Fluorescence Properties and Functional Roles of Tryptophan Residues 60d, 96, 148, 207, and 215 of Thrombin*

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Robert Bell‡, Willem K. Stevens§§, Zongchao Jia‡, John Samis¶, Hélène C. F. Côté**,**,
Ross T. A. MacGillivray§, and Michael E. Nesheim‡ ‡‡§§

From the Departments of ‡Biochemistry, §Pathology, and §§Medicine, Queen’s University, Kingston, Ontario K7L 3N6 and the ‡‡Department of Biochemistry, University of British Columbia, Vancouver, British Columbia V6T 1Z3, Canada

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Abstract

Conservative Trp-to-Phe mutations were individually created in human thrombin at positions 60d, 96, 148, 207, and 215. Fluorescence intensities for these residues varied by a factor of 6. Residues 60d, 96, 148, and 215 transferred energy to the thrombin inhibitor 5-dimethylaminonaphthalene-1-sulfonylarginine-N-(3-ethyl-1,5-pentanediyl)amide efficiently, but residue 207 did not. Intensities correlated inversely with exposure to solvent, and measured and theoretical energy transfer efficiencies agreed well. Function was measured with respect to fibrinogen clotting, platelet and factor V activation, inhibition by antithrombin, and the thrombin-activable fibrinolysis inhibitor (TAFI). All activities of W96F and W207F ranged from 74 to 154% of the wild-type activity. This was true for W148F, except for inhibition by antithrombin, where it showed 60% activity. W60dF was deficient by 30, 57, and 43% with fibrinogen clotting, platelet activation, and factor V cleavage (Arg1006), respectively. W215F was deficient by 90, 55, and 56% with fibrinogen clotting, platelet activation, and factor V cleavage (Arg1536), respectively. With protein C and TAFI, W96F, W148F, and W207F were normal. W60dF, however, was 76 and 23% of normal levels with protein C and TAFI, respectively. In contrast, W215F was 25 and 124% of normal levels in these reactions. Thus, many activities of thrombin are retained upon substitution of Trp with Phe at positions 96, 148, and 207. Trp215, however, appears to be very important for TAFI activation, and Trp215 appears to very important for clotting and protein C activation.

Thrombin is a trypsin-like serine protease that is generated upon vascular injury from its plasma precursor prothrombin following a cascade of zymogen-to-enzyme conversions (1, 2). A principal role of thrombin is to catalyze the cleavage of fibrinogen to fibrin (3), which spontaneously polymerizes to form the fibrin clot. Thrombin plays several other procoagulant roles, including activating platelets and up-regulating its own production through proteolytic activation of the essential, non-enzymatic cofactors of coagulation, factors V and VIII (4, 5).

Thrombin also stabilizes the newly formed fibrin clot through the activation of factor XIII (6), a transglutaminase that introduces isopeptide bonds between fibrin protomers, reinforcing the clot structure. In complex with the endothelial cell-surface protein thrombomodulin, thrombin acts as an anticoagulant through the activation of protein C (7) and as an attenuator of fibrinolysis through the activation of the plasma carboxyypeptidase zymogen TAFI (8, 9).

The amino acid sequence of thrombin is highly conserved throughout nature. The B-chains of thrombins from 11 vertebrate species share 75% amino acid identity (10). When the five known mammalian thrombin sequences are compared, 80% identity is observed. The seven cysteine and nine tryptophan residues in the B-chain of all 11 thrombins are invariant.

The crystal structures of thrombin in complex with numerous inhibitors disclose the spatial arrangement of the tryptophan residues (11, 12). Since thrombin is structurally similar to chymotrypsin, the nomenclature of its residues is based on that of chymotrypsin (11). Thus, the tryptophan residues of thrombin are designated Trp29, Trp51, Trp60d, Trp96, Trp141, Trp148, Trp207, Trp215, and Trp237. Four of these are intimately associated in space with the active site of thrombin. Trp60d and Trp148 are on loops that surround the active site and presumably control access to it; Trp215 is in the so-called aryld-binding pocket of the active site and lines the S2-binding site; and Trp96 is located in the active-site cleft. Trp215 is generally conserved in the family of serine proteases, whereas Trp60d and Trp148 are not.

Previous studies indicate that one or more of the tryptophan residues of thrombin are required for activity and are in some way associated with the formation of the active site upon conversion of prothrombin to thrombin. Uhteg and Lundblad (13) and Thompson and Salem (14) showed that limited oxidation of tryptophan residues with N-bromosuccinimide inactivates thrombin for both fibrinogen and small substrates. Other studies have shown that the conversion of prothrombin to thrombin is marked by increases in intrinsic tryptophan fluorescence (15–17). Analogous changes in energy transfer from tryptophan residues of (pro)thrombin to the dansyl moiety of the reversible thrombin inhibitor dansylarginine-N-(3-ethyl-1,5-pentanediyl)amide (DAPA) (18) are observed upon prothrombin conversion to thrombin (19, 20). In addition, differences in the environments of some of the tryptophan residues exist, as inferred by

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** Recipient of a Medical Research Council of Canada studentship.

*** To whom correspondence should be addressed: Dept. of Biochemistry, Botterell Hall, Rm. A212, Queen’s University, Stuart St., Kingston, Ontario K7L 3N6, Canada. Tel.: 613-533-2957; Fax: 613-533-2987; E-mail: nesheimm@post.queensu.ca.

1 The abbreviations used are: TAFI, thrombin-activable fibrinolysis inhibitor; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; DAPA, dansylarginine-N-(3-ethyl-1,5-pentanediyl)amide; PCR, polymerase chain reaction; TAME, p-toluenesulfonylarginine methyl ester; PAGE, polyacrylamide gel electrophoresis; EGF, epidermal growth factor.

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The mutated nucleotides are indicated in boldface.

| Primer | Sequence | Use |
|--------|----------|-----|
| B      | 5’-GGGACATAGAATACGCTGACCTGACGCAGGACCAGCTTGTGAA-3’ | Mutagenesis, first PCR |
| C      | 5’-GAGAAGACGATGTACGAC-3’ | Mutagenesis, third PCR |
| D      | 5’-GAGAAGACGATGTACGAC-3’ | Mutagenesis, third PCR |
| W60dF  | 5’-TACCGGCTTTCCGACAGAAGCT-3’ | W60dF |
| W96F   | 5’-CAGGTACACTCCGGAGCCACG-3’ | W96F |
| W145F  | 5’-CCTGCCCTGGAACGTTCCTCT-3’ | W145F |
| W207F  | 5’-AACGGCCGCTATGCAATAAGG-3’ | W207F |
| W215F  | 5’-ATCGTCTATTCCGGAGG-3’ | W215F |

**EXPERIMENTAL PROCEDURES**

**Materials and Proteins**—The reversible fluorescent thrombin inhibitor DAPA was synthesized as described previously (18). Factor V and human factor X were isolated according to previously published procedures (21, 22). Factor V (0.2 mg/ml) was activated immediately prior to use by the addition of human α-thrombin to a final concentration of 2 NIH units/ml and incubation at 37 °C for 5 min. Factor X was activated with purified Factor X activator from Russell’s viper venom, and Factor Xa was isolated by chromatography on benzamidine-Sepharose as described previously (22). Human protein C and TAFI were isolated as described previously (8, 17). Recombinant soluble thrombomodulin (Solulin) was a generous gift of Dr. John Morser (Berlex Biosciences, Richmond, CA).

**PCR Mutagenesis of Human Prothrombin**—Templates for PCR mutagenesis of the human prothrombin cDNA were generated by digesting the Bluescript-based plasmid pHII (17) with SstI and PstI (for W60dF, W96F, W145F, or with PstI and EcoRI (for W207F and W215F) and subcloning the fragments into Bluescript. Mutagenesis was carried out using the procedure described by Nelson and Long (23) with the primers listed in Table I; oligonucleotides B, C, and D are Bluescript-specific primers for the PCR steps (23). The PCR products were purified, cloned into Bluescript, and sequenced. The full-length prothrombin cDNA sequence was regenerated by ligating the mutant fragment with the corresponding wild-type fragments in a multiple part ligation and verification by restriction enzyme analysis and DNA sequencing. For expression in baby hamster kidney cells (BHK2-21), the mutant human prothrombin cDNAs were ligated into the pNUT vector, which encodes a modified dihydrofolate reductase gene and allows selection at high methotrexate concentrations (24).

**Cell Culture, Transfection, and Selection**—Baby hamster kidney cells were cultured in Dulbecco’s modified Eagle’s medium/nutrient mixture F-12 (1:1) supplemented with 5% newborn calf serum (Life Technologies, Inc., Burlington, Ontario, Canada). Cells were transfected with individual pNUT constructs using the calcium phosphate coprecipitation method (25, 26). Plasmid DNA (10 µg) was precipitated at pH 6.85 and added to 100-mm plates of baby hamster kidney cells at ~40% confluence. After 6 h, the cells were rinsed with one change of Dulbecco’s modified Eagle’s medium/nutrient mixture F-12 and 5% newborn calf serum and allowed to recover for 2 h before selection was initiated by changing the medium to Dulbecco’s modified Eagle’s medium/nutrient mixture F-12 and 5% newborn calf serum containing 1.44 mm methotrexate (David Bull Laboratories, Boucherville, Quebec, Canada). After ~2 weeks of selection, methotrexate-resistant colonies were subcultured into six-well plates and grown to confluence. The highest expressing clones (determined by enzyme-linked immunosorbent assay) were retained and seeded into triple flasks for large-scale production. After the cells became confluent, the medium was switched to serum-free low protein medium (Opti-MEM, Life Technologies, Inc.) supplemented with vitamin K₁ (10 µg/ml; Abbott Laboratories, Montreal, Quebec) and ZnCl₂ (50 µM). The medium was discarded and replaced after 24 h and harvested every other day thereafter.

**Purification of Recombinant Thrombins**—Prothrombin in the conditioned medium (400 ml) was concentrated by slurring the medium with 15 ml of the anion-exchange resin Q-Sepharose (Amersham Pharmacia Biotech, Baie d’Urfe, Quebec) for 45 min. The mixture was then allowed to settle; the supernatant was decanted; and the resin was transferred to a 20-ml Dispotech (Bio-Rad, Mississauga, Ontario). The resin was washed with 50 ml of 0.02 M HEPES and 0.15 M NaCl (pH 7.4), and the adsorbed prothrombin was eluted with 0.02 M HEPES and 0.5 M NaCl (pH 7.4). Following dialysis with 0.02 M HEPES to a final salt concentration of 0.15 M, the prothrombin in the media concentrate was activated to thrombin. The thrombin was then purified, and the active-site titers was determined as described previously (27).

**Measurements of the Contributions of Individual Tryptophan Residues to the Total Fluorescence of Thrombin and Energy Transfer to DAPA**—The absorbances of wild-type thrombin and the various mutants were read at 280 and 300 nm. The pH was then adjusted to 12 with a small aliquot of 5 M NaOH, and the absorbances were again read (to the fourth place after the decimal) with a Perkin-Elmer A6 spectrophotometer. The measurement at 280 nm (pH 7.4) represented primarily tryptophan residues, and the increment that occurred at 300 nm upon increasing the pH specifically measured the tyrosine content of the proteins (28). The measurements of tyrosine indicated that each of the measured tryptophan residues contributes 9.7 ± 3.3% of the absorbance at 280 nm. Thus, the assumption was made that all nine residues contribute equally to the absorbance, as with other proteins (29, 30); and the concentrations of the mutants were calculated from the absorbances at 280 nm, with the extinction coefficient adjusted for the loss of one tryptophan residue. Fluorescence emission spectra were collected between 290 and 500 nm with a Perkin-Elmer LS50B spectrofluorometer using an excitation wavelength of 280 nm and a 290-nm cutoff filter in the emission beam. The protein concentration was typically 40 ng in the cuvette. Spectra were then normalized to the same protein concentration. The spectra of the individual residues were obtained by subtracting the spectrum of the corresponding mutant from that of wild-type thrombin. To determine whether the difference measurements might be confounded by changes in the extents of Trp-Trp or Trp-Tyr energy transfer in wild-type compared with mutant thrombins, theoretical energy transfers for all possible pairs in thrombin were calculated from the distances between them and their orientations provided by the x-ray structure (Protein Data Bank code 1FPC).2 Remarkably, only two non-zero values for energy transfer were calculated despite the presence of nine tryptophan and 14 tyrosine residues in the molecule. The non-zero values were for the pairs Trp29/Trp207 and Trp51/Trp237, where efficiencies of 0.49 and 0.05 were calculated, respectively. To determine the extent of energy transfer from each residue to the dansylated, active site-directed inhibitor (DAPA), it was added to a final concentration of 5.0 µM, and the spectral analyses were repeated. Efficiencies of energy transfer (E) were calculated by the equation

\[ E = \frac{I(DAPA) - I(DAPA)/I(DAPA)}{1} \]

where I(+/DAPA) is the integrated intensity of a given residue in the presence of DAPA and I(−/DAPA) is the intensity in its absence. To assure saturation at 5.0 µM DAPA, wild-type thrombin and each of the variants were titrated with DAPA, and the decrements in thrombin emission at 340 nm were measured after each addition of DAPA. The data (ΔI(DAPA)) were fit to the equation

\[ ΔI = I_0 - \frac{Δmax[DAPA]}{K_D + [DAPA]} \]

by nonlinear regression to determine the K_D values for the binding of DAPA to the various forms of thrombin. I_0 is the initial intensity in the absence of DAPA. Theoretical energy

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2 I. I. Matthews and A. Tulinsky, unpublished data.
transfer efficiencies from several of the thrombin residues to the dansyl moiety of DAPA were also calculated (31) from the orientations and distances provided by the crystal structure of the thrombin-DAPA complex, the respective emission and absorption transition dipole of thrombin (32) and the dansyl moiety (33), and the Forster distance for the pairs (21 A) (34).

Amidolytic, Esterolytic, and Clotting Activities—Samples (100 μl) of wild-type or mutant thrombins were incubated for 5 min at 37 °C in the wells of a microtiter plate, and an aliquot (150 μl) of 0.5 mM S2238 (τ-p-phenylalanyl-t-pipeolyl-t-arginyl-p-nitroaniline dichlorohydrate) was added. The rates of change of absorbance at 405 nm were determined for amidolytic assays using TAME were carried out in 0.05 M Tris and 0.15 mM NaCl (pH 8.1) at 22 °C. The thrombin sample (30 μl) was pipetted into 870 μl of the assay buffer and incubated for 2 min, and 10 μl of 0.1 M TAME was added and mixed. The absorbance was determined at 247 nm was determined at 45-s intervals, and the initial rates of TAME hydrolysis were determined. To determine clotting activity, aliquots (100 μl) of various dilutions (5–160 nm) of wild-type and mutant thrombins were incubated at 37 °C for 5 min, and a 100-μl aliquot of a solution of 2 mM/ml human fibrinogen in 0.02 M HEPES, 0.15 mM NaCl, and 10 mM CaCl2 (pH 7.4) was added. The clotting times were measured by turbidity in a microtiter plate reader. Plots of the logarithms of the clotting time versus the logarithms of the thrombin concentrations were prepared. The plots were linear, and all thrombins gave the same slope. The clotting activities of the mutants, relative to wild-type thrombin, were measured from the displacements of the mutant lines on the concentration axis.

Competitive Inhibition of the Hydrolysis of S2366 by Fibrinogen—The ability of fibrinogen to act as a competitive substrate was analyzed by measurements of the steady-state kinetics of the hydrolysis of the synthetic peptide p-nitroanilide substrate S2366 (t-tyrosyl glutamyl-t-lysyl-t-arginyl-p-nitroaniline hydrochloride). Solutions (200 μl) consisting of 0.02 M HEPES, 0.02 mM sodium pyrophosphate, 0.15 mM NaCl, and 0.01% Tween 80 (pH 7.4) with S2366 at various concentrations with and without fibrinogen (2.5, 5.0, or 10 μM) were pipetted into wells of a microtiter plate which had been presoaked with 0.02 mM HEPES, 0.15 mM NaCl, and 0.1% Tween 80 (pH 7.4) for 1 h and then emptied (to minimize protein adsorption). The plate was placed in a plate reader thermostatted at 22 °C. Base-line absorbances were measured, and the reactions were initiated with a small aliquot of recombinant wild-type or mutant thrombins (final concentration of 5.0 nm). The reaction progress was monitored at 405 nm, and the initial rates of the reactions were calculated utilizing an extinction coefficient of 9961 M-1 cm-1 for p-nitroaniline (35). Rate data were globally fit to the Michaelis-Menten equation for competitive inhibition by nonlinear regression (17) with rate, kcat [thrombin] [S2366]/(1 + [fibrinogen])/Km and kcat [fibrinogen] as variables and kcat, Km, and Kf for fibrinogen as optimized parameters. The reaction equation was as follows: rate = kcat[thrombin][S2366]/Km(1 + [fibrinogen])/Kf + [S2366]).

Platelet Activation—Washed human platelets were prepared by a modification of the method of Mustard et al. (36) as described previously (17). Platelet counts were determined with a Bayer System 9000 cell counter. They were kept at 37 °C and used within 1.5 h of preparation. The thrombin concentration dependence of aggregation was determined for wild-type and mutant thrombins. Platelet aggregation was measured in siliconized glass cuvettes in an aggregometer. Gain settings were adjusted to give 100% transmittance for the buffer and 0% for the platelet suspension prior to the addition of thrombin. The platelet concentration was 1 x 10^8/ml, and the buffer was HEPES/Tyrode’s solution/albumin and 5 mM CaCl2 (17). The rate of aggregation was taken as the slope of absorbance versus time plot.

Analysis of Bovine Factor V Activation by Light Scattering, SDS-PAGE, and Densitometry—A 2.5-ml solution of single chain bovine factor V (100 μg/ml) in 0.02 M HEPES, 0.15 mM NaCl, and 5.0 mM CaCl2 (pH 7.4) was placed at 22 °C in a Perkin-Elmer 350B spectrophotometer. Light scattering at right angles (λmax = λabs = 320 nm) was monitored continuously. After determining the initial intensity value, thrombin (3.54 nM) was added, and cleavage to smaller species was monitored over time by the decrease in scattering intensity. Samples (50 μl) were removed at 0, 0.25, 0.5, 0.75, 1.0, 1.5, 2, 5, 10, and 15 min after adding thrombin. Each was immediately added to 2 l of 10 μM τ-phenylalanylarginyl chloromethyl ketone to stop the reaction and subsequently subjected to SDS-PAGE under reducing conditions and laser densitometry. Factor V activation occurs in response to cleavages at Arg5006, Arg5713, and Arg5386, in this order (21, 37). The first cleavage generates intermediates that do not dissociate (38); the second occurs in the lighter intermediate to yield the heavy chain of factor Va and to liberate an activation peptide; and the third occurs in the heavier intermediate to yield the light chain of factor Va and to liberate the second activation peptide. Thus, factor V (FV) conversion to factor Va (FVa) was modeled according to three sequential reactions through two intermediates, as indicated in Reaction 1.

\[
k_1 \text{FV} \rightarrow k_2 \text{FVI} \rightarrow k_3 \text{FVa}
\]

Although the first step cannot be visualized by light scattering because no fragments are released, the last two each involve release of an activation fragment and are therefore signaled by a decrement in light scattering. Since k1 was not accessible by light scattering, its value was deduced from the time course of cleavage of factor V itself as monitored by SDS-PAGE and densitometry. For wild-type thrombin and each of the mutants, values of k2 and k3 were inferred by nonlinear regression of the light scattering time course. The regression equation had three exponential terms plus a constant for the final scattering value when the reactions were over. The five best fit parameters were k1, k2, k3, and the scattering intensities of FV1 and FV2 (relative to that of FV)

In these analyses, the value of the coefficient for the first exponential (k1) was determined by SDS-PAGE and densitometry. The thrombin concentration dependence of aggregation was determined by incubating thrombin (6.67 nM) with anti-thrombin (250, 500, 750, or 1000 nM) at 22 °C in 0.05 M HEPES, 0.15 mM NaCl, and 0.01% Tween 80 (pH 7.4) in a total volume of 200 μl. At regular intervals, 10-μl aliquots were withdrawn and added to 190 μl of 0.4 mM S2238, and the absorbance at 405 nm was monitored over time to determine residual thrombin activity. The pseudo first-order rate constants for decay of thrombin were determined from slopes of plots of ln(activity) versus time. Second-order rate constants were then determined from slopes of plots of the pseudo first-order rate constants versus the anti-thrombin concentration.

Factor C and TAFI Activation by Thrombin-Thrombomodulin—Human protein C (final concentration of 0.1–2.0 μM) and recombinant soluble thrombomodulin (final concentration of 60 nM) were combined in 0.02 M HEPES, 0.15 mM NaCl, and 5.0 mM CaCl2 (pH 7.4) in a total volume of 40 μl in presoaked wells of a microtiter plate. Reactions were initiated by adding 20 μl of a solution of wild-type or mutant thrombins (final concentration of 1 nM). Ten minutes later, the reactions were quenched by adding 140 μl of a solution of DAPA (40.6 μM), S2366 (45.4 mM), KI (14.0 M), and EDTA (10 mM) in 0.02 M HEPES and 0.15 mM NaCl (pH 7.4). The absorbance at 405 nm was then measured at 1-min intervals for 30 min. Initial rates of 2366 hydrolysis were converted to activated protein C concentrations based on a standard curve made with activated protein C. Rates of protein C activation were then calculated, and data were fit by nonlinear regression (SYSTAT) to the Michaelis-Menten equation to determine the best values of Km, and kcat. TAFI (0.0–2.0 μM) was incubated with wild-type and mutant thrombins (1.0 nM) and soluble thrombomodulin (50 nM) at 22 °C in 0.02 M HEPES, 0.15 mM NaCl, 5.0 mM CaCl2, and 0.01% Tween 80 in a total volume of 20 μl in presoaked wells of a microtiter plate. After 10 min, reactions were quenched by adding 180 μl of a solution of τ-phenylalanylarginyl chloromethyl ketone (20 μM) and the carboxypeptidase B substrate furfurylacycloxylation in arginine (500 μM) in the same buffer. TAFIa concentrations were then deduced from the initial rates of decrease in absorbance at 340 nm. Experiments with mutant W60DF were repeated with thrombomodulin concentrations of 6.12, 12.5, 25, 50, and 100 nM. All rate data were fit to the Michaelis-Menten equation by nonlinear regression (SYSTAT) as described previously (8, 40).

RESULTS

Generation of Tryptophan-to-Phenylalanine Substitution Mutants of Human Thrombin—Clonal cell lines stably expressing each of the mutant prothrombins as well as wild-type human prothrombin-secreting prothrombin in the medium at levels between 2 and 10 μg/ml/24 h. The prothrombin in the conditioned medium was captured by batch chromatography on Q-Sepharose and was then quantitatively converted to thrombin. The thrombin produced was captured on and subsequently eluted from the cation-exchange resin Sephadex SP-C50. Analysis of the purified proteins by SDS-PAGE revealed each as a single band comigrating with wild-type thrombin (Fig. 1). All of
the thrombin species had >0.8 mol of active site/mol, with protein concentrations determined by the BCA protein assay (Pierce).

Fluorescence Properties of Selected Tryptophan Residues of Thrombin—The emission spectra and individual contributions of Trp60d, Trp96, Trp148, Trp207, and Trp215 to the total fluorescence intensity of thrombin and energy transfer to DAPA were determined by differences between wild-type thrombin and the mutants lacking each of the residues. The analysis was very similar to that described by Meagher et al. (41), who used mutagenesis to study the properties of the individual tryptophan residues of antithrombin. The individual spectra of Trp60d, Trp96, Trp148, Trp207, and Trp215 are shown in Fig. 2. The highest emitter of this group is Trp207, which contributed 34.9% of the total emission of wild-type thrombin (Table II). This is followed by Trp96, which contributed 11.1%. Trp60d, Trp148, and Trp215 contributed 6.5, 5.5, and 5.9%, respectively, and the average contribution for the group comprising Trp29, Trp51, Trp141, and Trp237 was 9.4%. The relative red shifts in emission maxima for Trp60d and Trp148 correlate with their contribution for the group comprising Trp29, Trp51, Trp141, and Trp237 each contributed on average 8% to the energy transfer to DAPA. The calculations yielded fluorescent for Trp60d and Trp148 correlate with their relatively high surface exposures of 0.73 and 0.78 (Table II). Measured energy transfer efficiencies for Trp60d, Trp207, and Trp215 agree very well with the theoretical values. The value for Trp60d (0.57 ± 0.2) does not agree as well (theory 0.98), suggesting that the orientation or position of Trp60d in the crystal structure differs from that in solution. A theoretical value for Trp215 is not available because this residue did not appear in the crystal structure of the thrombin-DAPA complex. The total tryptophan fluorescence intensity of thrombin decreased by 40% when DAPA was bound due to energy transfer from tryptophan to DAPA. As percentages of this decrement, 16% was due to transfer from Trp60d, 16% from Trp96, 7% from Trp148, 12% from Trp207, and 18% from Trp215. The residues in the group Trp29, Trp51, Trp141, and Trp237 each contributed on average 8% to the energy transfer to DAPA.

DAPA Binding and the Amidosyclic, Esterolytic, and Clotting Activities of Wild-type Thrombin and Mutants—The dissociation constants for DAPA binding of the mutants were very similar to the value for wild-type thrombin (Kd values as follows: wild type, 52 ± 3 nM; W60dF, 86 ± 6 nM; W96F, 57 ± 5 nM; W148F, 30 ± 3 nM; W207F, 53 ± 6 nM; and W215F, 43 ± 6 nM). Amidosyclic and esterolytic activities against S2238 and TAME were not appreciably affected either, with values ranging from 89 ± 3 to 118 ± 8% of the wild-type activity. Clotting activities with W96F, W148F, and W207F were 86 ± 3, 93 ± 3, and 90 ± 2% of the wild-type activity. Activity with W60dF was modestly reduced (30 ± 3%), whereas activity with W215F was only 10 ± 2% of wild-type thrombin. These results indicate that at each of the positions 60d, 96, 148, 207, and 215, phenylalanine substitutes for tryptophan very well in supporting DAPA binding and activity against TAME and S2238. When the substrate is fibrinogen, however, phenylalanine does not substitute for tryptophan equivalently at position 60d and only poorly at position 215.

To determine whether the catalytic defects of thrombin mutants W60dF and W215F in clotting are expressed in substrate binding or catalytic turnover, fibrinogen was examined as a competitive substrate for the hydrolysis of the small chromogenic substrate S2366. Mutant W207F, which had normal clotting activity, was also subjected to this analysis as a control.

In both the absence and presence of fibrin, reaction kinetics conformed to the Michaelis-Menten equation with the three enzymes, with results typical of competitive inhibition, where the inhibitor (fibrinogen) influences K_m (but not k_cat) for the small substrate. Thus, the K_f (i.e. K_m) for fibrinogen was found by nonlinear regression of the data to the equation for competitive inhibition as described under "Experimental Procedures." The calculations yielded K_m values for fibrinogen binding of 11.0 ± 0.7 μM for wild-type thrombin, 34 ± 4 μM for W60dF thrombin, 43 ± 6 μM for W215F thrombin, and 8.0 ± 2.0 μM for W207F thrombin (Table III). These data suggest that the catalytic defect in clotting activity, particularly with mutant W215F, cannot be attributed exclusively to an elevated K_m value because the elevation of K_m is insufficient to account for a 90% loss of clotting activity.

The data in Table III also indicate that mutants W96F, W148F, and W207F have catalytic efficiencies (k_cat/K_m) for the small substrate indistinguishable from that of the wild type. Mutant W215F has a k_cat/K_m value that is 51% that of the wild type; and perhaps remarkably, mutant W60dF has a value only 12% that of the wild type, even though its activities with TAME and S2238 are nearly identical to those of the wild type, and its clotting efficiency is 70% that of the wild type.

Platelet Activation—The ability of the thrombin mutants to activate platelets was measured by the extent of platelet aggregation at various thrombin concentrations (0.5–15 nM) in a suspension of 1 × 10^9 platelets/ml. The results are presented in Fig. 3. Substitution of Phe for Trp at any of the positions studied did not affect the maximal extent of aggregation observed at high concentrations of thrombin. The maximal extent of aggregation was saturable in the thrombin concentration; and in the case of the W96F, W148F, and W207F mutants, the concentration of thrombin required for the half-maximal extent...
not localized in the crystal structure.

activation of wild-type thrombin (2.7 ± 0.6 nM). In contrast, the W60dF and W215F mutants were approximately half as potent as the wild type in activating platelets, displaying a half-maximal effect at thrombin concentrations of 6.3 ± 0.2 and 6.0 ± 0.8 nM, respectively. These results support an important role of the tryptophan side chain at positions 60d and 215 in the cleavage of the platelet thrombin receptor.

Activation of Bovine Factor V—The kinetics of cleavage of bovine factor V were determined by SDS-PAGE for Arg1006 and by nonlinear regression of light scattering time courses for Arg713 and Arg1536. Residuals from light scattering data were randomly distributed, indicating that the model of sequential cleavages was valid (data not shown). First-order rate constants for the three cleavages are listed in Table IV, expressed relative to those obtained with wild-type thrombin. The rate constants for the first cleavage (Arg1006) were similar for all thrombin species, with values ranging from 78% of the wild type for mutant W60dF to 156% for W207F. The rate constants for the second cleavage (Arg713) by the mutants were higher than that of the wild type; values ranged from 140% for mutant W96F to 310% for mutant W148F. The rate constants for the third cleavage (Arg1536) were approximately equal to that of wild-type thrombin for mutants W96F and W207F, ~140% for mutants W60dF and W148F, and only 54% for mutant W215F. Thus, tryptophan-to-phenylalanine substitutions, depending on position, either only marginally impair or actually increase cleavage effectiveness at Arg1006, increase cleavage effectiveness at Arg713, and either have no effect or increase cleavage effectiveness at Arg1536, except for substitution at residue 215, which causes a 2-fold reduction. Since cleavages at Arg713 and Arg1536 were inferred from light scattering changes due to dissociation of activation fragments, the calculated values measure kinetics of dissociation. Thus, the values for the cleavage rate constants are minimum estimates.

Inhibition by Antithrombin—The second-order rate constants, calculated from the slopes of pseudo first-order rate constants versus the antithrombin concentration, indicated that the rate constant of inhibition of mutant W207F is virtually identical to that of wild-type thrombin; those of mutants W96F and W148F are 71 and 61%, respectively; and those of mutants W60dF and W215F are 46 and 41%, respectively. Thus, phenylalanine substitutions for tryptophan at positions near the active site (60d, 96, 148, and 215) lead to decreased

![Figure 3. Activation of washed platelets by wild-type and mutant thrombins. The extent of aggregation is plotted versus the concentration of wild-type thrombin (●) and mutants W60dF (○), W96F (■), W148F (◇), W207F (□), and W215F (□).](image-url)
kinetics of inhibition to varying extents, whereas that at the site remote from the active site (Trp207) has no effect. The value of the second-order rate constant with wild-type thrombin was

\[ \frac{k_{cat}}{K_m} = 6.9 \pm 0.4 \times 10^3 \text{ M}^{-1} \text{ s}^{-1} \]

**Protein C and TAFI Activation by Thrombin-Thrombomodulin**—The kinetics of activation of Protein C by wild-type and mutant thrombins were saturable in protein C concentration and conformed to Michaelis-Menten kinetics (Fig. 4). Nonlinear regression of the data yielded optimized parameters for \( k_{cat} \) and \( K_m \) for each thrombin-thrombomodulin complex (Table V). The catalytic efficiencies of \( k_{cat}/K_m \) for W96F, W148F, and W207F mutants were not sensibly different from that of wild-type thrombin despite slight differences in their individual \( k_{cat} \) and \( K_m \) values. The W60dF mutant displayed a slight reduction (23%) in catalytic efficiency for protein C, a defect expressed primarily in an increase in \( K_m \) for the reaction. The W215F mutant, however, showed a much larger reduction in catalytic efficiency for protein C activation (75%). This defect in protein C activation is a result of both an increase in \( K_m \) and a decrease in \( k_{cat} \). These results indicate that tryptophans at positions 215 and 60d and particularly at 215 are necessary for full effective Protein C cleavage in the presence of thrombomodulin.

Rates of activation of TAFI by wild-type and mutant thrombins also were saturable in the TAFI concentration and conformed to Michaelis-Menten kinetics (Fig. 5). Mutants W96F, W148F, W207F, and W215F, the latter which was very deficient in protein C activation, had the same or slightly higher catalytic efficiencies compared with wild-type thrombin. Mutant W60dF, which was close to normal in protein C activation, was substantially deficient in TAFI activation (\( k_{cat}/K_m \) was 23% that of wild-type thrombin). Although mutant W60dF had a \( k_{cat} \) almost twice that of wild-type thrombin, this was offset by an 8-fold greater \( K_m \). The data are summarized in Table V. The kinetics of activation by mutant W60dF were measured at several concentrations of thrombomodulin as described previously (8, 40) to determine whether thrombomodulin binding is impaired. The analysis indicated a dissociation constant for the interaction of W60dF thrombin with soluble thrombomodulin of 11 ± 1 nM, which is the same as that found for wild-type thrombin with either plasma or recombinant TAFI (8, 40). Thus, the catalytic defect of mutant W60dF in TAFI activation cannot be attributed to a defective interaction between the enzyme and thrombomodulin. Although the data are not shown, TAFI activation at a single concentration (1.0 \( \mu \text{M} \)) was measured in the presence of 200 nM wild-type or W60dF thrombin without thrombomodulin. Under these conditions, mutant W60dF was 19% as active as wild-type thrombin. Altogether, these data show that Trp215 appears to be highly important in the activation of Protein C, but not TAFI; and, conversely, Trp60d appears to be highly important in the activation of TAFI, but not protein C.

**TABLE IV**

| Thrombin | \( k_1 \) | \( k_2 \) | \( k_3 \) |
|----------|---------|---------|---------|
| WT       | 1.00 ± 0.06 | 1.00 ± 0.04 | 1.00 ± 0.01 |
| W60dF    | 0.79 ± 0.03 | 2.42 ± 0.14 | 1.40 ± 0.01 |
| W96F     | 1.21 ± 0.04 | 1.40 ± 0.04 | 1.07 ± 0.01 |
| W148F    | 1.41 ± 0.02 | 3.10 ± 0.16 | 1.44 ± 0.01 |
| W207F    | 1.56 ± 0.10 | 1.42 ± 0.06 | 0.97 ± 0.01 |
| W215F    | 0.88 ± 0.06 | 2.14 ± 0.06 | 0.54 ± 0.02 |

**FIG. 4.** Kinetics of protein C activation by thrombin-thrombomodulin. Initial rates of human Protein C activation were determined in the presence of Ca\(^{2+}\) (5.0 mM), soluble thrombomodulin (60 nM), and wild-type thrombin (●) or mutant W60dF (○), W96F (▼), W148F (▲), W207F (■), or W215F (▲). The thrombin concentration in each case was 1.0 nM. Data were fit by nonlinear regression to the Michaelis-Menten equation to find the best values for \( k_{cat} \) and \( K_m \). The indicated lines are from the regression analyses.

**DISCUSSION**

Analyses of fluorescence spectra disclosed a 6-fold difference in intensities among the residues for which individual measurements were available. The most intense is Trp207, and the least intense is Trp148. Analysis of energy transfer from the various residues to the dansyl moiety of the inhibitor DAPA in the active site indicated that the quenching of tryptophan fluorescence is due to transfer from most of the residues, although half the energy transfer is contributed collectively by Trp60d, Trp396, and Trp215. The meizothrombin-DAPA complex is more fluorescent than the thrombin-DAPA complex (17, 19, 22), and prothrombin conversion to thrombin is accompanied by an increase in intrinsic fluorescence (17). Although these observations indicate changes in local environments of one or more tryptophans upon conversion of prothrombin to meizothrombin and then to thrombin, identification of the residue(s) that experience these changes by DAPA fluorescence would likely be difficult because of the numerous contributions to energy transfer.

Mutants W96F and W148F are similar to wild-type thrombin with respect to all measured functional activities. Other investigators, however, found that deletion of the sequence ETW at positions 146–148 drastically reduces the clotting activity of thrombin (42). Subsequent studies, however, suggested that this is due to conformational changes in other regions of thrombin structure (43). Recent studies involving mutagenesis of tryptophan 148 to alanine (44) or glycine (45) indicated that these mutations at position 148 do not influence clotting activity, in agreement with the present results. In addition, mutagenesis of bovine thrombin indicated that replacement of the 148 loop with that of trypsin (and thus deletion of tryptophan 148) does not substantially influence clotting activity (46).

Le Bonnic et al. (47) found that deletion of tryptophan 60d along with adjacent proline residues 60b and 60c caused a 50-fold reduction in clotting activity. In addition, a recent surface mapping study showed that the thrombin activity in the conditioned medium of cells transiently expressing thrombin with a W60dA mutation was greatly reduced (44). This latter study and the present result indicate that tryptophan 60d is necessary for the clotting activity. Phenylalanine, however, can substitute for tryptophan quite well, as indicated by the retention of 70% of the activity of wild-type thrombin by mutant W60dF.

To further investigate the losses of fibrinogen clotting activ-
The influence of substitutions of phenylalanine for tryptophan on the two thrombomodulin-dependent reactions, protein C and TAFI activation, indicated that the structural requirements of thrombin in the two reactions are not identical. Substitution at Trp215 severely suppressed protein C activation, but was inconsequential in TAFI activation, whereas the opposite was the case with substitution at Trp604. These observations augment other studies that show that the structural requirements of thrombomodulin in the two reactions differ and that the surface residue requirements of thrombin also differ. The minimal structure of thrombomodulin required for efficient protein C activation comprises EGF domains 4–6 plus the small peptide connecting EGF3 to EGF4 (53). This is not sufficient for TAFI activation, however. Further elements of EGF3 are required (54, 55), comprising minimally the disulfide-bonded c-loop of EGF3. A recent study by Hall et al. (56), involving replacement of surface residues with alanine, identified three mutants with decreased ability to activate TAFI, but not protein C, and another three that had the opposite characteristics. In addition, 12 mutants were found that were deficient in both reactions, 11 of which involved mutations in exosite I. The other was an alanine substitution for tryptophan at position P9 of fibrinopeptide A which is replaced by tyrosine.

Table V

| Thrombin | Protein C | TAFI |
|----------|-----------|------|
|          | $K_m$ | $k_{cat}$ | $k_{cat}/K_m$ | $K_m$ | $k_{cat}$ | $k_{cat}/K_m$ |
| WT       | 2.3 ± 0.3 | 0.083 ± 0.007 | 0.036 ± 0.006 | 0.74 ± 0.12 | 0.64 ± 0.04 | 0.83 ± 0.14 |
| W60dF    | 2.9 ± 0.6 | 0.081 ± 0.010 | 0.028 ± 0.006 | 5.9 ± 1.7 | 1.14 ± 0.23 | 0.19 ± 0.07 |
| W96F     | 2.1 ± 0.4 | 0.068 ± 0.009 | 0.033 ± 0.008 | 0.83 ± 0.34 | 0.70 ± 0.12 | 0.85 ± 0.37 |
| W148F    | 2.6 ± 0.3 | 0.086 ± 0.006 | 0.035 ± 0.004 | 0.76 ± 0.33 | 0.81 ± 0.15 | 1.1 ± 0.5 |
| W207F    | 1.5 ± 0.2 | 0.053 ± 0.004 | 0.034 ± 0.006 | 0.63 ± 0.12 | 0.65 ± 0.05 | 1.0 ± 0.2 |
| W215F    | 2.8 ± 0.5 | 0.034 ± 0.003 | 0.009 ± 0.002 | 0.65 ± 0.21 | 0.68 ± 0.08 | 1.0 ± 0.3 |

Values are given as means ± S.E. and were obtained by nonlinear regression of the data shown in Figs. 4 and 5 fit to the Michaelis-Menten equation. WT, wild-type.
will substitute well in protein C (but not TAFI) activation. The present studies and previous studies involving site-directed mutagenesis of thrombin have suggested collectively that the structural requirements for effective catalysis of the myriad reactions in which thrombin participates vary from substrate to substrate and involve not only numerous charged or polar surface residues, but also the hydrophobic tryptophan residues in the vicinity of the active site.

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