A clotting scheme for 1984

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Abstract. *Two processes govern the generation of thrombin: proteolytic proenzyme activation and heterogeneous biocatalysis. The main reaction sequence of proenzyme activation is F VII → F X → F II. And there is a reinforcement loop constituted by the reactions F VII → F IX → F X. All activations in this central triangle of blood coagulation require a protein cofactor and phospholipid. The protein cofactors from the plasma (F V and F VIII) have to be activated by thrombin in order to function. This means that thrombin acts in a positive feedback loop. This is the more important because thrombin will cause platelets that stick to collagen to present their procoagulant phospholipids at the outside of their plasma membrane. The two most probable sites of action of heparin are: inhibition of thrombin and frustration of the feedback loops and/or inhibition of factor* X_a.

Le schéma de la coagulation 1984

Résumé. *2 processus servent de base à la formation de la thrombine : l'activation protéolytique des proenzymes et la biocatalyse hétérogène. La séquence principale d'activation des proenzymes est F. VII → F. X → F. II. Il existe une boucle de renforcement sous la forme des réactions F. VII → F. IX → F. X. Dans ce triangle central de la coagulation du sang toutes les activations requièrent des cofacteurs protéiques et des phospholipides. Les cofacteurs protéiques du plasma (F. V et F. VIII) requièrent pour fonctionner l'activation par la thrombine qui ainsi agit dans une boucle positive de rétroaction. Ceci est d'autant plus important que la thrombine agit au niveau des plaquettes pour leur permettre d'adhérer au collagène en présentant leurs phospholipides coagulants à la partie externe de la membrane plasmique. Les 2 plus probables sites d'action de l'héparine sont : l'inhibition de la thrombine et l'inhibition des boucles de rétroaction et/ou l'inhibition du facteur* X_a.

Key words : *Thrombin formation — Blood coagulation — Blood clotting factors — Anticoagulant therapy — Heparin*

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It is a tradition among specialists in the field of blood coagulation to present their knowledge and speculation in the form of complicated diagrams. This may be partly due to the fact that coagulation is a relatively complicated process, partly however these intricate schemes have a charm of their own. On the other hand there is also a certain challenge in trying to present and intricate mechanism in a simple way. Therefore we will here try our hand at a simple representation of the mechanism of thrombin formation, perhaps at the cost of oversimplification.

Elementary processes

Two elementary processes suffice to describe the mechanism of thrombin formation.

Activation

Activation indicates the conversion of a protein from an inactive into an active form by limited proteolysis. For proteolytic enzymes, especially the serine proteases such as trypsin, this process has been extensively studied in classical biochemistry. It is interesting to note however that the process as such was probably first discovered in the field of blood coagulation when Pekelharing discovered prothrombin [1]. In blood coagulation activation often is a proenzyme-enzyme conversion. This is the

case with the factors II, VII, IX, X, XI, XII and prekallikrein. In the case of the factors V and VIII however a protein that has no enzymatic action of its own but that acts as a cofactor to a serine protease is the product of limited proteolysis [2].

In the following we will adopt a short hand notation for proteolytic activation. X → II means that activated factor X exerts its proteolytic action upon factor II (prothrombin) so as to activate it. It is not to be confused with the usual chemical convention which would indicate that X is converted into II.

Heterogeneous biocatalysis

The main reactions that lead to thrombin formation do not take place between molecules in free solution, but occur when the reaction partners are adsorbed at an interface. Apart from the activating enzyme and the substrate that is to be activated there is usually a third reaction partner, the protein cofactor that is also adsorbed at the interface and that helps in making the reaction more efficient. Let us take prothrombinase as an example: the activation of prothrombin is brought about by a complex of the factors X_a and V_a, adsorbed at a phospholipid surface while the prothrombin itself is also adsorbed to the surface [2].

The proteolytic reaction chain

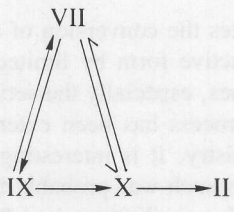
The core of the coagulation process is a chain of interactions between proteolytic enzymes and proenzymes. There is good reason to assume that the main trigger enzyme of blood coagulation is factor VII [3]. The most important reaction sequence of blood coagulation is then given by:

$$VII \rightarrow X \rightarrow II$$

As it has been demonstrated that factor X_a can activate factor VII, [4] the first activation step is reciprocal.

$$VII \leftrightarrows X \rightarrow II$$

Factor VII can also activate factor IX [5], that in its turn activates factor X as well as factor VII [6]. This leads to the following scheme:



Apart from factor VII and tissue factor there is an alternative way of activating factor IX via the contact activation mechanism. This mechanism starts via the mutual activation of prekallikrein and factor XII and then continues by the activation of factor XI [7].

$$PK \leftrightarrows XII \rightarrow XI$$

Inclusion of this activation pathway completes the proteolytic reaction chain. Also the activation of factor VII by factor XII can be taken into account [8] (Fig. 1).

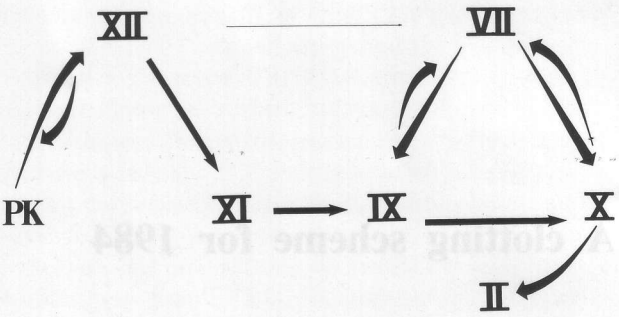


Fig. 1. The proteases of blood coagulation. The roman numerals indicate the respective coagulation factors. PK = prekallikrein. The arrows indicate activation by limited proteolysis (c) copyright Coen Hemker

Heterogeneous catalysis

The reactions in the VII → IX → X domain occur at a phospholipid-solute interphase. Negatively charged phospholipids (especially phosphatidyl serine, PS) are essential. Under physiological circumstances the cell membrane of triggered blood platelets and the phospholipids from the inside of wounded cells serve as the necessary surface. Cell membranes of intact cells and non triggered platelets *do not* provide the necessary phospholipids [9]. Each of the three activation reactions under discussion has its specific non enzyme protein cofactor [2].

1) Factor VII_a, activating factor X and factor IX needs the protein component of tissue factor (TF).
2) Factor IX_a activating factor X needs activated factor VIII [10].
3) Factor X_a acting upon prothrombin (factor II) needs activated factor V [11].

The contact activation reactions take place at negatively charged surfaces such as kaolin, glass or sulphatide micelles, phospholipids do not play a role here [12]. High molecular weight kininogen acts as a non enzymatic protein cofactor [13]. This completes our scheme of the reaction mechanism of prothrombin activation (Fig. 2).

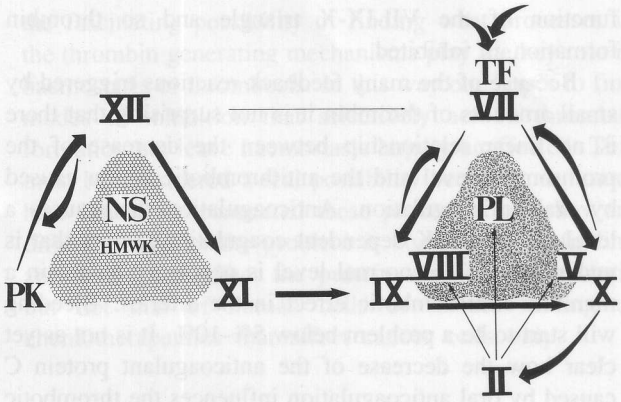


Fig. 2. A clotting scheme for 1984. In addition to fig. 1 the non enzymatic cofactors and the surfaces at which the reaction takes place are indicated. N.S. = negatively charge surface. HMWK = High Molecular Weight Kininogen. TF = Tissue Factor. © copyright Coen Hemker.

Triggering mechanisms

A wounded cell will provide *tissue thromboplastin*. Tissue thromboplastin consists of a non protein cofactor for factor VII firmly attached to membrane particles that provide a suitable phospholipid surface for the adsorbtion of factors VII_a and X [14].

It is interesting to note that the proenzyme factor VII has a measurable proteolytic activity even before being converted into factor VII_a [15]. This means that the adsorbtion of circulating factor VII into the tissue thromboplastin shed out by wounded wells will form a complex capable of activating factor X. Factor X_a will then convert factor VII into factor VII_a and thus the coagulation mechanism is started.

Factor X_a adsorbed onto phospholipid will be able to produce thrombin at a low rate even in the absence of factor V_a. Once the first molecules of thrombin have been formed a set of positive feedback reactions will set in. They will be discussed in detail below but it is important to note that the explosive triggering of thrombin formation in wounded tissue can be explained by these feedback reactions.

If blood from an ideal venipuncture is brought into contact with a foreign surface such as glass, coagulation starts rather by the mutual activation of prekallikrein and factor XII then by activation of factor VII. One has to assume a minute activity of at least either factor XII or prekallikrein in order to explain that the process gets started. Apart from glass and other surfaces rare in the human body a few other mechanisms are claimed to be able to activate the contact factors, such as bacterial lipopolysaccharides, structural elements found in subendothelium of blood vessels [16] and according to Walsh, platelets triggered by either ADP or collagen [17]. The triggering by contact activation is traditionaly known as the intrinsic pathway, whereas the extrinsic pathway is the one triggered by the thromboplastin from wounded tissue.

The importance of contact activation may have been overestimated in the early literature because it was recognised that the antihaemophilic factors (factors VIII and IX), the importance of which cannot be denied, have a place in the intrinsic pathway. It was observed relatively late that factor VII activates factor IX so that the antihaemophilic factors also form a reinforcement loop in the extrinsic pathway [18]. The importance of this fact, discovered as early as 1965 by Josso, may have escaped attention because in vitro one works either in complete absence of TF (recalcification time, APTT), so that clotting proceeds via a factor VII independent pathway *or* in the presence of an excess of tissue thromboplastin (Quick test and its modifications). Under these two circumstances the reinforcement loop is not important because the pathway VII → IX → X shows its importance only in the presence of *small* amounts of TF. When factor VII cannot immediately activate large amounts of factor X, the growing amount of factor IX_a becomes an important second factor X activator in the course of the reaction process [3]. In this respect it may be interesting to note that the bleeds of haemophiliacs are as a rule more important in thromboplastin poor tissues such as joints and muscle then in thromboplastin rich ones such as brain and lung.

Feedback mechanisms by thrombin

Probably blood coagulation, i.e. the formation of a fibrin clot, is by no means the most important action of thrombin in haemostasis. The fact that afibrinogenaemia does not automatically lead to a serious bleeding condition indicates this. As the important role of thrombocytes in haemostatic and thrombotic processes has become more and more recognised in the last decades one would rather think that the interaction of thrombin and platelets is the key reaction of haemostasis. Now this is not a one-way reaction. Thrombin is known to be the most potent platelet activator but also activated blood platelets obtain important procoagulant properties. In the first place thrombin induces in platelets the release reaction by which factor V becomes available [19]. Because the plasma concentration of factor V is ~25 nM whereas that of its partner, factor X is ~180 nM the contribution of platelet factor V, which after complete release in normal blood constitutes another 25 nM, is not negligible. It is hard to tell what the importance is under conditions where the proportions of platelets to plasma shifts in favour of the platelets, as

in the case in the haemostatic plug and thrombus. Anyhow the factor V contribution to the platelets will tend to become more important under these conditions.

In the second place low concentrations of thrombin (~1 nM, i.e. <1 % of the thrombin potentially generated in blood) will induce the so called *flip flop reaction* in platelets that are in contact with collagen [9]. This means that the procoagulant, negatively charged, phospholipids that in the resting platelet are to be found almost uniquely at the inner layer of the cell membrane are moved to the outer layer by a transbilayer movement [20]. Large concentration of thrombin alone will also cause some flip flop, whereas in platelet ghosts and debris of course the inside of the membrane is randomly accessible from the outside. Thrombin also makes platelets aggregate and the aggregate is reinforced by fibrin formation.

It is clear that coagulation and platelet reactions are not independent or parallel processes but are tightly interwoven in a positive feedback mechanism that will lead to an important amplification of both processes.

Two more positive feedback processes take place in the thrombin generation process. Thrombin converts factor V into a form that is able to be active as a cofactor of factor X_a and it likewise activates factor VIII-C, the cofactor of factor IX_a.

Apart from positive feedback there are also negative feedback loops involved in thrombin generation. It is interesting to see that these are separated from the positive effects in time and/or in space. By its reaction with thrombomodulin, a protein bound to the endothelial surface, i.e. outside the area of the wound, thrombin will become a potent activator of protein C [21]. Activated protein C breaks down activated factor V and probably also factor VIII [22]. Activated factor VIII, when not present in the phospholipid-IX_a-$VIII_a$ complex is unstable anyhow, be it that its breakdown is about tenfold slower than its activation. Thrombin can also attack prothrombin so as to make it a bad substrate for prothrombinase (phospholipid-Xa-Va) and the activation peptides of prothrombin, i.e. that part of the molecule that does not constitute thrombin, is an inhibitor of prothrombinase (product inhibition) [23].

Mode of action of oral anticoagulants

Oral anticoagulants inhibit a step in the synthesis of the vitamin K dependent coagulation factors, so that the γ-carboxyglutamic acids that normally occur in these factors are not present [24]. Because of this deficiency the factors cannot adsorb onto phospholipids and hence cannot take part in the activating complexes either as enzyme or as substrate. This causes a decrease of the function of the VII-IX-X triangle and so thrombin formation is inhibited.

Because of the many feedback reactions triggered by small amounts of thrombin it is not surprising that there is no linear relationship between the decrease of the prothrombin level and the antithrombotic action caused by oral anticoagulation. Anticoagulation that causes a level of vitamin K dependent coagulation factors that is under 20% of the normal level is necessary to obtain a manifest antithrombotic effect in the arteries. Bleeding will start to be a problem below 5%-10%. It is not as yet clear how the decrease of the anticoagulant protein C caused by oral anticoagulation influences the thrombotic tendency.

Mode of action of heparin

The heparin-antithrombin III complex is an efficient scavenger of thrombin and the factors X_a, IX_a, XI_a and XII_a. It is reported to be less active against kallikrein and inactive against factor VII_a [25]. It is important to note however that this pertains to the activated factors in free solution. It has been shown that as soon as factor X_a takes part in the prothrombinase complex it is no longer subject to the inhibitory action of antithrombin III with or without heparin [26]. This raises the interesting question of why and how heparin influences haemostasis and thrombosis in vivo. One possibility is that the action against free thrombin is important in an early stage of blood coagulation because the feedback mechanisms via thrombin are frustrated. Another possibility is that soluble factor X_a is attacked and that thus bound factor X_a is withdrawn from the prothrombinase complex. This seems less likely in view of the tight binding of factor X_a there [27]. It remains possible however that factor X_a is accessible to inhibitory action when it passes from its activating enzymes (i.e. VII_a-TF and IX_a-$VIII_a$PL) to the prothrombinase complex. We plan to study this problem in the near future.

Still another possibility is that the direct interaction of heparin with thrombin elicits its action on one or more high molecular weight substrates such as factors V and VIII or the platelet. The recent development of pure fractions from natural heparin and of synthetic heparins of precisely known composition [28] open the possibility of obtaining materials that selectively show one or more of the known actions of natural heparin. In this way heparin fractions that do not induce an increased antithrombin activity in antithrombin III have been found. Also a dissociation of the inhibitory capacity of heparin towards the different functions of thrombin has been observed [29]. Comparison of the detailed mechanism of action of specific purified heparins with their antithrombotic and antihaemostatic properties opens up

the fascinating possibility of finding what processes in the thrombin generating mechanisms play the key role in haemostasis or thrombosis. In this way one hopes to find modes of inhibition that selectively act on thrombus formation but leave haemostasis largely unaffected. This must be considered a real possibility because, no matter how intimately connected these processes are, they are different. In fact the problem is comparable to finding antibiotics that effect the protein synthesis of bacteria but not that of human cells or to the finding of chemotherapeutics that affect cancer cells only.

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