-independent inhibitory action on thrombin without having an antithrombin III-dependent one. Besides that, we demonstrated that when heparin is fractionated by charge density, the specific activities of the fractions in the thrombin-antithrombin III reaction are the same when the concentration of antithrombin III binding sites is taken into account.

INTRODUCTION

Heparin is a complex mixture of negatively charged glycosaminoglycans. Fractionation of heparin has led to the recognition of different functional binding domains. A defined sequence of an octasaccharide has been identified to contain the antithrombin III-binding domain (1,2). The binding of thrombin to heparin has been shown to be an essential feature in the catalytic function of heparin on the inactivation of thrombin by antithrombin III. By using covalently bound heparin-antithrombin III complexes, Hoylaerts et al. (3) demonstrated that the dissociation constant of thrombin binding to these complexes decreased three orders of magnitude with increasing the molecular weight of heparin from 3,200 to 15,000. They concluded that the binding of thrombin and heparin involved electrostatic interactions and that thrombin diffuses along the heparin chain to interact with antithrombin III bound to the same heparin chain. As yet, no defined sequence has been found for a thrombin-binding domain.

The major difficulty in studies on the structure-function relationship of heparin is to isolate by a fractionation technique one structural variable at a time. Hurst et al. (4) showed that fractionation of heparin according to its charge density followed by a further fractionation by affinity chromatography on antithrombin III-Sepharose offers a way to functionally

isolate the effects of the protease-binding properties of heparin from the effects of the antithrombin III-binding properties. In addition, Heuck et al. (5) could demonstrate that heparin acts on the antithrombin III-thrombin reaction through cooperative electrostatic binding to thrombin and non-electrostatic interaction with antithrombin III.

We have previously investigated the antithrombin III-independent effect of heparin on the thrombin-catalyzed reactions of platelet activation. Unfractionated heparin appeared to be a more potent inhibitor of these reactions than two low molecular weight heparin fractions obtained by ethanol extraction or depolymerization and a heparinoid (6). This effect was attributed to a direct action on thrombin resulting in the masking of its secondary substrate binding sites by heparin.

The work presented here was initiated to establish whether and to what extent the antithrombin III-independent action of heparin on the inhibition of thrombin is a function of its charge density. Our second aim was to establish whether or not heparin species lacking the antithrombin III binding domain were able to inhibit the activity of thrombin towards macromolecular substrates.

To this end we studied platelet activation by thrombin in the presence of heparin fractions obtained by ion-exchange chromatography according to Sache et al. (7) and a heparin fraction with a very low affinity to antithrombin III obtained by affinity chromatography of unfractionated heparin on antithrombin III-Sepharose.

MATERIAL AND METHODS

Materials. HD Phenylalanyl-L-pipecolyl-L-arginine-p-nitroanilide hydrochloride (S 2238) was purchased from AB Kabi Diagnostica, Stockholm, Sweden. Fatty acid free human serum albumin and dioleoyl phosphatidylcholine were obtained from Sigma, USA. D-phenyl-alanyl-prolyl-arginyl chloromethylketone (PPACK) was from Calbiochem, La Jolla, USA. All reagents used were of the highest grade commercially available.

Preparation of phospholipids and phospholipid vesicles. Phosphatidylserine (18:1cis/18:1cis) was prepared from phosphatidylcholine by enzymatic synthesis according to the method of Comfurius and Zwaal (8). Single bilayer

vesicle solutions were prepared as previously described (9). Phospholipid concentrations were determined by phosphate analysis according to Böttcher et al (10).

Proteins. Bovine prothrombin, factor X_a and factor V_a were purified according to established procedures (11). Bovine and human thrombin were prepared as previously reported (6). Bovine antithrombin III was prepared according to Thaler and Schmer (12).

Protein concentrations. Concentrations of thrombin, factor X_a and prothrombin were determined as reported earlier (13). Factor V_a concentrations were determined by kinetic analysis (11). The molar concentration of bovine antithrombin III was calculated using $E_{280}^{1\%} = 6.0$ and $M_r = 56,600$ (14).

Isolation of human platelets. Human platelets were isolated by differential centrifugation as described earlier (15). Platelets were kept at room temperature in a buffer containing 136 mM NaCl, 2.68 mM KCl, 2mM $MgCl_2$, 10 mM Hepes, 5 mM glucose and 0.1% fatty acid free human serum albumin, pH 7.5. No loss of platelet activity was found for at least 15 hours. Platelet concentrations were determined with a Coulter counter.

Heparin fractions. Porcine mucosal sodium heparin was fractionated by ion-exchange chromatography on DEAE-Sephacel with increasing salt molarities. The four fractions obtained, corresponding to the fractions A, B, C, and D described by Sache et al. (7), as well as an extra-sulfated fraction A (fraction A^S) were a kind gift from Choay Institute (Paris). High affinity and low affinity fractions were obtained from crude heparin by chromatography on an antithrombin III-Sepharose column. They were a kind gift from Pharmuka Laboratories (Paris).

Fluorescence studies. Fluorescence measurements were made in the ratio mode using an SLM/Aminco SPF-500C spectrofluorometer. Titrations of antithrombin III with heparin were performed at 25° C using λ_{ex} 285 nm and λ_{em} 345 nm. The excitation and emission bandpasses were set at 2 and 4 nm respectively. We used a buffer containing 0.05 M, Tris 0.03 M NaCl, pH 7.5. The antithrombin III concentration used was 1 μ M, a value far above the dissociation constants of the heparin-antithrombin III complexes. The fluorescence increase resulting from the heparin-antithrombin interaction was calculated as $(F - F_0)/(F_{max} - F_0)$ where F is the fluorescence intensity of the sample containing heparin, F_{max} that of the sample containing saturating heparin concentration, and F_0 that of the control solution without heparin (16).

This ratio represents the fraction of antithrombin III-bound heparin over the maximal antithrombin III-bound heparin and was plotted against the heparin concentration. It was used to determine the amount of antithrombin III high affinity material present in the heparin fractions as described by Nordenmann et al. (17).

Neutralization of thrombin activity by antithrombin III. Purified bovine thrombin (10 nM) was added to antithrombin III (100 nM) and varying amounts of heparin in 50 mM Tris, 175 mM NaCl, 0.5 mg/ml human serum albumin, pH 7.9 at 37° C. At intervals aliquots were removed and brought into 1 ml cuvette containing 1 mg/ml Polybrene in the Tris-NaCl buffer and were assayed for residual thrombin activity by using the synthetic substrate S2238 (0.24 μ mol). The amount of residual thrombin was measured by the change in absorbance recorded on an Aminco DW2 spectrophotometer in the dual wavelength mode ($\lambda_s = 405$ nm and $\lambda_r = 500$ nm) thermostated at 37° C. Pseudo-first order rate constants of inhibition of thrombin (k) were calculated from an exponential plot of residual thrombin concentrations versus time.

Inhibition by active site blocked thrombin. PPACK-thrombin was prepared by incubating purified bovine thrombin (8 μ M) with PPACK (1 mM) in 50 mM Tris-HCl, 175 mM NaCl, pH 7.9 at 37° C until no amidolytic activity was measurable. The solution was extensively dialyzed against the aforementioned buffer. Pseudo-first order rate constants of inhibition of thrombin were determined at fixed heparin concentration either in the absence or in the presence of varying PPACK-thrombin concentrations.

Determination of platelet factor Va generation. Washed platelet suspensions were incubated in the presence of varying heparin concentrations prior to activation with human thrombin (0.5 nM). After 2 and 10 min aliquots were taken and assayed for factor V_a activity and the amount of heparin causing 50% inhibition of the reaction (IC_{50} value) was calculated from double reciprocal plot as previously described (6).

RESULTS AND DISCUSSION

Characterization of heparin fractions obtained by ion-exchange chromatography. The physico-chemical properties and the anticoagulant activities of the four heparin fractions A, B, C and D, obtained by ion-exchange

Table I: Characteristics of heparin fractions obtained by ion-exchange chromatography on DEAE-Sephacel^a.

Properties	UFH ^b	Heparin fractions			
		A	B	C	D
Molecular weight	17,300	7,300	13,200	17,300	20,300
Elution position (M NaCl)	-	0.55	0.65	0.75	0.85
Fraction weight (mg)	1000	43	476	174	158
Yield ^c	1	0.10	0.62	0.17	0.13
Charge density (Z^2)	10.96	6.81	9.67	10.56	11.02
Antithrombin activity (U/mg)	304	4	133	396	538
% High affinity AT III	52	23	43	86	100
% High affinity AT III ^d	50	7	40	88	182

^a Data taken from Sache et al. (7)^b UFH, unfractionated heparin^c Moles of fraction/moles of heparin applied on the column^d This study

chromatography eluted with increasing salt molarities were taken from Sache et al. (7) and listed in Table I. The fractions exhibited respectively negligible, intermediate, high and very high activities in the antithrombin III-dependent inactivation of factor X_a and thrombin in plasma. Based on the data of Sache et al. (7) on uronic acid content (UA) and sulfate content (SO_4) of the different fractions, we calculated the anionic density (Z) from $Z = 1 + (SO_4/UA)$ as described by Hurst et al. (18). The Z^2 values are listed in Table I. It is worth noting that only the charge density of fraction A differs markedly from that of the other fractions and unfractionated heparin. When the amount of each fraction is calculated as the percentage of the total amount of heparin applied on the DEAE-Sephacel column from the average molecular weight and the mass (mg) recovered in each fraction, it is clear that the heparin species with low anionic

density only contributes to a small extent of the total amount of heparin (Table I). Moreover, it is evident that the low anionic density species are found in heparin fractions with rather low molecular weight (less than 13,000).

It has been assumed that the probability to find an antithrombin III-binding sequence on the heparin chain increases with the chain length of the heparin molecule (19). Indeed, from the data of Sache et al. (7) it can be concluded that the proportion of antithrombin III high affinity material that was found to bind to immobilized antithrombin III increased with the increase in average molecular weight of the heparin fraction. Because the concentration of antithrombin III-binding sites on heparin can be more accurately determined by the heparin-dependent increase in the intrinsic fluorescence of antithrombin III (17), we performed such experiments in the presence of 1 μ M antithrombin III. The amount of antithrombin III high affinity material present in each fraction as a percentage of the total heparin molar concentration is also listed in Table I. It is clearly shown that with respect to fraction A and D, our data differ markedly from the values reported by Sache et al. (7). Whereas fraction A contains one binding site for antithrombin III per 14 molecules of heparin, fraction D contains almost two antithrombin III-binding sites per molecule of heparin. Evidence that high molecular weight heparin may contain more than one antithrombin III-binding site per chain has already been reported (20).

Thus, besides a marked difference in anionic density between A and the other fractions, the differences in antithrombin III-binding sites are even more pronounced. This raises the question as to the ability of the fractions A, B, C and D to catalyze the thrombin-antithrombin III reaction. To this end, thrombin (10 nM) was incubated with antithrombin III (100 nM) in the presence of varying amounts of the heparin fractions. The pseudo first order rate constants of inhibition of thrombin were calculated from exponential plots of the residual thrombin concentration versus reaction time and are listed in Table II. It is shown that in order to obtain, for the heparin fractions, identical values of pseudo first order rate constants, the amount of each heparin fraction had to be varied widely.

It is of interest to compare our data (k values normalized for the heparin concentration) with the antithrombin activities determined by an amidolytic method in human plasma reported by Sache et al. (7). A comparison based on

Table II: Antithrombin activity of heparin fractions in the presence of antithrombin III.

Heparin Compound	Total conc. (ng/ml)	Active ^a conc. (ng/ml)	k ^b (min ⁻¹)	Activity ^c (min ⁻¹ .μg ⁻¹ .ml)	Activity ^d (min ⁻¹ .μg ⁻¹ .ml)
UFH	20	10	1.4	65	130
Fractions					
A	200	14	1.2	5	71
B	30	12	1.3	40	100
C	10	9	0.9	85	96
D	5	9	1.2	220	121

^a calculated for the percentage of heparin with high affinity for antithrombin III as determined by fluorescence titration.

^b corrected for the heparin-independent reaction ($k = 0.1 \text{ min}^{-1}$).

^c calculated from the pseudo first order rate constant of inhibition of thrombin by antithrombin III (k) and the total heparin concentration.

^d calculated from the pseudo first order rate constant (k) and the amount of antithrombin III affinity material in each fraction.

the respective activities reveals in our assay a 5-fold and 2-fold higher specific activity for fraction A and fraction D, respectively. When the molar concentration of antithrombin III-binding heparin is taken into account to calculate the specific activities of the fraction, then the differences between the fractions become much smaller.

Characterization of heparin fractions obtained by antithrombin III affinity chromatography. Pseudo first order rate constants of inhibition of thrombin (10 nM) and factor X_a (10 nM) by antithrombin III (100 nM) were determined in the presence of unfractionated heparin and low and high antithrombin III affinity heparin at varying concentrations. The rate constants of inhibition of factor X_a, corrected for the heparin-independent reaction, were

0.11 and 13.2 $\text{min}^{-1} \cdot \mu\text{g}^{-1} \cdot \text{ml}$ for low and high affinity heparin, respectively. The rate constants for thrombin were 0.9 and 137 $\text{min}^{-1} \cdot \mu\text{g}^{-1} \cdot \text{ml}$ for low and high affinity heparin, respectively. We conclude that the antithrombin III low affinity heparin fraction contained less than 1% of high affinity material.

Effect of heparin fractions on thrombin-induced platelet factor V_a formation. The effect of heparin fractions with low and high affinity for antithrombin III, as well as the effect of heparin fractions with different charge density were studied on the thrombin-induced platelet factor V_a formation. Washed platelets ($5 \times 10^6/\text{ml}$) were incubated with thrombin (0.5 nM) in the presence of varying amounts of the different heparin fractions.

Table III: Binding of heparin fractions to thrombin.

Compound	IC ₅₀ values	
	Thrombin Induced	Heparin-dependent
	Platelet Factor V_a	Thrombin Inhibition
	Generation (heparin conc, $\mu\text{g}/\text{ml}$)	by Antithrombin III (PPACK-thrombin, nM)
Unfractionated		
heparin	31	200
High Affinity	27	200
Low Affinity	660	n.d.
Fraction A	90	400
Fraction B	37	220
Fraction C	22	190
Fraction D	17	150

n.d.: not determined

Samples were taken at different time intervals and assayed for factor V_a activity. We have previously shown that under our conditions the rate of platelet factor V release and of platelet factor V activation, as well as the maximal amount of platelet factor V released are proportional to the thrombin concentration (6). Thus, when thrombin activity decreases as a result of inhibition by heparin, the rate of factor V_a formation and the maximal factor V_a concentration decrease. The heparin concentrations at which half the rate of factor V_a formation and half the maximal amount of factor V_a formed were obtained (IC_{50} values) are listed in Table III. It is apparent that the IC_{50} values correlate with the charge density of the heparin fractions. That is, the highest IC_{50} value is found for the heparin fraction, fraction A, with the lowest charge density. Interestingly, the antithrombin III low affinity fraction showed a very high IC_{50} value, indicating that this heparin fraction had hardly any affinity for thrombin. When compared with unfractionated heparin, the antithrombin III high affinity fraction was found to have an identical IC_{50} value. From this we concluded that the antithrombin III high affinity fraction contained most, if not all, of the thrombin-binding heparin species.

The effect of PPACK-thrombin on the heparin-dependent inhibition of thrombin by antithrombin III. The IC_{50} values reported above were obtained from a rather complicated reaction, i.e. the thrombin-catalyzed platelet factor V_a formation. Because an active-site-blocked thrombin, like PPACK-thrombin, has the same affinity for heparin when compared to thrombin, it can be used as a tool to compare the affinities of different heparin fractions for thrombin. That is, PPACK-thrombin is a competitive inhibitor of the heparin-dependent inhibition of thrombin by antithrombin III. The amount of PPACK-thrombin required to obtain half the maximal rate constant of the heparin-dependent antithrombin III-thrombin reaction (IC_{50} value) depends on the affinity of PPACK-thrombin for heparin. The results of such an experiment are depicted in Figure 1. Pseudo first order rate constants were determined for the antithrombin III-thrombin reaction in the presence of fraction D (5 or 10 ng/ml) and varying amounts of PPACK-thrombin. It is shown that the IC_{50} values are independent of the heparin concentration. The IC_{50} values for the other heparin fractions were obtained in the same way and are listed in Table III. The results are comparable to the ones obtained in the platelet experiments: the highest IC_{50} value of

Figure 1: Effect of PPACK-thrombin on the pseudo first-order rate constant of thrombin inactivation by antithrombin III and heparin fraction D. Thrombin (10 nM), antithrombin III (100 nM) were incubated with fraction D in the presence of varying PPACK-thrombin concentration. Aliquots were taken in time and the pseudo first order rate constants were determined at every PPACK-thrombin concentration. Heparin fraction D, 5 ng/ml (○) 10 ng/ml (●)

PPACK-thrombin is found for the heparin fraction with the lowest charge density.

Studies with extra-sulfated fraction A (fraction A^S). Fraction A was chemically modified by the incorporation of sulfate groups. Upon this modification the sulfate/carboxyl ratio increased from 1.31 to 1.90, thus the square of charge density increased from 5.3 to 8.41. We questioned whether this increase was sufficient to obtain a heparin fraction with a higher affinity for thrombin when compared with non-modified fraction A. To this end, we estimated the IC₅₀ values of fraction A^S in the thrombin-catalyzed platelet factor V_a formation reaction and the IC₅₀ value of PPACK-thrombin in the fraction A^S-dependent inhibition of thrombin by antithrombin III as described above. The IC₅₀ values were in both cases lower for the extrasulfated fraction A than for fraction A. In the thrombin catalyzed platelet factor V_a formation, the IC₅₀ value was 60 µg/ml for fraction A^S compared to 90 µg/ml for fraction A. An IC₅₀ value of 260 nM was obtained in the competition experiment with PPACK-thrombin, compared to 400 nM for fraction A. Despite the increased affinity for thrombin we found that this fraction A^S was less effective in the neutralization of thrombin

Figure 2: Effect of extra-sulfated fraction A^S on the pseudo first order rate constant of inhibition of thrombin by antithrombin III and fraction A. Thrombin (10 nM) and antithrombin III (100 nM) were incubated with varying amounts of fraction A, in the absence (●) or in the presence (○) of fraction A^S (200 ng/ml).

by antithrombin III. When normalized for the heparin concentration the pseudo first order rate constant of inhibition of thrombin by 100 nM antithrombin III was $2.9 \text{ min}^{-1} \cdot \mu\text{g}^{-1} \cdot \text{ml}^{-1}$, which is half the rate constant found with fraction A. Whatever caused the different rate constants, we were unable to demonstrate that the higher affinity for thrombin of fraction A^S resulted in an inhibitory effect of the fraction A-dependent thrombin-antithrombin III reaction. Pseudo-first order rate constants were determined for the antithrombin III-thrombin reaction in the presence of varying amounts of fraction A, either in the absence or in the presence of a fixed amount of fraction A^S (Figure 2). It is shown that when fraction A^S was added, the pseudo first order rate constant of inhibition of thrombin increased due the effect of fraction A^S alone.

CONCLUSION

The results of the present study indicate that the inhibitory action of heparin in the thrombin-catalyzed release and activation of platelet factor V depends on the affinity of heparin for thrombin and as such is a function of the charge density of the heparin molecule. A linear relationship was

Figure 3: Relationship between charge density and heparin-thrombin interaction. Replot of IC_{50} of heparin-thrombin interactions from Table III as a function of charge density Z^2 . Heparin dependent thrombin inactivation by antithrombin III in the presence of PPACK-thrombin; (●); thrombin induced platelet factor V_a generation (○).

found between the square of the charge density of heparin fractions obtained by ion-exchange chromatography and their IC_{50} values in the thrombin-catalyzed platelet factor V_a generation reaction (Figure 3), as well as in the effect of active-site-blocked thrombin on the heparin-dependent inactivation of thrombin by antithrombin III.

In view of the rather high IC_{50} values, we have to assume that when these heparin fractions also catalyze the thrombin-antithrombin III reaction, the antithrombin III independent pathway of thrombin inactivation might not be of physiological importance.

In this respect it is interesting to see that when crude heparin is fractionated on immobilized antithrombin III, the heparin fraction with low antithrombin III affinity has a 20-fold higher IC_{50} value in the above mentioned reaction when compared with crude heparin and the antithrombin III high affinity heparin fraction. This indicates that in unfractionated heparin the heparin species with affinity for thrombin also possess the specific polysaccharide sequence required for antithrombin III binding.

When crude heparin is fractionated on the basis of its charge density, thus indirectly of its affinity for thrombin, the same is observed. The heparin fraction with the lowest charge density has the highest IC_{50} value and

possesses almost no antithrombin III-binding sites.

Moreover, our study indicates that when a heparin fraction (fraction A) is chemically modified in such way that it acquires a higher charge density, it does increase the ability to inactivate thrombin in the absence of antithrombin III, whereas such a modification reduces the catalytic efficiency of the heparin fraction in the thrombin-antithrombin III reaction.

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CHAPTER VI

THROMBIN GENERATION AND INACTIVATION IN THE PRESENCE OF ANTITHROMBIN III AND HEPARIN

SUMMARY

We have determined the rate constants of inactivation of factor X_a and thrombin by antithrombin III-heparin during the process of prothrombin activation. The second order rate constant of inhibition of factor X_a alone by antithrombin III as determined by using the synthetic peptide substrate S-2337 was found to be $1.1 \times 10^6 \text{ M}^{-1} \text{ min}^{-1}$. Factor X_a in prothrombin activation mixtures that contained prothrombin, and either saturating amounts of factor V_a or phospholipid (20 mol % dioleoylphosphatidylserine/80 mol % dioleoylphosphatidylcholine, $10 \mu\text{M}$) was inhibited by antithrombin III with a second order rate constant that was essentially the same: $1.2 \times 10^6 \text{ M}^{-1} \text{ min}^{-1}$. When both factor V_a and phospholipid were present during prothrombin activation, factor X_a inhibition by antithrombin III was reduced about 10-fold: second order rate constant of $1.3 \times 10^5 \text{ M}^{-1} \text{ min}^{-1}$. Factor X_a in the prothrombin activation mixture that contained both factor V_a and phospholipid was even more protected from inhibition by the antithrombin III-heparin complex. The first order rate constants at 100 nM antithrombin III and normalized for the heparin concentration were $0.11 \text{ min}^{-1}(\mu\text{g heparin/ml})^{-1}$ and $6.1 \text{ min}^{-1}(\mu\text{g heparin/ml})^{-1}$ in the presence and the absence of factor V_a and phospholipid. When the prothrombin was varied widely around the K_m for prothrombin, this had no effect on the first order rate constants of inhibition. It is our conclusion that factor X_a when acting in prothrombinase on prothrombin is profoundly protected from inhibition by antithrombin III in the absence as well as in the presence of therapeutic concentrations of heparin. The second order rate constant ($4.5 \times 10^5 \text{ M}^{-1} \text{ min}^{-1}$) of inhibition of thrombin activity generated during prothrombin activation was found to be: 1) Constant during the time course of prothrombin activation, 2) Independent of the composition of the prothrombin-converting complex and 3) About 4-fold lower than the second order

rate constant of inhibition of purified alpha-thrombin ($1.9 \times 10^6 \text{ M}^{-1} \text{ min}^{-1}$). Surprisingly, heparin did not enhance the antithrombin III-dependent rate of inhibition of thrombin activity formed during the initial phase of the prothrombin activation. This is possibly caused by the fact that meizothrombin (des fragment 1) is the major product during the initial phase of prothrombin activation. A mathematical model is given which provides a relationship between the thrombin concentration and the pseudo first order rate constant of inhibition of factor X_a as well as that of thrombin when prothrombin is activated in the presence of antithrombin III-heparin.

INTRODUCTION

The plasma protein antithrombin III is an inhibitor of serine proteases involved in the process of blood coagulation. Its action is dramatically enhanced by heparin. The main targets of antithrombin III are activated blood coagulation factor IX (factor IX_a), factor X_a and thrombin (1). The activation of prothrombin to thrombin is a membrane-mediated process requiring the binding of both factor X_a and prothrombin to a negatively charged membrane surface (phospholipid vesicles). In addition, activated blood coagulation factor V (factor V_a) that interacts with the membrane, with factor X_a and with prothrombin, greatly increases the $V_{\max}$ of prothrombin activation and lowers the K_m for prothrombin when the membrane contains a low negative charge density (2). Because in vivo both factor V_a and phospholipids are present during factor X_a -catalyzed activation of prothrombin, a number of studies were undertaken to reveal the effects of these so-called accessory components on factor X_a inhibition by antithrombin III. It has been shown that the inhibition of factor X_a is reduced by phospholipids alone (3,4) and by phospholipids when factor V_a is also present (5).

Ellis et al. (4) used the rate of prothrombin activation as a measure for residual factor X_a activity. Quite unexpectedly, the antithrombin III carried-over in the prothrombin activation assay, as far as we understand, did not affect the thrombin activity, nor factor X_a . Walker & Esmon (6) reported that fragment 2 was found to slow down the rate of inhibition of alpha-thrombin about 3-fold. These observations incite to the investigation

of the kinetics of the antithrombin III-dependent neutralization of both factor X_a and thrombin activity under more physiological conditions. Therefore, our purpose was to assess the kinetics of the antithrombin III-dependent inhibition of factor X_a as part of the prothrombinase complex while acting on prothrombin, as well as the kinetics of the antithrombin III-dependent inhibition of thrombin activity as a product of the prothrombinase reaction. The basic assumption underlying the data analysis of the experiments presented here was that, at any time during the prothrombin activation reaction in the presence of antithrombin III, the observed rate of thrombin formation equals the actual rate of thrombin formation (proportional to the residual factor X_a activity) minus the rate of inhibition of the thrombin activity. The same experiments were also performed in the presence of unfractionated heparin.

Our results lead us to question the validity of a number of basic assumptions underlying the current ideas about the effects of factor V_a and phospholipid on the factor X_a -antithrombin III reaction. Furthermore, a dramatic discrepancy was found between the kinetics of the heparin-dependent inhibition of thrombin generated directly from prothrombin activation and the kinetics of the heparin-dependent inhibition of alpha-thrombin by antithrombin III.

MATERIALS AND METHODS

Materials. Phosphatidylcholine (dioleoyl), soybean trypsin inhibitor and fatty acid free human serum albumin were purchased from Sigma. H-D-phenylalanyl-L-pipecolyl-L-arginine-p-nitroanilide dihydrochloride (S-2238) and Bz-L-isoleucyl-L-glutamyl(piperidyl)-L-glycyl-L-arginine-paranitroanilide hydrochloride (S-2337) were obtained from Kabi AB, Sweden. Crude heparin (165 USP units/mg) was generously provided by Pharmuka Laboratoires, France. All reagents used were of the highest grade commercially obtainable.

Proteins. Bovine antithrombin III was prepared by the method of Thaler & Schmer (7). All other bovine clotting factors were prepared and quantitated as previously described (8).

Phospholipid Vesicle Preparation. 1,2-Dioleoyl-sn-glycero-3-phosphoserine (DOPS) was prepared from 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) as described by Comfurius & Zwaal (9). Sonicated vesicle solutions were prepared from mixtures of DOPS (20 mol %) and DOPC (80 mol %) in a buffer that contained 50 mM Tris-HCl, 175 mM NaCl, pH 7.9 as described by de Kruijff et al. (10).

Determination of Inhibition Rate of Purified Proteases. The neutralization of factor X_a and thrombin by antithrombin III was investigated under pseudo first order conditions. Factor X_a (5 nM) or thrombin (5 nM) was incubated at 37 °C in 50 mM Tris-HCl, 175 mM NaCl and 0.5 mg human serum albumin/ml, pH 7.9 with antithrombin III (50 to 300 nM). At timed intervals after the addition of the protease, 10 μ l aliquots were removed into 590 μ l of substrate solution (containing 50 mM Tris-HCl, 175 mM NaCl, 20 mM EDTA, 0.5 mg human serum albumin/ml, and 0.22 mM S-2238 or S-2237, pH 7.9) at 37 °C. After 4 min, 300 μ l of 50% acetic acid (v/v) was added and the absorbance of the mixture was measured at 405 nm against a blank by using an Aminco DW-2 spectrophotometer. The rates of thrombin and factor X_a inhibition by antithrombin III after sampling into the substrate solution were found to be sufficiently slow so as to not result in measurable enzyme inhibition. The residual enzyme activity was calculated from a standard curve constructed under the same conditions from known amounts of thrombin or factor X_a and exponentially plotted versus reaction time.

Neutralization of Factor X_a and Thrombin by Antithrombin III in Prothrombin Activation Mixtures. Prothrombin was incubated with antithrombin III in the presence of either factor V_a , phospholipid vesicles or heparin or a combination of these in 50 mM Tris-HCl, 175 mM NaCl, 5 or 10 mM $CaCl_2$ and 0.5 mg human serum albumin/ml, pH 7.9 at 37 °C for 4 min. Prothrombin activation was started by the addition of factor X_a . The final concentrations of the reaction components are listed in Table I. At timed intervals after the addition of factor X_a , 40 μ l aliquots were removed into 560 μ l of the aforementioned solutions of S-2238. Thrombin concentrations were determined as described before. In separate experiments, soybean trypsin inhibitor (20 μ g/ml) was added to prothrombin activation mixtures at known intervals after the addition of factor X_a , resulting in an instantaneous

Table I: Conditions of Prothrombin Activation.

Composition of the prothrombinase complex				Prothrombin concentration	Rate of thrombin formation
FX _a	FV _a	Phospho- lipid	Ca ⁺⁺		
(nM)	(nM)	(μM)	(mM)	(μM)	(M II _a min ⁻¹ M X _a ⁻¹)
1. 0.003	0.1	10	5	1.0	2000
2. 1.5	20.0	-	5	6.0	2
3. 4.0	-	5	10	0.5	0.6

The reactions were performed in a buffer containing 0.05 M Tris-0.175 M NaCl - 0.5 mg human serum albumin/ml, pH 7.9 at 37° C.

and complete inhibition of thrombin formation. After the addition of soybean trypsin inhibitor, 40 μl aliquots were removed at regular intervals and assayed for thrombin activity. Control experiments established that soybean trypsin inhibitor had no effect on the antithrombin III-thrombin reaction nor on the amidolytic activity of thrombin.

Kinetic Analysis of Antithrombin III-Factor Xa and Antithrombin III-Thrombin Reaction in Prothrombin Activation Mixtures. When thrombin is generated by prothrombinase-catalyzed activation of prothrombin in the presence of antithrombin III, the appearance rate of amidolytic active thrombin¹, v_r , will vary as a function of time t during prothrombin activation according to:

¹"Amidolytic active thrombin" or "thrombin activity" is used throughout this paper to indicate that this activity is not necessarily identical with the formation of alpha-thrombin

$$(v_r)_t = (v_a)_t - (v_i)_t \quad (1)$$

where $(v_a)_t$ is the velocity of thrombin generation at time t and $(v_i)_t$ is the velocity of the antithrombin III-thrombin reaction at time t . In all experiments, the initial antithrombin III concentration was significantly greater than the protease concentrations, so that the protease-antithrombin III reaction followed pseudo first order kinetics over the time course studied. Thus, when at any time interval prothrombin activation is blocked, $(v_i)_t$ can be calculated by multiplying the observed pseudo first order rate constant of the antithrombin III-thrombin reaction by the thrombin concentration.

It is worth noting that the antithrombin III-protease reaction is a two step reaction. The reversible interaction between antithrombin III and thrombin ($K_d = 1.4 \times 10^{-3}$ M in the absence of heparin and $K_d = 4 \times 10^{-6}$ M in its presence) is followed by a conversion into a stable antithrombin III-thrombin complex, resulting in the inactivation of thrombin with a pseudo first order rate constant, $k = 10.4 \text{ s}^{-1}$ (11). Thus, the reaction can be analyzed by pseudo first order kinetics only when both the antithrombin III and protease concentrations are far below the K_d value. This condition holds for our experiments.

The pseudo first order rate constant of the antithrombin III-factor X_a reaction can be obtained when $(v_a)_t$ is plotted exponentially versus t . The velocity of thrombin generation, v_a , at time t was calculated from Equation 1. The appearance rate of amidolytic active thrombin, v_r , at time t was determined from the slope of the thrombin generation curve at time t .

Electrophoretic Blotting Procedure. Proteins were subjected to slab gel electrophoresis in the presence of sodium dodecyl sulfate as described by Laemmli (12). The proteins were then transferred to nitrocellulose sheets according to the procedure described by Towbin et al. (13). The electrophoretic blots were soaked in 0.1% bovine serum albumin and 0.05% Tween in phosphate-buffered saline. In essence, the immunological detection of antithrombin III and antithrombin III-protease complexes were performed as described by Towbin et al. (13). The first antiserum was rabbit anti-human antithrombin III (Sigma). We used horseradish peroxidase-conjugated swine anti-rabbit IgG (Dakopatts, Denmark) as an indicator. For the color reaction, the blots were soaked in a solution of 0.5 mg diaminebenzidine

tetrahydrochloride/mL (Fluka) and 0.01 % H_2O_2 in 0.05M Tris-HCl, pH 7.5. This solution was freshly prepared and filtered before use.

RESULTS

The Activation of Prothrombin to Thrombin in the Presence of Antithrombin III. The experimental conditions of prothrombin activation that were used to study the inhibitory action of antithrombin III are summarized in Table I. The reactions were initiated by the addition of factor X_a . Under all conditions the rate of thrombin formation was proportional to the factor X_a concentration and when performed in the absence of antithrombin III linear during the time of the experiment. In the presence of antithrombin III the rate of appearance of thrombin activity decreased in time. We assumed that this was due to the inactivation of factor X_a and the inhibition of thrombin activity. A typical curve is shown in Figure 1.

Figure 1: Inhibition of thrombin formation and thrombin activity in prothrombin activation mixtures by antithrombin III. Prothrombin (0.5 M) was activated by [factor X_a (4.0 nM) - phospholipid (5 M) - CaCl_2 (10 mM)] either in the absence (●) or presence (▲) of 100 nM antithrombin III. At timed intervals samples were taken and assayed for thrombin activity. In separate experiments soybean trypsin inhibitor (20 $\mu\text{g}/\text{ml}$) was added 6 min after starting the reaction with factor X_a . The time courses of thrombin activity are indicated by the dashed lines.

Figure 2: Pseudo first order plots of inhibition of thrombin activity in prothrombin activation mixtures by antithrombin III. Conditions of prothrombin activation in the presence of antithrombin III were as given in Figure 1. After the addition of soybean trypsin inhibitor samples were taken and assayed for residual thrombin activity.

The expressions are various time intervals of addition of soybean trypsin inhibitor: (●), 2 min; (○), 4 min; (▲), 6 min; (△), 8 min; (■), 10 min

To distinguish between the antithrombin III action on thrombin and that on factor X_a we inactivated factor X_a by soybean trypsin inhibitor. Thus by preventing further thrombin formation the inhibition of thrombin activity can be studied separately. As shown in Figure 1, soybean trypsin inhibitor instantaneously and completely inhibits factor X_a inactivation, whereas it had no effect on the thrombin activity.

Soybean trypsin inhibitor was then added at timed intervals after the start of prothrombin activation and residual thrombin activity was exponentially plotted versus time. We found that the pseudo first order rate constants of inhibition of thrombin activity were the same at different time intervals of prothrombin activation (Figure 2).

Knowing the rate constant of inhibition of thrombin activity, the rate of inhibition of thrombin can be calculated at any known concentration of thrombin, i.e. at any point of the curve of thrombin activity versus time. From the same curve the rate of appearance of thrombin activity can be determined from the slope at time t . The sum of this rate and the rate of

Figure 3: The rate of thrombin formation in the presence of antithrombin III. Prothrombin activation in the presence of 100 nM antithrombin III was carried out as described in Figure 1. The rate of thrombin formation ($\bullet$, v_p) was calculated from the slope of the thrombin generation curve at the times indicated on the horizontal axis. The rate of thrombin formation, v_p , corrected for the rate of thrombin inacti

vation ($\blacksquare$, v_i) is depicted by the closed triangles (v_a). The inset to the figure shows the exponential plot of v_a versus the prothrombin activation time.

disappearance gives the actual rate of prothrombin activation. The latter then is a true measure for the residual factor X_a activity at time t (Figure 3). From an exponential plot of rate of prothrombin activation (prothrombinase activity) versus time, the pseudo first order rate constant of inhibition of factor X_a by antithrombin III can be estimated (Figure 3, inset).

The Inhibition of Factor X_a in Prothrombin Activation Mixtures. Following the aforementioned procedure, inhibition of factor X_a by antithrombin III was studied in prothrombin activation mixtures of different composition. In addition, the antithrombin III concentration was varied between 50 and 300 nM in order to obtain the second order rate constant of inhibition of factor X_a under these circumstances.

The dependency of the pseudo first order rate constant of inhibition of factor X_a under the different conditions of prothrombin activation on the antithrombin III concentration is shown in Figure 4. The validity of our measuring system is indicated by the observed linear relationships. In

Figure 4: The pseudo first order rate constant of inhibition of factor X_a as a function of the antithrombin III concentration. Prothrombin was activated by: (●), factor X_a -factor V_a -phospholipid- Ca^{++} ; (○), factor X_a -factor V_a - Ca^{++} ; (▲), factor X_a -phospholipid- Ca^{++} . The concentrations of the various reaction components are

listed in Table I, entries 1,2 and 3, respectively. The pseudo first order rate constant of inhibition of factor X_a was determined as described in Figure 3. The pseudo first order rate constants of inhibition of free factor X_a (■) were determined as described in Materials and Methods.

addition, the first order plots extrapolated to within 10% of the rate of thrombin formation in the absence of antithrombin III. The second order rate constants are listed in Table II. When compared with the second order rate constant of inhibition of factor X_a alone, as assayed with the synthetic peptide substrate S-2337, it is clearly shown that the inhibition of factor X_a is reduced only when both factor V_a and phospholipid were present. No reduction of factor X_a inhibition was found when prothrombin activation was performed in the presence of phospholipid and at a prothrombin concentration of $0.5 \mu M$, i.e. about 25-fold the K_m value (2). It is apparent that under these conditions, prothrombin is not a competitive inhibitor of the factor X_a -antithrombin III reaction. Also when factor V_a and phospholipid were present the second order rate constant of inhibition of factor X_a was found to be independent of the prothrombin concentration when varied between below and above the K_m (data not shown).

Table II: The Second Order Rate Constants of Inhibition of Factor X_a and Thrombin by Antithrombin III.

Condition	Second order rate constant ^a ($M^{-1} \text{ min}^{-1}$)	
	Thrombin	Factor X_a
Free proteases	19.0×10^5	11.0×10^5
Alpha-thrombin plus added components ^b	20.0×10^5	
Prothrombin activation ^c		
$X_a - V_a - PL^d$	4.3×10^5	1.3×10^5
$X_a - V_a$	4.4×10^5	11.0×10^5
$X_a - PL$	4.9×10^5	12.0×10^5

^a The second order rate constants were determined as described in Materials and Methods.

^b Inhibition of alpha-thrombin in the presence of prothrombin activation reactants.

^c The conditions of prothrombin activation were as listed in Table I. The rate constants are given for the inhibition of the thrombin activity formed during prothrombin activation and for factor X_a as part of the prothrombinase complex.

^d PL, phospholipid.

Heparin (30 ng/ml) increased the pseudo first order rate constant of inhibition of free factor X_a (5 nM) by antithrombin III (100 nM) from 0.096 min^{-1} to 0.28 min^{-1} . The synthetic peptide substrate S-2337 was used to measure residual factor X_a activity. When compared to the inhibition of free factor X_a , a 10-fold higher heparin concentration was required to obtain a measurable effect of heparin on factor X_a inactivation in the prothrombin activation mixture that contained 3 pM factor X_a , 1.0 μM prothrombin, 0.1 nM factor V_a and 10 μM phospholipid. The pseudo first order

Figure 5: The pseudo first order rate constant of inhibition of thrombin activity as a function of the antithrombin III concentration. Prothrombin was activated by: (●), factor X_a -factor V_a -phospholipid- Ca^{++} ; (○), factor X_a -factor V_a - Ca^{++} ; (▲), factor X_a -phospholipid- Ca^{++} .

The concentrations of the reactants are listed in Table I, entries 1, 2, and 3, respectively. Antithrombin III was present at various concentrations. The pseudo first order rate constants of inhibition of thrombin activity were determined as described in Figure 2. The pseudo first order rate constant of inhibition of alpha-thrombin (■) was determined as described in Materials and Methods.

rate constants at 100 nM antithrombin III were 0.024 min^{-1} and 0.056 min^{-1} in the absence and presence of heparin (300 ng/ml), respectively (Table III). Because the rate constants are proportional to the heparin concentration when corrected for the heparin-independent reaction, i.e. antithrombin III alone (data not shown), the rate constants of inhibition can be compared when they are expressed per unit heparin concentration. The pseudo first order rate constants of factor X_a inhibition are $6 \text{ min}^{-1}(\mu\text{g/ml})^{-1}$ and $0.11 \text{ min}^{-1}(\mu\text{g/ml})^{-1}$ for factor X_a alone and the complete prothrombinase complex, respectively. Whereas factor V_a and phospholipid caused a 4-fold reduction of the heparin-independent inactivation reaction between prothrombinase and antithrombin III, a 55-fold reduction was found for the heparin-dependent inactivation reaction.

The Inhibition of Thrombin Activity in Prothrombin Activation Mixtures.

Rate constants of inhibition of thrombin activity were determined after blocking prothrombin activation by soybean trypsin inhibitor (see Figures 1 and 2). The dependency of the pseudo first order rate constants of inhibition of thrombin activity on the antithrombin III concentration under different conditions of prothrombin activation is shown in Figure 5.

Table III: Pseudo First Order Rate Constants^a (k) of the Heparin-Dependent Antithrombin III-Protease Reaction.

Protease	Heparin conc. (ng/ml)	k (min ⁻¹)	(k-k ₀)/[heparin] ^b (min ⁻¹ μg ⁻¹ ml)
Free factor Xa	0	0.096	
	30	0.28	6.13
Alpha-thrombin	0	0.18	
	30	1.34	38.7
	100	3.86	36.8
	200	7.41	36.2
	300	11.58	38.0
Alpha-thrombin plus added components ^c	0	0.18	
	30	0.49	10.3
	100	2.73	25.5
	200	5.92	28.7
	300	8.48	27.7
Prothrombin activation ^d FX _a -FV _a -PL	0	0.024	
	300	0.056	0.11
Thrombin activity	0	0.040	
	300	0.039	0

^a The pseudo first order constants were determined as described in Materials and Methods. The antithrombin III concentration was 100 nM.

^b k₀, pseudo first order rate constant of the heparin-independent reaction.

^c Added components: prothrombin (1 μM), factor V_a (0.1 nM), phospholipid (10 μM) and Ca⁺⁺ (5 mM).

^d The conditions of prothrombin activation were as listed in Table I, entry 1. The rate constants are given for factor X_a as part of the prothrombinase complex and for the thrombin activity formed during prothrombin activation.

The second order rate constants of inhibition are listed in Table II. Under the different conditions of prothrombin activation we measured identical rate constants of inhibition ($4.5 \times 10^5 \text{ M}^{-1}\text{min}^{-1}$). However, the rate constants were markedly reduced when compared with the inhibition of alpha-thrombin ($1.9 \times 10^6 \text{ M}^{-1}\text{min}^{-1}$).

It might be questioned whether the prothrombin activation reactants (e.g., factor V_a , phospholipid, Ca^{++} and prothrombin) caused the reduced rate of inhibition of the thrombin activity. To this end, pure alpha-thrombin (10 nM) was added to a mixture containing factor X_a (3 pM), factor V_a (0.1 nM), phospholipid (10 μM), Ca^{++} (5 mM), prothrombin (1 μM) and soybean trypsin inhibitor (20 $\mu\text{g/ml}$). Because of the presence of soybean trypsin inhibitor no thrombin formation occurred. After an incubation of 5 min at 37°C , antithrombin III was added and samples were taken at different time intervals to determine residual alpha-thrombin activity. The antithrombin III concentration varied between 50 and 300 nM. The second order rate constant of inhibition of alpha-thrombin under these conditions was found to be $2.0 \times 10^6 \text{ M}^{-1}\text{min}^{-1}$, indicating that the prothrombin activation components had no effect on the rate constant of inhibition (Table II).

To our surprise, heparin concentrations that caused a dramatic increase in the rate constant of inhibition of alpha-thrombin had virtually no effect on the rate constant of inhibition of thrombin activity formed from prothrombin by the prothrombinase complex. The pseudo first order rate constant of inhibition of alpha-thrombin (5 nM) by antithrombin III (100 nM) in the presence of heparin (30 ng/ml) was 1.3 min^{-1} , whereas the rates of inhibition of thrombin activity in the prothrombin activation mixtures, 0.04 min^{-1} , remained the same whether or not heparin (300 ng/ml) was present.

One explanation that might account for the absence of a heparin effect is that some of the prothrombin activation components remove heparin from the thrombin-antithrombin III reaction. Therefore, we performed the antithrombin III-dependent inhibition of alpha-thrombin in a mixture containing 1.0 μM prothrombin, 0.1 nM factor V_a , 10 μM phospholipid, 5 mM Ca^{++} and varying amounts of unfractionated heparin. The pseudo first order rate constants are given in Table III. It is shown that in the presence of the added components the rate constants are lower, but proportional to the heparin concentration. It appeared that prothrombin competed with throm-

bin-antithrombin III for heparin. However, it is clear that because of the absolute absence of a heparin-stimulating effect on the inhibition of the formed thrombin activity, a different cause has to be found.

Identification of the Molecular Species with Thrombin Activity During Prothrombin Activation. It has been reported that prothrombin fragment 2 reduced the rate of inhibition of thrombin by antithrombin III about 3-fold. The second order rate constants for antithrombin III-mediated inhibition of thrombin in the presence of saturating amounts of fragment 2 and antithrombin III-mediated inhibition of meizothrombin (des fragment 1) appeared to be identical (6). Therefore, our results showing a reduced rate of inhibition of thrombin activity could be explained by the presence of prothrombin activation products fragment 1.2 and/or fragment 2. Another interesting possibility might be the formation of meizothrombin as an intermediate in prothrombin activation (14). In order to establish the presence of different molecular species with thrombin activity at nanomolar concentrations we used the following procedure. At timed intervals samples were removed from prothrombin activation mixtures containing prothrombin ($1.0 \mu\text{M}$), factor X_a (3 pM), factor V_a (0.1 nM) and phospholipid ($10 \mu\text{M}$). The samples were incubated with antithrombin III ($1 \mu\text{M}$) and heparin ($1 \mu\text{g/ml}$) at 37°C for 10 min, i.e. sufficiently to obtain complete inhibition of thrombin activity. Subsequently, the samples were analysed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, followed by a blotting procedure and detection of antithrombin III by antibodies conjugated with peroxidase, as described in the Materials and Methods section. The results are shown in Figure 6. In addition to a heavily stained band, comigrating with the large excess of free antithrombin III, two antithrombin III containing complexes are visualized: a major band comigrating with the meizothrombin (des fragment 1)-antithrombin III complex ($M_r = 110,000$) and a minor band comigrating with alpha-thrombin-antithrombin III complex ($M_r = 91,000$). Upon reduction the two antithrombin III-containing complexes were found at a position which corresponds to that of the thrombin B chain-antithrombin III complex ($M_r = 75,000$). We concluded that the major product of prothrombin activation under initial rate conditions was meizothrombin (des fragment 1).

Figure 6. Identification of molecular species with thrombin activity in prothrombin activation mixture. Prothrombin (1.0 M) was activated by [3 pM factor X_a - 0.1 nM factor V_a - $10 \text{ }\mu\text{M}$ phospholipid - 5 mM CaCl_2]. Samples were removed at the times indicated, incubated with antithrombin III ($1 \text{ }\mu\text{M}$) and heparin ($1 \text{ }\mu\text{g/ml}$) and subjected to SDS-gel electrophoresis. A, unreduced samples; B, disulfide reduced sample. Abbreviations used: AT III, antithrombin III; MDF 1, meizothrombin (des fragment 1); T, alpha-thrombin; B, B-chain of alpha-thrombin. Experimental details are given in Materials and Methods.

DISCUSSION

In the present communication we report the results of a study on the inhibition of factor X_a and thrombin by antithrombin III-heparin. Whereas other studies have used a synthetic peptide substrate or prothrombin to measure residual factor X_a activity, our approach was to study factor X_a inactivation under its actual working conditions, i.e. during prothrombin activation. An interesting advantage of this approach is that the effects of the reaction constituents like factor V_a , phospholipid and prothrombin (activation products) can be studied simultaneously on both the inactivation of factor X_a and the inhibition of thrombin activity.

In order to obtain a relationship between the thrombin activity at any time of the reaction and the pseudo first order rate constants of inhibition of factor X_a and thrombin, Equation 1 can be expressed as:

$$d/dt (T) = v_o \exp(-k_1 t) - k_2 T \quad (2)$$

where T is the thrombin concentration, v_o is the rate of thrombin formation in the absence of antithrombin III, k_1 is the pseudo first order rate constant of inhibition of factor X_a and k_2 is the pseudo first order rate constant of inhibition of thrombin.

Integration of Equation 2 leads to:

$$T_t = [v_o / (k_2 - k_1)] [\exp(-k_1 t) - \exp(-k_2 t)] \quad (3)$$

where T_t is the thrombin concentration at time t .

Equation 3 was utilized to calculate the thrombin concentration at different time intervals of the prothrombin activation reaction under the conditions of the experiment given in Figure 1. We have set $k_1 = 0.11 \text{ min}^{-1}$, $k_2 = 0.047 \text{ min}^{-1}$ and $v_o = 2.8 \text{ nM min}^{-1}$. The calculated thrombin activities are in excellent accord with those determined experimentally (Figure 7).

Inhibition of Factor X_a . The experiments presented here show that when prothrombin activation occurs at a phospholipid surface in the absence of factor V_a , factor X_a is not protected from inactivation by antithrombin III. Under this condition the second order rate constant of inhibition of factor X_a was found to be $1.2 \times 10^6 \text{ M}^{-1} \text{ min}^{-1}$ which is identical to the rate

Figure 7: Thrombin formation during prothrombin activation in the presence of antithrombin III. The conditions were as given in Figure 1. The experimental data (●) were taken from Figure 1. The data, calculated as described in the Discussion section, are presented by the closed triangles.

constant of inhibition of factor X_a alone as measured with a synthetic peptide substrate (Table II). Because in the absence of phospholipid, factor V_a did not protect factor X_a from inactivation either, it is evident that protection must arise from the formation of the phospholipid-bound factor X_a - factor V_a complex.

Ellis et al. (4) concluded that "the protective effect is probably due to the factor V_a that altered the binding of factor X_a on the phospholipid surface in such a way that the steric hindrance to antithrombin III-binding is increased, with the large bulk of the factor V_a molecule perhaps contributing to this effect". Walker & Esmon (3) came to the same conclusion. Ellis et al. (4) reasoned that since their assay only determined phospholipid-bound factor X_a and not free factor X_a , they have determined the kinetics of inhibition of phospholipid-bound factor X_a . Obviously, this can only be true if during the time of the experiment phospholipid-bound factor X_a did not exchange with free factor X_a . We note that this assumption is probably irreconcilable or incompatible with the current knowledge of both the equilibrium and dynamic properties of factor X_a -phospholipid interaction (15, 16). The kinetics of the factor

X_a -phospholipid interaction can be indicated by the second order rate constant of association and the first order rate constant of dissociation: $10^{-7} \text{ M}^{-1} \text{ s}^{-1}$ and 2 s^{-1} , respectively. Therefore, it is highly unlikely that Ellis et al. (4) have solely monitored the inhibition of phospholipid-bound factor X_a .

Our data support that notion. Using previously reported factor X_a -phospholipid binding parameters (17,18) it can be calculated that under our conditions of prothrombin activation in the presence of phospholipid the amount of factor X_a bound to phospholipid was at least 50% of the total amount of factor X_a present. In addition, the K_m values for prothrombin in the presence or absence of phospholipid being $0.02 \text{ } \mu\text{M}$ and $100 \text{ } \mu\text{M}$, respectively (2), we can calculate that because prothrombin was present at a concentration of $0.5 \text{ } \mu\text{M}$, all phospholipid-bound factor X_a is saturated with substrate, whereas in solution the amount of factor X_a -prothrombin complexes is negligible. Therefore, our assay virtually measures the thrombin formation by the factor X_a bound to the phospholipid surface only. It is worth noting that synthetic peptide substrates at concentrations above their K_m value have been reported to compete with antithrombin III for factor X_a (19). Thus, when factor X_a is saturated with prothrombin, we might expect to find such a competitive action of prothrombin in the factor X_a -antithrombin III reaction. However, the second order rate constant of factor X_a inactivation by antithrombin III during prothrombin activation in the presence of phospholipid was the same as that determined for free factor X_a (Table II). This observation, together with the known data on the kinetics of factor X_a -phospholipid interaction, strongly indicate that the hypotheses and conclusions of Ellis et al. (4) are untenable. That is, the effect of phospholipid on the kinetics of inhibition of factor X_a by antithrombin III cannot be explained by assuming that the enzymatic properties towards antithrombin III are altered when factor X_a is bound to the phospholipid surface. Moreover, because of the rapid kinetics of the factor X_a -phospholipid interaction, the inhibition of free factor X_a has to be considered as well. In this respect it is important to note the following.

Firstly, Pusey et al. (16,20) demonstrated that factor X_a has a very marked effect on the rate of dissociation of factor V_a from a phospholipid surface. The most likely explanation for this effect is that because of the

tight association between the two phospholipid-bound proteins a complex is produced with two membrane association sites (8). The overall result is a markedly reduced rate of dissociation of both factor V_a and factor X_a from the phospholipid surface. Therefore, the protection of factor X_a from inhibition by antithrombin III when both factor V_a and phospholipid are present might be caused by the reduced rate of dissociation of factor X_a from the phospholipid surface.

Secondly, we demonstrated that when prothrombin is activated by the complete prothrombinase complex, factor X_a is even more protected from inactivation by the heparin-dependent inactivation reaction than from the heparin-independent one. When compared with free factor X_a , a 55-fold and 4-fold lower pseudo first order rate constants were found in the presence and absence of heparin, respectively.

Thirdly, antithrombin III has no affinity for the phospholipid vesicles that were used throughout our studies (Lindhout, unpublished result).

In conclusion, our results argue strongly for a competition for factor X_a between a phospholipid surface and antithrombin III in solution. It is feasible that protection of factor X_a from inhibition by antithrombin III depends on both the rate of inhibition of factor X_a in solution and the rate of dissociation of factor X_a from the phospholipid surface.

Inhibition of thrombin. The second aim of this study was to reveal whether or not the kinetics of inhibition of alpha-thrombin differ from those of inhibition of thrombin activity formed during the initial phase of prothrombin activation. There has been a report on the effect of prothrombin fragment 2 on the inhibition of alpha-thrombin by antithrombin III (6). In addition, recent work from our laboratory has indicated that during the initial phase of prothrombin activation by factor X_a prothrombin is mainly cleaved between Arg₃₂₃ and Ile₃₂₄, resulting in amidolytic active meizothrombin (14). We found that the second order rate constant of inhibition of alpha-thrombin by antithrombin III was about 4 times the rate constant of thrombin activity in the prothrombin activation mixtures and independent of the composition of the prothrombinase complex. Walker & Esmon (6) reported that fragment 2 was found to slow down the rate of inhibition of alpha-thrombin about 3-fold. Therefore, the presence of fragment 2 and/or fragment 1.2 in our activation mixtures might be a plausible explanation for our findings. An alternative explanation is the

formation of meizothrombin, because covalently and non-covalently linking of fragment 2 to thrombin gave the same second order rate constants of inhibition (6). Therefore, we analysed our activation mixtures for the presence of meizothrombin. As shown, a considerable amount of meizothrombin (des fragment 1) was detected (Figure 6). Because meizothrombin appeared to be the main product under the conditions used, it can be questioned whether pure alpha-thrombin can be used as a standard to measure the reaction product of prothrombin activation. However, on basis of the functional molar concentrations as assessed by active site titration of meizothrombin preparations obtained by the activation of prothrombin by Echis carinatus venom and alpha-thrombin preparations, we found no differences between the reactivity of alpha-thrombin and meizothrombin towards the synthetic peptide substrate S-2238.

Figure 6 demonstrates that alpha-thrombin is present as a minor reaction product. Because the pseudo first order plots of inhibition of thrombin activity extrapolated to within 5% of the amount of thrombin activity originally present and assuming that alpha-thrombin is neutralized at a much faster rate than meizothrombin (Table II), it indicates that alpha-thrombin contributes to less than 5% of the total thrombin activity. In addition, our experimental data could be fitted according to Equation 3 with the experimentally determined pseudo first order rate constants of inhibition of both thrombin and factor Xa (Figure 7).

An effect of phospholipid, as described for the inhibition of factor X_a , on thrombin which might be bound to the surface via a covalently or non-covalently linking via fragment 1.2 could be ruled out. When soybean trypsin inhibitor was replaced by EDTA, the rate of inhibition of thrombin did not increase.

Intriguing is the observation that when heparin is studied in prothrombin activation mixtures, it had no effect on the inhibition of thrombin activity by antithrombin III. We ruled out that this phenomenon was caused by the heparin-binding properties of prothrombin. As yet, it remains to be established to what extent and in which mode fragment 2 and/or fragment 1.2 either covalently or non-covalently bound to alpha-thrombin affects the interaction between heparin and thrombin. Recently, we initiated a study on the interaction between meizothrombin and heparin. Our preliminary results

indicate that in contrast to alpha-thrombin, meizothrombin is unable to bind heparin.

Our work indicates that the mode of action of heparin and its derivatives on the process of blood coagulation can never be deduced from its actions on the purified free serine proteases.

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CHAPTER VII

GENERAL DISCUSSION

The work presented here concerns *in vitro* studies on the influence of heparin and heparin derivatives on the generation of thrombin. Our aim was to identify some important reactions of blood coagulation and platelet activation that are sensitive to the action of heparin. Thrombin is generated from its precursor prothrombin by the proteolytic action of the prothrombinase complex. The maximal rate of thrombin formation is increased 2000-fold in the presence of the non-enzymatic cofactor, factor V_a (1). Factor V_a is formed by the limited proteolytic action of thrombin on plasma factor V. Furthermore, thrombin catalyzes platelet activation reactions inducing the release of factor V and its activation. This illustrates how a trace amount of thrombin causes a considerable amplification of its own formation and that regulatory mechanisms are required in order to prevent the development of thrombosis as a result of such an explosive process of thrombin formation. It is important to note that heparin can inhibit thrombin by two different mechanisms, either antithrombin III-dependent or antithrombin III-independent. Whereas the role of heparin in catalyzing the antithrombin III-dependent inactivation of thrombin has been well documented, much less information is available on the significance of the antithrombin III-independent inactivation of thrombin by heparin (Chapter I).

Studies on thrombin-induced platelet factor V_a formation

In chapter II we describe a kinetic study on thrombin-induced factor V_a formation in suspensions of human platelets. The effect of thrombin in stimulating the release reaction could be separated from its effect on factor V activation by use of a stable prostacyclin analogue that blocked instantaneously and completely the release reaction without interfering with the activation reaction. The rate-limiting step in thrombin-induced platelet factor V_a formation was found to be the thrombin-catalyzed activation of the released non-activated factor V. The rate of platelet

factor V release was 10 times faster than the rate of factor V activation and as fast as that of β -thromboglobulin release, a specific platelet α -granule protein (2). Thus our results differ from previously reported rates of factor V release, that actually measured the rate of factor V activation (3). The rates of factor V release and factor V activation appeared to be proportional to the thrombin concentration whereas the maximal amount of released factor V was obtained at a thrombin concentration of 1 nM and was identical to the amount measured in Triton X-100 lysed platelets (0.3 nM factor V/ 10^8 platelets/ml). This amount represents half of the value as determined by a radioimmunoassay (4). However, it is identical to the value reported by the same group in a more recent report (5). That all factor V present in platelets can be released by thrombin is in agreement with previous reports (3,6). Whether other agents like collagen are able to completely release platelet factor V is not yet established, because thrombin had to be used to convert the released factor V in its biological active form (7,8). Our method, which offers a way to separate the release step from the activation step, might be used to solve that problem.

An important question that needs to be addressed is the contribution of platelet factor V_a generation over plasma factor V_a generation in the regulation of thrombin formation. Factor X_a and factor V_a interact stoichiometrically at the platelet surface (9,10). Locally high platelet factor V_a concentrations might be provided upon platelet stimulation. It has been reported that the presence of platelet factor V, rather than plasma factor V activity, correlates with the severity of bleeding (5,11). However, we found that the rate of activation of platelet factor V, once it is released by thrombin, is identical to the rate of plasma factor V activation, and that platelet factor V concentration is only 10% of plasma factor V concentration. Furthermore, in this respect, our study on the thrombin-induced platelet and plasma factor V_a formation in patients with a Gray Platelet Syndrome, characterized by an absence of α -granules in the platelets, revealed some interesting information (chapter III). These patients have a moderate bleeding disorder that we found on the one hand, to go along with an almost normal plasma V concentration and rate of plasma factor V activation, and on the other hand revealed a decreased amount of platelet factor V (less than 20% of normal), that required 4-fold higher

thrombin concentrations in order to be released than what is observed in normal platelets. Thus, our results suggest that a significant reduction of platelet factor V_a activity does not result in a severe bleeding disorder as long as the factor V_a formation is provided by plasma factor V.

The effect of heparin on thrombin-catalyzed reactions in the absence of antithrombin III. We have demonstrated that in the absence of antithrombin III, unfractionated heparin could inhibit four thrombin-catalyzed reactions: plasma factor V and plasma factor VIII:C activation, platelet factor V_a formation and generation of platelet prothrombin converting activity. The generation of the latter requires the combined action of thrombin plus collagen (chapter IV). The concentrations of unfractionated heparin required to reduce the rates of the previously mentioned reactions to 50% of those in the absence of heparin are in the same range as the therapeutic heparin concentration in plasma (1 USP unit/ml). However, low molecular weight fractions and fragments as well as a heparinoid were much less inhibitory in these thrombin-catalyzed reactions. Our results indicate that this effect of heparin is caused by the formation of a 1:1 molar complex with thrombin. In this complex the activity of thrombin towards small synthetic peptide substrates remained intact but was impaired on macromolecular substrates.

It has been reported that heparin is able to displace the blood coagulation proteins involved in the prothrombin or factor X activating complex, from the phospholipid surface (12-14). By equilibrium binding studies we have found that heparin did not alter the affinity nor the binding capacity of the proteins for phospholipid vesicles. Furthermore, we showed that, in the absence of antithrombin III, heparin did not inhibit prothrombin activation. Thus, heparin did not interfere with the assembly nor the functioning of the prothrombinase complex.

It might be questioned whether the inhibitory action of heparin in thrombin-catalyzed platelet activation is caused by a heparin-platelet rather than by a heparin-thrombin interaction. Salzman et al. (15) reported that high molecular weight heparin fractions irrespective of their antithrombin III affinity induced significantly more platelet aggregation than low molecular weight, antithrombin III high affinity fractions. However, Hirsh (16) showed that antithrombin III low affinity fractions

were unable to bind to platelets, whereas antithrombin III high affinity fractions did.

We found that the release of β -thromboglobulin could be inhibited by unfractionated heparin when thrombin was used as the platelet activator but not in the case of collagen-induced β -thromboglobulin release. Unless heparin binds to the very same platelet site as thrombin does, this result supports the notion that the thrombin-heparin interaction is the event that causes the inhibitory action of heparin on thrombin-catalyzed platelet activation reactions.

We further investigated the structural properties of heparin that are involved in the mechanism of inhibition of the thrombin-catalyzed reactions in the absence of antithrombin III (Chapter V). We studied the influence of heparin fractions obtained from unfractionated heparin by ion-exchange chromatography, on thrombin-catalyzed platelet factor V_a formation and on thrombin inactivation by antithrombin III in the presence of active-site-blocked thrombin. We observed a linear relationship between the IC_{50} values of heparin fractions and their charge density, indicating that their affinity for thrombin increased with their charge density. This finding is in accordance with those of Hoylaerts et al. (17) as to the electrostatic nature of the interaction between heparin and thrombin. However, we found that heparin fractions with increasing charge density also had an increasing percentage of antithrombin III-binding species. Furthermore, we found that an antithrombin III low affinity fraction hardly had any effect on thrombin-induced platelet factor V_a formation whereas the antithrombin III high affinity fraction had a similar effect as unfractionated heparin. We concluded that antithrombin III high affinity heparin contained most, if not all, of the thrombin-binding heparin species.

It is likely that the antithrombin III-independent mechanism of action of heparin is comparable to the anticoagulant properties of some sulphated heparinoids like the pentosan polysulphate, and related to their thrombin affinity (18). The effect of pentosan polysulphate on some thrombin-catalyzed reactions has been demonstrated in our laboratory (19).

An important question is whether or not this antithrombin III-independent effect of heparin on thrombin-catalyzed reactions of platelet activation and plasma factor V and factor VIII:C activation is of importance in the regulation of the hemostatic mechanisms. As yet, this question cannot be

answered. However, several studies have indicated that upon fractionation of crude heparin the in vitro antithrombin III-dependent activity of heparin can be dissociated from its in vivo antithrombotic and/or hemorrhagic effects. For instance, an antithrombin III low affinity fraction significantly increased bleeding without having any antithrombotic effect in vivo, as well as any antithrombin III-dependent anti-factor X_a or anti-thrombin effect in vitro (20). It has also been reported that when an antithrombin III low affinity fraction was added to an antithrombin III high affinity decasaccharide devoid of in vitro anti-thrombin activity as well as in vivo antithrombotic activity, the mixture significantly prevented thrombosis in an animal experimental model (21). Buchanan et al. (22) have suggested that whereas the antithrombotic properties of heparin are related to the antithrombin III-mediated inactivation of serine proteases, the hemorrhagic effects are due to an antithrombin III-independent inhibition of platelet aggregation and of thrombin formation at the platelet surface. We believe that such an interpretation of the antithrombin III-independent effect of heparin cannot be drawn from our studies which were limited to in vitro assays. However, our results clearly demonstrate that in studying the in vitro anti-thrombin activity of heparin(fractions), the antithrombin III-independent action should also be taken into account.

The antithrombin III-dependent action of heparin during factor X_a -catalyzed prothrombin activation.

The major question about the antithrombin III-dependent actions of heparin on thrombin, generated by the prothrombinase complex and on the prothrombinase complex itself, is whether the actions of antithrombin III-heparin can be inferred from studies on the inactivation of purified α -thrombin and factor X_a . It has been reported that when factor X_a was bound to phospholipid either in the absence or presence of factor V_a , its rate of inactivation by antithrombin III was much slower than that of free factor X_a (23-25). In addition, prothrombin fragment 2, an activation fragment generated during prothrombin conversion was found to slow down about 2-fold the rate of inhibition of α -thrombin by antithrombin III (26). No data are available on the effect of fragment 2 on the inactivation of α -thrombin by antithrombin III in the presence of heparin. In view of the physiological

importance of the aforementioned modulating effects on both factor X_a and α -thrombin inactivation by antithrombin III-heparin, a study on the efficiency of antithrombin III-heparin during the process of prothrombin activation is highly relevant. To this end, we developed a method which allowed us to study separately the rate of factor X_a inactivation by antithrombin III from the rate of thrombin inactivation during the conversion of prothrombin by the prothrombinase complex.

We have found that both factor V_a and phospholipid are required to obtain a protection of factor X_a from the inactivation by antithrombin III. In the presence of heparin, an enhanced protection was observed compared to the one in the absence of heparin. Kinetic studies on the interaction of factor X_a with factor V_a in the presence of phospholipid have shown that factor V_a facilitates the binding of factor X_a to the phospholipid surface, by decreasing the dissociation constant about 200-fold (26). Thus, the rate of inactivation of factor X_a by antithrombin III strongly depends on the way in which the equilibrium between free factor X_a and bound factor X_a is modified in the presence of factor V_a . It is likely that a protection of factor X_a from inhibition by antithrombin III depends on both the rate of inhibition of factor X_a in solution and the rate of dissociation of factor X_a from the phospholipid surface. This is in contrast with other studies where it has been suggested that the binding of factor X_a to phospholipid altered its enzymatic properties (24,25). Our study on the inactivation by antithrombin III of the thrombin activity generated during prothrombin activation revealed an important difference with the inactivation of the purified enzyme, α -thrombin. This difference resulted in a 4-fold lower second order rate constant of inactivation of the formed thrombin than the one for the inactivation of α -thrombin. Analysis of the molecular species which are formed during prothrombin activation showed that considerable amounts of meizothrombin (des fragment 1) were formed during the early stage of prothrombin activation. This is in accordance with the findings of Rosing et al. (28). Under our conditions we found that the amount of α -thrombin represented about 5% of the total thrombin activity. Because the rate of inactivation of meizothrombin is slower than that of α -thrombin, this finding thus explains why the rate of inhibition of the thrombin activity generated during prothrombin activation is lower than the rate of inhibition of purified α -thrombin.

Furthermore, we found that in contrast with its effect on the inactivation of α -thrombin by antithrombin III, heparin could not enhance the rate of inactivation of the thrombin activity formed by the prothrombinase complex. Preliminary results from our laboratory indicate that the affinity of heparin for meizothrombin is at least one order of magnitude lower than its affinity for α -thrombin.

Thus, the analysis of the inhibition by antithrombin III of thrombin formed by the prothrombinase complex, not only shows that this inhibitory effect differs from the one on the purified enzymes, but raises new questions on the mechanism of thrombin formation.

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SUMMARY

Heparin, a heterogeneous mucopolysaccharide existing in different molecular forms is widely used in antithrombotic therapy. The relationship between its in vivo antithrombotic and in vitro anticoagulant action is far from being understood. The aim of our work is to identify some reactions of blood coagulation that contribute to thrombus formation and that are sensitive to the action of heparin. In this respect thrombin has a central role in the blood coagulation process as it governs several positive feedback reactions. Heparin inhibits thrombin through two different mechanisms that are either dependent or independent of a plasma inhibitor, antithrombin III. Whereas the antithrombin III-mediated mechanism of heparin action has been extensively studied, the antithrombin III-independent one has been the subject of a very few investigations. These studies are reviewed in Chapter I.

The prothrombinase complex, which converts prothrombin into thrombin, consists of the enzyme factor X_a bound to the protein cofactor factor V_a , assembled on a phospholipid surface. Factor V_a is formed by the proteolytic action of thrombin on a biologically inactive procofactor, factor V, which is present in plasma and in the alpha-granules of platelets. Moreover, thrombin induces the release of platelet factor V. We were able to determine the kinetics of platelet factor V release and activation in thrombin-activated platelet suspensions by using a specific inhibitor of the release reaction, a semi-synthetic prostacyclin analogue (Chapter II). We found that the initial rate of factor V release is 10-fold faster than the rate of factor V activation which is the rate-limiting step of platelet factor V_a formation. The platelet factor V concentration is only 10% of the one in plasma. In this respect it can be questioned whether platelet factor V_a activity contributes to thrombin formation compared to plasma factor V_a activity. Although it may be important for the hemostasis and coagulation processes to obtain high factor V_a concentrations at the site of platelet activation, two arguments point to this contribution not being essential. First, factor V from plasma or platelets are functionally identical. Second, studies on platelets from patients with a hereditary alpha-granule deficiency revealed a factor V content which was about 1% of

their plasma factor V concentration. (Chapter III). Despite this platelet factor V deficiency these patients have a moderate bleeding disorder. In this case, plasma factor V which was found to be quantitatively and functionally normal, might account for a not severely impaired thrombin formation.

The inhibition of thrombin-catalyzed reactions such as plasma factor V activation and platelet factor V_a formation is a potential regulatory mechanism against thrombosis, as it results in an interruption of the amplification of thrombin formation. As a first step to study these complex reactions we showed that in the absence of antithrombin III, an inhibitory effect of heparin could be demonstrated on four thrombin-catalyzed reactions (Chapter IV). Besides the two previously mentioned reactions, plasma factor V activation and platelet factor V_a formation, thrombin converts plasma factor VIII:C to factor $VIII_a$ and, in the presence of collagen, induces the platelet prothrombin converting activity by providing a procoagulant phospholipid surface. This inhibitory action of heparin is due to the formation of a complex with thrombin, where the activity of thrombin towards its macromolecular substrates is impaired, but its enzymatic activity towards small synthetic peptide substrates is unaffected. We found that unfractionated heparin has a higher affinity for thrombin than the low molecular weight heparin fractions that we studied. The inhibitory action of heparin on platelet activation by thrombin was not due to an effect of heparin on platelet functions.

To better characterize the properties of heparin fractions involved in the inhibition of thrombin-catalyzed reactions in the absence of antithrombin III, we studied the effect of heparin fractions obtained by ion-exchange chromatography (Chapter V). We found that the affinity for thrombin increased with the charge density of heparin fractions, indicating an electrostatic type of interaction with thrombin. However, in parallel with the variation in charge density, the heparin fractions were found to differ in their percentage of antithrombin III-binding species. Furthermore, an antithrombin III low affinity fraction had no inhibitory effect on the thrombin-catalyzed platelet factor V_a formation. Our results suggest that in a heterogeneous heparin preparation no polysaccharide chains can be found which have exclusively an antithrombin III-independent action on thrombin.

It has been reported that the antithrombin III-dependent action of heparin on the inactivation of thrombin and factor X_a is modified by the presence of the components of the hemostatic system. However, the inhibitory mode of action of antithrombin III-heparin on factor X_a and thrombin during thrombin formation by the prothrombinase complex remained uncertain. Therefore we have developed a method in order to assess the effect of antithrombin III-heparin on the inactivation of factor X_a and thrombin during prothrombin activation by the prothrombinase complex. This inhibitory action was compared to the inactivation of each of the purified, free enzymes (Chapter VI). In the presence of phospholipid and factor V_a , factor X_a was protected from the inactivation by antithrombin III alone and in the presence of heparin. Moreover, independent of the composition of the prothrombinase complexes, the rate of disappearance of the amidolytic activity of the formed thrombin was considerably slower than the one of purified thrombin (alpha-thrombin). This was explained by the fact that, during prothrombin activation, the thrombin activity resulted mainly from the formation of meizothrombin and to a minor extent of alpha-thrombin. In contrast to thrombin, meizothrombin is virtually insensitive to the action of antithrombin III-heparin. Our conclusion is that the action of heparin on the antithrombin III-mediated inactivation of purified thrombin and factor X_a cannot be extrapolated to its effect in conditions that are more likely to reflect the in vivo situation.

RESUME

L'héparine, mélange de mucopolysaccharides sulfatés, est un agent antithrombotique largement utilisé en clinique humaine sous diverses formes moléculaires. Les relations existant entre son action antithrombotique in vivo et son action anticoagulante in vitro sont loin d'être entièrement élucidées. L'objectif de notre travail est de reconnaître, parmi les réactions de la coagulation qui sont importantes dans le développement de la thrombose, celles qui sont sensibles à l'action de l'héparine. A cet égard, on peut considérer que la thrombine occupe une position centrale dans la coagulation car elle contrôle plusieurs réactions conduisant à une amplification de sa propre formation. L'héparine peut inhiber la thrombine par deux mécanismes différents selon que son action est dépendante ou indépendante d'un inhibiteur plasmatique, l'antithrombine III. Alors que le mécanisme d'action de l'héparine médié par l'antithrombine III est le mieux connu, celui qui est indépendant de l'antithrombine III l'est beaucoup moins. Le chapitre I décrit l'état des connaissances sur l'effet inhibiteur de l'héparine sur la thrombine dans chacune de ces deux voies. Le complexe de la prothrombinase, qui permet la conversion de prothrombine en thrombine, est formé par un enzyme, le facteur X_a , associé à un cofacteur protéique, le facteur V_a , qui sont assemblés sur une surface phospholipidique. Le facteur V_a provient de l'action protéolytique de la thrombine sur un procofacteur biologiquement inactif, le facteur V, présent dans le plasma et dans les granules alpha des plaquettes. De plus la thrombine induit la sécrétion du facteur V plaquettaire. Ainsi après stimulation des plaquettes par la thrombine, deux réactions (sécrétion du facteur V, activation en facteur V_a) ont lieu simultanément. Nous avons pu déterminer séparément les cinétiques des deux réactions en ajoutant au cours du temps un inhibiteur spécifique de la réaction de sécrétion, un analogue semi-synthétique de la prostacycline (Chapitre II). Nous avons montré que la vitesse initiale de sécrétion du facteur V est 10 fois plus rapide que celle de son activation. Celle-ci représente donc l'étape limitante de la formation d'activité facteur V_a plaquettaire. De plus nous avons trouvé que la concentration du facteur V plaquettaire ne représente que 10 % de celle du plasma. Cela pose le problème de la contribution de l'activité facteur V_a plaquettaire par rapport à l'activité facteur V_a

plasmatique dans la formation de thrombine. Bien que l'obtention de concentrations élevées de facteur V_a au site même d'activation des plaquettes puisse avoir une importance dans les processus d'hémostase et de coagulation, deux arguments soulignent que cette contribution n'est pas essentielle. D'une part, sur le plan fonctionnel les facteurs V plasmatique et plaquettaire sont identiques. D'autre part, l'étude des plaquettes de patients présentant une absence congénitale en granules alpha a révélé qu'elles ne contiennent pas plus de 1% du facteur V présent dans leur plasma (Chapitre III). Malgré cette anomalie du facteur V plaquettaire, ces patients ne présentent qu'un syndrome hémorragique modéré. Ceci peut être attribué à la présence d'un facteur V plasmatique fonctionnellement et quantitativement normal.

L'inhibition des réactions catalysées par la thrombine, telles que l'activation du facteur V plasmatique et la formation du facteur V_a plaquettaire, représente un mécanisme régulateur potentiel de protection contre la thrombose car elle interrompt l'auto-amplification de la formation de thrombine. Notre première approche à l'étude de ces interactions complexes a été de démontrer qu'en absence d'antithrombine III, quatre réactions catalysées par la thrombine peuvent être inhibées par l'héparine (Chapitre IV). Outre les deux réactions décrites ci-dessus, l'activation du facteur V plasmatique et la formation d'activité facteur V_a plaquettaire, la thrombine active le facteur VIII:C plasmatique en facteur $VIII_a$ et, en présence de collagène, induit la formation d'une activité procoagulante de nature phospholipidique à la surface des plaquettes. L'action inhibitrice de l'héparine est due à la formation d'un complexe avec la thrombine, dans lequel l'activité de la thrombine vis-à-vis de ses substrats macromoléculaires est inhibée sans que son activité enzymatique sur des petits substrats peptidiques synthétiques soit modifiée. Nous avons observé que l'héparine non fractionnée a une affinité plus élevée pour la thrombine que ne l'ont les héparines de bas poids moléculaire étudiées. De plus l'action inhibitrice de l'héparine sur les réactions d'activation des plaquettes par la thrombine n'est pas due à l'inhibition des fonctions plaquettaires par l'héparine.

Pour préciser les caractéristiques de l'héparine et de ses dérivés intervenant dans l'inhibition des réactions catalysées par la thrombine en absence d'antithrombine III, nous avons étudié l'effect de fractions

d'héparine obtenues par chromatographie sur colonne échangeuse d'ions (Chapitre V). L'affinité de fractions d'héparine pour la thrombine augmente avec leur densité de charge, indiquant la nature électrostatique de la liaison héparine-thrombine. Cependant la variation de densité de charge de ces fractions va de pair avec la variation du pourcentage d'espèces moléculaires possédant le site de liaison à l'antithrombine III. De plus une fraction d'héparine de faible affinité pour l'antithrombine III est dépourvue d'action inhibitrice sur la réaction de formation d'activité facteur V_a plaquettaire catalysée par la thrombine. Nos résultats suggèrent qu'à partir d'une préparation d'héparine hétérogène on ne peut isoler de(s) chaîne(s) polysaccharidique(s) ayant une action anti-thrombine qui soit exclusivement indépendante de l'antithrombine III.

Un effet modulateur des composés du système hémostatique a été décrit sur l'inactivation des enzymes de la coagulation par l'antithrombine III-héparine. Cependant le mode d'action inhibitrice de l'antithrombine III-héparine sur le facteur X_a et la thrombine lors de la formation de celle-ci par le complexe de la prothrombinase n'est pas clairement établi. C'est pourquoi nous avons développé une méthode qui nous permet de déterminer l'action de l'antithrombine III-héparine, d'une part sur le facteur X_a participant au complexe de la prothrombinase, d'autre part sur la thrombine formée par la prothrombinase. Cette action a été comparée à l'inactivation de chaque enzyme purifié, libre en solution (Chapitre VI). En présence de phospholipides et de facteur V_a , le facteur X_a est protégé de l'inactivation par l'antithrombine III seule et en présence d'héparine. Par ailleurs l'action inhibitrice de l'antithrombine III-héparine sur la thrombine formée est beaucoup moins importante que son action sur la thrombine purifiée (alpha-thrombine). Ce phénomène est observé quelle que soit la composition des complexes de la prothrombinase. L'explication peut en être trouvée dans l'étude des produits d'activation de la prothrombine. Il semble que l'activité de la thrombine ainsi formée résulte non seulement de la formation d'alpha-thrombine, mais surtout de la formation de meizothrombine. Contrairement à la thrombine, celle-ci est pratiquement insensible à l'action de l'antithrombine III-héparine.

En conclusion il est clair que l'action de l'héparine médiée par l'antithrombine III, sur l'inactivation du facteur X_a et de la thrombine purifiés, ne peut pas être extrapolée à son action étudiée dans des conditions reflétant mieux la situation physiologique.

SAMENVATTING

Heparine wordt veelvuldig toegepast bij de behandeling en preventie van veneuze trombose en trombo-embolisme. Het is bekend dat heparine een bloedstollings-remmende werking heeft. Echter, het verband tussen de antistollings- en antitrombotische werking van heparine is nagenoeg onbekend. In het onderzoek naar deze relatie staan twee vragen centraal: welke processen (bloedstollingsreacties) hebben een sleutelpositie in het ontstaan van trombose en op welke wijze werkt heparine in deze reacties. In hoofdstuk I wordt op deze vragen nader ingegaan.

Omdat trombine, gevormd in de voorlaatste stap van het bloedstollingsproces, middels positieve tegenkoppelingsreacties, een grote rol speelt in zijn eigen vorming, hebben we ons vooral gericht op het bestuderen van een aantal trombine-afhankelijke reacties en het effect van heparine daarop.

Trombine wordt gevormd uit prothrombine door het zogenaamde prothrombinase complex. Dit complex bestaat uit het enzym factor X_a en de eiwit cofactor factor V_a , gebonden aan een membraan, een fosfolipide oppervlak. Factor V_a wordt gevormd uit een biologisch inactieve procofactor onder invloed van trombine. De procofactor, factor V, is een plasma eiwit, maar het komt ook voor in de alpha-granula van de bloedplaatjes. Trombine is in staat factor V uit de plaatjes te laten treden (release-reactie) waarna factor V vervolgens door trombine wordt omgezet in zijn actieve vorm. Hoofdstuk II beschrijft de kinetiek van de plaatjes factor V release-reactie en de plaatjes factor V activeringsreactie. Deze twee, simultaan verlopende, reacties konden apart bestudeerd worden door het gebruik van een remmer, een semi-synthetische prostacycline analoog, van de release reactie. We vonden dat de activeringsreactie de snelheidsbeperkende stap in de plaatjes factor V_a vormingsreactie is.

De hoeveelheid factor V aanwezig in circulerende bloedplaatjes is slechts 10% van de hoeveelheid factor V aanwezig in plasma. Dit feit en de waarneming dat plaatjes factor V en plasma factor V in alle opzichten identiek zijn, roept de vraag op of plaatjesfactor wel een specifieke fysiologische betekenis heeft, zoals in de literatuur gesuggereerd wordt. Uit ons onderzoek naar de factor V_a vorming in plasma en suspensies van bloedplaatjes van patienten met een erfelijke alpha-granula deficiëntie

(Gray-platelet syndroom) blijkt dat hoeveelheid factor V_a die uit hun plaatjes vrijgemaakt kan worden hooguit 1% is van de hoeveelheid te vormen plasma factor V_a . Interessant is dat deze patienten desondanks geen ernstige bloedingsneigingen vertonen. Wellicht komt dit door een bijna normaal plasma factor V gehalte (Hoofdstuk III).

Het remmen van de trombine-gecataliseerde reacties zoals plasma en plaatjes factor V_a vorming, activeren van factor VIII en het activeren van bloedplaatjes, kan op het tromboseproces een belangrijke invloed hebben. Immers, op deze manier wordt de vorming van trombine gereguleerd. In plasma bevindt zich het antitrombine III, een belangrijke remmer van trombine. Heparine speelt bij deze reactie een catalytische functie. Echter, ook zonder antitrombine III is heparine in staat trombine te inactiveren. De antitrombine III-onafhankelijke antistollingseigenschap van heparine is nog nauwelijks bestudeerd. In hoofdstuk IV tonen wij aan dat een aantal trombine-afhankelijke reacties ook zonder antitrombine III door heparine geremd worden, zoals plasma factor V en plasma factor VIII activering en het induceren van een plaatjes procoagulant oppervlak. Deze heparine werking is het gevolg van de vorming van een complex tussen trombine en heparine, waarbij de trombine zijn activiteit t.o.v. natuurlijke, hoog molecuulair gewicht, substraten verliest. We vonden dat niet-gefractioneerde, klinisch toegepaste heparine veel meer effect had dan gefractioneerde, laag molecuulair gewicht heparines.

Nader onderzoek naar het effect van heparine fracties verkregen na scheiding op een ionenwisselaar, laat zien dat de affiniteit van deze heparine fracties voor trombine en dus hun remmende werking, toeneemt met de ladingsdichtheid van de fracties (hoofdstuk V). Wij hebben tevens gevonden dat de fractionering naar ladingsdichtheid gepaard gaat met een verrijking in antitrombine III-bindende heparine fracties. Omgekeerd, wanneer heparine gefractioneerd wordt op basis van affiniteit voor antitrombine III zien we dat de fracties met lage affiniteit voor antitrombine III niet in staat zijn trombine te inactiveren. Hierbij willen we benadrukken dat het antitrombine III en trombine ieder hun eigen specifieke bindingsplaats heeft op het heparine molecuul. Dit betekent dat met, in de trombine-remmende polysaccharide ketens van een heterogeen heparine preparaat, zowel de antitrombine III-afhankelijke als antitrombine

III-onafhankelijke remming van trombine gevonden kan worden. Met andere woorden, er bestaat geen heparine molecuul dat uitsluitend antitrombine III-onafhankelijk werkzaam is.

De rol van heparine in de inactivering van gezuiverd trombine en factor X_a is door veel onderzoekers bestudeerd. In een aantal studies werden hierbij enkele componenten van het hemostase systeem betrokken. Echter, de vraag wat is het effect van heparine op de inactivering van trombine en factor X_a door antitrombine III tijdens het proces van de protrombine activering bleef onbeantwoord. Wij hebben een methode ontwikkeld waarmee dit probleem zeer goed benaderd kan worden (hoofdstuk VI). We laten zien dat in tegenstelling tot het vrijkomen factor X_a , factor X_a als onderdeel van het protrombinase complex tijdens de omzetting van protrombine in trombine in aanwezigheid van therapeutische hoeveelheden heparine nagenoeg ongevoelig is voor de inactivering door antitrombine III. Bovendien vonden we dat de trombine activiteit die gevormd wordt niet gevoelig is voor de stimulerende werking van heparine in de inactivering door antitrombine III. We stellen vast dat gedurende de vroege fase van de protrombine activering meizotrombine het belangrijkste reactieproduct is. In tegenstelling tot trombine, is meizotrombine nauwelijks gevoelig voor de werking van antitrombine III/heparine. Waarschijnlijk is dit het gevolg van het feit dat meizotrombin een geringe of geen affiniteit heeft voor heparine. Onze conclusie is dat de werking van heparine op de antitrombine III-afhankelijke inactivering van gezuiverde trombine en factor X_a niet geëxtrapoleerd kan worden naar de meer in vivo situatie benaderende condities.

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CURRICULUM VITAE

Dominique Baruch was born on August the 2nd, 1954 in Suresnes (France). She graduated from high school in 1971 (Lycée Fénélon, Paris). From 1971 till 1977 she studied medicine at the university of Medicine Necker-Enfants Malades, Paris. In 1977 she passed the "Concours d'Internat en Médecine des Hôpitaux de Paris" (competitive examination to hospital residency). From 1977 till 1982 she worked as "interne en médecine" (resident physician) in different departments of medicine in Paris, including those of internal medicine (Prof. J. Dormont, Hôpital Antoine Bécclère), infectious disease and intensive care (Prof. F. Vachon, Hôpital Claude Bernard), hypertension (Prof. J. Ménard, Hôpital Broussais) and hematology (Prof. R. Zittoun, Hôtel-Dieu; Prof. J. Caen, Hôpital Lariboisière; Prof. M. Seligmann, Hôpital Saint-Louis). In 1979 she passed the "Certificat d'Etudes Supérieures de Biologie Humaine d'Hématologie" and in 1982 the "Thèse pour le Doctorat en Médecine" (Doctoral Thesis in Medicine) on the subject of renin-secreting tumors (Prof. J. Ménard).

In parallel she studied biochemistry at the Faculté des Sciences, Paris from 1977 till 1980. She passed the "Maîtrise de Biochimie" in 1979 and in 1980 the "Diplôme d'Etudes Approfondies de Biochimie", mention structure et fonction des protéines at the Université Paris VII, (first year of post-graduate studies approximately equivalent to a Master in Biochemistry). The "Diplôme d'Etudes Approfondies" requires a year of laboratory training, that she did in 1979 in the hematology laboratory, Faculté de Médecine Necker-Enfants Malades, directed by Prof. F. Josso, where she wrote a dissertation entitled: "Modifications des conditions physico-chimiques optimales d'activité de la thrombine par la liaison à l'alpha-2-macroglobuline: exemple du pH".

Since October 1982, she is a recipient of a "poste d'accueil pour internes" from the Institut National de la Santé et de la Recherche Médicale (fellowship for resident physicians) in the Unité de Recherches de Thrombose Experimentale et d'Hémostase, directed by Prof. J. Caen, and on a leave of absence in the department of Biochemistry, University of Limburg, Maastricht, where this dissertation was prepared. Since October 1985, she is a recipient of a poste d'accueil in the Groupe de Recherches sur la physiopathologie de l'Hémostase et de la Thrombose, directed by Dr. D. Meyer.