Characterization of the P_2' and P_3' Specificities of Thrombin Using Fluorescence-Quenched Substrates and Mapping of the Subsites by Mutagenesis[†,‡]

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ABSTRACT: The importance of substrate residues P_2' and P_3' on thrombin catalysis has been investigated by comparing the hydrolysis of a series of fluorescence-quenched substrates. Each consisted of a 10-residue peptide, carrying a 2-aminobenzoyl (Abz) group at the N-terminus, and a penultimate 2,4-dinitrophenyl (Dnp) derivatized lysine. Cleavage of such a peptide relieves the intramolecularly-quenched fluorescence, allowing determination of the kinetic parameters. The nature of the P_2' residue was found to have a major influence on the rate of cleavage: the k_{cat}/K_m value for the hydrolysis of the Arg-Ser bond in Abz-Val-Gly-Pro-Arg-Ser-Phe-Leu-Leu-Lys(Dnp)-Asp-OH was nearly 3 orders of magnitude higher than that for the hydrolysis of the same substrate with aspartate instead of phenylalanine at the P_2' position. Comparatively, the P_3' side chain was less important: the k_{cat}/K_m value for the substrate with the least effective residue (aspartate) was only 33 times lower than that of the substrate with the most favorable amino acid (lysine). The role of thrombin residues Arg^{35}, Lys^{36}, Glu^{39} and Lys^{60f} in the putative P_2' and P_3' binding sites was also examined. Replacement of Lys^{60f} by glutamine improved the rate of cleavage for peptides with P_2' lysine or leucine. Compared with thrombin, mutants E39K and E39Q hydrolyzed faster substrates with an acidic residue in P_2' or P_3', but slightly slower those with a lysine at either position. Mutations R35Q and K36Q only improved the hydrolysis of substrates with an acidic P_2' residue. Overall, thrombin prefers bulky hydrophobic side chains in subsite S_2' and positively charged residues in S_3', whereas acidic residues are markedly antagonistic to both subsites.

Thrombin exhibits a specificity that is considerably more restricted than trypsin (Bode et al., 1992; Stubbs et al., 1992), despite having numerous macromolecular substrates (fibrinogen, thrombin receptor, blood clotting factors V, VIII, XIII, protein C) and inhibitors (antithrombin III, heparin cofactor II, protease nexin 1, hirudin, rhodniin). Part of thrombin's remarkable specificity must originate from within the catalytic groove, where subsites S_3 to S_3' of the protease accommodate residues P_3 to P_3' of the targeted ligand (Schechter & Berger, 1967). For many substrates and inhibitors, however, two exosites remote from the catalytic groove also govern the specificity of thrombin (Rydel et al., 1991, 1994; Bode et al., 1992; Stubbs et al., 1992; Vitali et al., 1992; Qiu et al., 1993; Mathews et al., 1994). These exosites often bind to a region of the substrate distant from the scissile bond and/or combine with a cofactor; these interactions appear to lessen the adverse effects of nonoptimal P_3-P_3' sequences (Le Bonniec & Esmon, 1991; Le Bonniec et al., 1995). This multiplicity in the regulation of catalysis is likely to account for the puzzling profile of the P_3 to P_3' sequences of thrombin's macromolecular substrates and inhibitors. Except for an obvious requirement for arginine in the primary binding pocket, and a preference for proline and serine as P_2 and P_1' residue, respectively, no consensus emerges from a sequence alignment. Thus, evaluation of the subsites contribution requires the use of small substrates which cannot interact with the exosites.

A number of chromophores, fluorophores, and chloromethyl ketones have been attached to the C-terminal arginine of various peptides, allowing the precise determination of thrombin's preferences in subsites S_2 and S_3 (Kettner & Shaw, 1981; Pozsgay et al., 1981; Lottenberg et al., 1983; Lottenberg & Jackson, 1983; Kawabata et al., 1988; Powers & Kam, 1992; Butenas et al., 1992, 1995). These studies confirmed that proline is, by far, the preferred P_2 residue and that several amino acids (threonine, serine, aspartate, and glutamate) are rather restrictive at this location. The P_3 preferences of thrombin are not as fully characterized, but, even if less efficient than its D counterpart, L phenylalanine appears to be one of the most favorable residues, whereas acidic side chains are clearly detrimental (Ehrlich et al., 1990; Le Bonniec et al., 1991).

The use of arginine derivatives does not enable the determination of the amino acid preferences in the S′ subsites of the enzyme. Hence, to delineate the influence of the P′ residues on catalysis, various options have been used. With small peptides, having amino acids spanning both sides of the scissile bond, the amount of product (or intact substrate) can be measured by reverse phase chromatography. Alter-

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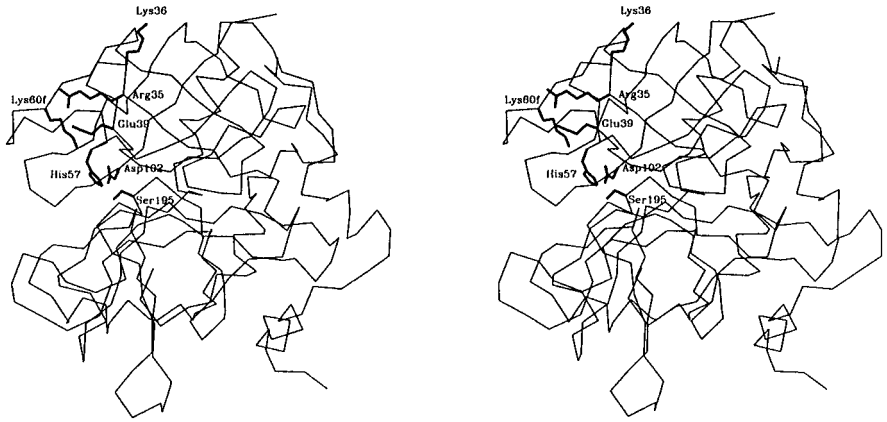

FIGURE 1: Stereo diagram of the thrombin catalytic groove. The coordinates are from Bode et al. (1992), and the structure is presented in the "typical" orientation. Side chains of the mutated residues (Glu^{39}, Arg^{35}, Lys^{36}, and Lys^{60f}) are fully drawn, as well as the catalytic residues (His^{57}, Asp^{102}, and Ser^{195}); all other amino acids are represented by their α-carbon chain.

natively, Schellenberger et al. (1993, 1994) successfully used acyl transfer to nucleophilic peptides to evaluate the P′ specificity in chymotrypsin, trypsin, α-lytic, and cercarial proteases. A number of studies have also reported characterization of the P′ preferences of proteases using intramolecularly-quenched fluorescence substrates (Matayoshi et al., 1989); examples include several kallikreins (Chagas et al., 1992) and subtilisins (Grøn et al., 1992) in the serine proteases families, together with various aspartyl proteases (Oliveira et al., 1992), cysteinyl proteases (García-Echeverría & Rich, 1992; Ménard et al., 1993), and metalloproteases (Knight et al., 1992; Anastasi et al., 1993).

To date, only a few studies have reported cleavage rates by thrombin of peptides comprising residues on both sides of the scissile bond (Chang, 1985; Chang et al., 1985; Le Bonniec et al., 1991, 1992). Thus the exosite-independent P′ specificity of thrombin remains largely unexplored. The importance of the P_1' residue had nevertheless been stressed through extensive site directed mutagenesis of antithrombin III: serine was found to be optimal, whereas threonine, glycine, and alanine were possible, though weaker, alternatives (Stephens et al., 1988; Theunissen et al., 1993; Olson et al., 1995). With respect to the P_2' specificity, little is known, other than that acidic side chains are detrimental (Chang, 1985). Aspartate is also detrimental in the P_3' position (Ehrlich et al., 1990; Le Bonniec et al., 1991; Richardson et al., 1992), and the dramatic impact of the P_3' Arg→Ser or Arg→Asn mutations in the fibrinogen Aα-chain suggests a positive contribution for a P_3' arginine (Ebert, 1991).

To document further the exosite-independent P_2' and P_3' preferences of thrombin, we have synthesized a series of fluorescence-quenched substrates and determined the k_{cat}/K_m values for their cleavage by thrombin. Overall, the results suggest that thrombin prefers bulky hydrophobic residues in P_2' and basic amino acids in P_3'. Based on various X-ray structures of thrombin,[1] subsites S_1' to S_3' could comprise Glu^{39}, Leu^{40}, Leu^{41}, Lys^{60f}, Phe^{60h}, Asn^{143}, and Gln^{151} (Figure 1). A complicated multicharge interaction network could also provide an indirect contribution from residues Arg^{35} and Lys^{36} (Qiu et al., 1992; Bode et al., 1992; Stubbs et al., 1992). In an attempt to delineate the residues in thrombin committed to its P′ specificity, we have determined the cleavage rate of the fluorescence-quenched substrates by a number of thrombins mutated in the presumed S_2' or S_3' subsites.

MATERIALS AND METHODS

Fluorescence-Quenched Substrates. Substrates were prepared via the Fmoc/*tert*-butyl strategy (Atherton & Sheppard, 1989), using a laboratory designed multiple synthesizer (Cammish et al., 1992). Briefly, 75 mg of Fmoc-Asp(Bu^t)-PEG-PS resin (Millipore) was packed into a 10 mm i.d. column fitted with a porous Teflon filter (Tessek, Prague) and washed with dimethylformamide (DMF).[2] Following deprotection for 10 min in piperidine/DMF (1/4 v/v) and washing in DMF, the resin was reacted for 45 min with Fmoc-Lys(Dnp)-OH using (benzotriazol-1-yloxy)tris(dimethylamino-phosphonium hexafluorophosphate, 1-hydroxybenzotriazole, and diisopropylethylamine (75 μmol each, in DMF). Subsequent synthesis steps were performed as above, using the appropriate protected amino acid (Novabiochem, England), and peptides were capped, following the same protocol, with 2-[(*tert*-butoxycarbonyl)amino]benzoic acid (Meldal & Breddam, 1991). Resins were washed with diethyl ether and dried *in vacuo* for 16 h, and the peptides were detached by a 2 h incubation with TFA/phenol/triethylsilane/ethanedithiol (91/3/3/3 v/v). Detached peptides were separated from the resin by filtration, flushed with N_2, precipitated with ice cold diethyl ether, and extracted 5 times with ether. Fluorescence-quenched substrates were purified by reverse phase chromatography using a Vydac 208TP1022 column and verified by amino acid analysis. Lyophilized substrates were resuspended in DMF, and the concentration of the stock solution (about 50 mM) was determined spectrophotometrically, assuming an absorption coefficient of 10^4 M^{-1} cm^{-1} at 360 nm. The intrinsic fluorescence of a 50 μM solution of the uncleaved peptides varied widely, but, consistent with the above absorption coefficient value, similar maximum fluorescences were attained after full cleavage by 0.1 μM trypsin (Worthington, Lorne Laborato-

[1] The amino acid sequence numbering of thrombin, as suggested by Bode et al. (1992), is based on its three dimensional topological identity with chymotrypsin; insertion residues are denoted by lower case letters in alphabetic order (e.g., Lys^{60f} is the 6th residue inserted at position 60).

[2] Abbreviations: DMF, *N*,*N*-dimethylformamide; Abz, *o*-aminobenzoyl; Dnp, 2,4-dinitrophenyl; k_{on}, association rate constant. The residues of the fluorogenic substrates are numbered from P_6 to P_6', where P_6 and P_6' refer to the sixth residue from the cleavage site on the amino and carboxyl side, respectively.

ries, Twyford-Reading, England). Depending upon the peptide, the fluorescence increased between 1.6- and 9.1-fold after full cleavage. Fluorescence of the cleaved peptides was proportional to their concentration (at least up to 0.5 mM); a plot of the fluorescence intensity as a function of dilution was linear. Accordingly, an increase in fluorescence could be equated to the increase in concentration of the cleaved substrate, allowing determination of the kinetic parameters. The C-terminal aspartate was added to improve the peptide solubility such that no precipitation was observed with concentrations between 0.5 μM (in 0.025% DMF v/v) and 0.5 mM (in 10% DMF). Intact substrates also appeared stable after dilution in kinetic buffer; without enzyme, fluorescence intensity did not change over a period of 4 h at 37 °C. Finally, small amounts of DMF did not interfere with thrombin catalysis; comparable rates of hydrolysis were detected in the presence of 0.025% and 0.5% organic solvent. During the progress curve kinetic experiments the concentration of DMF never exceeded 0.2%.

Proteins. Plasma-derived thrombin (Stone & Hofsteenge, 1986) and the thrombin mutants[3] E39K and E39Q were prepared as previously described (Le Bonniec & Esmon, 1991; Le Bonniec et al., 1991). The thrombin mutants R35Q, K36Q, and K60fQ were expressed in SF9 cells using the baculovirus system (Myles et al., 1993). The active site concentration of the thrombin variants was determined by titration with recombinant hirudin (Ciba-Geigy, Basel, Switzerland) as described in Wallace et al. (1989). Enzyme stability was assessed according to the method of Selwyn (1965), by comparing the time courses of H-D-Phe-pipecolyl-Arg-*p*-nitroanilide hydrolysis at three enzyme concentrations, as described (Le Bonniec et al., 1995).

Determination of the k_{cat}/K_m Values by Progress Curve Kinetics. Experiments were performed at 37 °C in 0.05 M Tris-HCl, pH 7.8, containing 0.1 M NaCl and 0.2% (w/v) poly(ethylene glycol) (M_r 6000). Hydrolysis of each substrate was monitored essentially according to Chagas et al. (1991), by measuring the fluorescence at $\lambda_{em} = 414$ nm (slit 4 nm) and $\lambda_{ex} = 325$ nm (slit = 10 nm) in a Perkin-Elmer spectrofluorometer (Model L550B). The cuvette (1×0.2 cm path length) containing 0.5 mL of the substrate solution was left in the thermostated cell holder until temperature equilibrium was attained (10−15 min). The enzyme solution (2−8 μL) was then added, and the increase in fluorescence with time was monitored. With most substrates, the change in fluorescence intensity was recorded every 6 s after the addition of 1 nM enzyme until a plateau corresponding to complete hydrolysis was reached. When the cleavage rate was slow (i.e., with k_{cat}/K_m values of less than 10^4 $M^{-1}\cdot s^{-1}$), the enzyme concentration (E) was increased (up to 50 nM) and the fluorescence intensity was followed for a maximum of 4 h (yielding at least 50% hydrolysis of the substrate). The first-order rate constant (k), the initial fluorescence of the uncleaved peptide (I_0), and the maximum fluorescence intensity (I_{max}) were estimated by nonlinear curve fitting of the fluorescence intensity (INT) dependence on time (t) using the equation:

$$\mathrm{INT} = I_0 + I_{max}(1 - e^{-Ekt}) \quad (1)$$

I_0 and I_{max} values obtained through the nonlinear curve fitting approach were always consistent with the fluorescence intensities measured before and after a 1 h incubation with 0.1 μM trypsin. The pseudo-first-order rate constant k will equal k_{cat}/K_m, provided that the initial concentration of substrate is much less than K_m. To assess whether this condition was met, progress curve kinetics were systematically performed at two initial concentrations of fluorescence-quenched substrate (between 0.5 and 5 μM). The results indicated that with all substrates assayed k was, within the experimental error, identical at the two substrate concentrations. Thus, in this concentration range, the rate of hydrolysis appeared independent of the amount of substrate, suggesting that k could be equated to the k_{cat}/K_m value.

RESULTS

Phenylalanine Is the Preferred P_2' Residue for Thrombin Catalysis. In natural substrates and inhibitors of thrombin, P_2' residues are very diverse: phenylalanine, leucine, valine, isoleucine, proline, glycine, histidine, glutamate, and asparagine can all be found. To evaluate the importance of the P_2' residue in thrombin catalysis, a number of fluorescence-quenched substrates were synthesized with various amino acids at this position. Each substrate was of the form (Abz)-Val-Gly-Pro-Arg-Ser-Xaa-Leu-Leu-Lys(Dnp)-Asp-OH, where Xaa represents any one of the 10 amino acids examined. Cleavage occurs at the Arg-Ser bond, and the P_2-P_1-P_1' sequence (Pro-Arg-Ser) constitutes a highly favorable combination for thrombin cleavage. In the intact peptide, the Dnp group quenches, by resonance energy transfer, the fluorescence of the N-terminal Abz group. Proteolytic cleavage of the connecting peptide relieves quenching, resulting in an increase in fluorescence proportional to the concentration of the released fluorophore fragment. For each substrate, the k_{cat}/K_m values were determined by following the cleavage under first-order conditions. Typical progress curves of fluorescence-quenched substrate hydrolysis are shown in Figure 2.

Unexpectedly, almost 3 orders of magnitude separated the highest value of k_{cat}/K_m, obtained with the substrate having phenylalanine in P_2' (2.1×10^6 $M^{-1}\cdot s^{-1}$), from the lowest value (3.2×10^3 $M^{-1}\cdot s^{-1}$), obtained with the substrate having aspartate in P_2' (Table 1). In general, substrates with bulky hydrophobic side chains (phenylalanine or tryptophan) exhibited greater k_{cat}/K_m values than those with polar side chains (lysine, glutamine, glutamate, or aspartate). Thus, with peptidyl substrates, the P_2' residue emerges as a prime determinant to govern thrombin's specificity. The lower k_{cat}/K_m value for the substrate with a P_2' proline compared to that with leucine was somewhat surprising, because the P_2' residue of the fibrinogen Aα-chain is a proline whereas a mutant fibrinogen where this proline is replaced by leucine is dysfunctional (Ebert, 1991). The mutation in fibrinogen may affect steps other than the proteolytic cleavage in the conversion of fibrinogen to fibrin. Alternatively, peptides and fibrinogen may use different modes of binding to the active site (Stubbs et al., 1992).

Positively Charged Residues Are Preferred in the P_3' Position. In natural substrates and inhibitors of thrombin,

[3] Mutations in thrombin are designated by the standard nomenclature where the first and last (capital) letters represent amino acid in the wild-type and mutant protein, respectively (e.g., K60fQ designs mutant where Lys^{60f} has been replaced by a glutamine).

Table 1: k_{cat}/K_m Values for the Cleavage of Quenched-Fluorescence Substrates by Thrombin (IIa) and Various Mutants[a]

	k_{cat}/K_m values ($M^{-1}\cdot s^{-1}$)					
substrate P_3-P_3'	IIa	E39K	E39Q	R35Q	K36Q	K60fQ
DPR-SFL	3.6×10^5	4.9×10^5	7.1×10^5	5.1×10^5	4.1×10^5	5.0×10^5
GPR-S**F**L	2.1×10^6	3.9×10^6	4.8×10^6	2.0×10^6	1.9×10^6	3.0×10^6
GPR-S**W**L	8.7×10^5	1.9×10^6	2.8×10^6	7.3×10^5	6.3×10^5	6.8×10^5
GPR-S**A**L	4.2×10^5	5.7×10^5	8.9×10^5	2.7×10^5	3.1×10^5	9.3×10^5
GPR-S**L**L	2.8×10^5	6.2×10^5	1.0×10^6	2.8×10^5	3.8×10^5	1.9×10^6
GPR-S**K**L	2.4×10^5	2.0×10^5	1.3×10^5	2.6×10^5	2.1×10^5	1.1×10^6
GPR-S**Q**L	1.9×10^5	2.4×10^5	2.8×10^5	9.2×10^4	1.5×10^5	3.7×10^5
GPR-S**G**L	9.9×10^4	5.0×10^5	4.4×10^5	1.2×10^5	1.7×10^5	6.5×10^5
GPR-S**P**L	6.3×10^4	2.0×10^5	2.6×10^5	8.8×10^4	1.1×10^5	5.3×10^4
GPR-S**E**L	4.5×10^3	4.2×10^4	3.6×10^4	1.4×10^4	1.6×10^4	1.5×10^4
GPR-S**D**L	3.2×10^3	2.7×10^4	3.1×10^4	1.0×10^4	2.6×10^3	1.4×10^4
GPR-SF**K**	8.6×10^6	6.6×10^6	7.3×10^6	9.2×10^6	8.4×10^6	1.2×10^7
GPR-SF**W**	4.7×10^6	4.8×10^6	4.1×10^6	4.9×10^6	4.1×10^6	3.2×10^6
GPR-SF**Q**	3.6×10^6	3.8×10^6	3.8×10^6	2.8×10^6	2.7×10^6	3.9×10^6
GPR-SF**P**	3.2×10^6	3.1×10^6	3.5×10^6	3.5×10^6	2.9×10^6	2.6×10^6
GPR-SF**A**	2.9×10^6	3.5×10^6	3.2×10^6	2.4×10^6	2.3×10^6	3.7×10^6
GPR-SF**F**	2.7×10^6	3.5×10^6	3.5×10^6	2.6×10^6	2.2×10^6	3.3×10^6
GPR-SF**G**	2.7×10^6	3.9×10^6	2.6×10^6	2.6×10^6	2.5×10^6	2.7×10^6
GPR-SF**L**	2.1×10^6	3.9×10^6	4.8×10^6	2.0×10^6	1.9×10^6	3.0×10^6
GPR-SF**E**	4.5×10^5	8.7×10^5	6.9×10^5	3.0×10^5	2.5×10^5	1.6×10^5
GPR-SF**D**	2.6×10^5	1.1×10^6	5.2×10^5	2.0×10^5	1.9×10^5	1.3×10^5

[a] Only residues from P_3 to P_3' are indicated and the amino acid which is unique to each substrate is in bold. All estimates of k_{cat}/K_m value $(k_{cat}/K_m)_i$ had a standard error (s_i) of less than 7%. The value given represents the weighted mean (calculated as $\sum[(k_{cat}/K_m)_i*(1/s_i)^2]/[\sum(1/s_i)^2]$) of a minimum of two determinations, each completed with two substrate concentrations (4 experimental values).

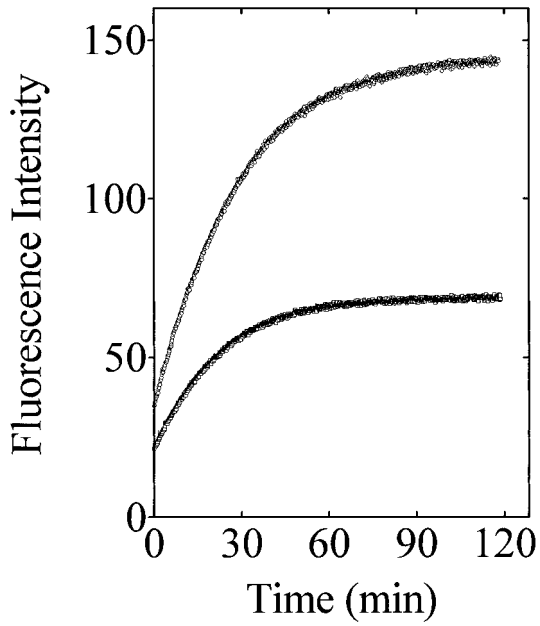

FIGURE 2: Typical progress curves of fluorescence-quenched substrate hydrolysis. Thrombin (3.4 nM) was added to 1 μM (lower curve) and 2 μM (upper curve) of Abz-Val-Gly-Pro-Arg-Ser-Gln-Leu-Leu-Lys(Dnp)-Asp-OH, and the fluorescence intensity was followed for 2 h at 37 °C. Solid lines represent the result of the nonlinear curve fitting according to eq 1, yielding k values of 2.0×10^5 and 1.8×10^5 $M^{-1}\cdot s^{-1}$, respectively.

P_3' residues as diverse as arginine, asparagine, histidine, leucine, glycine, and aspartate can be found. Following the same strategy as above for the P_2' investigation, we retained the most effective P_2' residue (phenylalanine) and synthesized 10 additional fluorescence-quenched substrates with representative amino acids in P_3' position. The potential of the P_3' side chain to alter thrombin's hydrolysis of peptides was less dramatic than that of the P_2' residue. Still, the substrate with the "best" P_3' amino acid (lysine) was cleaved with a k_{cat}/K_m value 33 times greater than the one having aspartate at this position (8.6×10^6 *versus* 2.6×10^5 $M^{-1}\cdot s^{-1}$, Table 1). In contrast, substrates with tryptophan, phenylalanine, leucine, alanine, proline, glycine, or glutamine were all cleaved with a less than 3-fold variation in their k_{cat}/K_m values (2.1×10^6 to 4.7×10^6 $M^{-1}\cdot s^{-1}$). Thus, the S_3' subsite of thrombin confers less limitation on catalysis than the S_2' subsite, and charge interactions seem to be the predominant element: lysine was the preferred residue, acidic side chains (glutamate and aspartate) were especially unfavorable, and uncharged residues were "neutral". A favorable contribution to thrombin catalysis of positively charged residues in the P_3' position is in accord with studies of natural variants of fibrinogen's Aα-chain: replacement of the P_3' arginine by either glycine, serine, or asparagine leads to bleeding disorders (Ebert, 1991). A detrimental effect of a P_3' aspartate occurs in thrombin's activation of protein C (Ehrlich et al., 1990; Richardson et al., 1992), in small peptide hydrolysis (Le Bonniec et al., 1991), and for its inhibition by antithrombin III variants (Theunissen et al., 1993).

Contribution of Lys^{60f} to the P′ Preference of Thrombin. The alignment of the thrombin sequence with that of chymotrypsin reveals an 8 amino acid insertion between residues 60 and 61. Part of this 60-loop insertion, namely, the Tyr^{60a}-Pro^{60b}-Pro^{60c}-Trp^{60d} motif, is implicated in the P_2 and P_3 specificities of thrombin (Bode et al., 1992; Le Bonniec et al., 1993; Guinto et al., 1994), whereas its C-terminal portion extends over the presumed S′ region of the catalytic groove (Figure 1). The sixth amino acid of this loop, Lys^{60f}, could conceivably interact with one or more of the P_1', P_2', and P_3' residues of a substrate. To clarify the potential contribution of Lys^{60f} to thrombin's specificity, the mutant K60fQ was prepared and its P_2' and P_3' preferences were determined. The K60fQ mutation had a notable impact on thrombin specificity, as did the K60fE mutation characterized by Wu et al. (1991). Although the "best" P_2' residue was still phenylalanine, substrates with lysine, glycine, or leucine in P_2' were hydrolyzed more efficiently by K60fQ;

compared with thrombin, the k_{cat}/K_m values were increased 4.6-, 6.6-, and 6.8-fold, respectively (Table 1). The "worst" P_2' residue for K60fQ also remained aspartate, but the k_{cat}/K_m value was increased 4.3-fold relative to thrombin.

The differences between thrombin and K60fQ in terms of their P_3' preferences were less pronounced than for the P_2' position, but the K60fQ mutation somehow enhanced thrombin's specificity: the substrate with lysine in P_3' was cleaved more efficiently by the mutant than by thrombin, resulting in the highest k_{cat}/K_m value obtained (1.2×10^7 $M^{-1}\cdot s^{-1}$). Conversely, the substrate with aspartate in P_3' exhibited a k_{cat}/K_m value 2-fold lower than that of thrombin (1.3×10^5 *versus* 2.6×10^5 $M^{-1}\cdot s^{-1}$). As with thrombin, however, K60fQ did not differentiate between P_3' residues with uncharged side chains.

Role of Segment 34−*41 in the P′ Specificity of Thrombin.* The inference that Glu^{39} and its supporting charge network (Arg^{35} and Lys^{36}) influence the P′ specificity of thrombin relies on kinetic (Le Bonniec et al., 1991) as well as crystallographic data (Bode et al., 1992; Stubbs et al., 1992). In particular, replacement of Glu^{39} by lysine (to give the E39K mutant) improves the ability of thrombin to cleave a peptide derived from the protein C activation site, which has aspartate in the P_3' position (Le Bonniec et al., 1991). Although the fluorescence-quenched peptides with acidic side chains in P_3' position remained poor substrates of E39K, they were hydrolyzed faster by the mutant than by thrombin (Table 1); specifically, the substrate with aspartate in P_3' was cleaved 4.2-fold faster (k_{cat}/K_m values of 1.1×10^6 *versus* 2.6×10^5 $M^{-1}\cdot s^{-1}$). The opposite was true for the substrate with lysine in P_3', which was cleaved slightly slower by E39K than by thrombin (k_{cat}/K_m values of 6.6×10^6 and 8.6×10^6 $M^{-1}\cdot s^{-1}$, respectively). The above data are consistent with a direct contribution of the charge carried by residue 39 to the P_3' specificity; thus this hypothesis was further investigated with the E39Q thrombin mutant, in which the charge of Glu^{39} was merely neutralized by isosteric substitution. Results were intermediate between those obtained with the positively charged E39K and the negatively charged thrombin: E39Q hydrolyzed substrates with P_3' aspartate or glutamate more efficiently than thrombin, but slower than E39K, and hydrolyzed the substrate with P_3' lysine less efficiently than thrombin, yet faster than E39K. As with thrombin and K60fQ, the Glu^{39} mutants were less sensitive to the other P_3' substitutions. Neutralization (or inversion) of the negative charge of Glu^{39} had an opposite effect to that of the K60fQ mutation: the P_3' specificity of thrombin became less restricted. Instead of the 92-fold difference between the k_{cat}/K_m values for the slowest and the fastest cleaved substrates observed with K60fQ (33-fold with thrombin), the difference was 14-fold with E39Q, and less than 8-fold with E39K. In fact, the Glu^{39} mutations lessened both the P_3' and the P_2' preferences of thrombin: while 660-fold separated the k_{cat}/K_m values of the "best" from the "worst" P_2' residue with thrombin, this difference was reduced to about 150-fold with E39K and E39Q.

In contrast to the S_3' subsite, electrostatic contribution cannot account alone for the alterations of the P_2' specificity caused by the Glu^{39} mutations. The most notable consequence of the E39K and E39Q mutations was the 8- to 10-fold more efficient cleavage of the peptides with aspartate or glutamate in the P_2' position; however, mutants also hydrolyzed substrates with P_2' tryptophan, leucine, glycine, or proline 2−5 times faster than thrombin (Table 1). Thus, thrombin's Glu^{39} participates to both the P_2' and P_3' specificities but in different ways. In the crystal structure of thrombin, Glu^{39} is hydrogen bonded to Arg^{35}, which, together with Lys^{36}, appears to participate in a charge network (Bode et al., 1992; Stubbs et al., 1992). Mutations R35Q and K36Q, which disrupt this charge network, had little impact on the P_3' specificity of thrombin, but they had a discernible effect on the P_2' specificity: substrates having glutamate or aspartate in P_2' exhibited k_{cat}/K_m values 3 times greater with the mutants than with thrombin.

DISCUSSION

By using fluorescence-quenched substrates, we have established that P_2' phenylalanine, and to a lesser extent tryptophan, promote the cleavage of peptide substrates by thrombin, whereas aspartate markedly inhibits catalysis. In the P_3' position, charge was found to be the predominant determinant, with positively charged residues promoting catalysis. Mapping of the S′ subsites by using site directed mutants also provided new insights into the molecular determinants of thrombin specificity.

The highest value of k_{cat}/K_m observed for a fluorescence-quenched substrate was 8.6×10^6 $M^{-1}\cdot s^{-1}$ with thrombin. This value approaches that for the release of fibrinopeptide A from the fibrinogen Aα-chain (1.1×10^7 $M^{-1}\cdot s^{-1}$; Higgins et al., 1983), but is lower than the value achieved for *p*-nitroanilide substrates hydrolysis (8.6×10^7 and 3.3×10^7 $M^{-1}\cdot s^{-1}$ with H-D-Phe-pipecolyl-Arg-*p*-nitroanilide and tosyl-Gly-Pro-Arg-*p*-nitroanilide, respectively; Stone et al., 1991). For each subsite, based on the current and previous studies, amino acids could be classified into one of three categories according to their effect on catalysis: enhancer, repressor, or impartial. For instance, acidic residues appear to be repressors in any of the P_3−P_3' positions. Overall, FPR-SFR represents an excellent, perhaps optimal, P_3−P_3' sequence. Surprisingly, none of the key cleavage sites hydrolyzed by thrombin exhibit such an "ideal" sequence. Still, a number of excellent substrates and inhibitors have at least 3 "optimal" residues within their P_3-P_3' sequence, whereas the key substrates having a "nonoptimal" sequence seem to require a cofactor and/or an exosite binding for productive interaction with thrombin. Consistent with this hypothesis, most P_3−P_3' activation sites of the coagulation zymogens resistant to thrombin cleavage fail to have an enhancer residue (other than the P_1 arginine) and often have one or more repressor residues.

Substrates and Inhibitors of Thrombin with "Near-Optimal" P_3−P_3' Sequences. The cleavage site of the thrombin receptor (D**PR-SF**L) has 4 out of 6 optimal residues (P_2−P_2'). The P_3' leucine is "impartial", neither favoring nor restricting thrombin catalysis, but the P_3 aspartate is rather detrimental (Table 2). Based on our results, the aspartate in P_3 would cause a reduction of about 6-fold of the k_{cat}/K_m value relative to an impartial residue such as glycine (Table 1). The thrombin receptor also possesses a region rich in negatively charged amino acids. This hirudin-like sequence, C-terminal to the scissile bond, binds to anion binding exosite 1 of thrombin (Liu et al., 1991; Mathews et al., 1994; Ishii et al., 1995). There is little doubt that the negative impact of the P_3 aspartate could be more than compensated for by the exosite contribution.

Table 2: Flanking Sequences of "Near Optimal" Thrombin Sites in Macromolecule Substrates or Inhibitors[a]

substrate or inhibitor	species	P_3-P_3' sequence
thrombin receptor	hum.	D**PR-SF**L
factor V (Arg^{1018})	hum.	S**PR-TF**H
factor V (Arg^{1006})	bov.	S**PR-SF**H
factor VIII (Arg^{1689})	hum.	S**PR-SF**Q
factor VIII (Arg^{740})	hum.	E**PR-SF**S
factor V (Arg^{709})	hum.	GI**R-SFR**
factor V (Arg^{713})	bov.	GL**R-SFR**
protein S (Arg^{49})	hum.	CL**R-SF**Q
prothrombin (Arg^{156})	bov.	I**PR-S**GG
prothrombin (Arg^{155})	hum.	T**PR-S**EG
prothrombin (Arg^{284})	hum.	N**PR-TF**G
antibody light chain	rabbit	L**PR-TF**G
α1-antitrypsin$_{(P1=Arg)}$	hum.	I**PR-S**IP

[a] Residues "optimal" for thrombin cleavage are in bold. The abbreviations used are: hum., human; bov., bovine. All sites have at least three "optimal" residues within the P_3-P_3' sequences.

Table 3: Flanking Sequences of "Nonoptimal" Thrombin Sites in Macromolecule Substrates or Inhibitors[a]

substrate or inhibitor	species	P_3-P_3' sequence
fibrinopeptide A	hum.	GV**R-GPR**
fibrinopeptide B	hum.	SA**R-GHR**
rhodniin	mosquito	C**P**H-ALH
heparin cofactor II	hum.	M**P**L-**S**TQ
antithrombin III	hum.	AG**R-S**LN
protease nexin 1	hum.	IA**R-S**SP
factor XI	hum.	K**PR**-IVG
factor XIII	hum.	V**PR**-GVN
protein C	bov.	D**PR**-IVD
protein C	hum.	D**PR**-LID
factor VIII (Arg^{372})	hum.	QI**R-S**VA
factor V (Arg^{1545})	hum.	YL**R-S**NN
factor V (Arg^{1545})	bov.	YL**R-S**NT
protein S (Arg^{70})	bov.	DL**R-S**CV
protein S (Arg^{70})	hum.	DL**R-S**CV
protein S (Arg^{52})	bov.	SF**R**-AGL

[a] Abbreviations are as in Table 2. In these thrombin substrates or inhibitors, fewer than three residues (in bold) are optimal within the P_3-P_3' sequence, but nearly all of them require a cofactor and/or utilize an exosite for productive binding. The high affinity of protease nexin 1 for thrombin remains however puzzling and cannot be explained by the P_3-P_3' sequence of its reactive site loop.

In spite of limiting plasma concentrations, factors V and VIII are activated very rapidly during the early stages of blood coagulation. Various cleavages have been reported to occur during activation, but only three of the proposed sites appear critical for triggering full activity in each cofactor (Toole et al., 1986; Pittman & Kaufman, 1988; Krishnan et al., 1991; Guinto et al., 1992; Bakker et al., 1994; Keller et al., 1995; Regan & Fay, 1995). In bovine factor V, the cleavage site within the B domain has the same optimal P_2-P_2' sequence as the thrombin receptor (S**PR-SF**H) and no repressor residues. The corresponding site in human factor V (S**PR-T**FH) has the less favorable P_1' threonine instead of the optimal serine. In human factor VIII, the cleavage between the B and A3 domains (S**PR-SF**Q) again has the optimal P_2-P_2' sequence, as does the site between the A2 and B domains (E**PR-SF**S), even though the P_3 glutamate is a repressor. In factor V, the cleavage site between the A2 and B domains (GI**R-SFR** in the human, and GL**R-SFR** in the bovine species) has an optimal P_1-P_3' sequence, instead of P_2-P_2'. Thus, 4 out of the 6 thrombin cleavage sites in factors V and VIII lack only 2 residues from the hypothetical "ideal" P_3-P_3' sequence. The other cleavage sites are between the A1 and A2 domains of factor VIII (QI**R-S**VA) and between the B and A3 domains of factor V (YL**R-S**NN). For these sites, only the P_1 and P_1' residues are optimal for thrombin cleavage (Table 3). However, following the site in factor VIII, there is an acidic sequence (YIAA**EEED**W**D**Y) resembling that of the carboxyl tail of hirudin and the thrombin receptor, which could bind to exosite 1 of thrombin. In spite of a very different topological location, the thrombin site in factor V is followed by the strikingly homologous sequence (YIAA**EE**ISW**D**Y). Binding of these hirudin-like sequences to exosite 1 of thrombin would rationalize the departure from an ideal P_3-P_3' sequence in these excellent thrombin substrates.

Prothrombin is another thrombin substrate (Krishnaswamy et al., 1987; Nesheim et al., 1988) which has three optimal residues within the P_3-P_3' sequence. The feedback cleavage site between kringle 1 and 2 (T**PR-S**EG in human and I**PR-S**GG in bovine prothrombin) has an optimal P_2-P_1' sequence; albeit the P_2' residue (glutamate or glycine) is a repressor. In human prothrombin, an additional thrombin cleavage site (N**PR-T**FG) removes 13 amino acids from the A-chain of the newly formed protease; this site has 3 optimal residues and no unfavorable residues. A nonphysiological thrombin substrate (antibody light chain) has been characterized by Chang (1985); it has a similar P_3-P_3' sequence (L**PR-TF**G), i.e., 3 optimal residues and no repressor. Finally, the serpin α1-antitrypsin does not normally inhibit thrombin, but if the natural P_1 methionine is replaced by arginine, to yield an optimal P_2-P_1' sequence (I**PR-S**IP), the resultant serpin inhibits rapidly thrombin: the association rate constant (k_{on}) becomes 1.2×10^6 $M^{-1}\cdot s^{-1}$ (Hopkins et al., 1995; Le Bonniec et al., 1995).

Thrombin Substrates and Inhibitors with "Nonoptimal" P_3-P_3' Sequences. Several important substrates and inhibitors of thrombin have P_3-P_3' sequences quite remote from our hypothetical "optimal" one, including the fibrinogen Aα-chain (GV**R**-GP**R**). Although the P_3' arginine is an enhancer, the P_3-P_3' sequence of the fibrinogen Aα-chain is an unlikely target of thrombin (Table 3). However, as with the thrombin receptor (and probably factors V and VIII), the fibrinogen Aα-chain binds to the anion-binding exosite 1 of thrombin. Furthermore, a phenylalanine in the P_9 position occupies the aryl-binding site of thrombin, ensuring effective binding (Stubbs et al., 1992). These additional contacts on both sides of the scissile bond may compensate for the interactions missing in subsites S_3 to S_3' or trigger an allosteric switch of the enzyme specificity. The scissile bond in the Bβ-chain (SA**R**-GH**R**) is also quite distant from our ideal sequence. Release of the fibrinopeptide B is slower than that of fibrinopeptide A, and a possible exosite contribution in this reaction has not been fully investigated. Similar to fibrinogen, the inhibitor rhodniin (Friedrich et al., 1993) is an extremely improbable ligand of thrombin, considering its P_3-P_3' sequence (C**P**H-ALH). This Kazal-type inhibitor, which apparently functions as a true canonical inhibitor, does not even have a P_1 arginine; the only promoter residue within the P_3-P_3' sequence is the P_2 proline. Yet, rhodniin inhibits thrombin with a K_i value in the subpicomolar range and a k_{on} value (7.6×10^8 $M^{-1}\cdot s^{-1}$) comparable to that of hirudin. In addition to the sparse contacts within the reactive site loop, this double-headed inhibitor interacts with Trp^{60d} and exosite 1 of thrombin, ensuring tight connections on both sides of the reactive site loop (van de

Locht et al., 1995). In this respect, it is interesting to note that the P_1 Arg→His substitution in the Aα-chain of fibrinogen does not totally impair thrombin cleavage (Higgins & Shafer, 1981; Southan et al., 1985).

Serpins also illustrate how additional binding and/or allosteric contributions can substitute for unfavorable interactions in the catalytic groove. In heparin cofactor II, the reactive site loop (M**P**L-**S**TQ) has ideal P_2 and P_1' residues, but the P_1 leucine obviously impairs the ability to inhibit thrombin in the absence of heparin: upon addition of the cofactor, the k_{on} value jumps from a modest 5.7×10^2 $M^{-1}\cdot s^{-1}$ to a rapid 5.0×10^6 $M^{-1}\cdot s^{-1}$ (Church et al., 1985; Rogers et al., 1992). After substitution of the unlikely P_1 leucine for the proper arginine, the k_{on} value in the absence of cofactor (1.0×10^5 $M^{-1}\cdot s^{-1}$) becomes consistent with the now P_2-P_1' optimal sequence of the mutant inhibitor (Derechin et al., 1990). The reactive site loop of antithrombin III (AG**R**-**S**LN) has only the P_1 arginine and the P_1' serine as optimal residues. This is consistent with a k_{on} value for thrombin inhibition of only 1.3×10^4 $M^{-1}\cdot s^{-1}$ in the absence of cofactor. While antithrombin III also utilizes the Pro^{60b}-Pro^{60c}-Trp^{60d} motif of thrombin for binding (Le Bonniec et al., 1995), potent inhibition (1.2×10^8 $M^{-1}\cdot s^{-1}$) is obtained only in the presence of heparin (Olson & Björk, 1992; Sheehan & Sadler, 1994). The sequence of the reactive site loop of protease nexin 1 (IA**R**-**S**SP) remains, however, quite puzzling. This serpin exhibits in the absence of cofactor a high k_{on} value for thrombin (1.5×10^6 $M^{-1}\cdot s^{-1}$), which cannot be rationalized by its P_3-P_3' sequence (only the P_1 arginine and the P_1' serine are optimal, as in antithrombin III). Whether binding involves an exosite of thrombin is unknown, but failure to transfer the properties of protease nexin 1 to another serpin by swapping their reactive site loops suggests that part of the high affinity of protease nexin 1 for thrombin resides outside the P_3-P_3' sequence (Hopkins et al., 1995; Djie, 1995).

Three substrates of thrombin, with poor promoting P_3-P_3' sequences, also require a cofactor for productive binding. In factor XI, the scissile bond (K**PR**-IVG) has only the P_2 proline and the P_1 arginine as optimal residues. Thus, it is not surprising that thrombin cleaves factor XI only slowly (2.7×10^3 $M^{-1}\cdot s^{-1}$), in the absence of dextran sulfate (Naito & Fujikawa, 1991; Gailani & Broze, 1991). The cleavage site in factor XIII (V**PR**-GVN) also offers only the P_2 and P_1 amino acids as optimal residues; high efficiency of activation (1.2×10^7 $M^{-1}\cdot s^{-1}$) is reached only in the presence of fibrin as a cofactor (Naski et al., 1991; Hornyak & Shafer 1992). Finally, the activation sites in human and bovine protein C (D**PR**-LID and D**PR**-IVD, respectively) retain only the P_2 and P_1 residues as enhancers and carry two repressors (the aspartates in P_3 and P_3'). Indeed, thrombin activates protein C very poorly in the absence of thrombomodulin, and several studies have established that neutralization of the adverse effects in P_3 and P_3' improves activation in the absence of cofactor (Ehrlich et al., 1990; Richardson et al., 1992).

In protein S, two thrombin cleavage sites have been described (Dahlbäck et al., 1986). The P_3-P_3' sequence of the first (DL**R**-**S**CV) has P_1 and P_1' enhancers, but the P_3 aspartate is a repressor; it is cleaved quite slowly by thrombin (2×10^4 $M^{-1}\cdot s^{-1}$).[4] The P_3-P_3' sequence of the second site differs in human and bovine protein S. In the bovine species, apart from the P_1 arginine, this site (SF**R**-AGL) has no enhancer residue and the P_2' glycine is a repressor; it is cleaved even more slowly by thrombin (2×10^3 $M^{-1}\cdot s^{-1}$). The corresponding site in human protein S is considerably more favorable (CL**R**-**SF**Q); there are no repressors, and the P_1-P_2' sequence is optimal (Table 2). It has been reported that human protein S is much more sensitive to thrombin that its bovine counterpart (Dahlbäck, 1983). Whether this relatively favorable thrombin site in human protein S accounts for the species specificity (Lundwall et al., 1986) remains to be explored.

Cleavage Sites That Are Resistant to Thrombin. It is interesting to examine the sequence of the major sites of proteolysis that occur during blood coagulation and are not cleaved by thrombin: their P_3-P_3' sequences suggest that they may hamper cleavage by thrombin. Excluding the universal P_1 arginine, only the factor IX activation site (**F**T**R**-VVG in the human, **F**S**R**-VVG in the bovine species) and the Arg^{306} (Kalafatis et al., 1994; Rosing et al., 1995) inactivation site in factor V (KT**R**-NL**K** in the human, KT**R**-NP**K** in the bovine species**)** exhibit a residue promoting thrombin catalysis (either a phenylalanine in P_3 or a lysine in P_3'). Yet, in both cases, a strong P_2 repressor (threonine or serine) probably deters thrombin from cleaving (Le Bonniec et al., 1992). Among the other cleavage sites, four have impartial residues only: the activation sites of plasminogen (PG**R**-VVG), of factor VII (QG**R**-IVG), and of bovine factor X (VV**R**-IVG), along with the Arg^{336} (Fay et al., 1991) inactivation site in human factor VIII (QL**R**-MKN). Five of the remaining major cleavage sites comprise one repressor residue (P_2 threonine or P_3 aspartate): the activation sites of kallikrein (ST**R**-IVG), human factor X (LT**R**-IVG), and human prothrombin (EG**R**-TAT), plus the inactivation sites at Arg^{506} in factor V (DR**R**-GIQ) and at Arg^{562} in factor VIII (DQ**R**-GNQ). The last three cleavages occur during the activation of prothrombin (DG**R**-IVE in the human and EG**R**-IVE plus EG**R**-TSE in the bovine species); they are protected by two repressors rather than one: the P_3 and the P_3' positions being occupied by acidic residues.

Residues of Thrombin Involved in Its P′ Specificity. According to the X-ray structures of a number of inhibitor−protease complexes, at least two surface loops of the protease (segments 34−41 and 60−64) are topologically positioned to interact with the $P_1'-P_3'$ residues of a canonical ligand. For example, His^{40} and Phe^{41} in trypsin contact the P_2' arginine and P_3' phenylalanine of the soybean trypsin inhibitor (Sweet et al., 1974). The P_2' arginine of the bovine pancreatic trypsin inhibitor develops similar contacts with Ser^{39}, Phe^{40}, and Gln^{41} in pancreatic kallikrein (Chen & Bode, 1983), and comparable contacts fasten the P_2' tyrosine and the P_3' arginine of the turkey ovomucoid third domain to Leu^{35}, His^{40}, and Phe^{41} of leukocyte elastase (Bode et al., 1992). In the complex of hirulog-3 with thrombin, the position of the P_2' and P_3' glycines suggests that a side chain could interact with Glu^{39}, Leu^{40}, and Leu^{41} (Qiu et al., 1992). Thus, a leucine at positions 40 and 41 is consistent with the hydrophobic nature of the S_2' subsite in thrombin, and the

[4] The k_{cat}/K_m values for protein S cleavage were estimated from Walker (1984) and Lundwall et al. (1986), assuming a first order reaction characterized by the reported $t_{1/2}$ of cleavage.

negative charge in position 39 is consistent with the preference for basic residues in P_3'.

Our data confirm that there is a link between the charge carried by thrombin's residue 39 and that of the P_3' amino acid of the substrate. The P_2' specificity was also altered by the Glu^{39} mutations, but the electrostatic hypothesis was insufficient to fully explain the consequences of the mutations. In thrombin, Glu^{39} is hydrogen bonded to Arg^{35}. It is conceivable that, in some instances, Arg^{35} substitutes for Glu^{39} in interactions with the P_3' residue, but Stubbs et al. (1992) have suggested that this may take effect only upon complex formation with a cofactor. In this scenario, neutralization of Glu^{39} by the cofactor would free Arg^{35} for productive interaction with a negatively charged residue in the P_2' or P_3' positions. This model could explain why replacement of Arg^{35} or Lys^{36} by glutamine had little impact on the hydrolysis of quenched-fluorescence substrates, except with the peptide having glutamate in P_2' position.

In several protease−inhibitor complexes, the loop around residue 60 also interacts with the substrate's leaving group. For example, in neutrophil elastase, Val^{62} contacts the P_3' arginine of the ovomucoid inhibitor, and in pancreatic elastase Leu^{62} contacts the P_3' threonine of a hexapeptide inhibitor (Bode et al., 1989). The charge of the P_3' arginine of the ovomucoid inhibitor (Fujinaga et al., 1987), as well as that of eglin (Frigerio et al., 1992), is counterbalanced through a water molecule by Asp^{64} in chymotrypsin. The 60-loop insertion (from Tyr^{60a} to Phe^{60h}) distinguishes thrombin from most other serine proteases. In the hirulog−thrombin complex, the P_2' glycine is positioned such that a side chain could interact with Lys^{60f} and/or Phe^{60h}. Consistent with a role for Lys^{60f} in the P_2' specificity of thrombin, its truncation to glutamine resulted in a mutant which cleaved more rapidly substrates with leucine or lysine as P_2' residue. The K60fQ mutation would leave more room for a leucine in the P_2' position and would remove a possible electrostatic repulsion between Lys^{60f} and a P_2' lysine.

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