Blood coagulation

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1. — CONFÉRENCE

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The central process in the sequence of blood coagulation is the enzymatic cleavage of prothrombin (factor II) into its activation peptide and thrombin (factor II_a). This reaction involves the splitting of two peptide bonds in the single chain prothrombin molecule. It is catalysed by a serine protease called factor X_a. Factor X_a is able to split prothrombin when both molecules meet in free solution. As soon as phospholipids and an accessory protein, called factor V, are present, the rate of the reaction can increase up to 100 000 fold.

It is one of the main aims in our laboratory to understand the mechanism of this reaction. Not only because it is of intrinsic biochemical interest but also because of its enormous medical importance. It has become clear that thrombin generation is a crucial step in the genesis as well as in the fatal late complications of atherosclerosis. Now this condition accounts for well over half of all deaths in western society so it can hardly be denied medical importance. Without exploring the link between thrombin generation and atherosclerosis in any detail, it can be indicated that there is good argument in favour of the view that the generation of platelet thrombi is essential in the development of atherosclerosis and that

thrombin plays the key role in the generation of irreversible thrombi. At the other end of this process occluding thrombosis on a (ruptured) atherosclerotic plaque often announces the final stage of atherosclerotic disease. Understanding of the mechanism of thrombin formation therefore is of both medical and scientific interest.

We approached the problem mainly by kinetic means, *i.e.* by analysing the relation between the activity of the prothrombin converting enzyme, its composition and the concentration of its substrate prothrombin. This prothrombinase complex in its complete form consists of the active site bearing serine protease called activated factor X ($f X_a$); an accessory protein factor (para-enzyme) called factor V_a ($f V_a$); phospholipid (PL) and calcium ions. Incomplete forms in which the active site bearing protease is present do show activity, however.

First, I will introduce the different components of the system under study and then discuss their interaction.

Prothrombin is a minor protein in plasma : about 2 μM (~ 150 mg/litre). It is a single chain protein of 72 000 daltons containing 582 amino-acid residues. It is synthesized by the liver in a two-step procedure. The first step is normal ribosomal protein synthesis. The second one is a vitamin K-dependent carboxylation of 10 glutamic acid residues in the N-terminal end of the molecule. VERMEER *et al.* (1978) in our laboratory recently purified the carboxylase responsible for this reaction. It is an interesting process, in many respects more akin to oxidative phosphorylation than to known mammalian carboxylations.

It needs O_2, CO_2, an electron donor like NADH as well as reduced vitamin K, but ATP or biotin are not involved in the reaction. Vitamin K deficiency inhibits the second step of prothrombin synthesis and causes the uncarboxylated precursor to appear in the blood stream.

In fact, it was by this phenomenon that we discovered the two-step synthesis (HEMKER *et al.*, 1963). γ-Carboxyglutamic acids are present in blood clotting factors II, VII, IX and X that are all known to be vitamin K-dependent, as well as in a fifth plasma protein, called protein C. This protein acts as an anticoagulant by breaking down factor V (STENFLO, 1976). The role of the γ-carboxyglutamic acid residues lies in binding the proteins *via* Ca^{2+} ions to the negatively charged surface of phospholipid bilayers. Two cuts have to be made into the prothrombin molecule in order to

produce thrombin; the first one after residue 274 yields the N-terminal activation peptide (called fragment 1.2) and an intermediate without enzymatic activity, called prethrombin 2; the second one is in prethrombin 2 after residue 318 and yields the A and B chains of thrombin, linked together by a disulfide bridge.

Factor X is a proenzyme like prothrombin. It shows marked homologies with prothrombin both in its heavy chain, that bears the active site and in the N-terminal part of the light chain that contains 12 gla residues. In order to be activated a small activation peptide has to be split off from the C-terminal part of the heavy chain. The activation of factor X can be caused by either factor IX_a or factor VII_a, both of which act in complexes with phospholipids and accessory proteins, very much like factor X_a does in activating prothrombin.

The phospholipids active in blood coagulation can be any mixture of negatively charged phospholipids presented at the outer surface of a bilayer, in a vesicle, liposome or cell membrane. In coagulating blood they will mostly be the phospholipids that normally form the inside of the platelet-plasma membrane. ZWAAL *et al.* (1980) in our laboratory have shown recently that a flip-flop of the platelet membrane, *i.e.* the presentation of a part of the interior face of the membrane to the outside is an essential part of the physiological thrombin forming process. In most of the kinetic experiments we used synthetic phospholipids usually equal amounts of di [^{14}C] phosphatidylserine and -choline.

Factor V is a trace protein in plasma (0.02 mg/ml) with a molecular weight of 240 000. It has no recognized enzymatic activity of its own. It is a one-chain molecule that is cleft by thrombin into the active form that accelerates factor X_a activity.

Early kinetic experiments (HEMKER *et al.*, 1967) had shown that the three constituents of the prothrombinase complex influence the prothrombinase activity in principle in the same way. When the concentration of two is kept constant increase of the concentration of the third increases the activity to a fixed upper limit that is approached by a saturation curve. Apart from this, phospholipid in high concentrations appeared to diminish the activity. This led to a model in which factors X_a and V_a, adsorbed in juxtaposition on the phospholipid surface, form the active enzyme. The saturation observed is the saturation of the sites binding factor X_a (or factor V_a) at the phospholipid. Increasing the amount

of phospholipid increases the number of binding sites. This situation will first draw protein molecules from the solution and increase the number of active enzyme complexes. Later the surface offered becomes so large that it becomes difficult for factor X_a and factor V_a molecules to find each other and achieve juxtaposition. This explains the inhibition at high phospholipid concentrations. Recently, it became possible to obtain both factor X_a and factor V_a in a pure state; also pure prothrombin could be prepared in substrate quantities. This, together with the advent of a chromogenic substrate for thrombin, which made it possible to determine thrombin generated with great precision by spectrophotometry, enabled us to carry out kinetic experiments with much more precision and to extend them substantially. Drs. ROSING, TANS and VAN DIEIJEN were able to determine K_m and k_{cat} of prothrombinase for prothrombin for various compositions of the enzyme complex. Their first finding was that the presence of phospholipids decreases K_m of prothrombin activation by a factor of about 1 000 but has hardly any effect on k_{cat}. Factor V_a on the other hand, increases k_{cat} by a factor of about 1 000 but does not influence K_m. This points to essentially different functions for both accelerators, and automatically prompted a search for a mechanistic explanation of these phenomena (ROSING *et al.*, 1980).

The first hint for the role of phospholipids was that the K_m determined in the experiments appeared to be a function of the phospholipid concentration. With the aid of the binding constant of prothrombin for the well defined phospholipid mixture we used and the number of binding sites reported in the literature (NELSESTUEN & BRODERIUS, 1977) we could calculate the number of phospholipid-bound substrate molecules at the apparent K_m found at a given phospholipid concentration. It then appeared that half maximal reaction velocity occurred at a fixed concentration of phospholipid-bound substrate molecules, independent of the amount of phospholipid present (apart from the dilution effect discussed above). So apparently only the concentration of phospholipid-bound substrate is of consequence for the enzyme, *i.e.* the reactions take place between molecules "skating" on the surface. "Skating" introduces the concept of lateral mobility which is essential in this model. Molecules immobilized on the surface would have no possibility to interact. It is known that molecules adsorbed onto, or in phospholipid bilayers do have such mobility. Calcula-

tion of the collision frequency between adsorbed molecules indicates that, although the lateral diffusion constant is less than that in free solution, still the number of collisions per unit time can be 100 to 1 000 times higher due to the concentration at the surface.

The explanation of the effect of factor V_a on V is less straightforward than that of phospholipid.

In the first place, we have to recognize that k_{cat} of serine proteases with small substrate is of the order of 10 to 100 per second. Factor X_a alone turns over prothrombin at the rate of about one per minute. When factor V is present this brings about a 1 000 fold increase of the turnover. So factor V enables the active centre of factor X_a to attain its normal achievement rather than causing an acceleration *per se*. A second interesting observation was made when a tetrapeptide and prothrombin were offered as competing substrates to factor X_a (in the presence or the absence of phospholipid). When both are present at a concentration equal to their K_m, one would expect 50 % inhibition of the conversion of each of them. It turned out that the tetrapeptide does inhibit the prothrombin conversion but unexpectedly prothrombin does not inhibit the splitting of the peptide, even at concentrations markedly above K_m. This can only mean that there are unproductive prothrombin-enzyme complexes (or product-enzyme complexes) that have their active centres free to handle the small substrate. It is tempting to conjecture that factor V serves to turn unproductive complexes into productive ones, that is to direct the active centre of the enzyme to the vulnerable sites in the substrate or, alternatively, to ensure a rapid dissociation of the products.

Kinetic experiments were also carried out with the factor-X-activating complex, that consists of a complex of factor IX_a bearing the active site and factor $VIII_a$ (thrombin turns factor VIII into its active form) again at the surface of a phospholipid bilayer. It became clear that this complex is in every respect comparable to prothrombinase (VAN DIEIJEN *et al.*, 1979). There is another factor X-activating complex that consists of factor VII_a, phospholipid and an intracellular protein. This enzyme has been studied in Nemersons laboratory and again — as far as can be judged at this moment — the same picture emerges (SILVERBERG *et al.*, 1977).

Recent research (ØSTERUD & RAPAPORT, 1977; GRIFFIN, personal communication) has revived the interest in a phenomenon already signaled by JOSSO & PROU-WARTELLE in 1965 pertaining to the

beginning of the coagulation process. It is customary to distinguish in coagulation the so-called extrinsic and intrinsic pathway of thrombin formation. The extrinsic pathway is started by wounded tissue. By the mechanism outlined in the paragraph above with factor VII_a this causes a factor X-activating enzyme complex to arise.

The intrinsic pathway is triggered by the adsorption of high molecular weight kininogen and factor XII onto a wettable surface. *Via* a mechanism that we will not discuss in detail, and in which factor XI and prekallikrein partake, this leads to the generation of active factor XI that can activate factor IX.

Intrinsic and extrinsic pathway are convenient in describing the situation in a test tube where either no, or an excess of tissue thromboplastin is added. It became clear that when small amounts of tissue thromboplastin are present factor VII is activated to a small extent and in its turn activates both factor IX and factor X. The active factor IX (together with factor VIII) and phospholipid substantially helps in activating factor X. The kinetic mechanism behind this phenomenon probably lies in the accelerating effect of the insertion of an extra step in the enzyme cascade. When factor VII_a itself converts 10 molecules of factor X per second and only one molecule of factor IX, then in the first second the direct action is more important than the indirect one. When factor IX_a is as good an activator of X as factor VII_a is, this advantage would be over after one second. After two seconds the direct and indirect pathway would be of equal importance, and later the indirect way *via* factor IX_a would gradually gain over the direct one. Kinetic experiments and precise mathematical modelling will eventually have to substantiate this gross numerical reasoning. Anyhow, it is clear from the experiments of GRIFFIN (personal communication) that, under circumstances where no contact activation takes place and small amounts of tissue thromboplastin are present, the activation of factor X is substantially retarded in plasmas deficient in factor IX or VIII but not in plasmas deficient in the contact factors (f XII, f XI). Now, small amounts of tissue thromboplastin and absence of contact activation very probably are much more like the situation in a wound *in vivo*, than are either excess of tissue thromboplastin or its complete absence. So a pathway *via* factor VII, and factors VIII and IX is a plausible candidate for physiological thrombin generation.

This gives a lead to the solution of one of the standing paradoxes in blood coagulation research : why is it that Mr. Hageman, the patient with factor-XII-deficiency, never bled, and died of thrombosis whereas haemophiliacs (patients with factor VIII-or factor IX-deficiency) have major haemostasis problems ? It is the charm and the curse of blood coagulation research that it switches back and forth between "pure" biochemistry, physiology and pathology. Those who are interested in it hope that the interdisciplinary character of their field of interest in the barren times ahead for biochemical research will aid keeping the subject alive, but they fear at the same time, both for themselves and for the patients, that an interdisciplinary field may be more vulnerable than established disciplines so that they may be left sitting between two chairs.

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