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Role of Accessory Components in the Activation of Vitamin K-Dependent Coagulation Factors

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Abstract. Kinetic studies of prothrombin activation and intrinsic factor X activation carried out in the absence and presence of phospholipids and the protein cofactors Va or VIIIa have provided insight in the mechanism by which the accessory components enhance coagulation factor activation. In intrinsic factor X and prothrombin activation, phospholipids cause a drastic drop of K_m for the substrates factor X and prothrombin, whereas the protein cofactors factor Va and factor VIIIa increase V_{max} of the prothrombin- and factor X-activating reactions. The mode of action of factor Va in prothrombin activation is however somewhat more complex. Besides its stimulatory effect on the catalytic activity of factor Xa, Factor Va also plays an important role in the assembly of the prothrombin-activating complex at phospholipid surfaces especially when the latter have a low affinity for vitamin-K-dependent coagulation factors. This effect is likely accomplished by promoting the binding of both prothrombin and factor Xa to the procoagulant surface.

Introduction

In the blood coagulation cascade, there are several reactions which require the presence of components, which by themselves have no enzymatic activity but which greatly stimulate coagulation factor activation [1]. These components, called nonenzymatic cofactors or accessory components, are divided in protein cofactors and negatively charged procoagulant surfaces. Enzyme, substrate and protein cofactors bind to the negatively charged surface and form an enzymatic complex that is optimally active in substrate activation. Thus, in the activation of the coagulation factors XII, XI and prekallikrein, enzyme-substrate interaction is promoted by their binding to negatively charged surfaces like kaolin, glass or sulfatides and by the protein cofactor high-molecular-weight kininogen. For the activation of the coagulation factors X and prothrombin the protein cofactors are: factor VIIIa for the intrinsic factor X activation, tissue factor protein for the extrinsic factor X

activation and factor Va for prothrombin activation. The procoagulant surface, which promotes prothrombin and also extrinsic and intrinsic factor X activation, consists of phospholipid bilayers that contain negatively charged phospholipids. It is likely that under physiological conditions activated blood platelets provide the procoagulant surface that promotes the assembly of the prothrombin- and intrinsic factor-X-activating complexes since upon stimulation with thrombin plus collagen they expose negatively charged phosphatidylserine in the outer monolayer of their plasma membranes [2, 3].

The improvement of purification methods of the proteins involved in intrinsic factor X and prothrombin activation and the development of chromogenic substrates specific for thrombin and factor Xa made it possible to obtain via a kinetic approach insight in the role of the accessory components in these reactions. In this paper we will summarize kinetic studies carried out in our laboratory on prothrombin [4, 5] and intrinsic factor X activation [6]. The data obtained allow a precise quantitation of the stimulatory effects of the accessory components and also provide information about their mechanism of action in coagulation factor activation.

Effect of the Nonenzymatic Cofactors on the Kinetic Parameters of Prothrombin and Factor X Activation

Prothrombin and intrinsic factor X activation are the most intensively studied vitamin-K-dependent clotting factor activation reactions. They have many features in common. Both the enzymes (factor Xa, factor IXa) and the substrates (prothrombin and factor X) are vitamin K-dependent proteins, which contain γ-carboxy glutamic acid residues that are important for the Ca^{2+}-dependent binding of these proteins to negatively charged phospholipids. Both enzymes are serine proteases. Also the protein cofactors factor Va and factor VIIIa share several properties. They have no enzymatic activity of their own and both have to be activated by thrombin to express their function in the coagulation factor activation [7, 8]. Also factor V (Va) and factor VIII bind with a high affinity to phospholipid bilayers [9, 10], although they do not contain γ-carboxyglutamic acids.

The importance of the nonenzymatic cofactors is illustrated in table I. We have summarized the effects of the accessory components on the rates of prothrombin and factor X activation. It is clear that phospholipids and the protein cofactor stimulate coagulation factor activation in a multiplicative way. Maximal rates of prothrombin and factor X activation are obtained in the presence of both phospholipids and the protein cofactor (Va or VIIIa). In order to get more insight in the mode of action of phospholipids, factor Va and factor VIIIa in the prothrombin and factor X activation, we have determined their effects on the kinetic parameters of coagulation factor activation [4, 6]. In the absence as well as in the presence of accessory components, Lineweaver-Burk plots for prothrombin and factor X activation were straight lines from which the kinetic parameters K_m and V_{max} could be determined. Table II summarizes the effects of the accessory components on the kinetic parameters for prothrombin and intrinsic factor X activation. These data indicate that the role of factor Va and factor VIIIa in prothrombin and factor X activation, respectively, is mainly restricted to an effect on V_{max}. Table II also

Table I. Effect of nonenzymatic cofactors on relative rates of prothrombin and factor X activation

Prothrombin activator[a]	Relative rate of prothrombin activation	Factor X activator[b]	Relative rate of factor X activation
Xa	1	IXa	1
Xa, Ca^{2+}	2	IXa, Ca^{2+}	8
Xa, Ca^{2+}, PL	5×10^2	IXa, Ca^{2+}, PL	2.3×10^3
Xa, Ca^{2+}, Va	2.3×10^3	IXa, Ca^{2+}, VIIIa	–
Xa, Ca^{2+}, PL, Va	7.7×10^5	IXa, Ca^{2+}, PL, VIIIa	5.3×10^6

PL = Phospholipid.

[a] 1 μ*M* prothrombin, 5 m*M* $CaCl_2$ 50 μ*M* phospholipid (phosphatidylserine 25%, phosphatidylcholine 75%, w/w) saturating factor Va.

[b] 1 μ*M* factor X, 5 m*M* $CaCl_2$, 50 μ*M* phospholipid (phosphatidylserine 25%, phosphatidylcholine 75%, w/w) saturating factor VIIIa.

reveals that the role of phospholipid in the factor-X- and prothrombin-activating complexes is restricted to an effect on K_m for factor X and prothrombin. In the absence of phospholipid, the K_m for prothrombin (84 μ*M*) and factor X (181 μ*M*) are considerably higher than their plasma concentrations (prothrombin 1.5 μ*M*, factor X 0.2 μ*M*), whereas upon addition of phospholipid to both complexes, the K_m drop to values well below the plasma concentrations. This allows both reactions to proceed at maximal velocity under physiological conditions, provided that a negatively charged phospholipid surface is present.

Mode of Action of Phospholipids in Prothrombin and Factor X Activation

Table III shows that the enzymatic complexes for factor X and prothrombin activation cannot be saturated by the addition of increasing amounts of phospholipid. K_m is dependent on the phospholipid concentration such that K_m for prothrombin and factor X increase with increasing phospholipid concentrations. Thus, K_m measured in the presence of phospholipid must be regarded as an apparent K_m ($K_{m_{app}}$), which is a function of the phospholipid concentration in the reaction mixture.

In figure 1, three models are presented which can explain the drop in K_m after phospholipid addition as well as the apparent character of this kinetic parameter. The models are given for prothrombin activation in the absence of factor Va and are presumably also valid for intrinsic factor X activation in the absence of factor VIIIa. Model 1 illustrates the situation in which a phospholipid-bound factor Xa molecule has a conformational state (asterisk) different from the factor Xa molecules in free solution. This conformational change of phospholipid-bound factor Xa causes an increase of the affinity of this molecule for prothrombin molecules which are present in free solution. This increased affinity between enzyme and sub-

strate will result in a lowered $K_{m_{app}}$ for prothrombin.

Also in model 2, prothrombin molecules originating from free solution interact directly with phospholipid-bound factor Xa molecules, but in this model the bound factor Xa molecules have not undergone a conformational change. Binding of prothrombin to phospholipid-bound factor Xa is promoted by the additional affinity of prothrombin to negatively charged phospholipid molecules in the direct environment of the factor Xa molecule (dashed circle). Both in model 1 and model 2, the saturation of factor Xa is directly dependent on the concentration of prothrombin free in solution. When the phospholipid concentration is increased, more prothrombin is bound to phospholipid leaving less prothrombin in solution available for interaction with phospholipid-bound factor Xa. To obtain half maximal saturation (i.e, K_m conditions) of factor Xa with prothrombin, more prothrombin must be added at higher phospholipid concentrations. Since K_m for prothrombin is expressed in terms of the concentration of total added prothrombin, $K_{m_{app}}$ will be higher at higher phospholipid concentrations (cf. table III).

In model 3, phospholipid-bound factor Xa activates phospholipid-bound prothrombin. In this model, half maximal saturation of bound factor Xa is determined by the local concentration of phospholipid-bound prothrombin. The fact that the local prothrombin concentration at the phospholipid surface is higher than that in free solution explains the phospholipid-dependent decrease of K_m for prothrombin. However, when the phospholipid concentration is again increased, the bound prothrombin molecules are diluted at the phospholipid surface and more prothrombin must be

Table II. Kinetic parameters of prothrombin and factor X activation

Prothrombin activator	K_m prothrombin, μM	V_{max}, IIa · $min^{-1} \cdot Xa^{-1}$
Xa, Ca^{2+}	84	0.68
Xa, Ca^{2+}, PL	0.11	2.56
Xa, Ca^{2+}, PL, Va	0.14	4,050
Factor X activator	**K_m factor X μM**	**V_{max}, Xa · $min^{-1} \cdot IXa^{-1}$**
IXa, Ca^{2+}	181	0.01
IXa, Ca^{2+}, PL	0.36	0.025
IXa, Ca^{2+}, PL, VIIIa	0.29	500

The kinetic parameters were measured at 50 μM phospholipid vesicles containing 25 mol% phosphatidylserine and 75 mol% phosphatidylcholine.

PL = Phospholipid.

Table III. Effect of phospholipid on K_m of prothrombin for prothrombin activation and K_m of factor X for intrinsic factor X activation

Prothrombin activation		Intrinsic factor X activation	
phospholipid[a] μM	$K_{m_{app}}$ μM	phospholipid[b] μM	$K_{m_{app}}$ μM
2.6	0.032	10	0.058
10.5	0.068	20	0.139
26.3	0.164	50	0.363
52.6	0.25	75	0.409
75	0.35	100	0.525
105	0.48	150	0.822
240	1.08	200	1.83
		300	1.76

In these experiments, neither factor Va nor VIIIa added.

[a] The phospholipid composition of the vesicles was 50 mol% phosphatidylserine and 50 mol% phosphatidylcholine.

[b] The phospholipid composition of the vesicles was 25 mol% phosphatidylserine and 75 mol% phosphatidylcholine.

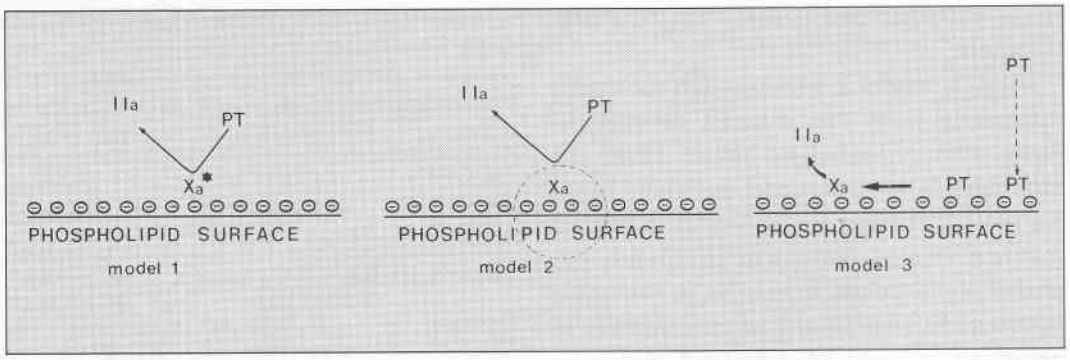

Fig. 1. Models for the mechanism of action of phospholipids in prothrombin (PT) activation in the absence of factor Va. In all three models, phospholipid causes a decrease of the $K_{m_{app}}$ for prothrombin. For further explanations, see text.

added to restore the local concentration of phospholipid-bound prothrombin required for half saturation of phospholipid-bound factor Xa. Thus, when K_m is expressed in terms of total prothrombin concentration, also in model 3 $K_{m_{app}}$ will increase at increasing phospholipid concentrations. In this model, it is expected, that, although $K_{m_{app}}$ increases with the phospholipid concentration, a K_m expressed in terms of prothrombin surface density would be constant. Using binding parameters reported by *Nelsestuen and Broderius* [11] we calculated prothrombin and factor X surface densities at the K_m presented in table III. Figure 2 compares the measured $K_{m_{app}}$ for prothrombin or factor X with the surface density of bound prothrombin and factor X, at the K_m concentrations. It is obvious that K_m expressed in terms of surface density is independent of the phospholipid concentration, as might be expected in model 3.

In model 1 and model 2, the concentration of free substrate at $K_{m_{app}}$ must be independent of the phospholipid concentration. Figure 2 shows that also the free prothrombin and factor X concentration at K_m are independent of the phospholipid concentration. Thus, on the basis of these calculations, we cannot distinguish between the three proposed models, since both the free substrate concentration (requirement of model 1 and 2) and the surface density of substrate (requirement of model 3) are independent of and constant at different phospholipid concentrations.

Table IV shows the effect of variation of the molar fraction of phosphatidylserine in phospholipid vesicles on the kinetic parameters of prothrombin activation. In the absence of factor Va, vesicles containing 40% phosphatidylserine exhibit a 40-fold lower $K_{m_{app}}$ than vesicles containing 2.5% phosphatidylserine. Prothrombin affinity for phospholipid vesicles has been shown to increase at increasing content of phosphatidylserine in these vesicles [11]. Therefore, the difference in $K_{m_{app}}$ measured with phospholipid vesicles containing varying molar percentages phosphatidylserine is likely caused by differences of the affininty of prothrombin for these vesicles. In models 2 and 3, a lower affinity of prothrombin for phospholipid results either in a lower affinity of the factor

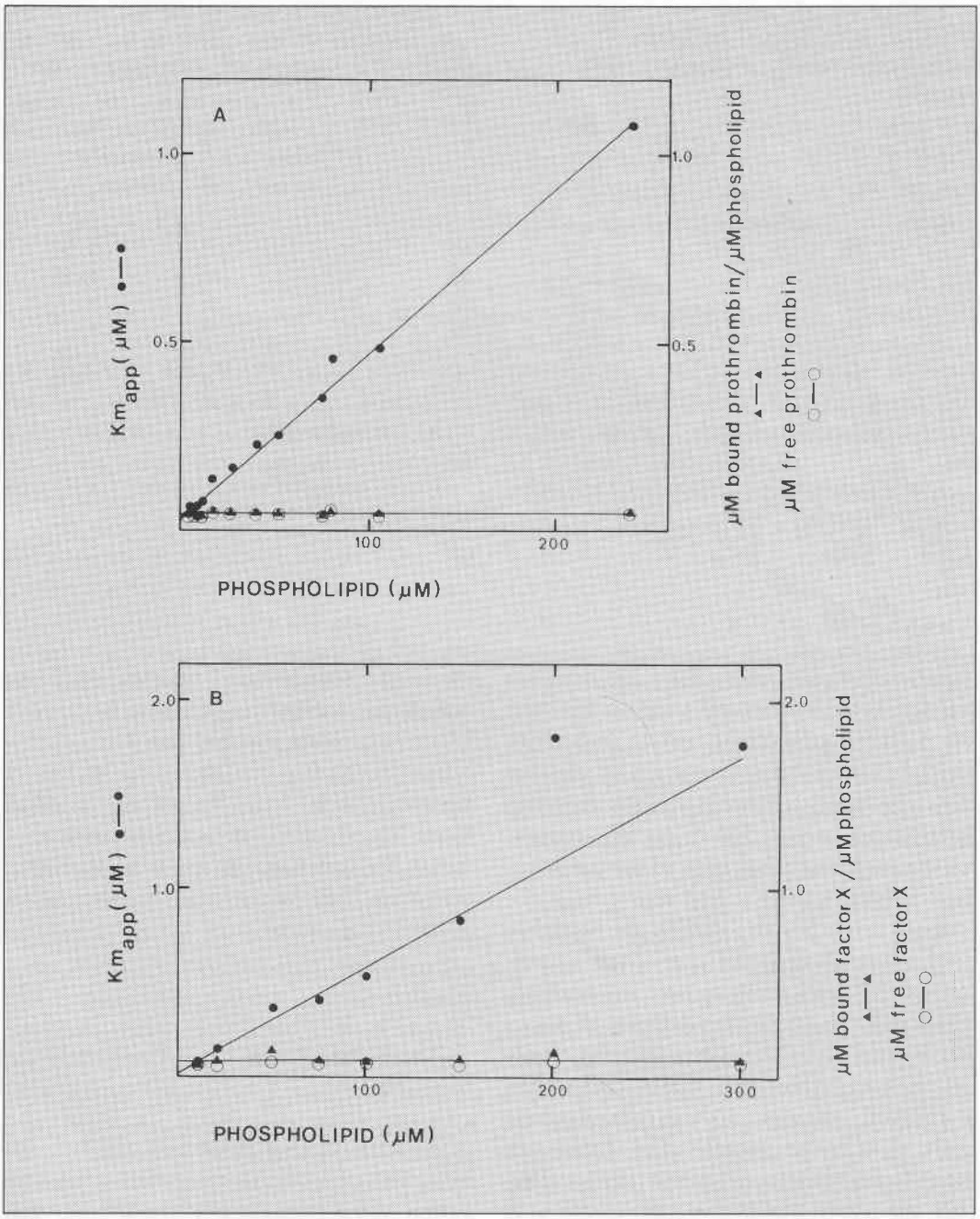

Fig. 2. Effect of increasing the phospholipid concentration on $K_{m_{app}}$ (●), Km expressed in terms of substrate surface density (▲) or K_m expressed in terms of free substrate concentration (○). **A** Prothrombin activation. **B** Intrinsic factor X activation.

Table IV. Effect of phospholipid vesicles with different phosphatidylserine content on the kinetic parameters of prothrombin activation in the absence of factor Va

Vesicle composition		K_m	V_{max}
PS, %	PC, %	μM	IIa·min^{-1}·Xa^{-1}
40	60	0.17	3.33
30	70	0.20	2.70
20	80	0.19	2.00
10	90	0.54	0.83
5	95	1.66	0.33
2.5	97.5	3.71	0.08

PS = Phosphatidylserine; PC = phosphatidylcholine.

Kinetic parameters were determined at a phospholipid concentration of 50 μM.

Table V. Effect of phospholipid vesicles with different phosphatidylserine content on the kinetic parameters of prothrombin activation in the presence of factor Va

Vesicle composition		K_m	V_{max}
PS, %	PC, %	μM	IIa·min^{-1}·Xa^{-1}
40	60	0.33	3,750
30	70	0.25	4,080
20	80	0.11	4,170
10	90	0.07	4,920
5	95	0.07	4,420
2.5	97.5	0.13	4,170

PS = Phosphatidylserine; PC = phosphatidylcholine.

Kinetic parameters were determined at a phospholipid concentration of 50 μM.

Xa-phospholipid unit for prothrombin (model 2) or in a decreased prothrombin density at the phospholipid surface (model 3), which in both cases will result in an increase of K_m. In model 1, the charge of the phospholipid surface is expected to be unimportant for the conformational state of the phospholipid-bound factor Xa, and moreover a decreased prothrombin binding to phospholipid will result in an increase of the concentration of prothrombin free in solution, which will cause a further decrease of $K_{m_{app}}$. We feel that on this basis and from the observation that acarboxyprothrombin, which has a reduced amount of γ-carboxyglutamic acids, gives in the presence of phospholipids much lower reaction rates than prothrombin [12], the conformational model (model 1) is less likely.

Also the V_{max} of prothrombin activation depends on the amount of phosphatidylserine in the phospholipid vesicles. At lower molar percentages of phosphatidylserine, lower V_{max} values are observed. This is likely caused by the fact that these vesicles have a lower affinity for factor Xa and thus bind less factor Xa, which will decrease the amount of factor Xa that participates in prothrombin activation. This will result in the observation of lower V_{max} values.

The Role of Factor Va in Prothrombin Activation

As we have seen in table II, the stimulatory effect of factor Va on prothrombin activation in the presence of phospholipid vesicles with a high molar percentage of phosphatidylserine must be attributed to an increase of V_{max} [cf. 4]. More recently it was observed

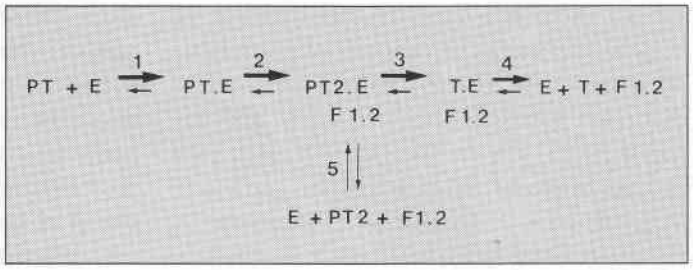

Fig. 3. Prothrombin activation pathway. PT = Prothrombin; E = prothrombin-activating complex; PT2 = prothrombin 2; T = thrombin; F1.2 = prothrombin fragment 1.2. Numbers 1–5 designate the reaction steps explained in the text.

that factor Va also lowers K_m for prothrombin for membranes with a low affinity for prothrombin [5, 13]. This effect of factor Va on K_m for prothrombin will be discussed later.

The increase of the V_{max} by factor Va is the result of three additive effects: (1) a shift in the reaction pathway of factor-Xa-catalyzed prothrombin activation [4], (2) an increase of a forward rate constant in the prothrombin activation pathway [14] and (3) an increase of the amount of factor Xa that participates in prothrombin activation [15, 16].

Figure 3 gives the minimal reaction mechanism for prothrombin activation that will be helpful to explain the factor-Va-induced shift in the prothrombin activation pathway. Gel-electrophoretic analysis has shown that in the absence of factor Va prethrombin 2 is the main product of prothrombin activation [4]. This shows that the major reaction pathway occurring under these conditions is that represented by steps 1, 2 and 5, and it also indicates that prethrombin 2 easily dissociates from factor Xa. In the presence of factor Va there is almost no prethrombin 2 formation, and thrombin is the major activation product. Thus, factor Va changes the pathway of prothrombin activation from one producing prethrombin 2 (steps 1, 2, 5) into one giving rise to thrombin (steps 1–4). Apparently, the presence of factor Va prevents the dissociation of prethrombin 2 from the prothrombinase complex, thereby increasing the rate of thrombin formation.

Based on the estimation of the rate constants of prethrombin 2 formation (without factor Va) and thrombin formation (with factor Va), *Tans* et al. [17] concluded that apart from the shift in the pathway factor Va must also increase at least one forward rate constant of the prothrombin activation pathway. This was indeed shown to be the case by *Nesheim and Mann* in 1983 [14]. From kinetic experiments with prothrombin and prothrombin activation intermediates they concluded that factor Va causes a 3,000-fold increase of the k_{cat} of the factor-Xa-catalyzed cleavage of the Arg_{323}-Thr_{324} bond in prothrombin (fig. 3 step 3).

The third effect of factor Va on V_{max} of prothrombin activation concerns its ability to promote the binding of factor Xa to negatively charged phospholipid vesicles [15, 16]. Especially for membranes with a low affinity for factor Xa, this property of factor Va will contribute to the increase of the V_{max}. This phenomenon becomes clear upon comparison of the effects of procoagulant surfaces with different phosphatidylserine content on V_{max} measured in the absence (table IV) or presence of factor Va (table V). Without fac-

tor Va there is a considerable decrease of V_{max} at low molar percentages of phosphatidylserine. This is likely caused by the fact that these membranes have a low affinity for factor Xa [11] hence less factor Xa will participate in prothrombin activation and lower V_{max} values will be observed. However, in the presence of factor Va there is hardly any effect of variation of the molar percentage of phosphatidylserine on V_{max} of prothrombin activation (table V). This indicates that under these conditions all added factor Xa participates in prothrombin activation irrespective of the affinity of the procoagulant membranes for factor Xa. This can be attributed to the fact that factor Va has the ability to promote the binding of factor Xa to the phospholipid vesicles.

Apart from the effects of factor Va on V_{max}, table V also shows an effect of factor Va on K_m for prothrombin when phospholipid vesicles with a low molar percentage phosphatidylserine are used as procoagulant surface. In the absence of factor Va, low molar percentages of phosphatidylserine gave high K_m values. This could be easily explained in models 1 and 2 (fig. 2), in which the weak affinity of prothrombin for the phospholipid surface of vesicles containing low molar percentages of phosphatidylserine [11] results in a high K_m for prothrombin. However, in the presence of factor Va the K_m for prothrombin is low and independent of the molar percentage of phosphatidlyserine in the vesicles. So apparently, factor Va compensates for the loss of binding affinity of prothrombin for this kind of membrane surfaces. Several explanations are possible for the observation that factor Va lowers K_m of prothrombin for procoagulant membranes with a low affinity for vitamin-K-dependent coagulation factors. This phenomenon can be accomplished by (1) a direct interaction of prothrombin with factor Va, (2) a factor-Va-induced clustering of negatively charged phospholipid molecules around the enzymatic unit that creates a better surface for prothrombin binding or (3) a factor-Va-induced increase of a rate constant in the pathway of prothrombin activation that simultaneously increases the k_{cat} and decreases the K_m. The third possibility can be explained with a simplified reaction scheme for serine proteases:

$$E + S \underset{}{\overset{K_s}{\rightleftharpoons}} ES \xrightarrow[P_1]{k_2} EA \xrightarrow{k_3} E + A$$

in which

$$k_{cat} = \frac{k_2 \cdot k_3}{k_2 + k_3} \quad \text{and} \quad K_m = K_s \cdot \frac{k_3}{k_2 + k_3}$$

In this mechanism it is possible that an increase of a single rate constant (k_2) by factor Va can cause an increase of k_{cat} and a decrease of K_m. At present we cannot give preference to either of the three models since proper kinetic data that would allow discrimination are not yet available.

Concluding Remarks

The most important contribution of the phospholipid surface in the activation of vitamin-K-dependent coagulation factors is to promote the interactions between the proteins involved in these reactions. For this stimulatory effect, the proteins must have the ability to bind to the procoagulant surface. The stimulation by surface is caused by the fact that the proteins have an affinity for the phospholipid surface and for each other, a combination which ensures efficient protein-protein interactions at the surface. Thus,

phopholipids stimulate prothrombin and factor X activation by facilitating enzyme-substrate complex formation – a phenomenon which is reflected in a large drop of K_m of their respective substrates.

The functions of the nonenzymatic protein cofactors, factors Va or VIIIa, are most easily explained on the basis of the effects of factor Va in prothrombin activation. It is shown that factor Va acts in prothrombin activation by enhancing the catalytic efficiency of factor Xa, and by assembling factor Xa and prothrombin to an enzymatic complex at the phospholipid surface.

The marked effects of factor Va on the kinetic parameters of prothrombin activation, which are found in the presence of phospholipid vesicles with a weak affinity for factor Xa and prothrombin, are likely of physiological importance. It is generally accepted that in vivo stimulated blood platelets provide the procoagulant phospholipid surface that serves the activation of prothrombin and factor X since activated platelets expose phosphatidylserine in the outer monolayer of their plasma membrane. After activation with physiological platelet activators (collagen plus thrombin), the platelet outer monolayer contains, about 5 mol% phosphatidylserine [1]. Such a membrane surface would show weak interactions with prothrombin and factor Xa in the absence of factor Va. Therefore, factor Va will also play an important role in the assembly of the prothrombin-activating complex at the surface of blood platelets. Since prothrombin and factor X activation have many common features, it is likely that phospholipids, factor VIIIa and activated blood platelets act in a similar way in intrinsic factor X activation as the corresponding cofactors in prothrombin activation.

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