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THE PHYSICAL SIGNIFICANCE OF K_m IN THE PROTHROMBINASE REACTION

Marc Lee Pusey and Gary L. Nelsestuen

Dept. of Biochemistry, University of Minnesota, St. Paul, MN 55108

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Abstract. Key kinetic parameters for the prothrombinase complex formed on membranes of phosphatidylserine (PS)/phosphatidylcholine (PC) (40/60) (K_m = 0.12 μM, k_{cat} = 11 s^{-1}) or PS/PC (2/98) (K_m = 0.40 μM, k_{cat} = 11 s^{-1}) differed only slightly. In contrast, the density of proteins on the membrane surface at the K_m differed greatly for the two membranes. The kinetics appeared unaffected by conditions where the number of phospholipid vesicles (2% PS) exceeded the number of protein molecules. These results establish that the K_m for the prothrombinase reaction is determined by the concentration of prothrombin in solution rather than its density at the membrane surface. This system can be treated as a dissociable enzyme acting on a soluble substrate.

The prothrombinase reaction is a complex process involving four macromolecular components (factor X_a, factor V_a, phospholipid membrane plus the substrate, prothrombin) plus calcium ions. All three of these proteins bind to membranes independent of one another. The membrane component is known to exert its major stimulatory effects on the reaction through tighter binding of factor X_a and a lowered K_m for prothrombin (1-5). Two potential explanations for these effects have been proposed: The membrane may serve as a large surface on which individual proteins bind to produce a dense shell of substrate and enzyme components; the apparent K_m for the substrate would be related to the density of substrate at the membrane surface (3-5). Alternatively, the membrane may provide tighter binding by virtue of the additive free energies of protein-protein plus protein-membrane interactions that occur at the active site; the K_m would be a function of the free prothrombin concentration (1,2).

Several lines of evidence indicate that assembly of the factor Xa-Va-phospholipid complex does not utilize a dense shell produced by individual protein-membrane associations (1-7). Despite several studies, the potential kinetic contribution of a dense shell of prothrombin substrate at the membrane surface has remained controversial (see 2,4,6). Relatively simple but definitive exper-

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iments to distinguish these hypotheses are reported here. The kinetic evidence demonstrated that the dense shell of prothrombin whch can be generated at the membrane surface was not kinetically important. The prothrombinase reaction can be treated kinetically as a three component enzyme (factors V_a, X_a plus membrane) with an active site that utilizes prothrombin from solution as substrate.

Materials and Methods

Prothrombin (1), factor X_a (8), and factor V (9) were prepared and quantitated by methods described previously. Phospholipids of defined composition were formed by mixing in organic solvent, drying under a stream of argon and sonicating by direct probe (10). Egg phosphatidylcholine (PC) and bovine brain phosphatidylserine (PS) were over 98 percent pure and were purchased from the Sigma Chemical Co.

Thrombin (designated II_a) generation was measured essentially by the method of Rosing et al. (3) except that activations were sampled continuously to obtain the rate of thrombin production. Factor V was pretreated with thrombin to produce the more active molecule designated factor V_a (11). Factor V_a was added in saturating amounts (0.5 µg/ml) so the limiting components of the prothrombinase complex were factor X_a (1.8×10^{-11} in all experiments) or phospholipid. Components were mixed in 0.05 M Tris-0.1 M NaCl-2 mM $CaCl_2$ (pH 7.5) at 25° and prothrombin, preequilibrated in the starting buffer, was added at zero time. Samples were removed at various times, the reaction stopped by diluting into 0.2 M Tris buffer (pH 8.4) containing 10 mM EDTA. Thrombin was then measured by hydrolysis of the p-nitroanilide peptide S-2288 (Kabi Group). Thrombin concentrations were obtained by comparison to the activity of pure thrombin (8). Duplicate activations were run and averaged for each point plotted. Velocities are expressed as the molar concentration of thrombin produced per second.

Results

Figure 1 shows the titration of prothrombinase activity as a function of phospholipid concentration for membranes of 40 and 2 percent PS. The PC used to

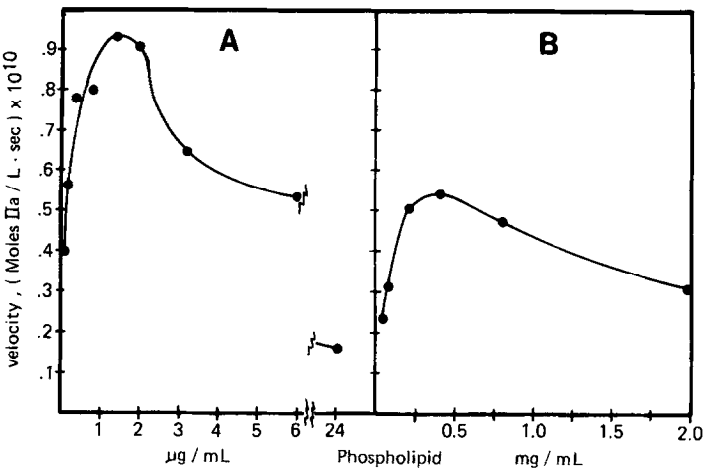

Figure 1. Prothrombinase kinetics as a function of phospholipid concentrations. The prothrombin concentration was 0.085 μM in all experiments. The phospholipids were PS/PC (40/60) in panel A and $\overline{PS}$/PC (2/98) in panel B. Other conditions were as outlined in Materials and Methods. II_a is the abbreviation for thrombin.

dilute the PS is inert in the prothrombinase reaction. Both titration curves show a stimulatory effect followed by inhbition at higher phospholipid concentrations. The stimulation is due to binding of factor X_a-V_a to the membrane thereby assembling the prothrombinase complex. The inhibitory effect of high phospholipid has been well documented previously and is due to binding of prothrombin (3) to the membrane surface thereby decreasing either the free prothrombin concentration (4) or, as phospholipid becomes more abundant, the density of prothrombin in the shell around the membrane surface (3). Either explanation can account for the results (4) although only one is the correct explanation. The data presented below demonstrate that the former explanation is correct.

The data in figure 1 show that determination of the K_m for the substrate by plotting total substrate concentration versus velocity requires selection of a phospholipid concentration where the inhibitory effects are minimized and the total prothrombin approximates the free prothrombin. The optimum phospholipid concentration was radically different for these two phospholipids. Much less 40 percent PS was needed than 2 percent PS. This is probably due to the affinity of the proteins for the membranes; the lower protein affinity for 2

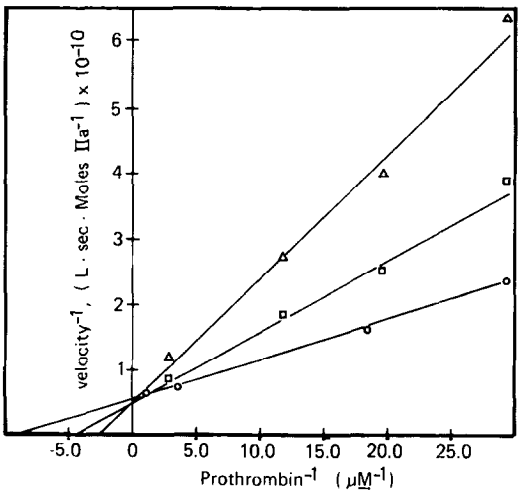

Figure 2. Lineweaver Burke plots of prothrombinase kinetics. The phospholipids consisted of PS/PC (2/98) (0.8 mg/ml (Δ)); PS/PC (40/60) (6 µg/ml (□)); PS/PC (40/60) (0.8 and 2.4 µg/ml (○)). Other components are as in Figure 1. The data were analyzed by the method of least squares and the kinetic constants obtained are given in Table I.

percent PS (4,9,12) requires that larger amounts be added to produce factor Xa-Va membrane complexes through mass action. This membrane composition also has much lower affinity for prothrombin (12) so that much higher concentrations are required to produce inhibition.

Figure 2 shows Lineweaver-Burk plots of prothrombinase kinetics for different phospholipids. The inhibitory effect of higher phospholipid is a K_m effect rather than a V_{max} effect (compare 0.8 versus 6 µg of 40 percent PS per ml, Fig. 2). This agreed with previous studies (3).

Table I compares some of the kinetic parameters obtained in this study with prothrombin-membrane binding data. Prothrombin binding to membranes of 2 percent PS could not be measured by the sensitive methods previously employed (12) and the K_D shown is for membranes of 6 percent PS. Based on observed trends, the binding to 2 percent PS would be much less tight. Despite these radical differences in binding, the kinetic parameters for the two phospholipids were similar (Table I). As a result, the density of prothrombin at the membrane surface at the K_m varied by about two orders of magnitude for the two phospholipids. In addition, the molar concentration of 2 percent PS vesicles exceeded the prothrombin concentration for all but one of the experiments

Table I

Comparison of Prothrombin-Membrane Binding and Kinetic Parameters

Phospholipid	Binding Parameters		Kinetic Parameters		Prothrombin bound per vesicle at K_m [b]
	K_D [a]	n [a]	K_m	k_{cat}	
PS/PC (40/60) 2 μg/ml (0.7 nM) [c]	0.5 μM [d]	70 [e]	0.12 μM	11 s^{-1}	7
PS/PC (2/98) 800 μg/ml (0.25 μM) [c]	>>20 μM [d]	7 [e]	0.40 μM	11 s^{-1}	<<0.2

a) K_D is the dissociation constant for the prothrombin-membrane complex while n is the maximum prothrombin-binding capacity of a typical vesicle of $M_r = 3 \times 10^6$. Taken from references 12 and 13.

b) This is per vesicle of $M_r = 3 \times 10^6$ (9).

c) The molar concentration is of phospholipid vesicles of average $M_r = 3 \times 10^6$ (9).

d) The K_D given is for prothrombin bound to membranes of 6 percent PS at 2 mM Ca^{2+}. Based on the observed trends (12), the K_D for binding to membranes of 2 percent PS would be much larger.

e) Seven sites per vesicle is based on extrapolation from membranes of higher PS composition (12).

used to estimate the K_m (Fig. 2 (Δ)). This condition obviously precludes the existence of a dense shell of prothrombin at the membrane vesicle surface. These results establish that the K_m for the prothrombinase reaction is determined by the free prothrombin concentration rather than the density of prothrombin at the membrane surface.

Discussion

The mechanism by which membranes exert stimulatory effects on enzymatic reactions is both an important and interesting question. In the case of the prothrombinase complex it was generally proposed that the membrane served as a concentrating devise to produce locally high protein concentrations on the two-dimensional membrane surface. Striking correlations between calculated protein density and reaction velocity have been presented (5). However, given that prothrombin-membrane binding affinity can be varied by more than two orders of magnitude by simply varying the membrane composition (12), it follows that some membrane composition will display a K_D similar to the kinetic K_m. A single correlation therefore does not establish that the concentration of prothrombin at the membrane surface is the kinetically important mechanism of membrane function.

A previous study which utilized a crude source of factor V reported that no apparent correlation existed between reaction velocity at a single prothrombin concentration and the density of prothrombin on the membrane surface (1,2). The present study, which utilized highly purified factor V and measured velocity over a large substrate range, conclusively showed that no correlation existed between the density of prothrombin at the membrane surface and the velocity of the reaction. Indeed, judicious selection of a membrane composition that bound prothrombin very weakly even allowed nearly optimum kinetics when the vesicles outnumbered the prothrombin molecules (2 % PS, Figure 1B and 2). A larger number of vesicles than factor Va and Xa molecules is commonly used in the reaction kinetics (1,2,4). Fewer than one protein molecule per vesicle obviously precludes the production of a dense shell of protein at the membrane surface.

The small difference in K_m observed for the two membrane compositions used here (Table I) can be explained by several factors. For example, if the K_m is related to a binding constant that results from simultaneous binding of prothrombin to protein (factors X_a and/or V_a) and membrane, a weaker prothrombin-membrane interaction will give a weaker overall interaction and a higher K_m. In an analogous situation, factor X_a binding to factor V_a-phospholipid was about 10- or 20-fold weaker when 5 percent PS was used than when 40 percent PS was used (4). The small change in the K_m for prothrombin observed here for an even larger variation in PS content is therefore well within the model presented. It is also quite possible that the K_m for prothrombin is diffusion controlled (13). This would not affect the conclusions drawn here.

From these results it is clear that the prothrombinase reaction consists of a dissociable three component enzyme (factor Xa-factor Va-membrane) that binds prothrombin from solution as its substrate. One function of the membrane-binding sites of these proteins is to enhance the overall binding affinity of proteins to each other at the active site and lower the free protein concentrations needed to saturate the active site. This model for the

prothrombinase complex may better fit biological membranes where the presence of many other membrane proteins may prevent formation of a dense shell of individual coagulation proteins at the biological membrane surface. The membrane also functions in other ways. For example, only cytosolic membranes contain the phospholipids needed for binding and their exposure may constitute an important control mechanism for onset of coagulation (12,14). In addition, the relevant membranes may be physically immobilized at the site of vascular injury and thus serve to localize the coagulation process.

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