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Biochemistry

Glu-192 → Gln substitution in thrombin mimics the catalytic switch induced by thrombomodulin

(serine proteinases/substrate specificity/mutagenesis/protein engineering/structure–activity relationship)

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ABSTRACT **In serine proteases, residue 192, three residues prior to the active site Ser-195, plays an important role in determining substrate specificity. In trypsin (EC 3.4.21.4) and most trypsin-like enzymes with relatively broad specificity, this position is occupied by Gln. In thrombin (EC 3.4.21.5), an enzyme with restricted specificity, position 192 is occupied by Glu. The potential importance of Glu-192 in restricting the specificity of thrombin was investigated by isosterically replacing Glu-192 with Gln. Unlike trypsin, thrombin cleavage of peptides with acidic residues in positions P3 and P'3 [where P3 and P'3 refer to three residues removed from the Arg (P1) cleavage site on the amino and carboxyl side, respectively] is inefficient. Protein C, an anticoagulant zymogen, has Asp residues in positions P3 and P'3. Thrombomodulin, an endothelial cell protein, complexes with thrombin to activate protein C rapidly thus altering the specificity of thrombin. Compared to thrombin, the Glu-192 → Gln mutant thrombin activates protein C 22 times more rapidly and cleaves the P7–P'5 peptide from the protein C activation site 19 times faster. Enhanced protein C activation results primarily from an increase in the catalytic rate constant rather than an improved Michaelis constant, a property that is shared by the thrombin–thrombomodulin complex. The Glu-192 → Gln mutation does not influence fibrinopeptide A release and only increases the rate of fibrinopeptide B release 2.7-fold. These results demonstrate that Glu-192 plays a critical role in restricting the specificity of thrombin and suggest that thrombomodulin may function in part by altering the enzyme–substrate interaction near residue 192 in thrombin.**

Thrombin (EC 3.4.21.5) is a serine protease that plays a central role in hemostasis. Thrombin clots fibrinogen, activates key blood clotting factors, and modulates platelet and endothelial cell function (1). The enzyme participates in the negative regulation of the clotting process by activating the anticoagulant zymogen protein C, a process that is dramatically accelerated by complex formation with the endothelial cell membrane protein thrombomodulin (2). Despite the multiple substrates that thrombin activates, the enzyme exhibits remarkable specificity with respect to the limited number of peptide bonds that it cleaves. Thrombin exhibits a strong preference for Arg in position P1 and Pro or Gly in position P2 (3, 4) (where P refers to the distance from the cleavage site moving toward the amino terminus of the protein where Arg at the cleavage site is defined as P1). Of particular importance, unlike trypsin, acidic residues in peptide position P3 dramatically reduce thrombin's activity.

Coagulation and complement proteases usually bind to regulatory proteins to form the efficient activation complexes responsible for subsequent zymogen activation. In blood coagulation, these regulatory proteins apparently function as allosteric modulators to alter the conformation and properties of the protease. For thrombin, interaction with thrombomodulin inhibits fibrinogen clotting and converts thrombin into an enzyme capable of catalyzing the rapid activation of protein C despite the presence of Asp in position P3 (2). Indeed, conversion of this Asp to either Gly or Phe improves the ability of thrombin to activate this zymogen (5).

Thrombin differs from most trypsin-like serine proteases by having Glu instead of Gln at position 192 (6), three residues from the active Ser-195 and located at position 522 of the prothrombin sequence. We have expressed and isolated normal recombinant human thrombin and a mutated thrombin, referred to as E192Q, in which Glu-192 was replaced with Gln. Our study shows that residue 192 contributes to the restricted substrate specificity of thrombin. Mutation of residue 192 also provides potential insights into the molecular basis of action for an allosteric modulator like thrombomodulin.

MATERIALS AND METHODS

The E192Q mutation was obtained by oligonucleotide-directed mutagenesis in M13 phage. Normal and mutated prothrombins were expressed using the pNUT vector (7). This vector includes a cDNA that expresses a mutated dihydrofolate reductase that is relatively insensitive to the inhibitory drug methotrexate. This allows for the selection of stable transformants. Wild-type prothrombin and the E192Q mutant were expressed under the control of the metallothionein I promoter in the presence of 50 μM zinc sulfate and of vitamin K_1 (5 μg/ml). A recombinant prothrombin yield of up to 10 μg per ml per 48 hr was obtained from baby hamster kidney cells (BHK-21) supernatant.

Normal and mutated recombinant prothrombins were isolated by diluting 4 liters of cell culture supernatant with 2 liters of distilled water containing 30 mM EDTA and 30 mM benzamidine hydrochloride, followed by batch adsorption at room temperature on 200 ml of swollen QAE-Sephadex (Sigma). The resin was packed into a column (2.5 × 60 cm) and bound proteins were eluted immediately with 50 mM Tris·HCl (pH 7.5) containing 0.5 M NaCl and 10 mM benzamidine hydrochloride. Recombinant prothrombins were purified by affinity chromatography with the human prothrombin-specific monoclonal antibody hPT-959 coupled at 4 mg/ml (final concentration) to Affi-Gel 15 (Bio-Rad). The column (1.5 × 25 cm) was washed with 300 ml of 50 mM Tris·HCl (pH 7.5) containing 0.5 M NaCl, and prothrombin was eluted with 50 mM Tris·HCl (pH 7.5) containing 3 M urea. Recombinant prothrombin was diluted 1:5 in 50 mM Tris·HCl (pH 7.5) containing 10 mM benzamidine hydrochloride and 5 mM EDTA and further purified by anion-exchange chromatography on a HR 5/5 Mono Q column (Pharmacia)

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Abbreviations: Cbo, carbobenzoxy; pNA, *p*-nitroanilide.

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developed with a 30-ml linear gradient from 0.1 to 0.5 M NaCl in 50 mM Tris·HCl (pH 7.5). Prothrombin was activated by the prothrombinase complex as described (8) and purified by cation-exchange chromatography on a HR 5/5 Mono S column (Pharmacia) developed with a 30-ml linear gradient from 0.05 to 0.4 M NaCl in 10 mM 2-(*N*-morpholino)ethanesulfonic acid (pH 6.5). Thrombin concentration was estimated by active-site titration using *p*-nitrophenyl *p'*-guanidinobenzoate.

Human prothrombin (8) and protein C (9), bovine protein C (10), fibrinogen (11), antithrombin III (12), and rabbit thrombomodulin (13) were prepared as described. Fibrinogen was further purified by gelatin-Sepharose chromatography and ammonium sulfate precipitation (34).

All kinetic studies were performed at 20°C in 50 mM Tris·HCl, pH 7.5/0.1 M NaCl containing either gelatin (0.1%) or bovine serum albumin (1 mg/ml). Amidolytic and clotting assays were performed on thrombin and E192Q before and after each experiment to ensure stability (14). Initial velocities were estimated by using experimental values below 15% hydrolysis of the substrate. Kinetic parameters were estimated by fitting the appropriate equation by nonlinear regression analysis using the Enzfitter computer program (15). The weighting for all analyses assumed proportional errors in the dependent variable.

Steady-state kinetics of *p*-nitroaniline substrate hydrolysis were measured in the presence of 0.1 nM enzyme when K_{cat} was higher than 80 s^{-1} and 1 nM enzyme otherwise. Commercially available synthetic substrates were purchased from Boehringer Mannheim, Sigma, KabiVitrum (Molndal, Sweden), or generously provided by American Diagnostica (Greenwich, CT). A minimum of 10 substrate concentrations were used to determine kinetic constants. Concentrations ranged from 10 times greater than K_m to 10 times less than K_m when possible (the highest concentration tested was 2 mM and the lowest concentration allowing initial velocity measurement was 4 μM).

The pseudo-first-order rate constant of inhibition (K') by dansyl-Glu-Gly-Arg chloromethyl ketone (Calbiochem) or bovine antithrombin III were estimated from the plot of the residual activities versus time. Apparent second-order rate constants were estimated from the linear plot of K' values against the initial concentrations of inhibitor (62.5, 125, 250, and 500 nM) assayed with 10 nM thrombin or 10 nM E192Q.

RESULTS

Fibrinogen hydrolysis by E192Q did not reveal any new peptide fragments compared to wild-type thrombin, as monitored by reverse-phase chromatography. The time courses of fibrinopeptide A release were similar for E192Q, normal recombinant, or plasma-purified thrombins (Fig. 1). In contrast, fibrinopeptide B was released more rapidly by E192Q than by wild-type thrombins. The time course of fibrinopeptide B release exhibited the characteristic upward curvature with either E192Q or thrombin. The kinetics are complex since two A fibrinopeptides and two B fibrinopeptides are released per fibrinogen molecule. Higgins *et al.* (17) have developed a kinetic model for fibrinopeptide release to estimate the apparent K_{cat} and K_m for fibrinopeptide A and the apparent K_{cat}/K_m value for fibrinopeptide B. They evaluated fibrinopeptide release at subsaturating fibrinogen concentrations in terms of a sequential model in which fibrinopeptide A must be released before fibrinopeptide B. When fibrinopeptide A release is analyzed according to this model, apparent K_m values of 8.8 ± 0.6 and 9.6 ± 1.6 μM and apparent K_{cat} values of 28 ± 4.5 and 33 ± 2.3 s^{-1} were obtained for thrombin and E192Q, respectively. The K_m values are derived from the concentrations of the Aα chain of fibrinogen. Analysis of fibrinopeptide B release yielded apparent K_{cat}/K_m values of 2.0 ± 0.5 and 5.4 ± 0.4 $\mu M^{-1} \cdot s^{-1}$ for thrombin and E192Q, respectively. Thus, replacement of Glu-192 with Gln in thrombin had little effect on the release of fibrinopeptide A but improved the K_{cat}/K_m of fibrinopeptide B release.

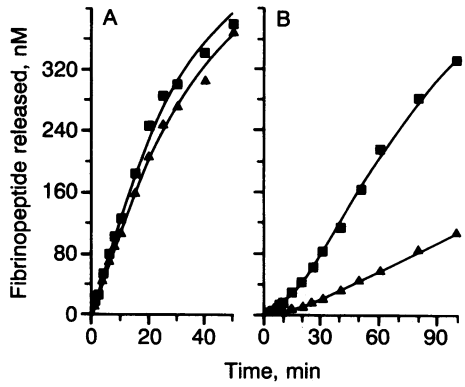

FIG. 1. Time course of release of fibrinopeptides A (*A*) and B (*B*) by 0.1 nM E192Q (■) or recombinant thrombin (▲), at an initial fibrinogen concentration of 0.25 μM in the presence of 4 mM $CaCl_2$. The reaction was stopped by the addition of phosphoric acid to 10% (vol/vol). Concentrations of fibrinopeptide were determined after separation on a reverse-phase C_{18} column (16) and identification by amino acid analysis. Apparent kinetic parameters of fibrinopeptide A release were estimated from six initial velocity measurements obtained at initial fibrinogen concentrations of 0.25, 0.5, 1, 2, 4, and 8 μM. Apparent K_{cat}/K_m value of fibrinopeptide B release were estimated from the time course of release obtained at initial fibrinogen concentrations of 0.25 and 0.50 μM by fitting the equation suggested by Higgins *et al.* (17). Solid lines in *B* represent the result of such analysis.

To provide a general comparison between thrombin and E192Q, we examined a series of chromogenic substrates (Table 1). For many of the substrates, the activity of thrombin and E192Q were very similar. With these 17 substrates, the K_m remained essentially unchanged, whereas K_{cat} was either unaltered or increased. E192Q had a tendency to hydrolyze substrates with Gly at position P2 and/or Glu at position P3 better than thrombin. Detailed interpretation was precluded because of variation in amino-terminal blocking agents and/or lack of identical residues beyond the P2 position. However, the suggestion that Gly in P2 might interact with E192Q better than thrombin was supported by examination of the relative rates of inhibition by antithrombin III. This natural inhibitor has the sequence Ala-Gly-Arg-Ser-Leu-Asn in the reactive center. The second-order rate constant for inhibition of E192Q with antithrombin III was 1.5-fold greater than that of thrombin (9.2 ± 0.2 versus 6.1 ± 0.2 $mM^{-1} \cdot s^{-1}$). In addition, consistent with a role of Gly in P2 and Glu in P3, dansyl-Glu-Gly-Arg chloromethyl ketone inhibited E192Q with a 3.4-fold greater second-order rate constant (38.4 ± 0.7 versus 11.3 ± 0.2 $mM^{-1} \cdot s^{-1}$).

Although not itself definitive, the above results suggested the possibility that substrates with acidic residues in position P3 might be processed more rapidly by E192Q than by thrombin. Protein C constitutes a thrombin substrate with Pro in P2 and Asp in P3. Relative to thrombin, the K_{cat} for protein C activation was increased 22-fold with E192Q (48.0 ± 2.9 versus 2.2 ± 0.1 min^{-1}, Fig. 2*A*). In contrast, the K_{cat} increase was less than 2-fold in the presence of thrombomodulin (149 ± 9.2 versus 80 ± 4.5 min^{-1}, Fig. 2*B*). The K_m was not altered between the mutated and the wild-type enzyme: 5.5 ± 0.6 and 4.7 ± 0.4 μM, in the absence of thrombomodulin, and 6.6 ± 0.6 and 5.9 ± 0.6 μM in the presence of thrombomodulin for E192Q and thrombin, respectively. E192Q is not totally thrombomodulin independent since K_{cat} was still increased by thrombomodulin (149

Table 1. Steady-state kinetics of *p*-nitroaniline substrate hydrolysis by recombinant thrombin and E192Q

Substrate	K_m, μM		K_{cat}, s^{-1}		EQ/W
	Wild	E192Q	Wild	E192Q	
(P3—P2—P1—pNA)					
D-Phe-Pip-Arg-pNA	8	9	277	254	0.9
d-Ile-Pro-Arg-pNA	<3	<3	149	144	1.0
D-Lys(Cbo)-Pro-Arg-pNA	5	5	275	282	1.0
D-Chg-Pro-Arg-pNA	<2	<2	42	48	1.1
Ts-Gly-Pro-Arg-pNA	10	12	368	472	1.3
D-Val-Leu-Arg-pNA	189	186	38	30	0.8
D-Hht-Ala-Arg-pNA	6	6	174	163	0.9
Bz-Phe-Val-Arg-pNA	69	83	87	229	2.6
Me-SO_2-D-Cht-Gly-Arg-pNA	29	24	248	379	1.5
Me-SO_2-D-Phe-Gly-Arg-pNA	79	80	46	123	2.3
MeO-CO-D-Hht-Gly-Arg-pNA	26	24	67	175	2.6
MeO-CO-D-Nle-Gly-Arg-pNA	70	65	31	99	3.2
Cbo-L-Glu(OBu^t)-Gly-Arg-pNA	51	43	14	45	3.2
MeO-CO-D-Chg-Gly-Arg-pNA	40	42	44	162	3.7
Me-CO-Lys(Cbo)-Gly-Arg-pNA	195	153	12	47	3.9
Bz-Ile-Glu-Gly-Arg-pNA	67	55	2	16	8.0
Bz-Val-Gly-Arg-pNA	>1000	>1000	>4	>57	—

EQ/W indicates the K_{cat} ratio between E192Q and thrombin (Wild). pNA, *p*-nitroanilide; Ts, tosyl; Bz, benzoyl; Nle, norleucyl; Pip, L-pipecolyl; Chg, cyclohexylglycyl; Cht, cyclohexyltyrosyl; Hht, hexahydrotyrosyl; Cbo, carbobenzoxy; OBu^t, *t*-butoxy. Standard errors were ±2% or less for K_{cat} and ±10% or less for K_m.

versus 48 min^{-1}). The Glu-192 → Gln substitution had no significant effect on the calcium dependence of protein C activation. As with thrombin, E192Q complexed with thrombomodulin activated protein C far better in the presence of calcium than in EDTA. Conversely, in the absence of thrombomodulin, E192Q activated protein C far better in EDTA than in calcium. Similar results were obtained when using human instead of bovine protein C.

The differences between thrombin and E192Q were confirmed and extended by analyzing the hydrolysis of synthetic peptides corresponding to P7–P'5 of the human protein C activation site. This peptide was cleaved by E192Q more than 19 times faster than by thrombin (650 ± 64 versus 33 ± 3 nM/min, Fig. 3*A*). When the Asp in position P3 was replaced by Gly to yield the sequence Gly-Pro-Arg, thrombin cleaved the substituted peptide 2.6 times faster than the normal peptide (86 ± 4 versus 33 ± 3 nM/min, Fig. 3*B*). In protein

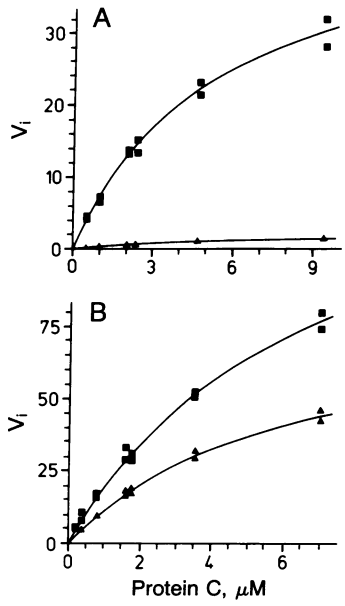

FIG. 2. Activation of bovine protein C in the absence (*A*) or in the presence (*B*) of thrombomodulin. Curves represent the initial velocities (V_i) of protein C activation (expressed as nM activated protein C per min per nM thrombin or E192Q) plotted against the initial concentration of protein C for E192Q (■) and recombinant thrombin (▲). Solid lines were obtained by fitting the Michaelis–Menten equation. Reaction mixtures contained 1 nM enzyme and 5 mM EDTA in *A* or 0.1 nM enzyme and 5 mM $CaCl_2$ in *B*. Activation was stopped by the addition of 4 μM antithrombin III. Concentrations of activated protein C were estimated from the steady-state kinetics of D-(Cbo)Lys-Pro-Arg-pNA hydrolysis by reference to a standard curve (18). A minimum of 15 activated protein C concentrations were estimated from initial velocity determination.

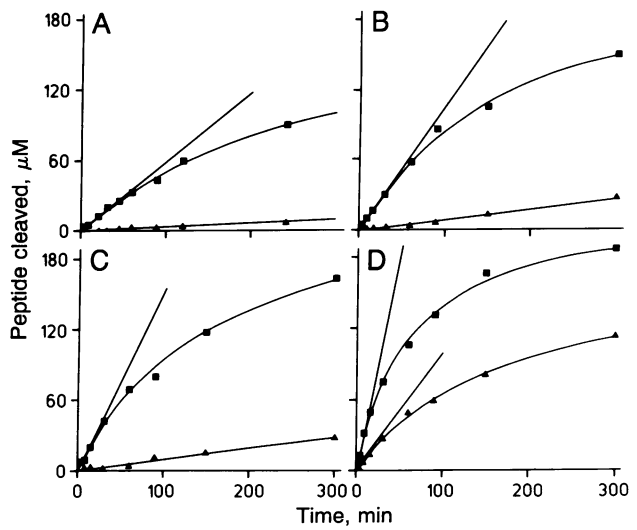

FIG. 3. Comparison of the ability of E192Q (■) and thrombin (▲) to cleave the P7–P'5 peptide corresponding to the activation site of human protein C (Glu-Asp-Gln-Val-Asp-Pro-Arg-Leu-Ile-Asp-Gly-Lys). Cleavage occurs at the Arg-Leu peptide bond. (*A*) Unsubstituted peptide. (*B*) Asp in position P3 was replaced by Gly. (*C*) Asp in P'3 was replaced by Gly. (*D*) Both Asp residues were replaced by Gly. The concentration of peptide cleaved is plotted against time. Reaction mixtures contained 50 nM enzyme and 0.2 mM peptide. The reaction was stopped by the addition of phosphoric acid to 10%. The concentration of intact peptide in the hydrolysates was determined after separation on a reverse-phase C_{18} column as described in Fig. 1.

C, the P'3 residue (where P'3 is defined as three residues carboxyl-terminal to the P1 Arg) is also an Asp. This is unusual since the P'1–4 residues are highly conserved in more than 50 serine protease zymogens with Gly in position P'3. Protein C, prothrombin, and chymotrypsin are among the few exceptions. When the Asp in P'3 of the protein C P7–P'5 peptide was replaced by a Gly, a small increase in the rate of cleavage by thrombin was also observed (92 ± 4 versus 33 ± 3 nM/min, Fig. 3*C*), but when Asp residues in both P3 and P'3 were replaced by Gly, the peptide was cleaved 30 times faster than the normal P7–P'5 peptide (993 ± 72 versus 33 ± 3 nM/min, Fig. 3*D*). These observations suggest that Asp residues in both P3 and P'3 contribute to the slow activation of protein C by thrombin in the absence of thrombomodulin. E192Q hydrolyzed all peptides better than thrombin, but the difference between E192Q and thrombin was decreased from more than 19-fold for the normal P7–P'5 peptide to less than 4-fold for the double-substituted peptide (3692 ± 215 versus 993 ± 72 nM/min). Single substitution in the peptide gave similar, intermediate, results with E192Q (1290 ± 81 and 1527 ± 356 nM/min for the P3- and P'3-substituted peptides, respectively). The effects of thrombomodulin on thrombin specificity are, however, more subtle than those observed with the Glu-192 → Gln substitution in that thrombomodulin does not alter the rate of hydrolysis by thrombin of the P7–P'5 peptide of protein C (data not shown).

The inhibitory influence of an acidic residue in the P'3 position may provide insights into the specificity of thrombin cleavage in fibrinogen and in protein C. After the release of fibrinopeptide A, the newly formed amino-terminal sequence is Gly-Pro-Arg-Val-Val-Glu. Surprisingly, the Arg-Val peptide bond is cleaved very slowly by thrombin (19). The P'3 position of this site contains an acidic amino acid that may contribute to the resistance of this bond to further proteolysis by thrombin.

DISCUSSION

These studies reveal that (*i*) Glu-192 plays a major role in the restricted substrate specificity of thrombin, (*ii*) replacement of Glu-192 with Gln allows thrombin to accommodate acidic residues in the P3 and P'3 positions, a property that is shared with trypsin, and (*iii*) both the P3 and P'3 residues are critical for restricting thrombin specificity. The latter observation is supported directly from analysis of protein C peptide hydrolysis and the impact of replacing Asp with Gly at the P3 and/or P'3 position. The Glu-192 → Gln substitution in thrombin results in an increase in K_{cat} for protein C activation, with little change in K_m. The observations that increased rates of cleavage by E192Q are associated primarily with the presence of acidic residues in P3 and P'3 and that the mutation results in charge neutralization would be easiest to rationalize in terms of improved enzyme–substrate interaction. That improved enzyme–substrate interaction can increase K_{cat} with little change in K_m suggests a role of residue 192 in stabilizing the transition state rather than a role in binding to form the Michaelis complex (20). By assuming no structural change from this isosteric substitution, the implications from this work would be that the Asp residues in positions P3 and P'3 of protein C interact with residue 192 in the transition state. These interactions are not favored in thrombin due to charge repulsions that are overcome either by association with thrombomodulin or by the Glu-192 → Gln substitution. The details of the molecular interactions responsible for the kinetic effects observed with this substitution are clearly beyond the scope of the current study.

The potential for Glu-192 conformational changes upon substrate binding is supported both by structural analysis of serine proteases in general and thrombin in particular. Since detailed information comparing the thrombin structure alone and in complex with transition state analogues is not yet available, the specific interactions in the transition state complex remain unknown. The possibility for specific interactions of residue 192 in the transition state is supported by the observation that, in other serine proteases, the side chain undergoes a major conformational change upon complex formation with pseudo substrates (21–28). In the complex, the side chain of residue 192 forms numerous contacts with the pseudo substrate. In the trypsin and chymotrypsin-like enzymes, regardless of the inhibitor, residue 192 contacts positions P2, P1, and P'1 but not P3 and P'3 of the substrate (21–25). In several structures, the side chain of amino acid 192 also contacts the residue(s) in P4 and/or P5. In contrast, position P3 does interact with residue 192 in elastase (26–28). Thrombin seems intermediate between the trypsin and elastase-like enzymes. In the thrombin–hirudin complex, the side chain of Val-2 in hirudin is located at the position occupied by the side chain of Glu-192 in the D-Phe-Pro-Arg-CH_2-thrombin structure (6, 29, 30). Thus, the conformation of Glu-192 in thrombin can be altered by interaction with pseudosubstrates. In the D-Phe-Pro-Arg-CH_2-thrombin structure, the Phe is not in contact with Glu-192. It should be noted that this is the D-isomer of Phe and, therefore, is not located in the normal S3 binding site of thrombin (6).

Although residue 192 is intimately involved in substrate interactions in all structures determined to date, the details of its role in enzyme specificity are yet to be totally elucidated. More than 60% of the trypsin-like enzymes have Gln at position 192. Only thrombin and protein C have a Glu. Several proteases, such as factors VII and XI, have basic residues, but the possible role of this basic residue in substrate specificity is unexplored. Residue 192 in the kallikreins is variable (Val, Met, Thr, or Lys). The presence of Met in position 192 correlates with the preference for bulky hydrophobic residues in position P2 (21). The above observation coupled with the variability of residue 192 and its interaction with substrates deduced from the crystal structures are consistent with the possibility that it may be an important determinant in enzyme specificity.

In the blood-clotting cascade, serine proteases are inefficient zymogen activators until they form complexes with specific regulatory proteins. Complex formation results primarily in increasing K_{cat} (31). Our data show that the Glu-192 → Gln mutation accounts remarkably well for the enhanced K_{cat} observed for protein C activation that results from association of thrombin with thrombomodulin. Given the multiplicity of substrate interaction sites that involve residue 192 in various serine proteases, the conformational changes in the specificity pocket that accompany thrombomodulin binding (32) may include Glu-192. Thrombomodulin (32) and hirudin (29, 30) are both known to alter the conformation of thrombin in the extended binding pocket. Further, the thrombin binding sites for thrombomodulin and hirudin overlap (33). Since hirudin alters the conformation of Glu-192, it is possible that thrombomodulin shares this property. Given the above arguments, thrombomodulin may function in part by altering the conformation of Glu-192 and alleviating the inhibitory interactions with the Asp in positions P3 and P'3. Consistent with this hypothesis, relative to thrombin, the Glu-192 → Gln mutation increases protein C activation 22-fold in the absence of thrombomodulin but only 2-fold in its presence. If the increase in protein C activation was due to a generalized alteration of the enzyme function that was totally independent of the changes induced by thrombomodulin, then one might expect to see thrombomodulin increase the activity of E192Q to the same extent that it increases the activity of thrombin.

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