THE PHYSIOLOGIC response to vascular damage culminates in the rapid generation of thrombin at the site of injury, leading to increased platelet deposition and the formation of the insoluble fibrin network. The response of the coagulation process is generally limited to the site of injury and is proportional in magnitude to the extent of vascular damage. These properties of the coagulation response require that the component reactions function in a localized, amplified, and modulated manner.

Localization of several reactions of the blood clotting cascade is achieved by the reversible binding of circulating coagulation proteins to damaged vascular endothelial cells, elements of the subendothelium, and peripheral blood cells such as platelets. These binding events lead to the assembly of blood clotting enzyme complexes that provide rapid delivery of products at the site of vascular injury. The cascade arrangement of the blood coagulation reactions is clearly one mechanism by which a small initiating stimulus is amplified to yield high levels of terminal product. However, amplification is also achieved at the level of the individual reactions by the formation of membrane-bound enzyme complexes and the membrane binding properties of the substrates. A profound enhancement in reaction rate is realized on assembly of the different coagulation complexes. For all intents and purposes, relative to their membrane bound equivalents, the coagulation serine proteases in solution display little or no activity toward their substrates on a biologically relevant time scale. In addition to the rate-amplifying aspects of enzyme complex formation on membranes, this process interferes with the regulatory effects of the potent inhibitory systems, which modulate and ultimately terminate the coagulation response.

The complexes involving vitamin K-dependent enzymes, which convert the vitamin K-dependent zymogens to the appropriate serine proteases, are illustrated in Fig 1. Each complex is composed of a vitamin K-dependent serine protease and an accessory or cofactor protein in association with a membrane surface. The presence of calcium ion is essential for the assembly and activity of these enzyme complexes. While each enzyme complex exhibits discreet substrate and proteolytic specificity, the complexes illustrated in Fig 1 share several common features: (1) the complexes are functionally analogous with structurally homologous constituents; (2) the enzyme complexes exhibit similar requirements for assembly and activity; and (3) in each case, enzyme complex assembly leads to a significant enhancement in the localized catalytic rate of activation of the substrate. In addition, there are three key regulatory events associated with the formation of the enzymatic complexes illustrated in this figure: (1) the conversion of a vitamin K-dependent zymogen to a serine protease; (2) the proteolytic activation of a plasma-derived profactor to an active cofactor (factor V and factor VIII) or the membrane expression of an integral membrane protein cofactor (tissue factor and thrombomodulin); and (3) the presentation of the appropriate membrane surface to accommodate the protein-binding interactions.

Most of the reactions illustrated in Fig 1 have been studied primarily using synthetic phospholipid vesicles. Membranes containing acidic phospholipids are required for the expression and activity of the prothrombinase and the intrinsic Xase complexes. In contrast, the complexes composed of integral membrane cofactor proteins appear to function on membranes composed entirely of phosphatidylcholine. While phospholipid vesicles appear to mimic the natural surfaces on which these complexes form in vivo, cellular membranes provide an additional opportunity for regulation. Cell-derived membrane sites are regulated by various agonists in vitro, thus cellular regulatory events at the membrane level contribute to the formation and expression of the complex enzymes described in Fig 1.

The coagulation cascade was initially conceived as being composed of a sequential series of proteolytic reactions. The accumulating evidence suggests that this simplification is probably inappropriate. Both thrombin and factor Xa are known to be multi-directional in their feedback reactions.
among these complexes. These proteases can enhance their own formation rates by catalyzing the activation of factor VII to factor VIIa, and by catalyzing the activation of factors V and VIII. In addition, thrombin plays an important role at the cellular level in platelet activation and the subsequent expression of cellular membrane binding sites.

It has also been generally assumed that the clotting enzymes exhibit absolute specificity with respect to the cofactor proteins necessary for complex formation. We have recently observed that factor Xa binds to thrombomodulin and can catalyze the proteolytic activation of protein C. Factor Xa appears to interact with thrombomodulin with an affinity that is similar to the affinity for the interaction between thrombin and thrombomodulin. The presumed fidelity of cofactor-enzyme interactions has not been adequately tested, and it is conceivable that the lack of absolute cofactor specificity observed in thrombomodulin may exist at some level for other cofactor-protein enzyme interactions.

VITAMIN K-DEPENDENT ZYMOGENS/ENZYMES

Three classes of vitamin K-dependent enzymes/zymogens participate in the reactions of the vitamin K-dependent enzyme complexes (Fig 2). The mature vitamin K-dependent proteins are characterized by two common domains. A highly conserved "Gla-domain" is found at the NH2-terminus of each protein and contains 9 to 12 γ-carboxyglutamic acid residues. Approximately half the mass of each protein is accounted for by the serine protease domain present at the COOH-terminus. This serine protease "zymogen domain" is equivalent in general structure to chymotrypsin and trypsin. Enzymes superimposed on the chymotrypsin/trypsin backbone are insertions of additional polypeptide material that are presumably responsible for the unique macromolecular substrate specificity of these enzymes and their ability to specifically associate with the appropriate cofactor.

There are two major types of vitamin K-dependent zymogens. Prothrombin represents the unique member of one class. In addition to the Gla and zymogen domains, this molecule has two "kringle" structures. Kringles were first identified in prothrombin by Maguson et al and are now known to exist in other proteins involved in coagulation, fibrinolysis, cytoadhesion, and lipid transport. Among vitamin K-dependent clotting proteins, prothrombin is unique...
in its manifestation of kringle-like structures. The other vitamin K-dependent zymogens, factor VII, factor IX, factor X, and protein C, contain two "epidermal growth factor like" domains in place of the kringle structures found in prothrombin. The first epidermal growth factor-domain contains the posttranslationally modified amino acid, β-hydroxy aspartic acid in the case of factors IX and X, and β-hydroxy asparagine in the case of protein S.25-37

Prothrombin, factor VII, and factor IX are synthesized and circulate in plasma as single chain proteins. Factor X and protein C, while synthesized as single polypeptide chains, circulate in plasma as two chain proteins as a consequence of proteolytic cleavages that occur during, or subsequent to, secretion from the liver. Both factor X and protein C give rise to an activation peptide on cleavage of a single bond in the two chain zymogens. The activation of factor IX involves two peptide bond cleavages and the release of an activation peptide from the single chain precursor. Factor VII activation does not give rise to an activation peptide.17

Early work on factor VII showed that uncleaved factor VII would incorporate diisopropylphosphofluoridate and displayed some minimal level of enzymatic activity.38 These observations led to the conclusion that factor VII in the single chain form was not a zymogen and possessed some low but significant level of activity. The "active zymogen" hypothesis for single chain factor VII has more recently been challenged in three types of studies: (1) Rao et al39,40 examined the activation of factor IX and the feedback activation of factor VII using congenitally abnormal factor IX as substrate and antithrombin III-heparin mixture to trap any active enzyme formed. From these studies, they concluded that factor VII in the uncleaved form is inactive. (2) In our laboratory, studies using fluorescent and biotinylated peptide chloromethylketones showed that while factor VIIa readily incorporated these active site-directed reagents, the single chain form of factor VII did not.41 Moreover, these peptide chloromethyl ketones that did not react with the single chain form of the protein could completely inhibit the conversion of factor X to Xa when present in reaction mixtures containing factor X, factor VII, tissue factor, and phospholipid. (3) Recent studies have made use of a recombinant factor VII mutant in which Arg152 was replaced with Glu, hence removing the scissile bond involved in the conversion of factor VII to VIIa.42 These studies concluded that the mutant single chain factor VII was inactive, although this mutant was not extensively characterized with respect to its ability to interact with the cofactor or to incorporate diisopropyl phosphofluoridate. Collectively, the data are most consistent with the conclusion that factor VII is indeed a zymogen, as are the other vitamin K-dependent enzyme precursors in blood.

Most of the vitamin K-dependent enzymes of coagulation are inactivated by antithrombin III and heparin, although mechanisms for inactivation are somewhat different in each case.43-44 Factor VIIa stands alone in not being susceptible to inactivation by antithrombin III-heparin. Factor VIIa is inactivated by the formation of a complex involving the lipid-associated coagulation inhibitor (LACI), tissue factor (TF), factor Xa, phospholipids, and calcium ion.45-47 While the LACI-TF-factor VIIa-factor Xa complex has the capacity of tightly binding and inhibiting factors Xa and VIIa, it is not certain whether the formation of this complex serves as an inactivation step or as a enzyme "buffer" that maintains dissociable and protected pools of factor Xa and factor VIIa in whole blood.

Most of our knowledge of the membrane and metal ion binding interactions of the vitamin K-dependent proteins have been extrapolated from detailed studies of prothrombin fragment 1, a proteolytic product of prothrombin that contains the Gla-domain and the first "kringle." This is a potentially dangerous extrapolation in view of the significant differences in molecular organization of prothrombin relative to the other vitamin K-dependent proteins (Fig 2). Jackson's laboratory was the first to show that prothrombin fragment 1 could bind to acidic phospholipids in the presence of Ca++.48 Subsequent studies identified that two, and perhaps three types of metal ion-dependent interactions were required to support prothrombin membrane binding.49,50 The occupancy of a limited number of sites (2 to 3) by a variety of multivalent metal ions leads to stabilization of an altered conformational state of the protein.51-53 The occupancy of additional metal ion binding sites (4 to 5) supports the binding of prothrombin to phospholipid membranes that contain acidic (negatively charged) phospholipids. The membrane-binding process is relatively specific for the calcium ion, although other alkaline earth and lanthanide metal ions may substitute less efficiently for this process.54,55 The lipid-binding interaction has been shown to be ionic strength-dependent and thus, in part, probably involves some ionic interactions between the protein and the membrane surface. Hydrophobic interactions have also been implicated and there are limited experimental data to support this suggestion.56

Recent crystallographic data from Tulinsky's laboratory has provided the structure of the metal ion stabilized conformation of the prothrombin fragment 1 Gla-domain at the atomic level (Fig 3).57-59 In contrast to the organized structure shown for the metal ion-dependent conformation, the domain corresponding to residues 1 through 35 is disordered in the metal ion-free structure, and thus is not represented in the x-ray structure of the metal-free protein. Therefore, the crystallographic data provides unequivocal corroboration for a metal ion-dependent structural transition in the Gla-domain of this protein. Recent studies in our laboratories using a metal ion-dependent, pan-specific antibody that reacted with the first 12 amino acid residues of prothrombin, protein C, factor X, and factor VII have illustrated that similar metal ion-dependent conformational transitions occur for each of these proteins.60-61 The location of this peptide epitope recognized by the conformation-specific antibody in prothrombin, factor X, protein C, and factor VII is highlighted in Fig 3. The similar antibody reactivity observed for these vitamin K-dependent proteins suggest that the first 35 residues of prothrombin Gla-domain represented in Fig 3 most likely is the appropriate molecular structure for all Gla domains.

The conformational transition required to generate the structure represented in Fig 3 requires the posttranslational carboxylation of almost all of the glutamate sites for the
prothrombin molecule. The elimination of as few as two Gla residues produces an abnormal (des Gla) prothrombin molecule, which cannot undergo the conformational transition (Fig. 3) or bind to acidic phospholipid membranes. The inhibition of the carboxylation process by either dietary vitamin K deficiency or by antagonists such as Warfarin produces protein molecules that are unable to develop the organized structure illustrated in Fig 3 in the presence of divalent cations, and hence are unable to interact with membranes. The elimination of these activities has significant consequences for the participation of these proteins as substrates or enzymes for the vitamin K-dependent enzyme complexes.

The plasma concentrations of each of the vitamin K-dependent proteins, their reported affinities for phosphatidylserine-phosphatidylcholine membranes, and binding stoichiometries for Ca$^{2+}$ are presented in Table 1. These data have been selected to represent the "most appropriate" values available in literature. Apart from factor VII (with an unusually high dissociation constant) and protein S (with an unusually low dissociation constant), the available dissociation constants for the protein-membrane interactions fall into two categories: prothrombin with a dissociation constant in the micromolar range in comparison with factor X, and protein C with dissociation constants with approximately a fivefold higher affinity. The somewhat lower affinity observed for the prothrombin-membrane interaction may result from the structural differences between "kringle" and "epidermal growth factor domains."

**COFACTOR PROTEINS**

The cofactor proteins for the coagulation complexes illustrated in Fig 1 fall into two classes.

**Plasma procofactor proteins.** Factor V and factor VIII are plasma proteins and circulate in plasma as inactive "pro" species.

**Cellular cofactor proteins.** Tissue factor and thrombomodulin are integral membrane proteins that are irreversibly anchored to the membrane surfaces of adherent cell populations.

The structures of the soluble plasma procofactors, factor V, and factor VIII are illustrated in Fig 4. Factors V and VIII are highly homologous proteins and share approximately 30% sequence identity. Both are synthesized as high molecular weight precursor proteins and circulate either as single chain species (in the case of factor V) or as a mixture of noncovalently associated polypeptide fragments (in the case of factor VIII). In addition, in humans approximately 25% of the factor V in blood is contained in a fragmented procofactor form in platelet α-granules. While the data suggest that factor V present in platelets is stored fragmented, proteolysis during isolation of this procofactor cannot be unequivocally eliminated. Factor V circulates in plasma at a concentration of approximately 20 nmol/L while factor VIII circulates in a tightly associated complex with von Willebrand factor (vWF) at a nominal concentration of 1 nmol/L.

Factors V and VIII share a common structural configuration of triplicated "A" domains and duplicated "C" domains. A structurally divergent "B" domain serves as a connecting region between the A$_1$ and A$_3$ domains. During proteolytic activation of both pro-cofactors, the "B" domain is excised, leading to the noncovalently associated collection of polypeptide chains that participate as the active cofactors in complex formation. Factor Va and factor VIIIa resulting from activation contain only "A" and "C" domains and display approximately 40% identity. In the case of factor Va, the active species generated by the action of thrombin is a heterodimer composed of an NH$_2$-terminal-derived heavy chain and a COOH-terminal-derived light chain that interact tightly and noncovalently in the presence of divalent

**Table 1. Properties of the Vitamin K-Dependent Proteins of Coagulation**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Plasma Concentration (nmol/L)</th>
<th>Protein/Membrane* (nmol/L)</th>
<th>Protein/Metal† (Ca$^{2+}$/protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>II</td>
<td>1.4</td>
<td>1.04</td>
<td>10-11</td>
</tr>
<tr>
<td>X</td>
<td>0.17</td>
<td>0.19</td>
<td>20</td>
</tr>
<tr>
<td>IX</td>
<td>0.09</td>
<td>&lt;1.0</td>
<td>12-13</td>
</tr>
<tr>
<td>VII</td>
<td>0.01</td>
<td>15</td>
<td>6</td>
</tr>
<tr>
<td>Protein C</td>
<td>0.08</td>
<td>0.23</td>
<td>16</td>
</tr>
<tr>
<td>Protein S</td>
<td>0.14</td>
<td>0.005</td>
<td>&gt;5</td>
</tr>
<tr>
<td>Protein Z</td>
<td>0.05</td>
<td>0.32</td>
<td>NA‡</td>
</tr>
</tbody>
</table>

* Dissociation constant for the protein-membrane interaction, measured using phospholipid vesicles composed of phosphatidylcholine and phosphatidylserine.
† Stoichiometry for the Ca$^{2+}$/protein interaction.
‡ NA, not available.
BLOOD COAGULATION ENZYME COMPLEXES

Fig 4. Structural organization of the plasma cofactor proteins. The illustrated structures of the procofactors (VIII and V) correspond to the gene products. The structures of the cofactors (VIIIa and Va) correspond to those obtained on the action of α-thrombin on the procofactor species. Both proteins contain homologous, internally triplicated A domains (A1 through A3) and internally duplicated C domains (C1 and C2). The B domains are not homologous and are released from the procofactor during activation. Factor VIIIa is a heterotrimer and factor Va is a heterodimer with the indicated subunit molecular weights (×10^3). Dashes represent noncovalent interactions within the subunits of the activated species.

Fig 5. Structural organization of the cellular cofactor proteins. The indicated domains of thrombomodulin (TM) and tissue factor (TF) correspond to an extracellular domain (E), a membrane-spanning domain (M), and a cytoplasmic domain (C). Thrombomodulin also contains six epidermal growth factor-like type B repeats (EGF 1 through 6) and a region containing glycosylation sites (G).
ity of the appropriate complex. Thrombomodulin is found principally on the surface of the vascular endothelial cells and platelets, but appears to be a constitutive product of only the endothelial cell. Functional thrombomodulin is also present at low concentrations in plasma, presumably due to proteolysis of endothelial cell, or platelet-derived thrombomodulin. Structure/function studies using fragments of thrombomodulin have demonstrated that a peptide comprising EGF repeats 2 through 6 contained sufficient information to accelerate the activation of protein C by thrombin. Similar studies also demonstrated that EGF repeats 5 and 6 contained the thrombin binding site of thrombomodulin.

Tissue factor is not found in the vascular endothelium under normal circumstances, but is abundant on the cells of extravascular tissue. Tissue factor can be expressed on the vascular endothelium and on circulating monocytes when induced by chemical, cytokine, and endotoxin stimulation. Tissue factor probably serves as an important trigger for coagulation within intravascular space owing to its constitutive expression on the cell surfaces.

MEMBRANE REQUIREMENT FOR COMPLEX ASSEMBLY

The presence of acidic phospholipids is an obligate requirement for the assembly of the enzyme complexes involving the soluble cofactors, factor Va, and factor VIIIa. In contrast, both thrombomodulin and tissue factor can form functional enzyme complexes on membranes that contain only neutral phospholipids. However, although enzyme complex formation can be demonstrated, the function of the tissue factor/factor VIIa complex is enhanced many fold when optimal amounts of acidic phospholipids are included in the membrane preparations.

Early studies by Papahadjopolous and Hanahan illustrated the importance of both the chemical and physical properties of the membranes on which coagulation reactions proceed. Sterzing and Barton used coagulation assays to demonstrate that the rate of coagulation reactions could be influenced by membrane fluidity. Our laboratory has examined the influence of membrane fluidity on the properties of the prothrombinase complex by using vesicles prepared from phospholipids with high- and low-phase transition temperatures. Prothrombin activation was characterized by a distinct lag when measured using nonfluid phospholipids. Because this lag could be eliminated by preassembling the enzyme before initiation with prothrombin, these studies were the first to demonstrate that the process of complex assembly could be rate-limiting under certain conditions.

The studies of prothrombinase complex assembly on fluid phospholipids were extended significantly by rapid reaction techniques. The application of this technique was made possible by the extensive development of fluorescent reporter groups designed to report selected aspects of complex assembly. The process of formation of the prothrombinase complex on a membrane surface proceeds through a mechanism in which factor Va and factor Xa initially bind independently to the membrane surface and subsequently rearrange (in two dimensions) to form the active prothrombinase complex. The kinetic mechanism deduced for prothrombinase assembly on synthetic phospholipid vesicles is illustrated in Fig 6 and the corresponding rate constants are presented in Table 2. Both factor Xa and factor Va bind to membrane surfaces at a significant fraction of the diffusion limited rate. The high collisional efficiencies (30% to 70%) for the protein-membrane binding events suggested by these data indicate that almost every collision between protein (either Xa or Va) and the phospholipid vesicle leads to the formation of a productive protein-membrane complex.

The high efficiency observed for the protein-membrane interactions that precede prothrombinase assembly raises the possibility of significant membrane cooperation in the initial binding event. One mechanism for such cooperation could involve a rearrangement of phospholipid head groups after the initial encounter of the protein with the membrane surface. The cooperative aspects of membrane binding from
the lipid perspective and the subsequent requirement for rearrangement in two dimensions on the membrane surface to form the prothrombinase complex provide additional opportunities for the regulation of prothrombinase assembly. For a natural (cell) membrane these regulatory opportunities could include the exposure of appropriate phospholipids on the outer leaflet, patching of head groups, and changes in membrane fluidity. Collectively, these observations also should caution investigators to interpret changes in gross catalytic rates of product (α-thrombin) production with caution since such expressed rates are the culmination of at least seven separable events. These include, in addition to potential membrane alterations, the binding of factor Va, the binding of factor Xa, complex formation, substrate (factor II) binding, two proteolytic cleavages, and release of α-thrombin.

Recent studies on the equilibria between membrane sites and factors Va and Xa within the prothrombinase complex suggest additional opportunities for the regulation of complex assembly when limiting concentrations of membrane sites are available. Factors Va and Xa are both bound to the membrane surface when assembled in the prothrombinase complex. These proteins mutually exclude each other for the initial binding reactions with membrane combining sites on synthetic phospholipid vesicles, suggesting that saturation of the membrane surface with one component would substantially reduce the maximum concentration of complex formed. However, equilibrium binding studies of complex assembly indicate that the protein-membrane interactions are linked to the protein-protein interactions within prothrombinase, such that the affinity of the individual interactions with the membrane surface is increased approximately 100-fold. As a consequence of this linkage, at limiting concentrations of membrane, factor Va is unable to displace prothrombinase at concentrations predicted from knowledge of the individual interactions. The observed linkage implies that complex assembly will still proceed to completion under physiologically relevant conditions represented by limiting membrane sites, ie, relatively high concentrations of cofactor (factor V plus Va) and low concentrations of factor Xa.

Binding studies of prothrombinase assembly indicate that factors Va and Xa interact on the membrane surface with a dissociation constant of approximately 1 nmol/L. In contrast, kinetic studies of prothrombin activation in the solution phase and direct binding studies in progress in our laboratories suggest that the protein-protein interaction between Va and Xa in solution is 1,000-fold weaker (kd ~ 10⁻⁵ to 10⁻⁶ mol/L, E. Pryzdiyal and K. G. Mann, unpublished results). Two possible explanations could account for the substantial increase in the affinity for this protein-protein interaction in the presence of membranes. The binding of factor Va and/or factor Xa to the membrane surface could cause a conformational change in one or both proteins that fundamentally changes the nature of the protein-protein interaction. Alternatively, the increased affinity could be explained by the substantial reduction the degrees of freedom for protein reorientation once both proteins are bound to the membrane surface. Hence, the required formation of protein-phospholipid binary complexes would increase subsequent reaction rates by limiting the dimensions required for translational diffusion and would increase the frequency of productive collisions between membrane-bound proteins by decreasing the permissible orientations of reacting species.

**LINKAGE OF COAGULATION REACTIONS AT THE MEMBRANE LEVEL**

The coagulation cascade, as initially described by Davie and Ratnoff and MacFarlane, is composed of a series of linked reactions, ie, the product of one reaction participates as the enzyme for the succeeding reaction. Based on the phospholipid binding properties of the zymogens and their respective enzymes for the reactions illustrated in Fig 1, the shuttling of product between reaction centers can be conceived to occur by two possible mechanisms. If the successive enzymes of the cascade individually assemble on different cell surfaces, the transfer of enzyme product to become the serine protease element for the following coagulation complex would require the dissociation of the product enzyme from the membrane surface on which it was formed, followed by rebinding on the appropriate membrane surface and incorporation into the succeeding enzyme complex. On the other hand, if successive reaction centers could assemble on the same membrane surface, the appropriately assembled complexes could "channel" products without requiring their dissociation from the membrane surface.

From a mechanistic standpoint, the latter scenario has appeal since it reduces the likelihood for an intermediate enzyme product to be swept away and "lost" to the flowing blood around to wound site. The total concentration of blood clotting enzyme inhibitors (antithrombin III, heparin cofactor 2, α2 macroglobulin, α1 antitrypsin), is extraordinarily high relative to any potential enzyme concentration that can be achieved in the vicinity of a blood clot. Additionally, most serine proteases involved in the procoagulant complexes are subject to rapid inhibition by antithrombin III/heparin as long as the reactants are present in solution. The cofactor proteins that make up the factor X (factor VIIIa) and prothrombin (factor Va) activating complexes are also more susceptible to inactivation by activated protein C if they are not complexed with their respective enzymes. Thus, the process of dissociation from the membrane surface or from the complexed state is contradictory to a sustained coagulation response as active coagulation components (serine proteases)
desorbing from the membrane surface or left behind (cofactors) would be rapidly inactivated. The organization of collections of reaction centers on a single membrane with channeling/transfer of reaction products in limited dimensions would be one mechanism by which the intermediates of the coagulation cascade could be protected from the hostile environment surrounding the blood clot.

The considerations for the two-dimensional and three-dimensional transfer processes are represented schematically using selected coagulation enzyme complexes in Figs 7A and 7B. In one case (Fig 7A) the factor Xa product would have to dissociate from the membrane on which it is produced: diffuse through solution to the membrane on which its “anchor,” factor Va, is bound; bind, and rearrange to give rise to prothrombinase. When both complexes assemble on the same membrane surface (Fig 7B), factor Xa could be transferred between reaction centers by diffusion in limiting dimensions along the membrane surface.

On a theoretical basis, the mechanism illustrated in Fig 7B is attractive when one considers the rate constants described in Table 2. The dissociation rate constants of factor Va ($k_{-1}$) and factor Xa ($k_{-2}$) from phospholipid surfaces are in the range of 10 to 100 million-fold lower than their respective association rate constants ($k_{+1}$ and $k_{+2}$). If factor Xa generated on one surface had to dissociate and rebind to another surface before its incorporation into prothrombinase (Fig 7A), the rate for prothrombinase assembly would be determined by the rate constant for the dissociation of factor Xa from the surface on which it is formed ($k_{-1}$) and not the association of factor Xa with the second membrane surface ($k_{+1}$). On the other hand, the two-dimensional transfer of factor Xa between enzyme complexes (Fig 7B) would occur several (greater than 8) orders of magnitude faster, being determined by the rate of the rearrangement of protein-membrane binary complexes to form prothrombinase ($k_{+3}$ or $k_{+4}$, Fig 6).

The numerical advantage of channeled reactions as illustrated in Fig 7 can be experimentally estimated by stopped flow studies of prothrombinase assembly. Assembly after the formation of protein-membrane binary complexes on the same vesicle was compared with complex formation initiated by reacting factors Va and Xa bound to separate vesicles. Under identical final reaction conditions, complex assembly initiated by mixing Va with the Xa-lipid binary complex
proceeded rapidly (t_{1/2} = 10.9 milliseconds), the rate-limiting step corresponding to the initial reaction between Va and free sites on the vesicle surface. When complex assembly was initiated by mixing Va bound to a separate set of vesicles with factor Xa bound to a separate set of vesicles, the rate of the process was decreased approximately 500-fold (t_{1/2} = 5.7 seconds). Under the latter conditions, the rate-limiting step corresponded to the dissociation of factor Xa from the membrane surface before its reassociation on the same vesicle containing factor Va. Thus, the 500-fold difference in membrane surface before its reassociation on the same vesicle containing factor Va may be inferred to exist for the transfer by channeling on a single membrane.

In addition to theoretical considerations, there is substantial evidence which suggests that natural cell membranes have the ability to bind multiple complexes. Studies from a variety of laboratories indicate that the platelet surface can directly demonstrate factor VIIIa binding to platelets. Thus, there is precedent to suggest that in addition to prothrombinase, the intrinsic factor IX and X activation complexes can also assemble and function on a single membrane. Hence, raising the realistic possibility of product channeling between these reaction centers.

**CATALYTIC ADVANTAGE OF ENZYME COMPLEX FORMATION**

The catalytic rates obtained with the vitamin K-dependent coagulation enzymes in solution are extremely low when measured at physiologically relevant concentrations of reactants. The addition of the accessory components and the assembly of the appropriate membrane-bound enzyme complex leads to a profound enhancement of reaction rate. For example, the activation of 10^{-6} mol/L prothrombin catalyzed by 10^{-8} mol/L factor Xa is enhanced 400,000-fold by the addition of saturating concentrations of factor Va and membranes containing acidic phospholipids. Recent data have directly demonstrated factor VIIa binding to platelets. Thus, there is precedent to suggest that in addition to prothrombinase, the intrinsic factor IX and X activation complexes may be inferred to exist for the transfer by channeling on a single membrane.

The kinetic constants that have been determined for the vitamin K-dependent enzyme complexes are listed in Table 3. The assembly of each enzyme into the appropriate complex causes a reduction in the apparent Km for substrate and an increase in the kcat for the reaction. For the most part, complex formation causes a decrease in the Km for the substrate to values comparable with the circulating concentrations of zymogen (Table 3). In addition, the measured apparent Km for substrate in each case is generally comparable with the dissociation constant for the interaction of the substrate with the membrane surface (Table 3). The exception to these trends in Table 3 is the activation of protein C catalyzed by the factor Xa/thrombomodulin/PCPS complex. In this instance, the measured Km is approximately 120-fold greater than the plasma concentration of protein C, and is approximately 40-fold greater than the dissociation constant for the protein C-membrane interaction. This discrepancy is unique among these complexes and raises questions regarding the physiologic relevance of protein C activation catalyzed by factor Xa. The estimated dissociation constants for enzyme complex formation (Table 3) are a consistent requirement for the conversion of approximately 1% to 10% of the required plasma zymogen to enzyme to provide sufficient enzyme for complex saturation.

The fundamental importance of complex formation can be realized by comparing the catalytic efficiencies (kcat/Km) of the enzyme complexes with the relevant serine protease acting on the same substrate in solution. Increases in catalytic efficiency ranging from 3 \times 10^4 to 1.2 \times 10^5 to 1.2 \times 10^{12}-fold have been reported to occur due to enzyme complex assembly (Table 3). The primary weakness in these comparisons is not the efficiency measured for the complex but the efficiencies measured for the free enzyme substrate pairs in solution where reaction rates are almost immeasurably slow. However, for the most credible values listed in Table 3, 10^{4}-10^{5}-fold increases in catalytic efficiency result from the

### Table 3. Kinetic Properties of the Vitamin K-Dependent Complexes of Blood Coagulation

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>Km* (μmol/L)</th>
<th>kcat† (min⁻¹)</th>
<th>kcat/Km (mol/L - min⁻¹)</th>
<th>kdS (nmol/L)</th>
<th>References</th>
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<tr>
<td>VIIa</td>
<td>IX</td>
<td>NA§</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>169</td>
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<tr>
<td>VIIa/TF/PCPS/Ca²⁺</td>
<td>IX</td>
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<td>6.42 \times 10⁷</td>
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<tr>
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<td>X</td>
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<td>0.024</td>
<td>4.93 \times 10³</td>
<td>—</td>
<td>170</td>
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<tr>
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<tr>
<td>IXa</td>
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<td>299</td>
<td>0.002</td>
<td>6.69</td>
<td>—</td>
<td>171</td>
</tr>
<tr>
<td>IXa/VIIa/PCPS/Ca⁺</td>
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<td>0.063</td>
<td>500.0</td>
<td>7.94 \times 10³</td>
<td>8.7</td>
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<tr>
<td>Xa</td>
<td>II</td>
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<td>4.68 \times 10²</td>
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<tr>
<td>Xa/Va/PCPS/Ca²⁺</td>
<td>II</td>
<td>1.0</td>
<td>1,800.0</td>
<td>1.80 \times 10⁸</td>
<td>0.8</td>
<td>118</td>
</tr>
<tr>
<td>Xa</td>
<td>PC</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>169</td>
</tr>
<tr>
<td>Xa/TM/PCPS/Ca⁺</td>
<td>PC</td>
<td>10</td>
<td>149.0</td>
<td>1.49 \times 10⁷</td>
<td>0.57</td>
<td>16</td>
</tr>
<tr>
<td>IIa</td>
<td>PC</td>
<td>60</td>
<td>1.2</td>
<td>2.00 \times 10⁴</td>
<td>—</td>
<td>104</td>
</tr>
<tr>
<td>IIa/TM/PCPS/Ca⁺</td>
<td>PC</td>
<td>0.1</td>
<td>214.0</td>
<td>2.14 \times 10⁹</td>
<td>0.62</td>
<td>104</td>
</tr>
<tr>
<td>APC/PCPS/Ca⁺</td>
<td>Va</td>
<td>0.02</td>
<td>24.0</td>
<td>1.20 \times 10⁹</td>
<td>—</td>
<td>172</td>
</tr>
<tr>
<td>APC/S/PCPS/Ca⁺</td>
<td>Va</td>
<td>0.03</td>
<td>35.0</td>
<td>1.17 \times 10⁹</td>
<td>0.8</td>
<td>172</td>
</tr>
</tbody>
</table>

*Michaelis constant for substrate utilization.
†Turnover number.
‡Apparent dissociation constant for enzyme complex assembly estimated from kinetic studies.
§NA, not available.
incorporation of the serine protease into the enzyme complex. This generalization does not apply to protein S/APC/PCPS complex that catalyzes the inactivation of factor Va. Values for this complex in the presence and absence of protein S are presented for comparison in the last two lines of Table 3. While there is a small increase in the turnover number for the reaction (~1.5-fold) in the presence of protein S, there is virtually no change in the Km. Thus, the overall change in reaction efficiency is slightly negative. This comparison calls into question the suggested role of protein S as a cofactor in the anticoagulant process.

The mechanism by which enzyme complex formation causes a decrease in the Km and an increase in the kcat has been the subject of significant controversy. Very little information is available to explain the origin of these increases in the catalytic efficiency. Studies with the prothrombinase complex suggested the change in kcat for the reaction is at least partially related to a direct influence of the cofactor (factor Va) on the active site of the serine protease (factor Xa). Some qualitative evidence suggests that factor Va may influence the active site environment of the Xa molecule. The active site-directed fluorophore (dansyl-Glu-Gly-Arg chloromethyl ketone) covalently incorporated in the active site of factor Xa is perturbed when factor Xa interacts with factor Va in the prothrombinase complex. These data suggest that the active site of factor Xa itself is directly influenced by the formation of the complex. However, the detected perturbation does not appear to lead to an increased reactivity of the catalytically active residues as the kinetic constants for the synthetic substrate S-2222 (Glu GlyArg p-nitroaniline) are unchanged for the complexed factor Xa when compared with the protein in solution.

The incorporation of factor Xa into the prothrombinase complex has a dramatic impact on the kinetic mechanism of the conversion of prothrombin to α-thrombin. This alteration in the kinetic mechanism of substrate utilization can be attributed to the effect of factor Va on the catalyst, and probably accounts for some aspect of the increase in catalytic efficiency observed on complex assembly. Early studies of prothrombin activation using solution-phase factor Xa suggested that prothrombin activation was preceded by an initial cleavage at Arg323-Le324 followed by cleavage at Arg329-Ile330 yields prethrombin 2 and fragment 1.2 as intermediates. A reaction proceeding via steps 3 and 4 (cleavage at Arg329-Ile330 followed by cleavage at Arg274). Thr279 yields meizothrombin as an intermediate. Prothrombin activation catalyzed by solution phase factor Xa proceeds exclusively via steps 1 and 2, while the reaction catalyzed by prothrombinase proceeds via steps 3 and 4.

Meizothrombin is a fully competent serine protease with a covalently bound Gla domain and two kringles (Fig 1). However, meizothrombin shares only a limited number of properties with α-thrombin. It is a competent protein C activator when bound to thrombomodulin and a potent vasoconstrictor with approximately five times the activity of α-thrombin. It is virtually inactive in a procoagulant role as a platelet activator, a factor V activator, or fibrinogen cleaving enzyme. Meizothrombin is also a poor substrate for inhibition by antithrombin-III–heparin as compared with α-thrombin. It is conceivable that meizothrombin provides a significant regulatory element in blood coagulation.

Recent studies have further dissected the contributions of factor Va to prothrombin activation by examining the kinetics of this reaction using solution phase Xa and Va. Prothrombin activation by solution phase catalyst also proceeds primarily via meizothrombin when saturating concentrations of factor Va are present. In solution, micromolar concentrations of the cofactor are required to saturate factor Xa, suggesting that under these conditions the Xa-Va interaction is characterized by the intermediate prothrombin-2 rather than meizothrombin. This generalization does not apply to protein S/APC/PCPS complex that catalyzes the inactivation of factor Va. Values for this complex in the presence and absence of protein S are presented for comparison in the last two lines of Table 3. While there is a small increase in the turnover number for the reaction (~1.5-fold) in the presence of protein S, there is virtually no change in the Km. Thus, the overall change in reaction efficiency is slightly negative. This comparison calls into question the suggested role of protein S as a cofactor in the anticoagulant process.

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In contrast, when membrane-bound prothrombin is activated by membrane-bound prothrombinase, prothrombin activation proceeds exclusively by the alternative pathway in which cleavage at Arg129 precedes cleavage at Arg274 (Fig 8), which associate tightly (kd = 10^-10 mol/L). Subsequently, the fragment 1.2-prethrombin 2 complex is cleaved at Arg275 to yield α-thrombin and the activation peptide, fragment 1.2.

The intermediate product of this reaction route is meizothrombin, a product that is identical to that produced by prothrombin activation by Echis carinatus snake venom. The exclusive production of meizothrombin as an intermediate of prothrombin activation is not only related to the influence of the cofactor on the reaction mechanism but is also related to the membrane binding properties of the substrate. Prothrombin, with altered membrane-binding properties, because of deletion of as few as two γ-carboxyglutamic acids, displays a greatly increased Km as compared with normal prothrombin, and is activated to α-thrombin by the mechanism characterized by the intermediate prethrombin-2 rather than meizothrombin. Meizothrombin is a fully competent serine protease with a covalently bound Gla domain and two kringles (Fig 1). However, meizothrombin shares only a limited number of properties with α-thrombin. It is a competent protein C activator when bound to thrombomodulin and a potent vasoconstrictor with approximately five times the activity of α-thrombin. It is virtually inactive in a procoagulant role as a platelet activator, a factor V activator, or fibrinogen cleaving enzyme. Meizothrombin is also a poor substrate for inhibition by antithrombin-III–heparin as compared with α-thrombin. It is conceivable that meizothrombin provides a significant regulatory element in blood coagulation.
altering the kinetic mechanism of substrate utilization and by stabilizing the enzyme-cofactor-substrate complex.

Extensive studies have been conducted in attempts to interpret the $K_m$ term observed in kinetic studies of prothrombin activation. As the apparent $K_m$ for prothrombinase varies with phospholipid concentration, interpretations of the $K_m$ term center about the origin of the prothrombin molecule, which becomes the substrate for prothrombinase. Two reasonable possibilities could describe the nature of the interaction of the substrate with the enzyme on the membrane surface. In one model, prothrombin interacts directly with factor $X_a$ and factor $V_a$ in forming the Michaelis complex without directly interacting with the membrane surface. In the second possible model, prothrombin would form the Michaelis complex by interacting with factor $V_a$, factor $X_a$, and a site on the membrane surface itself. Since the metal ion-dependent conformational transitions in prothrombin, its membrane-binding requirement and the lipid dependence of the $K_m$ observed for prothrombin activation are undisputed, it seems likely that the Michaelis complex must involve some interaction between prothrombin and the membrane surface.

The involvement of the membrane surface in the delivery of prothrombin to the assembled prothrombinase complex is the most controversial aspect of this reaction. The key dispute centers on whether the prothrombin molecule is delivered to the enzyme directly from bulk solution or whether prothrombin binds to the membrane surface before interacting with the prothrombinase complex itself. In the latter case, the substrate would be derived from the pool of membrane-bound prothrombin by a process that conceivably might involve the translational diffusion of prothrombin in two dimensions.

On the basis of accumulating information, our group subscribes to models that consider membrane-associated prothrombin as the preferred or "true" substrate for prothrombinase. Hence, the apparent $K_m$ term observed for prothrombin activation by prothrombinase is determined by the affinity for the prothrombin-membrane interaction and the degree of saturation of available sites on the membrane surface. The overall "bulk" rate of prothrombin activation, and hence the apparent kinetic constants for this process, therefore are dependent on the local concentrations of prothrombin in the vicinity of prothrombinase on the membrane surface. This notion has led to the development of a quantitative model for the function of prothrombinase based on a constant intrinsic $K_m$ for the assembled enzyme and the partitioning of reactants ($X_a$, $V_a$, and $II$) between solution phase and microvolumes surrounding the membrane surface. This mathematical model, termed "Clotspeed," is reasonably successful in predicting the dependence of reaction parameters on phospholipid concentrations and some of the other atypical kinetic properties of prothrombinase. Other workers have relied on studies of the phospholipid dependence of reaction rate to conclude that the principal source of substrate for prothrombinase is not derived from the membrane microenvironment but instead from bulk solution. However, the "Clotspeed" model which specifies that membrane-bound prothrombin is preferentially used as a substrate can successfully predict the observed phospholipid-dependence of reaction parameters.

The extrinsic factor $X$ activating complex (TF/VIIa/PCPS) is the only other enzyme complex where detailed studies by Forman and Nemerson have examined the role of the factor $X$-membrane interaction on the activation process. These investigators have provided data which suggest that solution phase factor $X$ and not membrane-bound factor $X$ is preferentially used by the enzyme complex. It is conceivable that the extrinsic tenase and the prothrombinase complex interact with their substrates in distinct ways. This hypothesis is reasonable since factor $V_a$ and tissue factor are substantially different with respect to structure, in their interaction with the membrane bilayer and in their requirements for the interaction with the appropriate serine protease.

**SUMMARY**

During the past 20 years contributions from many laboratories have led to the development of isolation procedures, delineation of primary structures, and more recently, to the expression of recombinant proteins associated with the coagulation cascade. In general, studies of coagulation proteins under defined conditions have demonstrated the presence of Davie and Ratnoff and MacFarlane in their proposals of the coagulation cascade. The more recent discovery of thrombomodulin by Esmon et al has led to the identification and characterization of components of the vitamin K-dependent anticoagulant pathway. In this review we have attempted to analyze and compare the functional properties of each of the vitamin K-dependent enzyme complexes associated with the procoagulant and anticoagulant phases of blood clotting. Although dissimilarities exist, the vitamin K-dependent complexes have analogous requirements and appear to function with a common general mode of organization. Membrane-bound cofactors serve as anchoring sites for the appropriate membrane-binding enzymes. This process localizes the complex on the membrane surface and increases the catalytic efficiency for substrate utilization. Complex formation provides extraordinary improvements in the catalytic efficiency for the complexes as compared with their soluble enzyme components. Membrane-bound complexes provide a mechanism that can be regulated at a site by membrane presentation, zymogen activation, and cofactor activation or presentation.

The kinetic constants obtained for the various coagulation reactions determined in vitro provide some insights into how these pathways may function in vivo. The catalytic efficiency ($kcat/K_m$) for factor $X$ activation by factor VIIIa/factor IXa is far in excess of the catalytic efficiency of activation of factor $X$ by tissue factor/factor VIIa (Table 3). This may provide a rational interpretation for the observation that patients with hemophilia A and B bleed even though they appear to have an alternative pathway to factor $X$ activation. In addition, tissue factor is not ordinarily presented by the vascular tissue that has direct access to blood. However, it appears that extravascular constitutive tissue factor is available once the blood vessel becomes disrupted.

The efforts to identify the initiating reactions of the blood
are the other proenzymes of the blood clotting process. We conclude that factor VII is most likely a zymogen, just as are the other proenzymes of the blood clotting process. In addition, it is difficult to rationalize the importance of the intrinsic pathway of coagulation involving factor XII, prekallikrein, and high molecular weight kininogen since the congenital absence of any one of these factors does not result in abnormal bleeding. A number of possible mechanisms may be involved in the initiation of the coagulation cascade. For example, the activation of factor XI on the platelet surface, or the action of other proteases, such as the cancer procoagulant responsible for factor X activation described by Falanga and Gordon, might provide the triggering event for coagulation. It is likely that the coagulation process functions continuously at some minimal "idling" level. The balanced flux between the procoagulant and anticoagulant pathways could then be tipped in favor of the procoagulant phase by the expression of membrane sites on the appropriate cells. It is also possible that molecules such as LACI provide "buffers" which help maintain active enzyme level in a releasable form in blood.

Coagulation complexes have generally been considered to be specific with respect to the components of the complex and substrate specificity. The concept of absolute substrate specificity has been eliminated by numerous investigators over the past 20 years. Factor Xa and thrombin are the best illustration of enzymes that act on diverse substrates in the coagulation scheme. The recent observation that factor Xa can form a complex with thrombomodulin suggests that fidelity of the components of coagulation complexes is not absolute. The physiologic relevance of the factor Xa-thrombomodulin complex is somewhat suspect in view of its relatively high Km for the substrate protein C. On the other hand, in animal studies it has been demonstrated that protein C activation occurs on infusion of factor Xa/phospholipid mixtures when only minimal thrombin generation can be demonstrated. Thus, it is possible that there are multiple initiators of protein C activation and possibly other proteases in blood clotting and in fibrinolysis. The mechanistic alterations that occur in prothrombinase activation of prothrombin to thrombin give rise to meizothrombin as a principal intermediate of the reaction. Meizothrombin may be an important participant in the anticoagulant pathway as it neither interacts with cellular or protein substrates involved in the procoagulant phase of coagulation, nor is it readily inhibited by antithrombin III and heparin. However, it is selective as a vasoconstrictor and as a protein C activator in the presence of thrombomodulin.

The localization of coagulation reactions on membranes is an illustration of a common conceptual notion in biology. At present, few examples of high order reactions (greater than second order) have been demonstrated in nature. The organization of multi-protein membrane complexes by a series of second order reactions eliminates the unfavorable statistical considerations for trinolecular reactions. It is likely that other processes in the anticoagulant and thrombolytic pathways are analogs of the membrane-bound coagulation complexes. Tissue plasminogen activator and plasminogen are known to interact with solid-phase fibrin, resulting in a diminished Km for the t-PA plasminogen activation reaction. Similarly, the antithrombin III neutralization of thrombin appears to function, at least in part, by the collection of the inhibitor antithrombin III and the enzyme thrombin on a heparin sulfate proteoglycan surface. The activation of plasminogen by t-PA and the reaction between antithrombin III and thrombin both take advantage of relevant multivalent surfaces for the assembly of the appropriate supramolecular structure. It is likely that many regulatory events in biology involving the localized assembly of multi-component complexes proceed by similar mechanisms.

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