The Prothrombinase Reaction: "Mechanism Switching" between Michaelis−Menten and Non-Michaelis−Menten Behaviors†

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ABSTRACT: Kinetic properties of prothrombinase were investigated as a function of composition and structure of the membrane component. The kinetic properties were quite diverse, giving linear or nonlinear Eadie−Hofstee plots and substrate concentrations at half-maximum velocity ($[S]_{0.5}$) that varied from 5 to more than 200 nM. This reaction might be described as a "catalytic system" in order to distinguish it from standard models that have been developed to describe the kinetics of soluble enzymes. The latter do not anticipate a key feature of prothrombinase and probably other membrane-bound enzymes, which is the presence of reaction steps that do not contain an enzyme (E) term. At least four kinetic mechanisms can arise from a logical series of steps that may occur during the prothrombinase reaction. All of these mechanisms appeared to contribute to reaction properties under some conditions. In some cases, one mechanism dominated at low substrate concentration and another at high substrate concentration. This change in the course of a titration was referred to as "mechanism switching". Only membranes of low phosphatidylserine (PS) content displayed Michaelis−Menten behavior. Transfer of substrate from the membrane surface to the enzyme was not important so that the enzyme was involved in capture of substrate directly from solution. As PS content increased, transfer of substrate from the membrane surface to the enzyme occurred. In these cases, multiple mechanisms contributed to the reaction so that K_M and apparent K_M, properties that describe an enzyme active site, were not appropriate, even when Eadie−Hofstee plots were linear. At high PS content, the enzyme captured every substrate molecule that became bound to the same vesicle. Reaction velocity was governed entirely by protein−membrane binding rather than by enzyme properties. Eadie−Hofstee plots were often nonlinear and/or V_{max} was less than $k_{cat}[E_t]$. A small impact from collision-limited kinetics was also detected. Small unilamellar vesicles (SUV, 30 nm diameter) gave higher $[S]_{0.5}$ values than large unilamellar vesicles (LUV, 100 nm diameter) of the same phospholipid composition. There appeared to be two bases for this behavior. First, LUV may provide a better relationship between the phospholipid surface and the enzyme, giving a better substrate binding site. Second, for membranes containing high PS, the number of substrate binding sites per vesicle contributed to the enhanced function of LUV. These studies showed that mechanism-switching was important to prothrombinase reaction *in vitro* and suggest that various mechanisms, generated by the nature of the membrane, may be an important regulator for prothrombinase behavior *in vivo*.

The prothrombinase reaction consists of a membrane-bound enzyme (factor VaXa) that acts on a substrate (prothrombin) that also interacts with the membrane [reviewed in Nelsestuen (1984a)]. These circumstances offer a number of possible routes that the substrate may take in binding to the enzyme, or which the product may take in leaving the enzyme. Several steps do not conform to the requirements of Michaelis−Menten or related kinetic models.[1]

A major concern for prothrombinase kinetic studies is the role of substrate−membrane binding and whether the substrate arrives at the enzyme from the population in solution or that on the membrane surface. While a direct relationship between prothrombin−membrane binding and prothrombinase kinetics has been reported (Nesheim et al., 1984), a single phospholipid composition was used, making coincidence possible. Other studies, which varied phospholipid composition and prothrombin−membrane binding properties, found no correlation between enzyme velocity or kinetic parameters such as K_M and prothrombin−membrane binding (Nelsestuen, 1978; Pusey & Nelsestuen, 1983; van Rijn et al., 1984). The latter suggested that the membrane did not function as a substrate-concentrating device and that substrate delivery to the enzyme occurred directly from the substrate population in solution. Direct participation of the enzyme in capture of substrate from solution provides a steady-state condition that should conform to Michaelis−Menten behavior. Indeed, many studies have reported K_M values for prothrombinase to reach important conclusions (e.g., Giesen et al., 1991b; Govers-Riemslag et al., 1994; Pusey & Nelsestuen, 1983; Rosing et

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[1] Michaelis−Menten behavior is defined broadly to include any situation where substrate titration can be characterized by a constant (e.g., K_M or apparent K_M) and V_{max}. This includes most single and multiple substrate situations. A property of these reactions, and cooperative enzymes as well, is that enzyme (E) is a component of every reaction step. Prothrombinase, and at least some other membrane-bound enzymes, provide the potential for non-Michaelis−Menten behavior of a radical type where some reaction steps do not contain an enzyme (E) term.

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al., 1980; van Rijn et al., 1984). Pusey and Nelsestuen (1983) restricted the use of K_M to very limited circumstances and substantiated mechanistic conclusions that were not dependent on Michaelis−Menten behavior. Concerns included the proper manner of incorporating the membrane component into the reaction description and the frequent observation of properties that were not explained by simple or more involved derivations related to Michaelis−Menten. For example, nonlinear steady-state kinetic plots are encountered. Unpublished studies by Abbott (1988) observed that "K_M" varied inversely with membrane vesicle size and increased with the number of enzymes per particle. Others have observed a similar relationship between K_M and particle size (Giesen et al., 1991b). While Smoluchowski theory, describing the rate constant for collision of particles in solution (k_{coll}), could produce some of the unexplained behaviors (Abbott, 1988; Giesen et al., 1991b), Abbott (1988) noted that k_{cat}/K_M ratios were only about 5−10% of k_{coll}, making it unlikely that the effect of vesicle size was due to k_{coll}, unless the reactants had substantial, unexplained behaviors. In addition, collision-limited reaction should not give linear Eadie−Hofstee or Lineweaver−Burk plots so that K_M should not apply at all (Martinez et. al., 1996). Extremely low K_M values (3−6 nM) were obtained when membrane particle size was increased to planar supported bilayers. This made it unlikely that the enzyme was directly involved in capture of every substrate from solution so that substrate must be transferred from the membrane surface to the enzyme (Billy et al., 1995; Giesen et al., 1991b). These combined properties appear to be in conflict with any single reaction mechanism.

Conditions which do produce collision-limited reaction kinetics involve situations where many enzymes are attached to infinitely large particles such as the surface of phospholipid-coated capillaries. Both the prothrombinase complex (Billy et al., 1995) and factor Xase reaction (Andree et al., 1994) appear to reach the state of limitation by substrate−surface collision under these conditions. Collision or transport-limited reactions may be common in many biological situations as illustrated by another recent study of enzyme kinetics in intact *Eschericia coli* (Martinez et al., 1992, 1996).

Another possible basis for vesicle size-dependent behavior of prothrombinase is the nature of the membrane surface. For example, rate constants for dissociation of prothrombin from supported phospholipid bilayers (Pearce et al., 1992, 1993) have been reported to be far lower than those from vesicles (Wei et al., 1982) even though the equilibrium binding constants appeared similar. A number of proteins, including blood clotting factor V, show high preference for binding to small unilamellar vesicles (SUV)[2] over large unilamellar vesicles (LUV) (Abbott & Nelsestuen, 1987). An accompanying study shows small differences between prothrombin binding to LUV versus SUV. The large differences between dynamic properties of prothrombin interaction with vesicles versus supported, planar phospholipid surfaces, may be an extension of this property. Thus, different interactions of substrate and the various phospholipids may underlay the large variations of prothrombinase behavior.

This study was initiated to provide explanations for the theoretically conflicting behaviors of the prothrombinase reaction. Four distinct kinetic mechanisms were identified from a logical series of steps which might contribute to the prothrombinase reaction. Only one of these mechanisms conformed to Michaelis−Menten behavior. The dominant kinetic mechanism was determined by reaction conditions, including membrane composition and particle size, substrate concentration, and other physical parameters. In some cases, one mechanism dominated at low substrate while another dominated at high substrate concentration. This process, referred to as "mechanism switching", appeared to be the basis of some of the most complex behavior of prothrombinase.

MATERIALS AND METHODS

Proteins. Bovine prothrombin, factor X (Nelsestuen, 1984b) and factor V (Pusey et al., 1982) were prepared and quantitated by published methods. Factor Xa was formed by action of Russell's Viper Venom on factor X which was then purified as described (Pletcher & Nelsestuen, 1983). Factor Va was formed by treatment of factor V with thrombin under conditions which produced maximum activity. Typically, 0.2 μg of thrombin were added to 10 μg of factor V in 30 μL of Tris buffer containing 1 mg/mL bovine serum albumin at 37 °C. After 15 min, the reaction was cooled and stored at 0 °C. Use was completed within 8 h. Factor Va activity was constant during this time period.

Phospholipids. The phospholipid vesicles used in this study were the same preparations described in the accompanying paper (Lu & Nelsestuen, 1996). This allowed the closest comparison of enzyme kinetics and protein−membrane binding properties. The membrane compositions included a low level of fluorescent phospholipids that did not appear to influence the enzyme reaction; similar reaction properties were obtained for membranes that contained only PS and PC.

Enzyme Velocity Measurements. Prothrombinase activity was determined essentially by the procedure of Bakker et al. (1992) using either a 30 or 60 s incubation. Briefly, the components of the prothrombinase complex were assembled in 0.25 mL of 0.05 M Tris buffer, pH 7.5, containing 2 mM calcium, 0.1 M NaCl, and 1 mg/mL bovine serum albumin (Sigma). Unless indicated, this included limiting factor Va (0.18 nM), excess Xa (5.1 nM), and the amount of phospholipid described for the experiment. After 60−90 min of assembly time, prothrombin, which had been incubated in the same buffer for at least 30 min at room temperature, was added to give the appropriate final concentration. These times for enzyme assembly were described by Ye and Esmon (1995), and both shorter and longer times were examined to ensure that reaction velocity was stable. In addition, the amount of Xa was varied to ensure that it was present at saturating levels for all experimental conditions. The reaction was allowed to proceed for 30 or 60 s at 25 °C. A solution of buffer containing 10 mM EDTA (0.75 mL) was added to stop the reaction. Thrombin was measured by hydrolysis of S2288. The assay was at 25 °C and contained 0.1 mL of the prothrombinase reaction in a total of 0.93 mL

[2] Abbreviations: $[S]_{0.5}$ is the substrate concentration at half-maximum enzyme velocity. This term is used instead of K_M when the reaction mechanism is not known and/or may proceed by non-Michaelis−Menten mechanisms; SUV, small unilamellar vesicles which have measured diameters of 25−30 nm; LUV, large unilamellar vesicles produced by extrusion technique and with average measured diameters of 110−120 nm.

Table 1: Comparison of Enzyme Kinetics and Prothrombin−Membrane Binding Characteristics

membrane composition	SUV or LUV	membrane binding properties[a]				enzyme properties[b]				
		Nk_1 (M^{-1} s^{-1})	k^{-1} (s^{-1})	N	K_D (nM)	$[S]_{0.5}$ (nM)	curve shape	n at $[S]_{0.5}$	$V_{max}/[S]_{0.5}$ (M^{-1} s^{-1})	surface density of proth at $[S]_{0.5}$ (n/μ^2)
10% PS	SUV	4.5×10^8	24	22	1600	174	linear	2	1.2×10^8	700
	LUV	2.2×10^9	17	560	3900	101	linear	14	2.1×10^8	370
25% PS	SUV	1.2×10^9	1.9	58	108	104	linear	28	2.0×10^8	10000
	LUV	6.1×10^9	1.0	1425	235	44	linear	220	4.8×10^8	5300
50% PS	SUV	1.4×10^9	0.15	79	8.3	6.5[c]	nonlinear	36	10×10^8	12700
	LUV	7.3×10^9	0.09	1600	20	5.6	nonlinear	350	38×10^8	7900

[a] Data from Lu and Nelsestuen (1996). [b] Values from this study. The vesicle preparations used for enzyme assay were the same as those used to determine prothrombin−membrane binding properties. [c] Midpoint of the linear portion of the plot (Figure 5B).

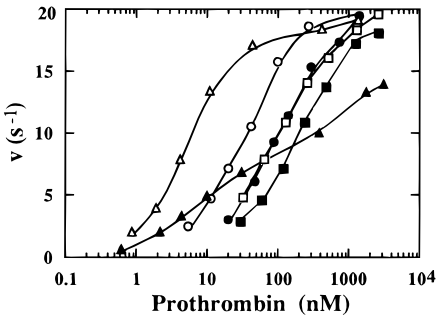


FIGURE 1: Comparison of prothrombinase kinetics with different phospholipids. Prothrombinase kinetics were determined by the procedures described in Materials and Methods. Free prothrombin concentration is plotted. This was calculated from total prothrombin, total phospholipid, and K_D and N values given in Table 1. Membranes (50 μg/mL) included LUV of 10 (□), 25 (○), and 50 (△) percent PS and SUV of 10 (■), 25 (●), and 50 (▲) percent PS.

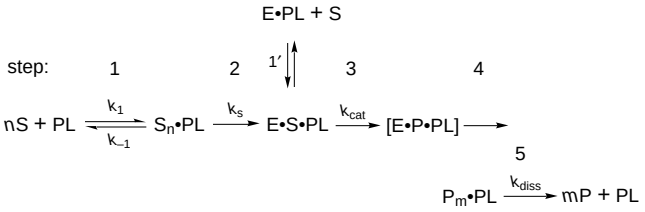
containing 60 μM S2288 and 1 mg/mL of bovine serum albumin. Thrombin concentration was estimated from an extinction coefficient of 1×10^4 M^{-1} cm^{-1} for reaction product (*p*-nitroanilide) and a k_{cat} of 100/s for thrombin. Background thrombin activity was determined with reactions that were treated just as described except that they contained no factor Va. Thrombin produced under these conditions was usually negligible and always less than 5% of the thrombin produced in the presence of Va. Unless indicated, error estimates report the average and standard deviation for at least three determinations. If no error bars appear, the standard deviation was within the dimensions of the symbol. Velocity is reported as mol of thrombin produced per second per mol of prothrombinase enzyme.

The maximum rate of product formation per prothrombinase enzyme complex (k_{cat}) was determined under reaction conditions that had limiting factor Xa. The assay was similar to that given above except that 0.10 nM Xa, a saturating level of prothrombin (0.5 μM), and optimum phospholipid (20 μg/mL of 10, 25, and/or 50% PS LUV and SUV) were titrated with factor Va to obtain the maximum velocity at 25 °C. Thrombin produced per second of incubation was divided by Xa concentration to give values of k_{cat}. Experimental values obtained with different phospholipids ranged from 16 to 21.4 s^{-1}. Since different phospholipids can reduce the apparent V_{max} by virtue of their mechanism, a k_{cat} of 21.4 s^{-1} was used when needed.

RESULTS

Theoretical Description of Steps That Contribute to the Prothrombinase Reaction. Figure 1 shows a general result encountered in study of prothrombinase reaction kinetics. It is apparent that K_M, or the midpoint of substrate titration ($[S]_{0.5}$), varied widely, V_{max} differed for some membranes, and curve shape also varied as a function of both membrane composition and vesicle size. These kinetic results are summarized in Table 1, and some results are described in greater detail below.

Scheme 1[a]



[a] Definitions: S, prothrombin; P, product (fragment 1.2 or fragment 1); PL can be either a protein binding site on a vesicle or an entire vesicle, depending on the situation described in the text; N, total number of binding sites on a vesicle; n, number of substrates bound per vesicle; m, number of products bound per vesicle. Step 1. k_1 = Second order rate constant for association of prothrombin with a binding site on the membrane; k_{-1}=dissociation rate constant; $K_D = k_{-1}/k_1$. When $k_1N \ll k_{coll}$, velocity of substrate binding to a vesicle = $k_1(N - n)$[S]; velocity of substrate dissociation from a vesicle = $k_{-1}n$. If $k_1N = k_{coll}$, mechanism 4 applies and velocity expressions differ (see text). Step 2. k_s = first order rate constant for transfer of substrate from the membrane surface to the enzyme. Forward velocity = nk_s. Although possible, reversibility of step 2 is not shown. Step 3. k_{cat} = rate constant for enzyme catalysis. Step 4. Product dissociation from the enzyme while still attached to the membrane. Normally, steady state kinetic properties cannot discriminate steps 3 and 4 and they can be expressed as a single step. Step 5. k_{dissn}= rate constant for product dissociation from the membrane. This is equal to k_{-1}. Step 1′. An alternative mechanism for addition of substrate directly to the enzyme.

Scheme 1 shows a logical series of reaction steps that can produce these behaviors through four basic kinetic mechanisms. Enzyme velocity is expressed per enzyme (v/E_t), and the following description is for ≤1 enzyme per membrane particle. At at any condition, total reaction velocity may include contributions from more than one mechanism.

Reaction Mechanism 1. Michaelis−Menten Behavior Requires That All Steps of the Kinetic Mechanism Contain the Enzyme Term. Experimental results are consistent with Michaelis−Menten behavior by step 1′. In this case, reaction 1 is an appropriate description.

$$S + E{\cdot}PL \rightleftharpoons S{\cdot}E{\cdot}PL \rightarrow P + E{\cdot}PL \quad \text{(reaction 1)}$$

This provides that K_M can be completely unrelated to K_D for prothrombin-membrane binding. Since the membrane surface, along with the enzyme, may contribute to the substrate binding site, K_M may still vary with membrane composition. This mechanism should provide no impact of particle size, unless size is accompanied by different membrane surface properties.

A Michaelis−Menten mechanism can also arise from steps 1−5, if steps 1 and 2 are combined. This can occur if $k_s \gg k_{-1}$ and $k_{dissn} \gg k_{cat}$. These conditions did not appear to apply to prothrombinase and this mechanism is not discussed in detail.

Reaction Mechanism 2. Non-Michaelis−Menten Kinetic Behavior with S_n·PL as the Intermediate. When step 2 of Scheme 1 is rate limiting, enzyme velocity is given by reaction 2, where PL is a membrane vesicle.

$$nS + PL \rightleftharpoons S_n{\cdot}PL \rightarrow P + PL \quad \text{(reaction 2)}$$

(steady-state parameters: $[S]_{0.5} = [S][PL]/[S{\cdot}PL] = k_{-1}/k_1 = K_D$; $v = k_s n$; $V_{max} = Nk_s \ll k_{cat}$)

The enzyme is not a component of these steps. In effect, it is not rate limiting because it is always present in excess. This is indicated by $V_{max} \ll k_{cat}$. K_M is inappropriate, and substrate concentration at half-maximum velocity ($[S]_{0.5}$) is used. Since reaction velocity is determined by substrate-membrane binding, steady-state kinetic plots such as Lineweaver−Burk or Eadie−Hofstee depend on this interaction and may or may not be linear. Since N is nearly proportional to vesicle surface area, velocity will be proportional to $4\pi r^2$ and $[S]_{0.5}$ will be proportional to $1/r^2$, where r is the radius of the vesicle.

Reaction Mechanism 3. Non-Michaelis−Menten Behavior Where P_m·PL Determines V_{max}. In this case, the steps in Scheme 1 combine to those in reaction 3, where PL represents a membrane particle.

$$nS + PL \rightarrow P_m{\cdot}PL \xrightarrow{k_{dissn}} mP + PL \quad \text{(reaction 3)}$$

(steady-state parameters: $[S]_{0.5} = [S][PL]/[P{\cdot}PL]$; $v = k_{dissn} m$; $V_{max} = k_{dissn} N \ll k_{cat}$)

Once again, kinetic rate plots will be dependent on the properties of substrate- and product−membrane interaction. Since substrate and product of the prothrombinase reaction show nearly identical membrane interaction properties (Lu & Nelsestuen, 1996), the complexities of $[S]_{0.5}$, V_{max} and the impact of vesicle size on velocity and $[S]_{0.5}$ are similar to those described for mechanism 2.

Reaction Mechanism 4. Non-Michaelis−Menten Behavior That Is Limited by the Rate of Collisions between Substrate and the Membrane. In this case, velocity is described by reaction 4.

$$S + PL \xrightarrow{k_{coll}} P + PL \quad \text{(reaction 4)}$$

This process will give velocity that is directly proportional to [S] so that Eadie−Hofstee plots will have a slope of zero, as shown in Figure 2A.

Mechanism Switching. Reaction 4 is a nonsaturable process and cannot be continued indefinitely. As the enzyme or binding sites on the membrane become filled, the reaction must switch to be rate limited by a saturable mechanism. The result is curvature of Eadie−Hofstee plots such as is shown in Figure 2. These reaction properties have been described more thoroughly (Martinez et al., 1992, 1996) and Figure 2 illustrates full and partial impacts of this mechanism on the general shape of Eadie−Hofstee plots. Nonlinearity occurs whenever the collisional limit impacts on a reaction. Since the collisional rate constant ($k_{coll} = 4\pi N_{av} D r/1000$, D = diffusion constant, N_{Av} = Avogadro's number, and r = particle radius) is proportional to vesicle size, while V_{max} is limited by a constant amount of enzyme per particle (one in this case), reaction velocity at low substrate is proportional to radius while $[S]_{0.5}$ is proportional to $1/r$.

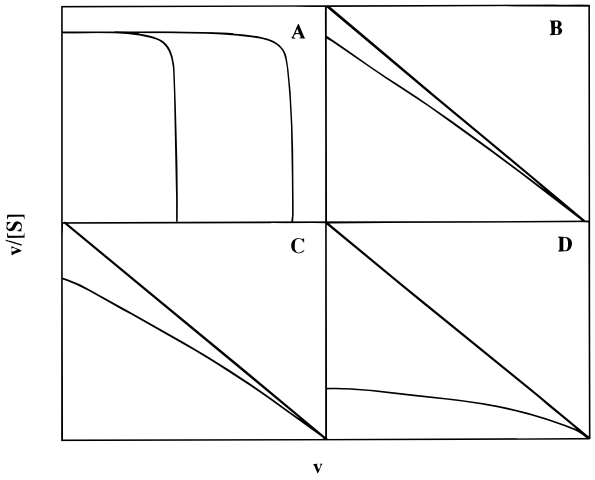


FIGURE 2: Impact of the collision-limited circumstance (mechanism 4) on enzyme kinetic behavior. The kinetic systems consist of particles which each contained many enzymes or binding sites. The theoretical curves were modified from those in Figures 1A and 1C of Martinez et al. (1996). (Panel A) Curve shapes for two solutions that have identical numbers of enzyme-containing particles but a 2-fold difference in the number of enzymes per particle. At low substrate concentrations, the enzyme captured >99% of the substrate that collided with each particle. (Panels B−D) Reactions that are partially limited by collision. The curved lines show the result for reactions of particles that contain many binding sites per particle, sufficient to proceed at 15% (panel B), 25% (panel C), and 75% (panel D) of the collisional limit at the lowest substrate concentrations. The straight lines show the theoretical result for reactions containing a similar amount of total enzyme that is free in solution and therefore unaffected by the collisional limit.

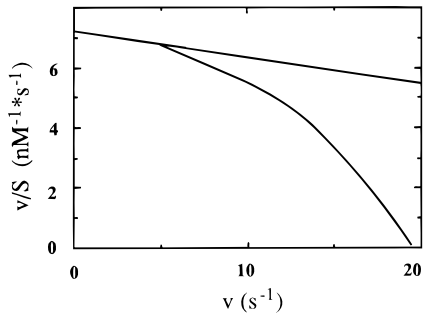


FIGURE 3: Theoretical results for mechanism 3 with a switch to mechanism 1 at high substrate concentration. The straight line shows the velocity of prothrombin binding to LUV as a function of prothrombin concentration, at equilibrium. If all substrates bound to the membrane are converted to product (mechanism 3), this curve should coincide with enzyme reaction velocity. This line eventually exceeds k_{cat} (about 20 s^{-1}), which is not possible. Thus, the plot must curve toward an intercept at 20 s^{-1}. The curved line shows this general result without a formal solution.

The other mechanisms can also participate in mechanism switching. An example is provided in Figure 3 where the kinetic mechanism is described by reaction 2 at low substrate concentrations but switches to be limited by step 3 at high substrate concentrations. In effect, k_{cat} is intermediate between k_s and Nk_s. Several types of mechanism switching between these four mechanisms can be proposed. Downward curvature of Eadie−Hofstee plots (Figures 2 and 3) is a

common feature of these mechanism switching events.

Experimental Studies: Mechanism I. Michaelis−Menten Kinetics. The kinetic properties of prothrombinase assembled on membranes of 10% PS conformed to the Michaelis−Menten mechanism (reaction mechanism 1). There was no suggestion that the population of membrane-bound substrates or products contributed to the reaction. This was shown by the lack of direct correlation between enzyme kinetic properties and prothrombin−membrane binding properties (Table 1). The lack of close relationship between K_D and K_M was reported earlier (Pusey & Nelsestuen, 1983; van Rijn et al., 1984), and this study extended this to individual rate constants. Eadie−Hofstee plots (not shown) were linear and did not show the concave downward shape characteristic of mechanism switching. Lack of concave downward curve shape also suggested that mechanism 4, a collision-limited reaction, did not contribute significantly to these results. In agreement with this conclusion, k_{cat}/K_M values for SUV (1×10^8 M^{-1} s^{-1}) were less than 2% of k_{coll} (about 7×10^9).

While enzyme is involved in every step of the pathway, including substrate capture from solution, the membrane may form a portion of the substrate-binding site. Its contribution appears to be relatively small and not highly influenced by charge density of the membrane or by K_D for prothrombin−membrane binding. For example, SUV of 10% PS gave a K_M of 174 nM (Table 1) while membranes of 2% PS gave a K_M of 400 nM (Pusey & Nelsestuen, 1983). In the absence of membrane, prothrombinase gave a K_M of about 30 μM (Rosing et al., 1980). A membrane contribution of only 3 kcal/mol would be adequate to lower the binding affinity to 0.2 μM (SUV, Figure 3). Low energy contributions may even be made by uncharged membranes where the prothrombin−membrane interaction is virtually undetected by commonly used methodology.

This Michaelis−Menten mechanism provided no apparent basis for an impact of membrane particle size, per se. However, the lower K_M displayed for LUV may arise from differences in surface properties. For example, the more planar surface of LUV may provide a more optimum spatial relationship between the membrane and the prothrombin binding site on the enzyme. Alternatively, the slower dissociation of prothrombin from LUV may suggest another slightly different interaction with prothrombin.

Reaction Mechanism III. Substrate Transfer from the Membrane to the Enzyme. Early studies showed that high membrane concentrations inhibited prothrombinase and that this was correlated with equilibrium binding of prothrombin to the membrane (Rosing et al., 1980). Since there was no correlation between substrate density on the membrane and reaction velocity, the important substrate pool was that in solution, not the protein on the membrane (Pusey & Nelsestuen, 1983; van Rijn et al., 1984). High phospholipid concentration reduced the prothrombin in solution (van Rijn et al., 1984). This lack of correlation between protein density on the membrane and enzyme reaction velocity was substantiated by the studies shown above. However, the following studies suggested that this mechanism and resulting conclusions were not universal. That is, mechanism switching allowed membranes of high PS content to function by an entirely different mechanism.

The results in Figure 4 compare the ability of membranes of various composition and size to inhibit the prothrombinase

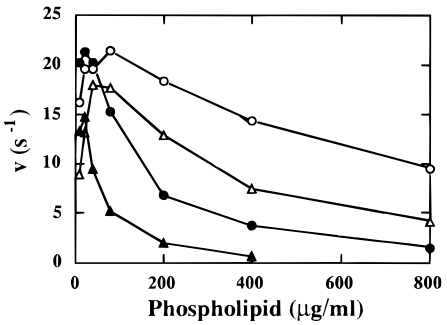


FIGURE 4: Summary of activation/inhibition of prothrombinase by different membranes. Velocity of the prothrombinase reaction (v, mol of thrombin produced per second per mol of prothrombinase enzyme) are shown at various concentrations of SUV of 50 (▲) and 25% (●) PS and LUV of 50 (△) and 25% (○) PS. Prothrombin concentration was 0.5 μM. Other conditions and procedures were as described in Materials and Methods.

reaction. For vesicles of the same size, the results correlated with earlier study (Rosing et al., 1980) and showed that inhibition correlated with K_D for prothrombin−membrane interaction. That is, potency of inhibition was greater for SUV of 50% PS than for SUV of 25% PS. Comparison of LUV and SUV differed from this pattern. That is, SUV of 25% PS were more potent for inhibition of prothrombinase than were LUV of 50% PS, despite tighter binding to the latter. LUV of 25% PS showed very low inhibition. The following results show how these behaviors are well described by various properties of prothrombin−membrane binding and prothrombinase kinetics.

Figure 5A shows that prothrombinase velocity correlated very closely with nk_{dissn}, the velocity of prothrombin (or fragment 1) dissociation from SUV of 50% PS. Since binding should be at equilibrium, enzyme velocity also equaled the velocity of prothrombin association with a vesicle. This suggested that the enzyme captured virtually every substrate molecule that bound to the same vesicle. In this situation, the substrate on the membrane surface would be the important precursor to enzyme-bound substrate.

As expected for this kinetic situation, prothrombinase kinetics were determined entirely by the characteristics of prothrombin−membrane binding (reaction mechanism 2 or 3). The results in Figure 5B show that $[S]_{0.5}$ was approximately equal to the K_D for substrate−membrane binding. As expected, V_{max} obtained by extrapolation of this linear portion of the Eadie−Hofstee plot (2.2−36 nM free substrate concentration) was less than k_{cat}. However, at very high substrate concentrations, the velocity curved toward k_{cat}. This was consistent with the approximately 2-fold increase in k_{dissn} as the membrane became very crowded with protein (Lu & Nelsestuen, 1996). At low substrate, a slight curvature of the Eadie−Hofstee plot was expected from a small contribution by the collisional limit. The value of $V_{max}/[S]_{0.5}$ (1.0×10^9) was about 15% of k_{coll} (6.7×10^9), and curvature should be approximately that shown in Figure 2B. The lack of greater curvature was also important since it suggested that k_{coll} did not make a larger contribution to the reaction.

In order to capture virtually every substrate molecule that bound to the membrane, k_s must be greater than k_{-1} or k_{dissn}. Thus, k_s should be $\gg 0.15$ s^{-1}. Estimates of k_s for membranes of other compositions (see below) were consistent with this value.

The results in Figure 6A show that LUV containing 50% PS showed a relatively close correlation between enzyme

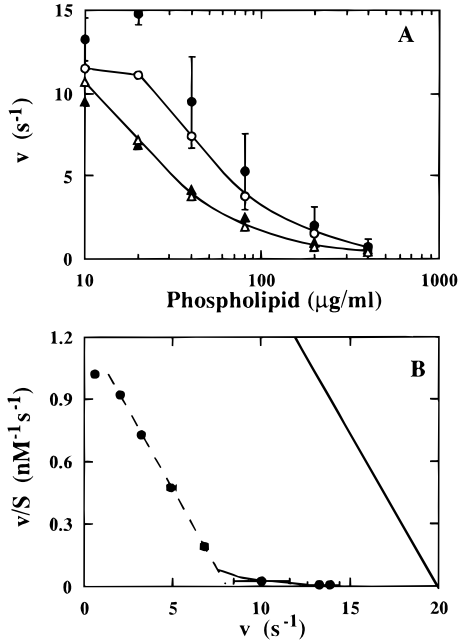


FIGURE 5: Prothrombinase assembled on SUV of 50% PS, rate limitation by product dissociation. (Panel A) Prothrombinase reaction velocity was determined as described in Materials and Methods and is plotted as a function of phospholipid concentration. The results for 500 nM total prothrombin concentration (●) are the average and standard deviation for three determinations, and those for 250 nM prothrombin (▲) are a single determination. The velocities of prothrombin dissociation from a vesicle at 250 (△) and 500 (○) nM prothrombin were estimated from nk_{dissn}, where n is the prothrombin/phospholipid vesicle ratio and $k_{dissn} = 0.15$ s^{-1}. (Panel B) Prothrombinase reaction velocity determined at constant phospholipid (50 μg/mL of 50% PS-SUV) and varied prothrombin concentrations. Prothrombin concentrations were varied from 70 to 4100 nM. [S] is free prothrombin concentration which was calculated from total prothrombin, a K_D for the prothrombin−phospholipid complex of 8 nM, and 80 prothrombin binding sites per vesicle of 4060 kDa. The straight line line drawn through the experimental points corresponded to a $[S]_{0.5}$ of 6.5 nM and a V_{max} of 8.3 s^{-1}. The theoretical straight line plot to the right corresponds to a $K_M = K_D = 8$ nM and $V_{max} = k_{cat} = 20$ s^{-1}. The latter is expected if velocity were limited by k_{cat}.

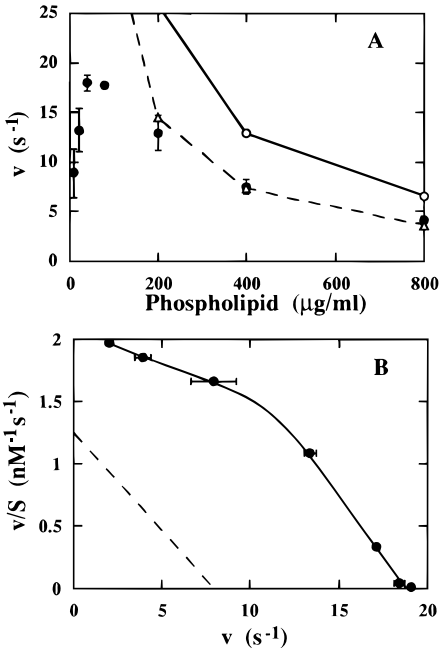


FIGURE 6: Prothrombinase assembled on LUV of 50% PS: mechanism switching. (Panel A) The velocity of prothrombinase reaction (●) is shown at 500 nM total prothrombin and the phospholipid concentrations given (LUV of 50% PS). The velocity of prothrombin dissociation from a vesicle (○) was calculated from nk_{dissn}, where n is the molar ratio of prothrombin to phospholipid vesicles and k_{dissn} was 0.09 s^{-1}. Also shown is the velocity of substrate transfer from the membrane to the enzyme (△) calculated from $nk_{dissn}[k_s/(k_s+k_{dissn})]$, where n is defined above, $k_s = 0.13$ s^{-1}. (Panel B) Prothrombinase velocity at constant phospholipid and varied prothrombin concentrations. Phospholipid was LUV of 50% PS (50 μg/mL), and total prothrombin was varied from 34 to 2070 nM. The concentration of free prothrombin, [S], was calculated from total phospholipid, total prothrombin, a K_D of 20 nM, and 1600 prothrombin binding sites per LUV. The dashed line corresponds to that in Figure 5B and is included to illustrate the large difference in kinetic properties of prothrombinase assembled on SUV versus LUV of a single composition.

velocity and protein−membrane dissociation (and association) rates at low protein/vesicle ratios. The fit at low reaction velocity (high phospholipid) was improved if a value of $k_s = 0.13$ s^{-1} was assumed. Since k_{dissn} was about 0.09/s, a k_s value of 0.13 provided that only a portion of the membrane-bound substrate molecules were captured by the enzyme. At high protein/vesicle ratios, the rate of substrate binding/dissociation exceeded enzyme velocity. These behaviors were consistent with the large number of proteins that bound to a LUV. The k_{cat} was insufficient to convert a high proportion of substrates before they dissociated from the membrane. As a result, at high substrate concentration, the reaction switched to be limited by step 3 (Scheme 1).

Mechanism-switching in the course of substrate titration was supported by observation of highly curved Eadie−Hofstee plots (Figure 6B). Enzyme saturation before the membrane became saturated with substrate should produce $[S]_{0.5} < K_D$. Again, this property was observed ($[S]_{0.5} =$ 5.6 nM; $K_D = 20$ nM, Table 1).

In further agreement with these general mechanisms, LUV were much less effective on a weight basis than SUV for inhibition of prothrombinase (compare Figures 5A and 6A). This could be explained by the fact that the total number of substrates per vesicle, not their density, was the important parameter for transfer to the enzyme. Thus, virtually all features of these reactions were in close agreement with behaviors predicted from Scheme 1 and its constituent reaction mechanisms.

It was interesting to note that a major contributor to the difference between LUV and SUV of 50% PS was the number of binding sites per particle, a factor determined by vesicle size. This differed from the case of 10%PS, where differences between SUV and LUV did not appear to be due to particle size per se, but to other properties. Thus, there did not appear to be a single explanation for an observed property of prothrombinase, even with respect to the enhanced function of LUV.

Mixed Mechanisms. At least two mechanisms may contribute to the kinetic properties of prothrombinase when it is assembled on vesicles of 25% PS. First of all, since $[S]_{0.5}$ was not greatly different from that for 10% SUV (104 vs 174 nM, Table 1), reaction mechanism 1 probably made a major contribution to enzyme kinetics. In addition, the close correlation between V_{max} and k_{cat} suggested that reaction mechanism 1, the Michaelis−Menten pathway, was dominant. Lower ability of LUV to inhibit the reaction (Figure 7A) could arise from the fact that they show lower $[S]_{0.5}$ and slightly higher K_D values (Table 1). As a result, higher

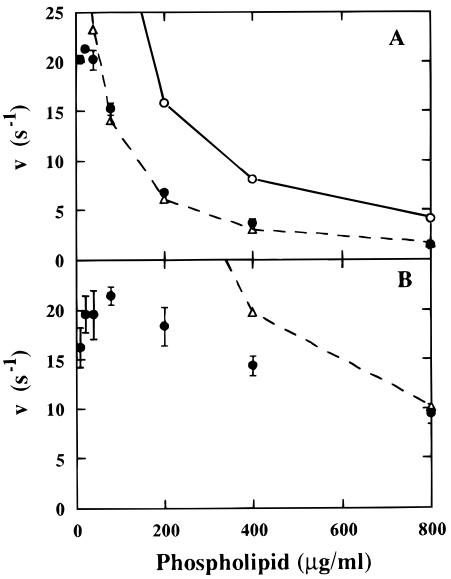


FIGURE 7: Comparison of phospholipid inhibition of enzyme with protein-membrane dynamics for SUV and LUV of 25 PS. (Panel A) Prothrombinase enzyme velocity (●) was determined at constant prothrombin (500 nM) and varied phospholipid concentration (SUV of 25% PS). The rate of prothrombin dissociation from a vesicle (○) was calculated from nk_{dissn}, where n is the molar ratio of prothrombin to vesicles and k_{dissn} is 1.9 s^{-1}. The rate of substrate transfer from the membrane to the enzyme (△) was calculated from $nk_{dissn}[k_s/(k_s+k_{dissn})]$, where n is as described above and k_s is 1.2 s^{-1}. (Panel B) Prothrombinase velocity (●) is shown as a function of the concentration of LUV of 25% PS. The velocity of prothrombin dissociation from these vesicles is displaced far above the portion of the graph shown. The rate of substrate transfer from the vesicle surface, $nk_{dissn}[k_s/(k_s+k_{dissn})]$, is shown (△). Once again, n is the ratio of prothrombin to vesicles and $k_s = 0.13$ s^{-1}.

concentrations of LUV would be required to reduce solution concentrations of prothrombin to levels that impact on enzyme velocity. Linearity of the Eadie−Hofstee plots (Table 1) was also consistent with reaction mechanism 1. As pointed out above, if transfer of substrate from the membrane surface to the enzyme is important (reaction mechanism 2), Eadie−Hofstee plots should not be linear unless $V_{max} < k_{cat}$. This did not appear to be the case.

However, other properties suggested some contribution by substrate transfer from the membrane to the enzyme (reaction mechanism 2). Figure 7A shows that, while enzyme activity for 25% PS did not correlate closely with the velocity of association/dissociation of substrate with the membrane, the curves at low substrate concentration (high PL concentration) became superimposible with theory when k_s was assigned a value of 1.2 s^{-1}. This value agreed with the limiting feature of k_s that was estimated for SUV of 50% PS (above, $k_s >$ 0.15 s^{-1}). In addition, $[S]_{0.5}$ (104 nM, Table 1) was similar to the K_D for prothrombin−membrane interaction (108 nM, Table 1). A close correlation between protein density on the membrane and reaction velocity, throughout substrate titration, would be consistent with a reaction that is described by reaction mechanism 2.

Lower efficacy of LUV versus SUV in prothrombinase inhibition (compare Figure 7 panels A and B) could also be explained by reaction mechanism 2. While the rate of prothrombin dissociation from the membrane was much greater than enzyme velocity, utilization of the k_s value obtained for LUV of 50% PS (0.13 s^{-1}) resulted in close correlation between predicted and experimental velocity at low substrate concentrations (high phospholipid, Figure 7B). Thus, the larger number of substrate binding sites on LUV could produce lower ability to inhibit the reaction. Earlier studies may also suggest rate limitation by reaction mechanism 2. That is, Van Rijn et al. (1984) observed that membranes of 10% PS had a slightly greater maximum velocity than membranes of 20% PS. This would be consistent with a small contribution from reaction mechanism 2 for membranes of 20% PS. Results conducted in this study did not show higher V_{max} for prothrombinase assembled on 10% PS versus 25% PS. However, the potential for small degrees of nonlinearity may complicate attempts to obtain highly accurate estimations of V_{max} by extrapolation of steady-state kinetic plots.

Overall, the results suggested that reaction kinetics with 25% PS arose from contributions by both reaction mechanisms 1 and 2. The lower $[S]_{0.5}$ value for LUV of 25% PS may arise for reasons outlined for 10% PS (improved spatial arrangement of the membrane surface and the enzyme binding site) or from another contributor to the improved function of LUV of 50% PS (a larger number of substrate binding sites per vesicle). While participation by two mechanisms should produce non-linear Eadie−Hofstee plots, the differences provided by the contributing mechanisms may be small and they may give superimposible plots. Curvature would not be detectable. Thus, although it may occur for membranes of 25% PS, substrate capture by the membrane followed by transfer to the enzyme (mechanism 2) did not appear to provide a substantial improvement over direct capture of substrate by the enzyme (mechanism 1).

DISCUSSION

This investigation used two approaches which both suggested that four kinetic mechanisms were needed to describe prothrombinase reaction kinetics. The simplest approach involved comparison of substrate exchange rates from the membrane with enzyme velocity (Figures 5B, 6B, and 7). The second method consisted of traditional kinetic measurements. An important outcome was the absence of a simple, universal expression that could describe the prothrombinase reaction. Even the basis for improved function of LUV over SUV appeared to vary with membrane composition. These several kinetic mechanisms appeared to explain much of the complex behavior of the prothrombinase reaction.

Switching between mechanisms has a number of ramifications. Changes of any experimental parameter may result in a switch of kinetic mechanism or in different contributions from the various mechanisms. Experiments that involve titrations of substrate, other proteins that compete for membrane surface binding, temperature, calcium concentration, ionic strength, number of enzymes per particle, membrane composition, surface properties, fluidity, curvature, size, or other parameter may encounter mechanism switching. Interpretation of results by standard methods that assume a constant mechanism (e.g., Arrhenius plots) may give incorrect conclusions. An alternative description might be that prothrombinase displays "system kinetics" so that models developed for soluble enzymes do not anticipate critical behavior. Inclusion of steps in the reaction pathway that are not enzyme-dependent is a key feature that distinguishes this reaction, and probably other membrane-bound enzymes, from virtually all soluble enzymes.

An important finding for membranes of 50% PS was the ability of the substrate to be transferred from the membrane surface to the enzyme and the product to be transferred back without dissociation. Results for membranes of 25% PS were less clear, partially due to the high efficacy of direct substrate capture by the enzyme (Michaelis−Menten behavior). As a result, membranes of very low PS content bound prothrombin poorly but gave K_M values that were not greatly different from those obtained with membranes of 25% PS. While transfer of substrate from the membrane surface to the enzyme may contribute to the reaction by membranes of 25% PS, the improvement over direct substrate capture by the enzyme was slight. However, a contribution of non-Michaelis−Menten mechanism to reaction velocity on membranes of 25% PS would make the mechanistic implications of a K_M or apparent K_M invalid, despite Eadie−Hofstee plots that appeared linear.

In this study, substrate transfer from the membrane to the enzyme was characterized by a first order rate constant, k_s. Expressed on the basis of surface area, k_s for LUV (0.1 s^{-1}) was 4.9×10^{-3} s^{-1} μ^{-2} and the value for SUV (1.2 s^{-1}) was 2.8×10^{-3} s^{-1} μ^{-2}. For membranes of 50% PS, the number of sites per vesicle was also an important factor in determining kinetic behavior, giving rise to larger differences in reaction velocity (although not necessarily in $[S]_{0.5}$) for LUV versus SUV than were observed for membranes of lower PS content.

Michaelis−Menten behavior requires enzyme participation in every step of the reaction. This can be achieved by steps 1−5, if k_s is large so that step 2 is never rate limiting. However, a k_s value of 1.2, estimated for SUV of 25% PS, would provide for transfer of only about two substrate molecules from the membrane surface to the enzyme at the $[S]_{0.5}$ value for 10% PS ($n = 2$, Table 1). Actual velocity at $[S]_{0.5}$ was about 10 s^{-1}. Thus, generation of Michaelis−Menten kinetics from steps 1−5 of Scheme 1 required that k_s increase dramatically as PS content of the membrane decreased. This seemed unlikely and step 1′ (Scheme 1) was proposed as the most likely basis for Michaelis−Menten behavior.

Use of k_s as a first order rate constant based on the entire vesicle ignored potential regional behaviors. Regional effects on a membrane would arise if the enzyme was exposed to only those substrates that bound in its vicinity and that substrates bound at distant locations would dissociate before encountering the enzyme. The lateral diffusion rate of prothrombin on supported membrane bilayers was estimated to be about 10^{-9} cm^2 s^{-1} (Huang et al., 1994). This would provide randomization of substrate on the surface of LUV (110 nm diameter) in about 0.25 s. Membranes of 25% PS or higher that show prothrombin dissociation rate constants of 1 s^{-1} or less (at 2 mM calcium) should therefore approach diffusional randomization of substrate and product on the surface before dissociation. There may be slight regional concentrations of product since randomization on the LUV surface may not occur between catalytic events ($k_{cat} = 20$ s^{-1}). This gradient should be negligible for SUV. The absence of significant regional behaviors was apparent for membranes of 50% PS where the enzyme captured a high proportion of substrates that bound to the vesicle. For membranes of 10% PS, a k_{dissn} of 15−20 s^{-1} would probably not provide diffusional equilibrium on the LUV surface and the enzyme would be exposed to a fraction of the substrate molecules that were bound to the vesicle. Since prothrombinase on 10% PS vesicles displayed Michaelis−Menten behavior, regional properties of substrate−membrane interaction would not be an important factor.

The enzyme captured only about one-third of the substrate molecules that bound to SUV of 25% PS. Simple enlargement of the surface area should not substantially improve enzyme exposure to substrate, unless other membrane properties were altered. Improved function of LUV may have been due to membrane structural properties rather than particle size, per se. It is possible that this trend extends to supported planar phospholipid bilayers which display K_M values of 3−6 nM (Billy et al., 1995; Giesen et al., 1991b), comparable to the $[S]_{0.5}$ value of 5 nM for LUV of 50% PS reported here. The k_{dissn} for prothrombin from supported phospholipid bilayers contained two components, one of about 0.5 s^{-1} and another of about 0.02 s^{-1} (Pearce et al., 1992, 1993). The average, about 0.1 s^{-1} was similar to the value obtained for 50% PS (0.1 s^{-1}) in the accompanying paper (Lu & Nelsestuen, 1996). These results make it possible that supported planar phospholipid membranes gave low K_M or $[S]_{0.5}$ values due to altered membrane properties and more extensive use of mechanism 2, transfer of substrate from the membrane surface to the enzyme. This may be a situation where alteration of a physical property of the membrane, other than composition, resulted in mechanism switching. Vesicles and supported planar bilayers may present quite different prothrombinase kinetics.

The results with membranes of 25% PS indicated little or no inhibition by membrane-bound product, except as it acted to displace either enzyme or substrate from the membrane. At V_{max}, the enzyme converted about one-third of the substrate molecules on the SUV to product. If this product bound to the enzyme as efficiently as substrate, V_{max} should be lowered by this proportion since no additional substrate can be added to the membrane. That V_{max} was similar to the highest values obtained indicated that membrane-bound product did not compete effectively for the enzyme active site. This property agreed with reports that fragment 1.2, the membrane-binding product of the prothrombinase reaction, is not a highly effective inhibitor of prothrombinase (Govers-Riemslag et al., 1985).

An interesting aspect of the various mechanisms outlined above was the limited number of situations where Eadie−Hofstee plots were linear. Direct participation of enzyme in every step, including capture of each substrate molecule from solution, produced mechanism 1 and linear Eadie−Hofstee plots. Situations limited by substrate-membrane binding (reaction mechanism 2) could give linear Eadie−Hofstee plots in the special case where substrate binding was characterized by a single K_D value. An additional requirement for linearity was a homogeneous mechanism throughout the titration so that enzyme is never rate limiting and $V_{max} \ll k_{cat}$. In contrast, any situation where the membrane contributed to substrate capture but where V_{max} equaled k_{cat} would produce nonlinear Eadie−Hofstee plots. These criteria can be used in partial diagnosis of situations where membrane capture of substrate contributes to the reaction kinetics.

The combined results of several studies may suggest a partial correlation between membrane surface properties that produce reduced k_{dissn} and those which provide greater efficiency of substrate transfer from the membrane to the

enzyme. An accompanying paper showed a small difference in the properties of prothrombin binding to SUV versus LUV (Lu & Nelsestuen, 1996). While K_D favored substrate binding to SUV, LUV showed a lower k_{dissn}. This correlated with the lower $[S]_{0.5}$ value of LUV. Very low k_{dissn} values (Pearce et al., 1992, 1993) may also correlate with the low K_M values (Billy et al., 1995; Giesen et al., 1991a) for these structures.

The mechanisms outlined in reactions 1−4 may be relevant to a number of other situations where protein components interact on a membrane surface. For example, mechanism 3, transfer of substrate from the membrane to the enzyme with capture of virtually every substrate molecule that was bound, may be similar to recent studies which showed that prothrombinase assembly occurred whenever factor Xa and Va were bound to the same vesicle (Ye & Esmon, 1995). Since factor Xa binds to the membrane with slightly higher affinity than prothrombin (Nelsestuen & Broderius, 1977), quantitative capture may occur at lower PS content. The transfer of proteins from the surface to a protein binding site, the equivalent of k_s, may be larger for the Xa−Va interaction as well. The ability of PC to support prothrombinase (Govers-Riemslag et al., 1994) may reflect the low impact of membrane composition on the K_M for prothrombinase when mechanism 1, direct substrate capture by the enzyme, is utilized.

Overall, the present study suggested that there was no simple, universal kinetic expression that could describe the prothrombinase reaction. Prothrombinase properties suggested several mechanisms, each arising from a different rate-limiting step from the series that might occur in the reaction pathway. For vesicles of low PS content, the reaction appeared to be Michaelis−Menten. However, membranes of intermediate and high PS content utilized mechanisms where the properties of protein−membrane interaction were partially or entirely responsible for the rate of product formation. Kinetic models, designed to characterize enzymes, are inadequate to describe this behavior. Important questions for further study include determination of the basis for large apparent differences in the dynamic properties of prothrombin binding to vesicles versus supported planar phospholipids and the relationship between these phospholipid structures and the membranes that support blood clotting *in vivo*. As shown by this study, the relevant kinetic mechanism and $[S]_{0.5}$, *in vivo*, may depend on properties (composition, curvature) of the membrane component.

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