The Role of Glu192 in the Allosteric Control of the S\textsubscript{2}' and S\textsubscript{3}' Subsites of Thrombin*

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Thrombin is an allosteric protease controlled through exosites flanking the catalytic groove. Binding of a peptide derived from hirudin (Hir\textsuperscript{52–65}) and/or of heparin to these opposing exosites alters catalysis. We have investigated the contribution of subsites S\textsubscript{2}' and S\textsubscript{3}' to this allosteric transition by comparing the hydrolysis of two sets of fluorescence-quenched substrates having all natural amino acids at positions P\textsubscript{2} and P\textsubscript{3}'. Regardless of the amino acids, Hir\textsuperscript{52–65} decreased, and heparin increased the k\textsubscript{cat}/K\textsubscript{m} value of hydrolysis by thrombin. Several lines of evidence have suggested that Glu\textsuperscript{192} participates in this modulation. We have examined the role of Glu\textsuperscript{192} by comparing the catalytic activity of thrombin and its E192Q mutant. Mutation substantially diminishes the selectivity of thrombin. The substrate with the "best" P\textsubscript{2}' residue was cleaved with a k\textsubscript{cat}/K\textsubscript{m} value only 49 times higher than the one having the "least favorable" P\textsubscript{2}' residue (versus 636-fold with thrombin). Mutant E192Q also lost the strong preference of thrombin for positively charged P\textsubscript{3}' residues and its strong aversion for negatively charged P\textsubscript{3}' residues. Furthermore, both Hir\textsuperscript{52–65} and heparin increased the k\textsubscript{cat}/K\textsubscript{m} value of substrate hydrolysis. We conclude that Glu\textsuperscript{192} is critical for the P\textsubscript{2}' and P\textsubscript{3}' specificities of thrombin and for the allostery mediated through exosite 1.

Thrombin (1, 2) is a multifunction serine protease finely tuned through: 1) restrictions fulfilled by the nature of the P\textsubscript{3} to P\textsubscript{3}' residues of the substrate, \(^*\) that the S\textsubscript{3} to S\textsubscript{3}' subsites of the protease must accommodate, 2) steric hindrance resulting from surface loops, that limit access to the active site, 3) secondary exosites (one apolar and two positively charged) that limit access to the active site, 3) secondary exosites flanking the catalytic groove. Binding of a peptide derived from hirudin (Hir\textsuperscript{52–65}) and/or of heparin to these opposing exosites alters catalysis. We have investigated the contribution of subsites S\textsubscript{2}' and S\textsubscript{3}' to this allosteric transition by comparing the hydrolysis of two sets of fluorescence-quenched substrates having all natural amino acids at positions P\textsubscript{2} and P\textsubscript{3}'. Regardless of the amino acids, Hir\textsuperscript{52–65} decreased, and heparin increased the k\textsubscript{cat}/K\textsubscript{m} value of hydrolysis by thrombin. Several lines of evidence have suggested that Glu\textsuperscript{192} participates in this modulation. We have examined the role of Glu\textsuperscript{192} by comparing the catalytic activity of thrombin and its E192Q mutant. Mutation substantially diminishes the selectivity of thrombin. The substrate with the "best" P\textsubscript{2}' residue was cleaved with a k\textsubscript{cat}/K\textsubscript{m} value only 49 times higher than the one having the "least favorable" P\textsubscript{2}' residue (versus 636-fold with thrombin). Mutant E192Q also lost the strong preference of thrombin for positively charged P\textsubscript{3}' residues and its strong aversion for negatively charged P\textsubscript{3}' residues. Furthermore, both Hir\textsuperscript{52–65} and heparin increased the k\textsubscript{cat}/K\textsubscript{m} value of substrate hydrolysis. We conclude that Glu\textsuperscript{192} is critical for the P\textsubscript{2}' and P\textsubscript{3}' specificities of thrombin and for the allostery mediated through exosite 1.

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\(^\dagger\dagger\) Lys60f is the 6th residue inserted at position 60).

\(^\ddagger\) Amino acid sequence numbering of thrombin, as suggested by Bode et al. (1), is based on its three-dimensional topological identity with chymotrypsin. Insertion residues are denoted by small letters in alphabetic order (e.g. Lys\textsuperscript{45} is the 6th residue inserted at position 60).

\(^3\) The abbreviations used are: BPTI, bovine (or basic) pancreatic trypsin inhibitor (Kunitz type 1, also called Trasylol or aprotinin); Abz, O-amino benzoyl; Dnp, 2,4-dinitrophenyl; Fmoc, N-(9-fluorenylmethoxycarbonyl).

\(^4\) Hirugen is a C-terminal analog of hirudin variant 2 (NGDFFEEIPEEYL). Hir\textsuperscript{52–65} (used in this study) is the corresponding C-terminal analog of hirugren variant 1 (NDGDFFEEIPEEYL). For both analogs, when Tyr\textsuperscript{28} is sulfated, the K\textsubscript{d} for thrombin decreases about 10-fold.
the cofactor on protein C activation (32, 33).

Binding of a ligand to the second positively charged exosite of thrombin also alters catalysis. In particular, heparin (a polydisperse mucopolysaccharide of $M_f$ ∼30,000) greatly increases the rate of thrombin inactivation by antithrombin and heparin cofactor II (34–36). Other ligands such as prothrombin fragment 2 or a synthetic peptide derived from its residues 63–116, as well as a monoclonal antibody recognizing an epitope that overlap with exosite 2, also induce conformational changes in the active site of thrombin (37–43).

The effect of the simultaneous addition of exosites 1 and 2 ligands on thrombin activity is not fully understood. Heparin decreases the rate of thrombin inactivation by antithrombin and fibrin (but not thrombin) (45–47). Finally, binding of heparin and fibrin contributes additively to the changes in the active site environment of thrombin, and fibrin (but not thrombomodulin) protects thrombin from inactivation by heparin-antithrombin (46–49).

Using a complete set of fluorescence-quenched substrates, we have compared the specificity of the “primed” subsites of thrombin with that of its E192Q mutant. Our results reveal that the mutation dramatically modifies the $P_2$ preferences, little is known about the possible outcome of the mutation with respect to its $P_2$ preferences. To fully characterize alterations in the $S_2$ and $S_3$ subsites, we have synthesized two series of fluorescence-quenched substrates, each having the sequence Abz-VOPR-SKL(Dnp)D, where the sequence Pro-Arg-Ser is optimal for thrombin cleavage, and X represents any one of the natural amino acids (except cysteine). For each substrate, we have estimated the value of $k_{obs}$ for its hydrolysis by thrombin, its E192Q variant, or trypsin (Table 1). We then deduced for substrates $S_2$ and $S_3$ of each protease a selectivity index, which is defined as the ratio of the $k_{obs}$ for the best substrate over that for the worst (higher indexes indicate greater selectivity). We reasoned that this approach might neglect possible cooperative effects between subsites but would at least disclose the importance of subsites $S_2$ and $S_3$. Precisely, results revealed a dramatic difference between thrombin and its E192Q variant with respect to their $S_3$ selectivity (Fig. 1); indexes were 636 and 49, respectively (20 for trypsin). Thus the simple exchange of the Phe residue could transform a peptide from being an excellent to a mediocre substrate of thrombin, whereas all substitutions have much less effect with E192Q and very little with trypsin. Hence, the

\[ INT = I_a + I_{max}[1 - \exp(-E \cdot k_{obs} \cdot t)] \]

(Eq. 1)

where $E$ represents the enzyme concentration. Initial estimates for the values of $I_a$ and $I_{max}$ (used in the nonlinear curve fitting analysis) were obtained independently by measuring the fluorescence in the absence of enzyme and after hydrolysis by 100 ng trypsin for 1 h, respectively. Two initial concentrations of substrate, varying by a factor of at least 2, were systematically assayed. With both concentrations, the values of $k_{obs}$ were within the experimental error (±10%), suggesting that $k_{obs}$ could be equated to the $k_{cat}/K_m$ value of the reaction (3).

\[ k_{cat} = \frac{[E][S]}{[I]} \]  

(Eq. 2)

$k_{cat}$ may also be expressed as a function of the total amount of enzyme ([E]+[I]).

\[ k_{cat} = \frac{[E][S]}{[I] + [E] + [I]} \]  

(Eq. 3)

where $a = k_{cat}[S]/[K_m + [S]]$ and $b = k_{cat}^* [S]/[K_m^* + [S]]$, such that $k_{cat}^*$ and $K_m^*$ are the $k_{cat}$ and $K_m$ values of the substrate ([S]) in the presence of saturating amount of the effector. On the other hand, according to the law of mass action (assuming a single binding site for the ligand on the enzyme), we composed the following equation.

\[ [E][S]/([E][S] + [I][S]) = \frac{3}{4} \]  

(Eq. 4)

Combining Equations 3 and 4 results in the following equation.

\[ k_{obs} = \frac{[E][S]}{[I] + [E] + [I]} - \frac{[E][S]}{[E][S] + [I][S]} - \frac{[E][S]}{[E][S] + [I][S]} \]  

(Eq. 5)

The $K_m$ values of $Hirs^{22-65}$ and heparin for thrombin or its E192Q derivative were estimated by nonlinear curve fitting analysis of the dependence of $k_{obs}$ on [I], using Equation 5. Initial estimates for the values of $a$ and $b$ were obtained independently by measuring $k_{obs}$ in the absence and in the presence of a saturating amount of the effector, respectively. Concentration of $Hirs^{22-65}$ was varied between 0.4 and 20 $\mu$M and that of heparin between 0.02 and 1.3 $\mu$M.

RESULTS

The E192Q Mutation Abolishes the Restrictions that Govern $S_2$ and $S_3$ Specificities of Thrombin—A number of studies suggest that residue 192 of serine proteases determines in part their specificity (4–9, 51–54). Although it is well established that substituting Gln for Glu at position 192 in thrombin alters its $P_2$ preferences, little is known about the possible outcome of the mutation with respect to its $P_2$ preferences. To fully characterize alterations in the $S_2$ and $S_3$ subsites, we have synthesized two series of fluorescence-quenched substrates, each having the sequence Abz-VOPR-SKL(Dnp)D, where the sequence Pro-Arg-Ser is optimal for thrombin cleavage, and X represents any one of the natural amino acids (except cysteine). For each substrate, we have estimated the value of $k_{obs}$ for its hydrolysis by thrombin, its E192Q variant, or trypsin (Table 1). We then deduced for substrates $S_2$ and $S_3$ of each protease a selectivity index, which is defined as the ratio of the $k_{obs}$ for the best substrate over that for the worst (higher indexes indicate greater selectivity). We reasoned that this approach might neglect possible cooperative effects between subsites but would at least disclose the importance of subsites $S_2$ and $S_3$. Precisely, results revealed a dramatic difference between thrombin and its E192Q variant with respect to their $S_3$ selectivity (Fig. 1); indexes were 636 and 49, respectively (20 for trypsin). Thus the simple exchange of the Phe residue could transform a peptide from being an excellent to a mediocre substrate of thrombin, whereas all substitutions have much less effect with E192Q and very little with trypsin. Hence, the

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where $a = k_{cat}[S]/[K_m + [S]]$ and $b = k_{cat}^* [S]/[K_m^* + [S]]$, such that $k_{cat}^*$ and $K_m^*$ are the $k_{cat}$ and $K_m$ values of the substrate ([S]) in the presence of saturating amount of the effector. On the other hand, according to the law of mass action (assuming a single binding site for the ligand on the enzyme), we composed the following equation.

\[ [E][S]/([E][S] + [I][S]) = \frac{3}{4} \]  

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Combining Equations 3 and 4 results in the following equation.

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TABLE I

| Substrate residues P$_1^\prime$–P$_3^\prime$ | Thrombin | E192Q | Trypsin |
|----------------------------------------|----------|-------|---------|
| SFL                                   | 2.1 × 10$^7$ | 4.3 × 10$^6$ | 4.2 × 10$^7$ |
| SYL                                   | 1.4 × 10$^8$ | 2.9 × 10$^7$ | 3.0 × 10$^8$ |
| SWL                                   | 6.9 × 10$^9$ | 1.7 × 10$^8$ | 2.6 × 10$^9$ |
| SGL                                   | 5.5 × 10$^7$ | 1.1 × 10$^6$ | 4.3 × 10$^7$ |
| SML                                   | 4.3 × 10$^7$ | 1.9 × 10$^6$ | 3.8 × 10$^7$ |
| SLY                                   | 3.6 × 10$^7$ | 1.6 × 10$^6$ | 4.7 × 10$^7$ |
| SLL                                   | 3.3 × 10$^7$ | 1.6 × 10$^6$ | 4.4 × 10$^7$ |
| SRS                                   | 3.3 × 10$^7$ | 1.7 × 10$^6$ | 3.9 × 10$^7$ |
| SIR                                   | 3.2 × 10$^7$ | 1.6 × 10$^6$ | 5.5 × 10$^7$ |
| SKR                                   | 2.9 × 10$^6$ | 6.6 × 10$^5$ | 2.3 × 10$^6$ |
| SHR                                   | 2.5 × 10$^6$ | 1.1 × 10$^5$ | 1.9 × 10$^6$ |
| SSL                                   | 2.1 × 10$^6$ | 1.5 × 10$^5$ | 3.8 × 10$^6$ |
| SRL                                   | 2.0 × 10$^6$ | 1.4 × 10$^5$ | 3.6 × 10$^6$ |
| SFL                                   | 1.5 × 10$^6$ | 9.9 × 10$^5$ | 1.4 × 10$^6$ |
| SFG                                   | 1.3 × 10$^6$ | 9.4 × 10$^5$ | 3.3 × 10$^6$ |
| SFP                                   | 5.9 × 10$^5$ | 4.0 × 10$^4$ | 9.3 × 10$^5$ |
| SFR                                   | 6.2 × 10$^5$ | 9.0 × 10$^4$ | 2.7 × 10$^5$ |
| SFD                                   | 3.3 × 10$^5$ | 8.7 × 10$^4$ | 6.4 × 10$^5$ |

Selectivity index (P$_1^\prime$) = 636 49 20

Selectivity index (P$_3^\prime$) = 52 3 2

Overall preferences of E192Q were similar to thrombin; the most favorable P$_3^\prime$ residue remained phenylalanine, and the most detrimental was aspartate; only the magnitude changed. Rather than switching specificity, the E192Q mutation appeared to remove the strong restriction normally conferred by the S$_3^\prime$ subsite of thrombin. The selectivity was lost primarily by lessening the strongest restrictions. The substrates with the most favorable residues (Phe and Tyr) were cleaved 2-fold faster by E192Q; those with the deleterious residues (Asp, Glu) were cleaved 15 and 26-fold faster, respectively. Nevertheless, the E192Q mutant no longer preferred arginine over aliphatic residues; from the fourth preferred residue with thrombin, arginine became the twelfth with E192Q. Thus, in thrombin, the negative charge of Glu$^{192}$ appeared both to prevent accommodation of negatively charged residues in P$_1^\prime$ and to facilitate that of cationic side chains. The preferences of trypsin were not only weak, they were also slightly different. Thrombin and E192Q preferred bulky aromatic side chains, trypsin preferred aliphatic side chains. Negatively charged residues were, as with thrombin and E192Q, the least favorable amino acids. Surprisingly, trypsin exhibited a modest preference for arginine (as thrombin) despite having a glutamine in position 192 (as the E192Q variant).

The outcome of the E192Q mutation on the P$_3^\prime$ preferences of thrombin conformed with the above observations concerning the S$_2^\prime$ subsite. The E192Q mutation also decreased by an order of magnitude the selectivity index (Table I), and the largest effects were observed with the least favorable residues (again, Asp and Glu). In essence, the E192Q mutation removed the constraints of the S$_3^\prime$ subsite; the selectivity index was only 3.4 with E192Q, a value comparable with that of trypsin (2.0), reflecting indifference toward the substrate P$_3^\prime$ side chain. Overall, the E192Q mutation specifically destroyed the ability of thrombin to attract or repel charged side chains in its S$_2^\prime$ and S$_3^\prime$ subsites, even though arginine was still the preferred P$_3^\prime$ residue and acidic side chains were still unfavorable. Consistent with this concept, the E192Q mutation also alters the P$_2$ and P$_3$ preferences of thrombin, but rather than switching its specificity, it appears to minimize the restrictions that thrombin normally applies at these positions (4, 5, 9).

Exosite 1 Modulation of the S$_2^\prime$ and S$_3^\prime$ Subsites—In a previous study (9), we challenged the hypothesis that the E192Q mutation mimicked the allosteric alteration induced by Hir$^{22-65}$ in the S$_3$ subsite of thrombin (4). The data were in favor of exosite binding, causing changes in the conformation of the S$_2^\prime$ and/or S$_3^\prime$ subsites of thrombin rather than mitigating unfavorable interactions between Glu$^{192}$ and acidic residues in P$_3$ position of the substrate. In the present study, we have extended our previous investigation to subsites S$_2^\prime$ and S$_3^\prime$ of thrombin. We first verified that affinities of Hir$^{22-65}$ for thrombin and E192Q were comparable by measuring the k$_{obs}$ values for H$_2$O-Phe-pipecolyl-Arg-p-nitroanilide hydrolysis by each enzyme in the presence of increasing concentrations of the effector. Likewise, similar K values for Hir$^{22-65}$ with either enzyme were obtained when the affinity was estimated using fluorescence-quenched substrates instead of the peptidyl-p-nitroanilide (Fig. 2). The effect induced by Hir$^{22-65}$, however, depended upon the substrate used to detect the binding. With thrombin and E192Q, Hir$^{22-65}$ increased the value of k$_{obs}$ for H$_2$O-Phe-pipecolyl-Arg-p-nitroanilide hydrolysis. In contrast, with most Abz-VGPRSSXKL(Dnp)D substrates, Hir$^{22-65}$ roughly halved the k$_{obs}$ value with thrombin but still increased that with E192Q (Fig. 2). The changes in the value of k$_{obs}$ were indeed the result of the effector binding to thrombin or E192Q and not of an interaction between the effector and the substrate or other...
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substrate with a $k_{\text{effector}}$; in contrast, with the E192Q mutant (Hir52–65. 7.6-fold that of thrombin in the presence of saturating concentrations of
those observed with the P 2
residue was an aspartate, the peptide was cleaved 64-fold more
rapidly by E192Q than by thrombin (versus 27-fold in the absence of Hir52–65).

Concerning the P 2
series of substrates, the changes induced by Hir52–65 on the catalysis of thrombin and E192Q matched those observed with the P 2
series. The $k_{\text{obs}}$ value of substrate cleavage in the presence of Hir52–65 was decreased with thrombin and increased with E192Q (Table II). Furthermore, Hir52–65 had little or no influence on the cleavage by E192Q of the peptide carrying a glutamate or an aspartate in P 2
, whereas it halved the $k_{\text{obs}}$ values of their cleavage by thrombin. Thus, the greatest difference between thrombin and E192Q was again on the hydrolysis of the substrate having an aspar
tate in P 2
(residue 52–65) that had been added to improve their solubility. Given the critical role of the negative charges in the allosteric regulation of thrombin, we wondered whether formation of the Michaelis complex with a thrombin carrying Hir52–65 was not hampered. A potential conflict existed between the C-terminal aspartate of the substrate and Hir52–65 which itself was heavily negatively charged. Such a conflict could explain the paradoxical allostery induced by Hir52–65. In contrast to our fluorescence-quenched substrates, the p-nitroanilide substrates having a proline in the P 2
position are cleaved with higher $k_{\text{obs}}$ values in the presence of Hir52–65 (9, 18). To test this hypothesis, fluorescence-quenched substrates lacking the C-terminal aspartate were synthesized. Solubility of Abz-VGPRSFLLK(Dnp) and Abz-VGPRSWLLK(Dnp) allowed estimation of the $k_{\text{obs}}$ values in experimental conditions that were the same as those for the peptides having a C-terminal aspartate. The values of $k_{\text{obs}}$ were similar, whether or not substrate carried a C-terminal aspar
tate, suggesting that residue P 2
of a substrate had little influence, if any, on thrombin catalysis. The allosteric modulation triggered by Hir52–65 was also of similar magnitude and in the same direction for both types of substrates. Therefore, the allosteric influence, if any, on the catalysis of trypsin, suggesting an absence of heterotropic effects between the substrates and the effectors. As in Table I, only the P 2
residues of each substrate are indicated, and the percentages given were calculated from the weighted mean of at least two determinations, each having a standard error of 7% or less.

**FIG. 2. Determination of the $k_{\text{obs}}$ values of the C-terminal fragment of hirudin (Hir52–65) for thrombin and its E192Q mutant, using the fluorescence-quenched substrate Abz-VGPRSFLLK(Dnp).** The solid lines represent the results of the nonlinear curve fitting analysis of the dependence of $k_{\text{obs}}$ (expressed in percentages of the value in the absence of effectors) on the concentration of Hir52–65 (Equation 5). Estimates of 0.8 and 2.0 $\mu$m were obtained with thrombin and its E192Q mutant, respectively. With thrombin (.), Hir52–65 decreased the $k_{\text{obs}}$ value of hydrolysis to 37% of that without effector; in contrast, with the E192Q mutant (○), Hir52–65 increased the $k_{\text{obs}}$ value up to 137%. Consequently, the E192Q mutant cleaved the substrate with a $k_{\text{obs}}$ value twice that of thrombin without effector but 7.6-fold that of thrombin in the presence of saturating concentrations of Hir52–65.

**TABLE II**

| Residues | Thrombin | E192Q | Trypsin |
|----------|----------|-------|---------|
| P 1
| P 2
| Hir52–65 | Hep | Both | Hir52–65 | Hep | Both | Hir52–65 | Hep | Both |
| SFL | 37 | 124 | 67 | 137 | 158 | 190 | 105 | 105 |
| SFL | 62 | 129 | 79 | 179 | 183 | 214 | 113 | 107 |
| SFL | 47 | 120 | 62 | 135 | 176 | 224 | 108 | 112 |
| SFL | 55 | 180 | 93 | 155 | 248 | 268 | 112 | 109 |
| SFL | 49 | 149 | 74 | 150 | 173 | 195 | 103 | 113 |
| SFL | 56 | 136 | 67 | 156 | 163 | 194 | 106 | 104 |
| SFL | 61 | 139 | 73 | 144 | 194 | 206 | 114 | 107 |
| SFL | 58 | 133 | 61 | 129 | 159 | 200 | 121 | 115 |
| SFL | 58 | 118 | 70 | 150 | 156 | 169 | 89 | 102 |
| SFL | 62 | 176 | 93 | 167 | 221 | 227 | 109 | 109 |
| SFL | 52 | 140 | 58 | 186 | 158 | 209 | 93 | 100 |
| SFL | 57 | 148 | 100 | 160 | 187 | 223 | 97 | 103 |
| SFL | 60 | 130 | 75 | 164 | 129 | 179 | 81 | 108 |
| SFL | 50 | 135 | 65 | 164 | 186 | 229 | 88 | 108 |
| SFL | 51 | 145 | 71 | 121 | 152 | 189 | 86 | 114 |
| SFL | 58 | 138 | 72 | 117 | 133 | 149 | 109 | 100 |
| SFL | 131 | 156 | 134 | 218 | 155 | 275 | 108 | 103 |
| SFL | 53 | 115 | 85 | 111 | 122 | 124 | 89 | 111 |
| SFL | 42 | 127 | 55 | 103 | 116 | 128 | 103 | 106 |
| SFR | 85 | 115 | 108 | 179 | 172 | 194 | 112 | 107 |
| SFR | 71 | 122 | 94 | 152 | 155 | 193 | 115 | 108 |
| SFR | 79 | 126 | 102 | 154 | 159 | 191 | 117 | 99 |
| SFR | 78 | 109 | 84 | 182 | 138 | 167 | 102 | 102 |
| SFR | 60 | 132 | 78 | 167 | 181 | 208 | 102 | 97 |
| SFA | 62 | 128 | 83 | 150 | 145 | 177 | 101 | 110 |
| SFA | 68 | 103 | 78 | 153 | 150 | 208 | 109 | 108 |
| SFA | 59 | 121 | 110 | 181 | 158 | 203 | 89 | 111 |
| SFA | 51 | 111 | 78 | 175 | 135 | 190 | 105 | 114 |
| Sofy | 50 | 132 | 71 | 157 | 134 | 201 | 96 | 100 |
| SFP | 77 | 119 | 92 | 161 | 143 | 174 | 110 | 114 |
| SFP | 75 | 139 | 107 | 143 | 176 | 189 | 117 | 100 |
| SFP | 48 | 115 | 78 | 171 | 155 | 186 | 109 | 109 |
| SFP | 42 | 104 | 65 | 167 | 170 | 189 | 105 | 100 |
| SFP | 50 | 105 | 73 | 142 | 142 | 176 | 89 | 104 |
| SFP | 45 | 112 | 82 | 158 | 135 | 185 | 109 | 106 |
| SFP | 37 | 124 | 67 | 137 | 158 | 190 | 105 | 104 |
| SFP | 42 | 127 | 52 | 111 | 115 | 121 | 113 | 100 |
| SFP | 44 | 148 | 71 | 108 | 121 | 127 | 99 | 108 |
Contribution of Glu\textsuperscript{192} in the Allostery of Thrombin

**E192Q**—Through various molecular mechanisms, heparin dramatically enhances the vulnerability of thrombin to a number of serpins. To evaluate possible modulation in the primed substrates of thrombin or E192Q that would be triggered through exosite 2 binding, we compared the values of \( k_{\text{obs}} \) for the hydrolysis of the two series of fluorescence-quenched substrates in the absence and presence of saturating amount of heparin.

As for the exosite 1 study, we first verified that the E192Q mutation did not grossly modify the affinity of heparin. \( K_d \) values of 0.21 and 0.36 unit/ml were obtained for thrombin and E192Q, respectively. Possible heterotropic effects between the substrate and heparin were also ruled out by comparing hydrolysis of the Abz-VGPRS\textsuperscript{LK(Dnp)}D substrates by trypsin in the presence or absence of heparin (Table II). On average, the value of \( k_{\text{obs}} \) for the hydrolysis of the substrates by thrombin was increased 1.4-fold by heparin, and that for the hydrolysis by E192Q was increased 1.7-fold. With both thrombin and E192Q, higher increases in the \( k_{\text{obs}} \) values were obtained with the substrates having either a lysine or an arginine in P\textsubscript{2} position (about 1.8-fold with thrombin and 2.3-fold with E192Q). Thus, the effect of heparin on E192Q catalysis was comparable with that on thrombin, albeit more pronounced. With the P\textsubscript{2} series of fluorescence-quenched substrates, the changes induced by heparin on the catalysis of thrombin and E192Q mirrored the above observations concerning the P\textsubscript{2} series; in the presence of heparin, substrates were cleaved by thrombin and E192Q with higher \( k_{\text{obs}} \) values (1.2- and 1.5-fold on the average, respectively; Table II). Thus, in contrast to the allostery mediated through exosite 1, the E192Q mutation did not overturn the effect induced by heparin. The relatively uniform effect of heparin on thrombin and E192Q catalysis also suggested that subsites S\textsubscript{2} and S\textsubscript{3} were not directly involved in this allostery. Therefore, our results confirm that a linkage exists between exosites 1 and 2, and the catalytic groove of thrombin but imply that this linkage is not mediated through Glu\textsuperscript{192}.

**Simultaneous Modulation of Thrombin and E192Q by Hir\textsubscript{52–65} and Heparin**—The full cofactor activity of thrombomodulin involves a chondroitin sulfate carried by this integral membrane receptor, suggesting that simultaneous binding to exosites 1 and 2 may occur. Hence, a study has reported that rather than simply circumscribing thrombin, thrombomodulin may also cross-link two molecules of thrombin, raising doubts on the bivalent binding hypothesis (55). In addition, a linkage exists between exosites 1 and 2, even if binding of heparin to exosite 2 does not exclude occupancy of exosite 1 (39, 40, 48). In an attempt to reconcile the puzzling consequences of the simultaneous presence of exosites 1 and 2 effectors on thrombin activity, we have determined the effect of a mixture of heparin and Hir\textsubscript{52–65} on the hydrolysis of the fluorescence-quenched substrates. With thrombin, the simultaneous presence of the two effectors resulted in values of \( k_{\text{obs}} \) that were intermediate between those obtained in the presence of each effector (Table II). Interpretation was difficult because opposing effects were induced by each effector separately. There was enough heparin and Hir\textsubscript{52–65} to saturate thrombin with either one or both, such that the concentration of free thrombin would be negligible during the assay. In a purely ternary complex model (i.e., heparin and Hir\textsubscript{52–65} bound simultaneously to thrombin), the progress curve of hydrolysis would simply reflect the simultaneous allosteric contribution of each exosite to a single species catalyzing a single reaction, which should be adequately represented by Equation 1. In fact, it was possible to analyze all data obtained in the presence of both heparin and Hir\textsubscript{52–65} according to Equation 1. In a strictly mutually exclusive binding model (i.e., thrombin bound heparin or Hir\textsubscript{52–65}, but not both), the progress curve of hydrolysis would reflect the independent contribution of two enzyme complexes (thrombin-Hir\textsubscript{52–65} and thrombin-heparin) catalyzing the same reaction (in parallel).

\[
\text{INT} = I_r + I_{\text{mol}} [1 - \exp(-E \cdot k_{\text{obs}(1)} \cdot t)] + (1 - \gamma) I_{\text{mol}} [1 - \exp(-E \cdot k_{\text{obs}(2)} \cdot t)] \quad (\text{Eq} \ 6)
\]

In this equation, \( y \) would be the percentage of the total thrombin concentration in complex with Hir\textsubscript{52–65}, the remaining (1 - \( y \)) being in complex with heparin; parameters \( k_{\text{obs}(1)} \) and \( k_{\text{obs}(2)} \) would be the \( k_{\text{obs}} \) values determined in the absence of the other effector. With only one exception, it was feasible to analyze the progress curve data according to this mutually exclusive model. The nonlinear curve fitting analysis consistently provided a value for \( \gamma \) comprised between 0.2 and 0.6 (i.e. 20–60% of thrombin in complex with Hir\textsubscript{52–65}, the remaining with heparin). Thus, most data were compatible with both the ternary complex as well as the mutually exclusive models, and it was not possible to discriminate between them by the nonlinear curve fitting approach. However, the fluorescence-quenched substrate having a proline in P\textsubscript{2} position was cleaved with a \( k_{\text{obs}} \) value higher than for thrombin (about 1.8-fold with thrombin and 2.3-fold with E192Q). Thus, the results were undoubtedly in favor of a simultaneous binding of the two effectors to a single molecule of E192Q. Therefore, our data suggest that heparin and Hir\textsubscript{52–65} can form a ternary complex with thrombin or E192Q, and it is reasonable to believe that the \( k_{\text{obs}} \) values obtained with thrombin for the hydrolysis of the fluorescence-quenched substrates...
in the presence of the two effectors reflected the activity of this ternary complex. Hence, the value of $k_{obs}$ obtained in the presence of the two effectors did not equate to the sum of those obtained with each effector separately, suggesting that a linkage exists between the two exosites (Fig. 4). This linkage did not involve a decrease of the affinity of the ligands for their exosite. In the presence of 2 units/ml heparin, the $k_{obs}$ values for the cleavage of the Abz-VGPRSFLLK(Dnp)D substrate were virtually identical whether Hir52–65 concentration was 10, 30, or 100 μM (3.6, 3.5, and 3.6 $10^6$ M$^{-1}$ s$^{-1}$, respectively), implying that the $K_d$ of Hir52–65 for the E192Q mutant did not increase upon binding of heparin to exosite 2.

**DISCUSSION**

Overall our data substantiate and extend to the substrate leaving group the concept that specificity of the catalytic groove of thrombin originates from an exclusion of the unfavorable P$_2$–P$_3$' side chains rather than from a positive selection of favorable ones. Thrombin cleaved its best fluorescence-quenched substrates with a $k_{cat}/K_m$ value comparable with that of trypsin and the other substrates with $k_{cat}/K_m$ values up to 15000-fold lower than trypsin. Thus, with respect to the catalytic groove, thrombin is a restricted trypsin. Glu$^{192}$ of thrombin emerged as a major determinant in this ability to repel unfavorable P$_2$' and P$_3$' side chains and also appeared to be involved in the allosteric mediated through binding of an effector to exosite 1. However, it is unclear whether the link between exosite 1 and residue 192 is direct, indirect, or fortuitous. In contrast, Glu$^{192}$ seemed to play a minor role in the allostery of thrombin mediated through exosite 2, and heparin exerted a similar effect on thrombin and E192Q catalysis.

A direct link between Glu$^{192}$ and exosite 1 would imply that upon binding of an allosteric effector, the glutamate changes its conformation. Consistent with this hypothesis, the side chain of Glu$^{192}$ adopts different orientations in the x-ray structures of thrombin (10–12, 56, 57). In particular, the side chain of Glu$^{192}$ occupies in free bovine thrombin a different conformation than when in complex with an inhibitor, an exosite ligand, or both. Compared with the d-Phe-Pro-Arg-CH$_2$Cl-thrombin structure, the binding of hirugen produces only minor alterations in the orientation of the side chains of the catalytic triad and of the exosite 1 residues, whereas changes occur along segment Ala$^{190}$-Glu$^{197}$, which reach a maximum near Glu$^{192}$ (10). Whether this conformational difference of residue 192 is a consequence of hirugen or of d-Phe-Pro-Arg-CH$_2$Cl binding remains unclear, but hirugen does not seem capable of inducing a conformational change when the active site is occupied (11). Several of the catalytic properties of E192Q thrombin suggest that the mutation mimics in part the modulation of thrombin by exosite 1 effectors. Compared with thrombin, the E192Q mutant activates protein C faster (4) and is inhibited by BPTI with a $K_i$ value 3 orders of magnitude lower (7). Thrombomodulin dramatically increases the rate of protein C activation by thrombin and increases 10-fold the $k_{cat}$ value of thrombin inhibition by BPTI (31). Nevertheless, α$_1$-antitrypsin neutralizes E192Q (6), and E192Q activates bovine factor X (5), two functions that thrombomodulin is unable to trigger in thrombin. Furthermore, thrombomodulin also modifies the catalytic properties of E192Q (4), and as shown in this study, Hir$^{52–65}$ has an effect opposite on thrombin and E192Q catalysis. Thus, even if some of the modifications introduced by the E192Q mutation mimic (in part) the allostery triggered by exosite 1 ligands, they cannot explain alone the catalytic switch of thrombin. Undoubtedly, the link between Glu$^{192}$ and exosite 1 is more elaborate.

The link between exosite 1 of thrombin and Glu$^{192}$ could be indirect; that the conformation of Glu$^{192}$ changes upon binding of hirugen does not preclude that other modifications occur simultaneously. If the allosteric effector induces one or several conformational changes that are shared upon mutation of Glu$^{192}$ in thrombin, then the E192Q mutant and the complex may share catalytic properties that differ from free thrombin. It is well established that the autolysis loop of thrombin, in the vicinity of the S$_2'$ subsite, adopts various conformations (1, 58). Docking to exosite 1 of the thrombin receptor peptide or of Hir$^{52–65}$, as well as covalent binding to the active site of d-Phe-Pro-Arg-CH$_2$Cl, all induce a reorganization of the autolysis loop (11, 59). In particular, inhibited thrombin is 95-fold less susceptible to chymotrypsin cleavage within this loop than free thrombin (60). Conversely, mutations in the autolysis loop of thrombin alter hydrolysis of p-nitroanilide substrates that are devoid of P$_3$ residues (61, 62). Thus, there is a link in thrombin between the autolysis loop and the subsites of the catalytic groove. Whether the E192Q mutation induces a similar alteration of the autolysis loop remains to be determined, but our study demonstrates a crucial role of Glu$^{192}$ to the P$_3'$ specificity of thrombin, raising the possibility that the S$_2'$ subsite is altered by the mutation. Crystallographic data, however, argue against this hypothesis; the conformation of the catalytic groove moiety comprising subsites S$_2'$ and S$_3'$ is the same in thrombin and in the complex of E192Q with BPTI (8). The side chain of Glu$^{192}$ also appears too distant to interact directly with the main chain of the P$_3'$ residue of Hirulog 3 (63). Thus, the link between exosite 1 and residue Glu$^{192}$ of thrombin must involve an alternate mechanism. A gross structural rearrangement of the 60-loop insertion occurs upon BPTI binding to E192Q; crystallographic data also demonstrate a structural link between the expulsion of the 60-loop and a reorganization of the 39-loop in the vicinity of exosite 1. It is therefore conceivable that the reverse process occurs with strong binding to exosite 1 facilitating expulsion of the 60-loop. Consistent with this hypothesis, the 60-loop insertion of thrombin forms in part the S$_b$ and S$_a$ subsites, and the E192Q mutation alters the P$_2$ and P$_3$ specificities (5, 9, 50). The deletion of three residues from the 60-loop insertion of thrombin (mutation des-PPW) also results in a nanomolar $K_i$ value for BPTI binding (64).
However, mutations E192Q and des-PPW are not interchangeable; they are additive with respect to BPTI binding, resulting in picomolar affinity (7). Furthermore, binding of fibrinogen, antithrombin, thrombomodulin, and hirudin is preserved by the des-PPW mutation. Thus the E192Q mutation in itself is unlikely to account for a major conformational change in the 60-loop; the mutation would rather favor a destabilization of the molecule triggered by the binding of BPTI.

Finally, the link between exosite 1 and Glu192 may be deceptive. The allosteric effector and the E192Q mutation may use unrelated alterations to change the $k_{obs}$ value of fluorescence-quenched substrate hydrolysis. The $k_{obs}$ value for the hydrolysis of a substrate depends on the binding energy, its turnover by the enzyme, and/or its approaching orientation. Glu192 results from such complementary electrostatic fields (65). The very high $k_{obs}$ value for the inhibition of thrombin by hirudin results from such complementary electrostatic fields (66, 67). Thrombin carries two strong positive patches that sandwich the catalytic groove, itself constituting a strong negative patch. Small substrates would not develop a strong electrostatic field, but the changes observed were small. In fact, the variations of the $k_{obs}$ values were related in part to the charge of the substrate; those carrying a lysine or an arginine in P3' were cleaved with higher $k_{obs}$ values by thrombin than by E192Q, and those with an aspartate or a glutamate were cleaved with higher $k_{obs}$ values by E192Q than by thrombin. All substrates of the P3' series were cleaved more efficiently by E192Q, but the increase of the $k_{obs}$ value was more pronounced for the substrates having negative P3' side chain. Thus, it is conceivable that an altered electrostatic field in E192Q allows for a better access/orientation to the negatively charged substrates or, conversely, impedes access/orientation to the positively charged substrates. On the other hand, the binding of Hir52–65 to exosite 1 of thrombin neutralizes in part the positive patch that follows subsite S3' of the catalytic groove. Again, there was a (small) link between the charge of the P3' residue of the substrate and the changes of the $k_{obs}$ value. Complexes of Hir52–65 with thrombin cleaved all substrates less efficiently than the free enzyme, but the decrease of the $k_{obs}$ value was less pronounced for the substrates having positive P3' side chain. In all these, however, the positive P3' side chain and was more pronounced for those having negative side chains. It is conceivable that neutralization of positive exosite 1 worsens the initial orientation of substrates negatively charged at P3'. Conversely, even if complexes of E192Q with Hir52–65 cleaved all substrates more efficiently than E192Q, the increase of the $k_{obs}$ value was more pronounced for the substrates having a positive P3' side chain and less pronounced for those having a negative side chain. These observations are consistent with the hypothesis that binding of Hir52–65 and the E192Q mutation influence the catalysis independently.

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