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ON THE ROLE OF PHOSPHOLIPIDS IN THE REACTION MECHANISM OF BLOOD COAGULATION

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ABSTRACT

In the mechanism of blood coagulation phospholipids function in the activation of factor II (1 x) and X (2 x). They create an interface at which the activation takes place. The kinetic effect is invariably a decrease in K_m of about 10^3 times. This can be explained by assuming that a) the enzymes (factors X_a, IX_a or VII_a) change properties upon adsorption (*the landing model*) or b) the local concentration of substrate is higher at the interface (*the concentration model*). It is shown that adsorption of the substrate at the interface and subsequent lateral movement (*the skating model*) is a plausible form of the concentration model. Experimental data up till now do not allow a discrimination between models.

KEYWORDS

Phospholipids, Blood Coagulation, Models, Prothrombin activation, Factor X activation, Factor IX_a, Factor VII_a.

INTRODUCTION

Presentation of the models

Phospholipids are recognized to play a role at three sites in the blood coagulation reaction sequence viz. in the activation of factor X by factor VII_a or factor IX_a and in the activation of prothrombin by factor X_a. Recent research has made it clear that phospholipids in all three cases exert their accelerating

effect by decreasing the K_m of the reaction (Mann and Taylor, eds. The regulation of coagulation. 1980; Rosing et al, 1980).(In the book of Mann and Taylor the great majority of all relevant references is to be found).K_m defines the ambient substrate concentration at which an enzyme is half saturated. A decrease in K_m can be brought about by (a combination of) two mechanisms: a) a change in the affinity constants of the binding between enzymes and substrate; or b) an increase in the local substrate concentration in the neighbourbood of the enzyme. The latter applies in cases where enzyme and substrate are not homogeneously distributed over the reaction volume. In that case the reaction between substrate and enzyme takes place under conditions that are not adequately represented by the concentrations of the reactants in bulk solution.

If case a applies the substrate, coming from the bulk solution meets an enzyme that has been adsorbed onto the phospholipid and the kinetic properties of which have changed by that adsorption. This is the "landing model" as elaborated by Nelsestuen and Broderius (1977).

In case b, it is thought that the enzyme reacts with a substrate molecule that is confined to a compartment on or near the phospholipid interface,in which compartment the substrate concentration is higher than in the bulk solution. This model, that can be called the "local concentration" model, comes in two varieties, differing in the mechanism that is thought to be responsible for the origin of the high local concentration.

In the "atmosphere model" (b1) the phospholipid draws substrate molecules from the solution into an atmosphere near its surface via electrostatic interaction between negatively charged phospholipid, Ca^{++} ions and (Gla residues of) substrate molecules (Dombrose et al, 1979; Jackson, page 117 in "The Regulation of Coagulation"; Nemerson, page 193 in "The Regulation of Coagulation"). This results in a high prothrombin concentration near the surface and hence near the adsorbed enzyme. Another possibility, depicted in the "skating" model, is that the substrate is bound onto the surface and, when bound, does retain a lateral mobility that enables it to meet an enzyme molecule that is adsorbed as well.

We can summarise the situation as follows:

OBSERVATION	MECHANISM	MODEL
K_m decreases upon adsorption	a. enzyme modification	a. landing
	b. substrate concentration	b. concentration
	b1.around the phospholipid	b1.atmosphere
	b2.on the surface	b2.skating

In the next paragraphs we will try to discriminate between possibilities a and b, and we will discuss the possibilities of choosing between b1 and b2. In the appendix it will be investigated whether known data on the interaction between proteins and lipids can rule out the skating model.

It should be stressed right from the beginning that the three models do not exclude each other. The concentration models do not rule out that changes in K_m are induced by the adsorption of the enzymes. They are not thought to play a major role though. Neither does the skating model exclude electrostatic interactions to be instrumental in attracting the substrate to the surface. Probably, all three mechanisms will reflect part of the truth. In selecting a mechanism we try to define which of the three is the main responsible for the observed drop in K_m.

Discrimination between enzyme modification and substrate concentration

If one calculates the amounts of free and bound prothrombin in the presence of phospholipid, one finds:

$$\left.\begin{aligned} P_b^2 - (P + K + S)\, P_b + PS = 0 \\ P_f^2 + (K + S - P)\, P_f - PK = 0 \end{aligned}\right\} \quad (1)$$

where:

P = concentration of prothrombin or factor X (substrate)
S = concentration of binding sites
K = dissociation constant

subscript f = free species
subscript b = bound species
no subscript = total concentration
subscript e = effective concentration

From equations (1) P_b and P_f can be calculated and the reaction velocities found in an experiment can be related to concentrations of the bound and free species. This has been done in Fig. 1.

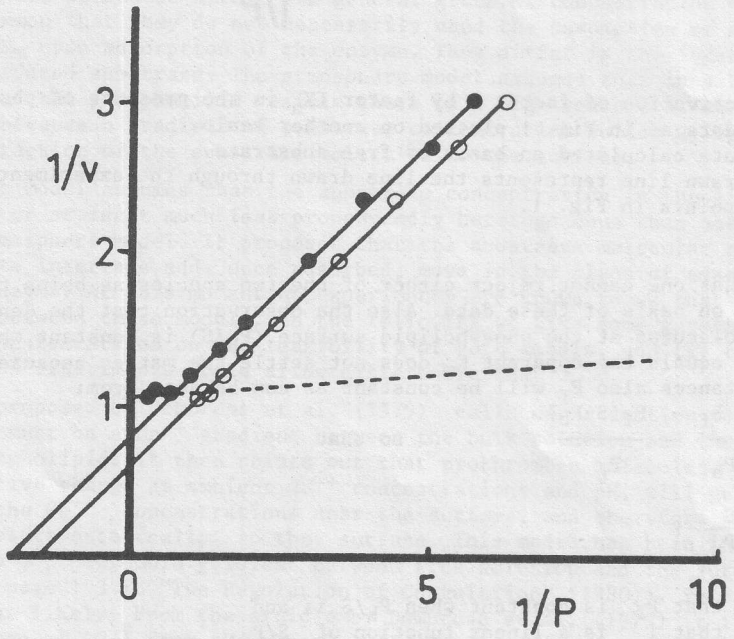

Fig. 1. The activation of factor X by factor IX_a in the presence of phospholipids. Incubation mixture: factor IX_a 0.01 µM; phospholipid (18:1 18:1 PS/ 18:1 18:1 PC) 100 µM; factor X (P) µM as indicated; Ca^{++} 10 µM; velocity is expressed as moles X_a/mole IX_a/min x 100.
The amount of factor X_a formed was estimated by subsampling into S 2222.
• Experimental data
o Same data calculated on basis of substrate adsorbed onto the phospholipid
--- Same data calculated on basis of free substrate (see Fig. 2).

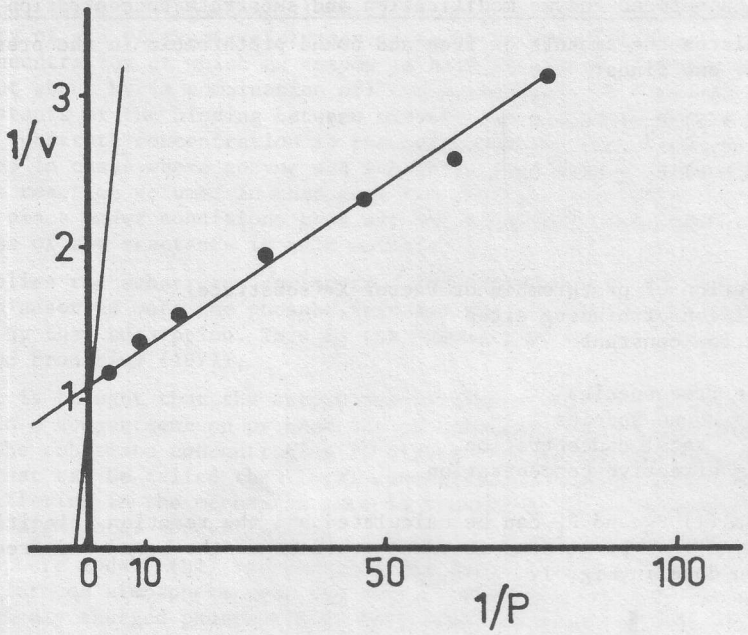

Fig. 2. The activation of factor X by factor IX_a in the presence of phospholipids. Same data as in Fig. 1 plotted on another scale.
• Data calculated on basis of free substrate.
The drawn line represents the line drawn through the experimental data points in Fig. 1.

It is clear that one cannot reject either of the two species as being the effective one on basis of these data. Also the observation that the density of prothrombin molecules at the phospholipid surface (P_b/S) is constant when the bulk concentration equals the apparent K_m does not settle the matter because under these circumstances also P_f will be constant as can be seen from:

$$K = \frac{P_f \cdot S_f}{P_b} = \frac{P_f(S-P_b)}{P_b} \qquad \text{so that}$$

$$\frac{K}{P_f} = \frac{S}{P_b} - 1$$

This shows a. that P_f is constant when P_b/S is and
b. that $\frac{1}{P_f}$ is a linear function of S/P_b,
so that any experiment that gives a linear Lineweaver-Burk plot with one species will do so equally well with the other.

P_b has been calculated to be 5 µmol/g phospholipid (2) when $P = K_m$ app. S is about 17 µmol/g phospholipid (3), therefore $S/P_b \approx 3.4$. If P_f is the effective concentration, then the K_m of the free species has to be about 4.10^{-8} M which is a low but acceptable value. Evidently, this type of approach will not result in discrimination between the models.

At this moment it may be useful to have a look at the experimental data that made others reject the concentration model in favour of the landing model (Nelsestuen in "The Regulation of Coagulation" (1980) page 31). The main issue is that with varying compositions of the lipid used, the prothrombin concentration at the surface varies but the rate of prothrombin activation does not vary. These experiments have been carried out at a prothrombin concentration of 3.3 μM. The apparent K_m for prothrombin varies between 0.03 and 1.0 μM (Rosing, 1980). The rate observed will therefore be pretty near theoretical maximal velocity anyhow, and cannot be expected to vary much with the substrate concentration. Indeed, no variation is explained by both models.

A second point brought forward in defense of the landing model is that phospholipid vesicle concentrations severalfold in excess of the enzyme concentration do not inhibit the reaction. As long as it is unknown how fast the substrate can exchange between vesicles it cannot be excluded that the substrate on a vesicle that does contain the enzyme is quickly replenished by the substrate from vesicles not occupied by an enzyme.

A possible method of discrimination would be working at low P and high S and varying the fluidity of the membrane. As diffusion in the membrane will influence the reaction rate in the skating model but not in the landing model, this might give an indication as to the model to be preferred.

Discrimination between different concentration models

The two models presented within the general group of concentration models have in common that they do not necessarily need the assumption of a large change in K_m upon adsorption of the enzyme. They differ in the location of the concentrated substrate. The atmosphere model assumes that in a limited space around the phospholipid vesicle there is a high concentration of substrate because a gradient of substrate concentration builds up by electrostatic attraction of the substrate molecules to the surface.

The skating model assumes that the substrate concentration in the liquid phase is uniform or at least much less pronouncedly heterogeneous than assumed in the atmosphere model. It proposes that the substrate molecules adsorb at the lipid solute interface and, once adsorbed, move in the plane of adsorption to meet the enzyme. At this moment no experiments are known to us that could discriminate between these models. In the following we give some general physicochemical considerations on the feasibility of both models, and try to find what type of experiments would allow differentiation.

The model proposed by Dombrose et al. (1979) calls our attention to the fact that there must be a Ca^{++} gradient between the bulk solution and the surface of the phospholipid. It then points out that prothrombin molecules, that have a net negative charge at ambient Ca^{++} concentrations and pH, will get a positive charge at the Ca^{++} concentrations near the surface, and therefore will be attracted electrostatically to that surface. This model has been interpreted as to cause a prothrombin gradient between free solution and the surface (Nemerson, page 193 in "The Regulation of Coagulation" (1980)). In our opinion this is less likely. From the article by Dombrose et al. (1979) it is seen that at a distance of 40 Å from the phospholipid surface, i.e. at about the diameter of a substrate molecule, the Ca^{++} concentration will be hardly higher than than in bulk solution. That gradient of Ca^{++} will, therefore, not be very effective in attracting substrate molecules from the solution. It does explain, however, why it is the Gla containing and hence Ca^{++} binding part of the substrate that binds to the phospholipid. As far as we can see the original proposition of Dombrose et al. was not meant to go beyond this. Also the lightscattering data from Nelsestuen (1977) indicate that the phospholipid vesicles in the presence of excess prothrombin can be adequately described as being covered by one layer of prothrombin molecules.

It may be mentioned that the highly specific effect of Ca^{++} in binding Gla containing substrates to phospholipid cannot be accounted for by electrostatic interaction only. Rather the liability of Ca^{++} and Gla residues to form cage complexes has to be taken into account (Furie, page 21 in "The Regulation of Coagulation" (1980). This again suggests interactions not extending beyond the diameter of one substrate molecule. The acceptability of the skating model will stand and fall with the possibility of adsorbed protein molecules to move in the plane of adsorption. As yet no data are available on the lateral mobility of adsorbed clotting factors. Such data do exist for various other types of proteins. In these cases the lateral mobilities of adsorbed proteins were shown to be 100 to 1000 times smaller than the mobilities in free solution (for a review see Cherry (1979). When this also can be applied to adsorbed coagulation factors, it can be readily demonstrated that indeed the number of meetings between enzyme and substrate can be considerably enhanced by adsorption onto phospholipid. This will be shown in the next paragraph. We conclude that the data at hand do not refute the skating model.

It has been argued that a substrate molecule has to remain adsorbed for a reasonable time in order for the skating model to be feasible (Nemerson, page 193 in "The Regulation of Coagulation" (1980)). This can be doubted, however. From the number of adsorbed molecules per unit surface and their velocity on that surface the area swept by these molecules in unit time can be calculated. It will be independent from the mean length of path covered by one individual molecule between adsorption and desorption until the mean length of path can be shown to be smaller than the mean diameter of a substrate or enzyme molecule.

APPENDIX

Collision rates of molecules in free solution compared to those of molecules adsorbed at an interface

Chemical reaction velocities are dependent upon the concentration of the reactants because the concentrations determine the number of collisions between the reactants. Energetic and steric factors determine what fraction of the collisions will result in a chemical reaction. Adsorption will affect the steric and energetic conditions under which enzyme and substrate meet. These effects are hard to evaluate. It is possible, however, to give an estimate of the influence of adsorption on the collision frequency of an adsorbed protein substrate and an enzyme. We define the following symbols:

$\underline{P}$ concentration of substrate (Moles)
$\underline{c}$ mean velocity of a protein molecule in free solution
c^+ mean lateral velocity of a protein molecule when adsorbed
d diameter of an enzyme or substrate molecule $40 \text{ Å} = 40.10^{-10}$ M
N Avogadro's number: 6.10^{23}
L concentration of lipid (Moles)
Q area of the outer surface of the phospholipid vesicles contained in one mole of phospholipid: 24.10^4 M^2

Q can be estimated on basis of the following estimates: the surface area of one phospholipid molecule is on the mean 60.10^{-20} M. With the vesicles used two thirds of the phospholipid molecules will face the solution. Therefore, one Mole of phospholipid presented in the form of such vesicles will present a surface of $0.67 \times 6.10^{23} \times 60.10^{-20}$ $M^2 = 24.10^4$ M^2.

An enzyme molecule in free solution will meet in unit time the substrate molecules contained in a cylinder with a volume of $\bar{c}.5.10^{-17}$ M^3. This cylinder contains $\bar{c}.P.N.5.10^{-17}$ molecules of substrate that are possible candidates for chemical interaction with the enzyme. Now we add L moles of lipid per litre and we assume that the enzyme molecule as well as all the substrate is adsorbed. The enzyme molecule now will sweep per unit time a surface of $\bar{c}^+.8.10^{-9}$ M^2. The total available surface is $L.24.10^{+4}$ M^2. The total number of possible candidates for chemical interaction met in the adsorbed state per unit time will be those

contained in the surface swept by the adsorbed molecule, i.e.:

$((\bar{c}^{+}.8.10^{-9})/(L.24.10^{+4}))6.10^{23}.P = 2.10^{10}.\bar{c}^{+}.P/L$

The ratio (R) of the number of substrate molecules met in presence and in absence of lipids is then easily calculated to be

$R = (\bar{c}.2.10^{10}.P/L)/(5.10^{-17}.P.N) = 700.(\bar{c}/\bar{c}^{+}).1/L$

The viscosity of bilayer membranes for proteins attached to it has been estimated to be between 1 and 10 poises. In solution the viscosity of about 1 centipoise. Via Stokes law it can be argued that the velocities of the proteins in the membrane will be 100 to 1000 times slower than in free solution. If we may use these data for the adsorbed clotting factors, then $R = 0.7/L$. The range of L used is from 10^{-6} M to 3.10^{-4} M. This would account for a decrease in K_{m_3} by a factor of roughly 7.10^{5} at the lowest phospholipid concentration to 2.10^{3} at the highest phospholipid concentration. The factors observed in practice are one or two orders of magnitude smaller. We conclude that adsorption at the surface of the phospholipid can easily account for the decrease in K_m that is observed but that more data on the lateral velocity of adsorbed clotting factors and on the influence of steric and energetic changes brought about by the adsorption are necessary.

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