ZYMOGENS AND COFACTORS OF BLOOD COAGULATION

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I. INTRODUCTION

Since the 1964 appearance of two papers likening blood coagulation to a linear sequence of proteolytic reactions (the "cascade" or "waterfall"),[1,2] much emphasis has been placed on the ordering of these reactions and on the chemical mechanisms of zymogen activation. Increasingly it has become clear that the zymogens involved in coagulation have extensive sequence homology with the zymogens of the pancreatic serine proteases and in this regard, coagulation is not unique.

Recently, however, it has also become clear that coagulation differs in several essential respects from the digestive proteases; e.g., most of the coagulation reactions require calcium ions for optimal function. In several reactions, membrane surfaces and protein cofactors are also needed. Further, the mechanisms by which coagulation is initiated are as yet obscure; i.e., no enzyme corresponding to enterokinase in function has been identified.

Classically, coagulation has been viewed as consisting of stages loosely corresponding to the initiating reactions, the formation of the fibrin clot, and those reactions occuring between these two stages. In this review we will not refer to these classical stages. We will, however, group certain reactions based on their biochemical mechanisms. Further, we will not attempt an encyclopedic review of the literature pertaining to this subject; we refer interested readers to a recent comprehensive treatise.[3]

II. AN OVERVIEW OF COAGULATION

When blood is put into a glass tube it forms a fibrin clot much faster than if it were collected into a siliconized or plastic tube. The proteins that interact with glass, and thereby accelerate coagulation have been referred to collectively as the "contact system". If small amounts of various tissues are added to the blood, it clots yet faster. The material which is present in tissues and leads to acceleration of coagulation has been called "tissue thromboplastin" or "tissue factor". The sequence of reactions

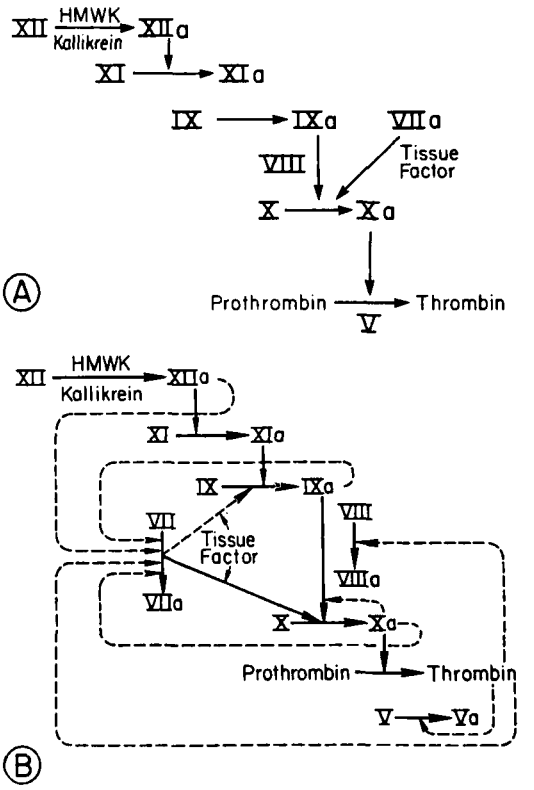


FIGURE 1. (A) A schematic view of blood coagulation. The reactions shown are simply updated from References 1 and 2. In addition, the tissue factor pathway has been indicated. (B) A view of blood coagulation in which most of the described reactions have been included. The physiological significance of the reactions departing from those shown in A has in the main not been evaluated.

initiated by glass contact is called the "intrinsic system", whereas the proteins responding to the addition of tissue factor have been termed the "extrinsic" or "tissue factor" system.

The contact system is now known to consist of at least four proteins: three zymogens, Hageman factor (XII), factor XI, and prekallikrein. A fourth protein, high-molecular-weight kininogen (a bradykinin precursor), is also involved but as a cofactor, not as a zymogen or an enzyme. The reactions of the contact system do not require calcium, so they may be studied in plasma anticoagulated by chelators. Formation of a visible clot, however, is prevented owing to the calcium requirements of later reactions.

The product of the contact system, activated factor XI (XI_a)*, initiates calcium-dependent reactions by proteolytically activating factor IX. Activated factor IX, in another calcium-dependent reaction, then activates factor X by limited proteolysis. This reaction, however, is extremely complicated owing to its requirements for blood platelets or phospholipids and a protein cofactor, factor VIII (the antihemophilic fac-

* By convention the activated form of a zymogen is noted by the suffix "a" (e.g., factor X_a) equivalent to activated factor X.

tor). An interesting aspect of this reaction is that factor VIII has little or no cofactor activity until it is exposed to thrombin, the last protease generated in the coagulation cascade. Prolonged exposure to thrombin renders factor VIII inactive. The combination of factor IX_a, factor VIII, phospholipids, and calcium ions converts factor X to factor X_a, the prothrombin activator.

In a reaction analogous to the preceding one, activated factor X converts prothrombin to thrombin in a reaction markedly accelerated by calcium ions, platelets or phospholipids, and a protein cofactor, factor V. Again, native factor V has little activity until exposed to thrombin. As with factor VIII, further exposure to thrombin generates inert factor V. Thrombin, the enzyme required for the clotting of fibrinogen, promotes gel formation by catalyzing limited proteolysis of fibrinogen. Thus the intrinsic system of coagulation consists of three stages: (1) the generation of activated factor XI; (2) formation of thrombin, and (3) limited proteolysis of fibrinogen resulting in the formation of the fibrin clot.

The addition of tissue factor to blood or plasma substantially enhances the rate at which fibrin forms, generally shortening the clotting time from minutes to about 12 sec. This acceleration is due to a "by-pass" pathway in which factor VII in the presence of tissue factor directly activates factor X. The chemistry of the activation of factor X is similar, whether it is activated via the intrinsic or tissue factor pathway. Similarly, the mechanism of prothrombin activation is independent of the activation pathway, although the yield of products may be under kinetic control, as will be discussed below.

The uniqueness of the coagulation system resides not only in the protein cofactor requirements alluded to above, but also in the calcium and membrane-dependence of some of the reactions. These reactions, which will be discussed in detail, involve the so-called vitamin K-dependent proteins, factors VII, IX, X, and prothrombin. Total synthesis of each of thse factors requires the animal be vitamin K-replete. It is now known that vitamin K is involved in the synthesis of γ-carboxyglutamic acid ("Gla"), which tend to be grouped towards the amino-terminus of these proteins and which are responsible for the calcium and lipid binding properties of these species. The carboxylation and its sequellae will be discussed in detail.

Thus in this review we will emphasize the difference between the serine proteases of coagulation and the digestive proteases. Further, we will emphasize mechanisms by which coagulation may be controlled which while not unique to this system, may regulate and thus limit coagulation to the sites of injury.

III. ASSAYS OF COAGULATION FACTORS: SOURCES OF ERROR

The enzymes, zymogens, and protein cofactors of the coagulation system have usually been assayed by their ability to shorten the clotting time of plasma deficient in a particular coagulation factor. These assays have been performed by using either one- or two-stage techniques. In the former, the factor to be assayed is added directly to the deficient plasma and the clotting time is determined. For example, to assay factor IX, a standard curve is constructed by adding known amounts of factor IX to factor IX-deficient plasma and determining the clotting time for each factor IX concentration. A plot of the logarithm of the clotting time vs. the logarithm of the factor IX concentration approximates linearity.

In a two-stage assay, the zymogen is first converted to the enzyme by prior incubation with an activating enzyme. For example, factor X is quantitatively converted to activated factor X by incubation with the coagulant protein derived from the venom of Russell's viper. The fully activated enzyme is then added to factor X-deficient plasma and the clotting time determined. A standard curve is constructed as above and again, a plot of the logarithm of clotting time vs. enzyme concentration approximates linearity.

While much of the current knowledge of coagulation has been derived from these assays, there are considerable practical and theoretical problems associated with them. First, fairly large changes in the concentration of coagulation components result in small changes in the clotting times. Thus, to generate statistically valid data, many replicate determinations must be done. Further, it would be most desirable if a statistical analysis of the results accompanied publication of the specific activity of a purified coagulation factor. Inasmuch as this is rarely done, one cannot rely on specific activity determinations to compare interlaboratory data.

While the problem of the accuracy and precision of coagulation assays can be dealt with in principle, there is a further difficulty which may not be soluble. Take, for example, an experiment designed to determine whether a zymogen, factor VII, has biological activity; i.e., whether the intrinsic proteolytic activity of the zymogen is sufficient to initiate coagulation or whether proteolytic activation of factor VII is an absolute requirement. The observation is that when the zymogen is added to factor VII-deficient plasma, an acceleration of coagulation follows. However, factor VII can be activated by at least three plasma enzymes: activated Hageman factor, activated factor X, and thrombin. Inasmuch as it would be extraordinarily difficult to determine with certainty that none of these enzymes had catalyzed the conversion of factor VII to factor VII_a during the assay, it follows that the observation of coagulant activity of factor VII is ambiguous. While it is theoretically possible to recover the zymogen from the reaction mixture to determine its physical state, the very high specific activity of the coagulation factors renders this approach unfeasible. The range at which factor VII is assayed, for example, is of the order of 5 to 50 ng/m*l*.

A straightforward way of obviating these problems would be to use synthetic substrates or pseudosubstrates for the assay of coagulation factors. This would require the conversion of each zymogen to its respective enzyme, which can be accomplished with little difficulty. The enzymes of the coagulation system, however, have different requirements for the hydrolysis of their natural protein substrates and for the hydrolysis of small esters or amides. For example, tissue factor increases the rate of the attack of factor VII_a on factor X some 3000-fold, whereas tissue factor has negligible effects on ester hydrolysis catalyzed by factor VII. Further, the enzymes can be manipulated so that they have full esterase or amidase activity, but have little or no activity towards their natural substrates. For example, thrombin can be acetylated, following which it will no longer clot fibrinogen but will possess full esterase activity.[4] A derivative of thrombin resulting from limited proteolysis has also been described with similar properties: it will catalyze hydrolysis of appropriate esters and amides but will not catalyze the cleavage of the bonds required for the clotting of fibrinogen.[5] An analogous set of experiments has demonstrated similar effects on activated factor using material that has been minimally proteolyzed by chymotrypsin or by factor X_a.[6,7] A most interesting result of proteolysis of factor X_a is a product which hydrolyzes prothrombin at rates more or less equivalent to the rates achieved with the intact enzyme. However, while the attack on prothrombin by native X_a is remarkably enhanced by the cofactors, factor V, calcium ions, and phospholipids, the attack by the derivative enzyme is not similarly regulated.

Analogously, a proteolyzed derivative of factor VII has been described which has about 50% of the esterase activity of VII_a, and which incorporates diisopropylflourophosphate (DFP) at about 50% of the rate of VII_a.[8] This enzyme, however, is virtually inert in coagulation assays. Thus, while the use of synthetic substrates and pseudosubstrates has yielded interesting information with respect to these enzymes, they cannot be used to determine the concentration of enzymes active in the coagulation system. It is, of course, of considerable theoretical interest that enzyme derivatives exist which have intact catalytic functions but which do not function in coagulation.

The lack of an active center probe for coagulation enzymes leads to potential errors in the kinetic analysis of coagulation reactions. The error, of course, will be restricted to those parameters dependent upon enzyme concentration, i.e., V_{max} and k_{cat}, but would not affect the determination of the K_m, a parameter independent of enzyme concentration.

To minimize some of the difficulties alluded to above, new assays have recently been designed utilizing the proteins normally hydrolyzed by the enzyme of interest. Although these assays have not been in general use, they appear promising for certain experiments. Their main advantage is that only that fraction of the enzyme population which is competent to function in coagulation is actually measured. One approach which has been effectively exploited by Kosow et al.[9], is to couple the activation of a zymogen with the hydrolysis of a specific amide by the product. Specifically, factor X was activated by a highly purified enzyme from Russell's viper. The reaction was performed in the presence of a chromogenic substrate for activated factor X. The nonlinear progress curves resulting from the coupled reactions were analyzed graphically for the contribution of the first reaction, the activation of factor X. Using this approach, these authors were able to evaluate quantitatively the activation of factor X. The latter would reflect only the specific proteolytic activity of the venom-enzyme. This general approach may be amenable to the study of many of the coagulation reactions. However, it must be shown that the chromogenic substrate acted as neither a substrate for, nor an inhibitor of, the enzyme catalyzing the zymogen activation. This constraint has not yet been dealt with.

Another approach which may have general use was recently developed by Silverberg et al.[10] These investigators developed a radiometric assay for the activation of factor X. The principle of the assay is to radiolabel the potential activation peptide of the zymogen substrate, to separate the activation peptide from the zymogen and other products after initiation of zymogen activation, and to estimate the radioactivity associated with the peptide. Owing to the fact that the bulk (about 80%) of the carbohydrate on factor X is located on the potential activation peptide, labeling was accomplished by reductive tritiation of periodate-generated aldehyde groups on sialic acid residues. The reaction is followed by adding trichloracetic acid at intervals and assaying the soluble tritium in a liquid scintillation counter. (Figure 2) This approach, while very sensitive and precise, does not obviate all ambiguities; the product of the reaction, activated factor X, catalyzes two cleavages in factor VII, one activating the enzyme, one destroying it.[8] Thus, proteolytic feedbacks are potentially as difficult to evaluate in these assays as in the use of assays based on plasma clotting times. To minimize the effects of feedback proteolysis, Silverberg et al. used a reversible inhibitor of factor X_a, benzamidine, which required the use of empirical corrections for the effects of the inhibitor on factor VII_a.[10]

IV. EVALUATING THE SIGNIFICANCE OF COAGULATION REACTIONS

In recent years a multiplicity of reactions have been described which depart from the classicial, linear cascade model. For example, thrombin has been shown to activate factors, V, VIII, and VII; activated factor X has been implicated in the activation of factor VII and factor VIII, and factor XII_a activates factor VII.[11-19] Owing to the structural similarities of the coagulation factors, it is not surprising that under defined conditions, these "side reactions" occur. The critical problem is determining which of these reactions is biologically significant in the coagulation sequence.

Several approaches have been used, but none are entirely satisfactory. Most often, investigators have interpreted "rapid" reactions at high substrate to enzyme ratios as

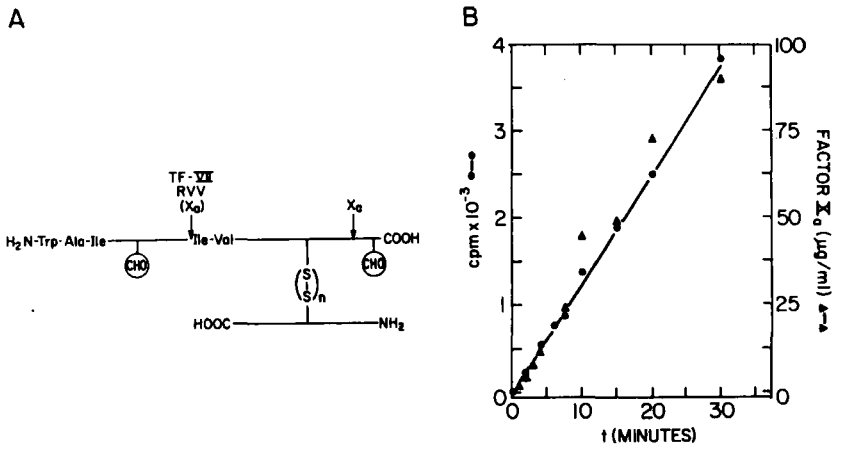


FIGURE 2. (A) Proposed sites of cleavage of factor X. All cleavages occur on the heavy chain. "CHO" indicates sites of attachment of the two carbohydrate chains. (From Jesty, J., Spencer, A.K., and Nemerson, Y., *J. Biol. Chem.*, 249, 5614, 1974. With permission.) (B) The release of soluble radioactivity from factor X with tritiated carbohydrate during the course of activation. (●) indicates TCA soluble radioactivity and (▲) indicates coagulant activity of factor Xa. (From Silverberg, S., Nemerson, Y., and Zur, M., *J. Biol. Chem.*, 252, 8481, 1977. With permission.)

indicative of significance. While for certain restricted purposes this approach may suffice, it ignores the effects of the K_m on reaction velocity. The reaction rate may be insignificant if the physiological level of the substrate is only a small fraction of the K_m.

To minimize this difficulty, it has now become common to set the substrate concentration at about its plasma concentration. This clearly eliminates the K_m problem, but ignores the effects of plasma protease inhibitors and competing substrates. The obvious way to evaluate these reactions would be to define all the kinetic parameters of each reaction, including those describing inhibition and the effects of competing substrates. Inasmuch as this would be a massive undertaking and prone to many errors, it is not clear that this approach is feasible.

An alternative approach, which has been exploited by several investigators, involves the addition of radiolabeled zymogens to plasma, initiation of coagulation by appropriate means, and analysis of the products. The latter most frequently has been accomplished by SDS-gel electrophoresis followed by counting of the sliced gel. Reliable information can be generated by this technique provided certain constraints are met. First, the concentration of added protein should be small compared to the concentration of the unlabeled protein in plasma. If the change in plasma concentration is kept to under about 10%, minimal errors attributable to changing the ratio of substrate concentration to the K_m will occur. Further, it must be shown that the activation of the labeled protein is kinetically indistinguishable from the activation of the native protein. This is somewhat difficult when trace-labeled substrates are used. For example, it is common to use radioiodinated protein in which the substitution is very much smaller than 1 g-atom/mol. In this situation, demonstrating the retention of full biological activity is not adequate: e.g., at molar ratios of iodine to protein of 1:100, the bioassays are inadequate to demonstrate a total loss of activity or change in the properties of the minority population. This difficulty, however, can be dealt with in a convincing manner simply by varying the degree of substitution on the protein and showing that the reaction patterns are the same irrespective of the substitution.

Another problem of potentially serious import and one which is rarely addressed is the effect of dilution of the plasma upon reaction rates. Typically, plasma is diluted anywhere from 10- to 30-fold owing to the addition of various reagents and also because many reactions apparently are faster in diluted plasma. If one is using radiolabeled substrates and the conditions alluded to above are met, valid data may be generated for a diluted plasma system. However, it is not valid to extrapolate from diluted to whole plasma (or vice versa) unless the kinetic parameters of the reaction and competing reactions are known. To take a ficticious but straightforward example, consider one enzyme, one substrate, and one inhibitor. Assume that the enzyme-substrate reaction follows Michaelis-Menton kinetics (that is, a plot of velocity vs. substrate concentration describes a rectangular hyperbola). Further assume that the inhibitor is present in concentrations much greater than the enzyme and that the inhibition kinetics are pseudo-first order. Now if the substrate in undiluted plasma is present at 10 times the K_m and the plasma is then diluted fivefold, the reaction rate will drop from 91 to 66% of V_{max}, a drop of 27%. In the same experiment, the rate of inhibition will fall as a linear function of the inhibitor concentration. Thus, upon dilution, the rate of inhibition will fall by 80%. If, on the other hand, the substrate concentration in plasma is near the K_m, the drop in the reaction rate will more closely approximate the drop in the inhibition rate. From these considerations, it follows that reactions which appear significant in diluted plasma may be much less prominent in whole plasma. Finally, it should be noted that whole blood clotting in vivo may yet differ from the plasma system owing to the presence of blood cells and the vascular endothelium.

V. THE CONTACT SYSTEM

$$\text{Factor XII} \xrightarrow[\text{High-molecular-weight kininogen}]{\text{Kallikrein}} \text{Factor XIIa}$$

$$\text{Factor XI} \xrightarrow[\text{High-molecular-weight kininogen}]{\text{Factor XIIa}} \text{Factor XIa}$$

$$\text{Prekallikrein} \xrightarrow[\text{High-molecular-weight kininogen}]{\text{Factor XIIa}} \text{Kallikrein}$$

For many years it was thought that adsorption of Hageman factor (factor XII) to glass or a glass-like surface would result in its becoming proteolytically active. It could then directly activate factor XI. However, in 1974 Schiffman and Lee in an important set of experiments showed that functionally pure Hageman factor would not activate factor XI even in the presence of kaolin.[20] (Kaolin and celite, both diatomaceous earths, are glasslike substances frequently used to study contact activation.) Addition of small amounts of normal plasma, however, allowed the reaction to proceed rapidly. They named the activity in plasma "contact activation cofactor". This cofactor was subsequently shown to be high-molecular-weight kininogen.[21,22]

Weupper and Cochrane also showed a relative requirement for prekallikrein, a zymogen of a serine protease, for optimal activation of the contact system.[23] Thus, it became clear that at least four proteins were involved in the contact system: Hageman factor, factor XI, prekallikrein, and "contact activation cofactor".

VI. THE PROTEINS OF THE CONTACT SYSTEM

Hageman factor (Factor XII) — Bovine and human Hageman factor have been

purified to apparent homogeneity.[24,25] Each consists of a single polypeptide chain of a molecular weight of about 80,000. An interesting aspect of the bovine protein is a region near its N-terminus that exhibits considerable sequence homology with several protease inhibitors although no function has yet been assigned to this domain of the molecule.[25] No sequence data have yet been published for the human protein. The sequence around the active site serine of bovine Hageman factor has been determined and it exhibits marked homology with the active center of trypsin.[25] From the purification data, the concentration of Hageman factor can be estimated to be 5 μg/m*l* of plasma (bovine) and 30 μg/m*l* (human).

Factor XI — Human, rabbit, and bovine factor XI have been purified to homogeneity by Weupper,[26] by Griffin,[27] and by Koide et al.,[28] respectively, using standard column chromatographic techniques. The molecular weight of the bovine preparation was estimated to be 124,000, as determined by sedimentation equilibrium ultracentrifugation.[28] The molecular weight of the human and rabbit proteins were estimated to be about 160,000, determined by SDS-gel electrophoresis. Owing to the presence of significant amount of carbohydrate on the protein, the molecular weight determined by SDS-gel electrophoresis may be artifactually high. The apparent molecular weight of each protein fell by approximately 50% when they were reduced prior to electrophoresis, thus indicating two subunits of similar molecular weight. Titration of bovine factor XI with antithrombin III, a plasma protease inhibitor which forms 1:1 complexes with many coagulation enzymes,[29] indicated that 2 mol of inhibitor were required to inhibit each mole of factor XI.[30] Thus, the enzyme appears to exist as a disulfide-linked dimer containing two active centers per mole. Whether this molecule circulates as a dimer or dimerization is due to in vitro disulfide interchange is uncertain.

Prekallikrein — The purification of this zymogen from human plasma was reported by Weupper and Cochrane.[31] They found two species with an apparent molecular weight of about 85,000. At this time it is not clear whether two closely related forms of prekallikrein are synthesized or whether one is a derivative of the other. Inasmuch as a prekallikrein antibody detected no antigen in a patient lacking this activity, it appears certain that the second species is not an unrelated, contaminating protein. The overall purification reported was about 1500-fold, implying a plasma concentration of about 50 μg/m*l*. Bovine prekallikrein has also been purified about 2300-fold.[32] These preparations were homogeneous when subjected to isoelectric focusing, disc-gel electrophoresis and ultracentrifugation. A 6000-fold purification of the rabbit protein has also been reported.[31]

High-molecular-weight kininogen — Komiya et al. have extensively purified this protein.[32a] It consists of a single polypeptide chain of mol wt 76,000. Under less rigorous conditions of isolation, a second species has been identified with the same molecular weight, but consisting of two disulfide-bridged polypeptide chains. The latter species quite likely arises from the former by limited proteolysis.

VII. ACTIVATION OF THE CONTACT SYSTEM

As a group, these proteins participate in the generation of factor XI_a. Hageman factor is absorbed to glass or a physiological substance such as collagen. Factor XI and prekallikrein are loosely associated as binary complexes with high-molecular-weight kininogen (HMWK) in plasma. Interestingly, while Hageman factor adsorbs directly to glass, factor XI adsorption requires the presence of HMWK. Using the protein concentrations employed in the absence of HMWK, only 14% of the available

factor XI binds to kaolin, whereas in the presence of HMWK, 87% of the factor XI is bound.[35] Likewise, under similar conditions 14% of the available prekallikrein was shown to bind to kaolin in the presence of HMWK, whereas in its absence, only 2% was bound. Further, the degree of cleavage of prekallikrein was markedly restricted in the absence of HMWK; 9% of the bound protein and 53% of the soluble species were cleaved in the presence of HMWK, whereas in its absence the degree of cleavage was under 1% for both. Thus, HMWK is clearly required for optimal binding and cleavage of prekallikrein and factor XI.

In the complete system, Hageman factor is cleaved into derivatives with differing biological activities. Cochrane and his collaborators have demonstrated what they call reciprocal activation,[36,37] in which activated Hageman factor activates prekallikrein which, in turn, then cleaves Hageman factor forming the more active two-chain species. Depending on the site of the cleavage, activated Hageman factor exhibits different characteristics. Hageman factor is a single polypeptide chain with at least one disulfide bridge. Kallikrein catalyzes two cleavages in the molecule, one inside a loop formed by the disulfide(s), forming a disulfide-linked, two-chain species; cleavage outside the loop results in a two-chain species lacking the disulfide bridge. In the former, the entire molecule remains adsorbed to kaolin; when the cleavage is outside the loop, a 28 K dalton fragment containing the diisopropylphosphofluoridate binding site is released into the supernatant.[38] The latter species rapidly activates prekallikrein but exhibits little coagulant activity. The disulfide-linked species remains adsorbed to kaolin and is thought to be an efficient catalyst of factor XI activation.[39] There are currently no data to suggest whether these cleavages occur in an obligatory or random order. The former species has been termed α-HFa and the latter, β-HFa. It is not clear whether β-HFa can catalyze the activation of factor XI. If it can, it is much slower than α-HFa owing to the fact that factor XI, the substrate, is bound to kaolin and thus is in low concentration in the fluid phase. Activated factor XI, as well, remains bound to kaolin.

A contrary view of Hageman factor activation has come from Ratnoff's work which suggests that the essential step in Hageman factor activation is conformational and not proteolytic. It is clear from these studies that Hageman factor does undergo a conformational transition upon binding. In an early experiment, Donaldson and Ratnoff showed that in the presence of ellagic acid, a soluble activator of Hageman factor, the protein behaved as a large aggregate on sucrose gradient centrifugation.[40] They also showed that following adsorption to barium carbonate and removal of the salt by conversion to the soluble acetate followed by dialysis, Hageman factor also behaved as a large aggregate. McMillin et al. have extended these findings using circular dichroism as a measure of change of protein structure.[41] Here they found a perturbation in the aromatic region of the spectrum upon mixing with ellagic acid or adsorption to quartz, a known Hageman factor activator. Their conclusion was that these conformational changes reflect activation of Hageman factor. While other workers have not dealt with the essential problem of what the initial proteolytic event is, the experiments of Ratnoff and his colleagues are also difficult to interpret because of the problem of correlating a spectral perturbation with proteolytic activity. In particular, the binding of the protein to a glass surface may in itself be associated with structural alterations which effect a spectral perturbation but are independent or unrelated to the generation of enzymatic activity.

In an attempt to obviate this difficulty, Ratnoff and Saito have utilized ellagic acid adsorbed to agarose to activate Hageman factor.[42,43] After interaction of Hageman factor with the agarose beads, Hageman factor was isolated by removal of the beads by centrifugation. This form of Hageman factor could activate factor XI in the presence of high-molecular-weight kininogen and possessed amidolytic activity toward syn-

thetic substrates. Most interestingly, the activated Hageman factor contained a single chain and showed no evidence of proteolysis, as evaluated by SDS-gel electrophoresis.

Although it is certain that cleavage of Hageman factor results in enhanced coagulant activity it is difficult to decide whether the intact molecule has intrinsic activity. For example, Fujikawa et al. found that bovine Hageman factor exhibited only a twofold increment in coagulant activity following cleavage to α-HFa.[44] The functional assay used by these investigators and, indeed, the one that is virtually in universal use, involves the ability of Hageman factor to shorten the clotting time of Hageman-factor-deficient plasma. The assay requires preincubation of all the reactants including kaolin and citrated Hageman factor-deficient plasma for 2 to 6 min prior to the addition of calcium. The observation that α-HFa has only twice the specific coagulant activity of native HF requires that either Hageman has significant endogenous activity or that during the performance of the assay, a considerable amount of Hageman factor has been converted to its activated form. At present, it is difficult to decide between these alternatives.

A considerable part of the confusion regarding the activities of Hageman factor is due to the multiple derivatives of the parent molecule and the subtle differences in their biological activities. As noted above, when attacked by kallikrein, Hageman factor generates two species termed α- and β-HFa. The former results from proteolysis within a disulfide loop in the parent molecule and consists of two chains with a disulfide bond(s). This species has been identified in human and bovine Hageman factor. It has potent clot-promoting activity and remains adsorbed to kaolin. It will also catalyze the activation of prekallikrein which reciprocally activates residual native Hageman factor. Kallikrein also catalyzes hydrolysis of a peptide bond lying outside the disulfide loop. The resultant species of this cleavage contains a free 28 K dalton fragment containing the active center of the molecule. This fragment does not bind to kaolin so it is recovered in the supernatant plasma. It has little or no coagulant activity, but rapidly activates prekallikrein. β-HFa appears to be rapidly inactivated by Cl INH,[45] the complement inhibitor, although no kinetic data have been published. Further, it is possible that as more kallikrein is produced, native Hageman factor and α-HFa will be attacked, generating β-HFa at the expense of α-HFa. If this were the case, the coagulant activities could be viewed as being limited by proteolysis and thus "self-damped".

Bagdasarian et al. have described another derivative of Hageman factor which seems distinct from the above species.[46] When Hageman factor was digested with plasmin, 22% of the protein was recovered as a single polypeptide chain with a molecular weight of 70,000, estimated electrophoretically. In contrast, digestion with kallikrein resulted in the two-chain species alluded to above. The 70,000-mol wt protein which these authors have termed "large activator" has minimal coagulant activity, but rapidly activates prekallikrein. It is not known whether "large activator" arises under physiological conditions. Finally, Stead et al. have described another species which was isolated from human blood.[47] This molecule is slightly smaller than native Hageman factor, having a molecular weight some 5000 less than the native protein as judged by SDS-gel electrophoresis. In contrast to Hageman factor, however, this species was inhibited by DFP and antithrombin III. This protein was composed of a single polypeptide chain; its relationship to the "large activator" isolated by plasmic digestion of Hageman factor by Bagdasarian et al. remains to be elucidated.

The fundamental dilemma facing workers in the field is that blood clots when shed from the body and that this clotting does not require the addition of proteolytic enzymes. From this it follows that either there is a low level of circulating enzymes which are capable of proteolytically activating Hageman factor to initiate clotting when the latter and other components of the contact system are adsorbed to a surface or, alter-

natively, that Hageman factor (the zymogen) can initiate proteolysis upon adsorption. There has been no demonstration of circulating enzymes which could initiate coagulation. Further, treatment of plasma with DFP to inhibit serine proteases does not prolong its clotting time. Thus, the hypothetical enzyme must not be attacked by DFP. At the moment, it would seem that a direct demonstration of this enzyme would be called for. Alternatively, experiments can be designed to test the endogeneous activity of Hageman factor by utilizing modified substrates such as prekallikrein and factor XI. The modification would have to allow these proteins to be adequate substrates, but would have to prevent the generation of enzymatic activity.

In summary, four plasma proteins participate in the contact system. Currently it is clear that three of these proteins are zymogens of serine proteases, that one protein is a cofactor, and that a surface is required for optimal generation of activated factor XI. The mechanism by which the contact system is initiated is unknown. Indeed, the absence of a hemorrhagic disorder in patients lacking Hageman factor, prekallikrein, and HMWK raises questions about the physiologic import of these proteins in the blood coagulation pathways.

VIII. ACTIVATION OF FACTOR IX

$$\text{Factor IX} \xrightarrow[\text{Ca(II)}]{\text{Factor XIa}} \text{Factor IXa}$$

Factor IX is activated in a two-step reaction catalyzed by factor XIa.[48] The first step, which appears to be rate-limiting, involves the cleavage of an Arg-Val bond resulting in a disulfide-bonded, two-chain intermediate which lacks enzymic activity. In a second, slower reaction, an additional peptide bond cleavage liberates an activation peptide from the heavy chain of the intermediate, resulting in the enzymatically active species, factor IX_a. Interestingly, an enzyme from the venom of Russell's viper also activates factor IX, but by a mechanism involving only the second cleavage.[49] That is, no activation peptide is released during activation. The product has about 50% of the activity of factor IX_a produced by the action of factor XIa. Factor VII also catalyzes the activation of factor IX; this will be discussed in the section on the tissue factor pathway of coagulation.

Activated factor IX catalyzes the activation of factor X in a reaction markedly accelerated by factor VIII, the antihemophilic factor. The rate enhancement attributable to factor VIII has been estimated to be in excess of 1000-fold, although precise measurements have not yet been made. Further, no mechanistic or kinetic data are currently available concerning this reaction, which exhibits a requirement for calcium ions and phospholipids.

Considerable confusion exists in the literature with respect to the relationship of factor VIII to the Von Willebrand factor. The latter is not a procoagulant, but is a protein required for the normal aggregation of platelets. The confusion relating to the identity of these proteins stems from the fact that two clearly distinct diseases share a phenotypic expression. Hemophilia A is a sex-linked disorder characterized by a marked reduction in factor VIII. This disease is virtually restricted to males. Von Willebrand disease, on the other hand, is autosomally transmitted and is characterized by a decrease in factor VIII as well as a decrease in the Von Willebrand factor. Thus, there was early confusion about these diseases which arose from the decrease in the procoagulant manifested by both. However, patients with Von Willebrand disease uniquely manifest reduction of Von Willebrand factor which is measured either by immunoassay or by the ability of platelets to aggregate in the presence of ristocetin.

The latter is an antibiotic which acts as a general protein precipitant but which, when used at low concentrations, interacts with platelets and Von Willebrand factor leading to platelet aggregation. Although it seems likely that these two activities, Von Willebrand factor and factor VIII, which are under clearly different mechanisms of genetic control, are properties of different gene products, there is evidence that these proteins are linked in some manner. Thus, Zimmerman et al. prepared an antibody to what probably was Von Willebrand factor and used it to determine antigen levels in plasma.[50] They reported that while patients with Von Willebrand disease had antigen levels which closely paralleled the factor VIII levels (when each was expressed as a fraction of normal population values), patients with hemophilia had antigen-to-procoagulant ratios higher than expected. Moreover, they showed that in obligate carriers of the disease, the ratio of antigen to factor VIII levels were also higher than expected. The latter allowed them to identify carriers, whereas the factor VIII levels alone were rarely diagnostic. The latter observation indicates reciprocal regulation of the levels of these proteins; the suggestion has been made that Von Willebrand factor serves as a carrier protein for factor VIII, perhaps thereby stabilizing it.[51]

Alternatively, however, factor VIII and Von Willebrand factor may simply be different expressions of the same protein. Thus when prepared from frozen plasma, Von Willebrand factor and factor VIII copurify and appear to be associated with a species with a molecular weight in excess of 1,000,000. Upon reduction, a molecular weight of about 200,000 has been reported for the human protein as determined by SDS-gel electrophoresis.[52-54] Similar preparations, when examined by sedimentation equilibrium, yield an apparent molecular weight of about 200,000.[54] Recently, one laboratory has succeeded in separating the two activities in very high yield utilizing fractional precipitation techniques with a solid resin, ethylene/maleic anhydride copolymers.[55] This observation strongly implies that two separate proteins are responsible for factor VIII procoagulant activity and the Von Willebrand platelet aggregating activity. Thus the chemical characterization of factor VIII referred to above may reflect the structure of Von Willebrand factor and *not* the structure of factor VIII.

It has been known since 1963 that coagulant activity of factor VIII is markedly increased by exposure to thrombin.[13] At low thrombin concentrations, a 25- to 50-fold enhancement has been observed which rapidly decays. At higher thrombin concentrations, only a rapid decay of factor VIII is observed. While thrombin stimulation and subsequent inactivation of factor VIII may play a significant role in controlling coagulation, the mechanism of activation by thrombin in this reaction has not been elucidated. Inasmuch as thrombin is a "good" proteolytic enzyme, it appears likely that it functions by virtue of catalyzing limited proteolysis of factor VIII. However, during the activation phase of factor VIII, neither a new NH_2-terminal amino acid nor changes on SDS-gels have been reported. This suggests that either thrombin is not functioning as a proteolytic enzyme during the activation of factor VIII or, alternatively, factor VIII has not been sufficiently purified to observe the expected changes. The latter seems the more likely explanation at the present time.

An intriguing paper recently appeared relating to the role of thrombin in factor VIII activation. Vehar and Davie reported that following thrombin activation,[56] the coagulant activity of factor VIII was inhibited by DFP and by antithrombin III. Inasmuch as DFP is known to inhibit some enzymes by "nonspecific" interactions, that is by other than acylating the active serine, one could attribute the inhibition of factor VIII to a similar mechanism. Inhibition by antithrombin III, however, is not readily explicable and indeed suggests the presence of an active enzyme. Factor VIII, however, either in its native or activated states, has no known substrate (including factor X), so that it is possible that active thrombin is required for the cofactor activity of this spe-

cies. Vehar and Davie interpret their data to show that a serine enzyme results from the action of thrombin on factor VIII.

At present, we could suggest an alternative, but speculative, interpretation of their experiment. It is possible that thrombin binds to, but does not hydrolyze factor VIII. It is also possible that as a consequence of this binding, factor VIII undergoes a change to a more active conformation. If this were the case, and assuming the factor VIII-thrombin complex is reversible, DFP and antithrombin III could compete for thrombin, thus removing it from the reaction. In this regard, it should be noted that DIP-thrombin does not bind to antithrombin III. While this formulation is purely speculative, examples of enzyme stimulation by antibodies, a situation analogous to enzyme-substrate binding, do exist.[57] This concept is consistent with the fact that there are no reports in the literature documenting the activation of factor VIII by thrombin bonded to a solid support, despite the fact that such preparations will clot fibrinogen.

At present, the mechanism of action of factor VIII is obscure. It is generally held, however, that the productive species involved in the activation of factor X is a complex composed of activated factor IX, factor VIII, calcium ions, and phospholipids. The evidence for this essentially rests on one qualitative binding study and one study using inhibition by antibodies. Hougie et al. showed that activated factor IX emerged in the void volume of a gel filtration column when co-chromatographed with phospholipids and calcium.[58] In the absence of these constituents, factor IXa was retarded by the column. Owing to the fact that factor VIII emerges in the void volume irrespective of the presence of other components, no conclusion can be validly reached about the incorporation of factor VIII into the complex. The coagulant activity of the putative complex, however, was significantly higher than that observed when the components were evaluated separately. This observation is consistent with the formation of a productive complex or with activation of factor VIII promoted by the presence of thrombin or a similar enzyme in these preparations. The latter was not rigorously excluded. Further, the demonstration of complex formation by physical analysis would not, in itself, indicate that this presumed enzyme-activator complex is a productive species. A careful kinetic analysis of this reaction will be required before a firm conclusion in this regard can be reached.

Østerud and Rapaport presented additional studies in support of the concept of complex formation.[59] In these experiments, antibodies were raised against factor VIII and factor IX. Addition of either antibody to the reaction inhibited the activation of factor X. The authors concluded that this observation supports the view that the active species was a complex of factors IXa and VIII. This conclusion, however, may be overstated. We feel that an equally valid explanation would be that both components are required for the optimal activation of factor X. That is, this experiment cannot distinguish between three alternative sites of action of factor VIII: (1) activation of the enzyme factor IXa, (2) activation of the substrate, factor X; or (3) dissociation of the enzyme-substrate complex, each of which is consistent with the observations.

IX. THE TISSUE FACTOR PATHWAY

$$\text{Factor X} \xrightarrow[\text{Factor VII}_a]{\text{Tissue factor, Ca (II)}} \text{Factor X}_a$$

Since the turn of the century, it has been known that the coagulation of blood is markedly accelerated by the addition of various tissues. For many years neither the active principle in tissue factor nor its mechanism of action was known, or indeed

studied. Howell in a most remarkable paper showed that ether or alcohol extraction of active tissues reduced their potency, although a weak activity was recoverable from the organic phase.[60] In retrospect, it is clear that the solvent extraction of tissue partitioned the thromboplastin into an inert protein component and a phospholipid component which did not function as tissue factor but accelerated coagulation by (presumably) satisfying the lipid requirement of other reactions. To put this into perspective, it should be noted that the complete lipoprotein (tissue factor) will promote fibrin formation in about 12 sec, whereas the lipid alone will shorten coagulation from a few minutes to perhaps 60 sec.

Chargaff and his coworkers extended these observations in the early 1940s.[61] By employing differential centrifugation and other precipitation techniques, these investigators partially purified tissue factor. In addition, they performed an experiment which ultimately led to techniques useful for the substantial purification of this material. In essence, they showed that when tissue factor was subjected to high-speed centrifugation in the presence of sodium deoxycholate, no activity was recoverable in the pellet. The supernatant was likewise relatively inert. However, when the pellet and supernatant were combined and dialyzed, subsequent centrifugation in the absence of detergent led to pelleting of a highly active material. Thus, it became clear that deoxycholate dissociated tissue factor into two components, both required for optimal activity.

Subsequently, Studer extended these observations using solvent extraction of active particles.[62] He also demonstrated that the extracted, insoluble fraction was inert in coagulation, but that full activity was restored when the lipid-soluble fraction was recombined with the insoluble residue. Hvatum and Prydz[63] and Nemerson,[64] using organic solvents and deoxycholate, each demonstrated in a more detailed manner the presence of a protein and lipid component in tissue factor.

Studies of patients who congenitally lack factor VII showed the requirement for the latter in the tissue factor pathway of coagulation. These patients, whose blood clotted normally in glass tubes, exhibited no acceleration of coagulation when tissue factor was added to their blood. Thus, the concept of a unique pathway of coagulation gained acceptance. It is now generally agreed that the tissue factor pathway consists of only a single, unique reaction: activation of factor X catalyzed by factor VII and tissue factor. The product of this reaction, activated factor X, is identical with activated factor X produced by the action of factors IXa and VIII. Recently, however, Østerud and Rapaport reported that tissue factor-factor VII catalyzes the activation of factor IX.

X. THE PROTEINS OF THE TISSUE FACTOR PATHWAY

Factor VII — Homogenous preparations of bovine factor VII have been reported by two laboratories.[14,65] Owing to the difficulty of separating this protein from prothrombin, multistep procedures including barium adsorption and elution, DEAE-chromatography, affinity chromatography, gel filtration, and preparative electrophoresis have been employed. The product has been extensively characterized, although the complete amino acid sequence has not been reported. Factor VII is isolated as a single polypeptide chain, although if inhibitors of proteolysis are omitted during the purification, *all* the factor VII is recovered as a two-chain molecule. Based on the purification data, estimates of the plasma concentration of factor VII range from 1 μg/m*l* down to about 150 ng/m*l*. This disparity may well be due to problems of assay and will be discussed in detail below. Factor VII is one of the vitamin K-dependent proteins and reportedly contains γ-carboxyglutamic acid residues. Factor VII from other spe-

cies, including man, have been purified extensively, but no reports of chemically homogenous protein have yet appeared. Of interest, however, is a report that factor VII purified from human serum has a lower molecular weight than that purified from plasma as judged from gel permeation studies.[66] If this represents a true change in molecular weight rather than in conformation this would indicate that human factor VII is activated by a mechanism different from that of bovine protein (see below).

Tissue factor — Although there are several reports in the literature of the protein component of tissue factor being purified to homogeneity, there are no convincing data that this has been accomplished. Inasmuch as tissue factor is a cell-bound lipoprotein, purification has been hampered by solubility problems and the tendency of membrane-like proteins to self-aggregate and to aggregate with other proteins having (presumably) extensive hydrophobic domains. Thus, all current attempts to purify this material stems from Chargaff's observation that deoxycholate dissociates the lipid and protein moieties of tissue factor. The basic strategy has been to remove the lipids with solvent or detergent, solubilize the protein in detergent, and then purify the protein using conventional techniques. Following purification, the protein is physically reassociated with lipid, thus restoring biological activity. Using preparative SDS-gel electrophoresis as a purification technique and analytical SDS-gel electrophoresis to document homogeneity and to determine molecular weight, Prydz and his colleagues concluded that monomeric tissue factor is the order of 60,000.[67] Unfortunately, these investigators did not publish a purification table so that it is impossible to compare these results to those obtained in other laboratories. One group has reported upwards of 1000-fold purification of this protein, which was a single band on acrylamide-gel electrophoresis but which demonstrated considerable heterogeneity upon SDS-gel electrophoresis.[68] Gonmori and Takeda have compared tissue factor isolated from a variety of tissues.[69] They report different molecular weight for each species of protein and therefore conclude that tissue factor is organ-specific, although each evidently functions in a similar manner in coagulation. This study, too, suffers from a lack of convincing data relating to the purity of the protein. It is clear that studies of this sort can be considered conclusive only when stringent criteria of purity have been met. In this regard, for the demonstration of homogeneity, the stained band must be correlated with activity and, certainly, more than one gel system should be employed as well as various loads of protein on the gels.

XI. THE INITIATION OF THE TISSUE FACTOR PATHWAY

The primary conceptual problem relating to the initiation of coagulation by tissue factor is similar to that discussed with respect to the contact system: the addition of a nonproteolytic substance, tissue factor, initiates a series of reactions in which proteolysis is the predominant mechanism of zymogen activation. Certainly it has been conclusively shown that factor X is proteolytically activated by factor VII_a in the presence of tissue factor.[70] Further, as expected, it has been shown that the product, factor Xa, is identical in all respects with that generated by the intrinsic system.[71] The predominant question, therefore, is really whether proteolysis, i.e., activation of factor VII, is required for the initiation of the tissue factor pathway.

Factor VII is isolated from plasma as a single-chain protein. It appears certain that this species is indeed the zymogen form of factor VII owing to the structural homology that exists between its NH_2-terminal region and that of prothrombin. It has been shown that at least three enzymes which arise during coagulation, thrombin, factor Xa, and

activated Hageman factor, catalyze the cleavage of an Arg-Ile bond in factor VII generating a two-chain, disulfide-bonded species.[72,73] When measured in a standard coagulation assay, the two-chain factor VIIa has some 50-fold greater activity than the precursor. An interesting and theoretically important problem is whether the activity of the zymogen measured in coagulation assays is intrinsic to the zymogen or whether it is a function of contaminating factor VIIa.

Somewhat circumstantial evidence favors the view that the endogenous activity of the zymogen is sufficient to initiate coagulation in the presence of tissue factor. First, the zymogen is uncommonly reactive with DFP. Activated factor VII incorporates ^{3}H-DFP with a pseudo first-order rate constant of 0.13 sec^{-1}, whereas the zymogen reacts with a rate constant of 0.03 sec^{-1}, approximately fourfold slower.[74] To place this in the perspective of other enzyme-zymogen pairs, it should be recalled that trypsinogen incorporates DFP at rates 4 to 5 orders of magnitude slower than does trypsin.[75] Further, it has been shown that both forms of factor VII incorporate DFP to the same extent, about 0.9 mol/mol of protein; following digestion with trypsin and thermolysin, the same peptide in each protein was found to be radiolabeled.[72]

While the experiments with DFP demonstrate the unique properties of factor VII, they do not prove that zymogen has proteolytic activity; one cannot equate reactivity with a pseudo-substrate with proteolytic activity. Accordingly, Zur and Nemerson studied the esterase activity of each species.[74] Using a highly sensitive substrate (a substituted nitrobenzyl ester), these investigators found the K_m of the zymogen-catalyzed hydrolysis to be greater than one order of magnitude higher than when the reaction was catalyzed by activated factor VII. Inasmuch as the K_m is independent of enzyme concentration, the catalysis cannot be attributed to contaminating activated factor VII. These results confirm the reactivity of the zymogen suggested by its ability to react with DFP. As noted previously, the enzymes of the coagulation system can be modified in a manner such that they exhibit esterase activity, react with DFP, but do not function proteolytically in coagulation. Because of this phenomenon, these experiments alone cannot prove the contention that factor VII is proteolytically active towards factor X.

In this regard, the demonstration that factor VII, the zymogen, accelerates coagulation is also inadequate. This is due to the intrinsic ambiguities of coagulation assays. Specifically, although factor VII is active in coagulation assays, it is clearly possible that following its addition to plasma, it is converted to activated factor VII by an enzyme in plasma. This problem led Silverberg et al. to design an assay which, in principle, could eliminate these ambiguities.[10] These investigators exploited the fact that the bulk of the carbohydrate on factor X resides near the NH_2-terminus of the molecule on the potential activation peptide. Thus when factor X is activated by cleavage of Arg_{s1}-Ile_{s2}, approximately 80% of the carbohydrate is released from the molecule. These investigators radiolabeled the carbohydrate by reductive tritiation of oxidized sialic residues. Owing to the fact that factor X and activated factor X are insoluble in TCA whereas the peptide is soluble, reaction rates are conveniently determined by following the appearance of TCA-soluble radioactivity.

Because activated factor X, the product of this reaction, can catalyze the activation of factor VII and also the hydrolysis of a bond near the carboxyterminus of the heavy chain of factor X (thus releasing the remaining 20% of the carbohydrate), benzamidine, a competitive inhibitor of activated factor X, was included in the reactions. Using this technique, the kinetic parameters of the reaction catalyzed by activated factor VII were determined (see below). However, when factor VII was used as the catalyst, it was found that it has kinetic parameters indistinguishable from those found for activated factor VII. The only discernible difference in the reactions was a lag period when the zymogen was used.

A similar phenomenon was recently reported by Østerud and Rapaport using human proteins and a somewhat different assay.[76] These investigators incubated factor VII or activated factor VII with tissue factor and factor X. At intervals, aliquots were removed from the reaction and the amount of activated factor X was estimated from the rate of hydroloysis of a chromogenic substrate. Again, although factor VII and activated factor VII exhibited considerably different activities in a coagulation assay, they gave similar results using this technique, the only observable difference being a short lag when the zymogen was used. The obvious interpretation of these results is that factor VII is converted to its activated form during the course of the reaction. However, this has not yet been demonstrated.

Until recently it was thought that the activation of factor X was the only reaction involved in the tissue factor pathway. Østerud and Rapaport, however, recently documented the activation of factor IX by factor VII and tissue factor.[77] This observation has been confirmed and extended to the bovine system by Zur et al.[78] Although this reaction appears to proceed rapidly, the evaluation of this pathway must await a kinetic analysis. It should be pointed out, however, that factor VII_a directly activates factor X whereas by activating factor IX, an enzyme is generated which exerts its biological activity by activating factor X in a factor VIII-dependent reaction, i.e., it is a step removed from the direct product of the tissue factor pathway. In addition, available evidence suggests that activation of factor X by activated factor IX and factor VIII is slow compared to the direct activation of factor X by factor VII_a and tissue factor. Thus, the significance of this pathway is not clear. It is possible, however, that this pathway could predominate if, for example, the K_m for factor X-activation catalyzed by factor IXa is well below that for the reaction catalyzed by factor VII.

XII. KINETICS OF THE TISSUE FACTOR PATHWAY; THE ROLE OF TISSUE FACTOR:

As noted repeatedly, one of the unique aspects of coagulation is the requirement for several protein or lipoprotein cofactors. It has been known for over 75 years that tissue factor accelerates coagulation and in recent years, it has become clear that the main site of action of tissue factor is the reaction in which factor VII (or VIIa) activates factor X. Until recently there have been no kinetic or mechanistic studies relating to the action of tissue factor.

Using radiometric assay detailed above, Silverberg et al. performed a kinetic analysis of this reaction.[10] In the presence of tissue factor, the K_m was found to be about 0.34 μM. Owing to the fact that the factor X concentration in plasma has been estimated to be about 0.15 μM, the reaction is clearly substrate-controlled under physiological conditions. The k_{cat} was found to be 32 sec^{-1}. In the absence of tissue factor, the K_m was 3.8 μM and the k_{cat} was reduced 2900-fold. From these data, it follows that at plasma concentrations of factor X, tissue factor would accelrate the reaction about 16,000-fold. It should be stressed that the bulk of this acceleration, 2900-fold, is attributable to an increase in the k_{cat}, whereas the decrease in the K_m contributes only a sevenfold rate enhancement. The site of action of tissue factor has not yet been determined, although a likely mechanism appears to be dissociation of the enzyme-substrate complex.

XIII. MECHANISM OF ACTIVATION OF FACTOR X

$$\text{Factor X} \xrightarrow[\text{Ca(II)}\ \text{Phospholipid}]{\text{Factor IXa}\ \text{Factor VIII}} \text{Factor Xa}$$

$$\text{Factor X} \xrightarrow[\substack{\text{Ca(II)}\\\text{Tissue Factor}}]{\text{Factor VIIa}} \text{Factor Xa}$$

Factor X is unique among the zymogens of serine proteases involved in blood coagulation in that it is composed of two polypeptide chains linked by disulfide bonds.[79,80] Bovine factor X, with a molecular weight of 56,000,[79,80] has a known amino acid sequence.[81,82] The heavy chain of factor X has a molecular weight of 38,000. The light chain, with a molecular weight of 18,000, contains γ-carboxyglutamic acid and is homologous with the N termini of the other vitamin K-dependent blood coagulation proteins.[83]

The activation of factor X can be catalyzed by activated factor IX, factor VIII, Ca(II), and phospholipid or by factor VIIa, tissue factor, and Ca(II). Alternatively, factor X may be activated in the presence of Ca(II) nonphysiologically by a protease from Russell's viper venom. The change in the covalent structure of factor X upon activation by any of these enzymes appears identical.[70,71] Activation of factor X is concomitant with the hydrolysis of the Arg_{51}-Ile_{52} bond on the heavy chain.[84] The activation peptide (M, 11,000) contains most of the carbohydrate associated with this protein. Activated factor X includes a heavy chain (M, 27,000) which contains the active site serine,[85,86] the unaltered light chain, and the noncovalently bound activation peptide.[87]

A second peptide bond is cleaved in activated factor X near the C terminus.[70,84] This modification, mediated enzymatically by activated factor X itself, has no effect on the esterase or coagulant activity of activated factor X. This reaction is accelerated significantly by the presence of phospholipid and Ca(II). This peptide does not appear to modulate any reaction.

Like trypsinogen conversion to trypsin, the activation of factor X to activated factor X involves important functional, but subtle structural changes.[88] A detailed spectroscopic study revealed that activation of factor X is associated with the partial exposure of a tryptophan and tyrosine residue to solvent. However, no apparent alteration in the circular dichroism spectrum was observed. These results emphasize that minor transitions in the three-dimensional structure can accompany peptide bond cleavage and the development of enzymatic activity in this and other zymogens.

XIV. MECHANISM OF ACTIVATION OF PROTHROMBIN

$$\text{Prothrombin} \xrightarrow[\substack{\text{Factor V}\\\text{Ca(II)}\\\text{Phospholipid}}]{\text{Factor } X_a} \text{Thrombin}$$

The substrate of activated factor X is prothrombin. Bovine prothrombin consists of a single polypeptide chain with a molecular weight of about 70,000.[89] The amino acid sequence of bovine prothrombin and much of human prothrombin is known.[90-92] The ten γ-carboxyglutamic acid residues are located near the N terminus.[93]

The activation of prothrombin to thrombin by activated factor X involves two obligatory peptide bond cleavages.[94-100] The overall reaction has a requirement for factor V, membrane surfaces, and metal ions for optimal rates of thrombin generation. In the conversion of bovine prothrombin to thrombin, initial cleavage of prothrombin by activated factor X yields an enzymatically inactive intermediate, prethrombin 2 (M, 41,000), and fragment 1-2 (M, 35,000). Prethrombin 2, which derives from the C ter-

minus of prothrombin, is a single-chain structure. Activated factor X converts prethrombin 2 to α-thrombin, a two-chain enzyme, by cleavage of a peptide bond within a loop formed by a disulfide bond. Thrombin has a molecular weight of 41,000.

The product of this reaction, thrombin, can also act upon prothrombin. Prothrombin is cleaved by thrombin to yield the thrombin intermediate, prethrombin 1 (M_r 51,000), and fragment 1 (M_r 23,000). Like prothrombin, prethrombin 1 can be activated to thrombin by activated factor X by two peptide bond cleavages. Furthermore, thrombin can cleave fragment 1-2 yielding fragment 1 (M_r 23,000) and fragment 2 (M_r 13,000).

Factor V, the protein cofactor in this reaction, is a single chain polypeptide with a molecular weight of 330,000.[100-103] In its native form it has minimal ability to accelerate the conversion of prothrombin to thrombin. However, treatment with thrombin enhances its activity up to 100-fold. Like factor VIII, factor V is both activated and degraded by thrombin. Recent experiments have shown that the activation of factor V by thrombin involves limited proteolysis.[102,103]

The mechanism of assembly of activated factor X, metal ions, phospholipid, factor V, and prothrombin is unknown. The rates of prothrombin and prethrombin 1 conversion to thrombin by activated factor X are accelerated by factor V while that of prethrombin 2 is not.[104] These observations suggest that the fragment 2 region of prothrombin is either a portion of the factor V binding site on prothrombin or is required to maintain the three-dimensional structure of prothrombin to facilitate factor V binding. In studies employing affinity chromatography as an analytical method, thrombin-activated factor V was found to bind to prothrombin while native factor V did not.[105] The recent availability of highly purified factor V should enable systematic study of the quaternary structure of the enzymatic machinery which activates prothrombin.

Although the consequences of prothrombin activation have been studied in chemically defined system, the pathways of prothrombin activation in vivo are unknown. Specifically, it is uncertain whether thrombin-mediated cleavage of prothrombin occurs in physiologic systems. Aronson et al. quantitatively recovered intact fragment 1-2 from whole clotted blood.[106] This observation suggests that the thrombin activity generated during clot formation neither acts upon prothrombin nor fragment 1-2. Furie et al. have examined the pathways and kinets of prothrombin activation of kaolin-activated human plasma containing either phospholipids or platelets.[107] In these experiments in which clotting is initiated through the contact system, fragments of radiolabeled prothrombin supplemented to plasma were identified and quantified by SDS-gel electrophoresis and radioactivity assay. Under the conditions employed, approximately 10% of the prothrombin is consumed during clot formation. Fragment 1 and prethrombin 1 are not observed prior to clot formation, indicating that prothrombin activation in plasma involves only the two activated factor X-mediated cleavages of prothrombin. Furthermore, although small quantities of thrombin appear in the early stages of clot formation, the major prothrombin conversion and thrombin generation take place in the final seconds prior to clot formation.[108]

XV. METALS AND MEMBRANES IN BLOOD COAGULATION

A unique feature of the enzymatic reactions of the blood coagulation cascade is the occurrence of many, if not all, of these reactions on membrane surfaces. Although a role for endothelial cell membranes cannot be excluded, the presumed major participant in blood coagulation is the platelet surface.[109] In vivo, circulating platelets collect at the site of vascular injury and undergo a series of events which are known collectively as "platelet activation". Through mechanisms which biochemically translate the

events of tissue injury into signals to initiate blood coagulation, the clotting cascade is activated. Participating coagulation proteins, in the presence of metal ions, appear likely to interact with the platelet surface in a highly organized array. These proteins are sequentially activated, promoting clot formation resulting from conversion of fibrinogen to fibrin.

Unlike the digestive proteases such as trypsin and chymotrypsin, conversion of the blood clotting proteins from their zymogens to their active enzymes requires cofactors. These cofactors almost certainly do not directly modify the catalytic activity of the serine proteases; rather it appears likely that they function by promoting assembly of the productive complexes or, perhaps, by accelerating product formation by interacting with enzyme-substrate complexes. A unique feature of the enzymatic reactions of the blood coagulation cascade is the occurence of many, if not all, of these reactions on membrane surfaces. As previously noted, activated factor X in the presence of Ca(II) activates prothrombin at a very slow rate; the reaction is enhanced 50-fold by the addition of phospholipid vesicles, 20,000-fold by the addition of factor V, and 300,000-fold if platelets are substituted for phospholipid vesicles in the complete system.[110] It would thus appear that on the time scale that blood coagulation occurs physiologically, the complete system is obligatory. Indeed, control of the amount of each cofactor available to participate in the reaction could allow for modulation of the rates of activation of the clotting cascade.

The discovery of γ-carboxyglutamic acid in the vitamin K-dependent proteins has intensified efforts to understand the interrelationship of the vitamin K-dependent blood coagulation proteins, metal ions, and the platelet membrane.[93,111,112] The metal-binding properties of these proteins involve, at least in part, γ-carboxyglutamic acid residues.[114,115] The vitamin K-dependent blood coagulation proteins represent a new biological class of Ca(II)-binding proteins which interact with the platelet membrane in the presence of metal ions.

XVI. METAL-BINDING PROPERTIES OF BLOOD COAGULATION PROTEINS

Calcium ions play a central role in blood coagulation, being required in the enzymatic activation of the four vitamin K-dependent clotting proteins — factor X, factor IX, factor VII, and prothrombin. For many decades it has been known that the vitamin K-dependent coagulation proteins bind certain insoluble salts, particularly those of Ba(II). This property has been the basis for partial purification of these proteins from plasma and is apparently related to the metal-binding properties of these proteins.

Before discussing specific details of metal-protein interactions involving the blood clotting proteins, we may consider some of the general features of the interactions of calcium ions with metal-binding sites of proteins. Calcium ions have a preference for oxygen ligands. In proteins, Ca(II) is bound to the oxygen atoms on the side chains of glutamic acid and aspartic acid residues and to the carbonyl oxygen in the peptide backbone. Since the interaction of Ca(II) with glutamic acid or aspartic acid is weak, physiologic Ca(II) concentrations in plasma (approximately 1 m*M*) are too low to support significant Ca(II) interactions with these amino acids alone. The metal-binding sites of Ca(II) binding proteins, therefore, are formed by several or more amino acid chains with donor oxygens, and by carbonyl groups which contribute, *in toto*, to the energy involved in metal-protein interaction. The geometry of the donor oxygens of the carboxylate and carbonyl groups is defined by the tertiary structure of the protein to optimize metal binding and is often modified by the bound metal itself. Furthermore, the affinity of Ca(II) for the metal-binding site is also determined by the oxygen-

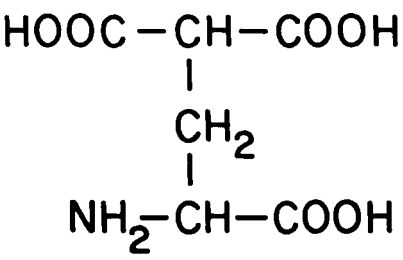


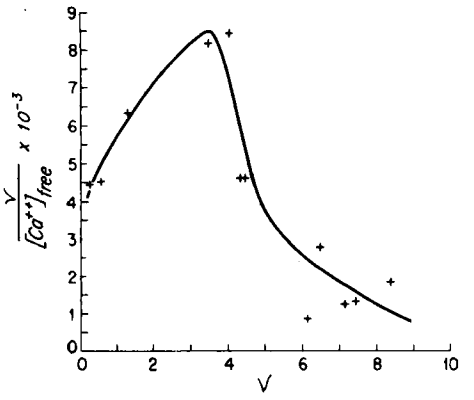
FIGURE 3. γ-Carboxyglutamic acid. This amino acid is found in the vitamin K-dependent blood coagulation proteins near the N terminus of prothrombin, factor IX, and the light chain of factor X. It is assumed to be present in factor VII. γ-Carboxyglutamic acid is formed from glutamic acid by a vitamin K-dependent hepatic carboxylase.

Ca(II) distances, the number of participating oxygen ligands, the Ca(II) coordination number of six to eight oxygen atoms, and the energy involved in altering the protein conformation to optimize the geometry of the metal binding sites in the metal-protein complex. Of the Ca(II) binding proteins studied, most have two to four anionic amino acids or carbonyl groups contributing to metal liganding. Of special interest in the vitamin K-dependent blood coagulations is the presence of γ-carboxyglutamic acid (Figure 3). The side chain of this amino acid, analogous to a malonic acid derivative, has a greater affinity for Ca(II) than does glutamic or aspartic acid residues. The interaction of Ca(II) with malonate is described by a binding constant K_D of about $3 \times 10^{-3} M$. At Ca(II) concentrations in blood, significant Ca(II)-γ-carboxyglutamate complex formation will occur in the absence of contributions from other anionic amino acid side chains. As might be expected, this amino acid plays a dominant role in defining the metal-binding properties of the vitamin K-dependent blood coagulation proteins.

To date, extensive metal-binding studies of the blood coagulation proteins have been restricted to factors IX, X, and prothrombin. For this reason, discussion of these proteins will be emphasized. However, it is likely that factor VII has metal-binding properties similar to prothrombin and factor X. It is also possible that some of the other coagulation proteins may also bind Ca(II).

Prothrombin and factor X have been found to have altered electrophoretic mobility in the presence of Ca(II).[116,117] Experiments employing equilibrium dialysis, rate dialysis, membrane filtration, and gel filtration have shown that Ca(II) binds to prothrombin weakly with a binding constant K_d of about 1 mM.[118-123] Although many of these studies have been performed with considerable care and interpreted with appropriate caution, basic questions as to the number of metal-binding sites, the number of classes of binding sites, the affinity of these binding sites for Ca(II), and the interaction between metal-binding sites remain controversial. Although variations in pH, buffers, and ionic strength may relate to some of these discrepancies observed, the primary problem may be associated with the difficulty of measuring weak ligand-protein interactions in which the proteins, at the high concentrations required for these studies, self-associate in the presence of metal ions.

In an experiment typical of those performed in many laboratories, Mann and colleagues employed equilibrium dialysis to evaluate the binding of Ca(II) to bovine prothrombin. The Scatchard analysis of these data is shown in Figure 4A. By inspection



(A)

FIGURE 4. Interaction of metal ions with bovine prothrombin. (A) Scatchard plot of the interaction of Ca(II) with prothrombin. Prothrombin, 4.5×10^{-5} *M*, Ca(II), 7.5×10^{-5} to 1×10^{-2} *M*, (From Bajaj, S. P., Butkowski, R. J., and Mann, K. G., *J. Biol. Chem.*, 250, 2150, 1975. With permission.) (B) Scatchard plot of the interaction of Gd(III) with prothrombin. Prothrombin, 1×10^{-6} *M*, Gd(III), 0.48×10^{-6} *M* to 6.5×10^{-6} *M*. (From Furie, B. C., Mann, K. G., and Furie, B., *J. Biol. Chem.*, 251, 3235, 1976. With permission.) (C) Scatchard plot of the interaction of Gd(III) with prothrombin. Prothrombin 1.1×10^{-5} *M*, Gd(III), 2.5×10^{-6} to 2.2×10^{-4} *M*.

it would appear that the interaction of Ca(II) with prothrombin is complex, characterized by multiple binding sites, a low association constant (K_A), and a nonlinear relationship between r/C vs. r, where r is the concentration of bound Ca(II) divided by the protein concentration and C is the concentration of free Ca(II). These data have been interpreted to suggest that at low Ca(II) concentrations characteristics of cooperative interaction between metal binding sites can be observed.[118,123] Although some investigators have concluded that there are at least two classes of metal-binding sites on prothrombin,[118] others have concluded that the class of binding sites cannot be unambiguously ascertained.[123] Because the binding studies have been performed at concentrations of protein that are 50- to 100-fold lower than the dissociation constant K_D, these experiments are inherently inaccurate. Thus, alternative interpretations are possible. Evidence exists indicating that prothrombin self-associates in the presence of metal ions to form dimers.[124] Although the details of the molecular basis for dimerization are not known, it is compelling to suggest that a metal ion bridges two γ-carboxyglutamic acid residues in two prothrombin molecules to form an electrostatic intermolecular bond. Geometric and symmetrical features permitting, a number of the γ-carboxyglutamic acid residues of one prothrombin could interact with those of another prothrombin molecule yielding a dimer with multiple weak, metal-dependent, electrostatic bonds involving γ-carboxyglutamic acid residues. We might predict that these metal-binding sites would exhibit positive cooperativity. Formation of one electrostatic bridge with a metal ion could constrain the ternary complex and the geometry of a second metal-binding site so as to decrease the energy required to ligand a second metal

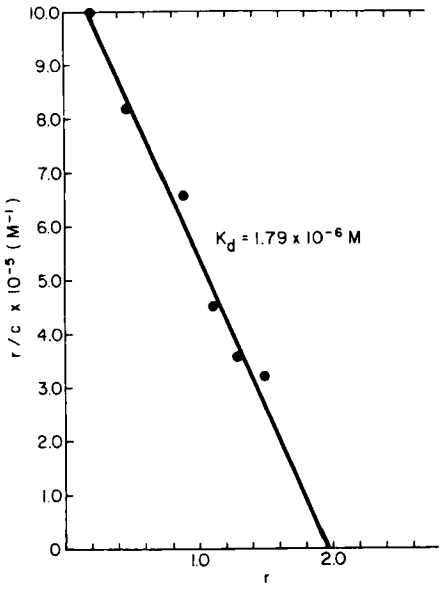


FIGURE 4B

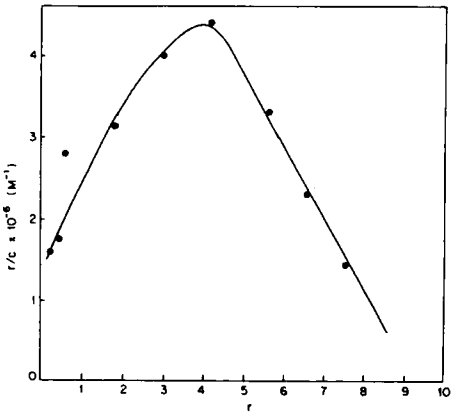


FIGURE 4C

metal-binding properties of a metal-dependent self-associating protein system or a protein with complex metal-binding characteristics becomes impossible.

To circumvent some of these problems, the interaction of other metal ions with prothrombin and factor X has been investigated. Like studies with Ca(II) these approaches have had both virtues and shortcomings. Trivalent lanthanide ions have been widely used to explore the metal-binding sites of Ca(II)-binding proteins since their introduction for this purpose a decade ago,[126,127] Lanthanide ions have ionic radii similar to Ca(II). Because they are trivalent, these metal ions tend to bind to metal-binding sites with a binding constant Kd about three orders of magnitude lower than Ca(II). Also, lanthanide ions are useful spectroscopic probes of the metal-binding sites of Ca(II) binding proteins because of their electronic and magnetic properties.

Using ^{153}Gd(III) and steady-state rate dialysis, two separate classes of binding sites have been identified on prothrombin and factor X.[125,128] Two high affinity metal-binding sites, which bind Gd(III) with a K_D of 0.75 μM do not show evidence of cooperative interaction between sites at the concentration of protein employed — 0.1 mg/mℓ or 1 $\times$ 10^{-6} M (Figure 4B). At higher concentrations of Gd(III), lower affinity metal-binding sites (K_D, 20 μM) can be identified. No evidence of complex binding is observed at low protein concentrations; at higher concentrations of protein, Scatchard plots of these data describing the interaction of Gd(III) with either factor X or prothrombin resemble those describing the interaction of Ca(II) with these proteins (Figure 4C). If the finding of cooperativity in solutions of purified proteins is dependent upon protein concentration, it becomes more likely that the molecular basis of this cooperativity is related to protein-protein association through metal bridges than to a metal-dependent intramolecular conformational rearrangement of the protein which alters the character of metal binding.

Thus we believe that these metal-binding studies offer no compelling evidence for cooperativity among the metal-binding sites of prothrombin. Two disclaimers are necessary, however. Firstly, the absence of observed cooperative metal-prothrombin interaction in these experiments does not preclude its presence. Rather, more sensitive and precise techniques might detect such an intramolecular phenomenon with considerable

ion. This metal binding reflects positive cooperativity but is perhaps of no significance to prothrombin function because the phenomenon is observed only at high nonphysiologic prothrombin concentrations. Indeed, a prediction of this hypothesis is that at physiologic concentrations of prothrombin (about 1.4 μM), complex binding of metal ions with prothrombin will not be observed. However, as noted, studies with calcium ions are difficult at low prothrombin concentrations because of the low affinity of Ca(II) for prothrombin.

Studies of the Ca(II)-binding properties of factor X have yielded data and interpretations similar to those observed for prothrombin. Henrickson and Jackson obtained complex data characterizing the interaction of factor X with Ca(II)[123]. These results were interpreted in terms of a complex model involving 20 Ca(II)-binding sites of factor X, of which some exhibit positive cooperativity. A binding constant K_D of 1.6 mM was estimated. Isolation of the light chain of factor X following reduction and alkylation (and concomitant destruction of the native three-dimensional structure) permitted investigation of the interaction of Ca(II) with this γ-carboxyglutamic acid-rich polypeptide. Although metal binding could be detected, the binding affinity of the metals for the light chain was 20-fold weaker than for native factor X.

Using steady-state rate dialysis at lower protein concentration, two high affinity Ca(II) binding sites on factor X can be detected.[125] These sites bind Ca(II) with a K_D of 2.3×10^{-4} M; many lower affinity metal-binding sites are also observed. These results are not consistent with those described above. Once again, distinguishing between the physiologic implications. Secondly, the presence or absence of cooperative metal binding in metal-prothrombin binary complex formation is unrelated to the presence or absence of cooperative metal binding in the formation of the prothrombin-metal-membrane ternary complex. Indeed, as will be indicated below, cooperative metal binding is expected in the metal-dependent bridging of prothrombin to anionic membranes via a mechanism similar to that described for prothrombin dimer formation.

Lanthanide ions cannot be substituted for Ca(II) in the metal-dependent activation of factor X by the coagulant protein of Russell's viper venom.[125] In fact, lanthanide ions show characteristic competitive inhibition kinetics of Ca(II)-dependent factor X activation, indicating that Ca(II) and lanthanide ions compete for the same metal-binding sites. Although lanthanide ions cannot replace Ca(II) in factor X activation, they do facilitate metal-dependent ternary complex formation of factor X-metal-venom coagulant protein. In contrast, lanthanide ions can substitute for Ca(II) in metal-dependent prothrombin activation catalyzed by activated factor X.[128] Maximal rates of activation are observed at lanthanide ion concentrations of 20 μM (compared to Ca(II) of 1 mM), but are only 25% of that observed with Ca(II).

Mn(II) has also been employed to study the metal-binding sites of prothrombin and factor X.[129,130] Mn(II) is divalent and has a coordination number similar to that of Ca(II). Although manganous ions tend to interact with nitrogen donors more than does Ca(II), they also interact with oxygen donors, specifically the carboxylate group. Most importantly, Mn(II) is paramagnetic with a single unpaired electron and can be employed in a facile manner using magnetic resonance spectroscopy. Using electron paramagnetic resonance to monitor the titration of prothrombin by Mn(II), two high affinity metal-binding sites (K_D, $2.2 \times 10^{-5} M$) and at least two lower affinity metal-binding sites (K_D, $2.5 \times 10^{-4} M$) were identified. Positive cooperativity in Mn(II) binding to prothrombin was observed for the high affinity sites, but is subject to the same interpretive uncertainties as similar data obtained with Ca(II) or Gd(III). Ca(II) and Mn(II) appear to compete for the same sites since Ca(II) can displace bound Mn(II) from prothrombin. An identical approach to the study of the Mn(II) binding properties of factor X revealed about eight metal binding sites which bind Mn(II) with a K_D of 9

$\times 10^{-5} M$. Ca(II) can easily displace six of eight bound Mn(II), but the two Mn(II) bound with highest affinity cannot be displaced with a Ca(II) at concentrations employed. This is presumably due to the fact that higher Ca(II) concentrations cause protein precipitation.

At least one of the metal-binding sites on factor X and prothrombin is in close proximity to a tryptophan residue. Tb(III), a fluorescent lanthanide ion, undergoes marked fluorescence enhancement when bound to either of these proteins when the protein-metal complex is irradiated with light which is absorbed by tryptophan or tryosine residues in the protein.[125] The absorption difference spectrum comparing factor X in the presence and absence of Ca(II) is consistent with a tryptophan perturbation;[88] similar studies have been performed on prothrombin.[131]

XVII. γ-CARBOXYGLUTAMIC ACID-A METAL BINDING AMINO ACID

The synthesis of γ-carboxyglutamic acid is dependent upon adequate concentrations of vitamin K in the mammalian liver. In the absence of vitamin K or in the presence of vitamin K antagonists such as dicoumarol or sodium warfarin, plasma may contain separate forms of these proteins which are characterized by the absence of coagulant activities but which are immunologically identical to their fully carboxylated counterparts.[116,117] The material which is antigenically related to prothrombin, termed abnormal prothrombin, is structurally similar to prothrombin except that it does not bind Ca(II),[121,122] does not bind phospholipid vesicles in the presence of metal ions,[132] and does not contain γ-carboxyglutamic acid.[111,112] It now appears that these proteins are synthesized in a precursor form and some of the glutamic acid residues near the N terminus are carboxylated by a vitamin K-dependent hepatic carboxylase to yield biologically active proteins containing γ-carboxyglutamic acid.[133]

The metal-binding properties of γ-carboxyglutamic acid have been studied in detail to describe further the special role that it plays in the vitamin K-dependent proteins.[134,135] The γ-carboxyl groups of γ-carboxyglutamic acid have a pK_1 of 4.3 and pK_2 of about 2.0. Like glutamic acid, the side chain of γ-carboxyglutamic acid is completely ionized at pH 7.4 At pH 6.5 γ-carboxyglutamic acid binds Tb(III) with a binding constant, K_D, of about 50 μM and a stoichiometry of 2 mol of γ-carboxyglutamic acid to 1 mol of metal. In contrast, a peptide (CBZ-D-Gla-D-Gla-OMe) binds Ca(II) or Mg(II) with a K_D of about 0.6 mM[135] γ-carboxygutamic acid and some of the carbon atoms within the metal-binding domain of the amino acid have been determined by paramagnetic relaxation enhancement studies;[134] a model of the Gd(III)-γ-carboxyglutamic acid complex has been generated (Figure 5). Several features of this complex may be considered. Firstly, the metal is bound symmetrically between the two γ-carboxyl groups about 3.3 Å from the carboxyl carbons. Secondly, the interaction of metal ions with γ-carboxyglutamate is weak, although considerably stronger than with aspartate or glutamate. Thirdly, the oxygens of the γ-carboxyl groups interact with half of the primary coordination sphere of the metal. For calcium or lanthanide ions, which can have a coordination number of eight, the other half of the coordination sphere is available for interacting with other ligands. This feature may account for the functional role of γ-carboxyglutamic acid in the presence of metal ions; the γ-carboxyglutamic acid residues on proteins are available to bridge the protein-membrane surfaces containing metal-binding ligands. Furthermore, it is conceivable that within the vitamin K-dependent proteins, some intramolecular metal-binding sites may be formed by two γ-carboxyglutamic acid residues in different regions of the protein sharing a single metal ion.

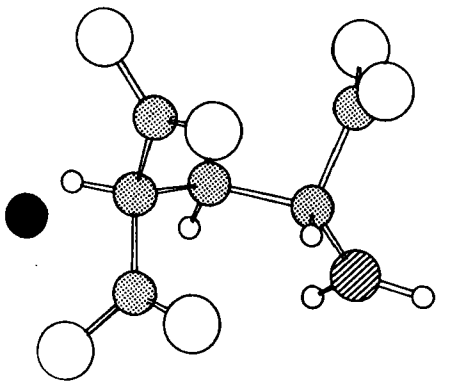


FIGURE 5. Structure of the metal-γ-carboxyglutamic acid based on distance measurements obtained by paramagnetic relaxation enhancement studies. γ-Carboxyglutamic acid interacts with half of the metal coordination sphere. The atoms are identified as follows: carbon (speckled), oxygen (white), metal (black), nitrogen (diagonal), and hydrogen (small white). (From Sperling, R., Furie, B. C., Blumenstein, M., Keyt, B., and Furie, B., *J. Biol. Chem.*, 253, 3898, 1978. With permission.)

XVIII. STRUCTURE OF THE METAL-BINDING SITES OF PROTHROMBIN

Bovine prothrombin has not been crystallized in a form suitable for X-ray crystallographic studies. Therefore most of the structural chemistry on these proteins involves application of X-ray crystallography and nuclear magnetic resonance spectroscopy to the study of prothrombin fragments. Fragment 1, a 23,000-mol wt polypeptide representing the N terminal one third of prothrombin, has been recently crystallized in the absence of metal ions and the preliminary diffraction patterns described.[136] These studies should yield important information about the γ-carboxyglutamic acid-rich domain of prothrombin in the not too distant future. Fragment (12-44), a γ-carboxyglutamic acid-rich 4,100-mol wt tryptic peptide of prothrombin has been amenable to study by natural abundance ^{13}C NMR spectroscopy using paramagnetic lanthanide ions to estimate the proximity of bound metal ions to various classes of carbon atoms in the peptide (Figure 6).[114,115] Fragment (12-44) binds metal ions and possesses two classes of binding sites; for Gd(III), one high affinity (K_D 0.4 μM) and six lower affinity sites (10 μM) have been detected. From the ^{13}C NMR studies direct evidence has been presented for the participation of γ-carboxyglutamic acid in metal binding. Furthermore, the lower affinity metal-binding sites can be distinguished and are likely individual γ-carboxyglutamic acid residues. The single high affinity metal-binding site has the following characteristics: (1) it involves two γ-carboxyglutamic acid residues; (2) the metal bound to this site is in very close proximity (7-8Å) to the ε-carbons of Arg_{16} and Arg_{25}; (3) reduction and alkylation of the disulfide bond connecting Cys_{18} and Cys_{23} eliminates the high affinity metal-binding site and the close proximity of bound metal ions to the arginine residues. Given the covalent structure of fragment (12-44), the features of metal binding by γ-carboxyglutamic acid, and the above data, a hypothetical model of the structure of fragment (12-44) has been proposed. In this model (Figure 7), the side chains of Gla_{15} and Gla_{26} face toward each other and bind a single metal ion. This is the high affinity metal-binding site, the tertiary structure of which is constrained by

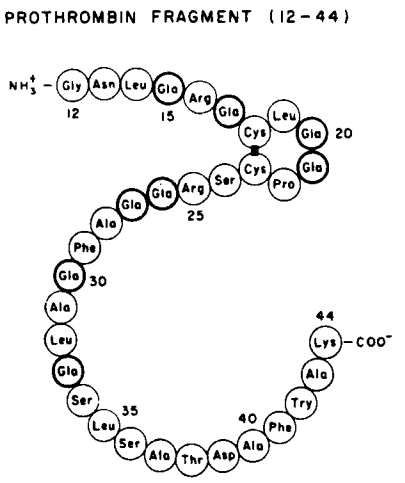


FIGURE 6. Amino acid sequence of prothrombin fragment (12-44). This peptide, representing residue 12 to residue 44 of bovine prothrombin, contains 8 of the 10 γ-carboxyglutamic acid residues of prothrombin. The two other γ-carboxyglutamic acid residues are at residues 7 and 8.

the nearby disulfide bond. The other γ-carboxyglutamic acid residues have low affinity for metal ions and possibly represent the domain in the protein which bridges prothrombin to membrane surfaces in the presence of metal ions. The high affinity metal-binding site is a metal-dependent reversible intramolecular bridge connecting two polypeptide chains in prothrombin. Structural changes in the vitamin K-dependent proteins associated with metal binding — a prediction of the model of the structure of the fragment (12-44)-metal complex — is that metal ions will alter the three-dimensional structure of this protein. In the absence of metal ions, the γ-carboxyl groups of the two γ-carboxyglutamic acid residues which participate in forming the high affinity metal-binding sites are ionized and repel each other. In the presence of metal ions, these γ-carboxyl groups converge on the single bound metal ion, altering the relationship of the polypeptide backbone near each γ-carboxyglutamic acid residue. Indeed, experiments employing fluorescence,[137,138] and immunochemical approaches have provided evidence indicative of changes in the three-dimensional structure of prothrombin associated with metal binding.[140,141] Monitoring the intrinsic fluorescence of tryptophan and tryosine residues in prothrombin and prothrombin fragment 1, several laboratories have observed significant quenching of this fluorescence in the presence of metal ions (Figure 8). About 6 and 40% of the intrinsic fluorescence of prothrombin and prothrombin fragment 1, respectively, are quenched by metal ions. This phenomenon is similarly observed for factor X.[142] Changes in the magnitude of the fluorescence may be due to direct perturbation of tryptophan or tryosine residues by metal ions, exposure of these fluorophores to aqueous solvent through changes in the tertiary structure of regions of the protein in close proximity to the fluorophores, or metal-dependent dimerization of these proteins. However, it appears most likely that this perturbation is associated with a general alteration of the three-dimensional structure of the protein. Because of the sensitivity of fluorescence methods, these studies are performed at protein concentrations in which artifacts due to prothrombin self-association do not occur. Thus, the pattern of fluorescence quenching associated with the

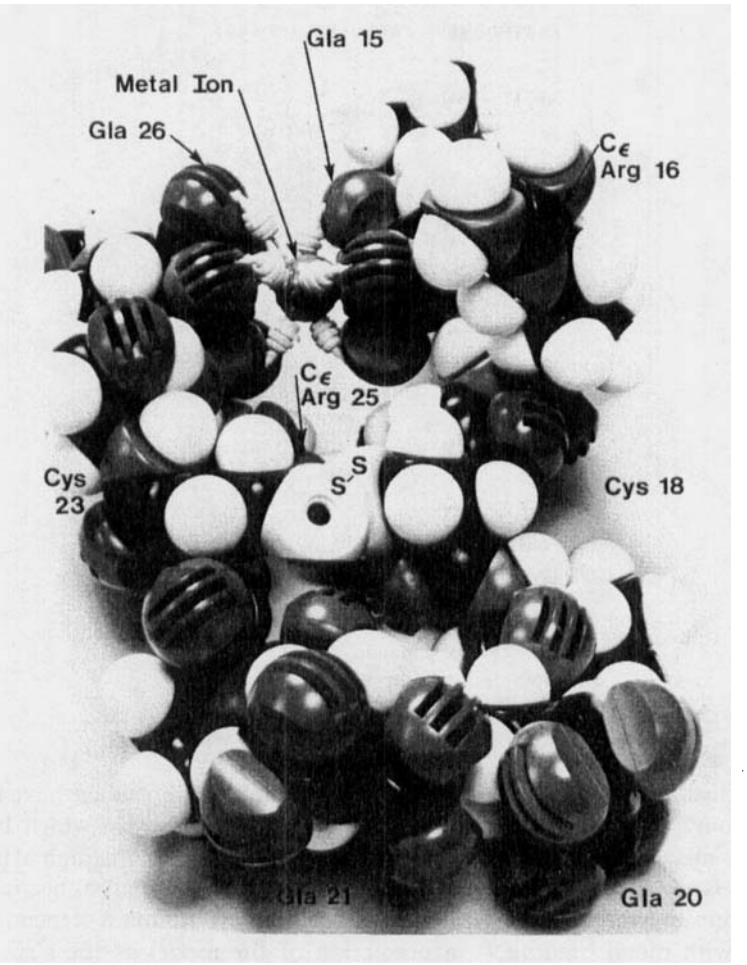


FIGURE 7. CPK model of prothrombin fragment (12-44). Two γ-carboxyglutamic acid residues form a high affinity metal binding site, which is shown occupied by a metal ion. The other γ-carboxyglutamic acid residues represent the low affinity metal binding sites and bridge the protein to membrane surfaces. (From Furie, B. C., Blumenstein, M., and Furie, B., *J. Biol. Chem.*, 254, 1252, 1979. With permission.)

addition of metal ions to prothrombin can be interpreted realistically in terms of cooperative interaction among some of the metal-binding sites. Half-maximal perturbation is observed at 0.3 m*M* Ca(II); fitting the data to the Hill equation yields a Hill coefficient of about 2. These experiments provide convincing evidence of positive cooperativity between two metal-binding sites on prothrombin in the absence of protein dimer formation.

Quenching of the intrinsic fluorescence induced by metal ions is unusually slow (100 min) at low temperature. This observation supports the interpretation that the perturbation of the fluorophore parallels a significant alteration of the tertiary structure. Furthermore, Nelsestuen has demonstrated that prothrombin cannot bind phospholipid vesicles,[137] even in the presence of metal ions, unless the slow metal-dependent conformational change has occurred. The metal ions that have been examined which can effect this structural transition include Ca(II), Mg(II), Fe(II), Fe(III), Gd(III), Zn(II), La(III), Tb(III), Pb(II), and Mn(II). High concentrations of Ba(II) are also effective. This structural transition obviously has little specificity for metal ions. The

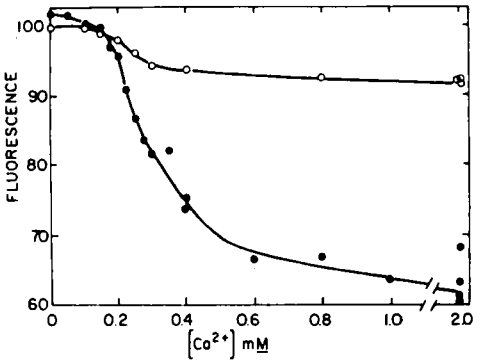


FIGURE 8. Metal ion-induced quenching of the intrinsic fluorescence of prothrombin fragment 1. Intrinsic fluorescence, monitored at 344 n*M* was measured as a function of calcium concentration. Fluorescence is plotted as the observed fluorescence divided by the fluorescence initially in the absence of Ca(II) × 100%. Prothrombin, -0-0-; prothrombin fragment 1, ●-●.

ability of metal ions to effect this transition relates, most likely, to the binding constant describing the interaction of the metal ions with the metal-binding sites on these proteins. In the absence of metal ions, mutual repulsion of γ-carboxyglutamic acid residues involved in the high affinity metal binding sites alters the structure of the protein compared to the structure in which a metal ion in the high affinity metal-binding site bridges two γ-carboxyglutamic acid residues. It might be predicted that any metal cation which can bind to the anionic oxygens in γ-carboxyglutamic acid will eliminate this repulsion and its concomitant distortion of the protein structure.

By independent criteria similar conclusions have been drawn from investigation of the effect of metal ions on the circular dichroism spectrum of prothrombin.[139] Metal-induced alterations in the CD spectrum in the far UV have been ascribed to changes in the secondary structure. The metal-protein binding data fit a model of cooperative metal binding, corroborating interpretation of the fluorescence data. As before, half-maximal perturbation is observed at about 0.3 m*M* Ca(II). Fitting the data to the Hill equation yields a Hill coefficient of 2.

An immunologic approach also corroborates the conclusion that prothrombin undergoes a major change in its three-dimensional structure with the addition of metal ions. Using quantitative immunoprecipitation, Stenflo determined that antiprothrombin antiserum reacts differently with prothrombin in the presence and absence of Ca(II).[116] These studies have been confirmed using purified rabbit antiprothrombin antibodies (Figure 9).[140] In further studies, a subpopulation of antibodies specific for the γ-carboxyglutamic acid-rich region of prothrombin, sequence (12-44), has been isolated. About two thirds of the high affinity subpopulation of this antibody interacts with prothrombin only in the presence of Ca(II).[140,141] These conformation specific antibodies must be directed against determinants whose structure on prothrombin are metal-induced and are located in the 12-44 region. Quantitative studies using Ca(II), Mg(II), and Gd(III) have shown that the structural transition induced by metal ions and recognized by the specific antibody correlate well with the data obtained by other physical methods. In addition, antibodies have been isolated which bind to prothrombin only in the presence of Ca(II).[143] These antibodies are directed against determinants on fragment 1, indicating that the metal induced structural change in prothrombin is

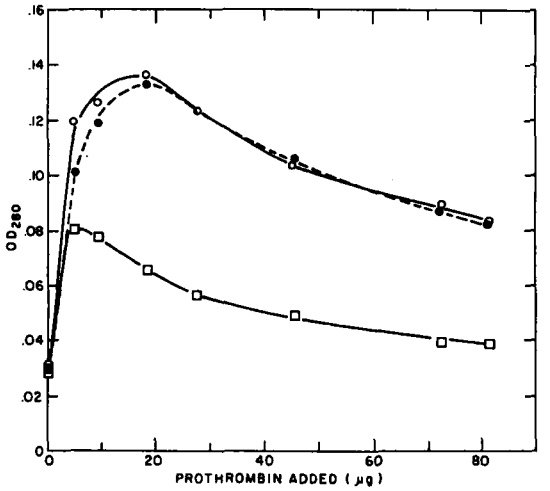


FIGURE 9. Quantitative precipitin curves indicating ligand induced conformational changes by immunochemical methods. Interaction of purified rabbit antiprothrombin and prothrombin in the absence (O) or presence of 1 m*M* $CaCl_2$ (●) or 3 MM EDTA (□).

localized to the N terminal one third of the molecule.

XIX. FUNCTIONAL IMPLICATIONS OF METAL BINDING

Prothrombin undergoes a conformational change which is metal-dependent before it can bind to phospholipid vesicles.[137] Owing to the fact that this structural transition proceeds very slowly at 0°C and that the binding of prothrombin to phospholipid dispersions can be monitored by gel filtration in a time period shorter than the time of the structural transition, it can be shown that prothrombin incubated briefly with Ca(II) at 0°C does not bind the phospholipid vesicles at 0°C. This conformer of prothrombin is the same as that observed in the absence of metal ions. Prothrombin incubated briefly with Ca(II) at 37°C does bind to phospholipid vesicles at 0°C; this form of prothrombin has undergone the metal-induced structural transition. These results support the concept that prothrombin in the absence of metal ion differs in its three-dimentional structure and phospholipid-binding properties from the prothrombin-metal binary complex.

These studies have been extended using a more direct approach to separate the metal requirements for the metal-induced structural transition of prothrombin and the prothrombin-phospholipid interaction.[142] The metal-dependent interaction of prothrombin with phospholipid vesicles was monitored by measuring the quenching of the intrinsic fluorescence of the protein when bound to a phospholipid emulsion in which a chromophore, a dinitrophenol-lipid derivative, was incorporated. Since the absorption spectrum of this liposome overlaps the emission spectrum of the intrinsic fluorescence of prothrombin, the quenching of the intrinsic prothrombin fluorescence may be monitored to measure prothrombin-phospholipid interactions. From this approach Ca(II), Sr(II), and Ba(II) were identified as metal ions capable of promoting prothrombin-phospholipid interactions while Mn(II) and Mg(II) could not. Trivalent lanthanide ions were not evaluated, but it is assumed that they will effectively support this interaction since they can be substituted for Ca(II) in prothrombin activation.[128]

Recently, metal binding studies have been extended to the evaluation of bovine fac-

tor IX.[144] As with prothrombin and factor X, factor IX possesses two relatively high affinity metal-binding sites and a larger number of lower affinity sites. The activation of factor IX by factor XIa is Ca(II)-dependent. Sr(II) can partially substitute for Ca(II) in this reaction, while Mn(II) cannot. The lanthanide ions Tb(III) and Sm(III) compete with Ca(II) for the metal-binding site, and are competitive inhibitors of factor IX activation.

Thus, two separate roles of metal ions in prothrombin and factor X function can be distinguished: (1) a metal-dependent conformational change, based presumably on the reorientation of the γ-carboxyglutamic acid residues which comprise the high affinity metal-binding sites and associated changes in the general structure of the protein; and (2) metal-dependent protein-phospholipid interactions, in which the γ-carboxyglutamic acid residues representing the low affinity metal-binding sites and metal ligands on the phospholipid vesicle surface are bridged by metal ions.

XX. SURFACE INTERACTIONS OF VITAMIN K-DEPENDENT BLOOD COAGULATION PROTEINS

Although activation of the vitamin K-dependent proteins may be mediated on platelet membrane surfaces, little is known about the chemical or structural characteristics of the sites on the platelet membrane which support blood coagulation. Previously, a phospholipid component or lipoprotein(s) in the platelet membrane, known functionally as platelet factor 3, was thought to be an important participant in blood coagulation in vivo. However, recent studies characterizing specific binding sites on the platelet suggest, at least in a preliminary way, that the receptor sites on the membrane are discrete entities which do not necessarily contain phospholipid.[110]

Investigating the interaction of ^{125}I-labeled human activated factor X with human platelets, Miletich et al. have established that each platelet contains about 200 receptor sites on its membrane which bind activated factor X with a binding constant, K_D, $3 \times 10^{-11} M$. This receptor is highly specific as it does not bind factor X in its zymogen form or DFP-inactivated active factor X. Of considerable import is that circulating platelets do not bind activated factor X; only after treatment with thrombin or the calcium ionophore A23187 — agents which effect platelet aggregation and the platelet release reaction — do the receptors appear. No structural details concerning these receptors are currently available. However, antifactor V antibodies inhibit the binding of activated factor X; further, platelets from patients with factor V deficiency do not bind activated factor X, thus implicating factor V as being at least part of the platelet receptor for activated factor X.[145]

Although platelets may contain the optimal surface for zymogen activation of the blood-clotting proteins, this surface is highly complex and its investigation is just beginning. Much of our current information concerning the physiochemical nature of surface structures which support blood coagulation relates to studies of phospholipid emulsions. Based on the original observations of Rouser and Schloredt,[146] and Wallach et al.[147] that phosphatidylethanolamine can accelerate the clotting of platelet-poor plasma, considerable effort has been expended in defining the physical and chemical properties of model lipid membranes relevant to coagulation. Although it is not clear whether the lipid component of the platelet membrane plays a significant role in blood coagulation, artificial lipid membranes have nonetheless been useful models for the systematic characterization of the salient features of surfaces upon which zymogen activation in blood coagulation can proceed.

Both prothrombin and factor X bind to phospholipid vesicles.[148-152] This interaction is reversible and metal-dependent. The physical properties of the liposomes, with specific regard to the component phospholipids, appears more important than the chemi-

cal composition of the liposomes for defining the suitability of the surface for protein binding and zymogen activation.[150] Liposomes composed of phosphatidylserine-phosphatidylcholine or phosphatidic acid-phosphatidylcholine bind prothrombin, but only phosphatidylserine-phosphatidylcholine dispersions both bind prothrombin and support surface-mediated activation.[151]

These protein-phospholipid vesicle interactions require the presence of acidic phospholipids, of which phosphatidylserine is the most effective. The protein-binding capacity of the phosphatidylserine-phosphatidylcholine vesicle is a function of the phosphatidylserine content; maximum binding capacity is realized in vesicles composed of 15% phosphatidylserine/85% phosphatidylcholine.[153] The average optimal molar ratio of phosphatidylserine to protein in each vesicle is about 9:1 for prothrombin and 5:1 for factor X. Phosphatidylserine content above this level is not associated with increased prothrombin binding, presumably due to the limits of packing of prothrombin on the vesicle surface.[154] An apparent dissociation constant K_D of about $2 \times 10^{-6} M$ is observed for the interaction of prothrombin or factor X with the phospholipid vesicles. It should be emphasized that in the absence of factor V this interaction is considerably weaker than that observed for the interaction of activated factor X with the platelet receptors — $2 \times 10^{-6} M$ vs. $3 \times 10^{-11} M$.

The domain of the vitamin K-dependent proteins responsible for phospholipid binding has been identified as the same region which contains a high content of γ-carboxyglutamic acid. Prothrombin can be proteolytically degraded by thrombin to yield two products: prethrombin 1 and prothrombin fragment 1. Prethrombin 1 (M_r 51000) is a zymogen of thrombin which can be activated by activated factor X; fragment 1 (M_r 23000) contains the ten γ-carboxyglutamic acid residues present on the native molecule. The activation of native prothrombin is markedly enhanced by phospholipid vesicles while the rate of zymogen activation of prethrombin 1 is unaltered by vesicles, indirectly suggesting that the fragment 1 region of prothrombin is the phospholipid-binding domain of the protein or that the fragment 1 region of prothrombin is required for stabilization of the three-dimensional structure of prothrombin to facilitate phospholipid interaction.[104,155,156] Direct evidence has supported the former interpretation. Using gel filtration techniques, Gitel et al.[157] determined that of the proteolytic products of prothrombin, only fragment 1 bound to the phospholipid vesicles. This interaction is Ca(II)-dependent, requiring 10-20 mM Ca(II). Phosphatidylserine-phosphatidylethanolamine dispersions bound both fragment 1 and prothrombin best, although phosphatidylglycerol-phosphatidylcholine and phosphatidylglycerol-phosphatidylethanolamine were also effective.

Isolation of the abnormal species of prothrombin from animals treated with a vitamin K antagonist yields a protein with a covalent structure identical to prothrombin except for the presence of glutamic acid in lieu of γ-carboxyglutamic acid. Although the three-dimensional structure of these proteins has not been solved, and certainly differences may exist, they are useful for examining the roles of γ-carboxyglutamic acid in liposome binding. Abnormal prothrombin does not bind to lipid dispersion in the presence of Ca(II),[132] thus implicating γ-carboxyglutamic acid in this binding. Owing to the possible effects of these residues on the tertiary structure of the molecule, however, these results are not entirely unambiguous.

The studies of Lim et al. support the contention that prothrombin and factor X bound to phosphatidylserine-phosphatidylcholine vesicles are extrinsic proteins.[158] That is, these proteins bind through electrostatic interactions on the membrane surface and do not become an integral part of the membrane. Using quasielastic light scattering as a method of estimating particle size, these workers have concluded that the average vesicle radius, 163 Å, increases to an average radius of 248 Å when saturated with prothrombin. The change in the effective radius, 85 Å, corresponds to the expected

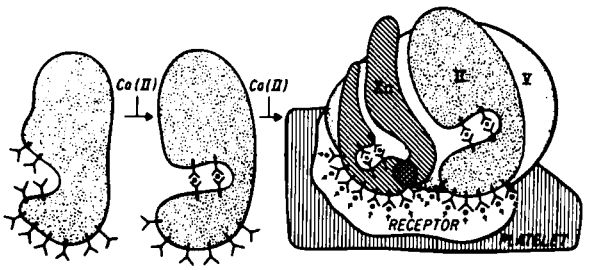


FIGURE 10. Model of the interaction of the vitamin K-dependent proteins with membrane surfaces. The Y-shaped structures represent γ-carboxyglutamic acid and the solid circles represent calcium ions. The protein undergoes a conformational rearrangement in the presence of metal ions, facilitating γ-carboxyglutamic acid-mediated metal bridging of the protein and membrane surface. Factor V binds to membranes in the absence of metal ions.

changes associated with the interaction of a macromolecule of 100 Å in length with the membrane surface. Prothrombin has been described as a prolate ellipsoid with an overall length of 110 Å. Therefore, the model proposed is consistent with one end of prothrombin interacting with the membrane in the absence of significant penetration of the membrane. This region of contact is the fragment 1 domain of prothrombin. Although the representation of the fragment 1, fragment 2, and prethrombin 1 domains of prothrombin as spheres is an obvious oversimplification and somewhat problematic, this model of the vesicle-prothrombin complex appears to be consistent with available experimental data. A similar description of factor X binding to phospholipid membranes has been proposed. In this construct, the estimated size of the factor X molecule is 93 Å over its length and the light chain of factor X interacts with vesicle surface.[158]

Jackson et al. have suggested that the major role of the phospholipid surface in accelerating blood coagulation may be to concentrate the participants of the reaction, activated factor X and prothrombin, into a single plane.[159] They estimate that in a solution containing 1 μM prothrombin, an increase of three orders of magnitude in the effective concentration of prothrombin to 1 mM may be realized if the prothrombin is bound to a membrane surface. On this basis, the acceleration of prothrombin activation by phospholipid vesicles in the presence of Ca(II) can be understood. The interaction of factor V with this surface further enhances zymogen activation; the mechanism of this acceleration is obscure.

Despite this detailed description of the interaction of the vitamin K-dependent proteins with artificial lipid membranes, it does not necessarily follow that lipids play a role in accelerating blood coagulation in vivo or when activated platelets are employed in vitro. For instance, polylysine can substitute for Ca(II) and factor V in facilitating conversion of prothrombin to thrombin by activated factor X in the presence of phospholipid vesicles.[160] We can assume that the protonated ε-amino groups of the lysine bind to both the γ-carboxyglutamic acid residues of prothrombin and activated factor X, thereby bridging these proteins to the anionic charges on the phospholipid membrane. Perhaps it would be fair to speculate that any mechanism which will organize the array of enzymes and substrates to optimize the geometry suitable for zymogen activation will accelerate blood coagulation. We thus have no reasons, *a priori*, to believe that the contribution of the platelet membrane to blood coagulation involves a lipid component. It is conceivable that the metal ligands on the platelet which are

involved in the bridge to the vitamin K-dependent proteins may be anionic side chains or amino acids in a platelet receptor protein or for that matter, any other molecular structure which can bind metal ions, including carbohydrate or lipid. These structures remain to be identified.

XXI. A GENERAL MODEL OF METAL-DEPENDENT MEMBRANE INTERACTION OF THE VITAMIN K-DEPENDENT BLOOD COAGULATION PROTEINS

Consolidating and unifying available information concerning the structural and functional features of the metal-mediated interaction of the vitamin K-dependent blood coagulation proteins with membrane surfaces, we propose the following construct (Figure 10). For purposes of this discussion, we will assume that all of the vitamin K-dependent proteins exhibit similar characteristics. Evidence now exists indicating that prothrombin and factor X have two classes of metal-binding sites which can be distinguished on the basis of their affinity and specificity for metal ions and the organization of γ-carboxyglutamic acid in forming the metal-binding sites. There are two high affinity metal-binding sites. Each of these sites are likely formed by two γ-carboxyglutamic acid residues whose four γ-carboxyl groups bind a single metal ion. In the absence of metal ions, the carboxylate groups electrostatically repel each other, significantly altering the relationship of the polypeptide chains in this region as well as other domains of the molecule. Six γ-carboxyglutamic acid residues represent six low affinity metal-binding sites. These sites, in the absence of metal ions, cannot interact with anionic surfaces. With the addition of Ca(II) (although many metal cations effect the same changes), the high affinity metal-binding sites become occupied with metal ions. This metal binding may be characterized by positive cooperativity inasmuch as occupancy of one site facilitates the occupancy of the second site. Formation of the high affinity metal binding sites via a metal-dependent ion bridge alters the local structure of the γ-carboxyglutamic acid-rich region of the protein as well as inducing alterations of the tertiary structure of the protein in general. This conformational change is metal-dependent and involves exposure of tryptophan residues to solvent and alterations of the secondary structure of the peptide backbone. Relative to other structural transitions of proteins, this transition is slow. The conformer of prothrombin which exists in the presence of metal ions contains a surface domain composed of γ-carboxyglutamic acid residues. As a result of the metal-dependent structural transition, these residues are aligned so as to meet the geometrical requirements of the anionic structures on the membrane surface. Prothrombin can bind to the membrane surface via six metal bridges; the binding energy describing the interaction of prothrombin with the membrane would equal the sum of these six electrostatic bonds. On the protein, the metal ligand is the carboxylate groups of the γ-carboxyglutamic acid. The metal ligand on the artificial phospholipid membranes is the phosphate group. However, there are currently no clues as to the identity of the metal ligand in the platelet receptor. Possibilities include aspartate or glutamate as part of a receptor protein, acidic structures or carbohydrates, phosphate groups on a lipoprotein or phosphoserine-containing protein, or even γ-carboxyglutamic acid residues in a protein of the platelet surface.

As a final comment, we note that this model of the role of cofactors in zymogen activation during blood coagulation offers implications with regard to the regulation of blood coagulation. Although circulating antithrombin III surely plays a critical role in preventing dissemination of the enzymatic machinery of blood clotting from the location of vascular injury into the general circulation, other regulatory mechanisms

must include a role for metal ions, protein cofactors, and platelet surface componenets in the modulation of hemostasis.

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