PREFACE

THROMBIN – APPLIED CLINICAL BIOCHEMISTRY OF THE MAIN FACTOR OF COAGULATION

Thrombin, the factor 2a (F2a) of blood coagulation, physiologically transforms soluble fibrinogen (F1) into insoluble glue-like fibrin at local areas of injured vessels. Thrombin is a 36000 Dalton-sized arginine-specific serine protease, i.e. a trypsin-like protease with an active catalytic Ser….His…Asp centre (ACC) in its B-chain (Fig. 1) [1]. The B-chain contains also the two major binding regions, called exosite-1 to bind lysine-rich fibrinogen and the positively charged exosite-2 to bind negatively charged sulphated glycosaminoglycans (such as heparin). Close to the pocket with the negatively charged catalytic centre and an apolar region [2] there is a switch region near Asp189 that binds cations, preferably Na⁺; the bound cation upregulates thrombin’s activity possibly via changing the molecular position of Glu192 [3], an amino acid close to Ser195 of the ACC: in normal human plasma the intrinsic thrombin generation is increased twofold by addition of about 4 mM Na⁺, 3 mM K⁺, or 6 mM Li⁺ [4,5]. Cations also activate other serine proteases via a similar mechanism as in thrombin [6].

Figure 1. Structure of thrombin (F2a).
α-Thrombin is a 36000 Dalton serine protease with two disulfide-linked chains, the A-chain has 36 amino acids, and the B-chain possesses 259 amino acids [7]. The B-chain contains a pocket with the negatively charged active catalytic centre with the catalytic triad Ser195…His57…Asp102 and an apolar region. Close to the arginine-specific active centre at Asp189, thrombin (B-chain) has a cation upregulator of its activity, the mostly binding cation is Na⁺. Thrombin (B-chain) has two exosites (Exo), Exo-1 binds Fibrinogen (F1), Exo-2 binds negatively charged sulphated glycosaminoglycans (such as heparin).

Figure 2. Intrinsic generation of thrombin.

Thrombin is generated out of prothrombin (F2; plasma conc. about 100 mg/l) via intrinsic or extrinsic activation of hemostasis. Intrinsic triggers are unphysiologic environments, often cell fragments, around factor 12 (F12; plasma conc. about 30 mg/l) or around pre-kallikrein (PK; plasma conc. about 50 mg/l) in vivo that could generate F12a or K, generators of the intrinsic tenase (F9a···F8a···PL···Ca++) (Fig. 2). Intrinsic generation of thrombin is of extraordinary pathophysiologic importance in clinical medicine [8]. The extrinsic trigger is tissue factor (TF) that generates the extrinsic tenase (F7a···TF···PL···Ca++). Both tenases transform factor 10 (F10; plasma conc. about 8 mg/l) into F10a. 1 mg human thrombin is
defined as 2525 IU (NIBSC standard). 1 nmol/l = 90.9 IU/l = 0.091 IU/ml; 1 IU/ml = 11 nmol/l.

Strange in vivo environments (changed matrices) in blood, e.g. the cell fragments phospholipid-microparticles (PL-MP) or free DNA, fold factor 12 (F12) of human plasma into F12a or prekallikrein into kallikrein [modified according to ref. 9]. The intrinsic F10ase (F9a with the cofactors F8a, phospholipids, Ca++) is assembled that generates F10a, together with its cofactors F5a, PL, Ca++, the generator of F2a = thrombin. Constantly, about 10% of generated thrombin reacts with the α2-macroglobulin transporter, 90% of generated F2a reacts with fibrinogen, fibrin, antithrombin-3, or heparin-cofactor-2. In an amplifying pre-phase of intrinsic hemostasis, kallikrein can directly activate F2 or F10, even without PL or Ca++.

Thrombin has very important physiologic functions [1], mainly in secondary hemostasis:

- Conversion of fibrinogen into fibrin (ACC and Exo-1)
- Activation of the accelerators of coagulation: F5 and F8 into F5a and F8a
- Activation of platelets via receptor cleavage (PAR-1, -3, -4) in primary hemostasis
- Activation of the stabilizer of fibrin: F13 into F13a
- Activation of the intrinsic factor F11 into F11a
- Activation of protein C (PC) complexed with thrombomodulin into PCa
- Reaction with its inactivator antithrombin-3(AT-3)/heparin complex (ACC and Exo-2)

The first 5 reactions are procoagulant. The last 2 reactions limit excessive in vivo thrombin generation.

Other functions of thrombin are [2]:

- Regulation of endothelial cells (NO-, endothelin, TF, t-PA)
- Contraction of smooth muscle cells
- Activation of fibroblast proliferation
- Activation of phagocytes (chemotaxis and cytokine generation [10])
- Cell growth (e.g. neurite growth)
- Seminal plasma is clotted by about 0.05 IU/ml of a thrombin-like protease and liquefied by about 0.1 IU/ml of a plasmin-like protease [11].

Thrombin’s main action is to avoid dangerous bleeding. Bleeding has to be limited, because major blood loss or even minor hemorrhage into critical organs such as the brain can threaten life. The generation of the adequate thrombin activity in such situations is of great physiologic importance. Recalcified normal citrated plasma is clotting by the action of about 0.05-0.2 IU/ml thrombin [12]. Therefore, physiologic tests for fibrinogen function should not work with unphysiologically high activities of thrombin [13], dysfibrinogens (e.g. in plasma of patients with PDIC) might then behave like normal fibrinogen.

Uncontrolled systemic generation of thrombin is of great pathophysiologic importance, because the systemically circulating thrombin generates systemically circulating micro-thrombi that might occlude small vessels or that might grow to macro-thrombi. Consequently, organs first get ischemic, then they get necrotic. Multi-organ failure is a feared complication
of increased systemic generation of thrombin. Many different mostly cell-fragmenting
diseases (e.g. sepsis, perioperatively, cancer, antiphospholipid-antibodies, placental
insufficiency, prostate traumata, polytrauma, atherosclerosis, diabetes mellitus, ischemia) or
drugs (e.g. valproic acid, asparaginase, immunosuppressants) can cause increased systemic
generation of thrombin [14-16].

Since many decades medical researchers have looked for a biomarker that diagnoses the
systemic activation state of blood coagulation, a biomarker that distinguishes the several
degrees of systemic acute or chronic coagulation activation, from normal to slightly
pathological to pathological to very pathological. A biomarker with similar characteristics as
blood creatinine for the kidney function or blood bilirubin for the liver function has been
wanted! With systemically circulating thrombin activity (F2a), i.e. thrombin reversibly
protected and transported by α2-macroglobulin (in absence of heparin about 10% of
systemically generated thrombin ends in the about 3 µM (about 2 g/l) α2M in human plasma
in a stable side reaction [17] (Fig. 3), the other 90% react with fibrinogen/
fibrin=antithrombin-1 (AT-1) [18], or antithrombin-3, or heparin-cofactor-2 in the main
reaction), we dispose for the first time of such a biomarker to diagnose the exact degree of the
activation state of coagulation in vivo [19-23]. F2a·α2M is a measure for the in-vivo function
of thrombin. The coagulation activation state of the individual patient can be staged according
to the plasma activity of the in the α2M encaged thrombin:

- 80-120 % = normal range (NIC)
- 121-150 % = pre-phase of pathologic intravascular (systemic) coagulation
  activation; (PIC-0); already life-threatening (very frequent)
- 151-200 % = pathologic intravascular (systemic) coagulation activation (PIC-1);
  very life-threatening (frequent)
- 200 % = most pathologic intravascular (systemic) coagulation activation (PIC-2),
  possibly with consumption of coagulation factors; extremely life-threatening
  (seldom)

Analogue to the main NIC-PIC classification of coagulation there is a NIF-PIF
classification of fibrinolysis, according to the activity of systemic plasmin, i.e. circulating
plasmin···α2-macroglobulin (Pli···α2M) complexes (100% of normal being 0.05 mU/ml = 0.2
mIU/ml plasmin; 1 U being the maximally inducible plasmin activity in normal citrated
plasma) [24,25]. The NIF-PIF classification is only valid, if the patient is in NIC, i.e. in
absence of pathological microthrombi - induced reactive activation of fibrinolysis:

- 80-120 % = normal range (NIF)
- 121-150 % = pre-phase of pathologic intravascular systemic fibrinolysis
  activation (PIF-0)
- 151-200 % = pathologic systemic intravascular fibrinolysis activation (PIF-1)
- 200 % = very pathologic intravascular systemic fibrinolysis activation (PIF-2).

Patients in PIC together with Pli···α2M > 200 % of normal are staged as being in PIC-3,
i.e. PIC with strong hyperfibrinolysis.
α2M is a 720 000 Dalton tube-like major blood glycoprotein, composed of 4 identical 180 000 Dalton subunits that form two symmetrical twin traps for one or two proteases, depending on their respective molecular mass [26-31]. The shape of α2M in section is similar to the letter H with the lateral sides of about 20 nm and the width of about 15 nm [32] (the ultrastructure is similar to the kyrillic letter Ж simplified like the latin letter H) with an upper and a lower trap, both containing an unspecific bait region for proteases. A protease such as thrombin (F2a) enters the interior of one of the two traps and cleaves the respective bait region. The cleaved bait region changes the structure of α2M, the entrance part of the tube nearly closes, the new entrance behaves like a molecular weight filter, the entrapped thrombin (MW = 36 000 Daltons) cannot leave the interior of α2M. Only small molecules such as arginine (Arg; □; MW = 174 Daltons) or chromogenic thrombin substrates (CS; e.g. CHG-Ala-Arg-pNA; MW = 625 Daltons) can enter the interior of the nearly closed trap. Large molecules such as fibrinogen (MW = 340 000 Daltons) cannot reach the entrapped F2a with its still fully functioning catalytic centre.

Thrombin in the F2a···α2M complex is still fully active towards small substrates of less than about 1000 Daltons. Synthetic thrombin inhibitors or hirudin cannot inhibit thrombin inside the α2M cage, possibly due to steric hindrance of thrombin folding when thrombin is inside the α2M trap [12]. Large molecules such as fibrinogen or AT3 cannot enter the nearly closed α2M cage. Therefore thrombin cannot cleave the symmetrical antenna-like N-terminal ends in the E-region of the two alpha-chains (and of the two beta-chains) of fibrinogen (Fig. 3). Cutting off mainly the two identical fibrinopeptides A (fpA; secondarily the two fibrinopeptides B) would make the resulting molecule (= fibrin monomer) sticky, which would be the first step in fibrin polymerization [33]. Thus, thrombin in the F2a···α2M neither can generate fibrin nor can it be irreversibly inactivated by 1:1 complexation with its specific serine protease inhibitor (serpin) AT3 [27]. The F2a···α2M complex is rather stable, but extreme conditions of oxidative or protease attack can break the α2M cage [34], and active thrombin is released. Thus, considering the thrombin activity, the F2a···α2M binding is reversible, whereas the serine protease/serpin binding (such as in TAT complexes) is totally irreversible [35]. However, only in less than 10% of septic patients α2M is inactivated to a significant amount, especially in patients with severe activation of the contact system of coagulation [36] with high plasmatic kallikrein activities [8]. Bait-cleaved α2M (α2M*)
exposes at its C-terminus [28] a lipophilic receptor recognition site for low-density lipoprotein receptor related protein (LRP); in vivo, the α2M*-protease complex is cleared by specific LRP-receptors on cells of the monocytes-phagocytes-system (MPS) or fibroblasts [37-40].

α2M has many more functions unrelated to its trapping – action against proteases: without converting to α2M* it binds the cytokines IL-1, IL-8, TNF-α and the growth factors TGF-β, PDGF, NGF-β, VEGF [31,41]. Often the binding site is the bait region of α2M [42]. Protease and cytokine/growth factor binding to the bait region seem to regulate each other [43,44]. α2M can act also as a chaperone, i.e. it folds denatured proteins into forms that do not aggregate [31]. So, α2M binds β-amyloid peptide and prevents fibril formation [45]. α2M - bound antigen facilitates presentation of macrophages (activated MØ) to T-cells [46].

In other species, such as the guinea-pig, α2M is the major plasma inhibitor against gram-negative sepsis [47]. Also in mice or rats α2M seems to be of major importance against pathologic inflammation [48,49]. Fortunately, α2M in humans -in contrast to rats- is not an acute phase reactant.

The result of the new thrombin activity test (Fig. 4) is reported (as for AT3) in % of normal. 5.5 mIU/ml thrombin is equivalent to 100% of normal. The normal range is 100±20% (mean value ± 1 standard deviation). The systemic thrombin activity (blood half time = about 0.5h; clearance by robust tissue macrophages, not by sensible hepatocytes as for TAT-complexes) reflects an acutely or a chronically increased in vivo generation of thrombin [21,38].

![Figure 4. Interaction of chromogenic substrates with thrombin (F2a).](image)

The catalytic centre of thrombin (Ser195…His57…Asp102) situated on two adjacent β-sheets is not freely accessible: the nearby negatively charged Glu192 (other serine proteases possess a Gln192), possibly with bound positively charged molecules (such as Arg (□) or
Na\(^{+}\) is an obstacle, especially for other positively charged molecules, to reach the catalytic centre [1,50]. The chromogenic substrate (CS; consisting of a chromophor such as para-nitroanilide (pNA) (O), linked to arginine (□) and cyclohexyl-glycine (◊)) finds the way through hydrophobic interactions to the catalytic center / apolar region of thrombin and is cleaved into the free chromophor and the 3-amino-acids-part of CS. Free pNA is measured at 405 nm. By contrast, the catalytic triad in kallikreins (Ser195…His57…Asp102) can be reached easily [51], even low concentrations of arginine in the range of about 25 mM suppress the amidolytic activity of kallikrein by about 50% [52]. About 30-40\(^{\text{th}}\) fold more arginine is needed to suppress thrombin’s amidolytic activity by 50% [53]. Additionally, arginine stops hemostasis activation and depolymerizes uncrosslinked (nascent) fibrin, making arginine an inhibitor of AT-1 [54,55]. So, free supra-molar conc. of arginine allow only the amidolytic activity of thrombin to be partly active, all other hemostasis interactions are completely inhibited [19,56].

Increased activities of systemic thrombin that generates increased numbers of circulating microthrombi are found in many different mostly cell-destructing diseases (e.g. sepsis, perioperatively, cancer, antiphospholipid-antibodies, placenta insufficiency, prostate trauma, polytrauma, atherosclerosis, diabetes mellitus, or ischemia) or drugs (e.g. valproic acid, asparaginase, immunosuppressors). Patients in PIC-0, PIC-1, or PIC-2 should be stronger anticoagulated. The preferred anticoagulant is low-molecular-weight-heparin (LMWH), for prophylaxis the target value of EXCA (extrinsic coagulation activity assay) should be about 20-40\(^{\%}\) of normal, for therapy or strong prophylaxis the target value should be 10-20\(^{\%}\) of normal [57,58]. This corresponds to about 0.5 IU/ml or 1 IU/ml LMWH, respectively [57,59].

The first 11 chapters of the book are dedicated to my new ultra-specific, ultra-sensitive assay techniques for plasmatic thrombin activity that are a breakthrough in hemostasis diagnostic. In cooperation with colleagues, the enormous importance of the new assay technique for systemic thrombin activity -that can discriminate plasma changes of 0.1 mIU/ml thrombin- is seen in intensive care patients with severe sepsis [60] or in unselected patients [61]. The recalcified coagulation activity assay (RECA) allows to quantify even discrete prothrombotic changes of the plasma matrix, such as occurring in elevated glucose concentrations [62,63], infused immunoglobulins [64], the drugs ethosuximide [65], folic acid [66], or diclofenac [67], or ultrasound exposure [68-71]. In conclusion, in future the newest thrombin activity assays (F2a test, RECA, INCA, EXCA in table 1) [23,72] will replace the rather blunted routine tests PT or APTT that measure thrombin activities in the range of 1-10 IU/ml.

Dickneite and Prags describe in their chapter “Impaired thrombin formation in experimental acquired coagulopathies can be corrected by the administration of a prothrombin complex concentrate” [73] that a therapeutic enhancement of thrombin generation can save human life, if the patients suffers from severe bleeding. The prothrombin complex used contained the coagulation factors 2, 7, 9, and 10. Usually these products contain also catalytic amounts of activated factors, e.g. F7a. Adequate infusion of coagulation enhancers by diagnostic surveillance is mandatory to avoid drug-induced circulating microthrombi [74]. Sometimes it might be sufficient to trigger intrinsic coagulation using a colloid such as HES instead of a crystalloid such as Ringer-solution. If intrinsic triggering of hemostasis is not wanted (patients without severe blood loss), a crystalloid or low dose human albumin of highest quality might be the preferred infusion [75-77].
Table 1. Advantages of innovative thrombin assays

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<tr>
<th>Assay</th>
<th>Best suited for testing</th>
<th>Clinical Indication</th>
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<tbody>
<tr>
<td>F2a test (F2a-α2M)</td>
<td>systemic (in vivo) thrombin</td>
<td>NIC – PIC diagnosis</td>
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<tr>
<td>RECA</td>
<td>procoagulant plasma changes</td>
<td>prothrombotic susceptibility to different drugs or clinical situations</td>
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<td>INCA</td>
<td>anticoagulant changes of intrinsic coagulation</td>
<td>therapeutic anticoagulation</td>
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<tr>
<td>EXCA</td>
<td>anticoagulant changes of extrinsic coagulation</td>
<td>therapeutic anticoagulation</td>
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Markwardt describes in his chapter “Historic perspectives in the development of thrombin inhibitors and their design as anti-thrombotic drugs” [78] the importance of drugs that inhibit the pathological action of thrombin. He outlines that the physiologic anticoagulant heparin is only a cofactor that is completely dependent on the plasmatic AT3 concentration of the individual patient. Therefore, he proposes that in clinical situations of extremely disturbed hemostasis such as PIC-1 or PIC-2 the physician might give one of the new direct thrombin (or F10a) inhibitors. However, it is suggested that exactly these patients need an aggressive and logical therapy that is as physiological as possible [14], i.e. supplementation of AT3 of high quality to supra-normal values combined with i.v. LMWH [79,80]. The synthetic thrombin (or F10a) inhibitors via their side chains might trigger intrinsic hemostasis, resulting in paradoxic thrombin generation especially in patients with insufficiency of hepatocytes that clear kallikrein and other activated coagulation factors out of the circulation. Thus, hirudin at the correct dosage – monitored with an innovative F10a/F2a generation assay – is a good alternative for patients with LMWH incompatibilities but only for them.

Bar-Shavit and co-workers describe in their chapter “Emerging tasks of thrombin and its receptors in epithelial tumour development” [81] cell signalling actions of protease activated receptors (PAR) in tumours. PAR-1, PAR-3 and PAR-4 are substrates for thrombin, PAR-2 is cleaved and activated by TF-F7a. PAR-1 seems to be a very important receptor for thrombin, not only in physiology but also in pathophysiology, promoting the growth of tumours.

Halldorsson and co-workers describe in their chapter “Thrombin transduction mechanisms mediating thrombin effects on vascular endothelium”[82] the signalling actions of thrombin via PAR (mainly PAR-1) on endothelial cells involves G-proteins, activation of phospholipase C (PLC), stimulation of the splitting of phosphatidylinositol bisphosphate, formation of the second messengers diacylglycerol (DAG) and inositol triphosphate (IP3), Ca++ release, activation of protein kinase C (PKC), stimulation of endothelial NO-synthase, eNOS. Even in healthy individuals thrombin is continuously generated in the systemic circulation (normal intravascular coagulation = NIC) guaranteeing vascular integrity. Pathologic activities of systemic thrombin (pathologic intravascular coagulation = PIC) participate in all stages of atherothrombosis, from endothelial dysfunction to diseased intima, plaque rupture and thrombosis. Shear stress modulates PAR-expression. The thrombin signal is only partly transduced at the level of gene transcription. The hemostasis pathophysiology includes changes in coagulation, endothelial cell shape and permeability, changes in vascular tone, angiogenesis, and inflammation [82].

Zhang and co-workers describe in their chapter „Thrombin and vagal dysfunction in inflammatory bowel disease” [83] the pathophysiologic importance of PAR-1 cleavage by thrombin in the dorsal motor nucleus of the vagus in the brainstem, possibly enhancing the vago-vagal reflex, e.g. in inflammatory bowel disease. Pathophysiologic activation of the
parasympathetic system can be life-threatening. In later disease phases, thrombin could also destroy cerebral neurons.

Baker and Falk describe in their chapter “Thrombin mediated regulation of gap junctional intercellular communication and physical cell-cell coupling” that thrombin can inhibit gap junctional intercellular communication (GJIC) and increase endothelial permeability with the consequence that blood vessels get “leaky” with tissue edemas, e.g. in lung edema or in adult respiratory distress syndrome (ARDS) [84]. Redox reactions seem to be involved in the regulation of GJIC. GJIC inhibition could be prevented by the pro-oxidant suramin. Physiological amounts of reactive oxygen species (ROS) such as singlet oxygen (\(1^\text{O}_2\)) generating \(\text{H}_2\text{O}_2\) can activate endothelial cells [85,86], pathophysiological amounts of ROS could destroy the endothelium.

Han and co-workers describe in their chapter “Maternal decidual cell-generated thrombin in abruption and preeclampsia [87]” that decidual vessels are surrounded by high TF-expressing decidual cells, i.e. here the maternal-fetal interface is highly coagulant, if TF of injured tissue enters the blood stream. Critical bleeding has to be avoided similarly as in the human brain, another organ rich in TF. It might be speculated that TF-generated fibrin protects cells from the immune attack, either physiologically in pregnancy (to avoid rejection of the fetus) or pathologically in tumours. Severely injured decidua (e.g. by abruption) can cause preeclampsia with pathologic activities of systemic thrombin and systemic microthrombi (PIC-0, PIC-1, PIC-2) [23] and can cause local thrombi that are massively invaded by neutrophils (PMN) [87], the main cells of cellular fibrinolysis [86,88].

Marchi Cappelletti describes in her chapter “Fibrinogen and fibrin: structure and functional aspects” the interaction of thrombin with its main substrate fibrinogen [89]. Thrombin interacts with many other substrates, e.g. in physiological concentrations (around 0.1-0.2 IU/ml) thrombin does not activate TAFI to an aggressive fibrin-degrading carboxypeptidase. Of particular interest are electron microscopic images of the D and E regions of fibrinogen.

The readers of the present book will understand a bit more the complicated thrombin generating system - called coagulation.

Understanding thrombin means understanding coagulation!
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