Prothrombin Activation by Platelet-associated Prothrombinase Proceeds through the Prethrombin-2 Pathway via a Concerted Mechanism

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*Running Title: Mechanism of platelet-associated prothrombinase

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Background: The key source of prothrombin activation in vivo is prothrombinase assembled on the activated platelet surface.

Results: Platelet-associated prothrombinase utilizes the prethrombin-2 pathway of prothrombin activation and a concerted enzyme mechanism.

Conclusion: Platelet-associated prothrombinase activates prothrombin with a concerted mechanism in which no anticoagulant intermediates are released.

Significance: Platelet-associated prothrombinase promotes coagulation by avoiding the release of catalytically-active meizothrombin.

SUMMARY

The serine protease α-thrombin is a key enzyme of the coagulation process as it is at the crossroads of both the pro- and anti-coagulant pathways. The main source of α-thrombin in vivo is the activation of prothrombin by the prothrombinase complex assembled on either an activated cell membrane or cell fragment—the most relevant of which is the activated platelet surface. When prothrombinase is assembled on synthetic phospholipid vesicles, prothrombin activation proceeds with an initial cleavage at Arg320 yielding the catalytically active, yet effectively anticoagulant intermediate meizothrombin, which is released from the enzyme complex approximately 30-40% of the time. Prothrombinase assembled on the surface of activated platelets has been shown to proceed through the inactive intermediate prethrombin-2 via an initial cleavage at Arg271 followed by cleavage at Arg320. The current study tests whether or not platelet-associated prothrombinase proceeds via a concerted mechanism through a study of prothrombinase assembly and function on collagen-adhered, thrombin-activated, washed human platelets in a flow chamber. Prothrombinase assembly was demonstrated through visualization of bound factor Xa by confocal microscopy using a fluorophore-labeled anti-factor Xa antibody, which demonstrated the presence of distinct platelet subpopulations capable of binding factor Xa. When prothrombin activation was monitored at a typical venous shear rate over preassembled platelet-associated prothrombinase neither potential intermediate, meizothrombin or prethrombin-2, was observed in the effluent. Collectively these findings suggest that platelet-associated prothrombinase activates prothrombin via an efficient concerted mechanism in which neither intermediate is released.

The serine protease α-thrombin (α-fIIa) is a key enzyme in the coagulation process, lying at the crossroads of the pro- and anti-coagulant pathways. The main source of α-fIIa is the activation of prothrombin (fII) by the prothrombinase complex, which consists of the serine protease factor (f) Xa and the protein cofactor fVα assembled on an appropriate membrane in the presence of Ca²⁺ (1). The prothrombinase complex activates fII 300,000-
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times more efficiently than does uncomplexed fXa in solution. The activation of fII to α-fIIa occurs via two cleavages at Arg271 and Arg320 (Figure 1). When the prothrombinase complex assembled on synthetic phospholipid vesicles activates fII, the initial cleavage is at Arg320, leading to the catalytically-active intermediate meizothrombin (mIIa) (Figure 1, pathway i). When fII is activated by fXa alone in solution or bound to a membrane (1), the initial cleavage of fII occurs at Arg271, leading to the zymogen intermediate prethrombin-2 (Pre-2) (Figure 1, pathway ii) (2). Both active thrombin species, α-fIIa and mIIa, can also carry out feedback cleavages at Arg155 and Arg284. Cleavage at Arg155 leads to the loss of fragment 1 (F1), resulting in the formation of prethrombin-1 (Pre-1) and mIIa (des F1) from fII and mIIa, respectively. Cleavage at Arg284 results in the formation of Pre-2 des 13-residues at the N-terminus (Pre-2') (3,4).

The primary biological surface for prothrombinase assembly is that of the activated platelet. A recent study by Wood et al. reported that when the prothrombinase complex is assembled on the activated platelet surface, the activation of fII proceeds via the Pre-2 pathway without the presence of the mIIa intermediate (5). Furthermore, this study showed preferential cleavage at Arg271 by platelet-associated prothrombinase, versus Arg320—the preferred initial cleavage site of prothrombinase assembled on synthetic phospholipid vesicles. An additional report by Fager et al. suggests that the structure of platelet-associated prothrombinase differs from that assembled on the surface of synthetic phospholipid vesicles (6), which may explain the differences in reaction pathways between the two systems. Some of these structural differences may arise from the presence of specific fVa and fXa receptors on the activated-platelet surface, unlike synthetic phospholipid vesicles (7-10). Furthermore, fXa cannot bind to the activated-platelet surface in the absence of the cofactor fVa (7,11), a pool of which, along with the procofactor fV, is released from the platelet’s α-granules upon activation (12,13).

Platelets are essential to both primary and secondary hemostasis, whereupon vascular injury exposes the subendothelium and a number of platelet recruiting agents—including fibronectin, collagen, and von Willebrand factor (VWF) (14). At low shear rates, such as those present in the venous system, platelets are recruited to collagen directly, through their glycoprotein (GP) VI and integrin α2β1 receptors (15-18). In addition to being vital for direct platelet adhesion to collagen, the GPVI receptor also serves as an important activator of platelets (19). Under high shear conditions, platelet adhesion to collagen is initiated by VWF-mediated interactions with the platelet’s αIIbβ3 integrin and the GPIIbα subunit of the GPIb-IX-V receptor leading to shear induced platelet activation (20-22).

We have recently demonstrated the production of the catalytically-active intermediate mIIa using a flow reactor when the prothrombinase complex was assembled on a supported phospholipid bilayer consisting of 75% dioleoyl phosphatidylcholine (PC) and 25% dioleoyl phosphotidylserine (PS) (23). Forty-percent of the thrombin species observed in the effluent consisted of mIIa, in agreement with previous work (24). This finding may be interpreted in one of two ways:

1. The activation of fII by the prothrombinase complex assembled on a synthetic phospholipid bilayer is accomplished by two successive cleavages by different prothrombinase complexes, where only 60% of the mIIa produced by the first cleavage is able to find a second prothrombinase complex where it is fully activated to α-fIIa before exiting the catalytically-active reactor.

2. fII is activated by a single prothrombinase complex, which only completes the second cleavage of fII to form α-fIIa 60% of the time, while 40% of the time the intermediate mIIa dissociates before being fully activated.

Although a previous study by Billy et al. investigated the activation of fII by prothrombinase assembled on activated platelets under flow, it did not take advantage of the system to address the question of the reaction pathway nor its mechanism (24). In the current study a flow chamber in which prothrombinase is assembled on the surface of activated platelets or a supported...
phospholipid bilayer is utilized to determine if the mechanism of prothrombin activation occurs via one of three catalytic processes:

1. A concerted reaction in which fII is efficiently activated to α-fIIa by a single prothrombinase complex through either the mIIa or Pre-2 pathway.

2. An inefficient reaction in which cleavage at Arg271 is carried out by one prothrombinase complex leading to the release of the non-catalytically active Pre-2, followed by its complete activation by a second prothrombinase complex.

3. An inefficient reaction that proceeds through the mIIa pathway—analogous to the pathway observed on synthetic phospholipid vesicles.

Thus, we propose that the use of a flow reactor will help to determine the mechanism by which fII is activated by prothrombinase assembled on the surface of activated platelets.

**EXPERIMENTAL PROCEDURES**

**Materials**—Phe-Pro-Arg-chloromethyl ketone (FPRck) and N-(3-ethyl-1,5-pentanediyl)amide (DAPA) were obtained from Haematologic Technologies (Essex Junction, VT). Arg-Gly-Asp-Ser peptide (RGDS) was synthesized by the protein core facility at the University of Vermont (Burlington, VT) or purchased from Bachem (Torrance, CA). Synthetic phospholipid vesicles (PCPS) containing 75% dioleoyl phosphotidylcholine and 25% dioleoyl phosphotidylserine from Avanti Polar Lipids (Alabaster, AL) were prepared as previously described (25). SpectrozymeTH and hirudin were purchased from American Diagnostica. Bovine serum albumin was purchased from AppliChem (Germany). The polyclonal burro-α-prethrombin-1 and monoclonal α-fX-27-5 antibodies were obtained from the University of Vermont Antibody Core (Colchester, VT). α-fXa-27-5 was conjugated to Alexafluor 488 using a commercially available kit from Invitrogen (Carlsbad, CA). CD62 (P-selection) antibody conjugated to phycoerythrin (PE) was purchased from Becton Dickinson (Franklin Lakes, NJ). Collagen (Type I) was purchased from ChronoLog (Havertown, PA). The fluorogenic substrate SN-7 was synthesized by and received as a gift from Dr. Saulius Butenas (26,27).

**Preparation of proteins**—Human fII, fV and fX were purified from plasma as described previously (28,29) or received as a gift from Haematologic Technologies. Human antithrombin (AT) was prepared in house as previously described (30). fII was activated to α-fIIa using a modification of the procedure of Lundblad et al. (31). fV was activated to fVa by incubating fV (1 µM) with α-fIIa (10 nM) for 20 min at 37 °C at which time the reaction was stopped with the addition of hirudin (12 nM). The concentration of hFVa was verified by its activity in hFV-deficient plasma with a prothrombin time clotting assay using TriniClot PT Excel S reagent (Trinity Biotech). Pre-1 and Pre-2/Pre-2' standards were prepared from a digestion of fII with α-fIIa (32) and purified using a BioCad 700E (Applied Biosystems, Foster City, CA) equipped with a HS20A column. The identity of the Pre-2/Pre-2’ standard was confirmed by N-terminal sequencing performed by the Protein Chemistry Core at the University of Texas Medical Branch (Galveston, TX). fII was radiolabeled with 125I as previously described (5,33) and has been previously shown to activated by prothrombinase in the same manner as unlabeled fII (5).

**Isolation and activation of washed human platelets**—All experiments were conducted at room temperature (~23 °C; RT) unless otherwise noted. Six parts blood was drawn from healthy, consenting adults into one part acid citrate dextrose (22 mM trisodium citrate, 14 mM dextrose, pH 4.5). Platelets were washed using a modification of the procedure of Mustard, et al. (34,35), followed by suspension in HEPES Tyrode’s Buffer (5 mM HEPES, 137 mM NaCl, 2.68 mM KCl, 11.9 mM NaHCO3, 0.42 mM Na2HPO4, 1 mM MgCl2, 5 mM CaCl2, 0.2% dextrose, pH 7.4; HT). Washed platelets were counted using a pocH-100i from Sysmex (Kobe, Japan) or a Coulter Z1 particle counter (Beckman Coulter). Washed platelets (3×10^8 mL^-1) were activated with α-fIIa (50 nM) in the presence of 3 mM RGDS for 5 min in HT. The reaction was stopped by the addition of hirudin (75 nM).

**Closed system prothrombinase time courses**—The prothrombinase complex was assembled by
incubating fVa (20 nM) and fXa (0.2 nM) with either PCPS vesicles (20 µM) or washed human platelets (1×10^8 mL^-1) for 5 min in HEPES-buffered saline (20 mM HEPES, 150 mM NaCl, pH 7.4; HBS) containing 0.1% polyethylene glycol 8000 (PEG) and 2 mM Ca^{2+} or HT respectively. The prothrombinase reaction was initiated with the addition of fII (1.4 µM). At 30 sec, 60 sec, 90 sec, 2 min, 5 min, 10 min, and 20 min time points, the reaction mixtures were quenched with FPRck (6 µM) and prepared for subsequent analysis by Western blotting.

**Western blotting**—Samples for Western blotting were prepared in sample preparation buffer (62 mM Tris, 2% SDS, 0.08% bromophenol blue, 10% glycerol; SPB), reduced with 5-10% β-mercaptoethanol (βME), analyzed by SDS-PAGE using 10% gels from Lonza (Basel, Switzerland), and transferred to nitrocellulose for Western blotting analysis. A polyclonal burro-α-human prethrombin-1 primary antibody was used to detect prothrombin fragments. Blots were developed using a LAS4000 from Fuji (Japan).

**Activation of prothrombin by prothrombinase assembled on activated platelets under flow**—Plasma cleaned capillaries were coated overnight with 0.5 mg/mL collagen diluted in 5% dextrose (pH 2.7). The capillaries were rinsed with HT for 5 min at a shear rate of 100 sec^-1, before a platelet-associated prothrombinase solution (1 mL) containing thrombin-activated platelets (1×10^3 mL^-1), fVa (20 nM), and fXa (0.2 nM) was incubated at RT for 5 min and subsequently flowed over the capillary at a shear rate of 100 sec^-1. A solution of fII (1.4 µM) and DAPA (500 nM) in HT was flowed over the capillary at 100 sec^-1 and the effluent was collected dropwise (~23 µL) into HBS containing 0.1% PEG and 20 mM EDTA to stop further Ca^{2+}-dependent reactions prior to determining the concentration of active thrombin species with a chromogenic assay. For fluorogenic assays to determine the presence of mIIa, the effluent (2 mL) was collected without further treatment.

**Activation of prothrombin by prothrombinase assembled on a supported PCPS bilayer under flow**—Plasma-cleaned capillaries were incubated for 1 hr with PCPS (100 µM) in HBS containing 2 mM Ca^{2+} at 4 °C to assemble a phospholipid bilayer as previously described (23,36-38). Following incubation, the capillaries were rinsed three times with HBS containing 2 mM Ca^{2+} and stored overnight in the same buffer at 4 °C. The capillaries were incubated for 15 min with fVa (20 nM) and fXa (0.2 nM) in HBS containing 0.1% PEG and 2 mM Ca^{2+} and then rinsed with HBS containing 0.1% PEG and 2 mM Ca^{2+} at 100 sec^-1 (~200-300 µL). A solution of fII (1.4 µM) and DAPA (500 nM) in HBS containing 0.1% PEG and 2 mM Ca^{2+} was flowed over the capillary at 100 sec^-1 as previously described (23) and the effluent was collected as described above.

**Assaying prothrombinase levels in PCPS coated capillaries**—Following each fII activation time course, PCPS coated capillaries were stripped in HBS containing 0.1% PEG and 20 mM EDTA (200 µM) for 15 min at RT (200 µM). The fXa levels were then assayed using a fluorogenic assay similar to that described previously (23): The capillary extract was diluted 1:4 in HBS containing 0.1% PEG in 20 mM EDTA and 50 nM hirudin to a total volume of 200 µL and incubated at 37 °C for 5 min at which point the SN-7 (50 µM) substrate was added and its hydrolysis was monitored using a Fluoromax-2 fluorometer from Jobin-Yvon-Spex (Edison, NJ) for 5 min at 37 °C (λexcitation, 350 nm; λemission, 470 nm; λfilter, 450 nm). fXa levels were determined by comparison to a standard curve.

**Determining the Concentration of active thrombin species**—Thrombin activity was assessed using the chromogenic substrate SpectrozymeTH (200 µM). The concentration of thrombin in the effluent was determined by comparison to a standard curve. The generation of active thrombin species as a function of time was fitted to Eq. 1 using the Graphpad Prism (v 5.02) software package as described previously (23) in which [P] is the concentration of product at any given time (T) or under final steady-state (ss) conditions, T_0 is the turning point of the function, and τ characterizes the rate of change upon approach to the final plateau (raw data not shown).

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P(T) = [P]_{ss} + \frac{[P]_{ss} - [P]_0}{(1+T/T_0)}
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**Differentiation of active thrombin species**—A differential fluorogenic assay distinguishing between α-fIIa and mIIa, based upon the work of
Cote et al. (39), was performed as described previously (23). Briefly, effluent containing DAPA (500 nM) was incubated with AT (1 µM) for 100 sec prior to heparin (6 U/mL) addition, and the DAPA fluorescence signal was monitored for an additional 300 sec using a Fluoromax-2 fluorimeter (λ<sub>excitation</sub>, 280 nm; λ<sub>emission</sub>, 545 nm; λ<sub>filter</sub>, 520 nm).

**Isolation of reaction intermediates**—Prothrombinase was assembled on PCPS and collagen-adhered, platelet-coated capillaries as described above, with the exception that 1 nM fXa was limiting to the assembly of prothrombinase. Effluent was collected and assayed as described above until a steady-state level of active thrombin species generation was obtained. The effluent was further collected (5 mL) into hirudin present at an approximate 10 nM excess over active thrombin species. After collection, the effluent was acetone precipitated, solubilized in SPB, and reduced with 5-10% βME for analysis by SDS-PAGE on a 5-15% gradient gel followed by Coomassie blue staining. Cleavage products were identified by mobility and comparison to fII fragment standards.

**Confocal and differential interference contrast microscopy**—After reactants were washed from the capillaries for 5 min at 100 sec<sup>−1</sup> with HT, platelets were subjected to fixation by rinsing with 2% paraformaldehyde (PFA) for 5 min at 100 sec<sup>−1</sup> and incubated for 20 min undisturbed. PFA was removed from the capillary by rinsing for 5 min at 100 sec<sup>−1</sup> with dd-H<sub>2</sub>O, and capillaries were stored overnight in dd-H<sub>2</sub>O at 4 °C. Platelets were immunostained with CD62 conjugated to PE and α-fX-27-5 conjugated to AlexFluor 488 for 30 min. Platelet coated capillaries were washed three times via capillary action with 2.7 mM KCl and 137 mM NaCl at pH 7.4, mounted to a microscope slide and visualized using a Zeiss 510 META confocal laser scanning microscope (Zeiss MicroImaging, Thronwood, NY) with immersion oil. All images were acquired as 12-bit multitrack mode using appropriate laser excitation and emission filters as previously described (40) and analyzed using MetaMorph Imaging Series 7.1 (Molecular Devices, Sunnyvale, CA).

**Isolation of non-platelet-associated prothrombin species under non-flow conditions**—Thrombin-activated platelets (1×10<sup>5</sup>) were incubated with plasma-derived fVa (5 nM) and fII (1.4 µM) with trace <sup>125</sup>I-fII (1000 cpm/µL) were incubated in HT for 5 min at RT. The reaction was initiated with fXa (5 nM) to saturate all potential platelet-associated prothrombinase binding sites. At 1, 5, and 10 min intervals the reaction mixture was removed and the reaction quenched by spinning through oil (Apiezen:N-butyl phthalate, 1:9 v/v) to separate platelet- and non-platelet-associated species at 10,000×g for 1 min (41,42). The non-platelet-associated supernatant was removed and analyzed by SDS-PAGE followed by phosphorimaging (5).

**Statistical analysis**—Statistical analyses (t-tests) were performed using the Graphpad Prism software package version 5.0 (San Diego, CA).

**RESULTS**

**Activation of prothrombin under non-flow conditions**—Reduced Western blot time courses of fII activation by prothrombinase assembled on PCPS-vesicles (Figure 2A) and activated human platelets (Figure 2B) are in agreement with the work of Wood et al. (5) and suggest different activation mechanisms for the two systems. When fII is activated by prothrombinase assembled on PCPS-vesicles, the initial product observed is fragment (F) 1.2 indicative of mIIa formation followed by the appearance of the B-chain. As with the activation of fII by prothrombinase assembled on PCPS-vesicles, we observe the appearance of Pre-1 after ~5 min indicative of cleavage at Arg155, leading to Pre-1 and mIIa des F1 as indicated by the appearance of the F2.A fragment. When fII is activated by prothrombinase assembled on activated platelets, the first fragment to appear is F1.2, indicative of cleavage at Arg271 followed by the near simultaneous appearance of Pre-2 and the B-chain. As with the activation of fII by prothrombinase assembled on PCPS-vesicles we observe the appearance of Pre-1 after ~5 min indicative of cleavage at Arg271. Under venous flow conditions (100 sec<sup>−1</sup>), in situ thrombin-activated platelets adhered to collagen fibrils (Figure 3A). Immunostaining with CD62 indicates that all adhered platelets are activated and express P-selectin on their membrane surface (Figures 3B

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and 3D, red staining). Procoagulant platelet subpopulation formation was also assessed using a fluorophore-labeled α-fX antibody (Figures 3C and 3D, green staining) that does not disrupt prothrombinase complex assembly as previously demonstrated in closed-system experiments (43). Overlay of the fluorescent images (Figure 3D) demonstrates that a majority of these platelets are binding fXa. As platelets do not bind fXa in the absence of fVa, we may assume that immunostaining for fXa is indicative of prothrombinase complex assembly and that there is not uncomplexed fXa associated with the activated platelet membrane (8,11).

Steady-state levels of active thrombin species generated under flow conditions—The average steady-state levels of active thrombin species generated at a shear rate of 100 sec⁻¹ were determined under two conditions of limiting fXa (0.2 and 1 nM): The steady-state levels of active thrombin species were 26 ± 4 nM (n=2) and 22 ± 12 nM (n=2) with 0.2 limiting fXa on PCPS and activated platelets respectively and were not significantly different. With 1 nM limiting fXa the steady-state levels of active thrombin species were significantly different (p<0.03)—103 ± 19 nM (n=3) on PCPS and 54 ± 24 nM (n=4) on platelets. However the prothrombinase densities measured from PCPS coated capillaries with 0.2 and 1 nM limiting fXa were not significantly different (1.8 ± 1.1 fmoles cm⁻² (n=3) and 2.3 ± 1.0 fmoles cm⁻² (n=2) respectively). We attribute this to uncomplexed fXa being washed out of the capillary in the 1 nM limiting fXa experiments over the ~1h long time course.

Distinguishing between active thrombin species—To identify the intermediates released under flow conditions during fII activation by prothrombinase assembled on a PCPS-bilayer and activated platelets, the method of Cote et al. (39) that takes advantage of the different susceptibilities of α-fIIa and mIIa to inhibition by AT-heparin was employed, wherein α-fIIa activity is rapidly inhibited by the AT-heparin complex while that of mIIa is not. As illustrated in Figure 4, 40% of the active thrombin species formed by prothrombinase assembled on PCPS is mIIa, while all of the active thrombin produced by prothrombinase assembled on activated platelets is α-fIIa.

Identification of reaction intermediates in the effluent—While evidence suggests that the activation of fII by PCPS and platelet-associated prothrombinase proceeds through two different pathways, the mechanism of the platelet-associated prothrombinase reaction remains unknown. To help identify the mechanism of the reaction, we employed a flow reactor as described previously. The collected effluent was acetone precipitated and analyzed by SDS-PAGE as shown in Figure 5. The large amounts of apparent unreacted fII is attributed to the bulk of prothrombin in the flowing solution not coming into contact with the catalytically active walls, consistent with previous results (23). Both PCPS and platelet-associated prothrombinase generated significant amounts of Pre-1 and Pre-2 resulting from thrombin feedback cleavages at Arg155 and Arg284. F1.2 is also apparent in both reaction systems; the doublet in the platelet-associated prothrombinase system is attributed to the formation of the F1.2-like fragment resulting from cleavage at Arg284.

PCPS prothrombinase generated both mIIa and Pre-2. As shown in Figure 2 and other closed system studies (2,5), the pathway of activation in this system is through the mIIa pathway. The formation of Pre-2 is most likely attributed to membrane-localized fXa, which is not associated with fVa—conditions in which the Pre-2 pathway is favored. However, no Pre-2 or mIIa is observed in the platelet-associated prothrombinase system, suggesting that the mechanism is more efficient than that of PCPS associated prothrombinase. The most likely explanation of this finding is a concerted reaction that is performed by a single prothrombinase complex in which no intermediate is released during the activation process (7,11).

Identification of fluid phase-associated activation species in a closed platelet system—The absence of the observation of a Pre-2 or meizothrombin intermediate in the platelet flow system is in contrast to the observation of prethrombin-2 in the closed platelet system (Figure 2). In the latter system, the total system was analyzed while in the former only the fluid phase is analyzed as platelet-associated species are not removed from the capillary via flow. To further investigate the association of intermediated species with the activated platelet surface, we also remove platelet-
membrane associated species via sedimentation through oil in a closed system (41,42). The resulting solution fraction of prothrombin activation on platelets in a closed system is depicted in the time course experiment shown in Figure 6. In this instance fII doped with trace $^{125}$I-fII was used and was analyzed by autoradiography. $^{125}$I-fII species in the supernatant after oil-separation of platelet-associated species consists primarily of F1.2, F1, and the B-chain as illustrated in Figure 6. Over the time course, the F1.2 concentration decrease is consistent with cleavage at Arg155 by active thrombin species, while concentrations of F1 and the B-chain increase consistent with current models of the enzymatic process. No Pre-2 or mIIa is apparent in the aqueous supernatant although in analogous experiments by Wood et al. and Figure 2 of the present study significant amounts of Pre-2 were observed at these time points when the entire reaction mixture is analyzed (5). Therefore, it can be concluded that the Pre-2 observed in closed, non-flow platelet-associated prothrombinase experiments can be attributed to that which is still associated with platelet-associated prothrombinase during sampling and revealed during denaturation in SDS prior to analysis. This also explains why the Pre-2 intermediate appears later in the activation time courses, as it is harder to trap than F1.2 which is released during the catalytic process (Figure 2).

**DISCUSSION**

The prothrombinase mechanism is an area of considerable interest within the coagulation community, despite over thirty-five years of active research. Recent work demonstrating that fII is activated via the Pre-2 pathway on platelets with a clear preference for initial cleavage at Arg271 suggests that during hemostasis the activated platelet surface is optimally suited for promoting a procoagulant response (5). Given this difference in the apparent activation pathways, we hypothesize that the mechanism of prothrombin activation most likely differs from that observed when prothrombinase is assembled in solution or on phospholipid vesicles.

Work by Nesheim and coworkers suggests that the catalytically active intermediate mIIa is released from non-membrane associated prothrombinase ~30% of the time, while ~60% of the time prothrombin processing is “channeled” through the mIIa intermediate via a concerted enzyme mechanism and is released as fully activated α-fIIa (44). Additional work from the same group also suggests that there are two prothrombinase configurations—one which channels fII through the mIIa pathway and another that channels it through the Pre-2 pathway—that interconvert after each successive catalytic event (45,46).

Other studies by Krishnaswamy and coworkers suggest that fII is activated by prothrombinase assembled on synthetic phospholipid membranes via a “ratcheting” mechanism in which after the initial cleavage at Arg320, mIIa undergoes a conformational rearrangement to a “proteinase-like” state which primes the substrate for cleavage at Arg271 and full conversion to α-fIIa (47). Lee et al. have shown experimental support for the ratcheting model of Krishnaswamy and coworkers with their ternary model of prothrombinase in complex with fII (48-50), although their conclusions have been questioned by Nesheim and coworkers (51).

The models of both the Nesheim and Krishnaswamy groups show that fII activation is performed, at least in part, through a concerted mechanism by prothrombinase assembled in solution or on synthetic phospholipid vesicles (45-47). However, studies have also indicated that significant amounts of mIIa (~30-40%), as well as some Pre-2, are released during the catalytic process (23,44,52). These findings indicate that fII activation by prothrombinase assembled under these conditions is relatively inefficient at channeling fII through its activation process, despite the increase in catalytic efficiency from that of uncomplexed fXa. As neither the inactive intermediate Pre-2 nor any active mIIa is observed in the effluent collected from fII activation by platelet-associated prothrombinase in the current study, we conclude that platelet-associated prothrombinase in the current study, we conclude that platelet-associated prothrombinase activates fII in a concerted manner in which the intermediate Pre-2 is formed but not released from platelet-associated prothrombinase prior to the final cleavage at Arg320. In conjunction with previous work (5,6), we believe that this observation may also support the conclusion that the platelet-associated
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The current studies of the activation mechanism of fII by platelet-associated prothrombinase are in agreement with those of Wood et al. and show Pre-2 generation in the absence of mIIa formation; titration studies by Wood et al. also support the conclusion that platelet-associated prothrombinase preferentially cleaves at Arg271 (5). Therefore, although the flow system presented within the current study does not permit the isolation of prothrombinase-associated intermediates, we speculate the Pre-2 pathway is utilized by platelet-associated prothrombinase. The potential utilization of the Pre-2 pathway by platelet-associated prothrombinase is significant as mIIa is considered to be a more anticoagulant enzyme than α-fIIa, with approximately the same reactivity towards protein C when complexed with thrombomodulin (93%) but only limited activity towards fibrinogen (7%) and platelets (2%) (39). We hypothesize that during hemostasis, promotion of the Pre-2 pathway by platelet-associated prothrombinase leads to a rapid procoagulant response and maintenance of hemostasis; however, we speculate that in the event of atherosclerotic plaque rupture, the lipids and cell-fragments that are released more closely resemble synthetic phospholipid vesicles, therefore promoting the mIIa pathway of fII activation (53). The generation of a mIIa population that is released from the prothrombinase complex during activation may help to ameliorate thrombus formation. Our current data demonstrating a concerted mechanism of fII activation by platelet-associated prothrombinase, further support these hypotheses—by only releasing fully activated α-fIIa, platelet-associated prothrombinase can efficiently induce a procoagulant response by avoiding the production of the enzymatically active and anticoagulant intermediate, mIIa.

Selective binding to collagen under flow also increases the procoagulant response of platelets. As previously demonstrated, platelets have distinct procoagulant subpopulations that support platelet-associated prothrombinase (43,54-58). Imaging work in our current study suggests that the subpopulation of procoagulant platelets also efficiently binds to collagen via their GPVI receptors. As illustrated in Figure 3, nearly all of the collagen adherent platelets bind fXa and consequently support prothrombinase assembly; however, studies of non-collagen-adhered platelets suggest that only ~30-50% of platelets are capable of binding fXa (43). Although GPVI expression by a procoagulant platelet subpopulation capable of supporting functional prothrombinase has not been quantified the expression of other adhesive receptors (integrin β3, integrin αIIbβ3, P-selectin, and GPIbα) is increased four- to six-fold within this subpopulation (43).

In conclusion, our model demonstrates that at a shear rate typical of the venous system (100 sec⁻¹) platelets adhered to collagen under flow elicit a procoagulant response that is not discernable in closed system experiments that utilize either washed platelets or synthetic phospholipid vesicles. These differences present themselves on two fronts:

1. The adhered platelet population is enriched with procoagulant platelets that support assembly of the prothrombinase complex compared to their abundance in the circulating plasma.
2. Prothrombinase associated with this adhered platelet population utilizes a concerted mechanism that only releases the procoagulant enzyme α-fIIa and not the more anticoagulant enzyme mIIa.
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Mechanism of platelet-associated prothrombinase

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The abbreviations used are: f, factor; fII, prothrombin; α-fIIa, α-thrombin; mIIa, meizothrombin; Pre-2, prethrombin-2; Pre-2', prethrombin-2 des 13 residues; Pre-1, prethrombin-1; F1, fragment 1; GP, glycoprotein; FPRck, Phe-Pro-Arg-chloromethyl ketone; DAPA, N-(3-ethyl-1,5-pentanediyl)amide; RGDS, Arg-Gly-Asp-Ser peptide; PCPS, synthetic phospholipid vesicles containing 75% dioleoyl phosphatidylycholine and 25% dioleoyl phosphatidylserine; HT, HEPES Tyrode’s Buffer; HBS, HEPES buffered saline; SPB, sample preparation buffer.

**FIGURE LEGENDS**

**FIGURE 1.** Prothrombin activation by prothrombinase may proceed through either one of two pathways dependent upon the conditions of enzyme assembly. Prothrombin consists four fragments (fragment 1, fragment 2, A-chain, and B-chain). Initial cleavage at Arg320 (pathway i) is characteristic of prothrombinase assembled on synthetic phospholipid vesicles and results in the formation of the catalytically active intermediate meizothrombin (mIIa). Initial cleavage at Arg271 (pathway ii) is characteristic of fII activation by fXa in the absence of fVa or prothrombinase assembled on activated platelets, and results in the formation of the non-catalytically active intermediate prethrombin-2 (Pre-2). The secondary cleavage at Arg271 or Arg320 respectively results in the formation of fully activated α-fIIa (pathways iii and iv, respectively).

**FIGURE 2.** Prothrombin is activated via two different pathways when prothrombinase is assembled on PCPS vesicles or activated platelets under non-flow conditions. Prothrombinase (20 nM plasma derived fVa and 0.2 nM fXa) was assembled on PCPS vesicles (20 μM, panel A) or thrombin activated washed human platelets (10^8 platelets/mL, panel B). The reaction was initiated with fII (1.4 μM) and aliquots for blotting were taken at predetermined time points as labeled at the top of each panel and quenched into FPRck before preparation for SDS-PAGE electrophoresis under reducing conditions using 10% acrylamide gels and analysis using quantitative Western blotting with a burro-α-Prethrombin-1 polyclonal antibody.

**FIGURE 3.** Confocal and DIC microscopy of collagen adhered platelets. Collagen adhered, thrombin activated platelets on which prothrombinase was assembled were fixed with 2% PFA and imaged as described in the Materials and Methods section. Adhered platelets align along collagen fibrils as shown in the DIC image in Panel A and are positive for CD62 (red staining) as shown in Panel B indicating that
the adhered platelets are activated and express P-selectin on their membrane surface. A significant number of the adhered platelets are also positive for fXa binding (green staining) indicating the presence of prothrombinase assemble as shown in Panel C and the overlay of fluorescent images in Panel D. All bars indicate 10 µm.

**FIGURE 4.** Differential analysis of active thrombin species in effluent by reactivities towards AT and heparin. Reacted effluent (2 mL) containing 500 nM DAPA and generated in the presence of 0.2 nM limiting fXa on PCPS (Panel A) and platelets (Panel B) under flow was incubated with AT (1 µM) for 100 sec at 25 °C before heparin (6 U/mL) was added (arrow) and the reaction was monitored an additional 300 sec (excitation wavelength = 280 nm, emission wavelength = 545 nm, broad pass filter = 450 nm).

**FIGURE 5.** Identification of prothrombin intermediates generated under flow conditions by platelet and PCPS prothrombinase. Effluents from platelet and PCPS prothrombinase (1 nM limiting fXa) experiments (5 mL) were acetone precipitated, analyzed by 5-15% SDS-PAGE under reducing conditions (5-10% βME), and stained with Coomassie blue. Lane 1 shows molecular weight markers (kDa) as labeled on the left-hand side of the figure. Lanes 2-7 show standards for prothrombin (fII), prethrombin-1 (P1, with Pre-2’), meizothrombin (mIIa), α-thrombin (αIIa), fragment 1.2 (F1.2, with fragment 1), and prethrombin-2 (P2, with Pre-2’ and the B-chain of thrombin). Lanes 8 and 9 show the platelet and PCPS prothrombinase effluents respectively. Prothrombin fragments are identified on the right-hand side of the figure. (F1.2.A, meizothrombin fragment F1.2.A; P2’, Arg284 cleavage product; B, B-chain; F2.A, meizothrombin fragment F2.A; F2, fragment 2).

**FIGURE 6.** Non-platelet associated prothrombin species in a closed system analyzed by autoradiography. Non-platelet associated species were removed by oil centrifugation and analyzed by 10% SDS-PAGE under reducing conditions (5-10% βME) followed by phosphorimaging of 125I-fII species. Apparent molecular weights are labeled on the left-axis, while fII species are identified on the right. No Pre-2 is observed at 1, 5, or 10 min indicating the Pre-2 intermediate remains platelet-associated during fII’s activation process.
Mechanism of platelet-associated prothrombinase

Figure 1

[Diagram showing the steps of prothrombin activation and the formation of different thrombin fragments.]

1. Prothrombin activation to Meizothrombin
2. Further activation to Prethrombin-2
3. Fragmentation to Fragment 1.2
4. Formation of α-Thrombin

[Steps i, ii, iii, iv indicated on the diagram.]
Figure 2
Figure 3
Mechanism of platelet-associated prothrombinase

Figure 4.
**Mechanism of platelet-associated prothrombinase**

Figure 5.

| MW | flI | P1/P2' | mIIa | αIIa | F1/2 | P2/P2' | Platelet Effluent (1 nM fXa) | PCPS Effluent (1 nM fXa) |
|----|-----|--------|------|------|------|--------|-----------------------------|---------------------------|
|    |     |        |      |      |      |        |                             |                           |
| 100|     |        |      |      |      |        |                             |                           |
| 75 |     |        |      |      |      |        |                             |                           |
| 50 |     |        |      |      |      |        |                             |                           |
| 35 |     |        |      |      |      |        |                             |                           |
| 25 |     |        |      |      |      |        |                             |                           |
Figure 6.
Prothrombin activation by platelet-associated prothrombinase proceeds through the prethrombin-2 pathway via a concerted mechanism
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