Structural Changes in the Protease Domain of Prothrombin upon Activation As Assessed by *N*-Bromosuccinimide Modification of Tryptophan Residues in Prethrombin-2 and Thrombin†

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ABSTRACT: Increases in intrinsic fluorescence (ΔI), reflecting changes in tryptophan environments, occur upon bond cleavages necessary for prothrombin (II) activation to thrombin (IIa) by prothrombinase. Cleavage at Arg^{274}-Thr^{275} (numbering based on bovine prothrombin sequence, with chymotrypsinogen numbering in braces) between the amino-terminal fragment 1.2 and protease (Pre2) domains of prothrombin yields ΔI = 5%, and cleavage within the Pre2 domain at Arg^{323}-Ile^{324} to form IIa yields ΔI = 35%, while cleavage at both yields ΔI = 25%. Since the change in fluorescence upon activation of prothrombin can be largely attributed to a change within the Pre2 domain, the susceptibilities of each of the 9 Trp residues of IIa and its immediate precursor Pre2 to oxidation by *N*-bromosuccinimide (NBS) were compared. Pre2 and IIa were titrated with increasing amounts of NBS (0.5–5 equiv of NBS/TRP), aliquots were removed and fully digested with trypsin, and tryptophan-containing peptides were separated and quantitated by RP-HPLC with fluorescence detection. Tryptic digests yielded 9 tryptophan-containing peptides, which were identified by amino acid composition. Tryptophan residues in IIa and Pre2 displayed a 10-fold range of sensitivity to modification. Tryptophans 337 and 360 {W29, W51} were modified less readily in IIa than in Pre2, while residues 373, 542, and 550 {W60D, W207, W215} were modified more readily, and other residues were equally susceptible. Residues 360 and 373 {W29, W60D} flank the active site histidine. From the crystal structure, residues 373 and 550 {W60D, W215} are implicated in substrate binding. These data suggest differences in structure between IIa and its precursor, both near the active site histidine and at the substrate binding site.

The process of blood coagulation produces in its final stages the components necessary to convert the zymogen prothrombin to the serine protease thrombin (Mann et al., 1990). These components are factor Xa (an active serine protease), factor Va (an essential cofactor), negatively charged phospholipid, and calcium ion, collectively known as the prothrombinase complex. More so than the other coagulation serine proteases, thrombin plays numerous diverse roles in the mechanisms of coagulation (Fenton, 1988). It catalyzes cleavage of fibrinogen to form fibrin which then polymerizes to form a clot; it catalyzes activation of two vital cofactors in the coagulation cascade (factors V and VIII); and it catalyzes activation of factor XIII (Hornyak et al., 1989), a transglutaminase which contributes to clot stabilization by catalyzing the cross-linking of fibrin polymers. In addition, in complex with the endothelial cell membrane protein thrombomodulin, thrombin negatively regulates blood coagulation by catalyzing the activation of protein C which then catalyzes the proteolytic inactivation of factors Va and VIIIa (Walker & Fay, 1992). Thrombin also contributes to platelet activation, provides growth factor like activity to fibroblasts, and is chemotactic for monocytes and neutrophils (Fenton, 1988).

The activation of bovine prothrombin to thrombin by prothrombinase involves cleavage at two sites within the molecule (Figure 1). Cleavage[1] occurs initially at Arg^{323}-Ile^{324}, forming the active intermediate meizothrombin (Krishnaswamy et al., 1987). Further cleavage by the prothrombinase complex at Arg^{274}-Thr^{275} liberates thrombin and the activation peptide fragment 1.2 (F1.2) from the respective

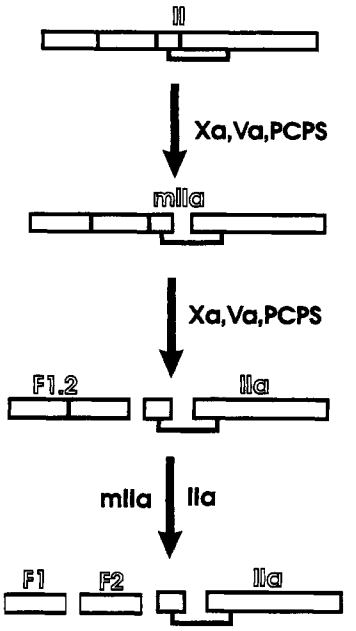

FIGURE 1: Schematic diagram of prothrombin (II) activation. Initial bond cleavage in the presence of prothrombinase [Xa, Va, PCPS (phospholipid vesicles, 75% PC, 25% PS), and Ca^{2+}] occurs at Arg^{323}-Ile^{324}, within the protease domain, forming meizothrombin (mIIa). Subsequent cleavage at Arg^{274}-Thr^{275} occurs between domains, producing fragment 1.2 (F1.2) and thrombin (IIa). Either IIa or mIIa can catalyze further cleavage at Arg^{156}-Ser^{157}, splitting fragment 1.2 (F1.2) into fragment 1 (F1) and fragment 2 (F2).

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[1] The numbering used is that of bovine prothrombin, with thrombin numbering also expressed in terms of chymotrypsinogen after Bode et al. (1989), in braces.

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carboxyl- and amino-terminal halves of the precursor. Thrombin is active as a two-chain protease, with the A and B chains covalently joined via a disulfide bond. Either thrombin or meizothrombin can catalyze cleavage of the F1.2 domain at Arg^{156}-Ser^{157} to yield fragment 1 (F1) and fragment 2 (F2). This cleavage is thought to be important in the release of thrombin from the membrane surface (Nesheim et al., 1988), since the F2 and protease domains remain tightly associated after cleavage at Arg^{274}-Thr^{275} (Myrmel et al., 1976). Cleavage of these bonds in the reverse order, as occurs when factor Xa alone is present (Krishnaswamy et al., 1987), produces the carboxyl-terminal fragment 1.2 and the activation intermediate prethrombin 2 (Pre2). Pre2 is chemically equivalent to thrombin except for the bond cleavage at Arg^{323}-Ile^{324} and unlike meizothrombin is not proteolytically active. Pre2 is therefore useful as a model for the protease domain in its unactivated state and allows for a rigorous comparison of tryptophan residues between both species.

The structure of thrombin has been very well characterized in that the crystal structures of thrombin in complex with five different inhibitors, as well as a peptide analog of fibrinogen, have been solved by three groups (Rydel et al., 1990; Bode et al., 1989, 1992; Banner & Hadvary, 1991; Martin et al., 1992). Relatively little information is available however, about the structure of the zymogen prothrombin, and the conformational changes that occur in the protease domain when prothrombin is activated. Thus, preliminary studies were performed to determine whether a change in structure upon activation could be inferred from changes in intrinsic fluorescence. The results indicated that the intrinsic fluorescence signal from a solution of prothrombin exposed to prothrombinase increases by 25% concomitant with cleavage of prothrombin to thrombin. The intrinsic fluorescence signal of prothrombin consists of a single peak with maximum intensity at ~335 nm, which can be attributed to the 14 tryptophan residues spread throughout the domains of prothrombin (3 in F1, 2 in F2, and 9 in the B chain of the protease domain). The change in intrinsic fluorescence signal upon activation implies both changes in tryptophan environments and changes in protein structure. In order to better understand the structural changes that convert the inactive precursor prothrombin into the multifunctional protease thrombin, prothrombin activation was examined using measurements of intrinsic fluorescence, controlled chemical modification of tryptophan residues with *N*-bromosuccinimide (NBS) (Lundblad & Noyes, 1984), and chromatographic analysis of tryptophan-containing tryptic peptides to identify specific tryptophan residues which undergo changes in environment upon conversion of the zymogen to the enzyme. Isolated Pre2 was used as a model for the protease domain of prothrombin in the chemical modification studies.

MATERIALS AND METHODS

Materials. Tris base, phosphatidylcholine (PC, egg), phosphatidylserine (PS, bovine brain), lyophilized *Echis carinatus* venom, bovine trypsin (tosylphenylalanyl chloromethyl ketone treated). DEAE-(A50-120) and SP-(C50-120) Sephadex ion-exchange resins, and crystalline *N*-bromosuccinimide (NBS) were obtained from Sigma Chemical (St. Louis, MO). The NBS was recrystallized from distilled water twice prior to use. To accomplish recrystallization, NBS (25 g) was dissolved in 100 mL of boiling H_2O and the mixture was cooled to room temperature. The resultant crystals were then separated from the mother liquor by filtration and washed with 10 mL of cold H_2O. PCPS vesicles (PCPS) were prepared from 3 parts PC and 1 part PS according to Barenholz et al. (1977), as described by Bloom et al. (1979). The chloromethyl ketone inhibitors PPACk and dEGRCk were obtained from Calbiochem (San Diego, CA). HPLC-grade acetonitrile was obtained from BDH (Toronto, ON), and TFA[2] was obtained from Pierce (Rockford, IL). The reversible fluorescent thrombin inhibitor DAPA was synthesized as previously described (Nesheim et al., 1979). Bovine blood used for the isolation of prothrombin, factor V, and factor X was obtained from McFedridge Meats, Glenburnie, Ontario, Canada.

Proteins. Bovine prothrombin, factor V, and factor X were isolated according to previously published procedures (Mann, 1976; Nesheim et al., 1981; Krishnaswamy et al., 1987). Prothrombin was further purified by gel filtration chromatography on ACA-34 as described by Boskovic et al. (1990). All proteins were determined to be pure by analysis by $DodSO_4$–PAGE on 5–15% gradient minigels according to Neville (1971) with staining by Coomassie Brilliant Blue. Factor V (0.2 mg/mL) was activated to factor Va (Va) immediately prior to use in 0.02 M Tris-HCl and 0.15 M NaCl, pH 7.4 (Tris–saline), by incubation with human α-thrombin at a final concentration of 2 NIH units/mL at 37 °C for 5 min. Factor X was activated with purified factor X activator of Russels viper venom, and factor Xa (Xa) was isolated by chromatography on benzamidine Sepharose as described previously (Krishnaswamy et al., 1987). The prothrombin activator from *E. carinatus* venom (ecarin) was isolated using steps including ion-exchange chromatography on DEAE-cellulose and preparative polyacrylamide gel electrophoresis as previously described (Hibbard et al., 1982). The prothrombin activation intermediates prethrombin 1 and fragment 1 were isolated according to Mann (1976). Prethrombin 2 and fragment 1.2 were prepared from 20 mg of bovine prothrombin that had been passed over a polyclonal burro anti-bovine factor V column [for description see Boskovic et al. (1990)], diluted to 1 mg/mL, and activated in 0.05 M sodium phosphate and 0.05 M NaCl in the presence of 20 μM DAPA with 1 μM factor Xa. After allowing the reaction to progress for ~45 min at 22 °C, the reaction was quenched with the addition of PPACk and dEGRCk to final concentrations of 10 μM (to inhibit thrombin and Xa activities, respectively). The reaction mixture was passed over SP-C50 and DEAE-Sephadex columns (15-mL bed volume in 30-mL disposable syringes) linked in tandem and previously equilibrated in the same buffer at 22 °C. In all cases the elution of proteins from the columns was monitored using measurements of absorbance at 280 nm of the collected fractions. After the reaction mixture had been loaded, the columns were washed with 50 mL of 0.05 M sodium phosphate and 0.05 M NaCl, pH 6.5, and separated. The flow-through fractions (~80-mL total volume), which contained Pre2, were pooled and loaded directly onto a Pharmacia Mono-S column equilibrated in 0.05 M NaCl and 0.05 M sodium phosphate, pH 7.0, at 4 °C, using a 50-mL superloop and two injections. Pre2 was eluted from the Mono-S column with a 20-mL gradient over 40 min, starting with the equilibration buffer, and ending with 0.45 M NaCl and 0.05 M sodium phosphate, pH 7.0. The absorbance of the eluate was monitored at 280 nm, and 1-mL fractions were collected. After separating the two columns and equilibrating the DEAE column in Tris–saline at 22 °C, F1.2 was eluted from the DEAE column with a 200-mL gradient from 0.02 M Tris-HCl and 0.15 M NaCl

[2] Abbreviations: TFA, trifluoroacetic acid; DAPA, dansylarginine *N*-(3-ethyl-1,5-pentanediyl)amide; GnHCl, guanidine hydrochloride; $DodSO_4$–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Pre1, prethrombin 1 (residues 156–582 of prothrombin); Pre2, prethrombin 2 (residues 275–582 of prothrombin).

to 0.066 M Tris-HCl and 0.5 M NaCl, pH 7.4. Prothrombin fragment 2 was isolated by activating 20 mg of bovine Prel (0.25 mg/mL) in Tris–saline with 2.5 nM Xa in the presence of 10 nM Va, 20 μM PCPS, 2.0 mM Ca^{2+}, and 10 μM DAPA. After incubation for ~30 min at 22 °C, the reaction mixture was diluted 2-fold by the addition of H_2O and passed over tandem SP-C50 and DEAE-Sephadex columns at room temperature as described above (previously equilibrated in 0.01 M Tris-HCl and 0.08 M NaCl, pH 7.4). After the reaction mixture had been loaded, the columns were washed with 50 mL of the equilibration buffer and separated. Following a wash with 1 column volume of Tris–saline, fragment 2 was eluted from the DEAE column with a 100-mL linear gradient of Tris–saline to 0.066 M Tris-HCl and 0.5 M NaCl, collecting 2.5-mL fractions. Bovine thrombin was prepared by incubating bovine prothrombin at 0.2 mg/mL in 0.02 M Tris-HCl and 0.1 M NaCl, pH 7.0, with 1 nM Xa, in the presence of 2 mM Ca^{2+}, 1 nM Va, and 10 μM PCPS. After incubating for ~50 min at 22 °C, EDTA (5 mM) was added, and the solution was passed over a 15-mL SP-C50 column (15 mL of resin in a 30-mL syringe), equilibrated in 0.02 M Tris-HCl and 0.1 M NaCl, pH 7.0, at room temperature. After a column wash with 0.05 M sodium phosphate and 0.1 M NaCl, the thrombin was eluted with a 100-mL linear gradient 0.1–0.5 M NaCl in 0.05 M sodium phosphate at pH 7.0.

Monitoring Prothrombin Activation via Intrinsic Fluorescence. An aliquot of bovine prothrombin (~200 μL) from a stock solution in 50% glycerol was diluted to 1 mL and dialyzed against 4 L of Tris–saline at 4 °C for 2 h. The prothrombin was diluted to a final concentration of 1.4 μM in the same buffer with 2.0 mM Ca^{2+}, and PCPS was added to a final concentration of 20 μM. A quartz cuvette containing 4 mL of this mixture and a micromagnetic stir bar was placed at 22 °C in the sample compartment of a Perkin-Elmer MPF-66 fluorescence spectrophotometer controlled by a Perkin-Elmer 7500 minicomputer using CLS software, and the solution was continuously stirred. The excitation and emission wavelengths were set at 280 and 340 nm, respectively, and 5-nm slit widths were used on both beams. A 290-nm emission filter was used to reduce artifacts from scattered light. After a stable base line was achieved, the activators, as specified in Results, were added, and fluorescence intensity at 340 nm was continuously monitored.

Determination of Molar Fluorescence Values. Small aliquots of purified prothrombin or its activation intermediates were diluted into Tris–saline and dialyzed against the same. Typically a 50-μL aliquot of protein from a stock solution in 50% glycerol was diluted to 1 mL and dialyzed vs 4 L of buffer for 2 h at 4 °C. Protein concentrations were determined from the molar extinction coefficients for the individual proteins (Mann et al., 1981) and measurements of absorbance at 280 nm, corrected for Raleigh scatter at 320 nm. The protein solution was diluted such that the A_{280} for each sample was approximately 0.05, and the fluorescence intensity was determined after a stable signal was achieved (λ_{ex} = 290 nm, λ_{em} = 340 nm, 5- and 3-nm slits, respectively, with a 290-nm long-pass filter in place, at 22 °C). Minor corrections for the internal filter effect were made as described by Boskovic et al. (1990).

Modification of Proteins with N-Bromosuccinimide. Single-point modifications of thrombin, PPACk–thrombin, and Pre2 were carried out in situ in a quartz cuvette maintained at 22 °C in the sample compartment of the MPF-66 fluorescence spectrophotometer. A small volume of a freshly prepared 25 mM stock solution of NBS corresponding to 3 equiv/tryptophan was added to the protein (2–3 μM) in 0.05 M sodium acetate buffer at pH 4.5, and the reactions were monitored to completion by tryptophan fluorescence as outlined above. In the case of titrations of thrombin and Pre2 with NBS, the proteins were present at 1 mg/mL in 0.1 M sodium phosphate buffer at pH 6.5. After each addition of NBS, when the fluorescence signal had decreased to a stable level (~2 min), a 250-μL aliquot was removed from the reaction and quenched by the addition of 250 μL of 6 M GnHCl, 0.02 M Tris-HCl, 0.15 M NaCl, and 2 mM tryptophan, pH 8.0.

Tryptic Digestion of Thrombin and Pre2. Samples of thrombin and Pre2 (0.25 mg) in 0.5 mL of 6 M GnHCl, 0.02 M Tris-HCl, and 0.15 M NaCl at pH 8.0 were incubated for 2 h at 22 °C with 25 mM dithiothreitol. The samples were then made 60 mM with respect to iodoacetic acid and incubated in the dark under N_2 at 22 °C for 2 h. Samples were dialyzed extensively with three changes of 2 L overnight against 0.1 M ammonium bicarbonate, pH 8.3. Tryptic digests were obtained by the addition of 5% w/w TPCK–trypsin from a stock solution (1 mg/mL in 0.1 M HCl) and subsequent incubation at 37 °C with shaking for 32–48 h. Small aliquots were lyophilized prior to HPLC analysis. Protein recoveries were monitored using trace amounts of [^{125}I]-labeled thrombin and determined by γ-scintillation counting of a 50-μL sample immediately prior to HPLC separation.

HPLC Analysis. HPLC separations of the products of tryptic digests of thrombin and Pre2 were made using a Perkin-Elmer Series 10 system, equipped with a Pharmacia Pep-S C2/C18 reversed-phase column (4 × 250 mm) run at 22 °C. Lyophilized samples were typically dissolved in 0.1 mL of buffer A [0.07% TFA (v/v) in HPLC-grade water], and 50-μL aliquots were injected (the remainder was used to determine recovery as outlined above). The column was developed with a 45-min gradient of 10–55% buffer B (0.07% TFA in 70% acetonitrile) following a 10-min wash with 10% B at 1 mL/min flow rate. Both absorbance and fluorescence of the effluent were monitored, using tandem detectors. Absorbance was monitored at 215 nm with a Perkin-Elmer LC-75 detector, and fluorescence was monitored with a Perkin-Elmer MPF-66 fluorescence spectrophotometer equipped with a 25-μL flow cell (λ_{ex} = 280 nm, λ_{em} = 345 nm, with 5- and 10-nm excitation and emission slits and a 290-nm emission filter). Further resolution of HPLC peak 5 (Figure 4) into two components (5a, 5b) was achieved by running a shallow gradient from 25% to 35% buffer B over 45 min.

The tryptophan-containing peptides isolated by HPLC were identified on the basis of amino acid composition, as determined using an Applied Biosystems Model 420H amino acid analyzer with on-line PTC derivitization and reversed-phase separation on a C18 column. Two peaks, 5a and 5b, were further characterized with 10 rounds of amino acid sequencing performed on an Applied Biosystems Model 475A protein sequencer with on-line PTH analysis, and reversed-phase separation with a C18 column.

Analysis of Prethrombin-2 and Thrombin by Gel Filtration. Aliquots of thrombin and Pre2 were analyzed by gel filtration chromatography on a Pharmacia Superose-12 HR 10/30 gel filtration column run by a Pharmacia FPLC system. The column was run at 4 °C at a flow rate of 0.25 mL/min, in 0.02 M HEPES and 0.45M NaCl. The column was calibrated using proteins of known hydrodynamic radius; these were chymotrypsinogen, ovalbumin, and bovine serum albumin [with hydrodynamic radii of 22.5, 27.6, and 36.1 Å, respectively (Tanford, 1961)]. The void volume and included volume were

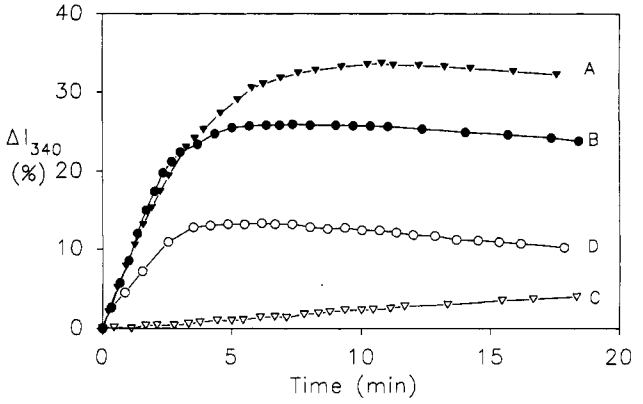

FIGURE 2: Path-dependent increases in intrinsic fluorescence. Changes in the intrinsic protein fluorescence during cleavage of prothrombin or Pre2 were monitored. The sample was continuously excited at 280 nm with a 2-nm excitation slit, and fluorescence intensity at 340 nm with a 5-nm emission slit was recorded. A 290-nm long-pass filter was in place to reduce artifacts from scattered light. The increase in intrinsic fluorescence is expressed as a percentage of the base-line signal. Prothrombin was cleaved at either Arg^{323}-Ile^{324} (using ecarin, trace A) or Arg^{274}-Thr^{275} (using factor Xa alone, trace C) or was cleaved at both sites using prothrombinase (Xa, Va, PCPS) as the activator (trace B). Cleavage of Pre 2 with ecarin is represented by trace D.

Table I: Relative Molar Intrinsic Fluorescence Values

Protein	Molar Fluorescence	Schematic
Prothrombin	1.00	
Pre1	0.99	
Pre2	1.52	
Thrombin	0.98	
F1	0.09	
F2	0.04	
F1.2	0.18	

determined by the elution positions of Blue dextran and benzamidine, respectively. Stokes radii were interpolated from a plot of partition coefficient $(V_e - V_0)/V_i$ vs log Stokes radius.

RESULTS

Changes in Intrinsic Fluorescence upon Selective Cleavages at the Two Prothrombinase Cleavage Sites in Prothrombin. Intrinsic fluorescence profiles obtained during prothrombin activation under various conditions are indicated in Figure 2. In trace B, cleavage was obtained using the full prothrombinase complex [factor Xa (2.0 nM), factor Va (2.0 nM), PCPS (20 μM), and Ca^{2+} (2.0 mM)] in order to effect cleavage at both Arg^{323}-Ile^{324} and Arg^{274}-Thr^{275}. Cleavage at both of these sites to produce thrombin was characterized by a 25% increase in intrinsic fluorescence. Alternatively, conditions were chosen to effect cleavage at either Arg^{323}-Ile^{324} or Arg^{274}-Thr^{275}. When factor Xa alone (25 nM) was used as the activator, the bond at Arg^{274}-Thr^{275} was selectively cleaved (Krishnaswamy et al., 1987), producing fragment 1.2 and Pre2, and only a 5% increase in intrinsic fluorescence was observed (Trace C). Conversely when the bond at Arg^{323}-Ile^{324} was preferentially cleaved with the snake venom-derived protease ecarin (Morita & Iwanaga, 1981), to produce meizothrombin, a 35% increase in intrinsic fluorescence was observed (trace A). In all cases, the reaction mixtures were intermittently sampled and aliquots were analyzed on 5–15% gradient minigels to ensure the expected cleavage events had occurred. This was uniformly the case. These results together indicate that the 25% increase in intrinsic fluorescence that was observed upon prothombin activation was primarily due to bond cleavage at Arg^{323}-Ile^{324} between the A and B chains of the protease domain of prothrombin. When the analogous cleavage was performed using the immediate precursor for thrombin, Pre2, as the substrate for ecarin, a similar increase in intrinsic fluorescence was observed, although the magnitude of change was less than half that seen with cleavage of prothrombin (Figure 2, trace D). This result suggests that the presence of the F1 and F2 domains is necessary to see the full increment in fluorescence, but the change that occurs can be largely attributed to changes in environments of tryptophan residues within the protease (Pre2) domain. The modest declines in fluorescence intensities observed at later times in traces A, B, and D were not due to photobleaching, as the same observation was made when the excitation beam was blocked and intrinsic fluorescence determined at discrete intervals. These declines likely reflect further subtle changes in structure of the corresponding species. Although the data are not shown, the fluorescence decline following the Pre2 to thrombin activation has been monitored over a period of an hour following cleavage, whereupon the signal returns to a value near that of the initial base line.

Molar Intrinsic Fluorescence Values for Prothrombin and Its Isolated Domains. The contributions of the isolated domains of prothrombin to its overall intrinsic fluorescence are indicated in Table I, where the fluorescence value of prothrombin was normalized to 1.0, and all other values are expressed relative to it. In the absence of subtle influences of the Pre2 and F1.2 domains on their respective structures, one would expect their molar fluorescence values to add up to that of prothrombin (1.0). This was not the case, as the summed values for Pre2 and F1.2 (1.52 + 0.18) clearly do not equal 1.0. Indeed, Pre2 alone, although it only has 9 of the 14 tryptophan residues of prothrombin, had a relative molar fluorescence value in excess of 1.0. This result suggests that some portion of the F1.2 domain quenches both the fluorescence of the Pre2 domain in intact prothrombin, and in Pre2 produced in situ (only a 5% increase was seen when prothrombin was cleaved to Pre2 and F1.2). Since the molar fluorescence value for Pre1 (Pre2 + F2) was near that of prothrombin, the F2 rather than the F1 domain was likely responsible for this quenching effect. In addition, the molar fluorescence of thrombin was roughly equivalent to that of prothrombin and significantly less than that of Pre2. Since activation of prothrombin or Pre2 in situ produces an increase in intrinsic fluorescence, these results suggest that thrombin as isolated may be structurally different than initially produced in situ.

Gel Filtration Analysis of Pre2 and Thrombin. Thrombin and Pre2 have identical molecular masses, and thus changes in global structure should be reflected in differences between the hydrodynamic radii for the two species. In order to assess the global extent of changes in structure occurring upon activation of Pre2 to thrombin, these two species were subjected to analysis by gel filtration on a calibrated column of Superose-12. The analysis indicated that Pre2 elutes slightly ahead of thrombin with an elution volume of 13.68 ± 0.02 mL compared to that of 13.82 ± 0.03 mL for thrombin from a column for which the included and excluded volumes are 26.85 and 6.73 mL, respectively. These results correspond to a change in Stokes radius from 24.9 ± 0.1 to 24.2 ± 0.2 Å and indicate a small but detectable change in overall structure upon the conversion of the zymogen Pre-2 to thrombin, suggesting that the enzyme is slightly more compact.

Modification of Thrombin, Pre2, and Active-Site-Blocked Thrombin with NBS. The time courses of tryptophan modification in thrombin, Pre2, and active-site-blocked

Table II: Identification of HPLC Peaks by Amino Acid Analysis, Comparing Obtained Composition to Expected Composition

residue	1 W572[a] {W237} W^{572}–K^{575}	2 W415 {W96} Y^{413}–K^{416}	3 W464 {W141} V^{461}–G^{468}	4 W360 {W51} W^{360}–C^{367}	5a W337 {W29} I^{324}–K^{344}	5b W471 {W148} E^{469}–K^{497}	6 W542/550 {W207/215} W^{542}–K^{559}	7 W542/550 {W207/215} W^{542}–R^{556}	8 W373 {W60d} L^{368}–R^{385}
D	0.1 (0)[b]	1.1 (1)	0.1 (0)	1.2 (1)	1.1 (1)	0.8 (1)	1.6 (2)	1.1 (1)	3.9 (4)
E	1.1 (1)	0.1 (0)	0.0 (0)	0.4 (0)	3.5 (4)	5.0 (5)	2.0 (2)	2.4 (2)	0.6 (0)
S	0.1 (0)	0.1 (0)	0.1 (0)	0.2 (0)	1.6 (1)	1.9 (2)	1.4 (1)	1.0 (1)	0.1 (0)
G	0.1 (0)	0.4 (0)	1.4 (1)	0.4 (0)	2.1 (2)	1.9 (0)	4.7 (4)	3.7 (3)	0.2 (0)
H	0.3 (0)	0.1 (0)	0.0 (0)	0.9 (1)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)
R	0.4 (0)	0.1 (0)	0.0 (0)	0.1 (0)	0.4 (1)	0.2 (1)	1.0 (1)	1.0 (1)	1.0 (1)
T	0.0 (0)	1.0 (1)	1.0 (1)	1.0 (0)	0.0 (0)	4.0 (3)	0.2 (0)	0.1 (0)	0.7 (1)
A	0.0 (0)	0.0 (0)	0.0 (0)	2.9 (3)	1.2 (1)	2.3 (2)	0.6 (0)	0.2 (0)	0.1 (0)
P	0.1 (0)	0.2 (0)	0.0 (0)	0.2 (0)	1.2 (1)	1.6 (3)	1.0 (1)	0.2 (0)	2.1 (2)
Y	0.0 (0)	1.2 (1)	0.0 (0)	0.1 (0)	0.0 (0)	0.0 (0)	0.8 (1)	0.4 (1)	0.6 (1)
V	0.3 (0)	0.1 (0)	1.0 (1)	1.0 (1)	1.1 (3)	3.5 (7)	1.2 (1)	1.0 (1)	1.9 (2)
M	0.0 (0)	0.0 (0)	0.0 (0)	0.1 (0)	0.0 (0)	0.0 (0)	1.0 (1)	1.0 (1)	0.0 (0)
C	0.0 (0)	0.1 (0)	0.0 (0)	+[c] (1)	0.0 (0)	+ (1)	+ (1)	+ (1)	0.0 (0)
I	0.7 (1)	0.0 (0)	0.0 (0)	1.4 (1)	0.6 (1)	0.0 (0)	1.3 (1)	0.8 (1)	0.0 (0)
L	0.0 (0)	0.0 (0)	0.0 (0)	3.1 (3)	2.3 (3)	2.6 (3)	1.5 (0)	0.4 (0)	3.9 (4)
F	0.0 (0)	0.0 (0)	0.0 (0)	0.1 (0)	0.0 (1)	1.0 (0)	0.0 (0)	0.1 (0)	1.1 (1)
K	1.0 (1)	1.0 (1)	0.0 (0)	0.5 (1)	0.4 (1)	0.6 (1)	1.0 (1)	0.1 (0)	1.1 (1)
W	+ (1)	+ (1)	+ (1)	+ (1)	+ (1)	+ (1)	+ (2)	+ (2)	+ (1)

[a] The numbering system for tryptophan residues is based on that of bovine prothrombin, with the numbering in braces after that of Bode et al. (1989), based on chymotrypsinogen. [b] Expected values based on the sequence of bovine prothrombin are in parentheses. [c] Plus sign (+) denotes the presence of Cm-cystine or tryptophan, which were not quantified.

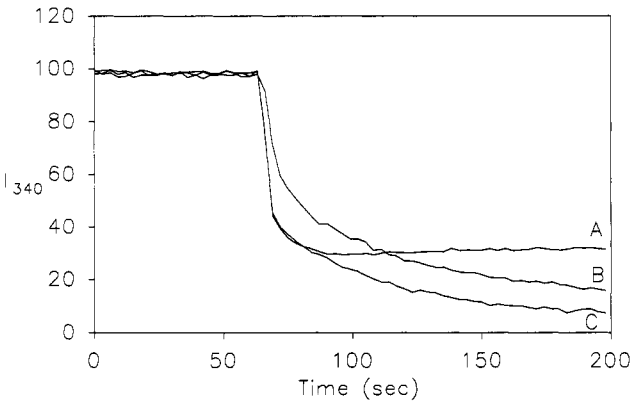

FIGURE 3: Profiles of tryptophan modification with NBS in Pre2, thrombin, and active-site-blocked thrombin. Fluorescence intensity was monitored (λ_{ex} = 280 nm, 5-nm slit, λ_{em} = 340 nm, 7.5-nm slit, 290-nm long-pass filter) during modification of Pre2, thrombin, and thrombin treated with Phe-Pro-Arg chloromethyl ketone. Protein concentrations were 2 μM, and a 3-fold molar ratio of NBS to tryptophan was used. A 25 mM stock of NBS was freshly prepared from NBS recrystallized from dH_2O, and the modifications were carried out in 0.05 M sodium acetate buffer, pH 4.5. Profiles for each of the proteins are as follows: trace A, Pre2; trace B, PPACk–thrombin; trace C, thrombin.

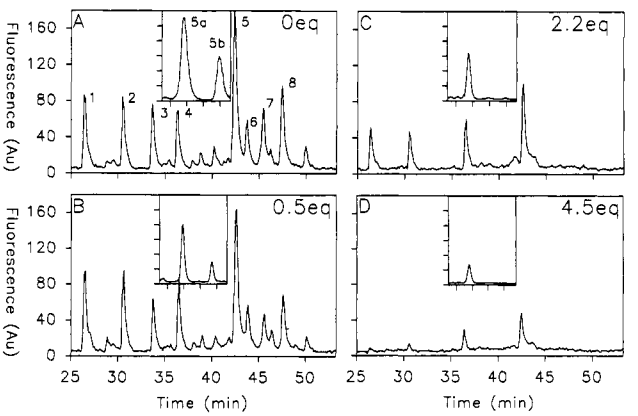

FIGURE 4: HPLC profiles of tryptic digests of thrombin and thrombin modified with NBS. A sample of thrombin (1 mg/mL) was titrated with NBS, and samples were removed at various intervals (between 0.5 and 4.5 equiv of NBS/Trp). Samples were denatured, reduced, carboxymethylated, and digested with trypsin as described in Materials and Methods. Aliquots of the digested material were lyophilized and resuspended in 0.07% TFA (buffer A) and loaded onto a Pharmacia Pep-S C2/C18 reversed-phase column. Following a 10-min wash with the loading buffer, the column was eluted with 10–55% buffer B (0.07% TFA in 70% acetonitrile). Tryptophan-containing peptides were detected by intrinsic fluorescence (λ_{ex} = 280, λ_{em} = 345). Representative panels are shown, comparing the elution profile of unmodified thrombin (A, 0 equiv) to thrombin modified with 0.5, 2.2, and 4.5 equiv of NBS/Trp (panels B–D). Peak 5 was further resolved into two components by a shallow gradient from 25% to 35% buffer B, as shown in the inset.

thrombin [irreversibly inhibited with Phe-Pro-Arg chloromethyl ketone (PPACk–thrombin)] are compared in Figure 3. These proteins (0.2 μM in 0.05 M sodium acetate, pH 4.5) were treated with 3 equiv of NBS/tryptophan, and the time courses of changes in intrinsic fluorescence were monitored. Modification of thrombin by NBS appeared to take place in at least three temporally resolved phases. There was an initial rapid modification in which 60% of the initial fluorescence signal was lost, followed by an intermediate phase in which a further 15% of the fluorescence signal was lost, and finally a slow phase occurred during which a further 17% of the signal was lost. Both Pre2 and PPACk–thrombin exhibited elements of this modification pattern. Pre2 was modified in a similar fashion to thrombin through the rapid and intermediate stages, but residues affected in the slow phase of thrombin modification appeared to be protected in Pre2. The active-site-blocked thrombin, on the other hand, was less susceptible to the rapid phase modification, but showed a curve parallel to that of thrombin for the intermediate and slow phases. These data indicate that, under these conditions, tryptophan residues of the three proteins are differentially susceptible to modification by NBS, implying subtle differences in the environments of the tryptophan residues between species.

Separation and Identification of Tryptophan-Containing Peptides from Tryptic Digestions of Thrombin and Pre2. Digestion of either Pre2 or thrombin with trypsin (5% by weight) for 32–40 h, followed by separation using reversed-phase HPLC on a Pharmacia Pep-S C2/C18 column with fluorescence detection, yielded identical patterns consisting of 8 major tryptophan-containing peaks (Figure 4A). Peak 5 was further resolved into two components, using a shallow water to acetonitrile gradient. These 9 tryptophan-containing peptides were identified on the basis of their amino acid

Table III: Equivalents of NBS Required for 50% Modification of Trp Residues in Pre2 and Thrombin

	residue							
protein	W373 {W60D}	W542/550 {W207/215}	W471 {W148}	W464 {W141}	W572 {W237}	W415 {W96}	W337 {W29}	W360 {W51}
Pre2	1.2	1.3	0.6	1.0	2.5	2.9	3.75	3.75
IIa	0.6	0.7	0.75	1.0	3.0	3.6	4.75	4.75

Table IV: Amount of Tryptophan Remaining Unmodified (%) after NBS Treatment

	residue in Pre2							
NBS/Trp	W373 {W60D}	W542/550 {W207/215}	W471 {W148}	W464 {W141}	W572 {W237}	W415 {W96}	W337 {W29}	W360 {W51}
0.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
0.56	62.42	67.80	52.58	59.74	82.77	81.97	83.84	82.18
1.11	50.94	55.91	26.62	46.30	94.86	91.81	103.3	98.12
2.23			7.94		57.62	68.44	89.61	83.58
3.34					20.33	37.48	64.12	60.65
4.46						16.03	28.49	25.06
	residue in thrombin							
NBS/Trp	W373 {W60D}	W542/550 {W207/215}	W471 {W148}	W464 {W148}	W572 {W237}	W415 {W96}	W337 {W29}	W360 {W51}
0.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
0.56	70.84	82.90	72.18	83.16	108.70	111.36	113.6	113.82
1.12			9.12	40.00	95.78	95.10	104.8	105.33
2.25				17.05	70.77	73.38	120.2	109.10
3.37					35.52	54.68	119.7	107.54
4.50					13.48	23.89	67.75	55.90

composition as presented in Table II. The identities of peaks 5a and 5b were further confirmed by 10 rounds of amino acid sequencing. Peak 5a yielded a single sequence matching that expected for the peptide I^{324}–K^{344}, while the sequence for peak 5b revealed the presence of a coeluting peptide corresponding to T^{288}–R^{299} in addition to expected sequence for the assigned peptide. This contaminant which is rich in glycine and contains phenylalanine explains the relatively poor match between expected and obtained compositions. The contaminating peptide does not contain tryptophan and therefore should not interfere with subsequent analyses. Peaks 6 and 7 represent the same two tryptophan residues (542/550 {W207/W217}) migrating on peptides with alternate cleavage of a carboxyl-terminal tripeptide. These peaks have been grouped together for analysis, and although we cannot distinguish between the two tryptophan residues, some inferences about their accessibility in both Pre2 and thrombin can nonetheless be made.

Determination of Extent of Modification at Individual Tryptophan Residues. A sample of either Pre2 or thrombin was titrated with increasing amounts of NBS (0.5–4.5 equiv/Trp), and the subsequent reactions were monitored by intrinsic fluorescence. When the signal was stable after each addition (~2 min), an aliquot was removed for analysis of the extent of modification at each of the tryptophan residues. Samples were reduced, carboxymethylated, and subjected to a full tryptic digestion. Peptides containing tryptophan were separated and quantitated using RP-HPLC with fluorescence detection. The chromatographic profiles of thrombin modified with increasing amounts of NBS are presented in Figure 4, panels A–D, with the quantitation, corrected for recovery, appearing in Table IV. Panel A of Figure 4 represents unmodified thrombin subjected to full tryptic digestion, while panels B–D display profiles obtained upon modification with 0.5, 2.2, and 4.5 mol of NBS/mol of tryptophan, respectively.

The tryptophan residues of Pre2 and thrombin displayed a 10-fold range of sensitivities to modification by NBS, where sensitivity is defined as the amount of NBS required for 50% modification. As shown in Table III, this extent of modification of the individual residues occurred between 0.5 and 5 equiv of NBS/Trp.

Differential Susceptibilities of Selected Tryptophan Residues in Thrombin and Pre2. In Figure 5, the relative sensitivities of the individual tryptophan residues to modification are presented. Values are expressed in fold differences in susceptibility of the individual residues in thrombin compared to those in Pre2. Positive values indicate residues modified less readily in thrombin, and negative values represent residues modified more readily in thrombin than in Pre2. These studies indicated that tryptophan residues 337 and 360 {W29, W51} were modified less readily in thrombin than in Pre2, suggesting that they change environments upon activation, becoming less accessible to the reagent. In contrast, tryptophan residues 542, 550, and 373 {W207, W215, W60D} were modified more readily in thrombin than in Pre2, indicating that they are more accessible to NBS. Although residues 542 and 550 {W207/W215} migrate on the same peptide, since both residues are completely modified at a lower level of NBS in thrombin than in Pre2, at least one and likely both of these tryptophans are more accessible in thrombin than in Pre2. A third group of residues, residues 415, 464, 471, and 572 {W96, W141, W148, W237}, are equally susceptible to modification in Pre2 and thrombin. Three of the tryptophan residues that are differentially susceptible to modification are located in and around the active site of thrombin. Tryptophans 360 and 373 {W51, W60D} bracket the active site histidine (366 {H57}). Since both of these residues change susceptibility to modification, in that one is modified less readily in thrombin and one more readily, a change in protein conformation apparently occurs proximal to H366 {H57} upon generation of an active site. In addition, tryptophan residues W373 and W550 {W60D, W215} have both been implicated in substrate binding (Rydel et al., 1990; Bode et al., 1989, 1992), and their increased susceptibility to modification in thrombin may represent increased accessibility of the substrate binding site upon activation.

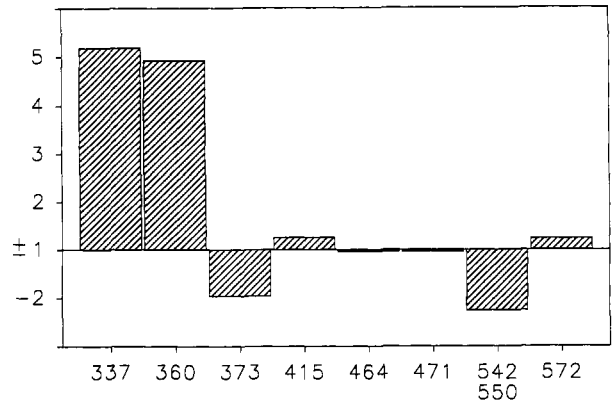

FIGURE 5: Relative susceptibility of tryptophan residues in Pre2 and thrombin to modification by NBS. Relative susceptibilities of individual tryptophan residues to NBS modification in Pre2 and thrombin were determined by a comparison of areas under plots of % modification vs [NBS]. Values are expressed as fold differences in susceptibility. Positive values indicate residues more susceptible in Pre2 than thrombin, while negative values indicate residues more susceptible to modification in thrombin than in Pre2.

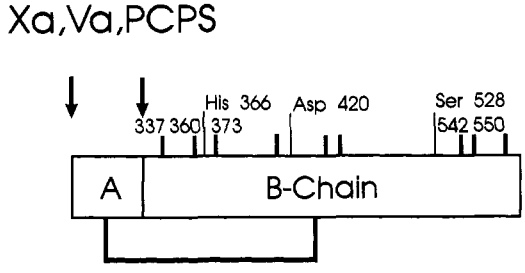

FIGURE 6: A schematic diagram of IIa, depicting prothrombinase cleavage sites, tryptophan residues, and active site residues. Prothrombinase (Xa, Va, PCPS) cleavage sites at Arg^{323}-Ile^{324} and Arg^{274}-Thr^{275} are indicated by arrows. Active site residues of thrombin (His 366, Asp 422, and Ser 528) are located as indicated. Tryptophan residues are indicated by the hatched marks. Those that change susceptibility to modification upon activation are identified by sequence number.

DISCUSSION

The results of the present study provide evidence for structural changes that occur during the creation of an active site upon activation of Pre2 to thrombin. Although Pre2 and thrombin both represent the same portion of the prothrombin backbone and differ only in that thrombin is cleaved at Arg^{323}-Ile^{324}, while Pre2 has this bond intact, the two proteins have very different activities. Pre2 is proteolytically inactive, while thrombin displays a range of proteolytic activities. The results obtained from the comparison of the modification characteristics of Pre2 and thrombin with NBS can be analyzed in light of the crystal structure of PPACk-inhibited thrombin (Bode et al., 1992). This crystal structure has been very well-defined and extensively analyzed. PPACk–thrombin is an active site derivative of thrombin, with the tripeptide chloromethyl ketone inhibitor PPACk covalently attached to the active site histidine. In the refined structure, the positions of both the enzyme and inhibitor are characterized. Since the other available crystal structures of thrombin are quite similar (with one notable exception which will be later discussed), and PPACk–thrombin is perhaps the best characterized, the positions of tryptophan residues are discussed in terms this structure.

Tryptophan residues in both thrombin and Pre2 displayed a range of susceptibilities to modification by NBS. Those residues which were most sensitive to modification are all in highly accessible locations in the crystal. Residues 373, 550, and 471 {W60D, W215, W148} are all at or near the active site, with residues 373 and 471 {W60D, W148} bracketing the entrance and residue 464 {W215} lying within the active site. Residue 471 {W148} occupies quite different positions in the crystal structures for PPACk–thrombin (Bode et al., 1992) and hirudin-inhibited thrombin (Rydel et al., 1990), suggesting the surface loop on which it resides is quite flexible. In both structures, however, this residue remains quite accessible. Two other tryptophans which were sensitive to modification, 542 and 464 {W207, W141}, also lie at or near the surface. Tryptophan 542 {W207} lies in a shallow groove at the interface between the A and B chains, and 464 {W141} at the fibrin secondary binding exosite. The least susceptible residues, tryptophans 337 and 360 {W29, W51}, are buried within the protein.

Tryptophan residues flanking the active site histidine, 360 and 373 {W51, W60D}, both change susceptibility to modification upon conversion of Pre2 to thrombin as indicated in Figure 6. Tryptophan 360 {W51} becomes less accessible to the reagent while 373 {W60D} becomes more accessible. Together, these changes suggest that the active site histidine may also experience a change in environment. Since it has been previously shown that Pre2 displays weak binding to DAPA, a reversible fluorescent active site inhibitor (Hibbard et al., 1982) (thrombin binds DAPA about 30 times more tightly than Pre2), but not enzymatic activity, this change at the active site may be responsible for making thrombin a competent enzyme. Alternatively, since tryptophan residue 337 {W29}, poorly accessible in Pre2, like tryptophan 360 {W51} becomes less accessible in thrombin, this change may indicate that thrombin has a more compact and stable structure than Pre2, which is consistent with the values for Stokes radii obtained by gel filtration. Two tryptophan residues that have been implicated in substrate binding, 373 and W550 {W60D, W215}, both become more accessible to modification with the development of an active site in thrombin and are modified more readily in thrombin than in Pre2. Since DAPA in the active site of thrombin is able to accept energy transfer from tryptophan, movement of these residues might explain the tighter binding and more efficient energy transfer to DAPA from thrombin than from Pre2. Unlike the other residues exposed at the active site, residue 472 {W148} is equally accessible to modification in Pre2 and thrombin.

The studies with intrinsic fluorescence revealed that the majority of the fluorescence change associated with prothrombin activation can be attributed to cleavage at Arg^{323}-Ile^{324}. Indeed, the increase seen with cleavage of prothrombin by Xa alone at Arg^{274}-Thr^{275} might be due to production of a small amount of thrombin as a side product. A comparison of the molar fluorescence data with the results of monitoring intrinsic fluorescence during cleavage of prothrombin at either Arg^{323}-Ile^{324} or Arg^{274}-Thr^{275} suggests that the F2 domain of prothrombin quenches the fluorescence of the Pre2 domain, but not that of thrombin. Pre2, although it has only 9 of the 14 tryptophan residues present in prothrombin, has a higher molar fluorescence than either Pre1 or prothrombin, suggesting that the F2 domain modulates the fluorescence of the Pre2 domain. Cleavage of prothrombin to Pre2 and F1.2 is not, however, accompanied by a large increase in fluorescence signal. Earlier studies indicate that F2 interacts tightly and noncovalently with both thrombin and Pre2 (Myrmel et al., 1976). Since cleavage of prothrombin to meizothrombin or thrombin is accompanied by an increase in intrinsic fluorescence, the F2 domain must interact differently with the protease domain when the bond at Arg^{323}-Ile^{324} is cleaved. Indeed, cleavage of prothrombin to meizothrombin is accompanied by almost 3 times the relative change in intrinsic

fluorescence that characterizes conversion of isolated Pre2 to thrombin. Part of this difference may also be explained by the fact that Pre2 has a higher initial molar fluorescence value than prothrombin (1.52 vs 1.0).

A comparison of the molar intrinsic fluorescence values of Pre2 and thrombin indicates that Pre2 has a 50% higher fluorescence value than thrombin. On the basis of these results one would expect the Pre2 to thrombin conversion to result in a net decrease in fluorescence. For this transition, however, an increase in signal is observed. This result suggests that thrombin adopts an intermediate conformation upon activation and slowly relaxes to a stable form with lower intrinsic fluorescence, over a period of hours.

Early studies utilizing chemical modification of thrombin with NBS (Uhteg & Lundblad, 1977) indicated that tryptophan is necessary for both amidolytic and clotting activity of thrombin, with function being lost at quite low levels of NBS modification (≤1 NBS/Trp). From the present study, residues 373, 471, and 550 {W60D, W148, W215} would be the obvious candidates for essential tryptophans. All three residues have been shown to be involved in substrate/inhibitor binding at the active site (Bode et al., 1989; Rydel et al., 1990), and all are modified at low levels of NBS. Further study is required to identify the specific residues, which, upon modification, correlate with the loss of functional activity.

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