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A Control Switch for Prothrombinase: Characterization of a Hirudin-Like Pentapeptide From the COOH Terminus of Factor VA Heavy Chain That Regulates the Rate and Pathway for Prothrombin Activation

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A Control Switch for Prothrombinase

*CHARACTERIZATION OF A HIRUDIN-LIKE PENTAPEPTIDE FROM THE COOH TERMINUS OF FACTOR Va HEAVY CHAIN THAT REGULATES THE RATE AND PATHWAY FOR PROTHROMBIN ACTIVATION**

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Membrane-bound factor Xa alone catalyzes prothrombin activation following initial cleavage at Arg^{271} and prethrombin 2 formation (pre2 pathway). Factor Va directs prothrombin activation by factor Xa through the meizothrombin pathway, characterized by initial cleavage at Arg^{320} (meizo pathway). We have shown previously that a pentapeptide encompassing amino acid sequence 695–699 from the COOH terminus of the heavy chain of factor Va (Asp-Tyr-Asp-Tyr-Gln, DYDYQ) inhibits prothrombin activation by prothrombinase in a competitive manner with respect to substrate. To understand the mechanism of inhibition of thrombin formation by DYDYQ, we have studied prothrombin activation by gel electrophoresis. Titration of plasma-derived prothrombin activation by prothrombinase, with increasing concentrations of peptide, resulted in complete inhibition of the meizo pathway. However, thrombin formation still occurred through the pre2 pathway. These data demonstrate that the peptide preferentially inhibits initial cleavage of prothrombin by prothrombinase at Arg^{320}. These findings were corroborated by studying the activation of recombinant mutant prothrombin molecules rMZ-II (R155A/R284A/R271A) and rP2-II (R155A/R284A/R320A) which can be only cleaved at Arg^{320} and Arg^{271}, respectively. Cleavage of rMZ-II by prothrombinase was completely inhibited by low concentrations of DYDYQ, whereas high concentrations of pentapeptide were required to inhibit cleavage of rP2-II. The pentapeptide also interfered with prothrombin cleavage by membrane-bound factor Xa alone in the absence of factor Va increasing the rate for cleavage at Arg^{271} of plasma-derived prothrombin or rP2-II. Our data demonstrate that pentapeptide DYDYQ has opposing effects on membrane-bound factor Xa for prothrombin cleavage, depending on the incorporation of factor Va in prothrombinase.

Prothrombinase is the enzymatic complex responsible for timely thrombin formation in response to vascular injury (1, 2). Activation of human prothrombin is the consequence of two cleavages at Arg^{271} and Arg^{320} in prothrombin by factor Xa. Depending on the order of peptide bond cleavage, different intermediates are formed (Fig. 1). Cleavage first at Arg^{271} produces fragment 1·2 and prethrombin-2, whereas initial cleavage at Arg^{320} results in the formation of meizothrombin, which has enzymatic activity (3–14). Whereas factor Xa alone can activate prothrombin following sequential cleavages at Arg^{271} and Arg^{320} to produce thrombin (Fig. 1, *pathway I, pre2 pathway*), its catalytic efficiency is poor in the absence of factor Va, and the overall reaction is incompatible with survival and the rapid arrest of bleeding. The prothrombinase complex, which is formed following the interaction of factor Va with membrane-bound factor Xa in the presence of divalent metal ions, catalyzes the activation of prothrombin following the opposite pathway (Arg^{320} followed by Arg^{271}; see Fig. 1, *pathway II, meizo pathway*), resulting in a substantial increase in the catalytic efficiency of factor Xa required for normal hemostasis (15). Although both cleavages are phospholipid-dependent, only initial cleavage of prothrombin at Arg^{320} is strictly dependent on factor Va. The increase in the rate of the overall enzymatic reaction is attributed to an increase in the k_{cat}, which in turn is solely credited to the interaction of the cofactor molecule with both the membrane-bound enzyme and the membrane-bound substrate (11–18). Thus, the activity of prothrombinase is limited and controlled by the presence of the soluble, nonenzymatic cofactor factor Va.

Proteolytic processing of factor V by α-thrombin at Arg^{709}, Arg^{1018}, and Arg^{1545}, resulting in the production of the active cofactor factor Va, which consists of a heavy chain (M_r 105,000) and a light chain (M_r 74,000), is required for the interaction of the cofactor with the members of prothrombinase (19–21). Factor Va heavy chain has an acidic hirudin-like region at the COOH terminus that has been implicated in cofactor activity (22–24). This region contains tyrosine residues that have the potential to be sulfated and have been reported to be important for both factor V activation by α-thrombin and cofactor function (23, 24).

Prothrombin and α-thrombin have two discrete electropositive binding exosites (anion-binding exosite I (ABE-I) and anion-binding exosite II (ABE-II)) that are responsible for the functions of the molecules (25–35). Proexosite I of prothrom-

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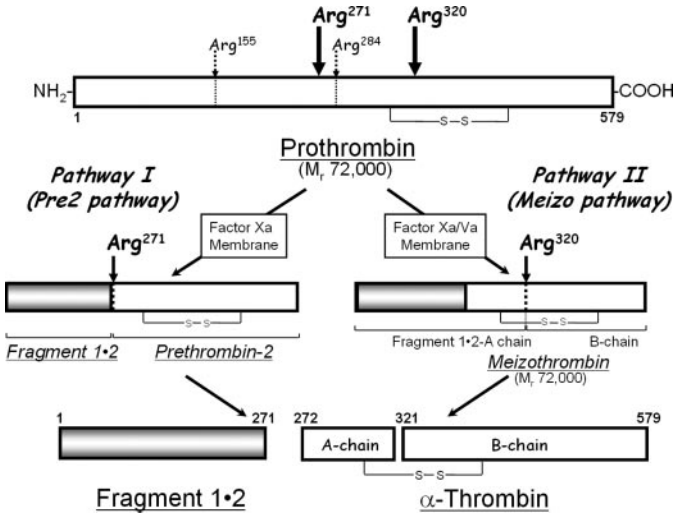

FIGURE 1. **Schematic of the two pathways for the activation of human prothrombin.** Two factor Xa-catalyzed cleavages convert prothrombin to thrombin. Initial cleavage at Arg^{271} by membrane-bound factor Xa in the absence of factor Va results in the generation of fragment 1·2 and prethrombin 2. Subsequent cleavage at Arg^{320} results in thrombin formation (*pathway I, pre2 pathway*). Initial cleavage at Arg^{320} by prothrombinase (factor Va bound to factor Xa on a membrane surface in the presence of Ca^{2+}) results in the production of an enzymatically active intermediate (meizothrombin). Cleavage of this intermediate at Arg^{271} produces thrombin and fragment 1·2 (*pathway II, meizo pathway*). Thrombin also cleaves prothrombin at Arg^{155} and at Arg^{284} (*dashed arrows*).

bin, which is present in a low affinity state on the molecule, is fully exposed following activation and formation of thrombin, and the affinity for its ligands increases by ~100-fold (36). The procofactor, factor V, interacts with immobilized α-thrombin but with a lower affinity than factor Va (25, 27). Finally, it has been repetitively shown by several laboratories that the role of ABE-I in prothrombinase is dependent on the incorporation of factor Va into the complex (28, 36–39).

We have recently identified a pentapeptide from the COOH terminus of the heavy chain of the cofactor (spanning amino acid residues 695–699, DYDYQ) as potent inhibitor of prothrombinase function (40). The peptide was found to be a competitive inhibitor of prothrombinase with respect to substrate. According to the mode of inhibition, we postulated that the peptide binds prothrombin in competition with the binding of the substrate to the enzyme and inhibits prothrombinase activity by substrate depletion. This mode of DYDYQ inhibition of prothrombin activation by the factor Va-factor Xa complex is similar to that demonstrated previously for sulfated hirugen (28). This uncommon mode of inhibition (41, 42) is an accepted mechanism of regulation of phospholipase A_2 by annexin 1 (lipocortin 1) (43, 44), as well as for the regulation of other important physiological processes (44–46). The present work was undertaken to elucidate the molecular mechanism underlying the inhibition of prothrombin activation by DYDYQ.

EXPERIMENTAL PROCEDURES

Materials, Reagents, and Proteins—L-α-Phosphatidylserine (PS)[2] and L-α-phosphatidylcholine (PC) were from Avanti Polar Lipids (Alabaster, AL). The chromogenic substrate Spectrozyme-TH was from American Diagnostica, Inc. (Greenwich, CT). Human α-thrombin and human prothrombin were from Hematologic Technologies, Inc. (Essex Junction, VT). The monoclonal antibody αhFV1 coupled to Sepharose was provided by Dr. Kenneth G. Mann (Department of Biochemistry, University of Vermont, Burlington, VT). Human factor Xa was from Enzyme Research Laboratories (South Bend, IN).

The pentapeptide DYDYQ that was previously shown to inhibit prothrombinase activity and delay factor V activation (40) was custom-synthesized under the form H_2N-DYDYQ-amide and purchased from New England Peptide, Inc. (Gardner, MA) and from American Peptide Co. (Sunnyvale, CA). In each case, the peptide was purified by HPLC to more than 96% homogeneity. Its molecular weight and composition were usually verified by mass spectrometry and amino acid composition analysis, respectively. DYDYQ is a highly negatively charged peptide, and all mass spectrometry analysis was conducted in a negative mode. The peptide was stored lyophilized at −20 °C in a dessicator. It is noteworthy that significant solubility problems were encountered when using the peptide. DYDYQ was insoluble at concentrations higher than 5 mM in water or in the assay buffer. DYDYQ at high concentrations slowly precipitated when incubated in an ice bucket, resulting in a gelatinous insoluble mass. Thus, for all experiments DYDYQ was made fresh in Milli Q (Millipore Corp., Bedford, MA) water at concentrations ranging between 2 and 4 mg/ml and kept at room temperature. DYDYQ was also frozen in small aliquots. All frozen peptide aliquots were thawed and used only once. Peptide solutions were always centrifuged prior to use to remove potential microaggregates and insoluble material. After the peptide was dissolved in water or buffer, the molar concentration of DYDYQ was initially calculated to a theoretical concentration assuming the degree of purity specified by the supplier and 100% peptide content. However, because DYDYQ is a highly negatively charged pentapeptide, theoretically it could adsorb water and counter ions during HPLC purification. Invariably, significant amounts of salt were present in the initial lyophilized, purified peptide preparations obtained from the manufacturers and used in this study. Thus, the exact concentration of each peptide solution used and provided throughout this study was determined following amino acid composition analysis of an aliquot in the laboratory of Dr. Alex Kurosky and Steve Smith (University of Texas Medical Branch, Galveston). Briefly, peptide samples were centrifuged for 5 min at 16,000 rpm to remove any precipitation that might be present in the tube. 10 μl of each supernatant was placed in a Wheaton 1-ml pre-scored vacule and dried (a vacule is a small thin-walled glass vial with a long neck that can be sealed with a torch while a vacuum is being applied). 100 μl of 6 N constant boiling HCl was added to each vacule. The vacules were sealed with a torch under vacuum and hydrolyzed for 20 h at 107 °C. After hydrol-

[2] The abbreviations used are: PS, L-α-phosphatidylserine; PC, L-α-phosphatidylcholine; HPLC, high performance liquid chromatography; rMZ-II, (prothrombin with the substitutions R155A, R284A, and R271A); rP2-II, (prothrombin with the substitution R155A, R284A, and R320A); ABE-I, anion binding exosite I; ABE-II, anion binding exosite II; DYDYQ, pentapeptide mimicking factor Va heavy chain sequence: Asp^{695}–Tyr^{696}–Asp^{697}–Tyr^{698}–Gln^{699}; DAPA, dansylarginine-*N*-(3-ethyl-1,5-pentanediyl)amine.

ysis the vacules were dried, and the contents were resuspended in 40 μl of 0.02 N HCl. 10 μl of each sample was subsequently injected into a Hitachi L-8800 amino acid analyzer. Average concentration (micromoles/ml) was determined by data analysis using the Hitachi L-8800 AAA System Manager. The mole/ml values obtained directly from the analyzer for each amino acid were multiplied by the molecular weight of the peptide (M_r 702) to determine the milligram/ml of peptide. Because of the harsh conditions of hydrolysis, the mole values found for Tyr were not taken into account for the determination of the final peptide concentrations. It is noteworthy that for each analysis, the average mole numbers of Asp divided by 2 was usually very close to the total mole number obtained with Gln, confirming the accuracy of the method. Usually the mole values obtained with Asp and Gln were averaged to obtain the exact concentration of peptide. This method determines the concentration of a peptide solution with an accuracy of 96 ± 6%. Under these conditions, the calculated starting concentration of peptide in each experiment was systematically found to be lower than its initial concentration calculated after the peptide was dissolved in water or buffer. It is important to note that in order to verify peptide bond integrity of DYDYQ during storage, several peptide solutions used in this study were randomly analyzed by NH_2-terminal sequence analysis in the same laboratory. Again, significant problems were encountered when attempting to sequence DYDYQ using the traditional method (polyvinylidene difluoride sample support). Under these conditions, the yield of amino acid per cycle was low as most of the peptide was washed off the polyvinylidene difluoride sample support. NH_2-terminal sequencing of DYDYQ was thus performed on a Biobrene Plus™-treated glass fiber filter sample support (Applied Biosystems, Foster City, CA.). Biobrene Plus is a cationic polymer used to immobilize peptide and protein samples on the glass fiber filter during Edman degradation. A much higher yield of amino acids per cycle was observed using these conditions. NH_2-terminal sequencing demonstrated that the majority of peptide remained intact following extensive incubation periods.

Recombinant prothrombin rMZ-II and rP2-II that have only one cleavage site for factor Xa were prepared and purified as described (47–49). Human factor V was purified using methodologies previously described employing the monoclonal antibody αhFV1 coupled to Sepharose (50) and activated to factor Va with thrombin as described recently (51). Phospholipid vesicles composed of 75% PC and 25% PS (referred to as PCPS vesicles throughout the study) were prepared as described previously (52).

Assay Measuring Thrombin Formation—Initial experiments performed to identify the best conditions allowing peptide inhibition of prothrombin activation revealed that preincubation of prothrombin with DYDYQ in a small volume is required to observe maximum inhibition of prothrombinase activity by the peptide. Thus, the ability of the peptide to inhibit prothrombin activation by either prothrombinase or factor Xa alone was conducted exactly as follows. In a typical experiment, a constant concentration of prothrombin was preincubated with increasing concentrations of peptide at room temperature in a 15-μl volume. Following a 10-min incubation period, the prothrombin/peptide sample was centrifuged, and the entire mixture was added to different tubes containing a solution composed of PCPS vesicles (1 or 10 μM), DAPA (50 μM), and factor Va (1–5 nM) in the presence of 5 mM Ca^{2+} in 20 mM HEPES, 0.15 M NaCl, pH 7.4. The reaction was started by the addition of factor Xa (0.5 nM) at room temperature. The final concentration of prothrombin was 1.4 μM or 300 nM, whereas the final concentration of peptide is provided in the text. All final concentrations of reagents provided in the text and relating to the measure of activity of prothrombinase in the presence or absence of peptide were calculated assuming a final reaction volume of 225 μl. At selected time intervals, aliquots of the mixture were diluted 2-fold in a buffer containing EDTA (50 mM) to quench the reaction. The assay verifying thrombin formation was conducted as described by measuring the initial rate of thrombin formation by the change in the absorbance of a chromogenic substrate at 405 nm (Spectrozyme-TH) monitored with a Thermomax microplate reader (Molecular Devices, Sunnyvale, CA) (51). Percent inhibition of thrombin generation was calculated by comparing the initial rates of thrombin formation obtained in the presence of peptide with the control reaction in the absence of peptide.

Inhibition of Prothrombin Cleavage by DYDYQ and Analysis of Prothrombin Activation by Gel Electrophoresis—Initial preliminary experiments carried out to determine the optimum conditions necessary to observe the effect of DYDYQ on prothrombin activation by prothrombinase by gel electrophoresis established that the best results (maximum inhibition) are obtained when the peptide is preincubated with prothrombin in a small volume. Thus, all samples analyzed by gel electrophoresis examining the effect of DYDYQ on prothrombin activation were prepared precisely as follows. In a typical experiment, plasma-derived prothrombin or recombinant mutant prothrombin at a constant concentration were preincubated with varying (increasing) concentrations of DYDYQ for 10 min in a 50-μl volume. Following centrifugation, the entire supernatant containing the prothrombin/peptide mixture was added to separated solutions containing PCPS vesicles (1 or 10 μM), DAPA (50 μM), and factor Va (1–5 nM) in the presence of 5 mM Ca^{2+} in 20 mM Tris, 0.15 M NaCl, pH 7.4. The reaction was started by the addition of factor Xa (0.5 nM) at room temperature. The final volume of each reaction mixture was 1,200 μl when the prothrombin concentration was 1.4 μM. The final concentration of DYDYQ reported throughout the study in experiments studying prothrombin activation by gel electrophoresis was always calculated using the final volume of the reaction mixture. The effect of the peptide on prothrombin activation by factor Xa alone (in the absence of factor Va) was studied in a similar manner (preincubation of prothrombin and peptide in a 50-μl volume for 10 min). The reaction was started by the addition of factor Xa. The final concentrations of reagents in this case were as follows: 2.5–5 nM enzyme, 1.4 μM prothrombin, and 50 μM PCPS in a final volume of 1,200 μl. Control experiments studying activation of prothrombin by factor Xa alone in the absence of factor Va and phospholipid were also performed in a similar fashion. At selected time intervals (indicated in the figure legends), aliquots from the reaction mixtures (60 μl) were removed and immediately diluted into 2

volumes of 0.2 M glacial acetic acid and concentrated using a Centrivap concentrator attached to a Centrivap cold trap (Labconco Corp., Kansas City, MO). The dried samples were dissolved in 0.1 M Tris base, pH 6.8, 1% SDS (final concentration), 1% β-mercaptoethanol (final concentration), heated for exactly 65 s at 90 °C, mixed again, and subjected to SDS-PAGE using 9.5% gels according to the method of Laemmli (53). Usually 6 μg of total protein per lane were applied. Protein bands were visualized following staining by Coomassie Brilliant Blue R-250 and destained in a methanol/acetic acid/water solution. After each experiment using a new peptide solution, an aliquot of the solution was stored at −20 °C. Following amino acid composition analysis, the exact initial concentration of each peptide solution was calculated for every experiment. The final concentration of peptide used was then back-calculated and is reported throughout the study. Finally, it is worth mentioning that despite all the problems encountered when working with DYDYQ, using the precise experimental protocol provided above, highly reproducible results were observed with 18 separate peptide preparations from two different suppliers (New England Peptide, Inc. and American Peptide Co.) representing three different batches of peptide.

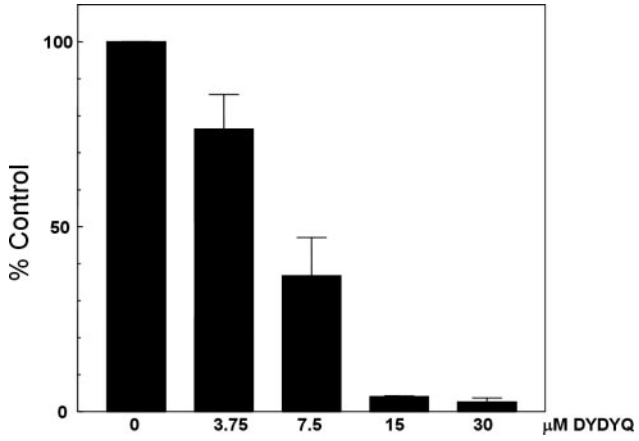

FIGURE 2. **Inhibition of prothrombinase function by DYDYQ.** Plasma-derived prothrombin (1.4 μM final concentrations) was preincubated with various concentrations of peptide DYDYQ shown on the *x* axis. The rate of thrombin formation by prothrombinase was assessed as described under "Experimental Procedures" and compared with the rate of thrombin formation determined in a control reaction in the absence of peptide. The data represent the average of the results found in three independent measurements.

Scanning Densitometry of SDS-PAGE and Calculation of the Rate of Prothrombin Consumption—Scanning densitometry of the gels was performed as described earlier (54) and recently with several modifications (51). The stained gels were scanned with a Lexmark printer/scanner; the final images were imported into Adobe Photoshop, captured as TIFF files, and subsequently imported into the software UN-SCAN-IT gel (Silk Scientific, Orem, UT). Following analysis, the numerical data were saved as a Microsoft Excel file, and the molar concentration of prothrombin as observed on the gels was calculated by normalizing its staining intensity to the initial prothrombin concentration. The data representing prothrombin consumption as a function of time (seconds) were subsequently plotted according to the equation representing a first-order exponential decay using the software Prizm (GraphPad, San Diego). The apparent first-order rate constant, k (s^{-1}), obtained directly from the graph was subsequently divided by the molar concentration of factor Xa used in each experiment (to obtain $s^{-1} \times$ molar factor Xa^{-1}). The number obtained was subsequently multiplied by the starting concentration of prothrombin used (1.4 μM or 300 nM). The final numbers reported throughout the study, characterizing the effect of DYDYQ on prothrombin cleavage by either prothrombinase or factor Xa alone, represent moles of prothrombin consumed per mol of factor Xa/s for a given experiment. All numbers reported in the study are representative of experiments performed at least in triplicate using 3–4 different peptide preparations.

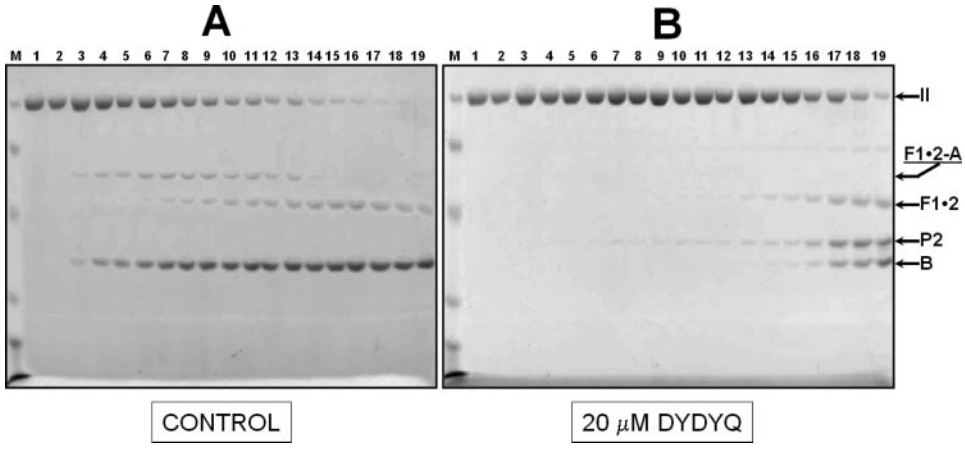

FIGURE 3. **Analysis of the activation of plasma-derived prothrombin by prothrombinase in the absence and presence of DYDYQ.** Plasma-derived prothrombin (300 nM) was preincubated with buffer or a peptide solution. The mixture was then added to a solution containing PCPS vesicles, DAPA, and plasma factor Va as described under "Experimental Procedures." The reaction was started by the addition of factor Xa. At selected time intervals, aliquots of the reactions were withdrawn and treated as described under the "Experimental Procedures." *M* represents the lane with the molecular weight markers (from top to bottom): M_r 98,000, M_r 64,000, M_r 50,000, M_r 36,000, and M_r 22,000. *Lanes 1–19* represent samples from the reaction mixture before (0 min) the addition of factor Xa and 20, 40, 60, 80, 100, 120, 140, 160, 180, 200, 220, and 240 s and 5, 6, 10, 20, 30, and 60 min, respectively, following the addition of factor Xa. *Panel A* control, prothrombinase assembled with plasma-derived factor Va in the absence of peptide; *panel B*, prothrombinase in the presence of 20 μM DYDYQ. The prothrombin-derived fragments are shown as follows: II, prothrombin (amino acid residues 1–579); F1•2-A, fragment 1•2-A chain (amino acid residues 1–320); F1•2, fragment 1•2 (amino acid residues 1–271); P2, prethrombin-2 (amino acid residues 272–579); *B*, B chain of thrombin (amino acid residues 321–579).

RESULTS

Effect of DYDYQ on the Pathway for Prothrombin Activation—We have shown previously that a pentapeptide containing amino acids 695–699 from the COOH terminus of factor Va heavy chain (Asp-Tyr-Asp-Tyr-Gln, DYDYQ) is a competitive inhibitor of prothrombinase with respect to the substrate, prothrombin (40). To ascertain the mechanism of inhibition of prothrombinase by DYDYQ, we have studied prothrombin activation by gel electrophoresis. Fig. 2 shows 50% inhibition of prothrombinase activity in the presence of 7.5 μM

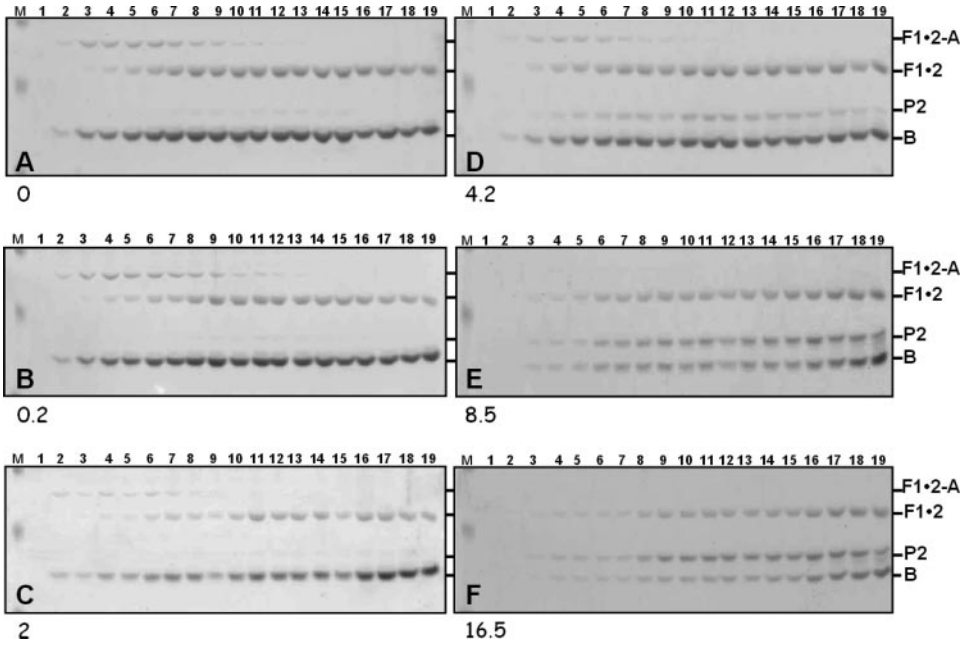

FIGURE 4. **Analysis of the activation of plasma-derived prothrombin by prothrombinase in the presence of increasing concentrations of DYDYQ.** Plasma-derived prothrombin (300 nM) was preincubated with buffer or several different peptide solutions and treated as described in the legend to Fig. 3 and under "Experimental Procedures." *Lanes 1–19* represent samples withdrew from the reaction mixture at time intervals detailed in the legend to Fig. 3. *Panel A*, control, prothrombinase assembled with plasma-derived factor Va in the absence of peptide; *panel B*, prothrombinase in the presence of 0.2 μM DYDYQ; *panel C*, prothrombinase in the presence of 2 μM DYDYQ; *panel D*, prothrombinase in the presence of 4.2 μM DYDYQ; *panel E*, prothrombinase in the presence of 8.5 μM DYDYQ; *panel F*, prothrombinase in the presence of 16.5 μM DYDYQ. Gels were submitted to scanning densitometry as described under "Experimental Procedures," and the rates of prothrombin consumption are reported in Table 1. For easier reading, the concentrations of peptide used in each experiment are also shown under each panel (in μM). Prothrombin-derived fragments are identified according to the legend of Fig. 3.

TABLE 1
Rate of activation of plasma-derived prothrombin by prothrombinase in the presence of peptide DYDYQ

Peptide concentration	Plasma-derived prothrombin[a]	Fold decrease[b]
μM	*moles consumed·s^{-1}·mol factor Xa^{-1}*	
0	8.2 ± 0.6	
0.2	5.4 ± 0.6	1.5
4.2	3.2 ± 0.4	2.6
8	0.87 ± 0.3	9.4
8.5	0.5 ± 0.2	16.4
16.5	0.4 ± 0.2	20.5
20	0.2 ± 0.2	41

[a] The rates of prothrombin consumption were obtained following scanning densitometry of the gels shown in Figs. 3 and 4. The final rate of prothrombin consumption in the presence of prothrombinase assembled with plasma-derived factor Va and various peptide concentrations was extracted following plotting of the data as described under "Experimental Procedures."

[b] Fold decrease is the ratio of the rate of prothrombin cleavage by prothrombinase in the absence of peptide compared with the rate prothrombin activation by prothrombinase in the presence of peptide.

DYDYQ. Almost complete inhibition of prothrombinase under the conditions employed required 15 μM DYDYQ.

We next assessed the effect of the pentapeptide on the pathway for prothrombin activation by gel electrophoresis. Fig. 3, *panel A*, shows a control experiment and demonstrates that in the absence of peptide activation of prothrombin by prothrombinase proceeds following initial cleavage at Arg^{320}, through the intermediate meizothrombin as confirmed by the appearance of fragment 1·2-A (meizo pathway). This fragment is then rapidly cleaved at Arg^{271} to produce thrombin. In the presence of 20 μM peptide (Fig. 3, *panel B*), no meizothrombin is evident as established by the lack of fragment 1·2-A. However, thrombin formation still occurs, as verified by the appearance of the B chain of thrombin, albeit with a considerably delayed rate, following the pre2 pathway as established by the presence of prethrombin 2. These data suggest that under the conditions employed DYDYQ selectively inhibits cleavage at Arg^{320} by prothrombinase and favors initial cleavage of prothrombin at Arg^{271}. This conclusion is inferred from the lack of meizothrombin and the increase in the concentration of prethrombin 2, both of which are the expected consequences of selective inhibition of cleavage of prothrombin by prothrombinase at Arg^{320}.

To determine at what peptide concentration a switch in the pathway for prothrombin activation occurred, we studied the profile of prothrombin activation by prothrombinase in the presence of increasing concentrations of peptide, and the data are presented in Fig. 4. For simplicity, we have focused on four characteristic fragments demonstrating cleavage of prothrombin through either the pre2 pathway (fragment 1·2 and prethrombin 2) or through the meizo pathway (Fig. 1, *fragment 1·2-A* and *B-chain*), and the rates of prothrombin consumption are reported in Table 1. In the presence of a membrane surface, under the conditions employed (0.5 nM factor Xa and 1 nM factor Va), and in the absence of peptide, prothrombin consumption proceeds with a rate of 8.2 mol·s^{-1}·mole factor Xa^{-1} through the meizo pathway (Table 1). In the presence of 2 μM peptide, fragment 1·2-A appeared readily and was quickly consumed following cleavage at Arg^{271}. As a result, thrombin formation occurred steadily as substantiated by the appearance of the B chain of thrombin (Fig. 4, *panels A–C*). At a concentration of 4.2 μM DYDYQ, both intermediates were observed (*i.e.* fragment 1·2-A and prethrombin 2) suggesting activation of prothrombin through both pathways (Fig. 4*D*). Under these conditions, the rate of prothrombin consumption was only decreased by 2.6-fold.

At 8.5 μM peptide, there was a significant reduction in both prothrombin consumption (~16-fold; Table 1) and the rate of thrombin formation (~60% by activity Fig. 2), which coincided with the complete disappearance of fragment 1·2-A and diminished rate of appearance of the B chain of thrombin (Fig. 4*E*). Thus, when prothrombin is saturated with peptide, no initial cleavage at Arg^{320} appears to occur; however, prothrombin activation still takes place following initial cleavage at Arg^{271} (pre2 pathway), albeit with a slower rate. These data demon-

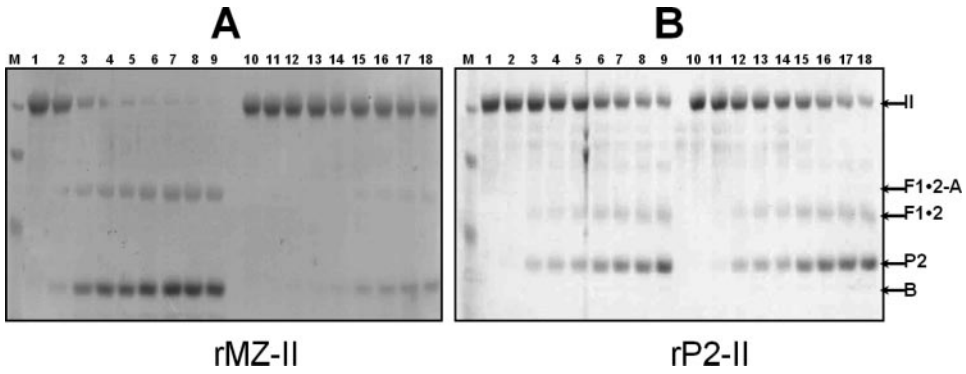

FIGURE 5. **Analysis of the activation of recombinant mutant prothrombin molecules by prothrombinase in the absence and presence of DYDYQ.** Mutant prothrombin molecules (300 nM) were preincubated with buffer or a peptide solution and treated as described in the legend to Fig. 3 and under "Experimental Procedures." *Panel A*, *lanes 1–9* represent samples of the reaction mixture following incubation of prothrombinase reconstituted with rMZ-II in the absence of DYDYQ, before (*lane 1*) or following 1, 4, 6, 10, 20, 30, 45, and 60 min incubation with factor Xa, respectively; *lanes 10–18* represent samples of the reaction mixture following incubation of prothrombinase reconstituted with rMZ-II in the presence of 10 μM DYDYQ, before (*lane 10*) or following 1, 4, 6, 10, 20, 30, 45, and 60 min of incubation with factor Xa. *Panel B*, *lanes 1–9* represent samples of the reaction mixture following incubation of prothrombinase reconstituted with rP2-II in the absence of DYDYQ at times points identical to *panel A*, *lanes 1–9*; *lanes 10–18* represent samples of the reaction mixture following incubation of prothrombinase reconstituted with rP2-II in the presence of 13 μM DYDYQ at times points identical to *panel A*, *lanes 10–18*. Positions of prothrombin-derived fragments are indicated at *right* as detailed in the legend to Fig. 3. Molecular weight markers (*M*) are (from top to bottom): M_r 98,000, M_r 64,000, M_r 50,000, and M_r 36,000. Gels were submitted to scanning densitometry as described under "Experimental Procedures," and the rates of prothrombin consumption are reported in Table 2.

TABLE 2

Rate of activation of recombinant prothrombin species by prothrombinase

Peptide concentration	rMZ-II (cleavage at Arg^{320})[a]	rP2-II (cleavage at Arg^{271})[a]
μM	*moles consumed·s^{-1}·mol factor Xa^{-1}*	
0	3.7 ± 0.6	0.34 ± 0.04
10	NS[b]	
13		0.2 ± 0.1
35		NS[c]

[a] The rates of prothrombin consumption were obtained following scanning densitometry of the gels shown in Fig. 5. The final rate of prothrombin consumption in the presence of prothrombinase assembled with plasma-derived factor Va and various peptide concentrations was extracted following plotting of the data as described under "Experimental Procedures."

[b] NS, not significant consumption. The concentration of prothrombin varied from 300 nM at time 0 to 280.3 nM following 3 h of incubation with prothrombinase and DYDYQ.

[c] NS, not significant consumption. In separate experiments, using a higher concentration of DYDYQ, the concentration of prothrombin varied from 300 nM at time 0 to 259.2 nM following 3 h of incubation with prothrombinase and DYDYQ.

strate complete inhibition of the pathway of prothrombin activation characterized by initial cleavage at Arg^{320} by DYDYQ (meizo pathway). Substantial inhibition of thrombin formation, which correlates with decreased appearance of fragment 1·2, prethrombin 2, and the B chain of thrombin, was observed in the presence of 16.5 μM peptide (Fig. 4*F*; Table 1). Under these conditions, prothrombin activation occurs slowly and exclusively through the pre2 pathway. The rate of prothrombin consumption under these conditions was decreased by 20-fold. In the presence of 20 μM peptide, the rate of prothrombin consumption was further decreased by 41-fold (Table 1). Finally, in the presence of very high concentrations of peptide ($\geq$100 μM), no significant prothrombin activation by prothrombinase was detected by gel electrophoresis (not shown). Overall, the data demonstrate a dual and differential effect of the peptide on both the rate and pathway for prothrombin activation by prothrombinase; at low concentrations of peptide, meizothrombin formation is attenuated, but thrombin formation occurs through the alternate pathway; at high peptide concentrations, both prothrombin consumption by prothrombinase and thrombin formation are severely impaired. It thus appears that although both cleavages are prone to inhibition by DYDYQ, cleavage at Arg^{320} by prothrombinase is more susceptible to peptide inhibition than cleavage at Arg^{271}. The combined data suggest that although inhibition of initial cleavage at Arg^{320} by DYDYQ can be explained by the interference of the peptide with the prothrombin interactive site of factor Va heavy chain, impaired initial cleavage at Arg^{271} by DYDYQ can be attributed to the fact that high concentrations of peptide also interfere with the prothrombin-binding site for factor Xa that in turn is responsible for accelerated cleavage of prothrombin at Arg^{271}.

To ascertain the effect of DYDYQ on each cleavage separately, we used recombinant prothrombin molecules that can only be cleaved at either Arg^{320} (rMz-II) or Arg^{271} (rP2-II) (Fig. 5). The data demonstrate that under similar experimental conditions cleavage of rMZ-II is severely impaired, whereas cleavage of rP2-II does not appear to be affected by low concentrations of DYDYQ. rMZ-II consumption was almost completely inhibited in the presence of 10 μM DYDYQ. In contrast, consumption of rP2-II was only marginally reduced in the presence of 13 μM peptide (Table 2). Higher concentrations of DYDYQ (up to 35 μM) were necessary to obtain significant inhibition of the rate of cleavage of rP2-II by prothrombinase (Table 2). These data confirm our findings and demonstrate that cleavage at Arg^{320} is much more sensitive to inhibition by DYDYQ than is cleavage at Arg^{271}, but at relatively high concentrations of DYDYQ, both cleavages can be substantially inhibited. Overall, the data clearly demonstrate that the specific obstruction of the interaction of prothrombin with elements of prothrombinase results in diminished thrombin production through the meizo pathway. However, because prothrombin has two possible pathways for activation, the interaction of DYDYQ with prothrombin favors the pre2 pathway.

Effect of DYDYQ on Prothrombin Activation by Factor Xa in the Absence of Factor Va—We next assessed the effect of the peptide on the ability of membrane-bound factor Xa, in the absence of factor Va, to activate prothrombin. High concentrations of peptide were necessary to partially impair thrombin formation under these conditions. However, complete inhibition of thrombin formation could not be observed even in the presence of high concentrations of DYDYQ (Fig. 6). Under the experimental conditions used, the extent of inhibition of the rate of thrombin formation by increasing concentrations of DYDYQ, in several separate experiments, varied between 50

and 70%. These data demonstrate that DYDYQ has an effect on prothrombin activation by membrane-bound factor Xa alone, in the absence of factor Va. Analyses of the pattern of the time course of prothrombin activation by membrane-bound factor Xa in the absence of factor Va by gel electrophoresis demonstrated accelerated cleavage of prothrombin at Arg^{271} by membrane-bound factor Xa in the presence of the peptide (Fig. 7). Similarly, whereas cleavage of rP2-II at Arg^{271} by membrane-bound factor Xa was significantly accelerated in the presence of the peptide, proteolysis of rMZ-II did not appear to be considerably affected (Fig. 8). Scanning densitometry of the gels shown in Figs. 7 and 8 provided a kinetic explanation for the observed phenomenon. In the absence of peptide, membrane-bound factor Xa alone cleaved prothrombin and rP2-II with similar rates (Table 3). Concentrations as high as 20 μM DYDYQ had minimal effect on rMZ-II consumption under the conditions employed (*i.e.* in the absence of factor Va). In contrast, a significant acceleration of cleavage of plasma-derived prothrombin and rP2-II by membrane-bound factor Xa alone at Arg^{271} was observed in the presence of 24 μM peptide (~50- and 71-fold, respectively; see Table 3) resulting in accumulation of prethrombin 2. Increasing the peptide concentration to 48 μM had no further accelerating effect on the rate of prothrombin consumption (Table 3). It is interesting to note for comparison that the interaction of factor Va with factor Xa and prothrombin on a membrane surface results in a rate of prothrombin consumption of 12 mol·s^{-1}·mol Xa^{-1}, which is ~200-fold faster than the rate of prothrombin consumption by membrane-bound factor Xa alone. Similarly, the interaction of DYDYQ with prothrombin results in a 50-fold increase in the rate of prothrombin consumption. However, with factor Xa and factor Va, prothrombin consumption results primarily in thrombin formation, whereas with factor Xa and DYDYQ prothrombin consumption results in accumulation of prethrombin 2. Overall our data demonstrate that the interaction of peptide DYDYQ with prothrombin has opposite effects on membrane-bound factor Xa for cleavage of the substrate depending on the incorporation of the cofactor into prothrombinase. In the presence of factor Va, the peptide decreases the rate of substrate consumption by prothrombinase by selectively inhibiting cleavage at Arg^{320} and directing factor Xa to the alternate pathway characterized by initial cleavage at Arg^{271}, resulting in delayed thrombin formation. In the absence of factor Va, the peptide induces a significant acceleration of initial cleavage at Arg^{271} by membrane-bound factor Xa alone, accelerating the rate of prothrombin consumption resulting in accumulation of prethrombin 2.

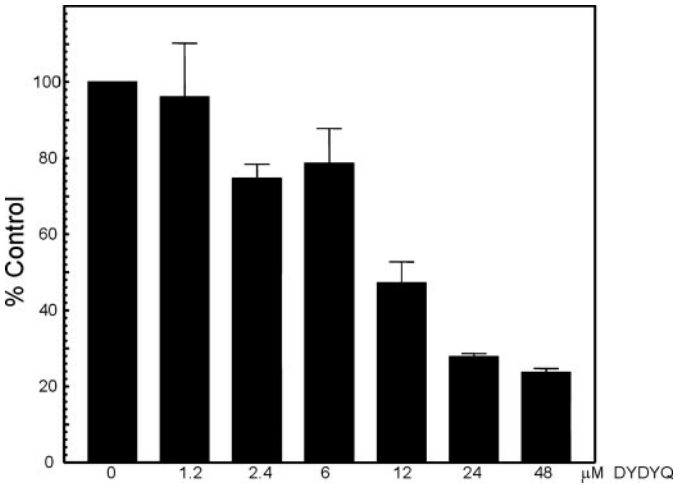

FIGURE 6. **Thrombin generation by membrane-bound factor Xa in the absence of factor Va.** Plasma-derived prothrombin (1.4 μM) was incubated with various concentrations of peptide shown on the *x* axis. The rate of thrombin formation by membrane-bound factor Xa alone, in the absence of factor Va, was assessed as described under the "Experimental Procedures" and compared with the rate determined in a control reaction in the absence of peptide. The data represent the average of the results found in three independent measurements.

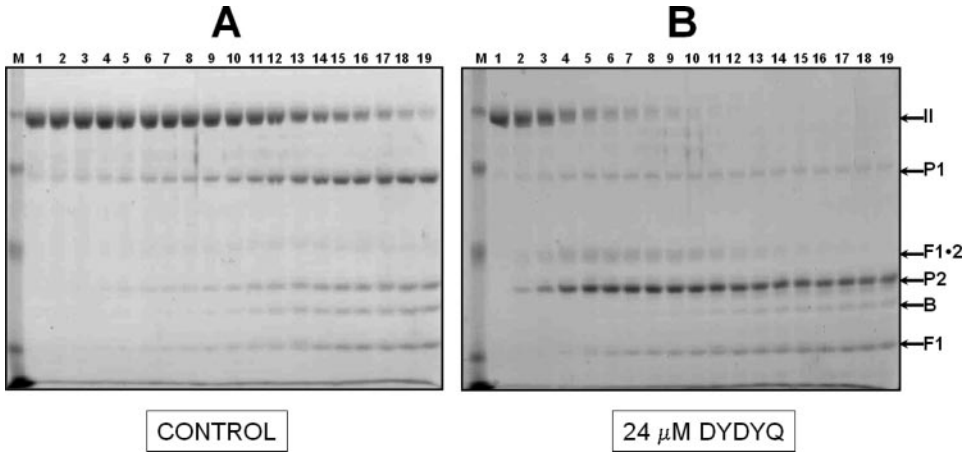

FIGURE 7. **Analysis of the activation of plasma-derived prothrombin by membrane-bound factor Xa alone in the absence and presence of DYDYQ.** Plasma-derived prothrombin (1.4 μM) was preincubated with buffer or a peptide solution. The mixture was subsequently incubated with PCPS vesicles and DAPA. The reaction was started by the addition of factor Xa (2.5 nM). At selected time intervals, aliquots of the reactions were withdrawn and treated as described under "Experimental Procedures." *Lanes 1–19* represent samples from the reaction mixture before (0 min) the addition of factor Xa and 30 s, and 1, 3, 5, 7, 10, 12, 15, 20, 30, 45, 60, 75, 90, 105, 120, 150, and 180 min, respectively, following the addition of factor Xa. *Panel A,* activation by membrane-bound factor Xa in the absence of peptide; *panel B*, activation by membrane-bound factor Xa in the presence of 24 μM DYDYQ. *M* represents the lane with the molecular weight markers (from top to bottom): M_r 98,000, M_r 64,000, M_r 50,000; M_r 36,000, and M_r 22,000. Prothrombin-derived fragments are identified according to the legend of Fig. 3. Other fragments are as follows: *P1*, prethrombin-1 (amino acid residues 156–579), and *F1*, fragment 1 (amino acid residues 1–155). Gels were submitted to scanning densitometry as described under "Experimental Procedures," and the rates of prothrombin consumption are reported in Table 3.

DISCUSSION

Our data demonstrate that preincubation of prothrombin with a peptide duplicating amino acid sequence 695–699 of factor Va, prior to its inclusion in a mixture containing prothrombinase, results in inhibition of initial cleavage at Arg^{320} by the enzyme (Fig. 9). During assembly of prothrombinase, factor Va binds factor Xa, which in turn is responsible for the catalytic event *per se*. Thus, factor Va has a dual effect within the complex, a receptor effect and an effector effect. Although the receptor effect is easy to understand (binding to the

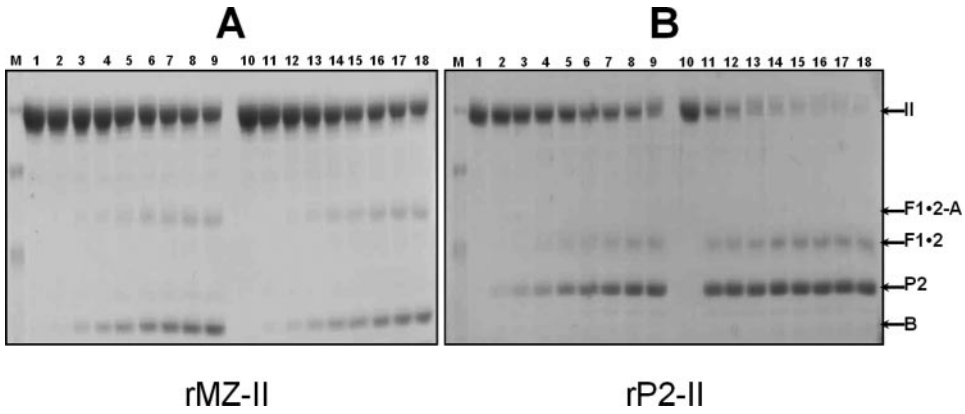

FIGURE 8. **Analysis of the activation of mutant prothrombin molecules by membrane-bound factor Xa alone in the absence and presence of DYDYQ.** Mutant prothrombin molecules (1.4 μM) were preincubated with buffer or a peptide solution and treated as described in the legend to Fig. 7. *Panel A*, *lanes 1–9* represent samples of the reaction mixture containing rMZ-II in the absence of DYDYQ, before (*lane 1*) or following 5, 15, 30, 60, 90, 120, 150, and 180 min of incubation with factor Xa, respectively; *lanes 10–18* represent samples of the reaction mixture following incubation of rMZ-II with 20 μM DYDYQ, before (*lane 10*) or following 5, 15, 30, 60, 90, 120, 150, and 180 min of incubation with factor Xa, respectively. *Panel B*, *lanes 1–9* represent samples of the reaction mixture containing rP2-II in the absence of DYDYQ at the same time points as in *panel A, lanes 1–9*; *lanes 10–18* represent samples of the reaction mixture containing rP2-II in the presence of 20 μM DYDYQ at the same time points as in *panel A, lanes 10–18*. Positions of prothrombin-derived fragments are indicated at *right* as detailed in the legend to Fig. 3. Molecular weight markers (*M*) are (from top to bottom): M_r 98,000, M_r 64,000, M_r 50,000, and M_r 36,000. Gels were submitted to scanning densitometry as described under "Experimental Procedures," and the rates of prothrombin consumption are reported in Table 3.

TABLE 3

Rate of cleavage of prothrombin molecules by membrane-bound factor Xa alone in the absence of factor Va

The rates of prothrombin consumption were obtained following scanning densitometry of several gels analyzing prothrombin consumption by membrane-bound factor Xa. Typical examples are illustrated in Figs. 7 and 8. The final rate of prothrombin consumption in the presence of membrane-bound factor Xa was extracted following plotting of the data to a first-order exponential decay as described under "Experimental Procedures."

Peptide concentration	Plasma-derived Prothrombin	rMZ-II (cleavage at Arg^{320})	rP2-II (cleavage at Arg^{271})
μM	*moles consumed·s^{-1}·mol factor Xa^{-1}*		
0	0.06 ± 0.03	0.08 ± 0.022	0.014 ± 0.005
12	2 ± 0.3		0.3 ± 0.09
20		0.06 ± 0.03	0.5 ± 0.09
24	3.1 ± 0.2		1 ± 0.4
48	3 ± 0.7		

membrane-bound enzyme and formation of a binary complex), the effector effect is more intricate and requires a full consideration of the molecular dynamics involved in prothrombinase complex assembly and function.

There is consensus in the literature that binding of prothrombin to prothrombinase involves the expression of an exosite that is provided to the substrate upon the incorporation of factor Va into the complex. One unanswered question is which molecule provides this extended exosite; is this exosite a consequence of the exposure of a hidden portion of factor Xa alone, which is expressed upon its interaction with the cofactor? Is this exosite provided by elements of factor Va alone, or is the exosite composed of discrete amino acids belonging to both factor Va and factor Xa? The data presented herein confirm the existence of the factor Va-prothrombin interaction within prothrombinase first demonstrated approximately 30 years ago (57) and suggest that both factor Va and factor Xa provide for an exosite for prothrombin. Data from the crystal structure of prethrombin-2 demonstrated that the Arg^{320}–Ile^{321} bond, which is not accessible to factor Xa in the absence of factor Va nor in the presence of both DYDYQ and factor Va (Fig. 9*B*), needs extensive rearrangement and must rotate ~150° around the Gly^{319}–Gly^{324} hinge points to be cleaved by factor Xa (58). Collectively, the data suggest that the interaction of prothrombin with amino acids from factor Va within prothrombinase may be part of a requirement for the rearrangement of amino acids around the Arg^{320}–Ile^{321} bond in such a manner that it becomes accessible for efficient cleavage by factor Xa as part of prothrombinase.

Hirudin and $Hir^{54-65}(SO_3^3)$ are specific proexosite I ligands that interfere with the factor Va-prothrombin interaction *in vitro* and in whole plasma (28, 59). Nonetheless, it has been demonstrated that $Hir^{54-65}(SO_3^3)$ is impaired in its inhibition of prothrombinase for prothrombin cleavage in the presence of phospholipid vesicles composed of 25% PS and 75% PC (28, 60). Although the inhibitory potential of $Hir^{54-65}(SO_3^3)$ on prothrombinase activity is partially recovered when similar experiments are performed with phospholipid vesicles composed of 5% PS and 95% PC, complete inhibition of prethrombin 1 activation by fully assembled prothrombinase on phospholipid vesicles composed of 25% PS and 75% PC was observed with $Hir^{54-65}(SO_3^3)$ (28). It has been also established that proteolytic elimination of fragment 1 in bovine prothrombin results in the elimination of the accelerating effect of the membrane surface for initial cleavage at Arg^{320} by prothrombinase (56). The accumulated data strongly suggest that binding of prothrombin to a membrane surface rich in PS confers fragment 1 the capability to interfere with the inhibitory effect of hirudin-derived peptides with (pro)exosite I, as well as the ability to regulate the interaction of factor Va with prothrombin. It thus appears that fragment 1 of prothrombin has a modulatory effect on the factor Va-prothrombin interaction. Data in support of this hypothesis include the demonstration that in the absence of PCPS membranes amino acids from both the Gla domain and kringle 1 of prothrombin inhibit thrombin generation in the presence but not in the absence of factor Va, suggesting that amino acids from the NH_2 terminus of prothrombin most likely regulate the factor Va-prothrombin interaction (61, 62).

The following has been well established: 1) fragment 1·2 of prothrombin interacts with prethrombin 2 with high affinity (K_d~0.1–33 nM) through its fragment 2 component (37, 55, 63, 64); and 2) this latter interaction alone leads to a large acceleration of the rate of cleavage at Arg^{320} of prethrombin 2 by fully assembled prothrombinase (~150-fold increase in the second-order rate constant in the presence of fragment 1·2 compared with the same reaction in the absence of fragment 1·2) (55). Our data demonstrate that at low concentrations of pentapeptide, no signif-

icant decrease in the concentration of B chain of α-thrombin is observed by gel electrophoresis because thrombin generation occurs with approximately the same rate via both pathways. Because fragment 1·2 accelerates cleavage at Arg^{320} by prothrombinase in prethrombin 2 in the pathway characterized by initial cleavage at Arg^{271} (55, 63, 64), more DYDYQ peptide would be necessary to sterically hinder the interaction resulting in delayed α-thrombin generation through this pathway (Fig. 9*C*).

Membrane-bound factor Xa alone is capable of activating prothrombin following sequential cleavages at Arg^{271} and Arg^{320} resulting in slow generation of α-thrombin (Fig. 1, *pathway I*). Our present data show that although DYDYQ inhibits initial cleavage at Arg^{320} by prothrombinase, the peptide accelerates significantly cleavage of prothrombin at Arg^{271} by membrane-bound factor Xa alone resulting in the accumulation of a noncovalent complex composed of prethrombin 2 and fragment 1·2 (Fig. 9*D*). Because DYDYQ is a specific factor Va-dependent inhibitor of prothrombinase, these data suggest that portions in/or around the factor Va-prothrombin interactive site may also be involved in prothrombin activation by factor Xa alone. Keeping in mind that fragment 1·2 accelerates significantly cleavage at Arg^{320} of prethrombin 2 by prothrombinase, whereas DYDYQ does not have this effect and because the peptide does not interact with factor Xa, we can speculate that once prothrombin is cleaved at Arg^{271} and fragment 1·2 interacts with prethrombin 2, the role of factor Va within the complex is exclusively reduced to the conversion of factor Xa in an enzyme form (prothrombinase) that can efficiently cleave prethrombin 2 at Arg^{320}. Consequently, results obtained with prethrombin 2 as a substrate for prothrombinase cannot be extrapolated and compared with the data obtained using prothrombin as the substrate for the enzyme. At this point we must note that the possibility that pentapeptide DYDYQ might create long range allosteric perturbations that in turn influence interactions distant from its site of binding cannot be excluded by our findings.

In line with all these findings, an alternative explanation for the acceleration of the rate of cleavage at Arg^{320} in prethrombin 2 was provided by earlier studies (55, 65, 66). All experiments in

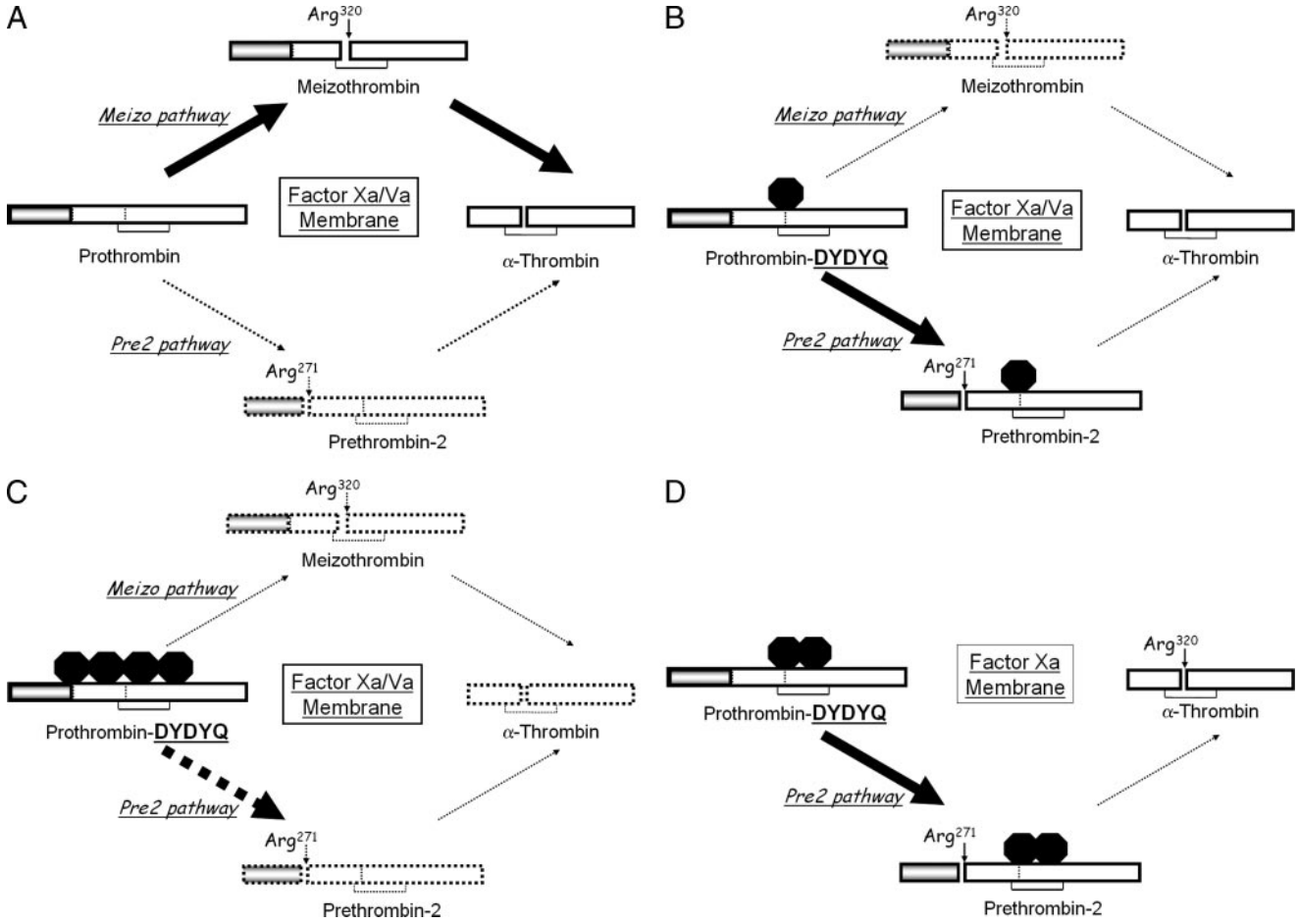

FIGURE 9. **Schematic of the effect of DYDYQ on prothrombin cleavage in the presence and absence of factor Va.** *Panel A,* initial cleavage of prothrombin at Arg^{320} by prothrombinase results in the production of meizothrombin (*meizo pathway*). Subsequent cleavage of this intermediate at Arg^{271} produces thrombin and fragment 1·2. This pathway is only observed in the presence of the nonenzymatic cofactor, factor Va. Initial cleavage of prothrombin at Arg^{271} by membrane-bound factor Xa in the absence of factor Va results in the generation of fragment 1·2 and prethrombin 2 followed by subsequent cleavage at Arg^{320} resulting in thrombin formation (*pre2 pathway*). *Panel B,* preincubation of prothrombin with low concentrations of DYDYQ (shown by the *filled octagon*) results in the inhibition of the meizo pathway and production of thrombin by prothrombinase through the pre2 pathway. *Panel C*, in the presence of high concentrations of DYDYQ (illustrated by the increased number of *filled octagons*), cleavage of prothrombin by prothrombinase through both pathways is significantly inhibited, resulting in notably impaired thrombin formation. *Panel D,* preincubation of prothrombin with DYDYQ results in a significant acceleration of the rate of cleavage at Arg^{271} by membrane-bound factor Xa alone, in the absence of factor Va, and accumulation of prethrombin-2. In this schematic the *dotted arrows* represent kinetically slow reactions, and the *heavy arrows* depict kinetically fast reactions. The *dotted molecules* depict intermediates (meizothrombin or prethrombin 2) or the final product (thrombin) that are generated with a very slow rate under the conditions described.

these latter studies were performed with saturating phospholipid concentrations composed of 25% PS and 75% PC and with prethrombin 2 as substrate for prothrombinase. These data support the hypothesis of the existence of a cryptic exosite for prothrombin docking exposed on membrane-bound factor Xa upon its interaction with the cofactor. Hence, the major role of factor Va in prothrombinase would be restricted solely to the exposure of the cryptic exosite(s) on factor Xa. This interpretation was based on data showing no interaction of factor Va with isolated fragment 2 (55) and from subsequent findings demonstrating inhibition of the acceleration of cleavage at Arg^{320} by prothrombinase in prethrombin 2 by both active-site blocked α-thrombin and ζ-thrombin (66). The latter thrombin derivative, which is produced following cleavage of the B chain of α-thrombin by chymotrypsin at Trp^{468} (sequence numbers in prothrombin represent numbers obtained following consecutive numbering of the 579 amino acid residues in mature human prothrombin) (67) is composed of two distinct fragments (68, 69) as follows: an NH_2-terminal polypeptide that contains ABE-I (ζ1-thrombin), and the COOH-terminal portion of the B chain that contains ABE-II (ζ2-thrombin). Using these fragments from α-thrombin and prethrombin 2, Betz and Krishnaswamy (66) identified ζ2-thrombin to be responsible for the inhibition of prethrombin 2 cleavage by prothrombinase. Coincidentally, it has been reported that a pentadecapeptide representing amino acids 557–571 from the COOH terminus of the B chain of α-thrombin inhibits factor Xa activity toward prothrombin in a factor Va-independent manner (70), whereas a pentadecapeptide also contained within ζ2-thrombin and corresponding to amino acid residues 473–487 of prothrombin inhibits prothrombinase activity as well, but in a factor Va-dependent manner (71). This latter group of amino acids is immediately adjacent to (pro)exosite I as shown in the crystal structures of human prethrombin 2 (58) and bovine meizothrombin desfragment 1 (72). Concurrent with all these findings, our data demonstrate that the pentapeptide DYDYQ has an effect on membrane-bound factor Xa for cleavage of membrane-bound prothrombin both in the presence and absence of factor Va. Collectively, the data imply that the amino acid region spatially surrounding proexosite I in prothrombin most likely has two contiguous interactive sites for the components of prothrombinase as follows: a factor Va-interactive site that is modulated by a portion of fragment 1 when prothrombin is saturated with a lipid bilayer rich in PS, and a factor Xa-binding site that is exposed (modulated) following incorporation of factor Va into prothrombinase and the interaction of prothrombin with factor Va and/or fragment 1·2. Existence of the former has been widely confirmed by direct binding assays (25, 28, 57, 61, 62, 73), by using prothrombin molecules mutated at specific residues in (pro)exosite I (38, 39) and by our recent findings demonstrating competitive inhibition of prothrombinase by DYDYQ (40), whereas direct evidence for the existence of the latter was provided by experiments using deletion mutants of prothrombin (74), monoclonal antibodies to fragment 2 (75), and synthetic peptides from fragment 2 (76, 77).

In conclusion, our data provide a logical explanation for many apparently conflicting results and, put in the context of the literature, demonstrate that binding of the cofactor to both prothrombin and factor Xa is required for optimum prothrombinase function under physiological conditions. Bearing all these qualifications in mind, the data presented herein are consistent with the interpretation that the leading mechanism by which factor Va physiologically increases the catalytic efficiency of factor Xa within prothrombinase is by binding both enzyme and substrate, and altering the structure and accessibility about the scissile bonds. Thus, following incorporation into prothrombinase, factor Va actively guides factor Xa for efficient prothrombin catalysis.

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