Allosteric modulation of the activity of thrombin

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Substrates containing a P_3 aspartic residue are in general cleaved poorly by thrombin. This may be partly due to an unfavourable interaction between the P_3 aspartate and Glu^{192} in the active site of thrombin. In Protein C activation and perhaps also thrombin receptor cleavage, binding of ligands at the anion-binding exosite of thrombin seems to improve the activity of thrombin with substrates containing a P_3 aspartate. To investigate the importance of Glu^{192} and exosite-binding in modulating thrombin's interactions with a P_3 aspartate, peptidyl chloromethanes based on the sequence of the thrombin receptor (containing a P_3 aspartate) have been synthesized and the kinetics of their inactivation of α-thrombin and the mutant $Glu^{192} \rightarrow Gln$ determined. The values of the inactivation rate constant (k_i) for the chloromethanes containing a P_3 aspartate were about two-fold higher with the $Glu^{192} \rightarrow Gln$ mutant. A peptide based on the sequence of hirudin ($rhir^{52–65}$), which binds to the anion-binding exosite of thrombin, was an allosteric modulator of the amidolytic activity of the $Glu^{192} \rightarrow Gln$ mutant; a 5-fold decrease in the K_m value for the substrate D-Phe-pipecolyl-Arg-*p*-nitroanilide was observed in the presence of saturating concentrations of $rhir^{52–65}$. This exosite-binding peptide also increased the k_i values of chloromethanes containing a P_3 aspartate with both α-thrombin and the $Glu^{192} \rightarrow Gln$ mutant. However, the increases in the k_i values were greater with the $Glu^{192} \rightarrow Gln$ mutant (5-fold compared with 2-fold for α-thrombin). Thus exosite binding does not seem to mitigate putative unfavourable interactions between Glu^{192} and the P_3 aspartate. Moreover, increases in the k_i caused by exosite binding were not unique to chloromethanes containing a P_3 aspartate; increases of the same magnitude were also observed when the P_3 position was occupied by the favourable D-phenylalanine in place of the unfavourable aspartate. The results obtained were consistent with exosite binding's causing changes in the conformation of the S_2 and/or S_1 sites of thrombin.

INTRODUCTION

Thrombin is a serine protease with a central role in haemostasis. Like trypsin, thrombin cleaves substrates on the C-terminal side of arginine residues, but its specificity is much more restricted. The structural basis for thrombin's specificity has been elucidated by crystallographic studies. Access to thrombin's active site is restricted by a surface loop that partly occludes this binding site and creates a particularly hydrophobic S_2 site. In addition, many of thrombin's natural substrates and inhibitors bind to a region outside the catalytic cleft termed the anion-binding exosite [1]. This exosite is a positively charged surface groove; negatively charged regions of specific substrates (fibrinogen and thrombin receptor) and inhibitors (hirudin and heparin cofactor II) as well as its cofactor thrombomodulin interact with this site [2]. In addition to providing increased binding energy, exosite interactions also allosterically modify the active site of thrombin. Exosite-binding fragments of hirudin, heparin cofactor II and the thrombin receptor modulate the cleavage of chromogenic substrates by thrombin [3–7]. Similar effects are also observed with thrombomodulin [8]. It has been proposed that the conformational change induced by exosite binding is important in the cofactor activity of thrombomodulin [9]. In the absence of thrombomodulin, Protein C is a poor substrate for thrombin. This is due at least in part to the presence of aspartic residues in the P_3 and P_3' positions in Protein C. Mutation of these residues greatly enhances the rate of activation of Protein C by thrombin [10,11]. In the presence of thrombomodulin, however, Protein C is efficiently activated by thrombin. An attractive hypothesis is that thrombomodulin's binding to the anion-binding exosite allosterically modifies thrombin's active site to allow more efficient binding of aspartate in the P_3 and/or P_3' positions. The thrombin receptor also contains an aspartic residue in the P_3 position as well as an exosite-binding sequence [12] and thus it also seems possible that exosite interactions modulate unfavourable active site interactions for this ligand. Indeed, the data of Ishii et al. [13] indicate that the exosite-binding sequence of the receptor overcomes the inhibitory effects of the P_3 aspartic residue on thrombin's cleavage. There is evidence to suggest that the unfavourable interactions between thrombin and the P_3 and/or P_3' residues of substrates involve Glu^{192} in the active site of thrombin. Mutation of Glu^{192} of thrombin to glutamine improves the ability of thrombin to cleave substrates with aspartate in the P_3 and P_3' positions [14].

To investigate the role of exosite interactions and Glu^{192} in modulating thrombin's cleavage of the thrombin receptor, we have synthesized peptidyl chloromethanes based on the thrombin receptor sequence and have determined the kinetics of inactivation of thrombin and a mutant (E192Q) in which Glu^{192} is replaced by glutamine by these peptidyl chloromethanes. In addition, the effects of exosite-binding on the rate of inactivation were examined. The results indicate that Glu^{192} might play a minor role in restricting thrombin's activity with substrates containing a P_3 aspartate, but allosteric interactions at thrombin's exosite do not, in themselves, improve its ability to bind ligands with a P_3 aspartic residue.

Abbreviations used: Fmoc, fluoren-9-ylmethoxycarbonyl; Mtr, 4-methoxy-2,3,6-trimethylbenzenesulphonyl; OtBu, t-butyl ester; pNA, *p*-nitroanilide; $rhir^{52–65}$, a peptide comprising residues 52–65 from the C-terminal region of recombinant hirudin.

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MATERIALS AND METHODS

Materials

All compounds used for peptide synthesis were purchased from Bachem (Bubendorf, Switzerland). Solid-phase peptides were synthesized with a Milligen 9050 PepSynthesizer and fluoren-9-ylmethyloxycarbonyl (Fmoc) strategy with the Fmoc amino acid pentafluorophenyl esters and Sasrin resin. D-Phe-Pip-Arg-pNA (S-2238) (where Pip is pipecolyl and pNA represents *p*-nitroanilide) was purchased from Quadratech (Epsom, Surrey, U.K.) and its concentration was determined from its absorbance at 342 nm [15]. Human α-thrombin and the mutant E192Q were prepared as previously described [14,16]. The concentrations of a peptide comprising residues 52–65 from the C-terminal region of recombinant hirudin (rhir$^{52-65}$) and the peptidyl chloromethanes were determined by amino acid analysis. The concentrations of α-thrombin and E192Q were estimated by active site titration with *p*-nitrophenyl-*p*′-guanidinobenzoate [17].

Synthesis of peptidyl chloromethanes

Ac-Asp-Pro-Arg-CH_2Cl was prepared by standard methods as previously described [18]. The blocked nonapeptide Ac-Ala-Thr-Asn-Ala-Thr-Leu-Asp-Pro-Arg-CH_2Cl was prepared by a '6 + 3' procedure in which the C-terminal tripeptide diazomethane, synthesized in solution, was coupled to the N-terminal hexapeptide, which had been obtained by solid-phase synthesis [18].

H-Asp(OtBu)-Pro-Arg(Mtr)-CHN_2

t-Butoxycarbonyl-Pro-*N*-hydroxysuccinimide was coupled with H-Arg(Mtr)-OH (where Mtr represents 4-methoxy-2,3,6-trimethylbenzenesulphonyl) by standard procedures. The t-butoxycarbonyl group of the resulting dipeptide was cleaved off with gaseous HCl in ethyl acetate to give H-Pro-Arg(Mtr)-OH, which was coupled with Fmoc-Asp(OtBu)-*N*-hydroxysuccinimide ester (where OtBu represents t-butyl ester) to yield Fmoc-Asp(OtBu)-Pro-Arg(Mtr)-OH. This was converted via the mixed anhydride with diazomethane to Fmoc-Asp(OtBu)-Pro-Arg(Mtr)-CHN_2. The Fmoc group was removed with piperidine/dimethylformamide (1:4, v/v).

Ac-Ala-Thr-Asn-Ala-Thr-Leu-Asp-Pro-Arg-CH_2Cl,trifluoroacetic acid

The N-terminal hexapeptide H-Ala-Thr(t-butyl)-Asn-Ala-Thr(t-butyl)-Leu-Sasrin from the solid-phase synthesis was acetylated with acetic anhydride and triethylamine. Then the peptide was cleaved off the resin with 1 % (v/v) trifluoroacetic acid in CH_2Cl_2. This was activated with 1-hydroxybenzotriazole/*N*,*N*′-diisopropylcarbodi-imide and coupled with H-Asp(OtBu)-Pro-Arg(Mtr)-CHN_2. The resulting nonapeptide was converted to the chloromethane with gaseous HCl in ethyl acetate. Deprotection was performed in trifluoroacetic acid containing 10 % (v/v) anisole. The product was purified by preparative reverse-phase HPLC with a 2.2 cm × 25 cm Vydac C_{18} column (218TP1022) and linear gradient of 10–50 % (v/v) acetonitrile in 0.1 % (v/v) trifluoroacetic acid over 30 min with a flow rate of 8 ml/min. The mass obtained for the purified product by fast atom bombardment mass spectrometry [*m*/*z* 1032 $(M+H)^+$] coincided with the calculated M_r of 1032.6.

Thrombin assays

These were performed in 50 mM Tris/HCl buffer, pH 7.8, containing 0.1 M NaCl and 0.2 % (w/v) poly(ethylene glycol), M_r 6000, as previously described [18]. Initial-rate studies to determine the effect of rhir$^{52-65}$ on the kinetic parameters for the cleavage of D-Phe-Pip-Arg-pNA used five different substrate concentrations and seven concentrations of rhir$^{52-65}$ (see Figure 1). For the determination of the inactivation rate constant for chloromethanes, assays contained a fixed concentration of substrate (100–400 μM) and seven different concentrations of peptidyl chloromethane.

Data analysis

The values of the inactivation rate constant (k_i) for the peptidyl chloromethanes were determined by progress-curve kinetics as previously described [18]. Progress-curve data obtained at seven different concentrations of peptidyl chloromethane (one assay did not contain inhibitor) were fitted by nonlinear regression to the following equation:

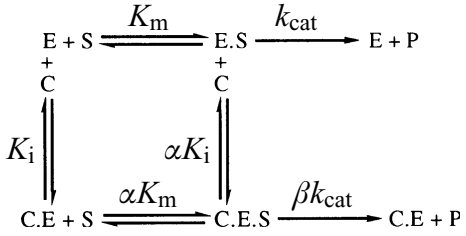
$$P = (v_0/k')[1-\exp(-k'[I]t)] \quad (1)$$

where P is the concentration of *p*-nitroaniline formed by cleavage of the substrate at time t, v_0 is the velocity of substrate cleavage in the absence of inhibitor, and k' equals $k_i/(1+[S]/K_m)$. Analysis with this equation yielded estimates of k_i together with the standard errors of these estimates.

RESULTS

Effect of rhir$^{52-65}$ on the cleavage of D-Phe-Pip-Arg-pNA by E192Q

The peptide rhir$^{52-65}$ acted as an allosteric modulator for the cleavage of D-Phe-Pip-Arg-pNA by E192Q. In the presence of rhir$^{52-65}$, the K_m value for D-Phe-Pip-Arg-pNA decreased, whereas the k_{cat} value remained constant. The results could be interpreted in terms of the following scheme, in which E, S and C represent E192Q, D-Phe-Pip-Arg-pNA and rhir$^{52-65}$ [19].



Because k_{cat} did not vary in the presence of rhir$^{52-65}$, it was assumed that β was equal to 1. In this case, the equation describing the dependence of the initial velocity (v) on the concentrations of D-Phe-Pip-Arg-pNA and rhir$^{52-65}$ is [19]:

$$\frac{v}{[E]_t} = \frac{k_{cat}[S]}{K_m(1+[C]/K_i)+[S](1+[C]/\alpha K_i)} \quad (2)$$

The initial-velocity data obtained with different concentrations of D-Phe-Pip-Arg-pNA and rhir$^{52-65}$ were analysed by nonlinear regression in accordance with this equation and the results of the analysis are shown in Figure 1. This analysis yielded values for k_{cat}, K_m, K_i and α that are given in Table 1; values for these parameters previously obtained for α-thrombin are given for comparison. In the presence of a saturating concentration of rhir$^{52-65}$, the K_m for D-Phe-Pip-Arg-pNA decreased 5-fold ($1/\alpha$); this decrease was twice that observed with α-thrombin (Table 1).

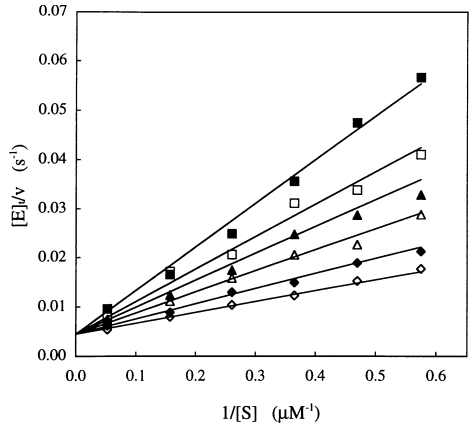


Figure 1 Effect of rhir$^{52–65}$ on the amidolytic activity of E192Q

Assays were performed as described in the Materials and methods section with 1.7–19.2 μM D-Phe-Pip-Arg-pNA and 7 pM E192Q. Initial velocities (v) were measured at five different substrate concentrations ([S]) in the presence of 0 (■), 0.49 (□), 0.97 (▲), 1.94 (△), 4.85 (◆) and 19.4 (◇) μM rhir$^{52–65}$. Nonlinear regression according to eqn. (2) yielded the estimates for K_m, α, k_{cat} and K_i given in Table 1. Five data points obtained at 9.7 μM rhir$^{52–65}$, which were used in the analysis, are not shown. The data are plotted in double-reciprocal form ($[E]_t/v$ against 1/[S]) and the lines are drawn to illustrate the fit of the data to eqn. (2).

Table 1 Kinetic parameters for the reaction of E192Q with D-Phe-Pip-Arg-pNA in the presence of rhir$^{52–65}$

Assays were performed as described in the Materials and methods section. The data (shown in Figure 1) were analysed with eqn. (2) to yield the estimates of the parameters given in the Table: ± values are standard errors derived from the variance–covariance matrix of the regression analysis. Values for α-thrombin are taken from [3].

	E192Q	α-thrombin
k_{cat} (s^{-1})	221 ± 4	205
K_m (μM)	19.9 ± 0.8	3.4
K_i (μM)	5.0 ± 0.6	3.2
α	0.21 ± 0.01	0.41
αK_i (μM)	1.04 ± 0.11	1.3
αK_m (μM)	4.2 ± 0.3	1.4

Inactivation of thrombin by chloromethanes based on the thrombin receptor sequence effect of rhir$^{52–65}$ and the E192Q mutation

To examine the ability of interactions with the anion-binding exosite and Glu^{192} to modulate the ability of thrombin to accept ligands containing a P_3 aspartate, peptidyl chloromethanes based on the sequence of the thrombin receptor were synthesized and the kinetics of the inactivation of α-thrombin and E192Q were determined. A chloromethane based on the P_3–P_1 sequence of the thrombin receptor (Ac-Asp-Pro-Arg-CH_2Cl) exhibited a relatively low inactivation rate constant (k_i) with α-thrombin (7.4×10^3 $M^{-1} \cdot s^{-1}$; Table 2); the k_i for Ac-Asp-Pro-Arg-CH_2Cl was about 3000-fold lower than that observed with D-Phe-Pro-Arg-CH_2Cl, one of the most rapid and best characterized peptidyl chloromethane inhibitors of thrombin [20]. The k_i of Ac-Asp-Pro-Arg-CH_2Cl with E192Q was 2-fold higher than with thrombin (Table 2). In contrast, D-Phe-Pro-Arg-CH_2Cl inactivated E192Q 5-fold more slowly than it did α-thrombin. Extension of the chloromethane to include the P_7–P_1 residues of the thrombin receptor (Ac-Ala-Thr-Asn-Ala-Thr-Leu-Asp-Pro-Arg-CH_2Cl) increased k_i 14-fold and 9-fold with α-thrombin and E192Q respectively.

The exosite-binding peptide rhir$^{52–65}$ caused an increase of 2–3-fold in k_i for the chloromethanes with α-thrombin. The magnitude of the increase did not depend markedly on the sequence of the chloromethane; it ranged from 2.2-fold with Ac-Asp-Pro-Arg-CH_2Cl to 2.9-fold with D-Phe-Pro-Arg-CH_2Cl. The binding of rhir$^{52–65}$ to the exosite caused slightly larger increases (approx. 5-fold) in k_i with E192Q; the observed increases with E192Q also did not depend on the sequence of the chloromethane.

DISCUSSION

In the present study we have investigated the role of exosite binding in modulating interactions with the active site of thrombin, and the involvement of Glu^{192} in this process. In the structure of D-Phe-Pro-ArgCH_2-thrombin [21,22], Glu^{192} is positioned such that it would make an unfavourable electrostatic interaction with a P_3 aspartic residue (Figure 2). Indeed, results obtained in this and other studies [14] suggest that the negative charge on Glu^{192} does seem to play a role in restricting the ability of thrombin to bind P_3 aspartic residues. Neutralization of the charge of Glu^{192} in E192Q resulted in a modest (2-fold) increase in k_i for peptidyl chloromethanes containing a P_3 aspartate. On binding of the hirudin-derived peptide hirugen to the exosite, the side chain of Glu^{192} moves from the S_3 site and occupies a solvent-exposed position [23,24] (Figure 2). Thus it seemed possible that exosite binding by the hirudin-like region of the thrombin receptor facilitated the binding of the P_3 aspartate in the active site by inducing a movement in Glu^{192}. A similar model could also explain the ability of exosite binding by thrombomodulin to promote the cleavage of Protein C. The results obtained in the present study, however, do not support this hypothesis. Exosite binding of rhir$^{52–65}$ caused an allosteric change in the active site of thrombin that increased the k_i values for peptidyl chloromethanes, but the same increase in k_i was observed irrespective of whether the P_3 position was occupied by aspartate or D-phenylalanine (Table 2). These results indicate that exosite binding does not specifically modify the S_3 site of thrombin to allow the accommodation of a P_3 aspartate. Moreover, our results indicate that movement in the position of Glu^{192} induced by exosite binding (Figure 2) does not play a role in modulating thrombin's aversion for aspartate in the P_3 position. Exosite binding by rhir$^{52–65}$ also increased the k_i of peptidyl chloromethanes containing a P_3 aspartate with E192Q. Because Gln^{192} would not make an unfavourable electrostatic interaction with a P_3 aspartate, its movement would not improve the binding of the P_3 aspartate.

Exosite binding also seems not to affect the conformation of the S_4 site. A 14-fold increase in k_i was observed on extension of the tripeptidyl chloromethane Ac-Asp-Pro-Arg-CH_2Cl by six amino acids to give Ac-Ala-Thr-Asn-Ala-Thr-Leu-Asp-Pro-Arg-CH_2Cl. Most of the increase can be accounted for by the binding of the P_4 Leu in the S_4 pocket of thrombin; NMR studies indicate that residues on the N-terminal side of the P_4 leucine are disordered and probably do not make any contacts with thrombin [25]. The S_4 site is also occupied by the D-phenylalanine residue of D-Phe-Pro-Arg-CH_2Cl. The fact that exosite binding caused the same increase in k_i irrespective of whether the S_4 was occupied (Ac-Asp-Pro-Arg-CH_2Cl compared with Ac-Ala-Thr-Asn-Ala-Thr-Leu-Asp-Pro-Arg-CH_2Cl) suggests that this site is not modified by exosite binding.

Our results are consistent with the hypothesis that the allosteric change induced by exosite binding has modified the S_2 and/or S_1 sites, leading to a better binding of proline and arginine to these

Table 2 Inactivation rate constants for peptidyl chloromethanes with thrombin and E192Q

Assays were performed as described in the Materials and methods section in the absence (−) or presence (+) of rhir$^{52–65}$ (30 μM). Progress-curve data were analysed with eqn. (1) to yield estimates of the inactivation rate constant (k_i). The values given represent the weighted means of two determinations.

	k_i ($M^{-1} \cdot s^{-1}$)			
	α-Thrombin		E192Q	
Chloromethane	− rhir$^{52–65}$	+ rhir$^{52–65}$	− rhir$^{52–65}$	+ rhir$^{52–65}$
D-Phe-Pro-Arg-CH_2Cl	$(2.00 \pm 0.06) \times 10^{7*}$	$(5.78 \pm 0.02) \times 10^{7*}$	$(4.00 \pm 0.02) \times 10^{6}$	$(1.86 \pm 0.01) \times 10^{7}$
Ac-Asp-Pro-Arg-CH_2Cl	$(7.44 \pm 0.03) \times 10^{3}$	$(1.67 \pm 0.01) \times 10^{4}$	$(1.70 \pm 0.01) \times 10^{4}$	$(7.74 \pm 0.06) \times 10^{4}$
Ac-Ala-Thr-Asn-Ala-Thr-Leu-Asp-Pro-Arg-CH_2Cl	$(1.05 \pm 0.01) \times 10^{5}$	$(2.58 \pm 0.01) \times 10^{5}$	$(1.52 \pm 0.01) \times 10^{5}$	$(8.25 \pm 0.08) \times 10^{5}$

* Taken from [3].

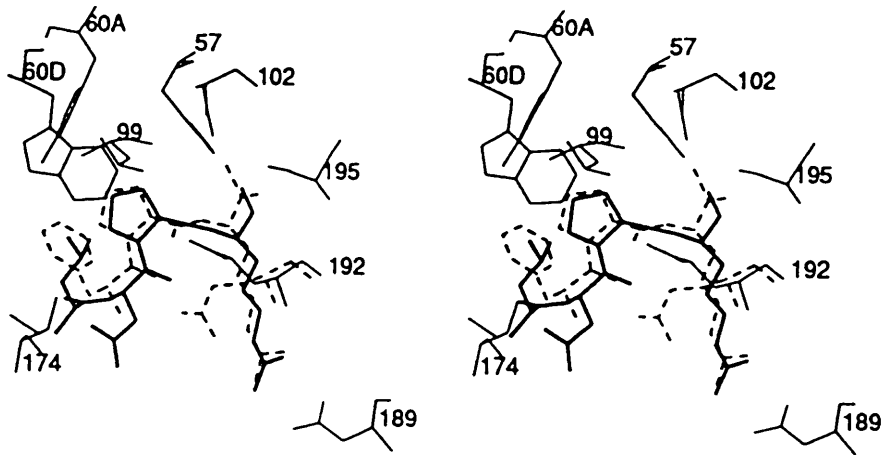


Figure 2 Stereo view of the active-site region of thrombin showing the conformational change in Glu^{192} induced by exosite binding

The receptor peptide Leu-Asp-Pro-Arg is shown in bold lines bound to the active site of thrombin (thin lines). The structure is that given by Mathews et al. [24]; in this structure, hirugen (residues 53–64 of hirudin) is bound to the exosite. The structure of D-Phe-Pro-ArgCH_2-thrombin [21,22] is superimposed in broken lines. Two conformations are observed for Glu^{192}. In the absence of exosite binding (broken lines), Glu^{192} would make an unfavourable electrostatic interaction with the P_3 aspartate. On exosite binding, Glu^{192} moves to assume a position where an unfavourable interaction with the P_3 aspartate is avoided.

sites. In particular, the results with E192Q support this hypothesis. Previous studies have demonstrated that the E192Q mutation dramatically affects the P_1 specificity of thrombin. The serpin α_1-antitrypsin contains a P_1 methionine residue and inactivates thrombin very slowly. On mutation of Glu^{192} to glutamine, the association rate constant for α_1-antitrypsin with thrombin is increased 1000-fold, indicating a striking change in thrombin's P_1 specificity caused by the E192Q mutation [26]. The E192Q mutation also results in a change in the P_2 specificity of thrombin [26,27]. Thus it seems likely that exosite binding has altered the S_2 and/or S_1 sites of E192Q, leading to a better binding of proline and arginine in these sites; exosite binding caused a 5-fold increase in the k_i values for the chloromethanes containing these P_2 and P_1 residues as well as a 5-fold decrease in K_m for the substrate D-Phe-Pip-Arg-pNA, which contains an analogue of proline in the P_2 position (Table 1). X-ray crystallographic data also indicate that exosite binding causes a change in the S_1 site of thrombin. The binding of both hirudin and receptor fragments to the exosite causes a small conformational change in the segment Ala^{190}–Gly^{197} [24] and residues 190–192 form part of the S_1 pocket of thrombin.

Although our data indicate that the alteration in the position of Glu^{192} caused by exosite binding (Figure 2) does not play a major role in allowing thrombin to accommodate an aspartate in the P_3 position, other data suggest that this conformation change might be more important when an aspartate is present in both the P_3 and P_3' positions. In the absence of thrombomodulin, the E192Q mutation caused a marked increase in the rate of cleavage of a peptide based on the Protein C cleavage site, which contains an aspartate in both these positions. Exosite binding by thrombomodulin, however, reduced the difference between α-thrombin and E192Q in the rate of cleavage of Protein C, suggesting that exosite binding mitigates unfavourable electrostatic interactions between Glu^{192} and aspartic residues in the P_3 and P_3' positions [14].

Our observations that exosite binding does not modulate the P_3 specificity of thrombin are in marked contrast with the results obtained by Ishii et al. [13]. These workers determined the EC_{50} values for the cleavage of mutant thrombin receptors expressed in Rat1 fibroblasts. EC_{50} estimates the concentration of thrombin that cleaves 50% of the receptors on the cell surface in 15 min; this value is related to the apparent second-order rate constant

(k_{cat}/K_m) for cleavage of the receptor on cells. The EC_{50} for cleavage of the wild-type receptor was 0.1 nM, and deletion of the receptor's hirudin-like domain resulted in a 200-fold increase in EC_{50} (20 nM). Replacement of the P_3 aspartate of the receptor with glycine did not affect the EC_{50} in the presence of the hirudin-like sequence, but led to a 20-fold decrease in EC_{50} in the absence of the hirudin-like sequence. On the basis of these results, Ishii et al. [13] proposed that exosite binding overcomes the adverse effects of the P_3 aspartic residue. According to this hypothesis, a P_3 aspartate is not inhibitory in the presence of exosite-binding and consequently the $P_3Asp \rightarrow Gly$ mutation has no effect. However, in the absence of exosite binding, the P_3 aspartate is inhibitory and its replacement improves the rate of receptor cleavage. It is difficult to correlate results obtained in solution in the present study with those obtained by Ishii et al. [13] for the cleavage of the receptor on the surface of cells. It should be noted, however, that the 20-fold inhibitory effect of the P_3 aspartate observed by Ishii et al. [13] in the absence of exosite binding was much larger than the 2–6-fold effect observed in previous studies for cleavage of peptides in solution [14,28]. A number of factors could influence the rate of cleavage of the receptor on the cell surface, including the conformation of the receptor and presence of other molecules that might modulate the interaction between the receptor and thrombin. Further studies will be necessary to resolve this discrepancy.

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REFERENCES

1 Stubbs, M. T. and Bode, W. (1995) Trends Biochem. Sci. **20**, 23–28
2 Stone, S. R. (1993) In Proteases and their Inhibitors: Fundamental and Applied Aspects (Avilés, F. X., ed.), pp. 125–140, Walter de Gruyter, Berlin
3 Dennis, S., Wallace, A., Hofsteenge, J. and Stone, S. R. (1990) Eur. J. Biochem. **188**, 61–66
4 Naski, M. C., Fenton, II, J. W., Maraganore, J. M., Olson, S. T. and Shafer, J. A. (1990) J. Biol. Chem. **265**, 13484–13489
5 Hortin, G. L. and Trimpe, B. L. (1991) J. Biol. Chem. **266**, 6866–6871
6 Liu, L. W., Vu, T. K., Esmon, C. T. and Coughlin, S. R. (1991) J. Biol. Chem. **266**, 16977–16980
7 de Cristofaro, R., Rocca, B., Bizzi, B. and Landolfi, R. (1993) Biochem. J. **289**, 475–480
8 Hofsteenge, J., Taguchi, H. and Stone, S. R. (1986) Biochem. J. **237**, 243–251
9 Ye, J., Esmon, N. L., Esmon, C. T. and Johnson, A. E. (1991) J. Biol. Chem. **266**, 23016–23021
10 Ehrlich, H. J., Grinnell, B. W., Jaskunas, S. R., Esmon, C. T., Yan, S. B. and Bang, N. U. (1990) EMBO J. **9**, 2367–2373
11 Richardson, M. A., Gerlitz, B. and Grinnell, B. W. (1992) Nature (London) **360**, 261–264
12 Vu, T. K., Wheaton, V. I., Hung, D. T., Charo, I. and Coughlin, S. R. (1991) Nature (London) **353**, 674–677
13 Ishii, K., Gerszten, R., Zheng, Y. W., Welsh, J. B., Turck, C. W. and Coughlin, S. R. (1995) J. Biol. Chem. **270**, 16435–16440
14 Le Bonniec, B. F. and Esmon, C. T. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 7371–7375
15 Lottenberg, R. and Jackson, C. M. (1983) Biochim. Biophys. Acta **742**, 558–564
16 Stone, S. R. and Hofsteenge, J. (1986) Biochemistry **25**, 4622–4628
17 Chase, T. and Shaw, E. (1970) Methods Enzymol. **19**, 20–27
18 Angliker, H., Shaw, E. and Stone, S. R. (1993) Biochem. J. **292**, 261–266
19 Segel, I. H. (1975) in Enzyme Kinetics, pp. 178–192, Wiley, New York
20 Kettner, C. and Shaw, E. (1981) Methods Enzymol. **80**, 826–842
21 Bode, W., Mayr, I., Baumann, U., Huber, R., Stone, S. R. and Hofsteenge, J. (1989) EMBO J. **8**, 3467–3475
22 Bode, W., Turk, D. and Karshikov, A. (1992) Protein Sci. **1**, 426–471
23 Skrzypczak-Jankun, E., Carperos, V. E., Ravichandran, K. G., Tulinsky, A., Westbrook, M. and Maraganore, J. M. (1991) J. Mol. Biol. **221**, 1379–1393
24 Mathews, I. I., Padmanabhan, K. P., Ganesh, V., Tulinsky, A., Ishii, M., Chen, J., Turck, C. W., Coughlin, S. R. and Fenton, II, J. W. (1994) Biochemistry **33**, 3266–3279
25 Ni, F., Ripoll, D. R., Martin, P. D. and Edwards, B. F. P. (1992) Biochemistry **31**, 11551–11557
26 Le Bonniec, B. F., Guinto, E. R. and Stone, S. R. (1995) Biochemistry **34**, 12241–12248
27 Le Bonniec, B. F., Guinto, E. R. and Esmon, C. T. (1992) J. Biol. Chem. **267**, 6970–6976
28 Le Bonniec, B. F., Myles, T., Johnson, T., Knight, C. G., Tapparelli, C. and Stone, S. R. (1996) Biochemistry **35**, 7114–7122

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