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INTRODUCTION

The role of factors V and VIII in the reaction sequence of blood coagulation has remained relatively obscure even after the general principles of the cascade mechanism had been recognised. First, it was thought that these factors participated in the sequence of proenzyme - enzyme transitions [1,2]. Later [3,4], it became evident that they do not belong to the zymogens that form the cascade *per se*, but enhance the activity of respectively the serine proteases factor X_a and factor IX_a. They exert this effect when participating in a ternary complex of e.g. factor X_a, factor V_a and factor II bound to a phospholipid water interface. In these complexes the activity of the enzyme is enhanced manyfold [5,6,7].

The kinetics of the formation of prothrombinase activity is compatible with a model in which both factor X_a and V_a adsorb reversibly onto the phospholipid surface and become active when they are adsorbed one beside the other [8]. The same results have been obtained with regard to the factor X activating enzyme [9]. It can be surmised that the protein component of tissue factor plays in the extrinsic factor X activation a role comparable to that of factors V_a and $VIII_a$ in the other complexes [10].

The fact that these accessory proteins, also called paraenzymes, are enhancing the reaction rates thus is firmly established. However, until recently no conclusions could be drawn as to the mechanism by which this rate enhancement was accomplished. This is an important question not only in the field of blood coagulation but also in other fields of biochemistry, as enzyme-paraenzyme complexes at interfaces have been recognised in the complement system, in oxidative phosphorylation and possibly in other membrane bound systems [11].

In order to obtain insight in the mode of action of the accessory proteins we determined their effect on the kinetic constants for the activation of both factor II and factor X in purified systems.

This became possible because purification of the clotting factors involved is now feasible and because chromogenic substrates provide accurate and sensitive methods to quantify thrombin and factor X_a. Since both factor X_a and IX_a belong to the class of serine proteases [12, 13], a model for the mode

of action of factors V_a and $VIII_a$ has to be compatible with the known mode of action of these enzymes.

ACCESSORY PROTEINS ENHANCE k_{cat}

Kinetics of prothrombin activation

The K_m's and k_{cat}'s obtained with prothrombin as a substrate and with different compositions of the prothrombin activating complex are summarized in Table I. These data explain the rate enhancements obtained by the addition of phospholipids and factor V_a observed earlier [5, 6]. The effect of phospholipids is mainly on the K_m for prothrombin whereas factor V_a almost exclusively enhances the k_{cat} of the reaction. A detailed description of the experiments leading to this conclusion is given elsewhere [17]. For a discussion of the effect of phospholipids the reader is referred to the same article and to the contribution of Zwaal et al [18] in this volume.

TABLE I

KINETIC CONSTANTS OF PROTHROMBIN AND FACTOR X ACTIVATION

Enzyme	Substrate	Accessory factors	K_m (μM)	k_{cat} (s^{-1})
X_a	II	Ca^{2+}	84	0.011
X_a	II	Ca^{2+}, V_a	34	6.22
X_a	II	Ca^{2+}, PL (7.5 μM)	0.06	0.038
X_a	II	Ca^{2+}, PL (7.5 μM), V_a	0.2	32.0
IX_a	X	Ca^{2+}	272	10^{-4}
IX_a	X	Ca^{2+}, PL (7.5 μM)	0.05	5×10^{-5}
IX_a	X	Ca^{2+}, PL (7.5 μM), $VIII_a$	0.05	1.85
VII_a	X	Ca^{2+}, tissue factor	0.34	32
VII_a	X	Ca^{2+}, PL	4.87*	3.95×10^{-4}*
VII_a	X	Ca^{2+}, tissue factor	0.45*	1.15*

* The constants were determined in the presence of 10 mM benzamidine.

The data for factor X activation via the extrinsic pathway are from Silverberg et al [10].

Kinetics of factor X activation

The experimental approach used for the prothrombinase complex was also applied in a study on the effects of accessory components (i.e. phospholipid and factor $VIII_a$) on the kinetic parameters of factor X activation by factor IX_a. The method for measuring the rate of factor X_a formation with different

factor X activating complexes is as follows: factor X is incubated with factor IX_a either in the absence or presence of Ca^{2+}, phospholipid and/or factor $VIII_a$. After different time intervals a sample is taken and essayed for factor X_a using the chromogenic substrate S 2222. From a calibration curve made with active-site titrated factor X_a the amount of factor X_a formed in the incubation mixture and the rate of factor X activation can be calculated.

It was established that the rate of factor X_a formation is linear in time and proportional to the amount of enzyme present. Straight Lineweaver Burk plots were obtained in all cases.

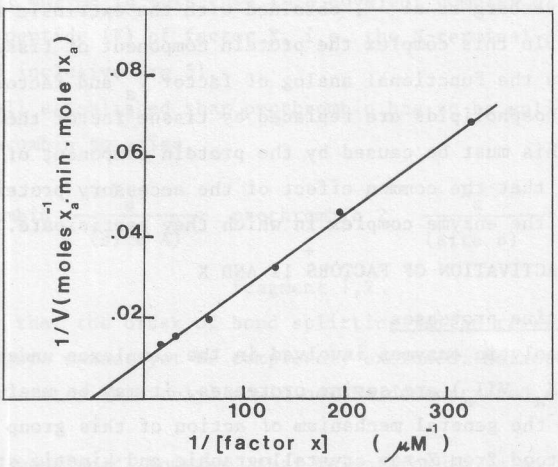

Fig.1. Lineweaver Burk plot for factor X activation in the presence of 9.2×10^{-9} µmole/ml factor IX_a, 0.3 mg/ml factor $VIII_a$, 10 µM phospholipid, 10 mM $CaCl_2$, 175 mM NaCl, 50 mM Tris-HCl pH 7.9 and 1 mg/ml ovalbumin. Factors X and IX were purified from bovine plasma [14,15]. Factor IX was activated with contact product and factor IX_a was purified on DEAE-Sephadex. The concentration of factor IX_a was determined by active site titration with paranitrophenylguanidinobenzoate. Bovine factor VIII, obtained by gelfiltration of a cryoprecipitate [16], was activated with 0.01 NIH-U thrombin/ml for 1 minute just before addition to the factor X activation reaction mixture. Phospholipids were single bilayer vesicles, prepared by sonication of equimolar amounts of phosphatidylcholine and phosphatidylserine. Further details can be found in the text and in the paper by Zwaal et al, this volume.

Fig. 1 shows the Lineweaver Burk plot for the complete complex, consisting of factor IX_a, Ca^{2+}, phospholipids and factor $VIII_a$. The values of K_m and k_{cat} determined for the different compositions of the activating complex are shown in Table I. It is seen that here, as in the prothrombinase complex, the effect of phospholipids is predominantly on K_m whereas factor $VIII_a$ greatly enhances k_{cat}. The 40,000 times increase caused by the addition of factor $VIII_a$ in this experiment is not even the maximum since we have not been able yet to saturate factor IX_a with factor $VIII_a$ (data not shown).

Similarities in the mode of action of accessory proteins

From Table I it is obvious that there is a striking similarity between the effect of phospholipids on both reactions studied, the effect being almost exclusively on K_m. It also is clear that the accessory proteins in both reactions act predominantly by increasing k_{cat}. Table I also contains the kinetic data of Silverberg et al [10], obtained with the extrinsic factor X activating enzyme. In this complex the protein component of tissue factor may be considered as the functional analog of factor V_a and factor $VIII_a$. It is seen that when phospholipids are replaced by tissue factor the main effect is again on k_{cat}. This must be caused by the protein component of tissue factor. We conclude that the common effect of the accessory proteins is an increase of k_{cat} of the enzyme complex in which they participate.

MECHANISMS FOR THE ACTIVATION OF FACTORS II AND X

The mechanism of serine proteases

Because the proteolytic enzymes involved in the complexes under study (i.e. factors IX_a, X_a, VII_a) are serine proteases, it may be useful to recall the details of the general mechanism of action of this group of enzymes as it is now understood from X-ray crystallographic and kinetic studies [19]. This mechanism can be depicted by the following scheme:

$$E + S \rightleftarrows E.S \rightarrow E\text{-}S \rightarrow E.P_1 \rightarrow E\text{-}P_2 \rightarrow E.P_2 \rightarrow E + P_2 \qquad (A)$$

$$\qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad \searrow \quad \nearrow$$

$$\qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad P_2 \qquad + P_1$$

First, the Michaelis complex (E.S) is formed by noncovalent interactions. Then a covalent tetrahedral complex (E-S) is formed by nucleophilic attack of the serine oxygen on the carboxyl group in the substrate. The reactivity of the seryl residue in this and the next step is enhanced by its participation in the charge - relay system formed by this residue and a sterically adjacent histidine and aspartic acid. The next step is the breakdown of the tetrahedral complex into the acyl-enzyme ($E\text{-}P_2$). If the substrate is a protein

or polypeptide, the new C-terminal will form the acyl bond, i.e. the polypeptide between the original N-terminal and the new C-terminal (P_2) will remain attached to the enzyme. The part between the new N-terminal and the old C-terminal end (P_1) is forced away from the enzyme. Finally, the reaction is completed by the hydrolysis of the acyl enzyme and the active site comes free again.

Factor X and prothrombin activation by serine proteases

Because factor IX_a (factor VII_a) is a serine protease the activation of factor X can be written as:

$$E + X \rightleftarrows E.X \rightarrow E{-}X \rightarrow E.X_a \rightarrow E{-}F \rightarrow E.F \rightarrow E + F \qquad \text{(B)}$$
$$\qquad\qquad\qquad\qquad \setminus F \qquad + X_a$$

From the work of the groups of Davie and Nemerson [20, 21], it can be concluded that the acyl enzyme in this case is a covalent complex of factor IX_a and the activation peptide (F) of factor X, i.e. the N-terminal part of the heavy chain up to and inclusive Arg 51.

It is now well established that prothrombin has to be split at two places in order for thrombin to arise.

$$\text{Prothrombin} \xrightarrow[\text{(site A)}]{X_a} \text{prethrombin 2} \xrightarrow[\text{(site B)}]{X_a} \text{thrombin}$$
$$+$$
$$\text{fragment 1,2}$$

The possibility that the order of bond splitting is the reverse of the order shown in the figure cannot yet be completely excluded. Meizothrombin (i.e. prothrombin split at site B only) could not be demonstrated as an intermediate of activation.

It has to be mentioned, however, that meizothrombin is an intermediate when Echis Carinatus venom is the activating enzyme [22], and in the activation of an abnormal prothrombin [23]. Since prethrombin 2 can qualify as an intermediate when factor X without factor V_a is the activating enzyme our working hypothesis for the reaction pathway of prothrombin activation is the one presented below [6].

$$E + P \overset{1}{\rightleftarrows} E.P \overset{2}{\rightarrow} E{-}P \overset{3}{\rightarrow} E.P_2 \overset{4}{\rightarrow} E.P_2 \overset{5}{\rightarrow} E.P_2 \overset{6}{\rightarrow} E{-}P_2 \overset{7}{\rightarrow} E{-}T \overset{8}{\rightarrow} E.T \overset{9}{\rightarrow} E + T \qquad \text{(C)}$$
$$\setminus F \qquad \vdots F$$
$$10 \downarrow\uparrow \qquad 11 \downarrow\uparrow \qquad 12 \downarrow\uparrow$$
$$E{-}F \qquad E{+}P_2 \qquad E{+}P_2$$
$$+P_2 \qquad +F$$

In this scheme a dot (.) denotes noncovalent binding in Michaelis complexes, and a hyphen (-) covalent binding in tetrahedral complexes and acyl enzymes. E = factor X_a; P = prothrombin; F = the activation peptide, i.e. fragment 1.2; P_2 = prethrombin 2; T = thrombin. E-P and $E-P_2$ are tetrahedral complexes, E-F and E-T are acyl enzymes. Prothrombin binds to factor X_a and after going through the Michaelis and tetrahedral complex the acyl enzyme consisting of factor X and the activation fragment is formed. Before the next bond can be split the acyl enzyme has to break down. The activation peptide dissociates and a new Michaelis complex between factor X_a and prethrombin 2 has to be formed which again goes through a tetrahedral complex and an acyl enzyme before thrombin is formed. There is ample possibility for prethrombin 2 to dissociate away from the complex. Indeed prethrombin 2 has been shown to pile up under certain conditions [24].

Influence of factor V_a on the pathway of prothrombin activation

In order to see whether the changes in kinetic parameters caused by accessory proteins and phospholipids are accompanied by a change in reaction pathway, we followed the time course of prothrombin cleavage by prothrombinase complexes of different composition by SDS gel electrophoresis. The reaction conditions were chosen on basis of our kinetic experiments, except that the amount of enzyme was raised and incubation times were prolonged in order to make the detection of the activation products in gels possible. The results are shown in Fig. 2.

In the absence of factor V_a with or without phospholipids, prethrombin 2 is the main end product during the initial phase of prothrombin activation. Only at a late stage of the activation sufficient amounts of thrombin are formed to be detectable on the gels. When factor V is present no prethrombin 2 is detected and thrombin is the main end product. Prethrombin 1 is formed as a result of the action of thrombin on the prothrombin present.

On the basis of these experiments we propose that in the absence of factor V_a prethrombin 2 dissociates readily from the enzyme. Reassociation which would offer a second chance for proteolysis is prevented by the excess amount of prothrombin competing for the same enzyme. Only small amounts of prethrombin 2 slip through and are converted into thrombin and measured in the kinetic experiments.

However, when factor V_a is present, the pathway of activation shifts to one resulting directly in thrombin. We conclude that the presence of factor V_a prevents dissociation of prethrombin 2 from the prothrombinase complex. This can be achieved in two ways. Either the dissociation of prethrombin 2 from the enzyme complex is prevented directly by lowering the dissociation rate constant

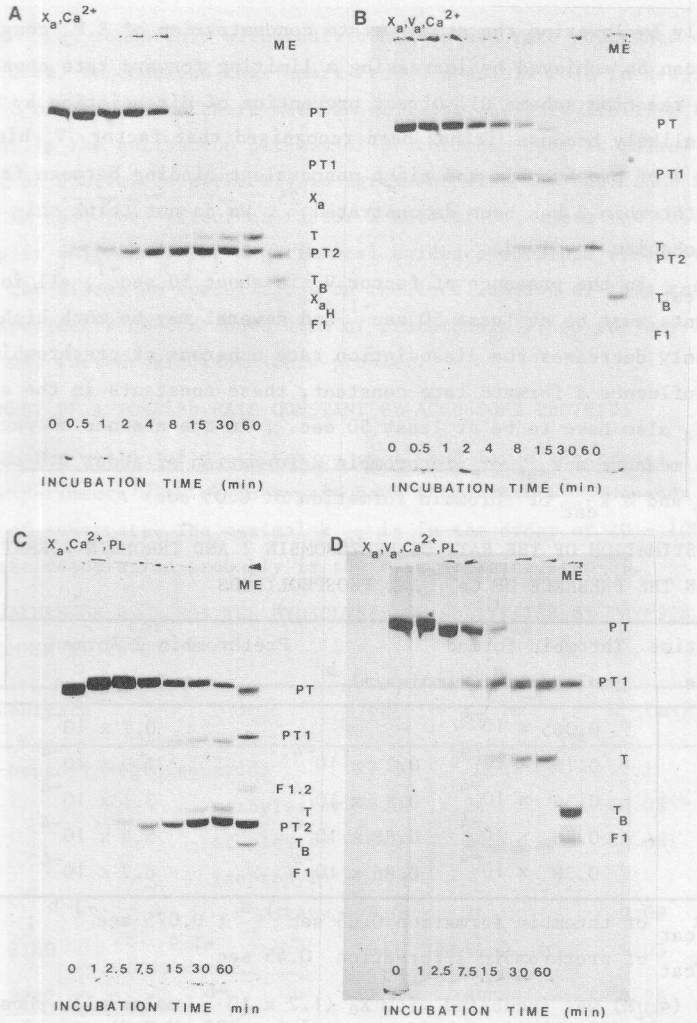

Fig. 2. Time course of activation of prothrombin with different activating mixtures.
The composition of the prothrombin activating mixture is shown on top of each series of gels. In order to slow down prothrombin conversion by thrombin, 2 mM diisopropylphosphofluoridate was included in the activation mixture.
Abbreviations used are: X_a = factor X_a; V_a = factor V_a; PL = phospholipid; PT = prothrombin; PT_1 = prethrombin 1; F 1.2 = fragment 1.2; T = thrombin; PT_2 = prethrombin 2; T_β = β-chain of thrombin; X_{a_H} = heavy chain of factor X_a and F_1 = fragment 1.
The last of each series (indicated ME) is a disulfide reduced sample of the reaction mixture taken after 60 min of incubation.

or indirectly by lowering the steady state concentration of $E.P_2$ complexes. The latter can be achieved by increasing a limiting forward rate constant (k_4 - k_9 in reaction scheme C). Direct prevention of dissociation by factor V_a is not unlikely because it has been recognised that factor V_a binds to fragment 2 of prothrombin and tight noncovalent binding between fragment 1,2 and prethrombin 2 has been demonstrated [25]. We do not think this can be the only mechanism involved.

As the k_{cat} in the presence of factor V_a is about 50 sec^{-1}, all forward rate constants must be at least 50 sec^{-1} and several may be much higher. If factor V_a only decreases the dissociation rate constant of prothrombin 2 and would not influence a forward rate constant, these constants in the absence of factor V_a also have to be at least 50 sec^{-1}. In the absence of factor V_a, however, we measure a k_{cat} of prethrombin 2 formation of about 0.5 sec^{-1} (Table II), and a k_{cat} of thrombin formation of 0.05 sec^{-1}.

TABLE II. ESTIMATION OF THE RATE OF PRETHROMBIN 2 AND THROMBIN FORMATION BY FACTOR X_a IN THE PRESENCE OF Ca^{2+} AND PHOSPHOLIPIDS

Incubation time minutes	Thrombin formed µmoles/ml [a]	µmoles/ml [b]	Prethrombin 2 formed µmoles/ml [c]
2	0.065×10^{-4}	-	0.7×10^{-4}
5	0.16×10^{-4}	0.2×10^{-4}	1.9×10^{-4}
10	0.34×10^{-4}	0.5×10^{-4}	3.1×10^{-4}
20	0.66×10^{-4}	0.88×10^{-4}	5.3×10^{-4}
30	0.78×10^{-4}	0.86×10^{-4}	6.2×10^{-4}

Estimated k_{cat} of thrombin formation 0.05 sec^{-1} [a] ; 0.075 sec^{-1} [b] ;
k_{cat} of prethrombin 2 formation 0.45 sec^{-1} [c]

Prothrombin (4 µM) was incubated with X_a (1.2×10^{-6} µmoles/ml), phospholipid (75 µM) and Ca^{2+} (30 mM), in a buffer containing 100 mM NaCl and 50 mM Tris-HCl at 37°C at pH 7.5. After different time intervals samples were taken and essayed for thrombin using S 2238 [a]. In order to get an estimate of the rate of prethrombin 2 formation, samples were taken and run on SDS-gels. Together with these gels, gels with known amounts of prethrombin 2 and active-site titrated thrombin were prepared. After staining and destaining of the gels, they were scanned on a Gilford Model 250 spectrophotometer. The bands on the gels with known amounts of thrombin and prethrombin 2 were integrated and from a calibration curve the amounts of thrombin [b] and prethrombin 2 [c] present in the activation mixture could be calculated.

This is in no way compatible with individual forward rate constants of 50 sec^{-1} or more. We conclude that apart from a possible role in keeping prethrombin 2 on the enzyme there must be at least one rate limiting forward rate constant in the activation pathway of prothrombin that is raised by factor V_a. This, of course, is perfectly in agreement with the fact that factor $VIII_a$ exerts an analogous effect on factor X activation, where the substrate has to be split only once. The experimental evidence obtained with both complexes thus far allows no speculations on the localization of this rate limiting step. Experiments on the conversion of prethrombin 2, on the way in our laboratory, may further elucidate this problem.

THE ENHANCEMENT OF A FORWARD RATE CONSTANT BY ACCESSORY PROTEINS

Comparison of the action of proteases on small and large substrates

Kinetic experiments with chymotrypsin have shown that k_{cat} depends strongly on the type of substrate. The maximal k_{cat} is in the order of 20 - 100 s^{-1} and in these cases deacylation probably is the rate limiting step [26].

TABLE III. VALUES OF k_{cat} FOR THE HYDROLYSIS OF SUBSTRATES BY TRYPSIN, FACTOR X_a, AND THROMBIN AT 25°C

Enzyme	Substrate		pH	k_{cat} (s^{-1})	K_m (mM)
Trypsin*	α-benzoyl arginine	amide	7.8	0.37	2.1
		methylester	8.0	24	~0.08
		ethylester	8.0	24	~0.08
		isopropyl-ester	8.0	24	~0.08
	S 2160		8.1	41.5	0.03
Factor X_a	S 2222		7.5	89	0.26
Thrombin	S 2238		7.5	84	0.009
	S 2160		8.3	17.3	0.08

* The data on the α-benzoyl arginine derivatives are taken from reference 27.

In Table III the k_{cat}'s of the splitting of various substrates by trypsin, thrombin and factor X_a are shown. The order of magnitude of k_{cat} of the conversion of chromogenic substrates suggests that deacylation is rate limiting. A remarkable feature stands out when the kinetic data for the conversion of prothrombin and S 2222 by factor X_a are compared (Table IV).

The k_{cat} for prothrombin in the absence of factor V_a is very low (0.01 s^{-1}), addition of factor V_a increases it by a factor 10^4 to the same level as that obtained with a small substrate.

TABLE IV. KINETIC CONSTANTS FOR THE HYDROLYSIS OF S 2222 AND PROTHROMBIN BY FACTOR X_a AT 37°C AND pH 7.5

Enzyme	Substrate	k_{cat} s^{-1}	K_m μM
X_a	S 2222	193	263
X_a	prothrombin	0.011	84
X_a , PL (75 μM)	prothrombin	0.065	0.35
X_a , PL (75 μM), V_a	prothrombin	45.8	1.7

Taking into account that two peptide bonds have to be split in the prothrombin molecule makes the high k_{cat} for that substrate as it is seen in the presence of factor V_a even more striking. It is tempting to speculate that both with S 2222 and with prothrombin in the presence of factor V_a the enzyme (factor X_a) works at or near its theoretically maximal capacity, i.e. in a situation where deacylation is the rate limiting step.

Why doesn't factor X_a alone function optimally with prothrombin?

We are left with a situation in which factor X_a, which is perfectly able to split a peptide bond in a small substrate with high efficiency, does not do so with its natural substrate but for the case that factor V_a is present. Obviously one or more of the forward rate constants is much smaller in the absence of factor V. Experiments on the competition between prothrombin and S 2222 for factor X_a appeared to shed some light on this question. From Table V it can be seen that S 2222 is a competitive inhibitor of prothrombin conversion. K_i equals K_m for S 2222 as would be expected for two substrates competing for one active site. To our surprise, in the reverse situation prothrombin does not inhibit the splitting of S 2222 to any appreciable extent. Yet at a concentration of 1 μM, which is about 8 x K_m under these conditions we expect about 90% of the enzyme to be bound to prothrombin. At the K_m half of the enzyme must be bound to prothrombin. This can only mean that forms of the enzyme exist that are bound to prothrombin but that are still able to catalyse the conversion of a small substrate; forms in which the active centre is not occupied.

TABLE V. EVALUATION OF COMPETITION OF PROTHROMBIN AND S 2222 FOR FACTOR X_a

Substrate	Inhibitor	K_m (μM)	k_{cat} s^{-1}	K_i (μM)
prothrombin	-	0.14	0.044	-
prothrombin	S 2222 (383 μM)	0.33	0.041	270
S 2222	-	263	193	-
S 2222	prothrombin (1 μM)	251	185	?
S 2222	prothrombin (0.1 μM)	275	195	?

Factor X_a (4.6×10^{-7} μmoles/ml) was incubated with 30 μM phospholipid in a buffer containing 25 mM $CaCl_2$, 100 mM NaCl, 50 mM Tris-HCl, 0.5 mg ovalbumin/ml at pH 7.5 and 37°C. After 3 minutes the substrate and inhibitor if present were added. S 2222 conversion was followed directly by measuring the absorbance change at 405 nm. Thrombin formation was measured by subsampling after different time intervals into a cuvet containing S 2238. Due to dilution S 2222 conversion by factor X_a could be neglected in the thrombin assay.

As there is no inhibition of S 2222 conversion, no prothrombin factor X_a complexes accumulate in which the active centre is occupied, therefore all forward rate constants listed in reaction scheme C must be within the normal range, i.e. the range that they show when factor V_a is present.

Unfortunately the same experiment cannot be repeated in the presence of factor V_a because thrombin then generates so quickly that S 2222 is split by this enzyme.

The postulate of open complexes

Neither the Michaelis complex, the tetrahedral complexes or the acyl-enzymes have their active centre available for the splitting of a small substrate. Yet as is shown in the preceeding paragraphs such complexes have to exist. We therefore postulate a new type of complexes in which prothrombin is bound to the complex by noncovalent interactions but still the active centre is left free. We will call these "open complexes". In formulas they will be indicated by brackets :(E.S) etc. Three types of open complexes can be imagined in the prothrombin activation sequence. One with prothrombin, one with prethrombin 2 and one with thrombin. The following reaction formula would result:

$$\begin{array}{ccccccccccccc} & 1a & & 1b & & 2,3,4,5a & & 5b & & 6,7,8 & & 9a & & 9b \\ E + P & \rightleftarrows & (E.P) & \rightleftarrows & E.P & \cdots\cdots & (E.P_2) & \rightarrow & E.P_2 & \cdots\cdots & E.T & \rightarrow & (E.T) \rightarrow E + T \end{array}$$

$$\begin{array}{ccc} & & \downarrow\uparrow 12b \quad \downarrow\uparrow 12a \\ & & E + P_2 \quad E+P_2 \end{array} \qquad (D)$$

This is essentially reaction scheme C with open complexes inserted at the places where Michaelis complexes arise or where the enzyme product complex breaks down. Intermediate steps are omitted at the places indicated by the dotted lines.

We do not say that all three of the open complexes have to exist. One would be sufficient to explain the data. What is necessary to assume is that the conversion of one or more of the open complexes into a Michaelis complex c.q. the dissociation of the (E.T) complex is a rate limiting step, that is enhanced by factor V_a to a velocity of 50 s^{-1} or more, so that deacylation then can become rate limiting.

At the moment we do not care to speculate about the localisation of the open complex that forms the site of factor V_a action.

It is clear that the role of accessory proteins is less obscure now than it has remained for a long time. Yet we are not able to pinpoint their role with certainty.

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