Role of Procoagulant Lipids in Human Prothrombin Activation. 2. Soluble Phosphatidylserine Upregulates and Directs Factor X_a to Appropriate Peptide Bonds in Prothrombin†

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ABSTRACT: Activation of human prothrombin to thrombin (II_a) by factor X_a during blood coagulation requires proteolysis of two bonds and thus involves two possible activation pathways (parallel-sequential activation model). Hydrolysis of Arg^{322}−Ile^{323} produces meizothrombin ($MzII_a$) as an intermediate, while hydrolysis of Arg^{273}−Thr^{274} produces prethrombin 2−fragment 1.2 (Pre2−F1.2). A soluble lipid, dicaproylphosphatidylserine (C6PS), enhances activation by 60-fold [Koppaka et al. (1996) *Biochemistry 35*, 7482]. We report here that C6PS binding to factor X_a not only enhances the rate of activation but also alters the pathway. Activation was monitored using a chromogenic substrate (S-2238) to detect both II_a and $MzII_a$ active site formation and SDS−PAGE to detect Pre2−F1.2 as well as II_a and $MzII_a$. Of the four kinetic constants needed to describe activation, two ($MzII_a$ and Pre2−F1.2 consumption) were measured directly, and two ($MzII_a$ and Pre2−F1.2 formation) were obtained by fitting the three time courses simultaneously to the parallel-sequential reaction model. The time courses of II_a, $MzII_a$, and Pre2−F1.2 formations were all well described below the C6PS critical micelle concentration (CMC) by this activation model. The rate of Arg^{322}−Ile cleavage leading to $MzII_a$ formation increased by 150-fold, while the rate of Arg^{273}−Thr cleavage leading to Pre2−F1.2 formation was inhibited slightly. At concentrations of water-soluble C6PS above its CMC, all four proteolytic reactions increased in rate by 2−5-fold at the C6PS CMC. We conclude that soluble C6PS differentially affects the rate of individual bond cleavages during prothrombin activation in solution such that activation occurs almost exclusively via $MzII_a$ formation. Finally, C6PS enhanced the rates of all proteolytic reactions to within a factor of 3 of the enhancement seen with PS-containing membranes. We conclude that PS-containing membranes regulate prothrombin activation by factor X_a mainly via interaction of individual PS molecules with factor X_a.

The activation of prothrombin to the serine protease thrombin is accomplished by the proteolytic cleavage of two peptide bonds in the zymogen. Because two bonds are cut, two pathways of activation are possible, each proceeding through distinct intermediates [see Figure 1 of the first paper in this series (*35*)]. In one possible pathway (Arg^{273}−Thr cleavage), the intermediates formed, fragment 1.2 (F1.2)[1] plus prethrombin 2 (Pre2),[1] has no proteolytic activity, since the Arg^{322}−Ile bond in the catalytic domain remains intact. Initial cleavage of this bond leads to a catalytically active intermediate, meizothrombin ($MzII_a$),[1] which also retains its membrane-binding domain.

We know that optimal prothrombin activation requires phosphatidylserine (PS)[1] (*9*, *13*, *24*), which is present in the cytoplasmic leaflet of the platelet surface membrane but becomes exposed on the extra-cytoplasmic face upon platelet activation (*2*, *6*, *28*). The role of platelet membranes in the functioning of the prothrombinase has long been described in terms of (i) assembly of the activation complex (*15*, *25*), (ii) funneling of substrate to the enzyme (*11*, *19*), and, more recently, (iii) conformational alterations of the enzyme and/or substrates (*8*, *12*, *33*, *34*). The first paper of this series (*35*) suggested two additional roles: (iv) directing activation via the $MzII_a$ intermediate and (v) channeling $MzII_a$ intermediate rapidly to thrombin without release into solution. This raised other questions about the role of membranes in prothrombin activation. First, are the effects seen with PS-containing membranes due to interactions of individual PS molecules with factor X_a or to interaction of the enzyme with a membrane surface? We have shown previously that a soluble form of phosphatidylserine (C6PS)[1] enhanced the rate of prothrombin activation by factor X_a in the absence of a membrane (*14*). Since the effects of C6PS on prothrombin had a different concentration dependence, it was argued that this concentration dependence reflected a specific role of PS molecules in regulating factor X_a (*14*). Can C6PS perform

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[1] Abbreviations: CMC, critical micelle concentration; C6PS, 1,2-dicaproyl-*sn*-glycero-3-phospho-L-serine; DAPA, dansylarginine-*N*-(3-ethyl-1,5-pentanediyl)amide; F1.2, fragment 1.2; $MzII_a$, meizothrombin; II_a, thombin; POPC, 1-palmitoyl-2-oleoyl-3-*sn*-phosphatidylcholine; Pre1, prethrombin 1; Pre2, prethrombin 2; PS, phosphatidylserine; S-2238, phenylalanylpipecolylarginine *p*-nitroanilide (synthetic substrate for thrombin); SDS, sodium dodecyl sulfate; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane.

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the role of directing activation via the $MzII_a$ intermediate? Second, does the proposed channeling of intermediates to thrombin require a membrane surface as implied by the channeling hypothesis (*35*) or does interaction of PS with factor X_a or possibly with the intermediates alter the interaction between substrate and enzyme, thus leading to channeling? Finally, we ask: what fraction of the roughly 500-fold membrane-associated increase in k_{cat}/K_M we observed for overall active site production from prothrombin (*35*) is due to the presence of a membrane surface and what fraction is due to the specific effects of the PS molecule?

EXPERIMENTAL PROCEDURES

Materials

1,2-Dicaproyl-*sn*-glycero-3-phospho-L-serine (C6PS) in the form of sodium salt was purchased from Avanti Polar Lipids (Birmingham, AL). The sources of other reagents are as described in the first paper of this series (*35*).

Methods

Short-Chain Phospholipid Preparation. C6PS stock solutions were prepared from a chloroform stock, after evaporating the chloroform under a stream of nitrogen, redissolving the lipid in cyclohexane, and then freeze-drying the frozen solution overnight. The resulting dry powder was dispersed in the appropriate volume of buffer and vortexed thoroughly to reach approximately the expected concentrations. Accurate stock concentrations were determined by an inorganic phosphate assay (*7*). The critical micelle concentration (CMC)[1] of C6PS under the exact conditions of our experiments has been previously determined by quasielastic light scattering and by two hydrophobic fluorescent probes (*14*).

Protein Preparation. Human prothrombin and factor X, as well as the prothrombin activation intermediates, prethrombin 2 (Pre2[1]), fragment 1.2 (F1.2),[1] meizothrombin ($MzII_a$),[1] and activated factor X (X_a), were obtained and assayed as described in the first paper of this series (*35*).

Kinetic Measurements. The activation of prothrombin was followed by measuring the generation of amidolytic activity (due to thrombin or $MzII_a$) toward a thrombin-specific synthetic substrate, S-2238. The appearance of the $MzII_a$ intermediate and thrombin, along with Pre2 appearance, was also followed by SDS−PAGE using unlabeled prothrombin. The differential fluorescence properties of danslylarginine *N*-(3-ethyl-1,5-pentanediyl)amide (DAPA)[1] complexed with the intermediates and products of human prothrombin activation were also exploited to study the kinetics of $MzII_a$, Pre2, and prethrombin 1 (prothrombin minus the N-terminal fragment 1 region, Pre1)[1] activation to thrombin. The details of all these measurements as well as a description of the use of these data to obtain kinetic constants for intermediate formation are given in the first paper of this series (*35*).

Kinetics for Thrombin-Catalyzed Prethrombin 1 Formation from Prothrombin. The initial rate of thrombin-catalyzed Pre1 formation was determined by SDS−PAGE.[1] Reaction mixtures (2 mL) contained prothrombin (3, 5, 7, and 10 μM) in 50 mM Tris, 170 mM NaCl, and 5 mM $CaCl_2$, pH 7.4, and was initiated at 37 °C by the addition of 80 nM thrombin. Aliquots (20 μL) were withdrawn at different time intervals and quenched by the addition of 10% SDS[1] and subjected to PAGE[1] (1.5 mm, 12% gels) without reduction according to the method described by Laemmli (*17*). Protein bands were visualized by staining with Coomassie Blue R-250, and the gels were analyzed for increasing band intensities of Pre1 formed with time using GelScan XL software (LKB UltraScan, Gathersburg, MD) to measure the initial rate of Pre1 formation at different substrate concentrations.

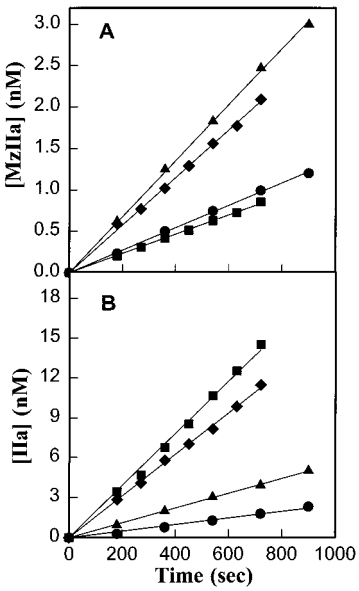

FIGURE 1: Time courses of meizothrombin appearance and thrombin formation at four C6PS concentrations. (A) The appearance of $MzII_a$ with time was monitored by the rate of S-2238 hydrolysis in the presence of heparin and antithrombin III. (B) Thrombin (II_a) formation was determined as the difference between the contribution of the active site detected by S-2238 in the absence and presence of heparin and antithrombin III (*26*, *35*). The reaction mixture contained 0.2 μM prothrombin and 5 nM factor X_a in 50 mM Tris, 175 mM NaCl, 0.6% poly(ethylene glycol), and 5 mM Ca^{2+} (pH 7.4) at 37 °C with different concentrations of C6PS: 75 μM (circles), 300 μM (triangles), 1 mM (diamonds), 1.5 mM (squares). Straight lines were obtained by performing a least squares linear regression analysis on all points except the zero time point.

Kinetic Modeling. Kinetic constants for reactions A (Pre2 formation) and C ($MzII_a$ formation) in Figure 1 of Wu et al. (*35*) were determined by fitting simultaneously three experimentally determined time courses (thrombin formation, $MzII_a$ appearance, Pre2 appearance), as described in detail in the first paper of this series (*35*). The kinetic constants k_b (Pre2 activation) and k_d ($MzII_a$ activation) were fixed at experimentally determined values. Consistent with our observations, a pseudo-second-order rate behavior was used for all four reactions in this scheme.

RESULTS

Effect of Soluble Phosphatidylserine on Prothrombin Activation. Figure 1 portrays the appearance of $MzII_a$ (panel A) and thrombin (panel B) as a function of time after addition of factor X_a to initiate proteolysis at four C6PS concentrations. Linear regression analysis of these data provided the initial rates of these two processes. The effect of C6PS on the initial rate of thrombin formation (circles) and $MzII_a$ (triangles) appearance are shown in Figure 2 as a function of C6PS concentration. The rate of thrombin formation increased markedly with the addition of C6PS, saturating at roughly a 60-fold enhancement in rate by 0.3 mM C6PS.

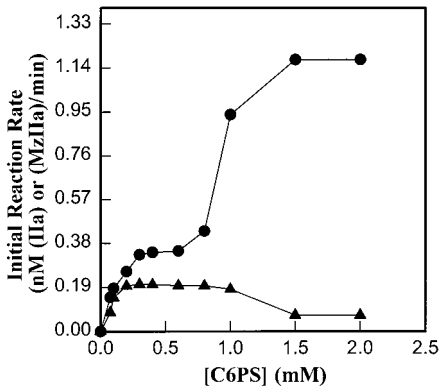

FIGURE 2: Initial rate of prothrombin activation as a function of phospholipid (C6PS) concentration. The initial rates of $MzII_a$ appearance (triangles) and of thrombin (II_a) formation (circles) were determined as described in the legend to Figure 1 and in Methods.

In addition to the increase in proteolytic activity that occurred at low C6PS concentration, a second sharp increase in the rate of thrombin formation occurred from 0.8 to 1.5 mM C6PS (Figure 2). We report elsewhere that the C6PS critical micelle concentration (CMC)[1] under our reaction conditions was 0.7−0.8 mM (*14*). This makes it clear that a soluble form of C6PS is responsible for the enhanced rate of thrombin formation at C6PS concentrations of 0−0.3 mM but that the second increase in rate (between 0.8 and 1.5 mM) was caused by the aggregation of C6PS.

The enhancements in the rate of appearance of $MzII_a$ and thrombin paralleled each other up to 0.6 mM C6PS. This explains why our earlier report of the overall increase in the rate of appearance of total active site in response to C6PS (*14*) was so similar to the current results. The rate of $MzII_a$ appearance was somewhat less than the rate of thrombin formation in this range, which is consistent either with the formation of a significant amount of thrombin via the Pre2 pathway or with a very rapid conversion of intermediate $MzII_a$ to thrombin. The nearly linear appearance of thrombin with time implies that, whatever the intermediate involved, its conversion to thrombin is rapid. Differences in the rate of appearance of thrombin and $MzII_a$ became more dramatic at concentrations of C6PS (0.8−1.5 mM) above the CMC, with the rate of $MzII_a$ appearance decreasing and the rate of thrombin production increasing roughly 3-fold (Figure 2). This indicates a contribution of C6PS micelle formation to the rapid conversion of $MzII_a$ to thrombin.

The initial rate of thrombin (panel B) or $MzII_a$ (panel A) formation is seen in Figure 3 to saturate with increasing substrate concentration with the exception of thrombin formation at 1500 μM C6PS. We fit these data to a standard hyperbolic expression to obtain apparent Michaelis−Menten kinetic constants. Although the constants obtained from such plots for overall active site formation cannot be rigorously interpreted in terms of the Michaelis−Menten model, it is instructive to compare these values with apparent kinetic constants for active site formation obtained elsewhere. The apparent k_{cat}/K_M we have obtained in the presence of 200 μM PS-containing membranes (2.2×10^4 M^{-1} s^{-1}) (*35*) is roughly 1.6-fold larger than obtained here for soluble C6PS below the C6PS CMC (1.36×10^4 $M^{-1}s^{-1}$). *Thus, the effect of a surface on the rate of prothrombin activation is less than a factor of 2*. Finally, the apparent K_M for active site

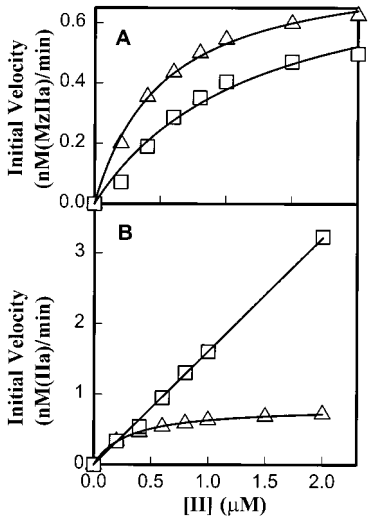

FIGURE 3: Meizothrombin and thrombin formation as a function of prothrombin concentration. The initial rates of $MzII_a$ appearance (A) or of II_a formation (B) were plotted as a function of prothrombin concentration. Reaction conditions were as summarized in the legend to Figure 1, with lipid concentrations of 300 μM (triangles) and 1.5 mM (squares). The data for all lipid concentrations were well described by a hyperbola.

formation obtained from Figure 3B (0.31 ± 0.01 μM) is much smaller than estimated by us [$K_M > 25$ μM (*35*)] or Rosing et al. [84 μM (*25*)] for activation of prothrombin by factor X_a in the absence of membranes. It is thus clear that the principal effect of C6PS on prothrombin activation is a major reduction in apparent K_M. Since there is no membrane surface present in our reaction mixtures to produce the commonly proposed reduction in dimensionality (*11*, *19*), this decrease in apparent K_M must reflect a C6PS-induced change in the enzyme that alters either its ability to bind substrate or to release product.

Kinetics of Activation of Pre2 and F1.2 (Reaction b), $MzII_a$ (Reaction d) to Thrombin. The intermediate $MzII_a$ contains an intact peptide bond at Arg^{273}−Thr, whereas Pre2−F1.2 contains an intact peptide bond at Arg^{322}−Ile. These intermediates were used to study individually the kinetics of the cleavage of these two peptide bonds by factor X_a in the presence of factor V_a and PS-containing membranes (*16*, *20*), as we do here in the absence of these cofactors but presence of C6PS. Figure 4 reports measurements of thrombin generation from Pre2 in the presence of equimolar amounts of F1.2 (panel A) and from $MzII_a$ (panel B) in terms of the initial rate of change in DAPA fluorescence intensity with time (*20*, *35*). The initial rates of thrombin formation in both cases were linear functions of respective substrate concentrations (inserts to Figure 4), the slopes of which yielded k_{cat}/K_M, the pseudo-second-order rate constants for Pre2 and $MzII_a$ activation to thrombin. Values of k_{cat}/K_M obtained below and above the C6PS CMC (300 and 1500 μM) are recorded in Table 1. For comparison to the activation of Pre2−F1.2, the initial rates of Pre2 and Pre1 activation were measured in the same way and were also linear functions of substrate concentrations (data not shown), with k_{cat}/K_M also obtained from the slopes of these curves. Interestingly, Pre2 was activated more slowly than Pre2−F1.2, while Pre1 was activated at a comparable rate, suggesting that both kringle domains of prothrombin are important for interaction either with factor X_a or with C6PS.

Table 1: Kinetic Parameters for Activation of Prothrombin and Its Intermediates[a]

reaction/activator	II → II_a, $MzII_a$ k_{cat}/K_M	II → Pre2, F1.2 k_{cat}/K_M, A	II → $MzII_a$ k_{cat}/K_M, C	Pre2, F1.2 → II_a k_{cat}/K_M, B	$MzII_a$ → II_a k_{cat}/K_M, D
factor Xa + Ca^{2+}	68 (*35*)[b]	$(1.1 \pm 0.04) \times 10^3$ (*35*)	44 ± 1 (*35*)	86 ± 7 (*35*)	$(1.5 \pm 0.1) \times 10^4$ (*35*)
factor X_a + Ca^{2+} + C6PS (<CMC; 300 μM C6PS)	1.36×10^4	$\leq 0.4 \times 10^3$ [c]	$(8.9 \pm 0.3) \times 10^3$ [d]	$(1.7 \pm 0.1) \times 10^5$	$(6.54 \pm 0.04) \times 10^5$
factor X_a + Ca^{2+} + C6PS (>CMC; 1500 μM C6PS)	6.4×10^3	$\leq 0.6 \times 10^3$ [c]	$(2.24 \pm 0.04) \times 10^4$ [d]	$(1.2 \pm 0.1) \times 10^5$	$(3.54 \pm 0.14) \times 10^6$

[a] All the rate constants are expressed in M^{-1} s^{-1}. [b] Prothrombin activation on a PS-containing membrane requires inclusion of direct conversion from prothrombin to thrombin, which is not shown in this table. $k_{cat}/K_M = (2.35 \pm 0.04) \times 10^4$, which corresponds to a channeling fraction of 0.54 (*35*). [c] Upper limits based on the limits of detection of our SDS−PAGE experiments. [d] These values adjusted to fit the parallel-sequential reaction model simultaneously to three data sets.

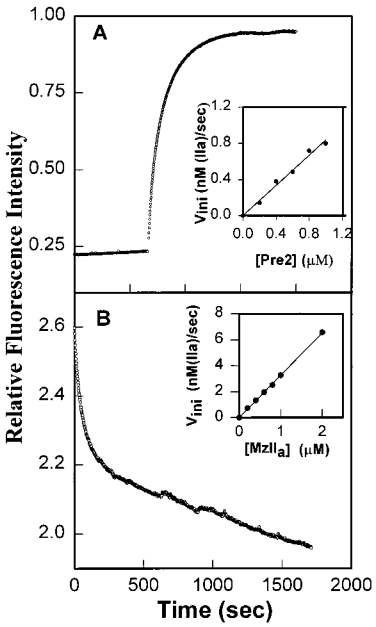

FIGURE 4: Time courses of meizothrombin and prethrombin 2−fragment 1.2 activation to thrombin. (A) For Pre2 and F1.2 activation, the increase in fluorescence intensity due to formation of the DAPA−thrombin complex was recorded as described in Methods. (B) For $MzII_a$ activation, the decrease in fluorescence intensity of DAPA was recorded (see Methods). The reaction contained 0.6 μM $MzII_a$, 3 μM DAPA, 5 nM factor X_a, and 300 μM C6PS in 50 mM Tris, 175 mM NaCl, and 5 mM Ca^{2+}, pH 7.4 at 37 °C. The initial rate of II_a formation was a linear function both of Pre2 and F1.2 and of $MzII_a$ concentrations (inserts in this figure). The slope of these curves yielded the pseudo-second-order rate constants, k_{cat}/K_M values for reactions B and D [Figure 1 of Wu et al. (*35*)].

Controls for Thrombin-Catalyzed Prethrombin 1 Formation. Thrombin-catalyzed feedback reactions are effectively inhibited by the presence of DAPA, an active site inhibitor of thrombin, and also by Ca^{2+} (*29*). Thus, these reactions were insignificant when the overall activation of prothrombin was monitored using the fluorescence of DAPA. However, while monitoring the rate of $MzII_a$ and thrombin appearance using S-2238, we found that the rates of these feedback reactions could become significant compared to the factor X_a-catalyzed reactions at the longest reaction times. To test for this, an estimate of the rate constant for thrombin-catalyzed Pre1 formation in the presence of 5 mM $Ca^{2+}$was obtained by SDS−PAGE as described in Methods. Measured band intensities of Pre1 formed were plotted against time, and the initial reaction rate of thrombin-catalyzed Pre1 formation was obtained from the linear portion of such curves. The initial rate of Pre1 formation was a linear function of prothrombin concentrations (data not shown), the slope of which yielded a pseudo-second-order rate constant of 55 ± 2 M^{-1} s^{-1}. This is somewhat slower than reported (80 M^{-1} s^{-1}) under similar ionic conditions but in the absence of lipid (*23*). This slightly lower rate of proteolysis may reflect the influence of C6PS on the proteolytic cleavage of prothrombin by thrombin. This possibility is supported by recent results in our laboratory showing that the rate of this proteolysis is reduced by roughly 2-fold and 20-fold in the presence of C6PS and PS-containing membranes, respectively (R. Majumder, unpublished observations). To ensure that thrombin-catalyzed consumption of prothrombin was minimal, we limited our kinetic modeling to time courses that produced less than 5 nM thrombin, so that the rate of thrombin-catalyzed proteolysis of prothrombin would be roughly 100-fold lower than the rate of factor X_a-catalyzed proteolysis. To confirm that this prediction was met, we analyzed a reaction mixture containing 2 μM prothrombin, 300 μM C6PS, 10 nM factor X_a, and 5 mM $CaCl_2$ by SDS−PAGE (results not shown). We could not detect prethrombin 1 or fragment 1 and fragment 2, indicating that the contributions of thrombin-catalyzed prothrombin proteolysis were minimal over the time period of our reaction conditions.

Estimation of the Rates of Pre2 and $MzII_a$ Formation. Since $MzII_a$ and Pre2 are consumed at rates comparable to those at which they form, we had to estimate the rate constants of their formation indirectly, assuming applicability of the parallel-sequential kinetic model discussed in the first paper of this series (*35*). To do so, we fit, for various C6PS concentrations from 0 to 2 mM, the measured time courses of Pre2 appearance, thrombin formation, and $MzII_a$ appearance up to times that corresponded to 1−8% of substrate consumption. Pre2 appearance was followed by SDS−PAGE, and this intermediate could not be detected at any C6PS concentration under our reaction conditions. The data were fit using the kinetic expressions and methods described by Wu et al. (*35*), while keeping k_b and k_d (pseudo-first-order rate constants = $k_{cat}/K_M[X_a]$ for Pre2 and $MzII_a$ activation) fixed at experimentally determined values (Table 1) and adjusting k_a and k_c to fit the kinetic model to the data. Figure 5 shows the best fits of calculated proteolysis time courses to the two observed prothrombin proteolysis data sets at C6PS concentrations; below (A, 300 μM) and above (B,1.5 mM) the C6PS CMC. It is evident that the data were fit well by the parallel-sequential model in both cases. The rate constants yielding these fits are given in Table 1.

Data taken below the C6PS CMC [ca. 0.7−0.8 mM under our reaction conditions (*14*)] clearly show a lag in thrombin formation, with $MzII_a$ initially appearing at a greater rate

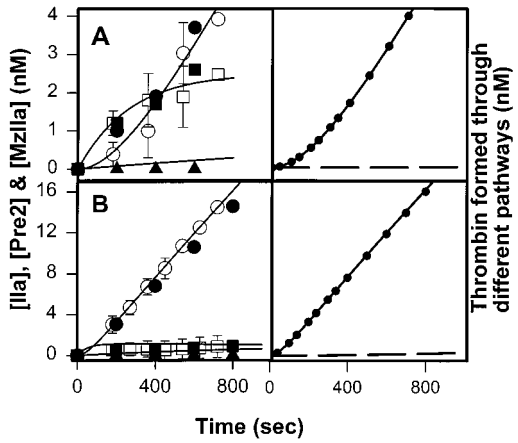

FIGURE 5: Best fit of the parallel, sequential model to observed prothrombin proteolysis data at different lipid concentrations. Left panel: Prothrombin (0.2 μM) proteolysis was catalyzed by factor X_a (5 nM) in the presence of different concentrations of C6PS and 5 mM $CaCl_2$ (see Methods) at 37 °C and in the presence of (A) 300 μM C6PS and (B) 1500 μM C6PS. We followed as a function of time thrombin (circles), $MzII_a$ (squares), and Pre2 (triangles) using activity assays (open symbols) or SDS−PAGE (closed symbols). Curves drawn for Pre2 represent upper limits to the rates of Pre2 formation based on the upper limits to k_{cat}/K_M given in Table 1. Right panel: Simulated (using methods in ref *35* and the best fit parameters given in Table 1) quantities of thrombin (solid lines) arising via $MzII_a$ (circles) or Pre2 (dashed line) are plotted versus time.

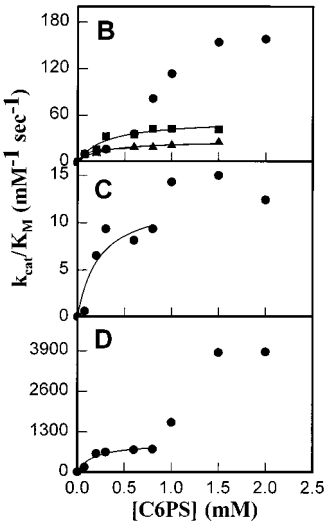

FIGURE 6: Second-order rate constants for three proteolytic reactions of prothrombin activation to thrombin as a function of phospholipid concentrations. Apparent second-order rate constants, k_{cat}/K_M, for three proteolytic reactions [B, Pre2 consumption; C, $MzII_a$ formation; and D, $MzII_a$ consumption; see Figure 1 in Wu et al. (*35*)] are plotted as a function of phospholipid concentration in panels B−D, respectively. The second-order rate constants for conversion of Pre2 and F1.2 to thrombin (B) and of $MzII_a$ to thrombin (D) were obtained experimentally as described in Figure 4. The rate constants for conversion of prothrombin to Pre1 and F1.2 (A) and to $MzII_a$ (C) were obtained as adjustable parameters as a result of fitting the data in Figure 5. In panel B, circles represent the reaction rate for the conversion of Pre2 and F1.2 to thrombin; triangles and squares indicate the rate constants for Pre2 and Pre1 conversion to thrombin, respectively. Only upper limits of the rate constants for Pre2 formation (reaction A) could be estimated, since this intermediate could not be detected at any C6PS concentration.

than seen for thrombin formation (Figure 5A). This is consistent with the behavior we have reported for prothrombin activation by factor X_a in the absence of membranes (*35*) and is expected for the parallel-sequential reaction mechanism. The parallel-sequential mechanism also predicts that, as $MzII_a$ is converted to thrombin, the rate of appearance of this intermediate should drop, which is as observed.

Figure 6 records, as a function of C6PS concentration, the second-order rate constants (k_{cat}/K_M) for three of the four proteolytic events leading to thrombin formation, with the values in panel C obtained by these regression procedures. Only upper limits to k_a could be estimated, since no Pre2 was detected. The k_b, k_c, and k_d rates increased in a saturable fashion up to the CMC and then again increased abruptly above the CMC, exactly as we have reported previously for the overall rate of thrombin production as stimulated by C6PS (*14*). The average apparent dissociation constant obtained from the hyperbola drawn through these few data points was 157 ± 75 μM, somewhat higher than but roughly consistent with the value reported for C6PS stimulation of overall factor X_a proteolytic activity [70 ± 10 μM (*14*)].

These rate constants demonstrate that C6PS alters the activity of factor X_a toward both bonds that must be cut in prothrombin to generate thrombin and does this in a substrate-dependent way. That is, reaction d (cutting Arg^{273}−Thr in $MzII_a$) was enhanced in rate by 44-fold, while there was a decrease in the rate of cutting this bond in prothrombin. Because no Pre2 was detected by SDS−PAGE, we could estimate only the upper limit of k_a (cutting Arg^{273}−Thr in prothrombin) based on the limits of resolution of the gels we used (<400 M^{-1} s^{-1}), considerably less than we observed for prothrombin activation by factor X_a in the absence of membranes or soluble lipids [1200 M^{-1} s^{-1} (*35*)]. On the other hand, the second-order rate constant for cutting Arg^{322}−Ile was increased roughly independent of substrate. Thus, reaction b (cutting Arg^{322}−Ile in Pre2−F1.2; 36000 M^{-1} s^{-1}) was enhanced by a factor of 400 relative to the rate in the absence of lipids [86 M^{-1} s^{-1} (*35*)], and reaction c (cutting the same bond in prothrombin to form $MzII_a$) was enhanced by approximately 200-fold. On the basis of these rate constants, we conclude that the differential effect of C6PS on the rates of $MzII_a$ or Pre2 formation is to switch the reaction from one that proceeds via either intermediate to one that proceeds exclusively via the $MzII_a$ intermediate. This is illustrated in the right-hand panel of Figure 5, wherein is plotted the concentration of thrombin formed via either the $MzII_a$ (small circles) or Pre2−F1.2 (dashed lines) intermediates.

Above the C6PS CMC, our data did not show the lag predicted by the parallel, sequential reaction mechanism. In addition, thrombin was formed at a much greater rate than seen for the appearance of $MzII_a$. These are characteristics that we have seen for prothrombin activation in the presence of PS-containing membranes under conditions for which we needed intermediate channeling to explain our data (*35*). Indeed, a good fit of the 1.5 mM C6PS data to the three-pathway model with channeling (*35*) was obtained (not shown). This description yielded a k_{cat}/K_M for $MzII_a$ release from the enzyme of $(2.24 \pm 0.04) \times 10^4$ M^{-1} s^{-1} and 0.3 as the fraction of prothrombin being converted directly to thrombin (*35*). However, we obtained a comparably good fit to these data with the parallel-sequential model, with this fit illustrated in Figure 5B and k_c given in Table 1. As must be the case for the parallel-sequential model, this showed the expected lag in thrombin formation but at reaction times

well below the shortest for which we have recorded data (Figure 5B). Since our experimental protocol was not designed to reveal such small differences at very short reaction times, we have no basis for distinguishing between the two descriptions of our data obtained above the C6PS CMC, so we have recorded the parameters obtained with the simpler parallel-sequential model.

DISCUSSION

The aim of this work was to answer three questions posed in the introduction. Our results offer the following answers or conclusions:

(1) Regulation of factor X_a-catalyzed prothrombin activation derives largely from the binding of PS molecules to regulatory sites on factor X_a. PS binding differentially alters the activity of factor X_a toward the two bonds that must be cut in prothrombin. The combination of a 200-fold increase in the rate of $MzII_a$ formation (reaction c) and a greater than 2.75-fold decrease in the rate of Pre2 formation means that C6PS dramatically increases the preference for the $MzII_a$ over the Pre2 pathway, effectively directing that activation occur exclusively via the $MzII_a$ intermediate.

(2) The time course of prothrombin activation by factor X_a in the presence of nonmicellar C6PS was well described by the parallel-sequential model, meaning that there is no evidence that C6PS causes channeling of intermediates to thrombin. Channeling in the absence of factor V_a seems to require a membrane surface.

(3) The presence of a membrane surface [200 μM membranes (*35*)] enhances the rate of prothrombin activation by about 2-fold over that reported here for soluble C6PS (300 μM).

Regulation of Factor X_a by PS Binding. Previous studies (*14*) unequivocally demonstrated that PS acts as a positive effector of prothrombin activation by upregulating the activity of the serine protease through the occupancy of specific PS regulatory sites on factor X_a. More recently, we have shown that factor X_a possesses two PS binding sites, one that requires a diacylglycerol moiety and one a glycerophosphoserine moiety (*1*). The activity of the enzyme toward its zymogen substrate, the structure around the enzyme's active site, the enzyme's overall secondary structure, and its state of aggregation in solution all are dependent on the specific occupancy of one or both of these two sites by phospholipids, with PS being the only lipid capable of eliciting a full change in proteolytic activity (*33*). The diacylglycerol-dependent site is located in the EGF domain pair and, when this is linked to the Gla domain by the presence of Ca^{2+} (*32*), specifically recognizes C6PS ($K_d \cong 70-100$ μM) to alter the factor X_a active site (*30*). The glycerophosphoserine-dependent site ($K_d \cong 150-2000$ μM) is not involved in regulation of activity but is actually an amine-sensitive site that is part of the substrate recognition site (*18*, *30*). These studies all make it clear that the effect of C6PS is not a nonspecific effect such as that reported for high concentrations (6 M) of certain alcohols (*31*).

Previous studies of the rates of $MzII_a$ and Pre2−F1.2 activation in the presence (*16*, *21*) or absence (*5*) of membranes have been interpreted to mean that factor V_a, the glycoprotein cofactor of factor X_a, directed activation via the $MzII_a$ intermediate. In support of this, $MzII_a$ activation by factor X_a bound to factor V_a in solution (in the absence of membranes) was estimated to occur at a rate within a factor of 10 of that seen for the full, membrane-bound prothrombinase (*5*). However, this result does not really address the relative extents to which factor V_a or a PS-membrane surface direct activation via the $MzII_a$ intermediate. To do that, it is essential to estimate the rates of Pre2 and $MzII_a$ formation, not just their consumption. Our results and those of the first paper in this series (*35*) demonstrate that prothrombin is a very different substrate for factor X_a, in solution, on a membrane, or bound to C6PS, than are Pre2−F1.2 or $MzII_a$. This is consistent with the demonstration that the overall shapes of prothrombin and $MzII_a$ on a PS-containing membrane are very different (*8*). The rate of prothrombin conversion to $MzII_a$ relative to the rate of conversion to Pre2 from our estimates of these rate constants (Table 1) was >22-fold. In the presence of PS-containing membranes, this ratio of rates was >55 (*35*), meaning that the preference of factor X_a for $Arg^{322}-Ile^{323}$ relative to $Arg^{273}-Thr^{274}$ is largely due to molecular PS with a 2−3-fold increase due to presence of a membrane surface. Recently, Boskovic et al. (*4*) have estimated this ratio as 1 for factor X_a in the presence of factor V_a without membranes.[2] In the absence of any cofactor, this ratio was only 0.04 (*35*), consistent with the fact that activation of prothrombin appears to occur through the Pre2 intermediate in the absence of PS membranes and factor V_a (*10*, *25*). This means that binding of PS to specific sites on factor X_a enhances the preference of the enzyme for the $MzII_a$ pathway for activation by >550-fold (>1400-fold if one includes the small effect of a surface; see above). By contrast, from the data of Boskovic et al. (*4*), factor V_a enhances the preference for the $MzII_a$ pathway by roughly 25-fold. Thus, the major factor directing activation via the $MzII_a$ pathway in vivo would appear to be appearance of PS on the surface of vesicles released from activated platelets (*3*, *13*, *27*). This clearly does not mean that factor V_a has no role in directing activation via the $MzII_a$ intermediate, but it leaves the exact role of factor V_a in prothrombin activation in more need of a clear definition than has been thought.

Direct Conversion of Prothrombin to Thrombin or Intermediate "Channeling" Requires a Membrane. In the first paper of this series, to explain the observed time courses of prothrombin activation at optimal membrane concentrations, we hypothesized that a fraction of prothrombin had to be activated directly to thrombin (*35*). We proposed that this would require a membrane surface to which intermediate could remain bound in juxtaposition to bound factor X_a. In support of this hypothesis, we find no evidence of intermediate channeling in the presence of soluble C6PS, even though the soluble lipid produced most of the bond-specific upregulation seen in the presence of membranes. Above the C6PS CMC, the data were fit equally well by the parallel-sequential model with the assumption of channeling as without. Since a micelle should provide a membrane-like surface, one might have expected to find the channeling hypothesis necessary to describe the data. However, protein-

[2] This estimate is only slightly clouded by the fact that factors V_a and X_a interact only weakly in solution, since roughly 10% of factor X_a was in complex with factor V_a in this study and the activity of the complex is roughly 4 orders of magnitude greater than the activity in the absence of any cofactor (*5*).

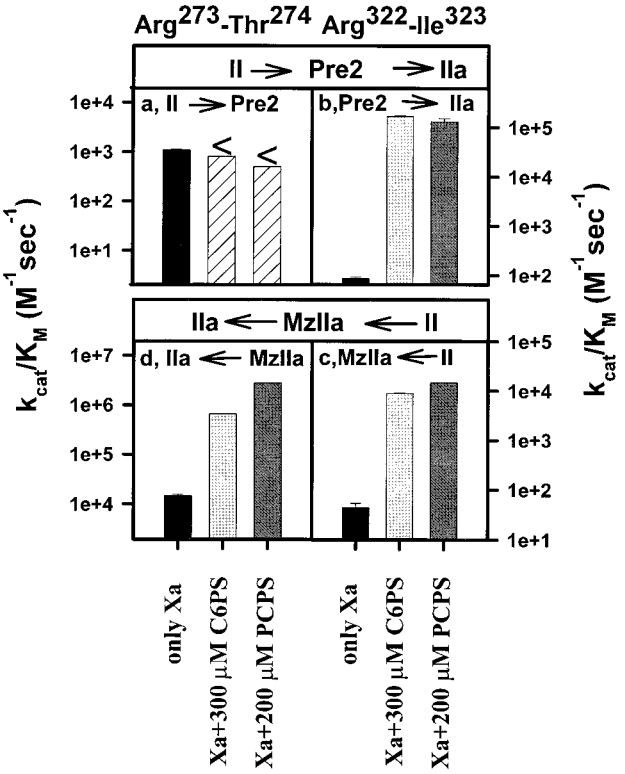

FIGURE 7: Pictorial summary of the rates of prothrombin bond cleavage under different conditions: X_a; X_a + 300 mM C6PS; X_a + 200 μM PS/PC membrane. The left panel represents the cut Arg^{273}−Thr^{274} (reactions a and d) while the right panel represents the cut Arg^{322}−Ile^{323} (reactions b and c). Activation via the Pre2 pathway proceeds from left to right in the top frames and via $MzII_a$ from right to left in the bottom frames. This figure represents k_{cat}/K_M in a logarithmic scale.

laden C6PS micelles just above the CMC are only about 10 nm in diameter and, as such, contain only about 150−200 C6PS molecules (*14*). At higher C6PS concentration, much larger aggregates form (*14*). Thus, at 1500 μM C6PS, the micelle concentration could be as high as 2 μM. Since our experiments contain 5 nM factor X_a, there could be at most a single factor X_a per micelle. The conditions that we have reported that lead to channeling had multiple factor X_a molecules bound per vesicle (*14*). It may be that the presence of two or more enzyme molecules on the same surface is a requirement for rapid association of released intermediate with a neighboring enzyme molecule. Alternatively, it may be that channeling did occur on C6PS micelles but could not be detected without much more accurate data at very short reaction times to distinguish between these two possible reaction models (parallel-sequential or three-pathway model with channeling). Since our goal was to define the influence of soluble rather than micellar C6PS, we have not pursued this further.

Effect of a Surface. The effect of PS-containing membranes on prothrombin activation has often been described in terms of a reduction in the reaction space from the full solution to the surface of the membrane (*11*, *21*, *22*, *25*). It is clear from comparison of the second-order rate constants collected in Figure 7 that, for Arg^{322}−Ile^{323} cleavage (reactions b and c; see Figure 7), the effect of gathering enzyme and substrate on a membrane surface was about the same as the effect of individual PS molecules binding to and activating factor X_a. For Pre2 formation (reaction a, Figure 7), C6PS actually seems to have inhibited the reaction relative to the rate in the absence of lipid or the presence of a PS-containing membrane (Figure 7). But proteolysis of the same Arg^{273}−Thr^{274} bond during $MzII_a$ activation (reaction d, Figure 7) was actually enhanced by about 4-fold more by a PS membrane than by individual PS molecules. Thus, a membrane surface has a small effect on the rate of prothrombin activation, mainly on the rate of $MzII_a$ activation. However, most of the effect of a PS-containing membrane seems to be that of individual PS molecules binding to regulatory sites on factor X_a, thereby directing the activation almost exclusively through the $MzII_a$ pathway. The dramatic effect of C_6PS and PS-containing membranes on the pathway of activation can be seen by comparing the $MzII_a$ pathway (lower panels of Figure 7) to the Pre2 pathway (upper panels).

REFERENCES

1. Banerjee, M., Drummond, D. C., Srivastava, A., Daleke, D., and Lentz, B. R. (2002) *Biochemistry* (submitted for publication).
2. Bevers, E. M., Comfurius, P., and Zwaal, R. F. (1983) *Biochim, Biophys, Acta 736*, 57−66.
3. Bevers, E. M., Roland, P. C., Tilly, H. J., and Zwaal, R. F. A. (1991) *Thromb. Haemostasis 65*, 688a.
4. Boskovic, D. S., Bajzar, L. S., and Nesheim, M. E. (2001) *J. Biol. Chem. 276*, 30.
5. Boskovic, D. S., Giles, A. R., and Nesheim, M. E. (1990) *J. Biol. Chem. 265*, 10497−10505.
6. Chap, H. J., Zwaal, R. F., and van Deenen, L. L. (1977) *Biochim. Biophys. Acta 467*, 146−164.
7. Chen, P. S., Jr., Toribara, T. Y., and Warner, H. (1956) *Anal. Chem. 28*, 1756−1758.
8. Chen, Q., and Lentz, B. R. (1997) *Biochemistry 36*, 4701−4711.
9. Comfurius, P., Smeets, E. F., Willems, G. M., Bevers, E. M., and Zwaal, R. F. (1994) *Biochemistry 33*, 10319−10324.
10. Esmon, C. T., and Jackson, C. M. (1974) *J. Biol. Chem. 249*, 7791−7797.
11. Giesen, P. L., Willems, G. M., and Hermens, W. T. (1991) *J. Biol. Chem. 266*, 1379−1382.
12. Husten, E. J., Esmon, C. T., and Johnson, A. E. (1987) *J. Biol. Chem. 262*, 12953−12961.
13. Jones, M. E., Lentz, B. R., Dombrose, F. A., and Sandberg, H. (1985) *Thromb. Res. 39*, 711−724.
14. Koppaka, V., Wang, J., Banerjee, M., and Lentz, B. R. (1996) *Biochemistry 35*, 7482−7491.
15. Krishnaswamy, S. (1990) *J. Biol. Chem. 265*, 3708−3718.
16. Krishnaswamy, S., Church, W. R., Nesheim, M. E., and Mann, K. G. (1987) *J. Biol. Chem. 262*, 3291−3299.
17. Laemmli, U. K. (1970) *Nature 227*, 680−685.
18. Monnaie, D., Arosio, D., Griffon, N., Rose, T., Rezaie, A. R., and Di Cera, E. (2000) *Biochemistry 39*, 5349−5354.
19. Nesheim, M. E., Eid, S., and Mann, K. G. (1981) *J. Biol. Chem. 256*, 9874−9882.
20. Nesheim, M. E., and Mann, K. G. (1983) *J. Biol. Chem. 258*, 5386−5391.
21. Nesheim, M. E., Taswell, J. B., and Mann, K. G. (1979) *J. Biol. Chem. 254*, 10952−10962.
22. Nesheim, M. E., Tracy, R. P., and Mann, K. G. (1984) *J. Biol. Chem. 259*, 1447−1453.
23. Petrovan, R. J., Goversriemslag, J. W. P., Nowak, G., Hemker, H. C., Tans, G., and Rosing, J. (1998) *Biochemistry 37*, 1185−1191.
24. Rosing, J., Speijer, H., and Zwaal, R. F. (1988) *Biochemistry 27*, 8−11.
25. Rosing, J., Tans, G., Govers-Riemslag, J., Zwaal, R., and Hemker, H. (1980) *J. Biol. Chem. 255*, 274−283.
26. Rosing, J., Zwaal, R. F., and Tans, G. (1986) *J. Biol. Chem. 261*, 4224−4228.

27. Sandberg, H., Bode, A. P., Dombrose, F. A., Hoechli, M., and Lentz, B. R. (1985) *Thromb. Res. 39*, 63−79.
28. Schick, P. K., Kurica, K. B., and Chacko, G. K. (1976) *J. Clin. Invest. 57*, 1221−1226.
29. Silverberg, S. A. (1979) *J. Biol. Chem. 254*, 88−94.
30. Srivastava, A., Wang, J.-F., Stenflo, J., Rezaie, A. R., Esmon, C. T., and Lentz, B. R. (2002) *J. Biol. Chem.* (in press).
31. Sturzebecher, J., Kopetzki, E., Bode, W., and Hopfner, K. P. (1997) *FEBS Lett. 412*, 295−300.
32. Valcarce, C., Holmgren, A., and Stenflo, J. (1994) *J. Biol. Chem. 269*, 26011−26016.
33. Wang, J., Majumder, R., and Lentz, B. R. (2002) *Biophys. J.* (submitted for publication).
34. Wu, J. R., and Lentz, B. R. (1991) *Biophys. J. 60*, 70−80.
35. Wu, J. R., Zhou, C., Majumder, R., Powers, D. D., Weinreb, G., and Lentz, B. R. (2002) *Biochemistry 41*, 935−949.

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