Regulation of thrombin generation by protein S : novel aspects of a cofactor protein

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Regulation of thrombin generation by protein S

Novel aspects of a cofactor protein

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Novel aspects of a cofactor protein

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1

Basic mechanisms of haemostasis

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Abstract

Haemostasis governs two essential processes of human life in that it maintains the fluidity of blood under physiological conditions and prevents excessive blood loss after injury. Haemostasis is regulated by components of the vessel wall and blood cells, and by humoral coagulation factors. Under normal conditions, these components are involved in an active equilibrium through activation, propagation, and termination of the haemostatic pathways. This equilibrium is disturbed upon vascular injury leading to a procoagulant response when needed. Unfortunately, pathological disturbances can occur as a result of inherited or acquired coagulation factor deficiencies that may lead to bleeding or thrombotic disorders.

Introduction

During the preparation of this article we commemorated Rudolf Virchow (1821-1902) at the 100th anniversary of his death. Virchow's pioneering achievements in recognizing venous thromboembolism (VTE) have led to his famous triad describing the interaction between the damaged vessel wall, hypercoagulability, and restricted blood flow in the development of VTE. His original work unveiled the finely tuned interactions between cellular and humoral components of haemostasis currently known as the haemostatic balance.

Although defined as a balance, quiescent haemostasis consists of a dynamic equilibrium between procoagulant and anticoagulant processes, without the occurrence of any thrombotic or bleeding tendencies. When triggered upon vascular damage, a cascade of events results in the formation of a thrombus at the site of injury, preventing excessive blood loss, pathogen invasion and allowing tissue repair. Pathological conditions may cause unwanted shifts of the balance by either hereditary or acquired defects in components of the hemostatic system causing thrombotic or bleeding complications.

Primary haemostasis: the platelet plug

The quiescent endothelial cell lining of the vasculature promotes the fluidity of blood by inhibiting blood coagulation and platelet adhesion and by stimulating fibrinolysis. However, upon vascular injury, the endothelial cell layer is disrupted and collagen in the subendothelium is exposed to the blood. Within seconds platelets will bind to the exposed collagen through their membrane glycoprotein (GP) receptors¹. The nature of binding is directed by vessel diameter and blood flow rates. In large vessels at concomitant low flow rates (e.g. veins), low shear forces enable platelets to directly interact with collagen through integrin $\alpha_2\beta_1$ ² and GPVI³ receptors. When blood flow rates increase (arteries) or when vessel diameters decrease (capillaries), shear forces increase and platelet adhesion becomes heavily dependent on the presence of von Willebrand factor (vWF), a large multimeric glycoprotein that contains binding sites for both collagen and platelets⁴. vWF also functions as the physiological carrier for factor VIII (FVIII), a cofactor of coagulation. Under high shear force conditions, platelets adhere

Figure 1.1 Procoagulant response to vascular injury. Platelets adherence to subendothelial collagen followed by activation, degranulation, and aggregation leads to the formation of a primary platelet plug. In conjunction coagulation is triggered by subendothelial tissue factor, leading to thrombin formation followed by fibrin polymerization resulting in a stable thrombus, covering the site of injury and preventing excessive blood loss.

to collagen-bound vWF through the GPIb part of their GPIb-IX-V receptors⁵⁻⁷. Platelet adhesion initiates activation (shape change), secretion and aggregation, thus promoting further platelet recruitment, stimulation and aggregation³. Release of thromboxane A₂ and ADP/ATP in the extracellular environment promotes platelet activation and induces vasoconstriction in an effort to reduce blood loss. Platelet integrin IIb β ₃ receptor function for fibrinogen and vWF is activated, enabling platelet interlinking and subsequent aggregation⁸. Phosphatidylserine, a negatively charged phospholipid confined to the inner leaflet of the resting platelet membrane, is exposed during platelet activation leading to a negatively charged outer lipid membrane that provides an ideal surface for coagulation to take place⁹. This initial response to injury is called primary haemostasis and leads to the formation of a platelet plug that is large enough to cover the injured vessel area. In conjunction, coagulation is triggered at the site of injury by tissue factor (TF) and propagated on the surface of activated platelets, eventually leading to fibrin formation and stabilization of the platelet plug (Fig. 1.1).

Secondary haemostasis: blood coagulation

The enzymes and cofactors that play a role in activation and regulation of blood coagulation circulate in the blood as inactive zymogen proteins, with the exception of TF, which is normally not exposed to the bloodstream. Blood coagulation proceeds via a cascade system^{10,11} that is initiated by a small trigger that causes activation of the first zymogen of the coagulation cascade into an active enzyme by selective peptide bond

cleavages. Subsequently, the activated enzyme with its assigned cofactor is able to activate the next coagulation factor to an active enzyme, which again assembles with a cofactor and activates the following coagulation factor and so on, until by sequential amplification a burst of thrombin occurs. Thrombin on its turn converts soluble fibrinogen into fibrin polymers that stabilize the primary platelet plug, prohibiting recurrent bleeding. The latter process is called secondary haemostasis.

Most of the blood coagulation enzymes belong to the serine protease family. They are primarily synthesized in the liver and require postribosomal modifications for proper functioning. The γ -carboxylation of N-terminal glutamic acid residues to γ -carboxyglutamic acid (Gla) under the influence of a vitamin K-dependent carboxylase is considered of key importance. The N-terminal Gla modules of the coagulation factors prothrombin, VII, IX, X, protein C and protein S contain between 9 and 11 Gla residues that are pivotal for calcium ion binding. The calcium-bound conformation of Gla modules is essential for binding of the coagulation proteins to negatively charged phospholipid (platelet) surfaces, and thus for initiation and propagation of blood coagulation. A widely used antithrombotic therapy intervenes with γ -carboxylation by administering vitamin K-antagonists (coumarins). Other posttranslational modifications in coagulation factors include β -hydroxylation, N- and O-linked glycosylation, and phosphorylation.

Initiation: tissue factor

Traditionally, the coagulation cascade is divided into two pathways: the intrinsic and the extrinsic pathway. The *in vivo* activation of blood coagulation is mediated exclusively through the extrinsic pathway and is initiated by the binding of the enzyme factor VIIa to TF. TF is an integral membrane protein that is exposed in addition to collagen whenever

Figure 1.2 Blood coagulation: initiation and propagation. Upon vascular injury, traces of factor VIIa (FVIIa) from the circulation bind to subendothelial tissue factor and generate the transient *extrinsic tenase* complex, the physiologic activator of blood coagulation. The extrinsic tenase will activate factor X (FX), and FXa on its turn will bind to negatively charged phospholipid surfaces (*i.e.* activated platelets) together with its cofactor FVa to generate the prothrombinase complex, the central prothrombin activator of coagulation. Small amounts of thrombin then start to generate insoluble fibrin polymers from soluble fibrinogen, activate platelets, and activate cofactors V and VIII. In another positive feedback loop, thrombin activates factor XI (FXI) from the intrinsic pathway of coagulation, which on its turn can activate factor IX (FIX). FIXa with its cofactor FVIIIa assembles on negatively charged phospholipids to form the *intrinsic tenase* complex, securing FXa generation for participation in the prothrombinase complex and thus thrombin formation and fibrin polymerization.

the endothelial cell lining is damaged. TF acts as a cofactor for FVIIa in the activation of FX (Fig. 1.2), a reaction that occurs on the negatively charged phospholipid surface contributed by the damaged tissue. Under normal conditions, the small amount of FVII (1%) that is present as FVIIa in plasma¹² will bind to TF upon exposure and generate the first traces of activated FX (FXa). FXa will activate more FVII, increasing the extrinsic tenase potential and therewith its own formation, but FXa is also able to generate small amounts of thrombin, which on its turn activates factor V (FV) into FVa, the cofactor of FXa in prothrombin activation. FXa and FVa assemble on negatively charged phospholipid surfaces (*i.e.* activated platelets) to form the prothrombinase complex, the central prothrombin activator.

Meanwhile, tissue factor pathway inhibitor (TFPI), an early negative regulator of blood coagulation, comes into action to shut down the extrinsic pathway of coagulation. TFPI is synthesized by endothelial cells and is normally present in plasma. When the extrinsic pathway produces FXa, TFPI can directly inhibit free FXa. The complex between TFPI and FXa is a very potent inhibitor for the membrane bound TF-FVII complex, generating a stable quaternary enzyme-inhibitor complex. This shutdown mechanism for extrinsic FXa generation is very effective and already occurs during the early stages of the procoagulant response. As thrombin generation should not yet be called a halt, an alternative route to FXa and thrombin generation is provided by a part of the classical intrinsic pathway of coagulation, the propagation process.

Propagation: thrombin feedback

Thrombin is the central enzyme of the haemostatic system, where its main task is to generate fibrin polymers from soluble fibrinogen, establishing a stable thrombus. In addition, thrombin is a key player in several positive and negative feedback reactions of the regulatory mechanisms of coagulation. The main positive feedback reaction of thrombin, which favors its own formation, is the activation of the intrinsic system of coagulation after the extrinsic system has been shut down by TFPI (Fig. 1.3). Thrombin can activate factor XI (FXI) into FXIa¹³⁻¹⁵, which in turn activates FIX. FIXa then assembles on negatively charged phospholipids with its cofactor FVIIIa that has already been activated by traces of thrombin and released from its carrier protein vWF in the process. The complex of FIXa and FVIIIa on negatively charged phospholipids is known as the intrinsic tenase complex and secures the generation of FXa necessary for continuation of thrombin formation.

An important observation was made that TF-FVIIa, the extrinsic tenase complex, could also activate FIX and thus contribute to intrinsic tenase activity¹⁶. As with FXa-generation by TF-FVIIa, this contribution is transient as a result of TFPI shutdown of the extrinsic tenase complex. The discovery of TFPI represented a strong argument in favor of links between extrinsic and intrinsic pathways of coagulation¹⁷.

In addition to activation of the FXI feedback loop, thrombin activates more platelets, promoting recruitment, degranulation, and aggregation. To stabilize the clot even further,

Figure 1.3 Blood coagulation: termination, inhibition and degradation. As soon as FXa is generated it can bind to tissue factor pathway inhibitor (TFPI) after which the bimolecular complex of TFPI-FXa inhibits TF-FVII by generation of a quaternary inactive enzyme-inhibitor complex. Following shutdown of the extrinsic tenase complex, free coagulation enzymes are inhibited by the serine protease inhibitor antithrombin (AT). Traces of generated thrombin activate not only positive feedback reactions but also a major negative feedback system that is initiated when thrombin binds to endothelial cell expressed thrombomodulin (TM), enabling protein C activation. Subsequently, activated protein C (APC) together with its cofactor protein S down-regulates the propagation phase of coagulation by proteolytic degradation of the cofactors of intrinsic tenase and prothrombinase complex.

thrombin bound to thrombomodulin that is expressed by endothelial cells inhibits fibrinolysis by activation of thrombin-activatable fibrinolysis inhibitor (TAFI)¹⁸⁻²⁰.

One of the last events in coagulation is the stabilization of the clot by cross-linking of fibrin polymers through a transglutaminase reaction by factor XIIIa (FXIIIa), which is activated from FXIII by thrombin^{21,22}.

There is no evidence that the contact system, which activates the intrinsic coagulation pathway, is important as a physiological activator of blood coagulation. *In vitro* activation of the contact system (FXII, high-molecular-weight kininogen (HMWK) and prekallikrein) results in the formation of FXIa, a serine protease that activates FIX into FIXa, which subsequently acts as enzyme in the intrinsic tenase complex. When blood is collected into a glass tube, this contact system causes the blood to clot during the preparation of serum, but *in vivo* it is not likely to have a function, at least not in triggering blood coagulation.

It should be emphasized that the three pivotal enzyme-cofactor complexes of blood coagulation, the extrinsic tenase, the intrinsic tenase and the prothrombinase complex, are assembled on negatively charged phospholipid surfaces. This serves multiple purposes in that (1) the powerful coagulation reactions will be restricted to the site of injury, and (2) locally very high concentrations of enzymes, cofactors and substrates are obtained, necessary for optimal coagulation activation and a quick response to a procoagulant stimulus. The negatively charged procoagulant surface of the activated platelet is recognized as the physiological surface supporting all phospholipid-dependent coagulation reactions in situations where clotting is needed. However, the same surface plays an important role as pathological breeding ground for development of thrombotic disease.

Termination and degradation: natural anticoagulants

Although a local procoagulant burst is required to stop bleeding from an injured vessel, a systemic procoagulant response is prevented by the presence of several inhibitors and negative feedback regulating systems.

Inhibition of active coagulation enzymes by the serine protease inhibitors (serpins) antithrombin (AT)^{23,24}, heparin cofactor II^{25,26}, and α_1 -antitrypsin (α_1 AT)²⁷ eliminate the free enzymes from plasma, preventing downstream activation of coagulation. A less clear role of α_2 -macroglobulin (α_2 M) is defined; it is proposed to be a general backup inhibitor in a massive procoagulant response in combination with serpin consumption²⁸. The inhibitory activity of AT towards free thrombin and FXa is markedly enhanced by heparan sulfates present on the surface of endothelial cells²⁹. The increased inhibitory activity of AT for FXa and thrombin in the presence of heparan sulfates forms the basis of the clinical application of heparins and low-molecular-weight heparins (LMWH). Currently, pentasaccharides, which are the smallest heparin analogues that specifically target AT to FXa but not to thrombin, are soon to be clinically applied.

In addition to the *free enzyme scavengers* described above, several negative feedback systems play a role in the down-regulation of coagulation. TFPI represents the first negative feedback system that comes into action after activation of coagulation. TFPI is a member of the Kunitz-type inhibitor family, has three Kunitz-type modules and is bound to low-density lipoproteins (LDLs) in plasma³⁰. The second Kunitz module of TFPI reacts with the active site of FXa, upon which the first Kunitz-type module can react with the active site of FVIIa in complex with TF, generating an inactive quaternary enzyme-inhibitor complex³¹. The role of the third Kunitz module is yet unknown. Although LDL-bound TFPI consists of a C-terminally truncated form, a minor fraction of TFPI in plasma still contains the C-terminal tail expressing a (high-molecular-weight) heparin-binding site. Under normal conditions, this C-terminal tail is suggested to be bound to heparan sulfates on the surface of the endothelial cells, and released systemically after administration of heparin, which might add to the anticoagulant potential of heparin³²⁻³⁴.

One of the major negative feedback systems is provided by the protein C system³⁵. The essential cofactor for activation of this pathway is the integral membrane receptor thrombomodulin (TM), expressed on the surface of quiescent endothelial cells³⁶. TM consists of an N-terminal lectin homology region, 5 successive epidermal growth factor (EGF)-like modules, an O-linked glycosylation region containing chondroitin sulfates, and a transmembrane region. When thrombin is generated, it can bind to EGF modules 5 and 6 next to the chondroitin sulfate-rich region, which improves the binding and orientation of thrombin in such a way that it is able to activate the zymogen protein C^{37,38}. The activation of TM-bound protein C by TM-bound thrombin requires EGF 4-6 of thrombomodulin^{39,40} and results in the release of the anticoagulant serine protease activated protein C (APC). TM not only contributes to the anticoagulant response by acting as a cofactor of protein C activation, but also inhibits the procoagulant properties of the bound thrombin by inducing a conformational change of its the active site⁴¹⁻⁴³. Meanwhile, APC generation is transient as TM-bound thrombin can be rapidly

inactivated by protein C inhibitor⁴⁴. On the other hand, APC in circulation has the longest half-life of blood coagulation proteases (> 15 min), effectively attaining its anticoagulant activity⁴⁵. Emphasizing the efficacy of protein C activation during the initial phases of thrombin generation is the observation that an early intermediate of thrombin generation, meizothrombin, is a more effective TM-dependent protein C activator than thrombin^{46,47}. The serpin protein C inhibitor (PCI) inhibits APC⁴⁸ or protein C activation by inhibiting thrombin bound to TM⁴⁹.

An endothelial cell protein C receptor (EPCR) was identified that binds protein C as well as APC⁵⁰. EPCR, which stimulates thrombin-TM mediated protein C activation⁵¹, is mainly expressed in large arteries where TM expression is low, and is almost absent in the microvasculature where TM expression is high⁵². A physiological role for the EPCR-APC complex is as yet unclear, but the EPCR-APC complex activation of protease activated receptor (PAR)-1⁵³ causing cell signaling and gene expression alteration has been reported⁵⁴.

APC inactivates the cofactors FVa⁵⁵ and FVIIIa⁵⁶ of blood coagulation with the help of its nonenzymatic cofactor protein S⁵⁷, shutting down the intrinsic tenase complex and the prothrombinase complex, calling a halt to thrombin generation and restoring the haemostatic balance.

APC can cleave factor FVa at three arginines (R) at positions 306, 506, and 679. A rapid cleavage at R506 results in a partly inactivated FVa that is subsequently completely inactivated by a slow cleavage at R306⁵⁸. It was observed that FVa is protected against inactivation by APC when present with FXa in the prothrombinase complex, and that protein S can counteract the protective effect of FXa⁵⁹. Later, this observation could be explained by a decrease of the rate of cleavage at R506 by the presence of FXa, and by a 20-fold increase of the rate of APC-catalyzed cleavage at R306 due to the presence of protein S⁶⁰, resulting in complete FVa inactivation. The cleavage at R679 is not considered of physiological importance because of its slow rate^{58,61}.

APC can cleave factor FVIIIa at R336 and R562^{62,63}, although it is believed that the activity of the intrinsic tenase complex of which FVIIIa is a cofactor is down-regulated as a result of the natural decay of FVIIIa^{64,65}.

A breakthrough discovery was made in 1993 when APC-resistance was reported⁶⁶, a phenomenon in which plasmas of individuals with a history of thrombosis did not respond efficiently to the addition of APC, unveiling an arginine 506 to glutamine mutation (R506Q) in the FV molecule⁶⁷⁻⁶⁹ that blocks proteolytic cleavage by APC and impairs inactivation of FVa. This mutation in the FV molecule, known as FV_{Leiden}, is one of the most common (moderate) risk factors of thrombosis^{70,71}. Conceptual contributions were made to understanding mechanisms of FV activation and inactivation after characterization of the FV_{Leiden} molecule.

Fibrinolysis: dissolving the clot

Within minutes after the formation of a clot, fibrinolysis is initiated as the final event of haemostasis, starting the process of clot lysis to avoid as much as possible restriction of blood flow within the vessel lumen. The central enzyme of fibrinolysis is plasmin that is generated after activation of its precursor plasminogen⁷². The two major plasminogen activators are tissue-type plasminogen activator (t-PA) and urokinase (urinary plasminogen activator, u-PA) that differentiate between fibrin-dependent and fibrin-independent activation of plasminogen.

Plasminogen binds to lysine residues that are exposed on fibrin upon proteolytic cleavage of fibrinogen by thrombin⁷³. t-PA, synthesized and released by endothelial cells, will also bind to fibrin and activate plasminogen. The active enzyme plasmin will subsequently proteolytically cleave fibrin into fibrin degradation products (FDP). In this negative feedback system, fibrin acts as a cofactor for its own degradation; in addition, partially degraded fibrin enhances degradation even more by extra stimulation of t-PA-mediated plasminogen activation⁷⁴. Plasmin can be inhibited by α_2 -antiplasmin but is protected against inhibition when bound to fibrin⁷⁵. One of the fibrin degradation products, D-dimer, has become of a prognostic value as a fibrinolysis activation marker in the diagnosis of thrombosis^{76,77}.

A physiologic role for the contact system of blood coagulation might be established in the fibrin-independent activation of fibrinolysis. The plasminogen activator u-PA has to be activated from its precursor prourokinase, also known as single-chain urokinase plasminogen activator (scu-PA), to activate plasminogen effectively. This reaction is mediated by the contact system components kallikrein and FXIIa⁷⁸ and an anticoagulant role for the contact system of coagulation is currently under debate. Plasminogen can also be activated by scu-PA bound to the urokinase receptor due to enhancement of intrinsic proteolytic activity of scu-PA⁷⁹.

Inhibition of fibrinolysis starts after the initiation of coagulation. At the generation of traces of thrombin, TAFI can be activated by the thrombin-TM complex¹⁹. Activated TAFI in its capacity as carboxypeptidase B cleaves off C-terminal lysine residues from fibrin, inhibiting plasminogen binding and thus fibrinolysis^{80,81}. Plasminogen activators t-PA and u-PA in turn can be inhibited by serpins PAI-1,^{82,83} PAI-2,⁸⁴ and to a lesser extent by PCI (PAI-3)^{85,86}.

Thrombotic and bleeding disorders: disturbing the balance

Blood coagulation is the major defense mechanism against excessive blood loss and pathogen invasion upon vascular injury. Coagulation can err, however, and various scenarios have been identified in which unwanted shifts of the hemostatic balance result in anticoagulant or procoagulant states. The causes of these shifts can either be acquired or inherited and can result in mild to severe bleeding or thrombotic complications. Well-known deficiencies in humoral coagulation factors are hemophilia A, B, and C, causing mild to severe bleeding as a result of decreased (functional) levels of FVIII, FIX, and FXI^{87,88}. Von Willebrand disease affects both platelet adhesion and

Table 1.1 Risk factors for thrombosis

Factor	Consequence
Hereditary	
Antithrombin deficiency ¹⁰⁵	Impaired enzyme inhibition
Protein C deficiency ¹⁰⁶	Impaired inactivation of FVa
Protein S deficiency ^{107,108}	Impaired anticoagulant activity
Factor V Leiden G1691A (Arg506Gln) ⁶⁸⁻⁶⁹	Impaired proteolysis by APC
Prothrombin G20210A ¹⁰⁰	Increased levels of prothrombin
Dysfibrinogenemia ¹¹⁰	
FVIII ¹¹¹	Increased levels of FVIII
Methylenetetrahydrofolate (MTHF) reductase C677T	Hyperhomocysteinemia ¹¹²⁻¹¹⁴
Acquired	
Age ¹¹⁵	
Immobilization ¹¹⁶ (air travel) ^{117,118}	Stasis of blood flow
Oral contraceptives ¹⁰⁰	Acquired APC resistance ^{103,104}
Hormone replacement therapy ¹¹⁹	Acquired APC resistance ¹²⁰
Systemic lupus erythematosus	Antiphospholipid antibodies ¹²¹
Malignancy ^{122,123}	Changes in coagulation factors
Folic acid deficiency	Hyperhomocysteinemia ^{113,114}

plasma levels of FVIII because of vWF carrier function impairment, resulting in a wide spectrum of mild to severe bleeding⁸⁹. For redefining fundamental concepts of blood coagulation, clinical symptoms presenting with clotting factor deficiencies have always been the driving force behind understanding blood coagulation. For example, the question why haemophiliacs bleed has emphasized the essence of the propagation phase of coagulation, and triggered the change of the rigid model of separate extrinsic and intrinsic pathways to the more interwoven feedback model that we use today.

The development of a procoagulant state that leads to either venous or arterial thrombosis is governed by different risk factors. For example, the risk factors for developing arterial thrombosis such as smoking⁹⁰ or hypercholesterolemia⁹¹ do not increase the risk for development of venous thrombosis.

With respect to venous thrombosis, most of the risk factors can be tracked back to reduced blood flow and qualitative or quantitative changes in humoral factors in blood coagulation^{92,93}. In table 1.1 some well-known risk factors and possible risk factors for development of thrombosis are summarized. For some of these it is quite clear that they

severely affect the hemostatic balance and tip it towards a prothrombotic state. For example, homozygous protein C deficiencies lead to the severe neonatal thrombotic disease characterized as purpura fulminans. For other risk factors a thrombotic outcome is less predictable, as with factor V_{Leiden}, which constitutes a moderate risk factor but is frequently (20-50%) found in populations with venous thrombosis^{70,94}. On the other hand, the mutation is present in 5% of the normal population⁹⁵, of which the greater part will never develop thrombosis. These and similar observations for other risk factors led to an important concept regarding thrombosis as a multi-factorial disease in which separate (mild) risk factors, acquired as well as genetic, are combined until a "thrombosis-threshold" is reached^{96,97}. Analogous to this, anecdotal evidence has been obtained in which a prothrombotic risk factor (*i.e.* FV_{Leiden}) moderated the severe bleeding phenotype associated with haemophilia A^{98,99}.

Currently, the increased risk of thrombosis associated with oral contraceptive use^{100,101} is at the focus of attention. The relative risk of developing thrombosis increases fourfold as a result of oral contraceptive use⁹². If we consider a 7-fold increased relative risk as a result of a heterozygous factor V_{Leiden} carriership^{100,102}, and we combine this with oral contraceptive use, which will not be rare considering the prevalence of either risk factor, a synergistic effect of both relative risks up to 35 is obtained¹⁰⁰. The procoagulant state associated with the use of oral contraceptives is correlated with impairment of the protein C pathway^{103,104}, as is the case with factor V_{Leiden}. However, the molecular mechanisms underlying APC-resistance as a result of oral contraceptive use remain unclear and have to be elucidated in the years to come by an effective combination of clinical and fundamental research.

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Natural anticoagulants

Introduction

When coagulation is activated upon vessel wall damage, anticoagulant processes will rapidly restore the haemostatic balance to prevent excessive clot formation. Various anticoagulant systems are ready to control coagulation at every level of the cascade. Protease inhibitors, such as antithrombin (AT), directly inhibit the proteolytically active coagulation factors. In addition, tissue factor pathway inhibitor (TFPI) which eliminates tissue factor (TF), the initiator of coagulation, and the protein C/protein S pathway which inactivates two important cofactors, factors Va and VIIIa, down-regulate the procoagulant response via negative feedback mechanisms. Deficiencies in one of these naturally occurring anticoagulant proteins results in an increased thrombotic risk. Moreover, the complete absence of one of the anticoagulant systems leads to extensive fibrin deposition as well as bleeding due to consumptive coagulopathy and is not compatible with life¹⁻³. Figure 3 in chapter 1 gives a schematic representation of the anticoagulant pathways.

There is increasing evidence that natural anticoagulants not only control TF-induced coagulation but also inflammatory responses induced via receptor activation by coagulation factors⁴. Under normal conditions, TF circulates in low amounts in blood^{5,6}. However, various pathological conditions such as sepsis, atherosclerosis or cancer are associated with increased TF levels⁷ which results in a hypercoagulable state that can lead to life-threatening situations⁸. Treatment with natural anticoagulants improves survival in animal models of sepsis⁹⁻¹¹ and APC was shown to reduce mortality in septic patients¹². It appeared from these studies that coagulation as well as inflammation are affected by anticoagulants.

In this chapter, the various anticoagulant mechanisms are briefly summarized and structure-function relationships of protein S and TFPI are discussed in more detail.

Protease inhibitors

Blood contains high concentrations of various direct inhibitors that enable fast inhibition of the large amount of thrombin that is generated during coagulation. Several of these molecules, like AT, heparin cofactor II (HcII) and protein C inhibitor (PCI) belong to the family of serine protease inhibitors (serpins) of which all members share the same mechanism of inhibition¹³. A serpin acts as a suicide substrate and interacts with the active site of the enzyme. During cleavage important conformational changes in both the inhibitor and the enzyme occur that block further processing and release of the cleaved serpin¹⁴. Thus, the enzyme is trapped in a stable 1:1 stoichiometric complex with the serpin and is cleared from the circulation.

Although all serpins belong to the same gene family, the preferred target enzymes are different for each serpin. AT is the major inhibitor of thrombin, but also other coagulation proteases like FXa, FIXa, FXIa and FVIIa are inhibited with various efficiencies. Protease inhibition by free AT is inefficient but the presence of the glycosaminoglycan heparin accelerates the reaction several orders of magnitude. Heparin induces a conformational change in AT which potentiates the inhibition of FXa

by AT¹⁵. In the case of thrombin, heparin primarily acts as bridging molecule and favours the encounter between protease and inhibitor¹⁶. Moreover, the susceptibility of the enzymes is influenced by their environment. AT is a poor inhibitor of free FVIIa, but when FVIIa is complexed with TF, inhibition is more efficient, probably due to a conformational change in the enzyme^{17,18}. FXa on the other hand, is protected from inhibition by AT when it participates in the prothrombinase complex¹⁹. HcII inhibits thrombin but no other clotting factors. As with AT, the HcII activity is significantly potentiated by heparin, mostly by inducing conformational changes in the inhibitor²⁰. PCI can react with most coagulation enzymes as well as with the anticoagulant activated protein C (APC). Heparin only moderately accelerates PCI activity whereas thrombomodulin largely promotes the inhibition of thrombin by PCI, suggesting that the thrombin-thrombomodulin complex is the main target of PCI^{21,22}.

α_2 -Macroglobulin (α_2 -M) is a member of the alpha macroglobulin family. α_2 -M is a broad range protease inhibitor²³ which covalently traps its target proteins in an irreversible complex. The mechanistic basis, like for serpins, is the conformational change that is induced after cleavage of the 'bait' region of α_2 -M by the protease. Part of the thrombin generated during coagulation is inhibited by α_2 -M. The α_2 -M-thrombin complex can still activate small peptide substrates²⁴, which is observed when such substrates are used to monitor thrombin generation in plasma.

Tissue factor pathway inhibitor

In 1985 it was first described that for the inhibition of TF/FVIIa the presence of FX was needed together with an additional plasma component²⁵. In 1987, a component with such TF-inhibitory capacity was purified from HepG2 hepatoma cells²⁶ and with the isolation of its cDNA a year later²⁷, insight into the structure of the protein and its mechanism of inhibition rapidly increased. Initially, the protein was called tissue factor inhibitor (TFI), lipoprotein associated coagulation inhibitor (LACI) or extrinsic pathway inhibitor (EPI). Later, the name tissue factor pathway inhibitor (TFPI) was assigned to it, indicating that the inhibitor regulates the initiation of coagulation by TF. Together with earlier findings like the activation of FIX by TF/FVIIa²⁸, the discovery of TFPI led to an altered concept of coagulation, no longer a cascade with intrinsic and extrinsic pathways converging in a common pathway, but a process that comprises different phases (*i.e.* initiation, propagation and termination phase) and that is initiated by TF but sustained by other mechanisms²⁹.

Physiology of TFPI

Synthesis – Although TFPI was first purified from cultured HepG2 cells, hepatocytes appear not to be the main source of TFPI in humans³⁰, which is in agreement with the observation that patients with severe hepatic failure have normal plasma TFPI levels³¹⁻³³. Endothelial cells were shown to be the main site of synthesis of TFPI³⁰. Under normal conditions, TFPI is produced by microvascular endothelium³⁴, however, in a more recent study it was reported that TFPI is also found in coronary arteries³⁵. In addition, mRNA

was detected in a megakaryocytic cell line³⁰, which explains the presence of TFPI in platelets³⁶, and to a minor extent in other cell types^{30,34,37-39}. In contrast to other proteins, inflammatory stimuli (TNF α , IL-1) do not down-regulate TFPI expression by endothelial cells⁴⁰ and even upregulate expression in other cells^{35,38,41}. The TFPI gene is located on chromosome 2 in region 2q32^{42,43}. Multiple potential binding sites for transcription factors are located in the 5' promotor region of the TFPI gene, though the exact (tissue specific) regulation of TFPI gene expression needs to be elucidated⁴⁴ and it is unclear how translation into protein might be regulated.

Distribution – In vivo, TFPI is distributed in three pools, plasma, platelets and the vascular endothelium. Plasma TFPI is a heterogeneous pool⁴⁵ of which only a minor part is the full length form (~43 kDa) which circulates free in plasma. The rest of plasma TFPI is diverse in size (34-40 kDa) and represents various C-terminally truncated forms of which the majority is bound to lipoproteins⁴⁵⁻⁴⁷. The mean total plasma concentration TFPI in a normal population is ~2-2.5 nM (~80-100 ng/ml) as measured with an immunoassay³² or functional assay⁴⁸. The reported amount of free, full length TFPI varies from 5-50%^{47,49}. Moreover, the total TFPI concentration is regulated by the LDL and cholesterol content of plasma^{50,51}, without affecting the free, full length TFPI concentration or anticoagulant activity. It is not clear what the exact mechanism of the TFPI-lipoprotein interaction is, nor is it clear where the truncated variants are formed and what their significance is.

Platelets contain a low amount of full length TFPI which is released upon stimulation with thrombin or calcium ionophore³⁶. The concentration TFPI released by 3×10^8 platelets is 7-8% of the plasma concentration TFPI.

The observation that the plasma TFPI concentration increased 2-4 fold following intravenous infusion of heparin^{32,33,52} suggested that a TFPI pool exists *in vivo* which is associated with glycosaminoglycans such as heparan sulfate. Since addition of heparin to blood *ex vivo* had no such effect, the vessel wall appeared to contain these intra- or extracellular TFPI stores. Heparin-releasable TFPI is the full length form⁵³ and might play therefore an important role in the regulation of TF activity. Endothelial cells constitutively synthesize and secrete TFPI^{30,40} which remains associated with the outer leaflet of the cells and with intracellular organelles⁵⁴. Thrombin causes a quick release and redistribution of intracellular TFPI⁵⁵. Heparin has a similar effect as thrombin, indicating that heparin not just displaces TFPI from the cell-surface but also induces a reorganization of the intracellular TFPI⁵⁵. Moreover, part of the cell-bound TFPI is heparin-resistant^{56,57}. As this resistance was overcome by phospholipase treatment, it appeared that this TFPI was attached to the cell via a GPI-anchor. It was shown that direct (TFPI) and indirect (other surface protein) GPI-anchorage was involved⁵⁸. In addition, TFPI appeared to be associated with lipid rafts as well as with caveolae on endothelial cells^{57,59}.

Structural properties of TFPI

With the isolation of its cDNA, it became clear that TFPI belongs to the Kunitz-type inhibitor family²⁷. TFPI consists of an acidic N-terminus, 3 sequential Kunitz-domains (K-1 to K-3) each having 3 disulfide bridges, and a basic C-terminus. TFPI contains three potential N-linked glycosylation sites and 1 (putative) phosphorylation of the serine residue at position 2⁶⁰. The secondary structure of TFPI is represented in Fig. 2.1. The mature protein arises after removal of a 28 AA signal peptide and consists of 276 amino acid residues, its molecular weight is ~43 kDa.

A review published in 2001 provides a detailed overview of the molecular interactions between Kunitz and protease domains⁶¹. All three Kunitz domains have a positively charged AA on position P1 (Lys for K-1 and Arg for K-2 and K-3), suggesting that they can all act as a protease inhibitor. Whereas K-1 and K-2 inhibit FVIIa and FXa, respectively, for K-3 however no inhibitor function has yet been reported. Almost all the residues involved in the interaction between Kunitz-domains and protease domains differ for K-1 versus K-2 and for FVIIa versus FXa, which likely underlies specificity. The linker regions between the Kunitz domains probably provide additional interactions sites with the proteases.

TFPI contains heparin binding sites in the basic C-terminal domain^{53,62,63} and in K-3^{62,64,65}. The same regions are essential for cell surface binding^{54,66}, likely via (GPI-anchored) proteoglycans^{67,68}, and, interestingly, also for optimal anticoagulant activity in plasma^{62,69-71}. In addition, the basic C-terminus interacts with negatively charged phospholipids⁷², (oxidized) lipoproteins⁷³⁻⁷⁵ and FXa⁷⁶, probably via the Gla-domain^{62,69}.

Figure 2.1 Structure of TFPI. Mature full length tissue factor pathway inhibitor (TFPI) is a Kunitz-type inhibitor of 276 amino acids. Starting at the N-terminus, the following domains can be distinguished: an acidic N-terminus with a putative phosphorylation at Ser2, 3 consecutive Kunitz-domains which each contain 3 disulfide bridges (the arrows indicate the presumed active site inhibitory cleft (P1-P1') of each Kunitz domain) and a basic C-terminus. TFPI carries 3 potential glycosylation sites, at Asn residues 117, 167 and 228, indicated by asterisks. (Figure adapted from Broze *et al*, *Biochemistry*. 1990; 29: 7539-7546).

For the negatively charged N-terminus, no interactions have been described yet.

TFPI undergoes slow, limited proteolytic cleavage by various proteases such as thrombin⁷⁷, FXa⁷⁸, plasmin⁷⁹, and matrix metalloproteases^{76,80-82}, which leads to inactivation.

An alternatively spliced variant of TFPI (TFPI β) has been identified⁸³. TFPI β is a 38 kDa protein which lacks K-3 and the C-terminal domain but instead has a GPI-anchor at its C-terminus which localizes the protein at the surface of cells⁵⁸. Moreover, a second tissue factor pathway inhibitor (TFPI-2) has been isolated which is structurally related to TFPI⁸⁴, and which appeared to be identical to placental protein 5⁸⁵. TFPI-2 also contains three Kunitz-domains, but markedly shorter linker regions, and has a molecular weight of ~32 kDa⁸⁴. TFPI-2 shows inhibitory potential towards TF/FVIIa, FXIa, plasmin and matrix metalloproteases but limited activity towards FXa^{86,87}. The role of TFPI-2 as inhibitor of TF-induced coagulation is questionable.

Function of TFPI

The critical role of TFPI in the regulation of TF-induced coagulation is illustrated in mouse models. Using targeted gene disruption, mice were generated that produce TFPI which lacks the K-1 domain and therefore has lost the capacity to inhibit TF/FVIIa. None of these mice survived to the neonatal period which was due to intravascular thrombus formation and bleeding phenomena consistent with consumptive coagulopathy¹. Heterozygous or homozygous FVII deficiency rescued the TFPI-deficient mice⁸⁸. Using other animal models (depletion of TFPI in rabbits), Sandset *et al* showed that TFPI plays a critical role in the down-regulation of TF-induced coagulation^{89,90}. In addition, various biochemical studies underscored the capacity of TFPI to inhibit TF activity. Data on the clinical significance of TFPI, however, are rare. Only recently an association between plasma TFPI levels and the risk of thrombosis has been reported⁹¹, which seems in contrast to the observations in mice and rabbits. Since cell-bound TFPI constitutes most of the inhibitory activity, it was hypothesized that the measurement of plasma TFPI might not reflect the *in vivo* situation⁹¹.

Regulation of tissue factor initiated coagulation

Early studies revealed that for the inhibition of TF activity FX(a), FVII(a), calcium-ions and an additional component (TFPI) were necessary⁹². Moreover, catalytically active FXa was needed and the inhibition of TF was shown to be reversible^{92,93}. Soon it appeared that the inhibition of TF occurred via a two-step mechanism, which is depicted in Fig. 2.2. First, TFPI binds to FXa and this binding does not depend on the presence of calcium-ions or the Gla-domain of FXa⁹⁴, although inhibition of Gla-domainless FXa occurred 7-fold slower compared to FXa⁹⁵. Second, the FXa/TFPI complex interacts with TF/FVIIa and for this interaction calcium-ions as well as the Gla-domain of FXa are necessary⁹⁵. This model was confirmed by site-directed mutagenesis of TFPI. It was shown that K-1 interacts with FVIIa and K-2 with FXa and that both Kunitz-domains were required for TF/FVIIa inhibition⁹⁶. Alteration of K-3 had no effect. In addition, a hybrid

Figure 2.2 Scheme of the inhibitory mechanism of TFPI. Upon vessel wall damage, the transmembrane protein tissue factor (TF) becomes exposed to flowing blood and binds circulating activated factor VII (FVIIa). This represents the initial event during blood coagulation. The resulting TF/FVIIa complex proteolytically activates factor X (FX) into activated FX (FXa). Tissue factor pathway inhibitor (TFPI) inhibits TF-initiated coagulation via a two-step, feedback mechanism. **Step 1** is the interaction of the Kunitz-2 domain (k2) of TFPI with newly formed FXa. This interaction results in a binary FXa/TFPI complex in which FXa is active site inhibited. In **Step 2** the FXa/TFPI complex interacts with the TF/FVIIa complex. A quaternary complex is formed in which also FVIIa is active site inhibited, by Kunitz-1 domain (k1) of TFPI. Blood coagulation is a surface-dependent process and thus a procoagulant phospholipid surface and calcium ions (Ca²⁺) are involved in clotting reactions. Dark phospholipids represent negatively charged phosphatidylserine (PS), N and C indicate the N- and C-terminus of TFPI, respectively. (Figure adapted from Saleminck, Thesis Universiteit Maastricht, 1999).

protein that consists of the light chain of FXa (Gla-domain plus 2 EGF-like domains) attached to K-1 effectively inhibited TF/FVIIa activity without the need of FXa, suggesting that FXa helps the correct positioning of TFPI. These findings provided evidence for the formation of a quaternary complex in which both FXa and FVIIa are active site inhibited⁹⁷. The quaternary complex appeared very stable suggesting high affinity interactions. Free TFPI is a poor direct inhibitor of the TF/FVIIa complex^{98,99}. In complex with FXa, however, TFPI efficiently inhibits TF/FVIIa activity^{93,95}. Such a product-dependent inhibition allows TF to initiate coagulation but warrants an efficient down-regulation of TF-activity once product is formed¹⁰⁰.

It has been suggested that the first step of the inhibition of the extrinsic coagulation pathway, which is the formation of the FXa/TFPI complex, is rate limiting^{101,102}. Inhibition of FXa by TFPI occurs via a slow tight-binding mechanism^{103,104}, which is typical for Kunitz-type inhibitors. Various reports describe the structural requirements for FXa inhibition by TFPI. It appeared that full length TFPI inhibits FXa most efficiently and that inhibitory activity of TFPI decreases with increasing truncation at the C-terminus^{62,69,71,105}. This is in agreement with the activity of various TFPI forms in clotting assays^{62,69-71}. Phosphatidylserine-containing phospholipids were shown to enhance FXa/TFPI complex formation, though only in the case of full length TFPI^{102,103}. Also TF/FVIIa-catalyzed FX activation is poorly inhibited by truncated TFPI, which is a direct consequence of the poor FXa-inhibitory capacity of these variants¹⁰². The interaction between TF/FVIIa and FXa/TFPI, however, is not influenced by the C-terminal domain of TFPI^{71,102,106} although phospholipid-binding of TF/FVIIa and FXa/TFPI is required for efficient quaternary complex formation^{95,107}. Taken together these data suggest that for optimal FXa/TFPI complex formation phospholipids, the Gla-domain of FXa and the C-terminal domain of TFPI are required. Based on the observation that quaternary complex formation occurs at near-diffusion limited rate, it was proposed that TFPI interacts with 'nascent' FXa *i.e.* when FXa is still in complex with TF/FVIIa¹⁰¹.

In the presence of calcium-ions, FXa/TFPI complex formation is stimulated by heparin^{62,101}. TFPI inhibits FXa also more efficiently when it is incorporated in the prothrombinase complex compared to free FXa^{70,103}. The presence of prothrombin, however, counteracts the inhibition of the prothrombinase complex by TFPI¹⁰⁸.

Protein C pathway – focus on protein S

The first report on protein C dates back to 1976¹⁰⁹. A year later, Discipio described the purification of another vitamin K-dependent protein, protein S¹¹⁰. It appeared that protein C is a zymogen and that cleavage by thrombin turns it into an active, anticoagulant serine protease which cleaves FVa and FVIIIa^{111,112}. The function of protein S remained a question until 1980, when Walker *et al* reported that activated protein C (APC) is regulated by protein S¹¹³. With the cloning and characterization of the (bovine and human) cDNAs coding for protein C¹¹⁴ and protein S¹¹⁵, the modular structure of both proteins was elucidated and resembled that of other vitamin K-dependent coagulation factors. Both proteins are synthesized as precursors and contain a preproleader-sequence, a gamma-carboxyglutamic acid-containing region and epidermal growth factor-like domains. In contrast to many other vitamin K-dependent coagulation proteins including protein C, protein S is not a precursor to a serine protease. Instead, the C-terminus of protein S encompasses a large domain with considerable homology to sex-hormone binding globulin (SHBG)¹¹⁶.

During the past decades, the protein C anticoagulant pathway is extensively studied and is shown to be highly important in the down-regulation of coagulation. More recent investigations focus on the anti-inflammatory and anti-apoptotic role of the protein C pathway and show that APC is involved in cell signaling via protease activated

receptors¹¹⁷. An overview of the different functions of the protein C pathway is given in recent reviews^{118,119}. The following paragraphs describe in more detail the structure and function of protein S.

Physiology of protein S

Synthesis – The major source of vitamin K-dependent coagulation factors is the liver. Patients on oral anticoagulation show a reduction of ~50% of plasma protein S antigen, comparable to the reduction of prothrombin and protein C antigen. In patients with liver disease, however, plasma protein S is reduced no more than ~25% whereas prothrombin and protein C levels are reduced ~50%. This observation suggests an extrahepatic site of synthesis of protein S, likely the endothelium¹²⁰. *In vitro*, protein S is continuously synthesized and secreted by various cell types including hepatocytes and endothelial cells^{121,122}. Treatment of these cells with warfarin, a vitamin K antagonist, induces undercarboxylation of protein S which results in 1) reduced secretion and intracellular accumulation of protein S and 2) reduced activity of the secreted protein S. Expression of protein S *in vitro* can be influenced by various (inflammatory) stimuli. Protein S mRNA and antigen are also present in human platelets¹²³.

The protein S locus is situated on chromosome 3¹²⁴. The human chromosome carries two copies of the protein S gene¹²⁵: *PROS1* which contains 15 exons is transcriptionally active, *PROS2* is a pseudogene. Both genes share ~97% homology¹²⁵. Regulation of tissue specific protein S gene expression is largely unknown but seems rather complicated.

Human protein S is synthesized as a precursor protein of 676 amino acids of which the 41 N-terminal residues represent a preproleader sequence, homologous to other vitamin K-dependent proteins¹²⁶. The leader contains a signal peptide and a propeptide. During translation, the signal peptide directs the protein into the lumen of the endoplasmic reticulum where it is cleaved off. The propeptide harbours a recognition site for the vitamin K-dependent carboxylase which carboxylates several glutamic acid residues in the adjacent Gla-domain. Before secretion of matured protein S, also the propeptide is proteolytically removed.

Distribution – The protein S concentration in plasma is ~25 mg/l (~350 nM)¹²⁷. About 60% of protein S is captured in a 1:1 complex with C4b binding protein (C4BP), the remainder circulates free. Protein S also seems to reside in intracellular stores, though in low amounts. Protein S which is released from the alpha-granules of platelets (3×10^8) upon stimulation with thrombin, represents ~2.5% of the plasma concentration¹²³. Also in endothelial cells protein S is present in granule/vesicle-like structures¹²² and can be released after stimulation with calcium ionophore and probably also with thrombin. The amount of stored protein S seems negligible, but local concentrations might become significant. So far, it is not known if the release of protein S from endothelial cells or platelets is of significance *in vivo* e.g. during thrombin generation.

Figure 2.3 Modular organization of protein S. Mature protein S consists of 635 amino acids which are organized in various, consecutive modules. Starting from the N-terminus (N), protein S is organized as follows: a γ -carboxyglutamic acid (Gla) domain with 11 Gla-residues which are indicated by γ ; a thrombin sensitive region (TSR) which contains one disulfide bridge, two potential thrombin cleavage sites (Arg49 and Arg70) and one potential FXa cleavage site (Arg60); 4 epidermal growth factor (EGF)-like domains of which each domain carries 3 disulfide bridges and one β -hydroxylated amino acid (Hya (Asp-OH) in EGF1, Hyn (Asn-OH) in EGF2-4); a sex hormone binding globulin (SHBG)-like region which contains three potential glycosylation sites at Asn residues 458, 468 and 489 (indicated by reversed triangles) and two disulfide bridges. (Figure adapted from Dahlbäck, Thrombosis Research. 1995; 77: 1-43).

Structural properties of protein S

The 635 amino acid residues of mature protein S are organized in multiple modules (Fig. 2.3). No structural data on protein S via X-ray crystallography or NMR are available yet and therefore a three dimensional model of the protein still lacks. Computational modeling using information of homologous domains, however, is a powerful tool for model building and has given insight into the potential three-dimensional conformation of the N- and C-terminal parts of protein S^{128,129}. Starting from the N-terminus, protein S is composed of a γ -carboxyglutamic acid (Gla) domain, a thrombin sensitive region (TSR), four epidermal growth factor (EGF)-like domains and a sex hormone binding globulin (SHBG)-like domain (Fig. 2.3).

Protein S demonstrates considerable homology with the product of a growth arrest specific gene (Gas6). Human protein S and Gas6 share 44% amino acid identity though Gas6 most likely has no anticoagulant activity due to low homology with protein S in the activated protein C (APC)-binding domains¹³⁰. Regions with highest homology between protein S and Gas6 are the Gla-domain and EGF 2-4.

The Gla-domain of protein S comprises 11 γ -carboxylated glutamic acid (Gla) residues. Gla-residues bind calcium-ions that induce a transition of the Gla-domain into a calcium-ion stabilized structure in which the Gla-residues and calcium-ions reside in the core of the molecule¹³¹. A hydrophobic surface loop (omega loop) at the N-terminus mediates interaction of the Gla-domain with membranes containing

phosphatidylserine^{132,128}. Although the Gla-domains of vitamin K-dependent coagulation factors are highly homologous (reviewed by Stenflo¹³³), there is a wide range in phospholipid membrane binding affinity. Protein S shows high affinity for anionic phospholipids^{134,135} compared to other vitamin K-dependent coagulation factors. The Gla-domain anchors proteins to the membrane surface, which is essential for biological activity. Interactions of the Gla-domain are not restricted to phospholipids and increasing evidence supports a role for Gla-domains in protein-protein interactions. A recent study describes the direct involvement of the protein S Gla-domain in APC-cofactor activity and based on structural and mutational analysis it was proposed that the Gla-domain has two opposite sides of which one side interacts with the phospholipid surface and the other with APC¹³⁶.

The TSR is a disulfide loop which carries two potential thrombin cleavage sites (at Arg49 and Arg70 in human protein S). Cleavage in the TSR converts protein S in a two-chain molecule, which has reduced calcium affinity¹³⁷, suggesting that the TSR is involved in calcium binding¹³⁸. Thrombin-cleaved protein S still binds negatively charged phospholipids with similar affinity as intact protein S¹³⁸ but does not exhibit APC-cofactor activity¹³⁹. *In vitro*, however, thrombin cleavage only occurs in the absence of calcium¹⁴⁰ suggesting that inactivation by thrombin is not a physiological regulatory mechanism of protein S activity. The TSR can also be cleaved by FXa at Arg60 on a phospholipid surface in the presence of calcium¹⁴¹, and moreover, FXa-cleaved protein S has been detected in plasma¹⁴².

Protein S contains four EGF-like domains which are involved in calcium-ion binding¹⁴³. The affinity of the calcium-ion binding sites of domains 3 and 4 is very high, with dissociation constants in the nanomolar range¹⁴⁴. In general, EGF-like domains are highly stable modules and are involved in protein-protein interactions¹³³. Various experimental approaches as well as mutational analysis provided evidence that EGF-like domain 1¹⁴⁵⁻¹⁴⁸ and EGF-like domain 2^{149,150} are important for APC-cofactor activity of protein S and are involved in the direct interaction between the two proteins. In addition, EGF-like domains 3 and 4 have been reported to be involved in the interaction of protein S with FXa¹⁴⁴.

The C-terminal domain of protein S shows sequence similarity with proteins such as ABP/SHBG¹¹⁶. There are two small disulfide loops and three potential N-linked glycosylation sites (Asn residues 458, 468 and 489) in the SHBG-like domain; glycosylation has no significant effect on APC-cofactor activity or C4BP binding¹⁵¹. The SHBG-like domain consists of two laminin G-type regions (LGR). The exact function of the SHBG-like domain of protein S is still unknown. The protein S homologue Gas6 is known to function via its SHBG-like domain as a ligand for the Axl/Tyro family of receptor tyrosine kinases¹⁵². Whether protein S also activates these receptors is somewhat controversial, as there are differences in species-specificity: human Axl is activated by bovine protein S but not by human protein S^{153,154}. The SHBG-like domain of protein S interacts with other proteins. Many reports describe the interaction of protein S with C4b binding protein (C4BP), a regulatory protein of the classic complement pathway. C4BP is

a large, multimeric protein which consists of 6 or 7 α -chains and 1 β -chain. The β -chain is present in ~80% of all C4BP molecules under normal conditions¹²⁷ and carries the protein S binding site¹⁵⁵. Current knowledge about the structure of C4BP and its various functions and interactions are summarized by Blom *et al*¹⁵⁶. Protein S and C4BP form a 1:1 stoichiometric complex and in the presence of calcium the affinity of this interaction is very high ($K_d = <10^{-10}$ M)¹⁵⁷. Initially the concentration of C4BP in plasma was overestimated (350 nM) and it was postulated that there could be no free protein S in blood¹⁵⁷. In a study from 1992 however, the plasma concentrations of protein S, C4BP and complex were re-evaluated and re-determined, and apparently, all β -chain containing C4BP (200 nM) is in complex with protein S. Free protein S (150 nM) is the amount that remains from total protein S (350 nM) after complex formation with β -chain containing C4BP¹²⁷. The exact binding site of C4BP on protein S is not yet fully identified but lies completely within the SHBG-like domain¹⁵⁸ and comprises both LGRs¹⁵⁹. Using various techniques, different areas have been predicted to be part of the binding site for C4BP on the protein S SHBG-like domain¹⁶⁰, including residues within the two disulfide loops^{161,162} and residues 447-460¹⁶³. Likely, a combination of these areas is needed, as mutation of single regions did not lead to complete abolishment of C4BP binding. Deletion of the C-terminal part of the SHBG-like domain (residues 583-635) resulted in a reduced affinity but not in complete loss of binding between protein S and C4BP¹⁶⁴ and no loss in APC-cofactor activity of protein S. In the same region, a binding site for FVa was identified, comprising residues 621-635¹⁶⁵. Protein S bound to C4BP still binds to phospholipids¹⁵⁷.

Function of protein S

Phospholipid binding is essential for optimal function of protein S. Impaired phospholipid binding due to one missense mutation in the Gla-domain for example resulted in a 15-fold reduction of APC-cofactor activity of protein S and severe functional protein S deficiency¹⁶⁶. Moreover, protein S anchorage to endothelial cells¹⁶⁷, activated platelets¹⁶⁸ and platelet microparticles¹⁶⁹ was shown to be a prerequisite for anticoagulant activity on cell surfaces. Also APC-independent anticoagulant activity of protein S depends on phospholipid binding^{170,171}.

APC-cofactor function

As outlined in chapter 1, APC inactivates FVa and FVIIIa by cleavage at specific Arg residues and protein S acts as a cofactor in these reactions. Protein S stimulates cleavage of human FVa by APC only moderately^{172,173}, however, detailed kinetic analysis of FVa inactivation showed a biphasic pattern¹⁷⁴ of which the second, slower phase (cleavage at Arg306), which is needed for full inactivation of FVa, is accelerated ~20 fold by protein S¹⁷⁵. The use of FVa variants that lack one or more cleavage sites allowed the specific analysis of APC/protein S activity on the individual cleavage sites^{176,177}. In addition, the phospholipid composition was shown to be important for activity of APC as well as for protein S cofactor activity. Recently it was reported that APC inactivates FVa even in the absence of all three cleavage sites¹⁷⁸. Taken together, these data suggest a

Figure 2.4 Schematic representation of the APC-cofactor activity of protein S. FVa inactivation by APC is a biphasic reaction: a rapid cleavage at Arg506 is followed by a slow cleavage at Arg306. Arg506 cleavage gives rise to an intermediate FVa (FVa_{int}) with partial activity, while cleavage at Arg306 fully inactivates FVa (FVI). Protein S specifically accelerates the APC-cleavage of FVa at Arg306.

rather complicated mechanism of FVa inactivation. Fig. 2.4 gives a simplified representation of the cofactor activity of protein S in FVa inactivation by APC.

The mechanism by which protein S influences APC-cofactor activity involves various steps and interactions. 1) It was shown that protein S relocates the active site of APC closer to the phospholipid membrane¹⁷⁹, which might reflect the positioning of APC from Arg506 to Arg306. Moreover, when the active site of APC was lowered by engineering methods, protein S did not enhance the Arg306 cleavage anymore¹⁸⁰. 2) Protein S increases the affinity of APC for the phospholipid surface¹⁸¹. Recent studies have shown that when the affinity of APC for negatively charged phospholipids was augmented by site directed mutations in the Gla-domain, the anticoagulant activity of APC was increased (in clotting assays). In addition, the rate of FVa inactivation by this APC-mutant was increased, especially in the absence of protein S. Thus, the need for protein S is reduced when the affinity of APC for membranes is increased^{182,183}. 3) FVa is protected from cleavage by APC when it is bound to FXa¹⁸⁴ and this protection is counteracted by protein S^{173,175}. Moreover, FXa was shown to specifically protect the Arg506 cleavage, and protein S was shown to stimulate specifically the Arg306 cleavage^{173,175}.

The role of protein S in complex with C4BP in APC-cofactor activity is not clear. It was reported that protein S-C4BP has no cofactor activity^{185,186}, however, binding of protein S to APC is not inhibited by C4BP nor does APC inhibit binding of C4BP to protein S¹⁸⁶.

APC-independent anticoagulant activity

The first study describing APC-independent anticoagulant activity of protein S dates back to 1988 when Mitchell reported the direct inhibition of prothrombin activation by protein S¹⁸⁷. Some years later it was shown that protein S directly interacts with FVa and FXa^{171,188,189}. The binding of protein S to FXa resulted in a reduced proteolytic activity of FXa towards chromogenic substrate¹⁷¹ as well as prothrombin¹⁸⁸, and the inhibition appeared non-competitive¹⁷¹. Regarding FVa it was reported that protein S and prothrombin competed for binding to FVa¹⁸⁹. Protein S in complex with C4BP did not bind FVa anymore, however, the protein S-C4BP complex still inhibited FXa activity as well as prothrombinase activity on a cell surface¹⁷¹. Thus, one mechanism by which protein S can down-regulate prothrombin activation by the prothrombinase complex appears to be the direct binding to the prothrombinase complex constituents (Fig. 2.5a). The identification of FVa and FXa binding sites on protein S supports this hypothesis^{144,165}.

In addition, it has been shown that binding to a phospholipid membrane is essential for the APC-independent anticoagulant activity of protein S^{170,171}. Since purified protein S binds to anionic phospholipids with a high affinity, it was suggested that occupation of the phospholipid membrane could be another mechanism by which protein S inhibits prothrombin activation (Fig. 2.5b). Moreover, it was observed that the APC-independent

Figure 2.5 Schematic representation of the APC-independent anticoagulant activity of protein S. Protein S (Pr S) inhibits activation of prothrombin (FII) into thrombin (FIIa) through interference with the formation of the prothrombinase complex, (A) through direct interactions with factor Va (FVa) and factor Xa (FXa) and (B) through competition for phospholipid binding sites.

anticoagulant activity of protein S was most pronounced at low concentration anionic phospholipids¹⁹⁰. In their study from 1999, van 't Veer *et al* even concluded that the competition of protein S with the prothrombinase complex for phospholipid binding sites could fully explain the APC-independent anticoagulant activity¹⁹⁰.

However, comparing the different studies on the direct anticoagulant activity of protein S indicates that the inhibitory capacity of protein S shows a wide variation with IC₅₀ values varying between ~5 nM¹⁷⁰ and ~400 nM¹⁸⁷ protein S¹⁸⁹. In addition, the affinity for anionic phospholipids varies substantially between different protein S preparations, with dissociation constants (K_d) varying from 7 to 74 nM in the case of synthetic phospholipids¹⁷⁰. The affinity of protein S for cell membranes appeared to be lower, as K_d values were 100-200 nM^{169,171}. Moreover, it was shown that there is a good correlation between the phospholipid affinity of protein S and its APC-independent activity. The influence of the protein S purification procedure on the activity of a preparation has been recognized^{170,187,189} and it was suggested that modifications in the protein S molecule occur during purification^{188,189} which might explain the wide variation in APC-independent anticoagulant activity of different protein S preparations.

The APC-independent anticoagulant activity of protein S has also been studied in plasma. Protein S inhibited prothrombin activation on TF-rich endothelial cells in plasma under flow¹⁹¹. Moreover, two papers describe an assay for the APC-independent anticoagulant activity of protein S^{192,193}. When inhibitory antibodies against protein S were added to plasma, the protein S activity was eliminated which resulted in a significant reduction of the TF- or FXa-induced clotting time. In both studies it was shown that protein S-deficient patients have a decreased APC-independent anticoagulant activity. The activity is also decreased in women taking oral contraceptives¹⁹⁴ as well as in patients carrying the prothrombin G20210A mutation¹⁹³, which causes increased plasma prothrombin levels. It was therefore hypothesized that other factors might be involved in the APC-independent anticoagulant activity of protein S in plasma.

Other functions

Recent investigations focused on functions of protein S that are not directly related with coagulation. One of these functions is based on the capacity of protein S to bind to negatively charged phospholipid membranes, and its ability to attract C4BP to these surfaces¹⁵⁷. An early event during apoptosis is the exposure of phosphatidylserine in the outer leaflet of the cell. Protein S brings C4BP to the surface of apoptotic cells and therefore seems to be involved in the (down-)regulation of the immune response at the surface of apoptotic cells¹⁹⁵. Anderson *et al* showed that protein S stimulates macrophages to take up apoptotic cells¹⁹⁶. The phagocytosis of apoptotic cells was inhibited when C4BP was bound to protein S, indicating that a receptor binding site on protein S might be blocked¹⁹⁷ (reviewed by Rezende *et al*¹⁹⁸). The proposal that protein S is a receptor ligand is not new, and the first observation dates back to 1995 when it was reported that Gas6 and protein S are ligands for the Axl/Tyro3 family of receptor tyrosine kinases¹⁵³. Cellular effects of protein S have also been described for vascular

smooth muscle cells (VSMCs). Protein S binds to VSMCs via a so far unknown receptor and induces mitosis^{199,200}.

Protein S deficiency

The physiological relevance of protein S as an anticoagulant is illustrated by the association of recurrent venous thrombosis with heterozygous protein S deficiency²⁰¹⁻²⁰³ or the severe complications (purpura fulminans) associated with homozygous protein S deficiency^{204,205}. Also some cases of protein S deficiency-related arterial thrombosis have been reported²⁰⁶⁻²⁰⁸.

The prevalence of protein S deficiency in the general population is low (0.2-1%)²⁰⁹⁻²¹¹. Although protein S deficiency is recognized as a risk factor for venous thrombosis, affecting ~5% of thrombophilic patients^{212,213}, it appeared difficult to find a clear association between protein S deficiency and venous thrombosis. Case-control studies suggest no or a mildly increased risk (2.4-fold) of thrombosis associated with protein S deficiency²⁰⁹. Family-based studies, including studies on the effect of specific protein S gene mutations, show a higher risk (5-12-fold) in affected relatives of protein S deficient patients compared to non-affected relatives^{214,215}. Moreover, these studies revealed that reduced levels of free protein S are a stronger risk factor for venous thrombosis than reduced levels of total protein S.

Diagnosis of protein S deficiency relies on accurate determination of protein S antigen levels or activity. The measurement of free protein S levels by ELISA can be affected by the dissociation of the protein S-C4BP complex during incubation, causing overestimation of free protein S which might lead to misclassification of protein S-deficient patients²¹⁶. Functional assays, measuring the APC-cofactor activity of protein S, on the other hand, might be influenced by conditions of APC-resistance such as FV_{Leiden} that could result in false positive diagnosis of protein S deficiency²¹⁷. The methodology of the measurement of protein S antigen and activity is summarized by Goodwin *et al*²¹⁸. Protein S levels of individuals also fluctuate in time and are influenced by various factors such as gender, age, hormonal status and oral contraceptive use (summarized by Rezende *et al*¹⁹⁸). These phenomena cause overlap between normal and heterozygously deficient individuals which results in difficulties with diagnosis and classification²¹⁹. Inherited protein S deficiencies are classified based on the phenotype in plasma and three types of deficiency are distinguished. Like other coagulation factor deficiency states, quantitative deficiency *i.e.* low total protein S, is classified type I whereas qualitative deficiency *i.e.* normal levels of total and free protein S but decreased activity, is classified type II. A second class of quantitative protein S deficiency was recognized (type III) which is characterized by normal total protein S levels but reduced free protein S. It has to be noted however, that type I and III, both quantitative deficiencies, are suggested to be phenotypic variants of the same underlying genetic defect. A recent review summarizes the currently known mutations in the protein S gene that lead to protein S deficiency as well as situations that lead to acquired protein S deficiency¹⁹⁸.

Aim of this thesis

The research presented in this thesis focuses on the APC-independent anticoagulant activity of protein S. As outlined above, studies in model systems using purified components revealed two possible mechanism via which protein S can directly inhibit prothrombin activation *i.e.* via specific interactions with FVa and FXa or via competition for phospholipid binding sites. It was assumed that protein S directly interferes with the prothrombinase complex which results in decreased thrombin formation. The relative importance of both mechanisms, however, was at the time that our study was initiated, a matter of debate. A second topic of the APC-independent anticoagulant activity of protein S was the reported variation in inhibitory capacity between protein S preparations as well as the variation in phospholipid binding affinity. Apparently, different purified protein S preparations exhibit different direct anticoagulant activities. These inconsistencies make a good interpretation of the potential importance of the APC-independent anticoagulant activity as well as of the underlying mechanism difficult. Thus, we initially studied the biochemical characteristics of purified protein S in relation to its APC-independent anticoagulant activity (chapter 3 and 4). The goal was 1) to clarify what lies at the basis of the wide variation between different protein S preparations and 2) to define which mechanism is underlying the APC-independent anticoagulant activity.

Secondly, we wanted to obtain more insight into the mechanism of direct inhibition of thrombin generation by protein S in plasma. So far, it was not clear whether the mechanistic data obtained in studies with purified components might be valid in plasma too. Plasma is a much more complicated system and it is very well possible that other plasma factors influence protein S activity. Thus, the aim of the second part (chapter 5 and 6) was to characterize the APC independent anticoagulant activity of protein S in plasma and to define under which conditions it is most pronounced.

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Purified protein S contains
multimeric forms with increased
APC-independent anticoagulant activity.

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Abstract

Protein S, the cofactor of activated protein C (APC), also expresses anticoagulant activity independent of APC by directly inhibiting prothrombin activation via interactions with factors Xa, Va, and phospholipids. In different studies, however, large variations in APC-independent anticoagulant activities have been reported for protein S. The investigation presented here shows that within purified protein S preparations different forms of protein S are present, of which a hitherto unrecognized form (<5% of total protein S) binds with high affinity to phospholipid bilayers ($K_d < 1$ nM). The remaining protein S (>95%) has a low affinity ($K_d = 250$ nM) for phospholipids. Using their different affinities for phospholipids, separation of the forms of protein S was achieved. Native polyacrylamide-gel electrophoresis demonstrated that the form of protein S that binds to phospholipids with low affinity migrated as a single band, whereas the high-affinity protein S exhibited several bands that migrated with reduced mobility. Size-exclusion chromatography revealed that the slower-migrating bands represented multimeric forms of protein S. Multimeric protein S (<5% of total protein S) appeared to have a 100-fold higher APC-independent anticoagulant activity than the abundant form of protein S. Comparison of purified protein S preparations that exhibited a 4-fold difference in APC-independent anticoagulant activity showed that the ability to inhibit prothrombin activation correlated with the content of multimeric protein S. Multimeric protein S could not be identified in normal human plasma, and it is therefore unlikely that this form of protein S contributes to the APC-independent anticoagulant activity of protein S that is observed in plasma.

Introduction

Human protein S is a vitamin K-dependent glycoprotein that functions as a non-enzymatic cofactor for activated protein C (APC)¹ and which accelerates factor Va inactivation by selectively enhancing APC-mediated cleavage of factor Va at Arg306². The importance of this anticoagulant protein C pathway is emphasized by the increased risk for thrombotic complications associated with protein S and protein C deficiencies^{3,4}.

In 1988, it was reported that protein S also exhibits anticoagulant activity in the absence of APC⁵ by inhibiting prothrombin activation via direct interactions with the components of the prothrombinase complex: factor Va, factor Xa, and phospholipids⁶⁻⁸. In addition, it was shown that protein S reduced the level of F1+2 generation in plasma during perfusion over tissue factor-rich endothelial cell matrices in the absence of APC⁹. However, up to 100-fold differences in APC-independent anticoagulant activities have been reported for different protein S preparations⁵⁻¹¹.

The ability of protein S to bind to phospholipids appears to be crucially important for the expression of anticoagulant activity in the absence of APC^{6,10-12}. This was demonstrated by the observation that a monoclonal antibody directed against the γ -carboxyglutamic acid (Gla) module of protein S inhibited its APC-independent anticoagulant activity^{10,13}. More than 10-fold differences have been reported for the dissociation constant of protein S binding to phospholipids^{6,10-13} and the APC-

independent anticoagulant activity of a protein S preparation was shown to be related to its affinity for phospholipids¹⁰. Furthermore, the APC-independent activity appeared to be the consequence of occupation of the lipid membrane by protein S, since increasing the amount of phospholipids abrogated the inhibitory activity of protein S¹¹.

In this study we investigated the relationship between the APC-independent activity of protein S and its phospholipid binding properties in order to find an explanation for the variation in APC-independent anticoagulant activity between different protein S preparations.

Experimental procedures

Materials

Hepes, Tris, EDTA, bovine serum albumin (BSA), and ovalbumin were purchased from Sigma. 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-*sn*-glycero-3-phosphoserine (DOPS) were obtained from Avanti Polar Lipids (Alabaster, AL). The chromogenic substrate D-Phe-(pipecolyl)-Arg-pNA (S2238) was supplied by Chromogenix (Mölnådal, Sweden). Column materials were obtained from Pharmacia Bio-Rad Uppsala, Sweden. Gradient polyacrylamide gels (4-15%) were from Biorad (Hercules, CA). Immobilon polyvinylidene fluoride (PVDF) membranes were from Millipore (Bedford, MA). Diaminobenzidine (DAB) was obtained from Sigma (St. Louis, MO). 3, 5, 3', 5'-tetramethylbenzidine (TMB) was obtained from Roche (Mannheim, Germany). Protein S was obtained from Enzyme Research Laboratories (ERL, South Bend, IN). Antibodies against protein S were obtained from DAKO (Glostrup, Denmark). Human activated protein C (APC) and factor Xa were obtained from Enzyme Research Laboratories (South Bend, IN). Prothrombin¹⁴ and factor Va¹⁵ were purified as described.

Protein S purification

Human protein S preparations were purchased or purified from 20 units of freshly frozen human plasma (5 l) as described¹⁴. Both preparations were purified according to the same procedure, *i.e.* barium citrate adsorption of human plasma followed by ammonium sulfate precipitation, anion exchange chromatography (QAE Sephadex), affinity chromatography (Blue-Sepharose), and anion exchange chromatography (Q Sepharose fast flow). The protein S preparations were analyzed for purity and for the presence of thrombin-cleaved protein S by SDS-PAGE under non-reducing and reducing conditions, respectively. Protein S concentrations were determined by measuring A_{280} using an $\epsilon_{1\text{cm},280\text{ nm}}^{1\%}$ of 9.6^1 .

SDS- and native polyacrylamide gel electrophoresis and Western blotting

Protein S preparations were analyzed using PAGE in the presence of SDS or under native conditions. After electrophoresis, the separated proteins were transferred to PVDF membranes for Western blotting. Blocking of the PVDF membranes was performed with Tris-buffered saline (TBS: 50 mM Tris, 100 mM NaCl, pH 7.4) with 5% Protifar (blocking

buffer), and protein S was detected using peroxidase-conjugated polyclonal antibodies against human protein S diluted 1/1000 in blocking buffer. DAB or TMB was used as a peroxidase substrate.

Phospholipid Liposome and vesicle preparation

Liposomes were prepared essentially as described¹⁶. Dried phospholipids were suspended in TBS and vortexed for 3 min in the presence of glass beads. The liposomes were centrifuged at 8000g and washed once with TBS to remove small lipid structures. Small unilamellar vesicles were prepared as described¹⁵. Phospholipid concentrations were determined by microphosphate analysis¹⁷.

APC-independent anticoagulant activity of protein S

The APC-independent anticoagulant activity of protein S was measured in a prothrombinase assay. Factor Va, factor Xa, phospholipid vesicles (20/80 DOPS/DOPC molar ratio), and protein S were preincubated at 37°C in Hepes-buffered saline (HBS: 25 mM Hepes, 175 mM NaCl, pH 7.7) containing 3 mM CaCl₂ and 5 mg/ml BSA. Prothrombin was added after 15 min, resulting in final concentrations of 10 pM factor Va, 10 pM factor Xa, 100 nM phospholipid vesicles, 500 nM prothrombin and various concentrations of protein S in a volume of 300 µl. Rates of prothrombin activation were determined with the chromogenic substrate S2238.

Ellipsometric determination of the extent of protein adsorption to lipid bilayers

Protein adsorption to planar bilayers was assessed by ellipsometry¹⁸. Briefly, planar bilayers deposited on silicon slides were prepared as described previously¹⁹ by immersion of the hydrophilic silicon slide for 5 min in a stirred suspension of small unilamellar lipid vesicles (30 µM) in binding buffer (50 mM Tris, 100 mM NaCl, 3 mM CaCl₂, pH 7.5). The instrument and data analysis have been described previously²⁰. Measurements were performed using silicon slides at an angle of incidence of the light beam (HeNe laser) of 68°²¹. Experiments were performed in binding buffer containing 0.5 mg/ml BSA at room temperature (20-22°C) with continuous stirring in a trapezoidal cuvette.

Separation of protein S forms by liposome centrifugation

Liposomes (200 µM) were incubated with 900 nM protein S (protein S-*total*) in 200 µl TBS containing 3 mM CaCl₂ and 5 mg/ml BSA for 45 min with continuous mixing. The mixture was centrifuged for 10 min at 8000g in a Hettich EBA 12 eppendorf centrifuge and the supernatant was collected (protein S-*supernatant*). The liposome pellet was washed with TBS containing 3 mM CaCl₂ and 5 mg/ml BSA and again centrifuged. Then, phospholipid-bound protein S was eluted by resuspending the pellet in 200 µl of TBS containing 3 mM EDTA, and the liposomes were quantitatively removed by centrifugation in a Beckman TLA-100 ultracentrifuge (25 min at 110,000g). The eluted protein S thus obtained was designated protein S-*pellet*. All steps during the separation were performed

at room temperature in siliconized tubes. Protein S concentrations were determined using an ELISA.

Protein S ELISA

An ELISA was used to determine protein S concentrations of the separated protein S forms. Microtiter plate wells were coated overnight with a polyclonal antibody against human protein S diluted 1/1000 in 0.1 M Na₂CO₃, pH 9.0 (50 µl/well) at 4°C. Further steps were performed at room temperature. The wells were subsequently blocked with 200 µl of HBS containing 30 mg/ml BSA for 2h. Protein S samples were diluted in HBS containing 5 mg/ml BSA and 3 mM EDTA and 50 µl of each dilution was incubated in the wells for 1h. After washing was performed 3 times with 200 µl wash buffer (HBS containing 0.02% Tween-20), 50 µl of a peroxidase-conjugated polyclonal anti-human protein S antibody in HBS containing 5 mg/ml BSA was added to the wells and incubated for 1h. After washing was performed 5 times with 200 µl wash buffer, peroxidase activity was determined with the chromogenic substrate (TMB enzymatic kit, Roche) according to the manufacturer's instructions. ERL protein S (# 1540L) was used as a standard.

Size-exclusion chromatography

Protein S-supernatant and protein S-pellet (10 nM, 100 µl) were injected separately on a Superose 6 size-exclusion chromatography column (Pharmacia) at a flow rate of 0.25 ml/min in 25 mM Hepes, 150 mM NaCl, 1 mM EDTA, pH 7.4. Fractions (0.25 ml) were collected and immediately diluted 1:1 in HBS containing 30 mg/ml BSA. Protein S in the column fractions was determined by ELISA.

Isolation of protein S multimers using phospholipid-coated capillaries

Glass capillaries (0.58 mm internal diameter and 127 mm length, volume 35 µl, Brand AG, Wertheim, Germany) were thoroughly cleaned before phospholipid coating was carried out as described previously²². The phospholipid bilayer was deposited by incubating the capillaries for 1 h with a solution of 2 mM 20/80 DOPS/DOPC vesicles. To remove unbound phospholipid, the capillaries were washed with TBS containing 5 mg/ml BSA and 3 mM CaCl₂ for 30 min at a flow rate of 200 µl/min. Defibrinated, recalcified normal pooled plasma (3 ml), with or without addition of 50 ng of protein S pellet, was recirculated over 3 capillaries in series for 1 h at a flow rate of 200 µl/min. Then, a 30 min wash was performed with TBS containing 5 mg/ml BSA and 3 mM CaCl₂ at the same flow rate and finally, protein S elution was performed with TBS (130 µl) containing 5 mg/ml BSA and 4 mM EDTA. Samples of eluted fractions (30 µl) and purified protein S-pellet (10 ng) were subjected to native PAGE (4-15%) and Western blotting. Protein S was detected with peroxidase-conjugated polyclonal antibodies directed against protein S, using TMB as a substrate.

Results

APC-independent anticoagulant activity of protein S

To investigate the nature of differences in APC-independent anticoagulant activities between protein S preparations, we studied protein S obtained from two different sources: a protein S isolated from freshly frozen plasma (protein S_A) and a commercially available protein S (protein S_B). Both protein S preparations were characterized by SDS-PAGE. No differences between these protein S preparations were apparent under non-reducing and reducing conditions. SDS-PAGE analysis under reducing conditions indicated that in both preparations no thrombin-cleaved protein S was detectable (Fig. 3.1).

Figure 3.1 Characterization of protein S by SDS-PAGE. Protein S (4 μ g) was applied to a gradient SDS-gel (4-15% polyacrylamide). Lanes a and c contained nonreduced protein S_A and protein S_B, respectively. Lanes b and d contained reduced and alkylated protein S_A and protein S_B, respectively. Reduction was performed with 10 mM dithiotreitol for 15 minutes at 37°C followed by alkylation of the reduced sulfhydryl side chains with 20 mM iodoacetamide. Coomassie Brilliant Blue was used for staining.

Protein S_A and protein S_B were tested for their ability to inhibit prothrombin activation. Factor Va, factor Xa and phospholipid vesicles were preincubated in the presence of various concentrations of protein S. After 15 min, prothrombin was added and its activation was followed over time (Fig. 3.2A,B). The extent of prothrombin activation was linear with time, and both protein S_A (Panel A) and protein S_B (Panel B) inhibited prothrombin activation in a dose-dependent manner, although to different extents. Panel C shows the rate of prothrombin activation as a function of the protein S concentration. Half-maximal inhibition was reached at concentrations of 7 nM protein S_A and 28 nM protein S_B, indicating that protein S_A expressed a 4-fold higher inhibitory activity than protein S_B (Fig. 3.2C).

Phospholipid binding properties of protein S

Since binding of protein S to phospholipid is essential to expression of its anticoagulant activity, we compared the phospholipid-binding properties of protein S_A and protein S_B using ellipsometry. The level of concentration-dependent adsorption of protein S to DOPS/DOPC bilayers containing 20 mol% DOPS was measured as a

Figure 3.2 Effect of protein S on prothrombin activation in the absence of APC. Factor Va, factor Xa, phospholipid vesicles (DOPS/DOPC, 20/80) and protein S_A (Panel A) or protein S_B (Panel B) were preincubated at 37°C in HEPES-buffered saline (25 mM HEPES, 175 mM NaCl, pH 7.7) containing 3 mM CaCl₂ and 5 mg/ml BSA. After 15 minutes, prothrombin was added resulting in final concentrations of 10 pM factor Va, 10 pM factor Xa, 100 nM phospholipid vesicles, 500 nM prothrombin and 0 (●), 2 (○), 4 (■), 8 (□), 16 (▲) or 32 (△) nM protein S. At the indicated time points, the amounts of thrombin formed were determined with the chromogenic substrate S2238. Panel C shows inhibition of prothrombin activation by protein S_A (●) and protein S_B (○) plotted as a function of the final protein S concentration.

function of time (Fig. 3.3). The adsorption of both protein S_A and protein S_B was biphasic. A fast initial adsorption phase which was complete within 50 sec after addition of protein S was followed by a secondary slow adsorption during which additional protein accumulated on the lipid layer until equilibrium was reached.

The extent of protein adsorption during the first phase of binding was concentration-dependent and comparable for both preparations. This became apparent when the amount of protein bound to the lipid surface 50 sec after addition of protein S was plotted as a function of the total protein S concentration in the cuvette (Fig. 3.3C). The Langmuir model was used to analyze the adsorption at this time point. No difference was found between the two preparations in this first adsorption phase, and therefore, the data were combined for further analysis. The solid line in figure 3.3C represents the best fit to the combined data. The maximal level of binding ($\Gamma_{\max}$) to the phospholipid bilayer was estimated to be $0.34 \mu\text{g}/\text{cm}^2$ and the dissociation constant K_d was 246 nM.

The fast initial phase of binding was followed by a second phase of slow, continuing adsorption. For protein S_A (Fig. 3.3A) and protein S_B (Fig. 3.3B), the adsorption rate during the second phase was concentration-dependent, as shown by faster saturation of the lipid layer at higher protein S concentrations (Fig. 3.3A,B). However, protein adsorption during the second phase was much slower for protein S_B than for protein S_A . At 25 nM protein S, the extra amounts of protein S bound during the second phase of adsorption (between 50 s and 3800 s after addition of protein S) were $0.20 \mu\text{g}/\text{cm}^2$ for protein S_A and $0.09 \mu\text{g}/\text{cm}^2$ for protein S_B .

After 3800 s, the cuvette was flushed with a calcium-containing buffer. Surprisingly, only very limited desorption was observed (see below), implying that at the end of the adsorption phase protein S was bound with a high affinity to the phospholipid surface. Addition of EDTA, however, caused an instantaneous and complete dissociation of protein S from the lipid bilayer (see below), indicating that the high affinity binding was Ca^{2+} -dependent.

The biphasic adsorption behavior can tentatively be explained by assuming that protein S preparations contain two forms of protein S with different phospholipid binding properties. The initial fast adsorption phase likely originates from a fraction of protein S that is present abundantly and therefore binds rapidly but with a relatively low affinity, while the fraction that causes the additional slower binding is present in a low amount, resulting in a slow but high affinity accumulation of protein S on the phospholipid bilayer.

Adsorption and readorption of protein S on a DOPS/DOPC bilayer

To test the hypothesis that the slow adsorption process is associated with a high-affinity binding fraction of protein S, we characterized the readorption of the bound protein to the planar lipid layer at 50 nM protein S (Fig. 3.4A) and at 300 nM protein S (Fig. 3.4B).

After the characteristic biphasic adsorption of protein S, nonbound protein S was removed by rapidly flushing the cuvette with 50 ml calcium-containing buffer at 4700 s (Fig. 3.4, black arrowheads). This depletion of protein S from the solution caused only a slow and limited desorption and most of the protein remained bound to the phospholipid bilayer. After addition of EDTA at 5000 s (Fig. 3.4, white arrowheads) all protein S dissociated from the phospholipid bilayer. The amounts of protein S present in the EDTA-containing buffer (Fig. 3.4A), as determined by ELISA, were $0.23 \mu\text{g}$ protein S_A

Figure 3.3 Concentration-dependent binding of protein S to a phospholipid bilayer. The mass Γ ($\mu\text{g}/\text{cm}^2$) of protein adsorbed to DOPS/DOPC bilayers containing 20 mol% DOPS was measured by ellipsometry as described under "Experimental Procedures". (A) At $t=200$ s, protein S_A (25, 50, 100, 200 and 400 nM in curves a to e, respectively) was added to the cuvette and adsorption was followed over time for 1 h. (B) The same experiment was performed with protein S_B (25, 50, 100, 200 and 400 nM in curves a to e, respectively). (C) The amount of protein bound to the bilayer during the first, fast phase (50 s after addition of protein S to the cuvette) is plotted as a function of the final concentration of protein S_A (●) and protein S_B (○). The solid line represents the best fit to these data according to the Langmuir model. Experiments were performed at room temperature (20–22°C) in binding buffer (50 mM Tris, 100 mM NaCl, 3 mM CaCl₂, pH 7.5) containing 0.5 mg/ml BSA.

(0.74 nM) and 0.13 μg protein S_B (0.43 nM), respectively, which are close to the amounts of protein S that were bound to the phospholipid surface (0.6 cm^2) before the addition of EDTA (0.18 μg protein S_A and 0.11 μg protein S_B). This indicates that the phospholipid-bound protein was protein S.

Figure 3.4 Adsorption and re-adsorption of protein S to phospholipid bilayers. Adsorption and re-adsorption of protein S to a DOPS/DOPC bilayer (20 mole% DOPS) was analyzed by ellipsometry at two protein S concentrations (Panel A: 50 nM, Panel B: 300 nM). In both panel A and B, curve a and b represent protein S_A and protein S_B, respectively. Protein adsorption was initiated by addition of protein S to the cuvette at t = 200s and was followed until maximal adsorption was reached. At t = ~5000s (filled arrows), the cuvette was rapidly flushed with 50 ml calcium-containing buffer. The reversibly bound protein was allowed to desorb and when the desorption leveled off, EDTA was added (3.2 mM, open arrows) in order to desorb the remaining protein from the lipid layer. When desorption was complete, the calcium level was restored to 3 mM to allow the re-adsorption of the dissociated protein. About 4000s later (at t = ~10000s), the cuvette was rapidly flushed with 50 ml calcium-containing buffer (filled arrows).

Subsequently, the free calcium concentration in the cuvette was restored to 3 mM and re-adsorption of protein S was observed (Fig. 3.4). However, the initial fast phase of adsorption was no longer present. This observation indicates that (1) the high affinity protein S was responsible for the secondary, slow binding to the phospholipid bilayer and (2) that the high-affinity protein S, which was adsorbed during the slow binding phase quantitatively replaced the low-affinity protein S that was bound during the first rapid phase. In agreement with this, washing the cuvette before the initially bound protein S was replaced by the high affinity form (300 s after protein S addition) resulted in desorption of more than 75% of the bound protein S (data not shown). As mentioned earlier, the concentration of protein S in the incubation mixture after the release of protein S from the phospholipid bilayer by EDTA was < 1 nM. The fact that, after recalcification of the medium, approximately 50% of maximal protein S re-adsorbed indicates that the high-affinity form of protein S did bind to the phospholipid bilayer with a K_d value < 1 nM.

At 300 nM protein S, the adsorption and re-adsorption characteristics were similar for both preparations (Fig. 3.4B) indicating that in this case both forms of protein S were present in excess of the number of binding sites on the phospholipid bilayer. This observation suggests a quantitative, but not a qualitative difference between the two protein S forms present in the protein S_A and protein S_B preparations.

Preincubation of protein S preparations with small unilamellar vesicles

Since a small fraction of total protein S binds with such a high affinity to the phospholipid bilayers that it can only be released by addition of EDTA, it was reasoned

Figure 3.5 Binding of protein S to a lipid bilayer after preadsorption to small unilamellar phospholipid vesicles. Protein S (500 nM) was preincubated for 45 minutes at room temperature with DOPS/DOPC vesicles at various concentrations (20 mol% DOPS) in binding buffer (50 mM Tris, 100 mM NaCl, 3 mM CaCl_2 , pH 7.5) containing 0.5 mg/ml BSA with incidental shaking. The preincubation mixture (0.5 ml) was added to 4.5 ml binding buffer in the ellipsometer cuvette, yielding final concentrations of 50 nM protein S (protein S_A in panel A, protein S_B in panel B) and 0, 62.5, 125 or 250 nM phospholipid vesicles (curve a to d respectively in both panels). The adsorption to the planar DOPS/DOPC (20 mol% DOPS) layer was quantified using ellipsometry. At ~4500s (marked with arrows), the cuvette was flushed with 50 ml calcium-containing buffer.

that a protein S preparation could be depleted from this high affinity fraction upon preincubation with phospholipid vesicles. To verify this, we preincubated protein S_A and protein S_B with different concentrations of small unilamellar DOPS/DOPC (20 mol% DOPS) vesicles in the presence of 3 mM CaCl_2 . The protein S/vesicle mixtures were subsequently transferred to the ellipsometer cuvette, yielding final concentrations of 50 nM protein S and 0, 62.5, 125 or 250 nM phospholipid vesicles (Fig. 3.5). For both preparations, the fast initial phase remained constant but, with increasing amounts of phospholipid vesicles present in the preincubation mixture, the amount of protein S that adsorbed to the phospholipid bilayer during the second, slow phase was reduced for more than 90% after preincubation of protein S with 250 nM phospholipid vesicles. This suggests that the protein S form that is responsible for the secondary, slow binding was bound to the phospholipid vesicles during the preincubation and was not available anymore for binding to the planar phospholipid layer in the ellipsometer.

At saturation of protein S adsorption to the phospholipid bilayer ($t = 4500$ sec), bound protein was allowed to desorb by rapidly washing the cuvette with 50 ml of CaCl_2 -containing buffer (Fig. 3.5A, B). Almost no protein S desorbed from the planar bilayer in the control experiment in which the preincubation mixture did not contain phospholipids (Fig. 3.5, curves a). However, with increasing amounts of phospholipid vesicles present in the preincubation mixture, increasing amounts of protein S desorbed after flushing the cuvette (Fig. 3.5). This indicates that incubation of the protein S preparation with DOPS/DOPC vesicles resulted in depletion of the protein S preparations from high binding affinity protein S.

Figure 3.5 also shows that, compared to protein S_B , more phospholipid vesicles were needed to deplete protein S_A from the high-affinity protein S fraction, which confirms the presence of larger amounts of high-affinity protein S in protein S_A .

Separation of protein S forms by liposome sedimentation

The experiments presented above suggest that phospholipid vesicles can be used to separate and subsequently isolate the low- and high-affinity binding forms of protein S. Since protein S_A contains more high-affinity protein S than protein S_B , an attempt to separate the two forms of protein S was only performed with protein S_A .

To separate the protein S forms, 200 μ M centrifugable phospholipid liposomes were incubated with 900 nM protein S_A in a CaCl_2 -containing buffer. After centrifugation of the suspension, the majority of the protein S (840 nM) remained in the supernatant as measured by an ELISA, and this fraction was designated protein S-supernatant. The phospholipid-bound protein S was isolated by resuspending the liposome pellet in an EDTA-containing buffer. The liposomes were removed by centrifugation, and the protein S present in the supernatant was designated protein S-pellet. In addition, an experiment was performed in which protein S was subjected to the same sample handling in the absence of liposomes. This preparation was called protein S-total. ELISA measurements showed that the protein S-pellet concentration was 26 nM, which constituted ~3% of protein S-total.

Figure 3.6 Separation of protein S forms by liposome centrifugation. Liposomes were used to separate different protein S forms present in the protein S_A preparation as described under "Experimental Procedures". At the end of the procedure, protein S-total, protein S-supernatant and protein S-pellet fractions were obtained. Panel A: Kinetics of binding of protein S fractions to DOPS/DOPC planar bilayers. At 200 s, 100 μ l of protein S-total (a), protein S-supernatant (b) or protein S-pellet (c) was added to the ellipsometry cuvette and adsorption to the DOPS/DOPC bilayer (20 mol% DOPS) was followed over time. The unbound protein was depleted from the cuvette at ~4500 s by washing with 50 ml CaCl_2 -containing buffer (black arrowheads). Adsorbed protein was released from the phospholipid bilayer by addition of 3.2 mM EDTA (white arrowheads). Panel B: APC-independent inhibition of prothrombin activation by protein S-total (O), protein S-supernatant ($\blacktriangle$) and protein S-pellet ($\bullet$) assessed as described in "Experimental Procedures".

Figure 3.6A shows the time courses of protein S-*total*, protein S-*supernatant* and protein S-*pellet* binding to phospholipid. Protein S-*total* showed the characteristic biphasic adsorption to the lipid bilayer (Fig. 3.6A, curve a). At ~4500 s, the cuvette was flushed with 50 ml of calcium-containing buffer, resulting in a minor desorption of protein S. The protein S-*supernatant*, however, only exhibited the first, rapid phase of protein S adsorption (Fig. 3.6A, curve b). No secondary slow adsorption was observed, indicating that the high-binding affinity form was absent. This was confirmed by the fact that during washing at ~4500 s, all the bound protein desorbed, indicative for reversible and low-affinity binding of protein S. The lipid binding pattern of protein S-*pellet* only exhibited the second, slow phase of protein S adsorption (Fig. 3.6A, curve c), and no protein S desorbed when the cuvette was flushed with calcium-containing buffer at ~4500 s.

When the protein S fractions were tested for their ability to inhibit prothrombin activation, the major part of the APC-independent activity of protein S was shown to be associated with the protein S-*pellet* fraction (Fig. 3.6B). It was observed that protein S-*pellet* is 100-fold more effective in inhibiting prothrombin activation than protein S-*supernatant* under the experimental conditions used (Fig. 3.6B).

Difference in mobility of protein S preparations on PAGE analysis

Although protein S_A and protein S_B preparations exhibited different APC-independent anticoagulant activities and phospholipid binding properties, they could not be distinguished by SDS-PAGE analysis (Fig. 3.1). However, using native PAGE in the absence of SDS, a difference between the preparations was observed. Both protein S preparations contained more than one form of protein S, which migrated with different rates during native PAGE. To confirm that all protein bands represented protein S, the separated proteins were transferred to PVDF membranes for Western blotting with polyclonal antibodies against protein S. As can be observed, different bands migrating with different rates on native PAGE stained positive for protein S (Fig. 3.7A). The form of protein S with high mobility appears to be the most abundant whereas the form of protein S with low mobility is present in a low concentration. Interestingly, the amount of low-mobility protein S is greater in protein S_A than in protein S_B (Fig. 3.7A).

Figure 3.7 Analysis of protein S by native PAGE and Western blotting. Panel A: Native PAGE immunoblot analysis of protein S_A and protein S_B. 75 ng protein S_A (lane a) or protein S_B (lane b) was subjected to native PAGE (4-15%). After being transferred to a PVDF membrane, protein S was detected with peroxidase-conjugated polyclonal antibodies against protein S. Panel B: Native PAGE immunoblot analysis of protein S-*total*, protein S-*supernatant*, and protein S-*pellet*. 75 ng protein S-*total* (a), protein S-*supernatant* (b) or protein S-*pellet* (c) was subjected to native PAGE (4-15%), transferred to a PVDF membrane and detected with peroxidase-labeled polyclonal antibodies against protein S.

Protein *S-total*, protein *S-supernatant* and protein *S-pellet* were also subjected to native PAGE and Western blotting (Fig. 3.7B). Protein *S-total* showed the characteristic pattern with the different migrating forms. Protein *S-supernatant*, however, contained only the form with highest mobility, which was abundantly present in protein *S-total*. Strikingly, protein *S-pellet* predominantly contained the slower-migrating bands.

To rule out the possibility of cross-staining of the polyclonal anti-protein S antibodies with other proteins, we used a monoclonal antibody against the Ca^{2+} -bound conformation of the Gla module of protein S (CLB-13) to detect purified protein S on Western blotting. The same multimeric patterns of protein S were observed on Western blot when CLB-13 was used in the presence of CaCl_2 (data not shown). Furthermore, in an ELISA in which monoclonal anti-protein S antibody CLB-13 was used as capture antibody and peroxidase-labeled polyclonal antibodies against protein S were used for detection, purified multimers of protein S (protein *S-pellet*) were detected in the presence but not in the absence of CaCl_2 (data not shown).

It is also possible that other protein components are incorporated in the protein S aggregates. However, on SDS-PAGE, only one band was visible on gel (Fig. 3.1). This rules out a major second component to the multimeric structures unless the second component has the same mass as protein S. Specifically, prothrombin had to be excluded as a possible component of the multimers because (i) it is frequently observed as a contaminant of protein S preparations and (ii) prothrombin has a molecular weight comparable to that of protein S on SDS-PAGE. The protein S preparations were tested for the presence of prothrombin with the prothrombin activator from *Echis carinatus* venom. No measurable levels of thrombin in the preparations could be detected which indicates that prothrombin is absent. In addition, protein *S-pellet* was applied to native PAGE and Western blot, and developed using peroxidase-labeled anti-prothrombin antibodies and DAB. No signal was obtained for the purified protein S sample indicating that prothrombin is not part of the multimeric structures of protein S. Furthermore, in a control sample of purified prothrombin, no multimeric forms of prothrombin were present, indicating that multimerization is specific for protein S (data not shown).

Characterization of protein *S-pellet* and protein *S-supernatant*

The ladder pattern of protein S that is visible on native PAGE Western blots (Fig. 3.7) might be caused by the existence of either multimers or charge variants of protein S. To discriminate between these two possibilities, both protein *S-supernatant* and protein *S-pellet* were subjected to size-exclusion chromatography on a Superose 6 column (Fig. 3.8A). The protein *S-supernatant* eluted as a symmetrical peak, suggesting a homogeneous protein S fraction. In the case of protein *S-pellet*, however, several smaller peaks eluted from the column; one at the same position of the protein *S-supernatant* peak and the others at earlier elution times. This indicates the presence of multimers of protein S in the protein *S-pellet* fraction. Native PAGE immunoblot analysis of the peak fractions confirmed that the protein S fractions that eluted earlier from the Superose 6 column represented protein S forms with low electrophoretic mobility (Fig. 3.8B).

Figure 3.8 Size-exclusion chromatography of protein S. 75 ng of protein S-supernatant and protein S-pellet were injected separately on a Superose 6 column. **Panel A:** Elution profiles of protein S-supernatant (solid line) and protein S-pellet (dotted line) in the column effluent as detected by ELISA. White arrowheads indicate the void volume of the Superose column (V_0), the elution positions of C4b-binding protein (C4BP) and BSA, and the total volume of the Superose column (V_1). **Panel B:** Native PAGE immunoblot analysis of the column fractions (lanes b-e) indicated by black arrowheads in panel A. Samples were subjected to native PAGE (4-15%), transferred to PVDF membranes, and detected with peroxidase-conjugated polyclonal antibodies against protein S using DAB (lane a) or TMB (lane b-e) as a substrate.

Prolonged incubation at 4°C for 2 weeks or repetitive freezing and thawing of protein S-supernatant or protein S-pellet did not result in generation or disappearance of the protein S multimers (data not shown). Dissociation of purified protein S multimers (protein S-pellet) could only be achieved by incubation with the anionic detergent SDS, resulting in a single band on a SDS-PAGE Western blot (data not shown), in accordance with the results of SDS-PAGE of protein S-total (Fig. 3.1).

Protein S multimers in normal human plasma

To investigate whether protein S multimers are present in normal human plasma, we recirculated defibrinated normal plasma (3 ml) through a thin glass capillary coated with DOPS/DOPC vesicles in the presence of 3 mM CaCl_2 . After 75 min, the capillary was washed with TBS containing 3 mM CaCl_2 and 0.5% BSA to remove unbound protein from the capillaries, and the proteins that had bound Ca^{2+} -dependent to the phospholipid bilayer in the capillary were eluted with TBS containing 4 mM EDTA and 5 mg/ml BSA. The feasibility of this technique in detecting multimeric forms of protein S in plasma was verified in a similar experiment with defibrinated normal plasma to which protein S

Figure 3.9 Isolation of protein S multimers. Normal human plasma and normal human plasma supplemented with 0.25 nM purified protein S multimers were recirculated through phospholipid-coated glass capillaries (DOPS/DOPC, 20/80 mole%) in the presence of CaCl_2 as described in "Experimental Procedures". Phospholipid-bound protein S was eluted with EDTA buffer and subjected to native PAGE and Western blot analysis using peroxidase-conjugated polyclonal anti-protein S antibodies. Lane a: purified protein S multimers; lane b: protein S from normal human plasma; lane c: protein S from normal human plasma supplemented with purified protein S multimers.

multimers were added to a final concentration of 0.25 nM (<1% of total protein S). Western blotting after native PAGE (Fig. 3.9) shows the characteristic pattern of multimeric protein S in a purified sample of protein S-*pellet* (lane a) and in normal human plasma that was supplemented with protein S-*pellet* (lane c). However, protein S multimers were undetectable in normal plasma (Fig. 3.9 lane b). The protein band on top of the blot (Fig. 3.9, lane b) likely represents the noncovalent complex between protein S and C4b-binding protein that is abundantly present in plasma (23). When purified protein S-C4b-binding protein complex was applied to native PAGE and Western blotting, an identical band on top of the blot was observed (data not shown).

Discussion

In this study, we have demonstrated that purified protein S preparations contain two forms of protein S that can be separated and that have different functional and biochemical properties. Characterization of the phospholipid binding properties of protein S and its ability to inhibit prothrombin activation revealed the presence of multimeric protein S that binds with high affinity to phospholipid bilayers ($K_d < 1$ nM). This multimeric form of protein S, which constitutes a small fraction (< 5%) of total protein S, has a 100-fold higher APC-independent anticoagulant activity than the residual protein S form (> 95%) that binds with low affinity to phospholipids ($K_d = 250$ nM).

Comparison of two different protein S preparations, which exhibited a 4-fold difference in APC-independent anticoagulant activities, showed that the preparation with the lower inhibitory activity contained substantially less of the multimeric form of protein S. This indicates that differences in APC-independent anticoagulant activities between protein S preparations reported in the literature⁵⁻¹¹ are likely caused by the presence of different amounts of multimeric protein S.

The kinetics of protein S binding to phospholipid bilayers show typical biphasic adsorption behavior. During the first rapid phase of adsorption, protein S molecules with a low affinity for lipids that are abundantly (~95%) present in protein S preparations reversibly bind to the phospholipid surface. During the second slow phase of adsorption,

these protein S molecules are subsequently replaced by the high-affinity-binding-form of protein S. This phenomenon of sequential binding on a phospholipid surface in which a relatively abundant ligand with low affinity is replaced in time by a much less abundant protein with higher affinity has been observed before in protein mixtures²⁴⁻²⁶.

We succeeded in separating the low-and high-affinity-binding-forms of protein S. Gel electrophoresis under native conditions (*i.e.* in the absence of SDS) indicated that the form of protein S that binds with a low affinity to phospholipids migrates as a single band, whereas the high affinity binding form contains multimeric protein S, as revealed by size-exclusion chromatography and native PAGE.

Multimerization of protein S can well explain the high affinity for phospholipids provided that it does not result in shielding of the phospholipid binding site in the Gla module of protein S. The availability of the Gla modules in protein S multimers was confirmed with a monoclonal antibody directed against the Ca^{2+} -bound conformation of the Gla module of protein S (CLB-13). This antibody detected purified protein S multimers in the presence, but not in the absence, of CaCl_2 , indicating that in protein S multimers the Gla modules are functionally exposed and accessible. If bundling of Gla modules offers a rationale for the observed high phospholipid binding affinity of protein S multimers, it is likely that protein S multimerization proceeds in an ordered fashion and that Gla modules in protein S multimers are not randomly oriented. We were not able to detect multimeric protein S in normal human plasma. It is therefore unlikely that multimeric protein S contributes to the APC-independent anticoagulant activity of protein S that is observed in plasma^{6, 9, 27}. However, it is important to realize that when studying APC-independent activities of purified protein S, the presence of multimeric protein S will significantly enhance the overall anticoagulant activity of the protein S preparation being investigated. Therefore, it is preferable to use the here described protein S-supernatant in studies that are aimed at unraveling the mechanisms underlying APC-independent activities of protein S.

Our study indicates that noncovalent association of protein S molecules, which changes its biochemical and functional properties, most likely occurred during the purification procedure. The suggestion that protein S multimerizes in the absence of calcium-ions²⁸ might play a role in the observed phenomenon as EDTA is frequently used during purification procedures. However, complete reversal of multimerization in the presence of CaCl_2 is not observed in the present study.

Ongoing investigations are focussed on how protein S multimers can be generated and on how to avoid protein S multimerization during protein purification. Identification of conditions that induce multimerization will benefit the unraveling of mechanisms by which protein S exerts its anticoagulant activities in the absence of activated protein C.

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4

Protein S multimers are generated
in vitro and affect protein S
structure-function analyses

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Abstract

Purified human protein S preparations contain small amounts of multimeric protein S. Protein S multimers are absent in plasma, suggesting that multimerization results from purification. Protein S multimers effectively inhibit phospholipid-dependent reactions at low phospholipid concentrations, and may therefore interfere during functional analysis of protein S. We have demonstrated that anion-exchange chromatography, as well as high ionic strength or low pH elution conditions used in immunoaffinity purification of protein S, induce protein S multimer formation. When protein S multimers were removed from protein S preparations by size-exclusion chromatography, multimers spontaneously reappeared in the protein S monomer fraction. In model systems, high phospholipid concentrations (>50 μM) completely abrogate the inhibitory effect of protein S multimers on prothrombinase complex activity. In addition, C4BP does not bind to protein S multimers. Thus, at low phospholipid concentrations, addition of C4BP to purified protein S will not affect the inhibitory activity of protein S multimers. In conclusion, to avoid misinterpretations during protein S structure-function analysis due to multimers present in purified protein S preparations, we recommend studying the anticoagulant activities of protein S either in plasma, where protein S is in its unmodified natural form, or at high phospholipid concentrations in model systems with purified protein.

Introduction

Protein S is a vitamin K-dependent protein that is present in human plasma at a concentration of 25 mg/L^{1,2}. Protein S is an important natural anticoagulant, since deficiencies in protein S are associated with thrombotic complications³⁻⁵. Approximately 60% of plasma protein S is present in a high affinity 1:1 stoichiometric complex with C4b-binding protein (C4BP), a regulator of the classic complement pathway⁶. The C-terminal domain of protein S consists of a sex hormone-binding globulin (SHBG) homology region that is involved in C4BP binding⁷. Amino acid residues in the second (C-terminal) laminin G module domain of SHBG have been shown to be essential for the interaction between protein S and C4BP⁸. The free protein S present in plasma is a cofactor of activated protein C (APC)^{9,10} in the inactivation of coagulation factors Va and VIIIa^{11,12}. In addition, protein S has been reported to inhibit prothrombin activation in the absence of APC¹³ via direct interactions with the components of the prothrombinase complex¹⁴⁻¹⁷. Identification of binding sites for FVa and activated factor X (FXa) on protein S support this theory^{18,19}. Complexation of protein S by C4BP has been shown to abolish its APC-cofactor activity but not its APC-independent anticoagulant activity^{14,20,21}.

Binding of the N-terminal γ -carboxyglutamic acid-rich (Gla) domain of protein S to phospholipid surfaces is essential for the expression of both the APC-cofactor and the APC-independent anticoagulant activities of protein S^{17,22,23}. Compared with other vitamin K-dependent proteins, the affinity of protein S for lipid surfaces is high²⁴, and reported K_d values for purified protein S preparations vary from 7 to 74 nM¹⁷. However, purified protein S preparations are a heterogeneous mixture of protein S monomers and multimers²⁵. A small percentage of the protein S molecules (<5%) exist in multimeric

forms that bind with a much higher affinity ($K_d < 1$ nM) to anionic phospholipids than do the protein S monomers ($K_d \sim 250$ nM). Therefore, the reported K_d values likely represent an average phospholipid binding affinity of multimeric and monomeric protein S forms in the preparations used.

It has been proposed that protein S inhibits prothrombin activation in the absence of APC by competing with prothrombin, FXa and FVa for binding to phospholipid membranes^{17,26}. Because of their high affinity for phospholipids, multimeric forms of protein S are potent competitors and therefore efficiently inhibit prothrombin activation at low phospholipid concentrations²⁵. However, the fact that multimers could not be detected in plasma suggests that, in plasma, a different mechanism must underlie the observed APC-independent activity of protein S²⁷. This is underscored by the observations that, in the absence of APC, protein S is an effective inhibitor of tissue factor-induced thrombin generation, and that the APC-independent anticoagulant activity of protein S in plasma is not affected by variation of the phospholipid concentration between 0.1 and 100 μM ²⁷. Considering the above, protein S multimers are not physiologically relevant and their presence in purified protein S preparations should be avoided.

The presence of multimeric forms in purified protein S preparations has been observed previously in lipid-binding and activity experiments^{17,26,28} and appears to be characteristic for purified protein S preparations. The present study describes the steps during protein S purification at which multimers are generated, and provides information on how to adjust experimental conditions to circumvent unwanted influences of protein S multimers on structure-function analyses of protein S.

Experimental procedures

Materials

HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), MES (2-[*N*-morpholino]ethanesulfonic acid) and Tris buffer, EDTA (ethylenedinitrilotetra acetic acid), Benzamidine-HCl, BSA (bovine serum albumin), ovalbumin, and DAB (diaminobenzidine) were purchased from Sigma-Aldrich (St Louis, MO). NaCl, CaCl_2 , NaN_3 , and ammonium sulfate were obtained from Merck (Darmstadt, Germany). 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate (CHAPS) was from ICN Biomedicals (Aurora, OH) and D-phenylalanyl-L-phenylalanyl-L-arginine-chloromethylketone (PPACK) was supplied by Behring Diagnostics (La Jolla, CA). 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-*sn*-glycero-3-phosphoserine (DOPS) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) were obtained from Avanti Polar Lipids (Alabaster, AL). The chromogenic substrate D-Phe-(pipecolyl)-Arg-pNA (S2238) was supplied by Chromogenix (Möndal, Sweden). Column materials were obtained from Amersham Biosciences (Uppsala, Sweden). CNBr-activated Sepharose 4B was from Pharmacia Biotech. Gradient polyacrylamide gels (4-15%) and SDS (sodium dodecyl sulfate) were from Biorad (Hercules, CA). Immobilon

polyvinylidene fluoride (PVDF) membranes were from Millipore (Bedford, MA). Factor Xa was obtained from Enzyme Research Laboratories (South Bend, IN). Prothrombin²⁹, factor Va³⁰ and beta-chain containing C4BP³¹ were purified as described previously.

Protein S purification

Purification of protein S was performed as reported by Koedam et al²⁹ with some modifications. Seven units (~2000 mL) of freshly frozen human plasma were thawed and pooled, enzyme inhibitors were added (2 μ M PPACK, 25 mM benzamidine-HCl) and 240 mL 1 M BaCl₂ was added drop-wise while stirring. The barium citrate precipitate was washed twice with 0.1 M BaCl₂ and 25 mM benzamidine-HCl and was suspended in 25 mM benzamidine-HCl. Following 30% ammonium sulfate precipitation, the supernatant, which contained protein S, was dialyzed against MES-buffered saline (MBS: 10 mM MES, 150 mM NaCl, pH 6.0) containing 2 μ M PPACK, 10 mM EDTA, 0.02% NaN₃, 1 mM benzamidine, and subsequently applied to a QAE Sephadex column (5 x 40 cm) for anion exchange chromatography. Proteins were eluted with a linear gradient of 150-500 mM NaCl in MBS. The fractions containing protein S were pooled, concentrated (70% ammonium sulfate vol/vol) and dialyzed in Tris-buffered saline (TBS: 50 mM Tris, 150 mM NaCl, pH 7.4) containing 2 mM EDTA and 0.02% NaN₃. Subsequent affinity chromatography on a Blue Sepharose column separated protein S from protein C. In the final purification step, protein S-containing fractions were subjected to anion exchange chromatography on Q Sepharose fast flow. Protein S was eluted with calcium-containing TBS (25 mM Tris, 110 mM NaCl, 20 mM CaCl₂, pH 7.4), dialyzed against HEPES-buffered saline (HBS: 25 mM HEPES, 175 mM NaCl, pH 7.7), and stored at -80°C. All steps were performed at 4°C except for Blue Sepharose and Q Sepharose fast flow chromatography, which were performed at room temperature (RT). Protein S concentrations were determined with an enzyme-linked immunosorbent assay (ELISA) using protein S from Enzyme Research Laboratories (#1540), the concentration of which was determined from the absorbance at 280 nm (A_{280}) using $\epsilon^{1\%}_{1\text{cm},280\text{nm}} = 9.6^1$, as a standard. Samples were taken throughout the purification procedure and analyzed on SDS-polyacrylamide gel electrophoresis (PAGE) or native PAGE immunoblot as described previously²⁵.

In addition, protein S was purified from plasma-derived protein S-C4BP complex essentially as described by Koenen et al³¹. Reversed phase high-performance liquid chromatography (HPLC) of the purified complex resulted in a complete separation of C4BP and protein S in a linear gradient of acetonitrile in water/0.1% trifluoroacetic acid (TFA)³¹. Neutralization of pH of the protein S-containing fractions caused precipitation of the protein. After centrifugation, the precipitate was resolved in 0.1 M Tris-HCl, pH 8.0, containing 6 M guanidine-HCl and the solution were dialyzed against HBS.

Stability and C4BP binding of protein S monomers and multimers

Protein S monomers and multimers, present in a purified protein S preparation, were separated by centrifugation of a mixture of protein S and liposomes in HBS containing 5

mg/mL BSA as described previously²⁵. Subsequently, the formation and/or stability of protein S multimers and monomers were tested by incubating 1) protein S multimers (75 μ L of 40 nM) with 10 mM CHAPS for 1 h at 37 °C (total volume of 100 μ L) and subsequent dialysis against HBS; 2) protein S monomers (20 μ L of 800 nM) with 50 μ L potassium thiocyanate (4 M KSCN, pH 7.4) for 15 min at RT, and dialysis (twice) against 1 L HBS containing 10 mM EDTA for 1 h at RT; 3) protein S monomers (15 μ L of 800 nM) with 30 μ L 0.1 M glycine pH 2.7 for 20 min at RT and subsequent neutralization of the pH to ~7.5 by addition of 3 μ L 1 M Tris-HCl pH 8.8; and 4) protein S monomers (15 μ L of 800 nM) with 33 μ L glycine/Tris-HCl pH ~7.5 for 20 min at RT (control). Complex formation of C4BP with protein S was achieved by incubating 220 nM protein S with an excess of C4BP (290 nM) in HBS containing 5 mg/mL BSA and 0.5 mM CaCl_2 for 30 min at RT.

Removal of protein S multimers using size-exclusion chromatography

Small unilamellar 20/80 DOPC/DOPE vesicles were prepared as described previously³⁰. A 4 mL aliquot of a 10 mM vesicle solution in HBS containing 3 mM CaCl_2 was applied to a HiLoad 26/60 Superdex 200 column and eluted with HBS containing 3 mM CaCl_2 at a flow rate of 1 mL/min. Four fractions of ~2 mL from the void volume were collected and incubated with purified protein S for 3 h at RT and the solution was gently mixed occasionally. Final concentrations were 19 μ M protein S and ~0.9 mM phospholipid vesicles in HBS with 3 mM CaCl_2 . Subsequently, 8.75 mL of the protein S/phospholipid mixture was loaded on the Superdex size-exclusion chromatography column and eluted with HBS containing 3 mM CaCl_2 at a flow rate of 1 mL/min. Fractions of 1 mL were collected and protein S concentration was determined by measuring A_{280} . Then, 0.02% NaN_3 was added to all fractions. Immediately after size-exclusion chromatography, the fraction with the highest concentration of protein S was analyzed for the presence of multimers. Protein adsorption on a phospholipid bilayer was quantified by ellipsometry as described in a previous report²⁵. This fraction was stored for several weeks at 4°C and occasionally tested in the ellipsometer. The remaining fractions of the size-exclusion column which contained high protein S concentrations were pooled. Aliquots (1 mL) of the pooled fractions (600 μ g/mL protein S) were incubated with BSA (5 mg/mL) and/or ammonium sulfate (70%) and stored at 4 °C. Diluted aliquots of the protein S pool (75 μ g/mL protein S) were incubated in the same way. After 48 h, the ammonium sulfate pellet was resuspended, and all preparations were analyzed for the presence of multimers by ellipsometry.

Ellipsometric determination of protein S adsorption to lipid bilayers

Protein adsorption to planar bilayers was measured by ellipsometry³². Briefly, planar bilayers deposited on silicon slides were prepared as described previously³³ by immersion of the hydrophilic silicon slide for 5 min in a stirred suspension of small unilamellar lipid vesicles (30 μ M) in binding buffer (50 mM Tris, 120 mM NaCl, 3 mM CaCl_2 , pH 7.5). The instrument and data analysis have been described earlier³⁴.

Measurements were performed using silicon slides at an angle of incidence to the light beam (HeNe Laser) of 68 °. Experiments were performed in binding buffer containing 0.5 mg/mL BSA at RT (20-22 °C) with continuous stirring in a trapezoidal cuvette.

Influence of phospholipid concentration on anticoagulant activity of protein S

The inhibition of prothrombin activation by protein S was measured in a prothrombinase assay. Factor Va, factor Xa, phospholipid vesicles (20/60/20 DOPS/DOPC/DOPE) and protein S were incubated for 15 min at 37°C in HBS containing 3 mM CaCl₂ and 5 mg/mL BSA. Prothrombin was added and, after various time intervals, aliquots from the activation mixture were diluted in ice-cold Tris-buffered saline (25 mM Tris-HCl, 175 mM NaCl, pH 7.9) containing 20 mM EDTA and 0.5 mg/mL ovalbumin. Thrombin concentrations were determined with chromogenic substrate S2238. Final concentrations in the prothrombin activation mixture were 10 pM FVa, 10 pM FXa, 0.1-50 µM phospholipid vesicles, 1500 nM prothrombin and 0 or 100 nM protein S.

Results

Standard protein S purification

In a previous study, we observed that purified human protein S preparations contain a small fraction (<5%) of multimeric protein S forms²⁵. However, no multimers could be identified in plasma, suggesting that multimerization occurs during purification of protein S from plasma or plasma concentrates. This hypothesis was tested by following the protein S multimerization process on native PAGE and Western blot through the full purification procedure of protein S from plasma. The starting material – pooled freshly frozen plasma from healthy donors – did not contain any detectable multimeric form of protein S (Fig. 4.1, lane a). Two protein S bands were visible, a band with a low mobility at the top of the gel representing protein S in complex with C4BP, and a higher mobility band representing monomeric, free protein S. The presence of abundant plasma proteins (e.g. albumin) likely hindered the electrophoretic migration of the free protein S monomers. Subsequently, vitamin K-dependent plasma proteins were isolated from

Figure 4.1 Protein S multimerization during purification. Protein S (250 ng) obtained at different stages of purification was subjected to native PAGE and transferred to a PVDF membrane and visualized with peroxidase-conjugated polyclonal antibodies against protein S. Freshly frozen pooled human plasma (lane a); protein S after barium citrate precipitation and ammonium sulfate fractionation (lane b); pool of protein S-containing fractions from the QAE Sephadex column (lane c); protein S-containing fractions from the QAE Sephadex column after ammonium sulfate fractionation (lane d); pool of protein S-containing fractions from the Blue Sepharose column (lane e); pool of protein S-containing fractions after Q Sepharose fast flow chromatography (lane f). For further details see Experimental procedures.

plasma by barium citrate precipitation, and further fractionated by ammonium sulfate precipitation. No protein S multimerization was observed after these purification steps as shown on native PAGE and Western blot (Fig. 4.1, lane b). The protein concentrate was subjected to anion-exchange chromatography (QAE Sephadex) and protein S was eluted with 250-300 mM NaCl in a linear NaCl gradient. During the anion-exchange purification step protein S multimers were generated (Fig. 4.1, lane c). Fractions containing protein S were pooled and subsequent precipitation (70% ammonium sulfate) followed by dialysis did not alter the relative amount of multimers in the protein S concentrate (Fig. 4.1, lane d). Blue Sepharose chromatography was used to further purify protein S and, under the conditions used, protein S was not retained by the column and was eluted in the void volume. No increase in the amount of protein S multimers was observed after Blue Sepharose chromatography (Fig. 4.1, lane e). The final purification step was anion-exchange chromatography on Q Sepharose and elution of protein S was based on Ca^{2+} -dependent conformational change of vitamin K-dependent proteins. Protein S-containing fractions were pooled and dialyzed against HBS and the protein S preparation was analyzed on native PAGE and Western blot (Fig. 4.1, lane f). An increase in protein S multimerization was observed after this final anion-exchange purification step. Based on a concentration determination by ELISA, all lanes of the native PAGE/Western blot shown in Fig. 4.1 contained equal amounts of protein S. The staining intensities of the various protein bands on the blot indicate that multimer formation occurred during the first anion-exchange QAE Sephadex step and increased after the second Q Sepharose Fast Flow anion-exchange step. Barium citrate precipitation, ammonium sulfate precipitation, and Blue Sepharose chromatography did not induce multimerization of protein S.

Immunoaffinity purification of protein S

As an alternative to ion-exchange chromatography, protein S can be purified by immunoaffinity chromatography using Sepharose-bound polyclonal antibodies against protein S. Elution of protein S from the antibody column requires stringent conditions such as 3 M KSCN at pH 7.4 or 0.1 M glycine at pH 2.7. To study the effect of high concentrations of chaotropic reagents or low pH on the multimerization of protein S,

Figure 4.2 Protein S multimerization by affinity chromatography elution conditions. Isolated protein S monomers were treated (a) with HBS for 20 min at RT; (b) with 4 M KSCN, pH 7.4, for 15 min at RT, then dialyzed against HBS containing 10 mM EDTA; (c) with 0.1 M glycine, pH 2.7, for 20 min at RT, then pH was restored to ~7.5 with 1 M Tris-HCl, pH 8.8; (d) with 0.1 M glycine, pH ~7.5, for 20 min at RT. After incubation, free CaCl_2 was complexed by the addition of HBS containing 10 mM EDTA (a, c, d). Protein S (300 ng) was subjected to native PAGE immunoblot and protein S was visualized with peroxidase-conjugated polyclonal antibodies against protein S. For further details see Experimental procedures.

purified protein S monomers (Fig. 4.2, lane a) were subjected to the following treatments: (1) protein S was incubated with 3M KSCN at pH 7.4 for 20 min at RT and dialyzed against HBS (Fig. 4.2, lane b); (2) protein S was brought to low pH by the addition of 0.1 M glycine, pH 2.7, and after incubation at RT for 20 min the mixture was brought to neutral pH with 1 M Tris buffer pH 8.8 (Fig. 4.2, lane c); (3) protein S was treated with 0.1 M glycine at neutral pH 7.5 (control, Fig. 4.2, lane d). When protein S was treated with the chaotropic agent KSCN or with glycine at low pH, multimers were readily generated (Fig. 4.2). The presence of glycine at neutral pH does not induce multimerization of protein S. Considering the above, it would be virtually impossible to purify protein S from plasma by classic ion-exchange chromatography or by affinity chromatography without generation of protein S multimers.

Reversing protein S multimerization by detergent treatment

As purification of monomeric protein S from plasma was unsuccessful, an attempt was made to dissociate the protein S multimers present in a purified protein S preparation by the addition of detergent. Protein S multimers are not SDS resistant and when isolated protein S multimers (Fig. 4.3, lane a) were applied to SDS-PAGE and Western blot analysis, a single protein S band was observed (Fig. 4.3, lane c), indicating that the interaction between protein S molecules that leads to multimer formation is non-covalent. Because detergent removal would be necessary for subsequent functional studies with purified protein S, zwitterionic CHAPS was used instead of anionic SDS because of the difficulties of removing SDS by dialysis. Isolated protein S multimers were incubated with CHAPS for 1 h and then dialyzed against HBS. Native PAGE and Western blot analysis showed that only some of the protein S multimers could be reverted to monomers and the majority of multimers remained (Fig. 4.3, lane b).

Separation of protein S multimers and monomers by size-exclusion chromatography

As conversion of protein S multimers into monomers by detergent treatment was unsuccessful, an attempt was made to remove protein S multimers from a protein S

Figure 4.3 Dissociation of protein S multimers. Isolated protein S multimers were incubated without (a) or with (b) 10 mM CHAPS for 1 h at 37 °C and then dialyzed against HBS. Protein S (30 ng) was subjected to native PAGE and transferred to a PVDF membrane by Western blotting. Lane c represents Western blotting of 30 ng protein S multimers (untreated) subjected to SDS-PAGE. Protein S was visualized using peroxidase-conjugated polyclonal antibodies against protein S. * For reasons of clarity, protein S monomer bands were aligned, as mobility in native- and SDS-PAGE can not be compared.

preparation by size-exclusion chromatography on Superose 6. Protein S multimers elute slightly earlier from the Superose 6 column than protein S monomers. Although protein S multimers were enriched in the early eluting fractions, no clear separation from monomers could be achieved²⁵.

In the next step, the high affinity of protein S multimers for anionic phospholipids was used to separate protein S monomers and multimers. A purified protein S preparation containing monomers and multimers was incubated for 3 h with small unilamellar phospholipid vesicles (20/80 DOPS/DOPC) in CaCl_2 -containing buffer under conditions where protein S multimers, but not monomers, quantitatively bind to the vesicles. Subsequently, the protein S/phospholipid mixture was loaded onto a size-exclusion chromatography column (HiLoad 26/60 Superdex 200). Absorbance measurements at 280 nm showed that 2 separate peaks eluted from the column (Fig. 4.4A). The phospholipid vesicles eluted in the void volume peak 1 (~110 mL) and caused a high absorbance at 280 nm likely due to light scattering by the vesicles (Fig. 4.4A). The protein S concentration in the column fractions was determined by ELISA. Less than 10% of the total protein S applied to the column was bound to the vesicles as multimers. The majority of protein S was retarded on the column and eluted in a symmetrical peak at ~180 mL. This peak only contained protein S monomers and 1 h after size-exclusion chromatography, the protein S peak 2 fraction (0.6 mg/mL) was tested for phospholipid binding in an ellipsometer and compared with the starting material.

Protein S adsorption ($\mu\text{g}/\text{cm}^2$) to a planar phospholipid bilayer (20/80 DOPS/DOPC) was followed over time at a final concentration of 25 nM protein S (Fig. 4.4B) using ellipsometry essentially as described²⁵. The starting material, which was a mixture of protein S monomers and multimers, showed the characteristic biphasic adsorption as observed before²⁵ (Fig. 4.4B, curve a). The abundant protein S monomers in the preparation (~95%) with a low affinity for phospholipids resulted in a fast, initial adsorption phase, which was completed within 50 s after addition of protein S to the ellipsometer cuvette. Following the initial adsorption of the protein S monomers, the fraction of protein S multimers (~5%) with a high affinity for phospholipids resulted in a second slow continuing adsorption of protein S to the phospholipid bilayer. During this second phase, the protein S multimers with a high affinity for phospholipids ($K_d < 1$ nM) quantitatively replaced the low-affinity protein S monomers ($K_d = \sim 250$ nM)²⁵. This was illustrated by the observation that almost no protein S multimers dissociated from the lipid bilayer when the non-bound protein S was removed by flushing the cuvette with buffer containing CaCl_2 (at ~3500 s). Binding of protein S multimers was Ca^{2+} -dependent as addition of EDTA resulted in complete desorption (at ~3700 s).

Adsorption of protein S from the second peak of the Superdex chromatography run only showed the fast, initial adsorption phase that was reversible upon flushing with buffer containing CaCl_2 at ~1800 s (Fig. 4.4B). This confirmed that the second protein S peak from the Superdex column was essentially free of protein S multimers and only contained protein S monomers. When, after 1 week, the monomeric protein S was analyzed on native PAGE and Western blot, traces of multimers were observed (not

Figure 4.4 Separation of protein S multimers and monomers by size-exclusion chromatography. Purified protein S (19 μM) was incubated with 0.9 mM small unilamellar phospholipid vesicles (20/80 DOPS/DOPC) in HBS containing 3 mM CaCl₂ for 3 h at RT. After incubation, the mixture was applied to a HiLoad 26/60 Superdex 200 column, which was equilibrated with HBS containing 3 mM CaCl₂. **(A)** The absorption profile at 280 nm (dashed line) and the protein S concentrations in the column fractions determined by ELISA (solid line). **(B)** Adsorption of protein S on a DOPS/DOPC phospholipid bilayer (20 mol% DOPS) was quantified using ellipsometry. Protein S (25 nM) was added to the ellipsometer cuvette at ~200 s and the mass of protein adsorbed to the lipid bilayer (Γ) was followed in time. The protein S starting preparation (a) and the Superdex fraction 2 (b) are shown. After binding, the cuvette was flushed with HBS containing CaCl₂ (open arrowheads) and HBS containing EDTA (filled arrowheads) **(C)** Fraction 2 was stored at 4°C and analyzed by ellipsometry at 1 h (a), 1 week (b), 2 weeks (c) and 5 weeks (d) after size-exclusion chromatography. Ellipsometry experiments were performed at RT in a buffer (50 mM Tris-HCl, 100 mM NaCl, 3 mM CaCl₂, pH 7.5) containing 5 mg/mL BSA.

shown), which raised the question of whether multimer formation occurred spontaneously within purified (monomeric) protein S preparations.

In an additional Superdex chromatography experiment, the fraction containing monomeric protein S (peak 2) was kept at 4°C and tested in the ellipsometer after 1, 2 and 5 weeks of storage (Fig. 4.4C). New formation of multimeric protein S forms was clearly observed. The phospholipid-binding rate of protein S on the phospholipid bilayer during the second slow adsorption phase increased with storage time, indicating that there was a time-dependent increase in the formation of protein S multimers (Fig. 4.4C). In addition, the amount of bound protein S that was removed upon washing with CaCl₂-

containing buffer decreased with storage time, confirming the time-dependent formation of protein S multimers. Calculation of the initial adsorption slope of the second slow phase of binding showed that, after 1, 2, and 5 weeks of storage, the protein S preparation contained 9%, 17%, and 25%, respectively, of the amount of protein S multimers present in the starting protein S preparation (Fig. 4.4B).

In an attempt to prevent generation of protein S multimers in the purified protein S monomer preparation, freshly isolated monomeric protein S (600 µg/mL) was stored in a buffer containing BSA (0.5 %), precipitated with ammonium sulfate (70%), or both. Under all conditions, protein S multimers were formed (not shown). In addition, dilution of monomeric protein S fractions to 75 µg/mL prior to addition of BSA and/or ammonium sulfate still resulted in multimer formation. Therefore, we were not able to obtain a purified protein S preparation devoid of multimeric forms that could be used for structure-function analysis, unless used immediately (<1 day) after size-exclusion chromatography in the presence of small unilamellar phospholipid vesicles as described above.

Inhibition of prothrombin activation by protein S at various concentrations of phospholipid

Protein S multimers bind with high affinity to anionic phospholipids ($K_d < 1$ nM) and effectively inhibit phospholipid-dependent enzyme processes at low phospholipid concentrations. Purified protein S has been reported to inhibit prothrombin activation at phospholipid concentrations < 1 µM^{17,26} and differences in the extent of inhibition between protein S preparations could be attributed to differences in the multimer content of the protein S preparations²⁵. In order to find experimental conditions under which protein S multimers cannot exert their inhibitory activity, 2 protein S preparations were tested for their ability to inhibit prothrombin activation as a function of phospholipid concentration. Protein S₁ was purified from plasma as described in the Experimental procedures section, and contained multimeric protein S forms (Fig. 4.1, lane f). Protein S₂ was derived from plasma-purified protein S-C4BP complex and separated from C4BP by reverse phase HPLC as described in the Experimental procedures section. FXa, FVa and phospholipids were pre-incubated in Ca²⁺-containing buffer for 15 min in the

Figure 4.5 Inhibition of prothrombin activation by protein S multimers at varying phospholipid concentrations. FVa, FXa and phospholipid vesicles (20/80 DOPS/DOPC) were incubated with or without protein S in HBS containing 3 mM CaCl₂ and 5 mg/mL BSA for 15 min at 37°C. Prothrombin was added and thrombin formation was followed in time as described in the Experimental procedures section. Final concentrations were 10 pM FVa, 10 pM FXa, 1.5 µM prothrombin, 0 or 100 nM protein S and 0.2-50 µM phospholipid vesicles. Inhibition of prothrombin activation by protein S₁ (●) and protein S₂ (○) is presented as a function of the phospholipid concentration.

absence or presence of 100 nM protein S (final concentration). Thrombin formation was initiated by addition of prothrombin to the reaction mixture. At 0.2 μ M phospholipid, thrombin generation was nearly completely inhibited by 100 nM protein S₁ (Fig. 4.5). Increasing the phospholipid concentration resulted in a gradual loss of the inhibitory activity of protein S₁. The protein S₂ preparation contained a higher amount of multimeric forms than protein S₁ due to the exposure of protein S to low pH during HPLC purification procedures (data not shown), and was a more effective inhibitor of thrombin formation than protein S₁ (Fig. 4.5). Half-maximal inhibition of prothrombin activation was reached at 3.2 μ M phospholipid for protein S₂ and 0.7 μ M phospholipid for protein S₁, suggesting an ~4-fold higher multimer-concentration in protein S₂. At high phospholipid concentrations (>50 μ M), both protein S preparations only slightly inhibited prothrombin activation (<10%).

C4BP-protein S complex formation

C4BP has a profound influence on protein S function^{14,20,21} and is often used to modulate protein S activity in model systems. Because of the actual risk of misinterpretation of protein S activity at low phospholipid concentrations due to the presence of protein S multimers, it is important to test whether C4BP is able to bind to protein S multimers. A protein S preparation that contained multimers (Fig. 4.6, lane a) was incubated with excess β -chain-containing C4BP in a buffer with CaCl₂. Subsequent analysis on Western blot showed a shift of the major protein S monomer band to a low mobility protein S-C4BP band on top of the gel (Fig. 4.6, lane b). No change in intensity or mobility was observed for bands corresponding to protein S multimers. When isolated protein S monomers (Fig. 4.6, lane c) were incubated with C4BP, a shift of the protein S monomer band was again observed as a result of complex formation between protein S and C4BP (Fig. 4.6, lane d), but protein S-C4BP complex formation was not accompanied by protein S multimerization.

Figure 4.6 Protein S-C4BP complex formation. Purified protein S preparations (220 nM) were incubated with or without β chain-containing C4BP (290 nM) in HEPES-buffered saline (25 mM HEPES, 175 mM NaCl, pH 7.7) containing 5 mg/mL BSA and 0.5 mM CaCl₂ for 30 min at RT and subsequently subjected to native PAGE (4-15% acrylamide), transferred to a PVDF membrane by Western blotting and visualized with peroxidase-conjugated polyclonal antibodies against protein S. Lane a: total purified protein S; lane b: total purified protein S with C4BP; lane c: isolated protein S monomers; lane d: isolated protein S monomers with C4BP.

Discussion

Purified human protein S preparations contain 2 forms of protein S: a major fraction consisting of protein S monomers and a minor fraction (<5%) of protein S multimers²⁵. These multimers are absent in plasma, implying that multimerization occurs during the purification of protein S. We have shown that protein S multimers were generated during standard anion-exchange chromatography that is used for isolation of protein S from plasma. In addition, high ionic strength as well as denaturing conditions like low pH and treatment with chaotropic reagents, used in immunoaffinity purification of protein S, induce multimer formation.

When multimers were quantitatively removed from a protein S preparation by applying a pre-incubated mixture of purified protein S and phospholipid vesicles to size-exclusion chromatography, spontaneous generation of multimers occurred in the isolated fraction of protein S monomers. This observation contradicts our earlier report, where it was suggested that a multimer-free protein S preparation was stable for several weeks²⁵. In that study, liposome centrifugation was used to separate protein S multimers bound to the liposomes from the monomers in the supernatant. We now believe that this procedure likely introduces phospholipid remnant structures into the monomeric protein S fraction, that tightly bind traces of protein S multimers. These multimers can subsequently be missed in phospholipid binding analyses using ellipsometry, and in functional protein S studies.

The question remains as to what initiates multimer formation. *In vitro* purification conditions clearly induced protein S multimerization. When protein S multimers were removed from a purified protein S preparation, they spontaneously reappeared. The presence of either BSA or ammonium sulfate did not prevent multimer formation. As no contaminants could be detected in the purified monomeric protein S preparation, the origin of multimer formation seems to lie in the monomeric protein S molecule itself. This might imply a limited proportion of protein S molecules acting as nuclei to propagate multimerization.

Partly denatured intermediates of proteins that are relatively stable may reflect the so-called "molten-globule" state³⁵. Such intermediates have the same secondary structure as the native counterpart but their amino acid side-chains are more disordered. The latter results in (partial) loss of tertiary structure and in increased hydrophobicity. Exposure of hydrophobic residues to the solvent will likely result in aggregation of proteins. Such partial unfolding can be induced in denaturing conditions like low pH or increased temperature³⁶. Loss of tertiary structure can also occur under physiologic conditions when the stabilizing environment of a protein is absent. Thus, the lack of the plasma (protein) environment may induce minor modifications in the protein S molecule resulting in (partial) aggregation of purified protein S. Interestingly, the aggregation of recombinant human factor VIII was described as originating from "a small transition in the tertiary structure of the protein which produced a nucleating species that led to the formation of inactive soluble aggregates"³⁷. The observations for factor VIII and protein

S seem to be similar and emphasize the need for prudence with the use of purified proteins in structure-function analysis.

Prevention of protein S multimerization during purification from plasma appears virtually impossible, and it is therefore likely that the presence of protein S multimers is a general characteristic of purified protein S preparations. Protein S multimers have also been observed in previous reports. In 1990, Schwalbe *et al*²⁸ reported that the binding of purified human protein S to phospholipids had a biphasic nature and that multiple protein layers were formed on the phospholipid surface. Van 't Veer *et al*²⁶ reported that the APC-independent activity of protein S increased with prolonged incubation times, which is likely a result of the time-dependent increase in adsorption of the high-affinity, low-abundance protein S multimers to the phospholipid surface. Since the first report on the anticoagulant effect of protein S in the absence of APC, up to 10-fold differences in APC-independent activity of protein S have been reported^{15,17}, and the activity of a protein S preparation has been correlated with its affinity for anionic phospholipids¹⁷. Today we know that these observations can be ascribed to variable amounts of protein S multimers present in different protein S preparations²⁵. In addition, the influence of the protein S purification procedure on the activity of a preparation has been recognized^{15,38} and it has been suggested that modifications in the protein S molecule occur during purification^{15,16}.

At low phospholipid concentrations, the multimeric protein S present in purified protein S preparations will likely inhibit all lipid-dependent coagulation processes, not only in model systems of factor X or prothrombin activation^{17,25,26}, but also in plasma reconstitution experiments¹⁷. Moreover, it is possible that other processes occurring on a negatively charged phospholipid surface will be influenced by multimers as well. For example, we have observed that protein S multimers introduce cross-linking of liposomes (unpublished observations). The possibility that this also occurs with cells expressing phosphatidylserine on their surface cannot be excluded, which might be interesting in view of the stimulatory effect of purified protein S on phagocytosis of apoptotic cells³⁹.

Multimerization not only influences the interaction of protein S with a phospholipid surface; protein S multimers no longer form a complex with C4BP. Apparently, the binding site for C4BP on the protein S molecule is altered or obstructed in protein S multimers. Moreover, multimerization of protein S is not a result of C4BP-protein S interactions. The loss of the C4BP binding site on protein S might be due to steric hindrance or a specific alteration in the protein S molecule. The presence of C4BP did not affect the ability of purified protein S to inhibit prothrombin activation in the absence of APC¹⁴. This observation can now be explained by the fact that protein S multimers present in the protein S preparations were responsible for the inhibition of prothrombin activation, because C4BP, which does not form a complex with protein S multimers, does not affect their inhibitory activity.

Protein S multimers bind tightly to phospholipids ($K_d < 1$ nM)²⁵ and therefore can effectively compete for phospholipid binding sites with prothrombin, the substrate of the prothrombinase complex, and/or interfere with the assembly of the prothrombinase

complex. Consequently, the inhibitory activity of protein S multimers on prothrombin activation can easily reach 100% at concentrations of phospholipids $<1 \mu\text{M}$ (Fig. 4.5). The use of high phospholipid concentrations is therefore a prerequisite for studying the anticoagulant activity of purified protein S in model systems. The fact that protein S monomers have a low affinity for phospholipids suggests that monomeric protein S inhibits thrombin formation via a mechanism other than competition for binding sites on the phospholipid surface. Direct interactions of protein S with FXa and FVa may cause inhibition of prothrombin activation, and it has been hypothesized that such interactions could explain the APC-independent anticoagulant activity of protein S in plasma²⁷. However, in our model system using purified components, 100 nM protein S hardly inhibited prothrombin activation at phospholipid concentrations $>50 \mu\text{M}$, whereas in plasma experiments protein S considerably inhibited thrombin formation independent of the phospholipid concentration²⁷.

The results of the present study indicate that protein S structure-function analysis in model systems using purified proteins should be performed at phospholipid concentrations $\geq 50 \mu\text{M}$ to ensure that unwanted activities of protein S multimers are avoided. Furthermore, the inhibition of prothrombin activation by protein S in the absence of APC in model systems using purified proteins at low phospholipid concentrations is non-physiologic and a result of *in vitro* purification procedures: that mechanism is different from the mechanism by which protein S inhibits thrombin generation in plasma in the absence of APC.

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Inhibition of thrombin generation by protein S at low procoagulant stimuli: implications for maintenance of the haemostatic balance

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Abstract

The APC-independent anticoagulant activity of protein S on tissue factor-induced thrombin generation was quantified in plasma. In absence of APC, protein S significantly decreased the endogenous thrombin potential (ETP) in a concentration-dependent manner. The APC-independent anticoagulant activity of protein S in plasma was not affected by phospholipid concentrations, but strongly depended on tissue factor concentrations: protein S inhibited the ETP from 6% at 140 pM tissue factor to 74% at 1.4 pM tissue factor. Plasma with both 60% protein S and 140% prothrombin showed an ETP of 240% compared to normal plasma, suggesting an APC-independent protective role of protein S in the development of thrombosis as a result of protein S-deficiency and the prothrombin-G20210A mutation. At high tissue factor concentrations, protein S hardly expressed APC-independent anticoagulant activity but exerted potent APC-cofactor activity when thrombomodulin or APC were added to plasma. Neutralization of protein S under these conditions resulted in a 20-fold reduction of the anticoagulant activity of APC. The present study shows that protein S effectively regulates coagulation at two levels: at low procoagulant stimuli protein S *maintains* the haemostatic balance by directly inhibiting thrombin formation, and at high procoagulant stimuli protein S *restores* the haemostatic balance via its APC-cofactor activity.

Introduction

Human protein S was first described by DiScipio et al^{1,2} as a vitamin K-dependent glycoprotein that circulates in plasma. Protein S down-regulates blood coagulation via different mechanisms. Protein S is a non-enzymatic cofactor for activated protein C (APC) in the anticoagulant protein C pathway^{3,4} in which it accelerates the APC-mediated inactivation of factor Va^{5,6} and factor VIIIa^{5,7}. Protein S also expresses APC-independent anticoagulant activity by directly inhibiting prothrombin activation, a phenomenon that was observed both in purified systems⁸⁻¹⁰ and in plasma^{9,11}. Identification of interaction sites between protein S and factor Va¹² and Xa¹³ support a direct effect of protein S on prothrombin activation. Binding of protein S to negatively charged phospholipid surfaces is a prerequisite for protein S activity as antibodies directed against the phospholipid-binding Gla-module of protein S completely abolish the anticoagulant activity of protein S both in the absence and in the presence of APC^{9,14,15}. On the basis of these experiments, it was proposed that competition for phospholipid binding sites, not a direct interaction with factor Va and factor Xa, is the main mechanism by which protein S inhibits prothrombin activation in the absence of APC^{16,17}. However, this hypothesis was based on experiments performed with purified protein S preparations that contain *in vitro* generated multimeric forms of protein S¹⁸. Due to high phospholipid-binding affinity ($K_d < 1$ nM), protein S multimers inhibit prothrombin activation at limiting phospholipid concentrations by occupying the phospholipid surface. In plasma, however, protein S multimers are absent, and native protein S is unlikely to effectively shield phospholipid surfaces due to its low affinity for phospholipids ($K_d \sim 250$ nM)¹⁸.

Protein S deficiency has been shown to be a risk factor of venous thrombosis^{19,20}. Since then, several reports confirmed the importance of protein S as a cofactor for APC in regulating thrombin formation. There are no reports yet that demonstrate the physiologic importance of an impaired activity of protein S in the absence of APC.

Protein S has been shown to increase the clotting time in plasma stimulated with both tissue factor and factor Xa in the absence of APC^{9,21,22}. This suggests that protein S inhibits the initiation of coagulation, but it is not clear to what extent protein S can control the propagation and/or termination phase. Thrombin generation measurements provide a useful tool to study coagulation as it allows quantification of the total amount of active thrombin generated during the coagulation processes (the endogenous thrombin potential: ETP).

In the present investigation we quantified the anticoagulant activity of native protein S in plasma. We examined the ability of protein S to inhibit thrombin formation and we also examined how the inhibitory effect of protein S depends on the phospholipid concentration and procoagulant stimulus. In addition, we studied the effect of protein S on thrombin generation in a plasma system mimicking two conditions that affect the anticoagulant activity of protein S in the absence of APC: heterozygous protein S deficiency and the prothrombin G20210A polymorphism.

Materials and methods

Materials

Hepes-buffer was obtained from Sigma (St Louis, MA), bovine serum albumin (BSA) from ICN (Aurora, Ohio). Fluorogenic substrate Z-Gly-Gly-Arg-AMC.HCl (I-1140) was from Bachem (Switzerland). Recombinant tissue factor (thromboplastin) was from Dade Innovin (Dade Behring, Marburg, Germany). 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-Dioleoyl-*sn*-glycero-3-phosphoserine (DOPS) and 1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) were obtained from Avanti Polar Lipids (Alabaster, AL). Phospholipid vesicles (20% DOPS, 20% DOPE, 60% DOPC) were prepared as described previously²³. Polyclonal anti-protein S (4.1 mg/ml, 27.3 μ M) and anti-protein C (3.7 mg/ml, 24.7 μ M) antibodies and normal rabbit serum (~2 mg IgG per ml) were obtained from DAKO (Glostrup, Sweden). The monoclonal antibody CLB-PS13, which is directed against the Gla-domain of protein S, was a kind gift from Dr. Jan van Mourik, Sanquin Research, Central Laboratory of the Blood Transfusion Service (CLB), Amsterdam, The Netherlands. Protein S-depleted plasma was obtained from Affinity Biologicals (Hamilton, Ontario, Canada). Human prothrombin was purified as described²⁴, human factor Xa and APC were obtained from Enzyme Research Laboratories (South Bend, IN). Recombinant human thrombomodulin (TM) was a kind gift of Synapse BV (Maastricht, The Netherlands).

Normal pooled plasma

Blood was drawn from 74 healthy volunteers (45 men, 29 non-pregnant women not using oral contraceptives; mean age, 40.0; SD 8.9) on 3.8% citrate. The first few milliliters of blood were discarded. The residual volume was centrifuged for 15 minutes at 3000g, resulting in platelet-rich plasma, which was centrifuged again for 10 minutes at 11000g. The platelet poor-plasmas were pooled and snap frozen.

Measurement of thrombin generation

Tissue factor and phospholipid vesicles were incubated at 6.25 times the final concentration for at least 1 hr at 37°C in Hepes buffered saline (HBS: 25 mM Hepes, 175 mM NaCl, pH 7.7) containing 5 mg/ml BSA (HBS-BSA) and 2 mM CaCl_2 . 1 ml of this tissue factor/phospholipid/ CaCl_2 mixture was mixed with 0.25 ml HBS-BSA containing 400 mM CaCl_2 , right before use. Plasma was incubated with antibodies directed against (activated) protein C (1.23 μM IgG) and with or without antibodies directed against protein S (2.73 μM IgG) for 15 min at 37°C. Subsequently, 80 μl plasma samples were transferred into a microtiterplate at 37°C, 20 μl of a prewarmed fluorogenic substrate solution (1875 μM I-1140 in HBS-BSA) was added and coagulation was started by addition of 25 μl of a prewarmed phospholipid/tissue factor/ CaCl_2 mixture. Thrombin generation was followed continuously in time using a microtiterplate SPECTRAMax GEMINI XS fluorometer (Molecular Devices Corporation, Sunnyvale, CA) with excitation and emission wavelengths set at 368 and 460 nm, respectively.

To study the contribution of the APC-cofactor activity of protein S to the inhibition of thrombin generation, APC was included in the phospholipid/tissue factor/ CaCl_2 mixture (see previous paragraph) to yield a final APC concentration in plasma of 7.5 nM. In an additional experiment, purified thrombomodulin was added to plasma before coagulation was initiated. In these experiments thrombin generation was followed continuously in time by measuring fluorogenic substrate conversion in a microtiterplate Fluorskan Ascent fluorometer (Thermo Labsystems, Helsinki, Finland).

A calibrator was used to allow calculation of the amount of thrombin formed and correction of the obtained relative fluorescence units for inner-filter effects and fluorogenic substrate consumption²⁵. In addition, the contribution to substrate conversion by thrombin- α_2 -macroglobulin complexes was subtracted. Finally, the first derivative of the data was taken that yielded the thrombin generation curve, allowing calculation of the total area under the curve, the endogenous thrombin potential (ETP). In experiments (Fig. 5.1 inset, 4, 6 and 7) in which the Fluorskan Ascent fluorometer was used, these corrections were performed automatically by means of the calibrated automated thrombogram (CAT) computer software²⁵ provided by Synapse BV (Maastricht, The Netherlands). In all other experiments thrombin generation curves and ETP were obtained after manual correction.

Results

APC-independent anticoagulant activity of protein S

In order to investigate the APC-independent anticoagulant activity of protein S on thrombin generation in plasma, it had to be excluded that protein S contributes to the down-regulation of thrombin formation via its ability to act as a cofactor of APC. Because plasma may contain trace amounts of APC²⁶ and since the high concentrations of thrombin that are formed during the clotting of plasma may activate protein C, APC-enzymatic activity was blocked by inhibitory antibodies against (activated) protein C.

Normal pooled plasma was incubated with polyclonal antibodies against protein C for 15 min at 37 °C. As a reference, an identical incubation was performed in which the antiserum was replaced by HBS-BSA. Subsequently the thrombin-specific fluorogenic substrate I-1140 was added to plasma and coagulation was started by addition of a mixture of tissue factor, phospholipids and CaCl₂, resulting in final concentrations of 3.5 pM tissue factor and 10 µM phospholipid vesicles. Figure 5.1 demonstrates that the addition of antibodies against protein C caused a moderate increase in thrombin generation compared to control plasma in which buffer was added instead of antibody, illustrating the presence of APC during the thrombin generation process. However, from this experiment it could not be concluded that APC was fully inhibited by the antibodies. Therefore, 5 nM APC was added to normal pooled plasma in the presence or absence of antibodies against protein C. In the absence of antibodies against protein C, APC drastically reduced thrombin generation, whereas in the presence of antibodies the

Figure 5.1 The influence of APC and protein S on thrombin generation in plasma. Thrombin generation was initiated with 3.5 pM tissue factor, 10 µM phospholipid vesicles (20/60/20 DOPS/DOPC/DOPE) and 16 mM CaCl₂ (final concentrations) in normal pooled plasma in the absence of APC and antibodies against protein C and protein S (open circles) or in the presence of antibodies against protein C (filled circles), antibodies against both protein C and protein S (filled squares), 5 nM APC (filled triangles) or 5 nM APC and antibodies against protein C (open triangles). Thrombin generation was quantified with the fluorogenic substrate I-1140 as described in 'Materials and methods'. **Inset:** Thrombin generation was initiated under the same conditions in plasma in the presence of antibodies against protein C without (filled circles) or with (filled squares) monoclonal antibodies CLB-PS13 against the Gla-domain of protein S.

inhibitory effect of APC was completely abolished (Fig. 5.1). The fact that thrombin generation curves measured in normal pooled plasma with or without added APC were identical when antibodies against protein C were present (Fig. 5.1, open triangles and filled circles, respectively) and that the antibodies did not affect thrombin generation in protein C deficient plasma (data not shown) demonstrates that the protein C antibodies specifically and fully inhibited APC in plasma. Although the presence of APC and/or *in situ* activation of protein C in plasma had a moderate effect on thrombin generation, it was decided to add antibodies against protein C to plasma in all further experiments.

Subsequently, in the absence of APC, inhibition of protein S in plasma by addition of polyclonal antibodies against protein S resulted in a large increase in thrombin generation (Fig. 5.1). Both the lag time and the time required to reach the thrombin peak were shorter, and the ETP was increased to 156% when compared to plasma in which protein S was not inhibited by antibodies. In order to exclude non-specific effects of the protein S and protein C antibodies on thrombin generation, an experiment was performed in which the antibodies were replaced by an equal amount of rabbit IgG. The fact that rabbit IgG had no effect on thrombin generation in normal pooled plasma and that the addition of the polyclonal antibodies against protein S and protein C did not affect thrombin generation in protein S- and protein C-depleted plasma, respectively, indicates that the increase in thrombin generation upon addition of antibodies to plasma is due to specific depletion of the protein of interest.

The use of the monoclonal antibody CLB-PS13 confirmed that protein S directly inhibits thrombin generation in plasma. The epitope recognized by CLB-PS13 is located in the Gla-domain of protein S. Binding of this monoclonal antibody to protein S prevents interaction of protein S with negatively charged phospholipid surfaces and abolishes both the APC-cofactor and APC-independent activity of protein S^{15,17}. The inset in Fig. 5.1 shows that, like the polyclonal antibody, CLB-PS13 significantly increased thrombin generation.

Since many vitamin K-dependent proteins undergo conformational changes upon ligating calcium ions^{27,28} and thrombin generation in our experiments was initiated by the addition of calcium ions, we tested whether rate-limiting Ca^{2+} -dependent conformational changes might affect the direct regulation of thrombin generation by protein S. Therefore, plasma was incubated with CaCl_2 (16 mM final concentration) for 15 minutes at which, under the conditions used, no clotting occurred yet. Subsequently, tissue factor and phospholipids were added. It appeared that, except for a slight decrease in lag time, preincubation of plasma with calcium ions had no effect on thrombin generation and the ETP, either in the absence or presence of antibodies against protein S (data not shown).

Influence of protein S concentration on thrombin generation

In the previous experiment it was shown that protein S suppresses thrombin generation in plasma in the absence of APC. However, the amount of antibodies against protein S added to plasma resulted in complete neutralization of protein S in the plasma studied. Because homozygous protein S deficiencies are rare in nature and

Figure 5.2 The effect of varying protein S levels on thrombin generation in plasma in the absence of APC. **A:** Normal pooled plasma was incubated with antibodies against protein C and 2.73 μM (triangles up), 1.37 μM (triangles down), 0.68 μM (squares), 0.34 μM (diamonds), 0.17 μM (open circles) or no (filled circles) antibodies directed against protein S. **A inset:** ETP as function of the concentration antibodies against protein S. **B:** Protein S-depleted plasma was mixed with varying amounts of normal pooled plasma resulting in protein S levels of 0% (triangles up), 40% (triangles down), 55% (squares), 70% (diamonds), 85% (open circles) and 100% (filled circles). **B inset:** ETP as function of the protein S concentration. All plasma mixtures contained antibodies against protein C. Thrombin generation was initiated with 3.5 pM tissue factor, 10 μM phospholipid vesicles (20/60/20 DOPS/DOPC/DOPE) and 16 mM CaCl_2 (final concentrations) and quantified with the fluorogenic substrate I-1140 as described in 'Materials and methods'.

heterozygous protein S deficiencies are more common in populations presenting with thrombotic events, the dose-dependent effect of protein S on thrombin generation was studied. The protein S concentration in plasma was varied in two ways: (1) by adding increasing amounts of polyclonal antibodies against protein S to normal pooled plasma, or (2) by mixing protein S-depleted plasma with normal pooled plasma.

Polyclonal antibodies against protein S were added to normal pooled plasma resulting in final IgG concentrations ranging from 0 to 2.73 μM and the plasma/antibody mixtures were incubated at 37°C for 15 min. Coagulation was initiated as described, resulting in final concentrations of 3.5 pM tissue factor and 10 μM phospholipids. A clear increase in thrombin generation was observed at increasing concentrations of antibodies against protein S (Fig. 5.2A). Compared to thrombin generation in normal plasma that did not contain antibodies (ETP = 100%), neutralization of the APC-independent protein S activity in plasma by increasing the antibody concentration gradually increased the ETP to 161% at 2.73 μM IgG (inset, Fig. 5.2A). Concentrations of more than 2.73 μM did not further increase the ETP (data not shown). In addition, the time required to the onset of thrombin generation, which coincides with the clotting of plasma, increased with increasing protein S concentration (Fig. 5.2A).

In a separate experiment, the amount of protein S present in plasma was varied by mixing normal pooled plasma with protein S-depleted plasma. Varying the plasma

Figure 5.3 The effect of varying concentrations of tissue factor on thrombin generation in plasma in the absence of APC and presence or absence of protein S. Thrombin generation was initiated in normal pooled plasma with indicated concentrations of tissue factor, 10 μ M phospholipid vesicles (20/60/20 DOPS/DOPC/DOPE) and 16 mM CaCl_2 (final concentrations) in the presence (filled circles) or absence (open circles) of antibodies against protein S. **Inset:** Inhibition of the ETP by protein S as function of the tissue factor concentration. All plasma mixtures contained antibodies against protein C. Thrombin generation was quantified with the fluorogenic substrate I-1140 as described in 'Materials and methods'.

protein S concentration from 100% to 0% resulted in a protein S-dependent effect on thrombin generation in which the lag time decreased (Fig. 5.2B) and the ETP gradually increased from 100 % in normal plasma (100% protein S) to 160 % in protein S-depleted plasma (0% protein S) (inset, Fig. 5.2B).

APC-independent activity of protein S depends on the concentration of the procoagulant stimulus

In the previous experiments, coagulation was started with 3.5 pM tissue factor, at which the anticoagulant effect of protein S was clearly visible. To investigate the effect of the strength of the procoagulant stimulus on the inhibition of thrombin generation by protein S, coagulation was initiated with varying concentrations of tissue factor (1.4 pM to 140 pM) at a constant phospholipid concentration (10 μ M). In the absence of antibody, *i.e.* in the presence of protein S, a clear dose-dependent effect of the tissue factor concentration on thrombin generation was observed (Fig. 5.3, open symbols). At 140 pM tissue factor, after a short lag time a large amount of thrombin was formed. Lowering the tissue factor concentration resulted in a dose-dependent increase of the lag time and decrease of both the height of the thrombin generation peak and the ETP.

The effect of protein S on thrombin generation varied greatly with the tissue factor concentration. Neutralization of protein S with antibodies (Fig. 5.3, filled symbols) had virtually no effect on the lag time and the ETP at high tissue factor concentrations (140 pM and 14 pM). At these tissue factor concentrations protein S inhibited thrombin generation ~6%. The effect of protein S became more pronounced when the tissue factor concentration was further decreased. At 7 pM and 3.5 pM tissue factor, protein S neutralization resulted in an increase of the ETP to 121% and 143% of the ETP determined in the absence of antibody, respectively (inset, Fig. 5.3). At the lowest tissue

factor concentration (1.4 pM), the presence of protein S antibodies increased the ETP to a vast 380% (*i.e.* protein S inhibits thrombin generation ~74%) and considerably reduced the lag time compared to plasma that did not contain protein S antibodies.

The effect of phospholipid concentration on the APC-independent anticoagulant activity of protein S

Binding of protein S to an anionic lipid surface is essential for the expression of its anticoagulant activity^{9,29}. Moreover, it has been speculated that the mechanism underlying the APC-independent anticoagulant activity of protein S is competition for phospholipid binding sites^{16,17}. According to this hypothesis, the inhibitory effect of protein S on thrombin generation should be maximal at low phospholipid concentration and decrease at increasing concentrations of phospholipids.

To investigate the effect of the phospholipid concentration on the APC-independent anticoagulant activity of protein S, thrombin generation was measured in normal pooled plasma with and without antibodies against protein S, in which clotting was initiated with a fixed amount of tissue factor (3.5 pM) at varying phospholipid concentrations (1-100 μ M). Figure 5.4A demonstrates that the anticoagulant effect of protein S in the absence of APC was virtually independent of the phospholipid concentration. Calculation of the ETP of plasmas with and without protein S showed no substantial variation in the ability of protein S to inhibit thrombin generation at varying amounts of phospholipids.

The same results were obtained when a lower tissue factor concentration (1.4 pM) was used as procoagulant stimulus. Although the overall thrombin generation was lower and the inhibitory activity of protein S was more pronounced, there was also no influence

Figure 5.4 The effect of varying phospholipid concentrations on thrombin generation in plasma. Thrombin generation was initiated in normal pooled plasma with 3.5 pM tissue factor (A) or 250 pM factor Xa (B), 16 mM CaCl_2 and indicated concentrations of phospholipid vesicles (20/60/20 DOPS/DOPC/DOPE; 100, 25, 5 or 1 μ M final concentrations) in the presence (filled circles) or absence (open circles) of antibodies against protein S. All plasma mixtures contained antibodies against protein C. Thrombin generation was quantified with the fluorogenic substrate I-1140 as described in 'Materials and methods'.

of the amount of phospholipids on the APC-independent anticoagulant activity of protein S at a low tissue factor concentration (data not shown).

To address the question whether the APC-independent anticoagulant effect of protein S depends on the nature of the procoagulant trigger, tissue factor was replaced by factor Xa to initiate thrombin generation. When 250 pM factor Xa was added to plasma, thrombin formation started immediately while the amount of thrombin formed was lower compared to the tissue factor-initiated thrombin generation (Fig. 5.4B). Nevertheless, as for tissue factor-initiated coagulation, the ability of protein S to inhibit thrombin formation initiated with factor Xa did not decrease at higher phospholipid concentrations.

Significance of the APC-independent anticoagulant activity of protein S

The experiments presented above demonstrate that in the absence of APC, protein S has a pronounced inhibitory effect on the thrombin generation in plasma at low procoagulant stimuli. However, the relevance of the APC-independent anticoagulant activity of protein S *in vivo* remains unclear.

Heterozygous (Type I) protein S deficiency and the prothrombin G20210A mutation are two pathophysiologic conditions that are associated with an increased risk of venous thrombosis. To analyze the effect of these conditions on the APC-independent anticoagulant activity of protein S, their phenotypes were modeled in plasma. Protein S deficiency was modeled by mixing normal pooled plasma with protein S-depleted plasma resulting in 60% total protein S levels. The increase in prothrombin levels associated with the prothrombin G20210A mutation was mimicked by the addition of purified prothrombin to normal pooled plasma, resulting in a prothrombin level of 140%. Final concentrations

Figure 5.5 Significance of the APC-independent anticoagulant activity of protein S in plasma. Thrombin generation was initiated with 3.5 pM tissue factor, 10 μ M phospholipid vesicles (20/60/20 DOPS/DOPC/DOPE) and 16 mM CaCl_2 (final concentrations) in normal pooled plasma (filled circles) and in plasma containing 140% prothrombin (triangles), 60% protein S (open circles) or 140% prothrombin and 60% protein S (squares). All plasma mixtures contained antibodies against protein C. Thrombin generation was quantified with the fluorogenic substrate I-1140 as described in 'Materials and methods'.

of 3.5 μM tissue factor and 10 μM phospholipid vesicles were used to initiate coagulation and thrombin generation was followed with the fluorogenic substrate I-1140.

An increase in plasma prothrombin concentration to a level representative for carriers of the prothrombin G20210A mutation increased the ETP to 160% of that of normal plasma (Fig. 5.5). There was no effect on the lag time. Reduction of the protein S concentration to 60% of normal plasma resulted in an increase of the ETP to 130% and in a slight decrease of the lag time (Fig. 5.5). Thrombin generation was also quantified in plasma containing 140% prothrombin and 60% protein S. In this plasma (Fig. 5.5) the ETP was substantially higher (ETP = 240%) than in normal plasma (ETP = 100%) and than in plasma containing 60% protein S (ETP = 130%) or 140 % prothrombin (ETP = 160%).

APC-independent versus APC-cofactor activity of protein S

In the absence of APC, protein S inhibited thrombin generation most effectively when the procoagulant stimulus was low ([tissue factor] = 1.4 μM), whereas at high tissue factor concentrations (14 μM) the ability of protein S to inhibit the ETP in the absence of APC became minimal. These findings prompted us to compare the APC-independent- and APC-cofactor activities of protein S by measuring the effect of protein S on thrombin generation initiated in plasma with 1.4 μM and 14 μM tissue factor in the absence and presence of APC or thrombomodulin. When thrombin formation was initiated with 14 μM tissue factor in plasma in which both protein S and protein C were neutralized with antibodies, a maximal ETP was obtained (open circles, Fig. 5.6A).

Figure 5.6 APC-cofactor versus APC-independent anticoagulant activity of protein S. A. Thrombin generation curves were initiated with 14 μM tissue factor, 10 μM phospholipid vesicles (20/60/20 DOPS/DOPC/DOPE) and 16 mM CaCl_2 (final concentrations) in normal pooled plasma with anti-protein S and anti-protein C antibodies (open circles), anti-protein C antibodies (filled circles), 40 nM thrombomodulin (open triangles), 40 nM thrombomodulin and anti-protein S antibodies (filled triangles). B, C. Endogenous thrombin potentials (ETP) calculated from time courses of thrombin generation initiated with either 14 μM tissue factor (B) or 1.4 μM tissue factor (C). ETP were determined in plasma without added APC or thrombomodulin (black bars), addition of 40 nM thrombomodulin (white bars) or 7.5 nM APC (hatched bars) and presented in % of ETP in plasma with antibodies against protein S and protein C.

Omission of antibodies against protein S resulted in a small decrease in the ETP (filled circles, Fig. 5.6A), representing the low anticoagulant activity of protein S in the absence of APC under these conditions. Subsequently, antibodies against protein C were also omitted and activation of plasma protein C during thrombin generation was promoted by the addition of thrombomodulin to plasma (40 nM final concentration). As a result, the ETP was significantly reduced to 60% of its original value (open triangles, Fig. 5.6A). This reduction of the ETP could almost exclusively be attributed to the APC-cofactor activity of protein S, as the inhibitory effect of *in situ* activated protein C on the ETP was completely abolished by the addition of anti-protein S antibodies (filled triangles, Fig. 5.6A). The quantitative aspects of the effects of protein S and thrombomodulin (APC) on the ETP are summarized in Fig. 5.6B. This bar diagram also depicts the effect of APC added to plasma. Like thrombomodulin, APC considerably inhibits thrombin generation in a protein S-dependent manner.

This experiment was also performed at 1.4 pM tissue factor (Fig. 5.6C). At this tissue factor concentration the APC-independent anticoagulant activity of protein S was very pronounced and in the absence of APC (that is in the presence of protein C antibodies), protein S inhibited thrombin generation by 80%. Addition of thrombomodulin or APC resulted in an extra inhibition of thrombin generation to >95%. Under these conditions, anti-protein S antibodies abrogated the inhibition of the ETP by the APC-independent as well as the APC-cofactor activity of protein S (Fig. 5.6C).

APC-cofactor activity of protein S in plasma

Interestingly, the presence of anti-protein S antibodies almost completely abolished the anticoagulant activity of APC. An experiment was performed to quantify the contribution of protein S in the anticoagulant protein C pathway in plasma. The effect of APC on thrombin generation was measured in plasma in the presence or absence of protein S (Fig. 5.7). APC dose-dependently inhibited the ETP in the presence of protein

Figure 5.7 APC-cofactor activity of protein S. ETP calculated from thrombin generation curves initiated with 14 pM tissue factor, 10 μ M phospholipid vesicles (20/60/20 DOPS/DOPC/DOPE), 16 mM CaCl_2 and indicated concentrations of APC (final concentrations), in normal pooled plasma with (open circles) or without (filled circles) anti-protein S antibodies. ETP are presented as % of the ETP determined in plasma without added APC.

S, but became a poor inhibitor in the absence of protein S. Linear fits through the initial parts of the curves indicated that protein S enhances the anticoagulant activity of APC in plasma 21.5-fold.

Discussion

In this study we quantified the APC-independent anticoagulant activity of protein S in its natural environment, in plasma. Although it was previously recognized that protein S prolongs the clotting of plasma in the absence of APC, clotting times do not offer information about the entire process of thrombin formation. We therefore studied the influence of protein S on thrombin generation in plasma, which provides insight in the effect of protein S on the initiation, propagation and termination of thrombin formation. To investigate the anticoagulant activity of protein S in the absence of APC, we quantified thrombin generation in plasma with or without functional protein S by determining the ETP. Protein S present in plasma was functionally inhibited by addition of polyclonal antibodies against protein S. To rule out a contribution of APC, all experiments were performed in plasma to which a polyclonal antibody against (activated) protein C was added.

In the absence of APC, plasma protein S inhibited thrombin generation in a dose dependent manner, independent of the phospholipid concentration. However, the extent of inhibition varied considerably with the amount of tissue factor used for initiation of coagulation. When tissue factor concentrations decreased, the inhibitory potential of protein S increased more than 10-fold. Apparently, in the absence of APC, protein S controls coagulation efficiently when thrombin generation is limited. When the procoagulant stimulus is higher, the action of protein S could be overruled by the procoagulant driving force, and without APC, protein S is unable anymore to down-regulate thrombin generation. A possible explanation for this phenomenon might be that at high tissue factor concentrations large amounts of factor Xa are formed which will result in high concentrations of prothrombinase complexes. As a consequence, prothrombin activation will become diffusion limited³⁰, a condition at which inhibitors work less efficiently than in the case of an enzyme-controlled reaction.

Although at high tissue factor concentrations virtually no APC-independent anticoagulant activity of protein S was observed, protein S still expressed APC-cofactor activity under these conditions, since *in situ* generated or externally added APC effectively inhibited the endogenous thrombin potential in a protein S-dependent manner.

There is as yet no consensus about how protein S exerts its APC-independent anticoagulant activity. Several studies indicated that protein S may directly inhibit prothrombin activation via interactions with the protein components of the prothrombinase complex⁸⁻¹⁰. However, it was also proposed that the APC-independent anticoagulant activity of protein S is due to occupation of phospholipid binding sites^{16,17}, thereby preventing the assembly of the prothrombinase complex and the interaction with its substrate, prothrombin. The latter model may explain why purified protein S is very active at low concentrations of phospholipids and has reduced ability to inhibit

prothrombin activation at high concentrations of phospholipids. It should be emphasized, however, that the majority of the studies in which the APC-independent anticoagulant activity of protein S was investigated were performed with purified protein S preparations. In a previous report, we have shown that purified protein S preparations contain a small fraction of multimers that bind with a very high affinity ($K_d < 1$ nM) to anionic phospholipids and which effectively inhibit prothrombin activation at low phospholipid concentrations¹⁸. Multimer-free protein S binds with much lower affinity ($K_d = 250$ nM) to phospholipids, and inhibits prothrombin activation also at higher phospholipid concentrations.

As protein S multimers are absent in plasma¹⁸, we studied the native form of protein S in our experiments. The observation that the APC-independent anticoagulant activity of protein S in plasma is not influenced by variation of the phospholipid concentration suggests that the inhibition of thrombin formation is not due to occupation of phospholipid binding sites, but involves direct interactions of protein S with components of the prothrombinase complex. The observation that the monoclonal antibody CLB-PS13, which is directed against the Gla-domain of protein S, abrogates the direct anticoagulant activity of protein S in plasma indicates that protein S has to bind to phospholipid in order to interact with protein components of the prothrombinase complex and to inhibit prothrombin activation.

Currently, thrombosis is considered a typical multifactorial disease in that combinations of separate risk factors most often will lead to thrombotic complications. We have developed a plasma model in which we were able to study the effect of a combination of heterozygous protein S deficiency and carriership of the prothrombin G20210A mutation on thrombin generation. In the present study, its phenotype was modeled by supplementing plasma containing either 60% or 100% protein S with prothrombin to a final plasma level of 140%. In addition to the individual procoagulant effects of heterozygous protein S deficiency and the prothrombin G20210A mutation, which cause increases of the ETP by 30% and 60%, respectively, we demonstrated that a combination of these risk factors has a synergistic effect on thrombin formation. The large increase of the ETP (140%) in plasma containing the combined defects indicates that impairment of protein S function in the absence of activated protein C particularly comes to expression at high prothrombin levels.

During homeostasis, the haemostatic system is in balance. It has been reported that tissue factor circulates at low concentration in blood³¹, providing a constant procoagulant stimulus. Intravascular clotting caused by these small amounts of tissue factor is an unwanted phenomenon that has to be controlled by the anticoagulant system. On the basis of our observations, we propose that protein S plays a major role in maintaining the haemostatic balance by inhibiting thrombin generation independent of APC at low tissue factor stimuli. When a more severe disturbance of the haemostatic balance occurs, the APC-cofactor activity of protein S becomes important in the down-regulation of coagulation. Strikingly, the present data show that the anticoagulant effect of APC is for more than 95% dependent on the presence of protein S. Hence, native multimer-free

protein S effectively regulates thrombin formation at two levels: independent of APC at small disturbance of the haemostatic balance when insufficient amounts of APC are generated, and as essential cofactor of APC at stronger procoagulant forces. Failure to restore the haemostatic balance at low procoagulant stimuli might add to the development of thrombotic disease and therefore, an impaired APC-independent anticoagulant activity of protein S may contribute to the increased thrombotic risk associated with heterozygous protein S deficiency^{19,20}.

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6

Protein S stimulates inhibition of
the tissue factor pathway by
tissue factor pathway inhibitor

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Abstract

Tissue factor (TF) plays an important role in haemostasis, inflammation, angiogenesis and in the pathophysiology of atherosclerosis and cancer. In this paper we uncover a mechanism in which protein S, which is well known as the cofactor of activated protein C, specifically inhibits TF activity by promoting the interaction between full-length tissue factor pathway inhibitor (TFPI) and factor Xa (FXa). The stimulatory effect of protein S on FXa inhibition by TFPI is caused by a 10-fold reduction of the K_i of the FXa/TFPI complex, which decreased from 4.4 nM in the absence of protein S to 0.5 nM in the presence of protein S. This decrease in K_i not only results in an acceleration of the feedback inhibition of the TF-mediated coagulation pathway, but it also brings the TFPI concentration necessary for effective FXa inhibition well within range of the concentration of TFPI in plasma. This mechanism changes the concept of regulation of TF-induced thrombin formation in plasma and demonstrates that protein S and TFPI act in concert in the inhibition of TF activity. Our data suggest that protein S deficiency not only increases the risk of thrombosis by impairing the protein C system but also by reducing the ability of TFPI to down-regulate the extrinsic coagulation pathway.

Introduction

Tissue factor (TF) is a multifunctional protein that is not only involved in haemostasis and thrombosis¹ and atherosclerosis², but also participates in cell signaling activities^{3,4} that play an important role in inflammation⁵ and angiogenesis^{6,7}. Historically, TF was identified as the protein component from tissue extracts responsible for the initiation of blood coagulation. Upon exposure to blood, TF binds the circulating coagulation factor VIIa (FVIIa). The resulting phospholipid-bound TF/FVIIa complex converts the zymogen factor X (FX) into the active serine protease, factor Xa (FXa). Together with its cofactor factor Va (FVa), FXa subsequently incorporates into the prothrombinase complex and activates prothrombin to thrombin.

Coagulation is finely tuned, and during thrombin formation several anticoagulant reactions are initiated to prevent systemic activation of coagulation. Impaired activity of the anticoagulant systems results in a hypercoagulable state and increases the risk of venous thrombosis⁸. This paper deals with two natural anticoagulant proteins, tissue factor pathway inhibitor (TFPI) and protein S, deficiencies of which are associated with venous thrombosis^{9,10}.

TFPI is a Kunitz-type inhibitor that inhibits TF/FVIIa-initiated coagulation¹¹ via a two-step feedback mechanism through formation of a bimolecular FXa/TFPI complex that subsequently interacts with TF/FVIIa, yielding an inactive quaternary complex and resulting in termination of TF/FVIIa-catalyzed FX activation¹².

Protein S is an essential component of the protein C pathway that down-regulates thrombin formation¹³. Activated protein C (APC) is a serine protease that inhibits thrombin generation via inactivation of the coagulation factors Va and VIIIa. Protein S is a cofactor in these reactions that enhances the anticoagulant activity of APC up to twenty-fold^{14,15}.

Protein S can also down-regulate thrombin generation in the absence of APC via a mechanism that is as yet not fully understood. Because protein S directly inhibits prothrombin activation in model systems, it is generally thought that protein S exerts its anticoagulant activity in the absence of APC via direct interactions with FXa, FVa and phospholipids¹⁶⁻¹⁸. However, we have recently shown that the APC-independent anticoagulant effect of protein S in plasma is particularly observed at low TF concentrations¹⁹, which suggests that protein S may have a direct effect on TF-mediated FX-activation. This article describes an interplay between TFPI and protein S in the inhibition of TF activity.

Materials and methods

Materials

Hepes-buffer was obtained from Sigma (St Louis, MA), bovine serum albumin (BSA) from ICN (Aurora, Ohio). Fluorogenic substrate I-1140 was from Bachem (Switzerland). Recombinant tissue factor (thromboplastin) was from Dade Innovin (Dade Behring, Marburg, Germany). 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-Dioleoyl-*sn*-glycero-3-phosphoserine (DOPS) and 1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) were obtained from Avanti Polar Lipids (Alabaster, AL). Phospholipid vesicles (20% DOPS, 20% DOPE, 60% DOPC) were prepared as described¹⁹. Polyclonal anti-protein S and anti-protein C antibodies were obtained from DAKO (Glostrup, Sweden). Human FXa was obtained from Enzyme Research Laboratories (South Bend, IN). TFPI was kindly provided by T. Lindhout (Cardiovascular Research Institute Maastricht)²⁰. Full-length TFPI was produced in *Escherichia coli*, the truncated variant of TFPI (amino acid residues 1-161) was expressed in *Saccharomyces cerevisiae*. Purification and analysis of both forms of TFPI has been described^{21,22}. The TFPI concentration was determined as described²³. Recombinant FVIIa (NovoSeven) was obtained from Novo Nordisk (Copenhagen). TFPI-depleted plasma was a kind gift of R. van Oerle (Cardiovascular Research Institute Maastricht). The plasma was depleted from TFPI as described by van 't Veer *et al*²⁴: normal pooled plasma was applied to an antibody column containing rabbit polyclonal antibodies directed against the N-terminal region of TFPI. The remaining TFPI activity (<1%) was determined with an in-house assay which is based on the chromogenic assay described by Sandset *et al*²⁵.

Measurement of thrombin generation

Thrombin generation was initiated in normal pooled plasma with 1.4 pM TF, 10 μ M phospholipid vesicles (20/60/20 DOPS/DOPC/DOPE) and 16 mM CaCl_2 (final concentrations) and continuously followed with the fluorogenic substrate I-1140 (Z-Gly-Gly-Arg-AMC.HCl) as described^{19,26}. Interference by APC-activity was excluded in all experiments by addition of inhibitory anti-(activated) protein C antibodies (1.23 μ M IgG) sufficient to completely block both activation of endogenous protein C and the effect of 5 nM APC added to plasma. Protein S was inhibited in plasma by addition of saturating

amounts of polyclonal antiserum against protein S (2.73 μM IgG) and preincubation of plasma during 15 min at 37 °C before the initiation of thrombin generation as described¹⁹. Where indicated, C4BP was added to plasma to a final concentration of approx 575 nM (approx 200 nM endogenous C4BP and 375 nM exogenous C4BP) and incubated for 30 min prior to the addition of anti-protein C antibodies with or without antibodies against protein S. The endogenous thrombin potential (ETP = area under the curve) was calculated from thrombin generation curves by means of the calibrated automated thrombogram (CAT) computer software provided by Synapse BV (Maastricht, The Netherlands)²⁶.

Inhibition of TF/FVIIa-catalyzed activation of FX by TFPI and protein S

TF (1 pM) was incubated with 500 pM recombinant FVIIa (NovoSeven) in the presence of 15 μM phospholipids (20/60/20 DOPS/DOPC/DOPE) at 37 °C in Hepes-buffered saline (HBS: 25 mM Hepes, 175 mM NaCl, pH 7.7) containing 3 mM CaCl_2 and 5 mg/ml BSA. FXa generation was started by addition of 160 nM human FX either in the absence or presence of 1 nM TFPI and/or 100 nM protein S (final concentrations). After different time intervals, aliquots taken from the reaction mixture were diluted 10-fold in ice-cold stop-buffer (TBS: 50 mM Tris-HCl, 175 mM NaCl, pH 7.9) containing 20 mM EDTA and 0.5 mg/ml ovalbumin, and FXa present in the diluted aliquots was determined with the chromogenic substrate S2765 (Z-D-Arg-Gly-Arg-pNA.2HCl).

Inhibition of FXa by TFPI

Conversion of the chromogenic substrate S2222 (Bz-Ile-Glu(γ -OR)-Gly-Arg-pNA.HCl) by FXa was monitored in a Ultra Microplate Reader (Bio-Tek Instruments). A reaction mixture containing CaCl_2 , S2222, phospholipid vesicles (20/60/20 DOPS/DOPC/DOPE) with or without protein S was preincubated during 7 min at 37 °C. After FXa was added, the increase in absorbance at 405 nm was followed in time. After ~5 min, TFPI was added to the reaction mixture and the reaction was followed until the rate of chromogenic substrate conversion became constant. Final concentrations in all experiments were 0.2 nM human FXa, 500 μM S2222, and 0 or 10 μM phospholipid vesicles in Hepes-buffered saline containing 5 mg/ml BSA and 3 mM CaCl_2 . The dose-dependent effect of TFPI on FXa inhibition was measured in the absence or presence of 100 nM protein S: final concentrations were 0-3.9 nM and 0-7.7 nM TFPI with and without phospholipid vesicles, respectively, and 0-12.7 nM TFPI₁₋₁₆₁.

Kinetic analysis

Progress curves of FXa inhibition by TFPI were fitted to the integrated rate equation (d) for slow binding inhibition, generating values for v_o , v_s and k_{obs} : v_o and v_s are the initial and steady-state velocities of pNA formation, respectively, and k_{obs} is the apparent rate constant for the transition from v_o to v_s (FXa/TFPI to FXa/TFPI*, equation c). FXa inhibition at varying TFPI concentrations was measured and K_i values were calculated from a plot of V/v_o versus the concentration of TFPI, in which V is the rate of pNA

formation by FXa in the absence of TFPI^{27,28}. The x-intercept of this line is $-K_i(1+[S]/K_m)$, in which [S] the concentration chromogenic substrate S2222 in the reaction mixture (0.5 mM). Under the conditions used, the K_m value for S2222 conversion by FXa was 1.065 mM. Similarly, K_i^* was determined from a plot of V/V_s versus the concentration of TFPI. Subsequent application of equations (a and b)

$$k_{-2} = k_{obs} \cdot (v_s/v_o) \quad (a)$$

$$K_i^* = K_i \cdot k_{-2}/(k_2+k_{-2}) \quad (b)$$

yielded k_2 and k_{-2} ^{27,29}.

Results

Effect of protein S and TFPI on thrombin formation

In plasma, in which coagulation was initiated with TF, inhibition of protein S with polyclonal antibodies considerably increased thrombin generation (Fig. 6.1A). This effect of protein S was independent of APC since all experiments in plasma were performed in the presence of inhibiting antibodies against APC. Calculation of the area under the thrombin-generation curves, which yields the so-called endogenous thrombin potential (ETP), indicated that protein S inhibited thrombin generation approximately two-fold from 735 nMIIa.min in the absence of protein S to 285 nMIIa.min in the presence of protein S. To explore whether the effect of protein S is limited to its free form, plasma was saturated with C4b-binding protein (C4BP). Addition of a molar excess of purified C4BP to free protein S in plasma resulted in a 40% decrease of the anticoagulant activity of protein S, independent of the concentration of tissue factor used for initiation of coagulation (Table 6.1).

Next, the APC-independent effect of protein S was determined in TFPI-depleted plasma. Thrombin generation in TFPI-depleted plasma was increased compared to normal pooled plasma, which likely reflects increased FXa generation due to the lack of inhibition of the TF/FVIIa complex. In contrast to normal plasma, antibodies against protein S had no effect on thrombin generation in TFPI-depleted plasma. This finding

Table 6.1 Effect of C4b-binding protein (C4BP) on the inhibition of FXa by TFPI and protein S.

Tissue Factor (pM)	Inhibition of ETP by protein S (%)	Inhibition of ETP by protein S-C4BP (%)	Activity of protein S- C4BP complex (%)
3.5	12.6	7.4	59
1.4	41.1	23.0	56
0.7	57.1	33.4	58

ETPs were determined at varying concentrations of TF. The inhibitory effect of protein S-C4BP complex on the ETP was determined by preincubating normal pooled plasma with saturating amounts of purified C4BP (see Materials and methods).

Figure 6.1 Thrombin generation in plasma. **Panel A:** Thrombin generation was initiated in plasma (in the presence of APC-inhibiting antibodies) with 1.4 pM TF, 10 μ M phospholipid vesicles and 16 mM CaCl_2 (final concentrations) and followed continuously with the fluorogenic substrate I-1140 (Z-Gly-Gly-Arg-AMC.HCl). (●) normal plasma with protein S; (○) normal plasma without protein S; (▲) TFPI-depleted plasma with protein S; (△) TFPI-depleted plasma without protein S. A typical experiment is shown. **Panel B:** ETP values of TFPI-depleted plasma reconstituted with varying amounts of full-length TFPI (●,○) or TFPI₁₋₁₆₁ (▲,△) in the presence of protein S (closed symbols) or absence of protein S (open symbols). The averages of two independent experiments are shown.

indicates that protein S does not express APC-independent anticoagulant activity in the absence of TFPI (Fig. 6.1A) which led to the hypothesis that protein S enhances the ability of TFPI to down-regulate FXa and thrombin formation during TF-initiated coagulation.

To gain more insight in the interaction between TFPI and protein S, TFPI-depleted plasma was reconstituted with varying amounts of recombinant full-length TFPI or a truncated form of TFPI (TFPI₁₋₁₆₁) that lacks the Kunitz-3 domain and the C-terminus (Fig. 6.1B). In plasma that contained protein S, thrombin generation decreased with increasing concentrations of full-length TFPI (IC_{50} of ~ 1.7 nM) and was fully inhibited at 3.1 nM TFPI, whereas TFPI₁₋₁₆₁ did not show an inhibitory effect. In the absence of protein S, neither full-length TFPI nor TFPI₁₋₁₆₁ affected the ETP.

Inhibition of TF/FVIIa by TFPI and protein S

TFPI inhibits extrinsic coagulation via a feedback mechanism that requires the presence of FXa, the product of extrinsic FX activation¹². The first step, in which TFPI binds to and inhibits FXa, is rate-limiting³⁰. The second step, in which FXa/TFPI reacts with TF/FVIIa and forms an inactive quaternary complex, has been reported to proceed at near diffusion-limited rate³⁰. Because protein S did not inhibit thrombin generation in TFPI-depleted plasma and TFPI lost its anticoagulant activity in the absence of protein S, we hypothesized that protein S stimulates the inhibition of TF/FVIIa-catalyzed FX activation by TFPI.

This hypothesis was tested in a model system containing purified proteins. FX-activation by TF/FVIIa was followed in time in the absence and presence of TFPI and/or protein S (Fig. 6.2). In the absence of TFPI, TF/FVIIa-catalyzed FX-activation was linear

Figure 6.2 Inhibition of TF/FVIIa-catalyzed FX activation by full-length TFPI and protein S. Activation of 160 nM FX by 1 pM TF/FVIIa was followed in reaction mixtures that contained 15 μ M phospholipids, 3 mM Ca^{2+} and (◆) no TFPI and no protein S, (■) 100 nM protein S, (●) 1 nM TFPI, (▲) 1 nM TFPI and 100 nM protein S. Averages of three independent measurements $\pm$ standard deviation are shown.

in time and was not affected by protein S. In the presence of TFPI, the generation of FXa progressively decreased and was fully inhibited after 2 min. When both TFPI and protein S were present, virtually no FXa was generated, which indicates that protein S indeed accelerates the inhibition of TF/FVIIa-catalyzed FX activation by TFPI.

Inhibition of FXa by TFPI and protein S

Theoretically, protein S can accelerate the inhibition of TF/FVIIa-catalyzed FX activation by TFPI by stimulating the formation of the FXa/TFPI and/or the FXa/TFPI/TF/FVIIa (quaternary) complex. Since the formation of the quaternary complex is very fast and diffusion-limited³⁰ it is unlikely that this step is affected by protein S. Hence, we quantified the effect of protein S on FXa/TFPI complex formation by measuring progress curves of FXa inhibition by TFPI. These progress curves were analyzed according to a slow tight-binding mechanism that describes the inhibition of FXa by TFPI³¹ (equation c).

In this mechanism enzyme (FXa) and inhibitor (TFPI) are in rapid equilibrium and form a complex (FXa/TFPI) with a dissociation constant K_i ($K_i = k_{-1}/k_1 = [\text{FXa}][\text{TFPI}]/[\text{FXa/TFPI}]$). The FXa/TFPI complex subsequently slowly isomerizes into a tight complex (FXa/TFPI*) which at final equilibrium results in an overall dissociation constant K_i^* that is much lower than K_i ($K_i^* = [\text{FXa}][\text{TFPI}]/[\text{FXa/TFPI} + \text{FXa/TFPI}^*]$).

Rate constants and dissociation constants for the interaction between TFPI and FXa were determined in reaction mixtures that contained FXa, TFPI and the FXa-specific chromogenic substrate S2222 for monitoring the loss of FXa activity in time. For the mechanism presented in equation (c), the progress curves of S2222 conversion by FXa are described by the integrated rate equation (d)²⁷:

$$A_t = A_o + v_s t + (v_o - v_s) \{1 - \exp(-k_{obs} t)\} / k_{obs} \quad (d)$$

in which A_t and A_o are the absorbance values at time t and time zero; v_o and v_s are the initial velocity and final steady-state velocities of S2222 conversion, respectively, and k_{obs} is the apparent rate constant for the transition from v_o to v_s .

S2222 conversion by FXa was inhibited upon addition of TFPI (1.5 nM), and this inhibition was strongly potentiated by protein S (Fig. 6.3A). In the absence of TFPI, protein S had no effect on S2222 conversion by FXa, indicating that protein S enhances the inhibition of FXa by TFPI. Estimates of free FXa concentrations, obtained from the first derivative of the curves (Fig. 6.3A), showed that the addition of TFPI resulted in an immediate decrease of FXa activity by ~10% (Fig. 6.3B). This finding indicates that a rapid binding equilibrium was attained in which ~10% of the FXa present was incorporated in the FXa/TFPI complex. The further decrease of free FXa with time reflects the slow isomerization of FXa/TFPI into the tight FXa/TFPI* complex which causes a continuous re-establishment of equilibrium until finally more than 95% of FXa ended up in a complex with TFPI. In the presence of protein S, the fraction of FXa that was rapidly inhibited by TFPI increased to ~60% with a similar final equilibrium. This result demonstrates that protein S primarily stimulates the formation of the FXa/TFPI

Figure 6.3 Influence of protein S on FXa inhibition by full-length TFPI. **Panel A:** Conversion of 0.5 mM S2222 by 0.2 nM FXa was monitored in reaction mixtures containing 10 μ M phospholipids, 3 mM CaCl_2 and either no protein S (dashed line) or 160 nM protein S (dotted line). Without TFPI, S2222 conversion by FXa was linear in time (with or without protein S present, solid line). At the time indicated 1.54 nM TFPI was added. The absorbance data were fitted to equation d. **Panel B:** First derivatives of the fitted curves representing the change in free FXa with time. A typical experiment is shown.

Figure 6.4 Effect of protein S on v_o and v_s calculated from time courses of FXa inhibition by TFPI. Progress curves of S2222 conversion by FXa were measured at varying concentrations of TFPI in the absence and presence of protein S. Fitting of the progress curves to equation (d) yielded values for v_o (A) and v_s (B) as function of the TFPI concentration. Final concentrations were 0.2 nM FXa, 10 μ M phospholipid vesicles (20/60/20 DOPS/DOPC/DOPE), 3 mM CaCl_2 , 0.5 mM S2222, 0–3.9 nM TFPI and no protein S ($\circ$) or 100 nM protein S ($\bullet$). The average of two independent experiments is shown.

complex and has less effect on the isomerization of FXa/TFPI into FXa/TFPI*.

The effect of protein S on FXa inhibition by TFPI was further explored by measuring progress curves of FXa inhibition at varying TFPI concentrations (0–3.9 nM) both in the absence and presence of 100 nM protein S. Fitting the experimental data to equation (d) yielded values for v_o , v_s and k_{obs} at each TFPI concentration from which the rate constants k_{+2} and k_{-2} and the dissociation constants K_i and K_i^* were calculated (see Materials and methods).

Both in the presence and absence of protein S, the initial velocity v_o decreased with increasing concentrations TFPI (Fig. 6.4A). However, the decrease of the v_o was more pronounced in the presence of protein S, supporting the concept that protein S promotes the formation of the FXa/TFPI complex. The dissociation constant K_i of this complex decreased from 4.4 nM in the absence of protein S to 0.5 nM in the presence of protein S (Table 6.2). The final equilibrium rate (v_s) also decreased with increasing concentrations TFPI, but protein S had much less effect on v_s than on v_o , (Fig. 6.4B). From the variation of v_s as function of the TFPI concentration, K_i^* values were calculated which were 0.05 nM in the absence and 0.02 nM in the presence of protein S, respectively (Table 6.2). Comparison of k_{+2} determined in the absence ($k_{+2} = 2.5 \text{ min}^{-1}$) and presence of protein S ($k_{+2} = 0.72 \text{ min}^{-1}$) indicated that protein S actually slowed down the transition of FXa/TFPI into FXa/TFPI*. The reverse reaction described by k_{-2} was not influenced by protein S (Table 6.2).

The stimulatory effect of protein S on FXa inhibition by TFPI required the presence of anionic phospholipids (Table 6.2). In the absence of phospholipid, protein S hardly influenced the K_i (8.3 nM without and 10.9 nM with protein S, Table 6.2). The finding that TFPI was a relatively poor inhibitor of FXa in reaction mixtures containing calcium ions

Table 6.2 Kinetic constants for the inhibition of FXa by TFPI with or without protein S.

	TFPI	K_i (nM)	K_i^* (nM)	k_2 (min ⁻¹)	k_{+2} (min ⁻¹)
-	TFPI _{fl}	8.3	0.08	0.018	1.84
protein S	TFPI _{fl}	10.9	0.12	0.009	0.75
PL	TFPI _{fl}	4.4	0.05	0.030	2.49
PL + protein S	TFPI _{fl}	0.5	0.02	0.028	0.72
PL	TFPI ₁₋₁₆₁	39.0	0.21	0.010	1.88
PL + protein S	TFPI ₁₋₁₆₁	42.3	0.10	0.013	5.52

PL = phospholipid vesicles 20/60/20 DOPS/DOPC/DOPE; TFPI_{fl} = full-length TFPI; TFPI₁₋₁₆₁ = TFPI that lacks the third Kunitz domain and the C-terminus.

but no phospholipids is in agreement with literature³¹. The K_i values determined for FXa inhibition by TFPI₁₋₁₆₁ explain the observed lack of inhibitory activity of TFPI₁₋₁₆₁ on thrombin formation in plasma (Fig. 6.1). The fact that protein S had no effect on complex formation between FXa and TFPI₁₋₁₆₁ (Table 6.2) indicates that the Kunitz 3 and/or the C-terminal domain of TFPI are involved in protein S-dependent stimulation of TFPI-activity. The effect of protein S on initial FXa/TFPI complex formation in a model system using fixed amounts of purified FXa was half-maximal at 45 nM protein S (data not shown) and reached optimal levels around the free protein S concentration present in plasma (150 nM). However, in a more physiologic plasma model system in which FXa was generated by TF/FVIIa¹⁹, a dose-dependent decrease of the ETP was observed with increasing concentrations of protein S over the whole possible range (0-100%) of protein S concentrations in plasma. In this respect, any change of protein S concentration in plasma will be able to affect the regulation of thrombin generation.

Discussion

The results presented in this study provide insight in the mechanism through which TF-activity is regulated in plasma. Protein S inhibits TF activity by enhancing the interaction between TFPI and FXa thereby accelerating the feedback inhibition of the extrinsic TF/FVIIa pathway by TFPI. This observation not only underscores the important role of protein S in the down-regulation of coagulation, but also provides a mechanistic basis of the APC-independent anticoagulant activity of protein S in plasma¹⁹.

Since the first report on the inhibition of prothrombin activation by protein S in the absence of APC³², this inhibition was explained by direct interactions of protein S with the components of the prothrombinase complex FVa, FXa and phospholipids¹⁶⁻¹⁸. Since then, in purified protein S preparations *in vitro* generated protein S multimers have been identified that bind with a high affinity to phospholipids ($K_d < 1$ nM) and account for the

effective inhibition of prothrombin activation by protein S in model systems³³. However, protein S multimers are absent in plasma³³, and it was proposed that the APC-independent inhibition of thrombin generation in plasma by protein S is not due to competition between protein S and other coagulation factors for binding to procoagulant membrane surfaces¹⁹.

We demonstrate that the APC-independent inhibition of thrombin generation by protein S in plasma is also not explained by inhibition of prothrombin activation through direct interactions of protein S with FXa and FVa. The observations that protein S does not inhibit thrombin generation in TFPI deficient plasma (Fig. 6.1A) and that TFPI is a very poor inhibitor of thrombin generation in the absence of protein S (Fig. 6.1B) led to the hypothesis that protein S acts as a cofactor of TFPI in the inhibition of TF/FVIIa-catalyzed FX activation. The partial activity (60%) of the protein S-C4BP complex is not yet understood, and can originate from a change in phospholipid-binding affinity of the complex or sterical hindrance by C4BP when protein S is in complex with C4BP.

Experiments in a model system confirmed that protein S enhances the inhibition of TF/FVIIa-catalyzed FX activation by TFPI (Fig. 6.2). The inhibition of FX activation by TFPI involves the formation of a FXa/TFPI complex which slowly isomerizes into a tight FXa/TFPI* complex³¹ that subsequently forms an inactive quaternary complex with TF/FVIIa³⁰. Detailed kinetic analysis showed that protein S enhances the formation of the FXa/TFPI complex and has a minor effect on the subsequent isomerization step.

The stimulatory effect of protein S on FXa inhibition by TFPI is caused by a 10-fold reduction of the K_i of the FXa/TFPI complex, which decreased from 4.4 nM in the absence of protein S to 0.5 nM in the presence of protein S. We were not able to test whether protein S may also have an effect on the formation of the quaternary complex. However, because the formation of this complex is very fast and diffusion-limited³⁰, it is unlikely that this step is affected by protein S.

We have limited information on the molecular mechanism by which protein S enhances the formation of the FXa/TFPI complex. The fact that protein S only acts as a cofactor of TFPI in the presence of phospholipids suggests that co-localization and/or juxtaposition of protein S, TFPI and FXa on the phospholipid surface is a prerequisite for the fast protein S-mediated inhibition of FXa by TFPI. Protein S did not stimulate the inhibition of FXa by truncated TFPI (TFPI₁₋₁₆₁), a form of TFPI that lacks the Kunitz-3 domain and the C-terminus. It was reported that Kunitz-3 and the C-terminus of TFPI are involved in the binding of full-length TFPI³⁴ to cell surfaces and that the C-terminus interacts with anionic phospholipids³⁵ and with the Gla-domain of FXa³⁶. Hence, the loss of these interaction sites of TFPI₁₋₁₆₁ likely explains why protein S does not stimulate the inhibition of FXa by TFPI₁₋₁₆₁ (Table 6.2) and why truncated TFPI lacks inhibitory activity in plasma (Fig. 6.1B)³⁶⁻³⁸.

The important role of TFPI and protein S in the *in vivo* regulation of coagulation is illustrated by the observations that mice with a mutant form of TFPI that did not bind FVIIa died intra-uterine or during the neonatal period because of consumptive coagulopathy³⁹ and that homozygous protein S deficiency, which is also lethal if left

untreated, presents with a similar phenotype of consumptive coagulopathy⁴⁰. Furthermore, population-based studies indicated that low levels of protein S⁹ and TFPI¹⁰ are associated with an increased risk of venous thrombosis. In view of the pivotal role of TFPI and protein S in the regulation of coagulation, it is not surprising that our observations have important physiological implications. Considering its effect on the K_i for the inhibition of FXa by TFPI, which decreases from 4.4 nM in the absence of protein S to 0.5 nM in the presence of protein S, protein S brings the TFPI concentration necessary for efficient down-regulation of extrinsic FX activation well within the range of the free TFPI concentration in plasma (0.25-0.5 nM)⁴¹. Using the equations for a simple binding equilibrium, it can be calculated that, during the initiation of coagulation (where $[FXa] < [TFPI]$), protein S reduces the free FXa concentration from 90% to 45%, and thus increases the concentration of FXa/TFPI complex approximately 5-fold. Thus, protein S enhances the down-regulation of thrombin formation by 1) reducing the amount of FXa that can participate in prothrombin and FVII activation and 2) by increasing the amount of FXa/TFPI complex available for inhibition of the TF/FVIIa complex.

The extent of inhibition of the extrinsic coagulation pathway by TFPI depends on the TF concentration, and the amount of FXa that escapes regulation by TFPI linearly increases with the TF concentration³⁰. This observation indicates that at increasing amounts of TF, TFPI will ultimately fail to keep the FXa concentration below the threshold required for thrombin formation⁴², which explains why protein S hardly inhibits thrombin generation at high TF concentrations¹⁹. Thus, protein S and TFPI likely play a prominent role in suppressing the procoagulant activities at low TF concentrations e.g. of the small amounts of TF (~3 pM) circulating in plasma⁴³. Our data demonstrate that protein S deficiency affects the two cofactor activities of protein S: the TFPI-cofactor activity at low TF concentrations and the APC-cofactor activity at high TF-concentrations¹⁹. On the basis of our observations we propose that the increased risk of venous thrombosis associated with protein S deficiency may in part be explained by an impaired down-regulation of the extrinsic coagulation pathway by TFPI at low protein S concentrations.

In addition to its role in haemostasis, TF is involved in inflammation⁵, angiogenesis^{6,7} and tumor metastasis⁴⁴, processes that are likely modulated through TF/FVIIa- and TF/FVIIa-FXa-dependent PAR signaling^{3,4,45-47}. Recently, a selective role for TFPI was proposed in the inhibition of TF signaling through PAR1 and PAR2, in which PAR1 signaling appeared less sensitive to inhibition by TFPI than PAR2⁴⁸. Whether protein S also affects these functions of TF, especially the inhibition of TF-mediated PAR1 signaling by TFPI, remains to be elucidated.

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Summary and general discussion

Introduction

In 1988, Mitchell reported that protein S directly inhibited prothrombin activation in the absence of APC¹. The mechanism of this anticoagulant activity, however, was unclear. Later studies revealed that protein S interacts with factors Va (FVa) and Xa (FXa), the constituents of the prothrombinase complex, and that these interactions result in a direct inhibition of prothrombin activation²⁻⁴. In addition it was shown that binding of protein S to a phospholipid surface is essential for the APC-independent anticoagulant activity^{3,5}. Purified protein S binds to anionic phospholipids with high affinity⁶, thus competition for phospholipid binding sites could be another mechanism by which protein S directly inhibits phospholipid-dependent processes. Moreover, in 1999 van 't Veer and coworkers reported that the APC-independent activity of protein S was caused solely by occupation of phospholipid binding sites, since no reduction of prothrombin activation was observed at high concentrations of lipids⁷. However, large differences in affinity for phospholipids^{5,7-9} as well as in APC-independent anticoagulant activity^{1-5,7,10} have been reported for different purified protein S preparations. Van Wijnen *et al* showed that the APC-independent anticoagulant capacity (IC_{50}) of a purified protein S preparation strongly correlates with its affinity for phospholipids (K_d)⁵. The fact that the assumption in the study of van 't Veer was based on only one protein S preparation with high affinity for phospholipids ($K_d < 10$ nM) raises the question whether their conclusion is valid for all protein S preparations, including those which have a low affinity for phospholipids. Nevertheless, the origin for the ~10-fold differences in activity between different protein S preparations has been a mystery for a long time.

In summary, at the time our study was initiated, it was assumed that protein S directly inhibits prothrombin activation by interfering with the formation of the prothrombinase complex on a phospholipid surface, but there was no consensus on how protein S might accomplish this: via specific interactions with FVa and FXa, via competition for phospholipid binding sites, or via both mechanisms. Thus, we tried to clarify which of these mechanisms are important for the inhibition of thrombin formation by protein S in the absence of APC. To our opinion, a clear definition could only be made if we knew why purified protein S preparations differ so much in activity and in phospholipid binding affinity. Therefore, we initially focused on the biochemical characteristics of purified protein S in relation to its APC-independent anticoagulant activity.

APC-independent anticoagulant activity of purified protein S

In **chapter 3** we demonstrated that purified protein S preparations are not homogeneous and that in addition to monomeric protein S molecules a small amount of multimeric protein S is present. The phospholipid binding affinity of monomers and multimers is clearly different, indicated by dissociation constants K_d of ~250 nM and <1 nM, respectively. When such a monomer/multimer protein S mixture is incubated with anionic phospholipids in the presence of calcium ions, initially the abundant, low affinity monomers bind to the phospholipid surface but in time they are replaced by the high

affinity multimers. This phenomenon is registered in the ellipsometer as a biphasic adsorption: the second phase represents the accumulation of protein S multimers and after prolonged incubation, the phospholipid bilayer is (fully) occupied with multimeric forms.

Because preincubation protocols are used with functional assays to quantify the direct inhibitory effect of protein S on prothrombin activation, slow, tight binding of protein S multimers can occur next to the assembly of prothrombinase complexes on the phospholipid membrane. Thus, at the time prothrombin is added to start thrombin formation, a considerable amount of multimers has already bound. Comparison of the affinity of the phospholipid-prothrombinase interaction ($K_d = 1 \text{ nM}$)¹¹ and the phospholipid-multimer interaction ($K_d < 1 \text{ nM}$, **chapter 3**) indicates that protein S multimers are efficient competitors for phospholipid binding sites in cases where these binding sites are limited. It should be noted that in assays used for quantification of the

(a) Protein S monomers

(b) Protein S multimers

Figure 7.1 Effect of protein S monomers and multimers on prothrombin activation. (a) Protein S (Pr S) monomers have low affinity for anionic phospholipids (PL) and therefore do not inhibit prothrombin activation neither at low nor at high phospholipid concentrations. (b) Protein S multimers have high affinity for anionic phospholipid. Therefore multimers are efficient inhibitors of prothrombin (FII) activation at low concentration phospholipids by occupying the binding sites on the membrane and prohibiting the prothrombinase complex to assemble. At high concentration phospholipid, protein S multimers have no effect on the activation of prothrombin into thrombin (FIIa) as sufficient membrane is available to support normal thrombin formation.

APC-independent anticoagulant activity of protein S, often low concentrations of phospholipids are used.

Moreover, we showed that the APC-independent anticoagulant activity of a purified protein S preparation in model systems using purified proteins could be attributed to the presence of the multimeric forms of protein S since prothrombin activation is hardly reduced by isolated protein S monomers. This observation is underscored by the fact that thrombin formation is not inhibited by protein S if it is omitted during preincubation and added together with prothrombin⁷. Under such conditions, no multimers have bound to the phospholipid surface yet and protein S monomers do not compete for binding sites due to their low phospholipid binding affinity. Figure 7.1 depicts the capacity of protein S monomers and multimers to inhibit prothrombin activation at low and high phospholipid concentration.

The presence of protein S multimers also provides an explanation for one of the unresolved issues on purified protein S *i.e.* the variation of APC-independent anticoagulant activities between different purified protein S preparations. Van Wijnen *et al* already reported that the affinity of different purified protein S preparations for anionic phospholipids varies considerably ($K_d = 7\text{--}74$ nM) and that the APC-independent anticoagulant activity varies in concert with it ($IC_{50} = 6\text{--}39$ nM for prothrombinase; $10\text{--}91$ nM for intrinsic tenase)⁵. In **chapter 3** we demonstrated that it is the multimer content of a protein S preparation which determines its APC-independent anticoagulant activity. The K_d values reported by van Wijnen, which represent an average of monomers and multimers, are also an indication of the multimer content. Thus, IC_{50} and K_d values decrease with increasing amounts of protein S multimers present in purified protein S preparations.

However, in plasma we could neither detect protein S multimers nor were we able to directly isolate multimers from plasma, which strongly suggested that they arise during purification of protein S. Indeed, the experiments presented in **chapter 4** reveal that during standard purification procedures, protein S multimers are generated. This observation has important consequences because it implies that the multimer-related effective inhibition of phospholipid-dependent reactions by purified protein S that we observe in our model system is merely an artifact. Any attempt, however, to obtain a stable multimer-free protein S preparation failed. In this regard it is interesting to note that after quantitative removal of multimers from a protein S preparation, immediate regeneration occurs in the isolated monomers. Therefore it seems virtually impossible to obtain purified multimer-free protein S.

We can only speculate on what drives protein S molecules to aggregate. The spontaneous regeneration of multimers out of previously monomeric molecules suggests that the origin of the multimerization lies within the protein S molecule itself. Clearly, the isolation of a protein out of its natural environment (plasma) can induce destabilization of the molecules' conformation. Subsequent exposure of hydrophobic regions on the surface likely causes aggregation. Such phenomenon might be amplified by temporarily non-physiologic or denaturing conditions during the purification procedure, as we

observed increased protein S multimer formation at conditions like low pH and high ionic strength. Puzzling however is the observation that although every single protein S molecule seems to contain the intrinsic capacity to aggregate with other protein S molecules, multimerization is always a partial process (no more than ~5% multimers in purified preparations).

Aggregation is not unique for protein S. Interesting in this respect is the observation of Grillo *et al* on the aggregation of recombinant FVIII originating from "a small transition in the tertiary structure of the protein which produced a nucleating species that led to the formation of inactive soluble aggregates"¹², which resembles our observation with protein S. Protein aggregation also takes place *in vivo* e.g. the disordered accumulation of proteins in inclusion bodies or the ordered aggregation of β -amyloid in Alzheimer disease¹³. It is not clear whether multimerization of purified protein S occurs in an ordered way. Likely, the Gla-domains of aggregated protein S molecules remain freely accessible as phospholipid binding of multimers is not hampered. Moreover, the high affinity for phospholipids suggests multiple protein-phospholipid interactions, probably via bundling of Gla-domains. The interaction site for C4b binding protein (C4BP), which resides in the sex hormone binding globulin (SHBG)-like domain of protein S¹⁴, however, is lost after multimerization. It is not clear whether other protein-protein interactions of protein S (with APC, FVa or FXa) are also abolished upon multimerization. Protein S multimers do not bind C4BP, in contrast to monomers (**chapter 4**) and therefore the incubation of purified protein S with purified C4BP results in a mixture of monomers complexed with C4BP and free protein S multimers. As a consequence, the reported APC-independent activity of protein S-C4BP might at least in part be ascribed to the presence of multimers.

During our study on protein S multimers, a paper of Pauls *et al* was published describing the self-aggregation of purified protein S in the absence of calcium¹⁵. The authors showed that at high concentration, protein S existed in a dimer form. The dimerization could be reversed by adding calcium (as well as by dilution), which makes these protein S dimers clearly different from the multimers described in **chapters 3 and 4**. We observed multimer formation in the presence and absence of calcium and neither dilution, carrier protein (BSA), chaotropic agent (ammoniumsulfate) nor detergent (CHAPS)-treatment could reverse or prevent it (**chapter 4**). Moreover, although it is not clear why, the multimerization we observe is under all conditions a partial process whereas the self-aggregation reported by Pauls reached 100% at high protein S concentrations¹⁵. Although we did not detect stable multimers in plasma and although the aggregation reported by Pauls *et al* likely does not occur *in vivo* since calcium is present, it can be speculated that the non-covalent dimerization of protein S occurs in plasma. SHBG is a homodimeric plasma protein¹⁶ and its dimeric structure might be related to its function which involves signal transduction via receptor binding and dimerization. Also Gas6, a protein that shares 44% amino acid identity with protein S¹⁷, functions as a ligand for receptors¹⁸. Whether protein S can induce signaling is not clear as conflicting results are reported in literature^{19,20}.

In vitro generated multimeric forms are likely present in all purified protein S preparations. In retrospect, their presence has been observed in previous studies. Schwalbe *et al* studied the phospholipid binding of vitamin K-dependent proteins and, unlike the other proteins, protein S showed a biphasic binding behavior²¹. The authors described the accumulation of protein S on the surface which they ascribed to self-aggregation. Size-exclusion chromatography immediately before lipid binding experiments prevented the aggregation. Our own experiments showed that protein S eluted from a Superose 6 size-exclusion column in one peak. Although protein S multimers were enriched in the early eluting fractions, no clear separation from monomers was achieved (**chapter 4**).

Likely, protein S multimers will effectively inhibit all phospholipid-dependent enzyme processes at low phospholipid concentrations ($< 1 \mu\text{M}$)^{5,7}, in purified systems as well as in plasma reconstitution experiments⁵. The use of high phospholipid concentrations is therefore a prerequisite for studying the anticoagulant activity of purified protein S in model systems. In **chapter 4** we observed that inhibition of prothrombin activation by protein S indeed decreased with increasing concentration phospholipid vesicles and at $>10 \mu\text{M}$ phospholipids, protein S did not inhibit prothrombin activation anymore. Apparently, the reported interactions between protein S and FVa and FXa do not interfere with the formation of the prothrombinase complex. As protein S multimers are excellent lipid binders ($K_d < 1 \text{ nM}$), they will most probably adsorb to all surfaces that contain anionic phospholipids, including *e.g.* apoptotic cells. Therefore, protein S multimers might influence all lipid-dependent processes in an aspecific manner and thus it is possible that multimers interfere with *e.g.* the recently reported novel function of purified protein S, the stimulation of phagocytosis of apoptotic cells²². In this regard, our observation (unpublished results) that multimers cross-link phosphatidylserine-containing liposomes might be of importance.

APC-independent anticoagulant activity of protein S in plasma

Previous studies showed that protein S, in the absence of APC, inhibits the clotting of plasma. In these studies, coagulation was initiated with TF or FXa and the effect of protein S on the clotting time was quantified^{3,23,24}. One report described that protein S inhibits thrombin generation in plasma under flow on an endothelial cell matrix¹⁰. In **chapter 5**, we measured the entire time course of thrombin generation in plasma to get also information on the APC-independent anticoagulant effect of protein S on the initiation, propagation and termination phases of coagulation. Thrombin generation curves were obtained via automated registration of substrate conversion by thrombin in full plasma. The area under the thrombin generation curve can be calculated (endogenous thrombin potential, ETP) and reflects the total amount of active thrombin that has been present during coagulation.

We observed that in the absence of APC, protein S dose-dependently inhibited thrombin generation (ETP) and that this effect of protein S depended on the TF concentration used to initiate thrombin generation. It was reasoned that the lack of

inhibition of the ETP by protein S at high TF stimulus could be caused by high concentrations of prothrombinase complex in the plasma. Due to local depletion of the substrate, the rate of prothrombin activation will become dependent on the rate of prothrombin diffusion towards the prothrombinase complexes, a condition at which thrombin formation becomes less susceptible to inhibition of the prothrombinase complex. In addition, the observation that the APC-independent anticoagulant activity of protein S in plasma does not depend on the phospholipid concentration confirmed our hypothesis that the mechanism by which protein S inhibits prothrombin activation in plasma is not due to occupation of the phospholipid surface by protein S molecules. However, because in a model system using purified components, protein S monomers were unable to inhibit prothrombinase complex activity in the absence of APC (**chapter 4**), direct interactions of protein S with the components of the prothrombinase complex (FVa and FXa) were likely not responsible for the observed decrease in thrombin generation in plasma. We therefore reasoned that yet another mechanism and presumably another plasma factor is involved in the APC-independent inhibition of thrombin generation by protein S. As presented in **chapter 5**, protein S inhibited the ETP by ~75% at 1.4 pM TF and <10% at 14 pM TF. This TF-dependency of the protein S effect lead to the hypothesis that protein S interferes with the initiation of coagulation. Tissue factor pathway inhibitor (TFPI) plays a prominent role in down-regulating TF activity and we wondered whether both anticoagulant proteins might act in concert on the regulation of thrombin generation. The plasma experiments on this subject were reminiscent for an interplay between protein S and TFPI: inhibition of thrombin generation by TFPI was strongly reduced in the absence of protein S whereas protein S completely lost its APC-independent anticoagulant activity in the absence of TFPI (**Figure 1, chapter 6**).

The plasma concentration TFPI is 2-2.5 nM²⁵. Most of the TFPI in plasma (80-90%) is truncated at the C-terminus^{26,27}. Prolongation of the clotting time of plasma is only achieved by full-length TFPI and not by the various truncated forms²⁸⁻³⁰. Our thrombin generation experiments in TFPI-depleted plasma (**Figure 2, chapter 6**) were in agreement with this: full-length TFPI (AA residues 1-276) dose-dependently prolonged the lag time and decreased the ETP whereas a truncated TFPI variant (AA residues 1-161), which lacks the C-terminus and Kunitz-3 domain, had virtually no effect on thrombin generation. In addition, we showed that the anticoagulant activity of full-length TFPI in plasma strongly depends on the presence of protein S.

In a study of 1997, van 't Veer *et al* quantified the effect of full-length TFPI on TF/FVIIa induced prothrombin activation, though not in plasma but in a reconstituted reaction system containing plasma concentrations of purified FX, FIX, FV, FVIII and FII, but no protein S. It was concluded that full-length TFPI (2.5 nM used) prolongs the clotting time 2-fold and reduces the maximal rate of thrombin formation by 50-75%³¹. These results closely approach our own thrombin generation experiments, which are presented in figure 6.1: further analysis of the thrombin generation curves shows that addition of 2.5 nM full-length TFPI to double (TFPI and protein S)-depleted plasma (Fig. 7.2 open circles) increases the lag time ~1.4-fold (panel B) and reduces the maximal rate

Figure 7.2 Effect of TFPI and protein S on thrombin generation in plasma. Thrombin generation is measured, as described in chapter 6, in TFPI-depleted plasma, reconstituted with varying amounts of full-length TFPI in the presence (filled circles) or absence (open circles) of protein S. ETP, lag time and maximal rate of thrombin generation are determined for each of the thrombin generation curves. **Panel A:** ETP values. This panel corresponds to Fig. 6.1B, circles. **Panel B:** Lag time. **Panel C:** Maximal rate of thrombin generation, determined by calculating the first derivative of the thrombin generation curves.

of thrombin generation by ~50% (panel C). In the presence of protein S (Fig. 7.2, filled circles), however, we observe that the same amount TFPI increases the lag-time more than 4-fold (panel B) and reduces the maximal rate of thrombin formation by >95% (panel C). Panel A of Fig. 7.2 shows corresponding ETP values, similar to Fig. 6.1. This underscores the potentiating effect of protein S on the TFPI-activity and suggests that when TFPI is studied in a model system without protein S, its inhibitory activity is underestimated. In the reconstituted reaction system of van 't Veer and in our plasma system a similar, low concentration TF is used, 1.25 and 1.4 pM, respectively.

Free TFPI is a poor direct inhibitor of the TF/FVIIa complex. In complex with FXa, however, TFPI efficiently inhibits TF/FVIIa activity^{32,33}. Such a product-dependent inhibition allows TF to initiate coagulation but guarantees a quick down-regulation of TF-activity once FXa is formed³⁴. Following the experiments in plasma, we hypothesized that protein S stimulates the inhibition of TF/FVIIa-catalyzed FX activation by TFPI. This was confirmed in a model system, using purified components. TFPI alone progressively decreased FXa formation, though inhibition of TF/FVIIa activity was clearly accelerated in the presence of protein S resulting in a large reduction of FXa formation. Protein S alone did not influence FX activation by TF/FVIIa. Apparently, TFPI contains the inhibitory activity and protein S only stimulates this activity. We therefore concluded that, next to being a cofactor to APC, protein S is also a cofactor to TFPI. Subsequently we tried to obtain more insight into the mechanism of the TFPI-cofactor activity of protein S.

TFPI-cofactor activity of protein S

Mechanism

Broze and coworkers elucidated the two-step feedback mechanism by which TFPI inhibits coagulation^{35,36}. *Step 1* is the binding and inhibition of FXa by TFPI. This FXa/TFPI complex interacts in *step 2* with TF/FVIIa (Fig. 2.2). The resulting quaternary

FXa/TFPI/TF/FVIIa complex has no proteolytic activity. Theoretically, protein S could influence *step 1* (FXa/TFPI complex formation), *step 2* (quaternary complex formation) or both. However, *step 1* is the rate-limiting step whereas *step 2* is very fast and proceeds at near-diffusion limited rate³⁷. A stimulatory effect of protein S on TFPI activity could therefore be expected on *step 1*, the direct inhibition of FXa by TFPI, which we confirmed as shown in figure 3 of **chapter 6**. In the presence of Ca^{2+} and PS-containing vesicles, protein S promotes the binding of TFPI to FXa by decreasing the inhibition constant K_i of this FXa/TFPI interaction ~ 10 fold, from 4.4 nM to 0.5 nM. Figure 7.3 schematically represents the influence of protein S on *step 1* of the two-step inhibitory mechanism of TFPI.

In a previous kinetic study, Huang *et al* showed that in free solution full-length TFPI is a potent inhibitor of FXa³⁸, indicated by a K_i of 1.24 nM. Calcium ions, however, counteract the inhibition, as K_i increases ~ 40 -fold³⁸. It has been suggested that the basic

Figure 7.3 Effect of protein S on TFPI activity. Protein S (Pr S) promotes the binding/inhibition of FXa by TFPI by reducing the inhibition constant (K_i) of the FXa/TFPI complex ~ 10 -fold. Therefore, more FXa/TFPI complex will be formed during the initiation of coagulation which results in a faster inhibition of the TF/FVIIa complex, indicated by the bold arrows.

C-terminus of TFPI interacts with the Gla-domain of FXa^{28,39} and it can be speculated that Ca^{2+} disturbs this interaction. In the absence of Ca^{2+} , the Gla-residues of vitamin K-dependent coagulation factors are solvent-exposed rendering the surface of the Gla-domain negatively charged and freely accessible for positively charged patches of other proteins e.g. the C-terminus of TFPI. In the presence of Ca^{2+} , however, the conformation of the Gla-domain is very different and the Ca^{2+} -bound Gla-residues are buried in the core of the molecule⁴⁰. Under such conditions, TFPI might have lost an important interaction site on FXa, what results in an increase in K_i . Huang *et al* further showed that phospholipids counteract in part the adverse effect of Ca^{2+} . As it has been reported that TFPI binds negatively charged phospholipids via its C-terminus⁴¹, it can be reasoned that co-localization of FXa and TFPI on a phospholipid surface favors the interaction between FXa and TFPI in the presence of Ca^{2+} . Still, the dissociation constant of 14.6 nM³⁸ for the FXa/TFPI complex in the presence of Ca^{2+} and phospholipids is high, taken into account that the plasma concentration full-length TFPI does not exceed 0.5 nM (10-20% of total TFPI in plasma)²⁷. This predicts that TFPI is a poor inhibitor of FXa under such conditions and that the concentration of FXa/TFPI complex formed during initiation of coagulation will be low, implying an ineffective feedback inhibition of TF/FVIIa. Such theory, however, is in clear contrast to the observed importance of TFPI *in vivo*⁴² and its effect in plasma (**chapter 6**). Huang and colleagues countered this problem by indicating that in the presence of FVa or heparin, K_i further decreased³⁸. However, it can be questioned whether FVa really supports TF/FVIIa inhibition by favoring FXa/TFPI complex formation. 18.7 nM FVa, the concentration used in this paper, will likely not be reached during the initiation phase and rather indicates that TFPI efficiently inhibits FXa when it is in the prothrombinase complex. Heparin on the other hand is a therapeutic agent and although a large reserve of full-length TFPI is bound to endothelial cells via heparin-like structures²⁵, kinetics of FXa inhibition by endothelium-bound TFPI has not been studied yet.

During coagulation *in vivo*, Ca^{2+} and phospholipids will be present. In this regard it can be argued that our observation that protein S stimulates the FXa inhibition by full-length TFPI under these conditions, certainly has physiological relevance. Protein S is a natural anticoagulant (unlike heparin) that needs no activation (unlike FV). We observed a half-maximal effect at 45 nM protein S, indicating that at plasma concentration free protein S (150 nM), the TFPI-cofactor activity of protein S will be pronounced.

Regulation of thrombin generation

In 1996, Mast and Broze reported a poor effect of TFPI on prothrombinase activity in a purified system. This observation prompted them to predict that plasma concentrations of full-length TFPI will have a minimal effect on thrombin generation²⁹. Our plasma experiments disagree with this prediction, moreover, the unraveling of the mechanism underlying the TFPI-cofactor activity of protein S allows us to discuss on how and when the TFPI/protein S system might cause a decrease in thrombin generation. Protein S reduces the K_i for FXa inhibition by TFPI from 4.4 nM to 0.5 nM. The concentration TFPI needed for efficient inhibition of FXa, therefore, comes close to the concentration full-

length TFPI in plasma (0.25-0.5 nM). Thus, protein S increases the amount of FXa/TFPI complexes during initiation phase ~5-fold. Two consequences of this increased FXa/TFPI concentration can be envisioned, which both result in a reduced thrombin generation: first, less FXa is available for procoagulant activities and second, the feedback inhibition of TF/FVIIa by FXa/TFPI is accelerated.

A. Inhibition of FXa and the prothrombinase complex by TFPI and protein S

The observation that protein S increases the affinity of FXa for TFPI, suggests that TFPI might be an important direct inhibitor of FXa during coagulation. In plasma stimulated with moderate amounts of TF (~10 pM), the FXa concentration reached is limited (~1 nM) and not much higher than the plasma concentration full-length TFPI and under such conditions TFPI/protein S might inhibit a considerable part of the FXa produced. TFPI prolongs the clotting time in TF- as well as in FXa-stimulated plasma^{29,30} and in addition, the APC-independent anticoagulant activity of protein S has been described in plasma stimulated with FXa, including **chapter 5**^{23,24}. Although these observations indeed suggest that FXa is directly inhibited by TFPI/protein S in plasma, it is not clear whether this inhibition occurs when FXa is incorporated in the prothrombinase complex or not.

In a chromogenic assay, using a peptide as substrate for FXa, inhibition of phospholipid-bound FXa by TFPI was enhanced in the presence of FVa or FV^{29,38}. In a prothrombinase assay using the physiologic substrate prothrombin, on the other hand, TFPI-mediated inhibition of thrombin formation was obstructed by FVa though not by FV²⁹. These observations suggest that TFPI is a good inhibitor of FXa before the enzyme is incorporated into the prothrombinase complex. Prothrombin and TFPI compete for the active site of FXa, but apparently also for other sites on the prothrombinase complex for example a binding site on FVa which is not present on FV. Franssen and colleagues extended the study of Mast *et al* and concluded that in the presence of the plasma concentration prothrombin, the prothrombinase complex is almost fully protected from inhibition by TFPI⁴³. It can be speculated, however, that due to the absence of protein S under these conditions the direct inhibition of prothrombinase activity by TFPI is underestimated. Preliminary studies of our group indeed suggest that in the presence of plasma concentration free protein S, TFPI significantly inhibits preassembled prothrombinase complex. The fact that protein S increases the affinity of FXa for TFPI (decrease in K_i) is a possible explanation for this observation. Alternative explanations may be the direct binding of TFPI to protein S or the interaction of protein S with one of the components of the prothrombinase complex which eliminates the protective effect of PT and/or FVa. It should be emphasized, however, that protein S alone does not influence prothrombin activation and therefore does not disturb the interactions between FXa, FVa, prothrombin and phospholipids (**chapter 4**). Interestingly, FXa protects FVa from inactivation by APC⁴⁴ by blocking cleavage at Arg506 whereas protein S stimulates the cleavage of FVa by APC at Arg306^{45,46}. Protein S, however, does not abolish the protective effect of FXa on FVa inactivation by APC^{45,46} nor affects the interaction between FXa and FVa in the prothrombinase complex^{45,46}. Thus, protein S might

stimulate the inhibition of the prothrombinase complex by TFPI and APC via an essentially similar mechanism.

In relation to this, it is interesting to mention the report of Koenen *et al* in which a novel FXa-based assay was presented to quantify the APC-independent anticoagulant activity of protein S in plasma²⁴. It was observed that the assay was sensitive to prothrombin levels. In plasma of patients with increased prothrombin levels due to the prothrombin G20210A mutation, the protein S activity was significantly decreased, moreover, this effect of prothrombin was more pronounced at lower concentrations protein S. In **chapter 5** we confirmed these observations in a model system mimicking the prothrombin mutation or protein S deficiency. In both situations thrombin generation was increased and combination of the two defects caused a synergistic effect on thrombin generation. A possible explanation for this observation is that the decreased protein S concentration as well as the increased prothrombin concentration impair TFPI activity. It is probable that the protein S/prothrombin ratio gives an indication of the anticoagulant capacity of TFPI in plasma.

B. Inhibition of TF/FVIIa by TFPI and protein S

During the past decades, the relative importance of TFPI and AT in the inhibition of TF/FVIIa has been matter of debate. Lawson *et al* for example argued that, due to the high plasma concentration, AT might be of equal importance compared to TFPI⁴⁷, moreover, it was suggested that the serpin might be the 'first defense', as it inhibits TF/FVIIa directly, without a requirement for FXa. In **chapter 6**, we demonstrated in a purified model system that in the presence of protein S, TFPI efficiently inhibits FX activation by TF/FVIIa, much better than TFPI alone. The role of TFPI in TF/FVIIa inhibition is most likely more important than previously thought.

Van 't Veer indicated that in the reconstituted reaction system TFPI and AT act in concert during TF-initiated thrombin formation³¹. TFPI prolonged the lag-time during the initiation phase whereas AT did not; both TFPI and AT decreased the rate of thrombin formation during the propagation phase. The authors suggested that TFPI potentiates the activity of AT by reducing the amount of FXa and FIXa formed through TF/FVIIa thus enabling AT to more efficiently inhibit these limited amounts of activated proteases.

In a recent study, Lu *et al* shed new light on the initiation phase of coagulation. These authors postulated that in the presence of TFPI and AT, the main product of TF/FVIIa is FIXa, not FXa⁴⁸. In their model, FXa produced by TF/FVIIa is rapidly inhibited by AT whereas FIXa is not. Moreover, they propose that FIXa-catalyzed FX activation is the actual source of FXa during initiation and propagation. This is in contrast with earlier reports that predict 'distinct roles in subsequent events' for the initially formed FXa (initiation) and FIXa (propagation)⁴⁹. Although it is difficult to compare the results of Lu *et al* with ours, the proposed concept is challenging. It implies that FXa produced by TF/FVIIa plays an anticoagulant role, in the feedback inhibition via TFPI. In this respect it would be interesting to examine whether also in the presence of TFPI and protein S, the main product of TF/FVIIa will be FIXa and not FXa. The theory of Baugh *et al*, that FXa is

still in complex with TF/FVIIa at the moment that it is inhibited by TFPI³⁷, together with our observation that protein S improves FXa/TFPI complex formation, supports the hypothesis that the FXa produced by TF/FVIIa is merely needed as an anticoagulant.

It should be noted that Lu *et al* used a high concentration TF/FVIIa (0.2-1 nM) to initiate activation of FX and FIX, what rapidly gives rise to high amounts of activated factors, even in the presence of inhibitors⁴⁸. Van 't Veer *et al* reported that thrombin generation is a threshold-limited event and that in the presence of TFPI and AT the threshold for explosive thrombin formation is ~20 pM TF/FVIIa³¹. In addition, Baugh *et al* reported that due to the fast inhibition of TF/FVIIa by FXa/TFPI, the amount FXa that escapes regulation by TFPI linearly increases with the TF stimulus³⁷. Taken together, these observations imply that at higher amounts of TF, the regulation of FXa by TFPI (and protein S) will be insufficient to prevent explosive thrombin formation. Van 't Veer *et al* omitted protein S in their reconstituted reaction system, but used >5 times the plasma concentration full-length TFPI, which likely explains why our observations in plasma (**chapter 5**) are in agreement with the reported threshold value (20 pM TF/FVIIa³¹): the APC-independent anticoagulant effect of protein S disappeared at TF concentrations higher than 14 pM. Thus, at small disturbances of the haemostatic balance, TFPI and protein S likely play an important role in suppressing thrombin generation.

Molecular basis

We have little information on the molecular mechanism underlying the TFPI-cofactor activity of protein S. The presence of phospholipids and calcium are essential for the stimulating effect of protein S on FXa inhibition by TFPI, indicating that phospholipid binding of all components is necessary for optimal inhibition of FXa. TFPI lacking the C-terminus and Kunitz-3 domain has been shown to be a poor inhibitor of FXa. We observed that protein S does not show cofactor-activity for this TFPI variant. Although these observations do not provide evidence for a direct interaction between TFPI and protein S, it can be speculated that the positioning of the TFPI C-terminus on the phospholipid surface allows interaction with FXa, protein S, or both. It has been shown that in the presence of calcium, heparin has a similar effect on FXa inhibition by TFPI as protein S: heparin increases the affinity of the FXa/TFPI complex ~10-fold³⁸. The function of heparin seems more than merely bridging since the effect of heparin is also observed in the presence of phospholipids. This suggests that heparin induces a conformational change in TFPI and/or FXa. Heparin binds to both the C-terminus and the Kunitz-3 domain of TFPI. One may question whether protein S has a similar effect as heparin, moreover whether protein S has similar interaction sites. In this respect it might be interesting to test if the stimulatory effects of heparin and protein S are synergistic or not.

Likely, during the process of FXa inhibition by TFPI and TF/FVIIa inhibition by FXa/TFPI, protein S is somehow included in complex formation. However, due to the fact that protein S alone does not influence the enzymatic activity of TF/FVIIa, FXa or the prothrombinase complex, it can be reasoned that if protein S indeed directly interacts with one of the molecules in these complexes, protein S will not disturb other

interactions. Not much information is available on the molecular interactions of protein S. It was reported that FXa binds the EGF-like domains 3 and 4 of protein S⁵⁰. This interaction may be important for the TFPI-cofactor activity of protein S. Also a FVa binding site on protein S was detected⁵¹, and it can be speculated that a FVa-protein S interaction might occur during APC-cofactor activity of protein S (cleavage of FVa) but also during inhibition of FXa by TFPI in the prothrombinase complex.

Physiologic relevance of the TFPI/protein S system

Activated platelets contribute to coagulation by providing the membrane surface on which enzymatic reactions take place as well as by releasing the content of their granules. Novotny *et al* described the release of TFPI from platelets that were stimulated with thrombin or calcium ionophore⁵². Two platelet proteins were chosen as control, platelet factor 4 (PF4) and protein S. Whereas release of PF4 was faster compared to TFPI, the time course of protein S and TFPI release was similar. The amount of protein S and TFPI stored in platelets is low, 2.5% of the plasma concentration protein S⁵³ and 7-8% of the plasma concentration TFPI⁵². It can be envisioned, however, that during thrombus formation, the local concentration of activated platelets and thus released proteins significantly increases. Interestingly, platelet TFPI is the active, full-length form. Recent evidence indicates that TF-containing microparticles are recruited into a growing thrombus to maintain the initiation of coagulation⁵⁴. It may be possible that the (platelet released) TFPI/protein S system is important for the down-regulation of this blood-borne TF. In addition, TFPI/protein S might down-regulate FXa activity, originating from extrinsic (TF/FVIIa) or intrinsic (FIXa/FVIIIa) pathways in a growing thrombus. Moreover, in quiescent situations, the activity of circulating TF should be suppressed. The amount of blood borne TF under normal conditions is low (~3 pM)⁵⁵ and protein S and TFPI will be able to play a prominent role in suppressing this procoagulant activity.

Vascular endothelium is the main source of TFPI *in vivo*^{25,56}. A large amount of full-length TFPI is associated with endothelial cells via glycosaminoglycans⁵⁷. In addition, cultured endothelial cells were shown to synthesize protein S⁵⁸. Thus, also on the vessel wall, both proteins might be involved in the down-regulation of local, cell-based coagulation induced by TF which is exposed due to vessel wall damage or expressed by perturbed endothelial cells. Protein S might support other endothelial cell-based functions of TFPI, like clearance of FXa from the circulation⁵⁹ or endocytosis of the TF/FVIIa/FXa complex on endothelial cells⁶⁰.

In this respect it is interesting to mention the study of Hackeng and coworkers in which they showed that protein S inhibits prothrombinase activity on endothelial cells and platelets in the absence of APC³. It is possible that at least part of the inhibitory effect of protein S that was observed might be the result of TFPI-cofactor activity of protein S, since both cell types contain TFPI.

The vital importance of protein S and TFPI in the down-regulation of coagulation is illustrated *in vivo*. Mice that carry a mutant form of TFPI in which the FVIIa binding is abolished, die intra-uterine due to consumptive coagulopathy⁴². Case reports indicate

severe complications associated with homozygous protein S deficiency^{61,62}. In addition, reduced plasma levels of protein S and TFPI are associated with an increased risk of venous thrombosis^{63,64}. An example of the importance of the concerted TFPI/protein S activity in the regulation of thrombin generation *in vivo* might be oral contraceptive use. A recent report showed that protein S and TFPI concentration as well as OC use determine the sensitivity for APC in a thrombin generation based assay⁶⁵. The authors suggested that the reduced APC response in women taking OC might be related to the regulation of the TF pathway. This suggests that not only an impaired APC/protein S system (due to low levels of protein S) but also an impaired TFPI/protein S system (due to low levels of protein S and TFPI) adds to the increased risk of thrombosis associated with OC use. This is supported by a recent report of Koenen *et al*, in which it was reported that in plasma of women taking OC, there was a discrepancy between the APC-independent anticoagulant activity of protein S and the protein S antigen levels, indicating that another plasma factor might influence the protein S activity⁶⁶. It can be speculated that the decreased plasma TFPI levels during OC use explain that discrepancy.

Future perspectives

So far, our information on the TFPI-cofactor activity of protein S is limited. It remains to be elucidated, for example, whether protein S in complex with C4BP or thrombin-cleaved protein S stimulates TFPI activity. We have observed that addition of purified C4BP to plasma reduces the effect of protein S on thrombin generation by ~40%, suggesting that the protein S-C4BP complex has a lower TFPI-cofactor activity than free protein S (**chapter 6**). So far, however, we have no information on why free protein S loses only part (and not all) TFPI-cofactor activity when it is bound to C4BP but experiments in model systems using purified components might add to this understanding. An interesting question is also whether protein S is a cofactor to alternatively spliced TFPI (GPI-anchored TFPI-beta). Moreover, we can only speculate on the molecular interactions underlying the TFPI-cofactor activity of protein S. By use of various tools such as mutated molecules, peptides, monoclonal antibodies etc, insight may be obtained on which domains of protein S and TFPI are involved. Mechanistic information may also be used to develop a test that discriminates between both cofactor activities of protein S.

It can also be questioned whether other functions of TFPI are influenced by protein S as well. For example, it has become clear that coagulation and inflammation are two closely linked biological systems. Inflammatory mediators influence the expression of proteins involved in coagulation, including the upregulation of TF. TF-bound activated coagulation proteases, on the other hand, affect the inflammatory response via activation of protease-activated receptors (PARs) on endothelial cells^{67,68}. It has been shown that this process is regulated by TFPI⁶⁹.

Determining the *in vivo* relevance of our *in vitro* observations is an interesting challenge. Such information often originates from studies using genetically engineered mice that show a modified function and/or expression pattern of a protein. So far, no

such data are available for protein S. For example, the cell-type-specific knock out of protein S expression might provide information on the relative importance of hepatic, endothelial cell or platelet-derived protein S in coagulation but also during inflammation. Whether an impaired TFPI/protein S system is a risk factor of thrombosis also needs to be evaluated. In addition, it can be questioned whether protein S might be an interesting therapeutic agent. In septic patients for example, natural anticoagulants are used in an attempt to reduce mortality. It was shown that APC indeed improves the outcome of these patients⁷⁰. TFPI, however, did not have a significant beneficial effect⁷¹, although an interim analysis did predict a positive outcome. Since it has been reported that protein S concentrations are decreased in septic patients^{72,73}, which might be due to consumption, one may question whether acquired protein S deficiency lies at the basis of the poor protective effect related with TFPI administration⁷¹.

In summary

Ever since the first report describing the inhibition of prothrombin activation by protein S in the absence of APC, it was assumed that protein S caused this inhibition by interfering with the prothrombinase complex through direct interactions with FVa, FXa and phospholipids. The data presented in this thesis demonstrate that the inhibition of prothrombin activation by protein S in a model system using purified components, can be fully ascribed to the presence of *in vitro* generated protein S multimers. Therefore, it can be concluded that protein S does not influence the prothrombinase complex directly and that the APC-independent anticoagulant activity of protein S in plasma will be due to a different mechanism. Subsequently, we demonstrated that protein S acts as a cofactor of

Figure 7.4 Schematic representation of the dual role of protein S in hemostasis. At a high concentration tissue factor (TF), protein S is important for restoring the hemostatic balance as cofactor of activated protein C (APC). At low concentration TF, protein S plays a role in maintaining the hemostatic balance as cofactor of TFPI.

TFPI in the down-regulation of thrombin generation in plasma. Further analysis showed that protein S influences the binding of TFPI and FXa by decreasing the inhibition constant ~10-fold.

Taken together, our observations add to the understanding of the anticoagulant activity of protein S and propose a dual role of protein S in the inhibition of thrombin generation. At low procoagulant stimulus, protein S will maintain the haemostatic balance as a cofactor of TFPI, whereas at high procoagulant stimulus, protein S will restore that haemostatic balance as cofactor of APC (Fig. 7.4).

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Samenvatting

Wanneer een bloedvat beschadigd wordt, treden verschillende mechanismen in werking om het bloedverlies als gevolg hiervan te stoppen. Dit proces wordt ook wel hemostase genoemd. Het stollen van bloed (coagulatie) speelt hierbij een essentiële rol. Net als andere biologische systemen is de hemostase sterk gereguleerd. Bij beschadiging van de vaatwand moet bloedstolling accuut en massaal optreden, echter wel beperkt in plaats en tijd. Onder normale omstandigheden dient juist de vloeibaarheid van bloed gewaarborgd te zijn. Verschillende pro- en anticoagulante processen maken een strikte regulatie mogelijk. Hemostase wordt vaak voorgesteld als een balans tussen deze pro- en anticoagulante processen waarbij het voor de hand ligt dat bij bloedverlies de balans doorslaat naar de procoagulante zijde. De balans kan ook ongewenst doorslaan naar de procoagulante (bv bij trombose) of naar de anticoagulante (bv bij hemofilie) zijde.

In hoofdstuk 1 wordt besproken dat zowel cellulaire als niet-cellulaire (humorale) componenten bijdragen aan hemostase. Bij vaatwandbeschadiging binden bloedplaatjes aan componenten in het vrijgekomen, onderliggende weefsel. Hierdoor worden ze geactiveerd en vormen op de plaats van de beschadiging een plaatjesaggregaat. Op die manier werpen ze een eerste dam op om de bloeding te stelpen. Tegelijkertijd wordt de stolling in gang gezet. Dit resulteert in de vorming van een fibrinenetwerk dat het initiële plaatjesaggregaat verstevigt. De stolling wordt gekenmerkt door een reeks opeenvolgende reacties (cascade) waarbij stolfactoren, die in bloed aanwezig zijn als niet-actieve precursors, omgezet worden in hun actieve vorm.

De stollingscascade wordt geïnitieerd doordat weefselfactor (tissue factor, TF), dat vrijkomt bij de vaatbeschadiging, bindt aan de geactiveerde stolfactor VII (FVIIa), die in kleine hoeveelheden in bloed circuleert. Het TF/FVIIa complex activeert FX tot FXa en FIX tot FIXa, welke op hun beurt cascadegewijs een reeks van reacties in gang zetten. Tijdens de stolling worden meerdere macro-moleculaire complexen gevormd welke bestaan uit een actief enzym en een geactiveerde cofactor geassocieerd op een negatief geladen membraanoppervlak. Het product van één complex is op zijn beurt een onderdeel van een ander complex. Zo vormt het product van het TF/FVIIa complex, FXa, samen met zijn cofactor FVa het protrombinase-complex dat protrombine (FII) activeert tot trombine (FIIa), het centrale enzym van de stollingscascade.

Wanneer stolling is opgetreden, moet de hemostatische balans snel weer hersteld worden. Dit gebeurt onder invloed van verschillende anticoagulante processen. In het bloed zijn hoge concentraties enzym remmers aanwezig die de geactiveerde stolfactoren direct kunnen remmen. Daarnaast bestaan er twee negatieve feedback systemen die zorgen dat trombinevorming niet uit de hand loopt: 1) tissue factor pathway inhibitor (TFPI) remt met de hulp van FXa specifiek het initiërende TF/FVIIa complex; 2) het proteïne C/proteïne S systeem wordt geactiveerd wanneer proteïne C door trombine wordt omgezet in geactiveerd proteïne C (APC). In hoofdstuk 2 wordt een overzicht gegeven van de verschillende anticoagulante mechanismen en met name proteïne S en TFPI worden in detail besproken.

In hoofdstuk 3 tot en met 6 wordt de remming van trombinevorming door proteïne S beschreven. Als cofactor van APC stimuleert proteïne S de inactivering van FVa en FVIIIa, twee belangrijke cofactoren van de stolling. Deze samenwerking tussen APC en proteïne S leidt tot een efficiënte remming van de trombinegeneratie in plasma. Over de APC-cofactor activiteit van proteïne S zijn verscheidene gedetailleerde studies verschenen. Bijgevolg is aanzienlijke structurele en mechanistische informatie hierover aanwezig. Proteïne S vermindert trombinegeneratie ook in de afwezigheid van APC. De APC-onafhankelijke anticoagulante activiteit van proteïne S werd reeds in 1988 beschreven. Toch is er tot op heden maar weinig gekend over het mechanisme van de directe remming van trombinevorming door proteïne S. Algemeen wordt aangenomen dat proteïne S het protrombinase-complex (FVa, FXa en negatief geladen fosfolipiden) 'verstoort'. Er bestaat echter vooralsnog geen consensus over de manier waarop proteïne S dat doet. Enerzijds worden FVa en FXa direct beïnvloed door proteïne S wat aanleiding geeft tot een gereduceerde trombinevorming. Anderzijds bindt proteïne S, in vergelijking met andere stofactoren, met hoge affiniteit aan fosfolipiden. Dit suggereert dat proteïne S trombinevorming ook kan remmen door het afschermen van het voor enzymreacties noodzakelijke membraanoppervlak. De interpretatie van tot dusver gerapporteerde bevindingen wordt bemoeilijkt door het feit dat verschillende gezuiverde proteïne S preparaten duidelijke verschillen in APC-onafhankelijke anticoagulante activiteit vertonen.

De onduidelijkheid over de APC-onafhankelijke activiteit van proteïne S vormde het uitgangspunt voor het onderzoek beschreven in dit proefschrift. De hypothese was dat, hoewel de interactie met fosfolipiden essentieel is voor de directe werking van proteïne S, ook de interacties met de eiwitcomponenten van het protrombinasecomplex belangrijk zijn. Duidelijkheid over het mechanisme van de APC-onafhankelijke activiteit van proteïne S zou o.i. pas verkregen kunnen worden als ook duidelijk was waarom er een grote variatie in activiteit bestaat tussen de verschillende gezuiverde proteïne S preparaten.

In hoofdstuk 3 werd de binding van gezuiverd proteïne S aan een negatief geladen fosfolipidenoppervlak beschreven. Die binding bleek niet monofasisch te zijn, zoals verwacht wordt voor een zuiver eiwitpreparaat, maar bifasisch wat duidt op een niet-homogeen eiwit mengsel dat tenminste twee vormen van proteïne S bevat. Er kon inderdaad aangetoond worden dat een gezuiverd preparaat naast monomeer proteïne S ook een kleine hoeveelheid (<5%) multimeren proteïne S vormen bevat. Deze multimeren binden aan negatief geladen fosfolipiden met zeer hoge affiniteit (dissociatie constante $K_d = <1 \text{ nM}$). Het overgrote deel van de proteïne S moleculen zijn monomeren, die met een veel lagere affiniteit aan fosfolipiden binden ($K_d = \sim 250 \text{ nM}$). Het bleek dat de multimeren vormen nagenoeg volledig verantwoordelijk zijn voor de directe remming van de protrombinase-activiteit door gezuiverd proteïne S. Wanneer multimeren en monomeren van elkaar gescheiden en apart getest werden op hun capaciteit om protrombineactivering te remmen, vertoonden de multimeren proteïne S vormen een ~ 100 maal hogere APC-onafhankelijke activiteit dan de proteïne S monomeren. De aanwezigheid van multimeren verklaart ook de grote variatie in activiteit tussen

verschillende proteïne S preparaten. Aan de hand van Western blot kon worden aangetoond dat een preparaat met relatief lage APC-onafhankelijke anticoagulante activiteit, relatief weinig multimeren bevat, en *vice versa*.

In plasma konden geen proteïne S multimeren aangetoond worden, hetgeen sterk suggereerde dat deze vormen van proteïne S ontstaan tijdens de zuivering van het eiwit uit plasma. Om dit uit te zoeken werd de zuiveringsprocedure van proteïne S onder de loep genomen. De resultaten van dit onderzoek staan beschreven in hoofdstuk 4. Proteïne S multimeren worden inderdaad gevormd tijdens de zuiveringsprocedure die standaard voor proteïne S gebruikt wordt. Anionen wisselaar chromatografie in het bijzonder bleek een uitgesproken multimeer-vormend vermogen te bezitten. Ook minder fysiologische omstandigheden, zoals lage pH en hoge zoutconcentratie, versterkten de multimeervorming. Het bleek nagenoeg onmogelijk om de vorming van multimeren tijdens proteïne S zuivering te voorkomen; evenmin werd erin geslaagd eenmaal gevormde proteïne S multimeren te dissociëren. Verwijdering van multimeren uit een proteïne S preparaat d.m.v. liposoomextractie bleek effectief, maar in het resulterende monomere preparaat werden zeer snel nieuwe multimeren gevormd. Blijkbaar leidt de isolatie van proteïne S uit de plasma omgeving tot een situatie die multimeervorming stimuleert. Aangenomen kan worden dat in gezuiverd proteïne S altijd multimeren aanwezig zijn. Dientengevolge moet dus rekening worden gehouden met hun aanwezigheid wanneer de anticoagulante activiteit van gezuiverd proteïne S bestudeerd wordt. Reactie-omstandigheden moeten zo worden gekozen dat multimeren geen invloed hebben op het verloop van het bestudeerde proces. Eén mogelijkheid daartoe is de fosfolipidenconcentratie in functionele tests hoog genoeg te nemen. Zo wordt voorkomen dat een belangrijk percentage van het oppervlak bezet wordt door proteïne S multimeren. In hoofdstuk 4 werd aangetoond dat de remmende activiteit van proteïne S op protrombineactivering snel afneemt bij toenemende concentratie fosfolipiden. Vanaf ~10 μM fosfolipiden bleek de protrombineactivering vrijwel niet meer geremd te worden door gezuiverd proteïne S. Deze resultaten suggereerden dat proteïne S de trombinevorming niet remt door een direct effect op het protrombinasecomplex: noch door afscherming van het fosfolipidenoppervlak, noch door directe interacties met de eiwitcomponenten van het protrombinase complex.

Omdat in plasma geen multimeren aanwezig zijn, kan, om effecten van multimeren te vermijden, de APC-onafhankelijke anticoagulante activiteit van proteïne S ook bestudeerd worden in een plasma systeem in plaats van een modelsysteem met gezuiverde eiwitten. De anticoagulante activiteit van proteïne S in plasma werd bepaald aan de hand van trombinegeneratie metingen, en dit is beschreven in hoofdstuk 5. De oppervlakte onder de trombinegeneratie-curve (endogenous thrombin potential, ETP) is een maat is voor de totale hoeveelheid trombine die gevormd is tijdens het stollingsproces. De APC-onafhankelijke anticoagulante activiteit van proteïne S in plasma kan gekwantificeerd worden door de ETP, bepaald in normaal plasma, te vergelijken met de ETP in datzelfde plasma waaraan antilichamen zijn toegevoegd die proteïne S neutraliseren. Om zeker te zijn dat de APC-onafhankelijke activiteit van proteïne S gemeten werd en niet de APC-cofactor activiteit, werden in alle experimenten

ook neutraliserende antilichamen tegen APC toegevoegd. Toevoeging van de anti-proteïne S antilichamen aan plasma verhoogde de ETP tot ~160%, vergeleken met de ETP in plasma zonder anti-proteïne S antilichamen. Dit duidt erop dat actief proteïne S, in afwezigheid van APC, in staat is trombinegeneratie (ETP) te remmen. Er werd in kaart gebracht onder welke omstandigheden proteïne S optimaal de trombinegeneratie remt. De APC-onafhankelijke activiteit van proteïne S bleek niet te worden beïnvloed door de fosfolipidenconcentratie. Echter, de hoeveelheid TF die werd toegevoegd aan plasma om de stolling te initiëren bleek wel bepalend voor de proteïne S activiteit. Bij een lage concentratie TF (1.4 pM) werd de ETP door proteïne S met ~75% geremd. Wanneer de TF concentratie werd verhoogd daalde de APC-onafhankelijke anticoagulante activiteit van proteïne S: bij een concentratie van 3.5 pM TF werd de ETP met ~30% gereduceerd, terwijl bij hoge TF concentratie (14 pM) de ETP nauwelijks nog geremd werd door proteïne S. Bij deze hoge concentratie TF (14 pM) functioneerde proteïne S daarentegen uitstekend als cofactor voor APC: de remming van trombinegeneratie door APC werd onder die omstandigheden door proteïne S meer dan 20 keer versterkt.

Deze waarnemingen leidden tot een nieuw concept wat betreft de anticoagulante werking van proteïne S, waarbij de sterkte van de stollingstrigger (TF) bepalend is. Wanneer bloed echt moet stollen, bv bij vaatwandbeschadiging, is de TF concentratie hoog en dus de procoagulante respons sterk. Onder die omstandigheden helpt proteïne S als cofactor van APC de hemostatische balans te herstellen. In situaties waarbij stolling juist vermeden moet worden en de balans bewaard dient te blijven, remt proteïne S de trombinevorming efficiënt in de afwezigheid van APC. Lage concentraties TF blijken altijd te circuleren in bloed maar mogen onder normale omstandigheden stolling niet in gang zetten. Mogelijk speelt de APC-onafhankelijke anticoagulante activiteit van proteïne S hierbij een belangrijke rol.

In hoofdstuk 6 is er verder ingegaan op het feit dat de APC-onafhankelijke activiteit van proteïne S het grootst is bij lage concentraties TF. Dit leek erop te wijzen dat proteïne S een rol speelt tijdens de initiatie van stolling, welke voor zover bekend onder controle staat van TFPI. Daarom werd nagegaan of proteïne S en TFPI samenwerkten in de regulatie van stolling. Dit werd getest door middel van trombinegeneratie metingen in plasma. Wanneer TFPI uit plasma verwijderd werd, was proteïne S niet meer in staat om trombinegeneratie te remmen. Omgekeerd bleek dat de remming van de trombinegeneratie door TFPI sterk verminderd was in proteïne S deficiënt plasma. De experimenten in plasma suggereren inderdaad een samenwerking tussen proteïne S en TFPI. Dit werd bevestigd in een modelsysteem met gezuiverde componenten. De invloed van TFPI, proteïne S of beide remmers op de activering van FX tot FXa door TF/FVIIa werd bestudeerd. Zonder één van beide remmers wordt FX met een constante snelheid geactiveerd. De aanwezigheid van TFPI tijdens deze reactie zorgt ervoor de snelheid van FX activering afneemt in de tijd – TFPI is immers een TF/FVIIa remmer – en uiteindelijk helemaal stilvalt wanneer alle TF/FVIIa geremd is. Wanneer zowel TFPI als proteïne S in deze reactie aanwezig waren, werd TF/FVIIa veel sneller geremd dan met TFPI alleen, met als gevolg dat de hoeveelheid FXa die gevormd werd vele malen

lager was. Proteïne S alleen had geen effect op de TF/FVIIa activiteit waaruit geconcludeerd kon worden dat proteïne S fungeert als een cofactor voor TFPI.

TFPI remt het TF/FVIIa complex in twee stappen. In stap 1 bindt TFPI met FXa (het product van TF/FVIIa) waarbij de activiteit van FXa geremd wordt. In stap 2 interageert het FXa/TFPI complex met TF/FVIIa. Deze interactie resulteert in een hecht quaternair complex waarin ook FVIIa geremd is. Kinetische analyse toonde aan dat proteïne S de remming van FXa door TFPI (stap 1) beïnvloedt, door de remmingsconstante (K_i) van het FXa/TFPI complex ~ 10 keer te verlagen. Hierdoor is er dus enerzijds meer FXa/TFPI beschikbaar, waardoor de remming van TF/FVIIa sneller verloopt. Anderzijds is er minder FXa beschikbaar voor procoagulante reacties zoals de activering van protrombine. Verdere analyse toonde aan dat de aanwezigheid van een negatief geladen fosfolipiden oppervlak essentieel is voor de TFPI-cofactor activiteit van proteïne S.

In hoofdstuk 7 hebben we de resultaten, beschreven in hoofdstuk 3 tot en met 6, in verband gebracht met reeds gerapporteerde bevindingen en in een breder perspectief geplaatst. Het onderzoek beschreven in dit proefschrift heeft een bijdrage geleverd aan de opheldering van het mechanisme van de APC-onafhankelijke anticoagulante activiteit van proteïne S. Een belangrijke waarneming is dat proteïne S de stolling niet remt door verstoring van het protrombinase-complex, zoals eerder gedacht, maar fungeert als een cofactor voor TFPI en op die manier de initiatie van stolling beïnvloedt. Deze bevinding onderschrijft het belang van proteïne S als anticoagulante factor. Meer onderzoek is nodig om het moleculaire mechanisme waarmee proteïne S de interactie tussen FXa en TFPI bevordert, te karakteriseren.

Curriculum Vitae

De auteur van dit proefschrift werd op 14 september 1973 geboren te Antwerpen. De kleuter- en lagere school werden doorlopen in Hove, aan het Regina Pacis instituut, en tijdens de middelbare school werd de richting latijn-wetenschappen gevolgd aan het instituut van de heilige familie in Antwerpen (Berchem). In oktober 1991 begon zij met de studie industrieel ingenieur aan het De Nayer instituut te Sint Katelijne Waver, en koos voor de richting chemie met als optie biochemie. Na het voltooien van deze studie werd in oktober 1995 begonnen met de opleiding tot licentiaat in de biotechnologie aan de Universiteit Gent, welke 3 jaar later succesvol werd afgerond. Op 1 maart 1999 werd binnen het Cardiovascular Research Institute Maastricht (CARIM) aan de Universiteit Maastricht gestart als assistent in opleiding. Bij de capaciteitsgroep Biochemie deed zij in de daaropvolgende jaren onder leiding van Prof. Dr. J Rosing (promotor) en Dr. TM Hackeng (copromoter) onderzoek naar het mechanisme van de remming van trombine vorming door proteïne S, waarvan de resultaten beschreven staan in dit proefschrift. De auteur kreeg voor haar werk de Novo Nordisk Haemostasis Award 2005, die werd uitgereikt door Dr Harold Roberts tijdens het XXe congres van de ISTH in Sydney, Australië. Sinds oktober 2005 is zij werkzaam als postdoctoraal onderzoeker aan de RWTH Aachen bij de vakgroep celbiologie onder leiding van Prof. Dr. M Zenke.

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Awards

Young investigator award, XVIIIth congress of the International Society on Thrombosis and Haemostasis, 2001, Paris, France.

Young investigator award, XIXth congress of the International Society on Thrombosis and Haemostasis, 2003, Birmingham, UK.

Novo Nordisk Haemostasis award 2005, presented at the XXth congress of the International Society on Thrombosis and Haemostasis, 2005, Sydney, Australia.

Epiloog

...de laatste bladzijde, het is AF!!...een zucht van opluchting, en niet alleen voor mij. Blijkbaar duren laatste loodjes nog steeds het langst... en in mijn geval nog net wat langer... heerlijk het idee om zodadelijk dat laatste punt te kunnen zetten, en me vanaf dan enkel nog bezig te hoeven houden met de toekomst ... Maar eerst wil ik nog even stilstaan bij de afgelopen periode, bij de dingen die me zullen blijven van mijn biochemie-tijd. Een periode met ups en downs, waarin lang niet alles van een leien dakje liep; een periode ook waarin veel mensen, elk op een eigen manier, eraan hebben bijgedragen dat ik nu, achterovergeleund, door m'n boekje kan bladeren en met een tevreden gevoel kan zeggen, het is AF!!... Al deze mensen wil ik graag bedanken!

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De overige collega's van de vierde verdieping zuid, van wie ik sommigen ook buiten het werk heb leren kennen, in theater of in een huisje ergens in Zeeland (Simone, Theo), op verkenning in de Limburgse heuvels (Mieke en de 'meiden van Heemskerk' (sorry meiden)) of als familiemens (Marjo)... en natuurlijk alle anderen die ik trof voor een inhoudelijk gesprek of gezellig tussendoor-praatje, tijdens dagjes-uit en borrels of onder het genot van een stuk vlaai of een kop koffie...

Ook een verdieping lager, in een kamertje met een ietwat vreemd apparaat, trof ik, zeker in de beginjaren van m'n AIO-periode, wel vaker twee mensen die een belangrijke bijdrage hebben geleverd aan dit proefschrift; Rita, met engelengeduld mat je al die sampletjes door waarmee ik keer op keer naar beneden kwam hollen, bij jou kon ik ook altijd m'n verhaal kwijt; George, vanonder je wenkbrauwen keek je alles met een erg kritische blik aan, jouw andere invalshoek op eenzelfde probleem zette me wel vaker aan het denken...

Toen ik in maart '99 van Hove naar Maastricht trok, dacht ik dat mijn verblijf in 'Holland' slechts tijdelijk zou zijn, wist ik toen veel. Zeven jaar later, ik heb Maastricht ondertussen geruild voor een dorpje iets meer in het noordoosten, weet ik wel beter. Ik heb m'n plek hier in dit 'stukje Nederlands Vlaanderen' gevonden en daar ben ik blij om, want er is zoveel meer dan werk alleen... Ik wil daarom ook vrienden, kennissen en familie bedanken voor de heerlijke afleiding en gastvrijheid, waarmee ze wellicht onbewust hebben bijgedragen aan dit proefschrift. Met plezier denk ik terug aan alle gedeelde momenten die we doorbrachten al wandelend, spelletjes-spielend, klimmend, rugzaktrekkend, etend, bij-kletsend en niet te vergeten, al roeiend, ergens dobberend tussen Maastricht en Eysden, genietend van het avondzonnetje... Theo en Leni, Yvon en Leon, Kevin, Jeffrey en natuurlijk Lianne, jullie hebben een belangrijk aandeel in mijn 'inburgeringscursus', fijn dat jullie me nemen zoals ik ben (inclusief de grillen...).

Pap en mam, jullie hebben me altijd m'n gang laten gaan, erop vertrouwend dat het wel goed zou komen (en dat moet niet altijd even gemakkelijk geweest zijn); ik kan voor alles op jullie rekenen, altijd, en dat waardeer ik heel erg; Wouter en Liesbeth, de afstand is met m'n vertrek naar Maastricht letterlijk wat groter geworden, maar de betrokkenheid niet minder, voor jullie breekt een spannende tijd aan, waar ook ik een beetje naar uitkijk... vet cool gaaf dat je paranimf wil zijn, Wouter (wel ernstig kijken, he)!

Een speciaal plekje in dit dankwoord is voor jou, Marion; ergens, een jaar of vier geleden geloof ik, heb je me net op tijd opgevist en me met je alles-relativerende humor, heel veel 'positieve energie' en af en toe een schop onder m'n... de goeie kant uitgeduwd, zonder jou had ik nooit gestaan waar ik nu sta... dankjewel... enne, de zomer komt eraan, het wordt stilaan tijd om er weer eens op uit te trekken!