Evidence for common structural changes in thrombin induced by active-site or exosite binding

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The γ -loop of thrombin is a flexible, surface-accessible loop in free thrombin that appears to be one of several sites participating in the interaction of the enzyme with macromolecular substrates and inhibitors. Using limited proteolysis and intrinsic fluorescence measurements, we have studied changes in thrombin structure induced by small, site-specific ligands. Binding of a C-terminal peptide of hirudin to the anion-binding exosite of thrombin induced a structural change in the γ -loop, which caused a 6-fold reduction in the susceptibility of the enzyme to limited proteolysis by elastase and chymotrypsin. Binding of several active site-specific thrombin inhibitors conferred an even greater protection from proteolysis at the γ -loop. For example,

the covalent complex of thrombin with D-Phe-Pro-Arg-CH₂Cl was 95-fold less susceptible to cleavage by chymotrypsin than the free enzyme. Furthermore, binding of either exosite or active-site probes induced a common intrinsic fluorescence change in thrombin (a fractional increase of 0.13). These results are surprising because crystallographic studies indicate that direct contact between the bound probes and relevant residues of the γ -loop is very unlikely. Thus we have identified an allosteric interaction that couples the active site of thrombin to the γ -loop. An interaction of this nature may be one way in which thrombomodulin modulates the reactivity of thrombin.

INTRODUCTION

Thrombin plays a central proteolytic role in haemostasis. It cleaves fibrinogen to fibrin and activates procoagulant factors V, VIII and XIII [1]. In contrast, when bound to thrombomodulin it can function as an anticoagulant by activating protein C [2]. Thrombin also induces a range of cellular responses, including platelet aggregation [3,4], chemotaxis of monocytes [5] and mitogenesis of lymphocytes [6]. In addition to its trypsin-like catalytic site, thrombin appears to contain several recognition sites which influence the specificity of its interaction with macromolecular substrates and inhibitors [1]. One such recognition site is the anion-binding exosite, a surface region remote from the active site, which is involved in the interaction of thrombin with fibrinogen [1], the platelet thrombin receptor [7–9], thrombomodulin [10–12] and hirudin, a potent thrombin inhibitor from leeches [13,14]. Another potential recognition site in thrombin is the surface-accessible γ -loop which comprises the sequence Thr¹⁴⁷-Trp-Thr-Ala-Asn-Val-Gly-Lys^{149E}. This loop includes a six residue insertion compared with the trypsin sequence. A peptide containing this sequence was shown to be a competitive inhibitor of the interaction of thrombin with thrombomodulin [15,16]. Interestingly, several different conformations of the γ -loop have been observed in crystal structures of thrombin bound to hirudin [17–19] or to the small, active-site-specific inhibitors, D-Phe-Pro-Arg-CH₂Cl (FPRCK), benzamide, (2*R*,4*R*)-4-methyl-1[*N*²-(3-methyl-1,2,3,4-tetrahydro-8-quinolyl)sulphonyl]-L-Arg]-2-piperidinecarboxylic acid (MD805) and *N*²-(2-naphthylsulphonyl)glycyl-DL-*p*-amidino-Phe-piperidine (NAPAP) [20–23]. A further crystal

structure has been reported in which thrombin is bound to an analogue of the C-terminal domain of hirudin [24]. In this structure the anion-binding exosite is occupied but the active site of thrombin is vacant. However, the γ -loop was found to have a disordered structure. It is therefore unclear whether the different structural modes seen for the γ -loop represent real responses to ligand binding or whether they simply result from crystal-packing interactions. In this respect, structural information for free (unbound) thrombin might be of value, but has so far proved elusive.

In view of current interest in the possible function of the γ -loop and its structure in free and completed thrombin, we have studied the reaction of thrombin with molecules which bind specifically either to its active site or to the anion-binding exosite. Structural changes were probed using the intrinsic fluorescence of thrombin and its susceptibility to limited proteolysis. Structural changes known to be induced by the C-terminal peptide of hirudin [25,26] were localized to the γ -loop of thrombin. Similar structural changes could also be induced by the reaction of a range of small, active site-specific thrombin inhibitors. These structural changes greatly reduced the susceptibility of residues in the γ -loop to proteolytic digestion. Possible biological implications of these findings are discussed.

EXPERIMENTAL

Materials

Human α -thrombin was prepared as previously described [13] and was fully active as determined by titration with methylumbelliferyl *p*-guanidinobenzoate [27]. Porcine pancreatic elast-

Abbreviations used: FPRCK, D-Phe-Pro-Arg-CH₂Cl; MD805 (also called MQPA, Argatroban or Argipidine), (2*R*,4*R*)-4-methyl-1[*N*²-(3-methyl-1,2,3,4-tetrahydro-8-quinolyl)sulphonyl]-L-Arg]-2-piperidinecarboxylic acid; NAPAP, *N*²-(2-naphthylsulphonyl)glycyl-DL-*p*-amidino-Phe-piperidine; pNA; *p*-nitroanilide; SDZ 115–358, acetyl-D-Phe-Pro-boroarginyl pinanediol ester; 3-TAPAP, *N*²-(4-toluenesulphonyl)-DL-*m*-amidino-phenylalanyl-piperidine; rhir, recombinant hirudin variant 1; rhir-(1–51), the N-terminal fragment of rhir comprising residues 1–51; rhir-(52–65), the C-terminal residues 52–65 of rhir. Numbering of thrombin residues is that of Bode et al. [20].

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ase, bovine pancreatic α -chymotrypsin and trypsin [L-(tosyl-amido-2-phenyl)ethyl-CH₂Cl treated] were obtained from Worthington (Freehold, NJ, U.S.A.). The substrate D-Phe-piperidyl-Arg-*p*-nitroanilide (D-Phe-piperidyl-Arg-pNA) was from Pentapharm (Basel, Switzerland); acetyl-Ala-Ala-Pro-Phe-pNA and succinyl-Ala-Ala-pNA were from Bachem (Bubendorf, Switzerland). MD805 was from Mitsubishi. The following compounds were gifts: FPRCK and Ala-Ala-Pro-Thr-CH₂Cl from Dr. E. Shaw (Friedrich Miescher-Institut, Basel); Eglin C from Dr. M. Grütter (Ciba Geigy, Basel); NAPAP and acetyl-D-Phe-Pro-boroarginyl pinanediol ester (SDZ 115-358) from Drs. C. Tapparelli and R. Metternich (Sandoz Pharma, Basel); *N*^α-(4-toluenesulphonyl)-DL-*m*-amidino-phenylalanyl-piperidine (3-TAPAP) from Dr. J. Stürzebecher (Medizinische Akademie, Erfurt). The hirudin peptides recombinant hirudin variant 1 (rhir)-(1-51) and rhir-(52-65) were prepared as previously described [26] and their composition confirmed by amino acid analysis.

Reaction conditions

Unless otherwise specified, all experiments were performed at 37 °C in amidolytic assay buffer [50 mM Tris/HCl, pH 7.8, containing 0.1 M NaCl and 0.1 % poly(ethylene glycol) *M*_r 6000].

Covalent modification of thrombin by FPRCK

A sample of α -thrombin (15.8 μ M, 1.5 ml) was incubated with a 3-fold excess of FPRCK for 30 min at 25 °C in amidolytic assay buffer containing 0.5 M NaCl. The residual amidolytic activity of this sample after incubation was less than 0.1 %. The covalent complex was separated from unreacted FPRCK on a Sephadex G25 gel filtration column (1.5 cm $\times$ 25 cm) equilibrated with 0.5 M NaCl and was concentrated to 13 ± 0.5 μ M using Centricon-10 microconcentrators (Amicon). The protein concentration was determined using the Bio-Rad protein assay with unmodified thrombin as a standard. Incubation of unmodified thrombin (5 nM) with the FPRCK-inactivated sample (6.5 μ M) for 10 min at 37 °C caused no detectable decrease in amidolytic activity, consistent with an upper limit of 2 nM free FPRCK present in the stock solution of FPRCK-modified thrombin.

Fluorescence measurements

An Applied Photophysics SF17.MV stopped-flow fluorimeter was used as described in detail elsewhere [26]. The excitation wavelength was 280 nm (12 nm band width) and a 320 nm cut-on filter was employed. The instrument dead time was 2.2 ± 0.2 ms. Reactant concentrations were as follows: thrombin, 0.5 μ M; rhir-(52-65), 27 μ M; 3-TAPAP, 16 μ M; all other inhibitors, 5 μ M. These conditions ensured that the enzyme was ≥ 95 % saturated by inhibitor according to published *K*_i values [22,26,28,29] and that pseudo-first-order kinetic traces were obtained. Data shown are averages of six or more traces from at least two experiments. In reporting equilibrium fluorescence changes (ΔF) the intrinsic fluorescence contribution of the inhibitors (*F*_i) was subtracted. At the concentrations employed, *F*_i amounted to less than 5 % of the fluorescence of free thrombin, with one exception: *F*_i for NAPAP was 37 % of that of free thrombin. In control experiments, plots of intrinsic fluorescence against concentration were linear, both for free thrombin and for thrombin-inhibitor mixtures, at concentrations up to twice those used. Inner-filter effects could therefore be neglected.

Limited proteolysis

The pancreatic enzymes elastase, α -chymotrypsin and trypsin were used. Reaction mixtures contained either free or FPRCK-modified α -thrombin (4.0 μ M), or thrombin (4.0 μ M) pre-incubated with either rhir-(52-65) (27 μ M) or MD805 (4.0 μ M). The reaction was started by addition of proteinase (0.2–2.0 % by weight). Control runs were performed in the absence of proteinase. At specified times, 25 μ l portions were removed and proteolysis was terminated by adding a 2.5 μ l aliquot of one of 2.8 mM Ala-Ala-Pro-Thr-CH₂Cl (for elastase), 125 μ M Eglin C (for chymotrypsin) and 1.47 mM FPRCK (for trypsin). Reaction times were chosen such that the extent of proteolysis did not exceed 25 %. Aliquots of 25 μ l from the proteolytic digestions were injected into an LKB TKW-3000 column (0.5 cm $\times$ 60 cm) equilibrated in 0.5 % trifluoroacetic acid. The flow rate was 0.5 ml/min. Eluted peptides were detected at 220 nm. For sequence analysis, the relevant peak fractions were immediately frozen and concentrated by lyophilization. N-Terminal sequence determination was performed using standard methods [30].

Amidolytic assays

These were performed essentially as described [13] in the absence of thrombin, using 500 μ M succinyl-Ala-Ala-pNA (for elastase) or 200 μ M acetyl-Ala-Ala-Pro-Phe-pNA (for chymotrypsin). Conditions were otherwise identical with those of the proteolytic digestions described in the previous section.

RESULTS

Intrinsic fluorescence changes

A number of studies have demonstrated that the C-terminal domain of hirudin binds specifically to a surface region of thrombin encompassing the anion-binding exosite [17–19,24,25,31]. This explains why the C-terminal peptide of hirudin and its analogues do not compete with small molecules which bind specifically to the active-site cleft of thrombin [13,32]. In this study we have used the intrinsic fluorescence of thrombin to probe the structural changes which result from binding at one or both of these sites.

Figure 1(a) compares the binding of rhir-(52-65) to free thrombin and to the thrombin–MD805 complex. The data shown were collected using a split timebase of 50 ms and 500 ms. Further traces collected at longer timebases confirmed that binding was complete within 100 ms. The equilibrium fluorescence change on binding of rhir-(52-65) to free thrombin was $+0.20 \pm 0.01$ [26]. The stopped-flow trace for this reaction could be fitted to an exponential curve with an observed rate constant (*k*_{obs}) of 300 s^{−1} [26]. A reaction with this rate constant has a half-life of 2.2 ms. Extrapolation of the exponential curve back to the start of the dead time yields a fluorescence amplitude for this curve of $+0.13 \pm 0.01$. The remaining amplitude occurs in a rapid initial bimolecular step [26]. When thrombin was premixed with MD805 before binding to rhir-(52-65), the total fluorescence change was reduced from $+0.20$ to $+0.04 \pm 0.02$ (Figure 1a). In addition, an exponential curve could no longer be fitted to the trace. Comparable results were obtained using a range of other active site-specific inhibitors. In each case, the magnitude of the equilibrium fluorescence change for reaction with rhir-(52-65) was reduced by 0.13–0.16 (Figure 1b) and the exponential curve seen for binding of free thrombin to rhir-(52-65) could no longer be detected. The affinity of thrombin for MD805 was found to increase 1.6-fold in the presence of 27 μ M rhir-(52-65) (*K*_i values of 7.8 ± 0.5 nM compared with 12.1 ± 0.7 ; M. A. A. Parry and

Figure 1 Changes in the intrinsic fluorescence of thrombin on reaction with the C-terminal peptide of hirudin

(a) Stopped-flow traces for the reaction of rhir-(52–65) (CP) with free thrombin (T) or a pre-incubated mixture of thrombin and MD805 (T[MD]). The exponential decay curve observed for the reaction with rhir-(52–65) could no longer be detected if thrombin was pre-incubated with MD805. This was accompanied by a smaller equilibrium fluorescence change (ΔF_{eq}). Reactant concentrations: thrombin, $0.5 \mu\text{M}$; rhir-(52–65), $27 \mu\text{M}$; MD805, $5 \mu\text{M}$. (b) Comparison of ΔF_{eq} for the reaction of rhir-(52–65) with free thrombin (solid bar) or thrombin pre-incubated with saturating concentrations of a range of active site-specific inhibitors (open bars). ΔF_{eq} values are reported relative to the fluorescence of free thrombin under the conditions described in the Experimental section.

S. R. Stone, unpublished work). It follows from the principle of micro-reversibility that the affinity of thrombin for rhir-(52–65) should not be reduced when MD805 is bound. It would therefore appear that binding at either the active site or the exosite induces a fluorescence increase of 0.13 – 0.16 in thrombin. This effect is independent of the net ΔF for inhibitor binding (see below). These results are surprising since there is no physical overlap between the binding sites for these ligands.

The changes in intrinsic fluorescence observed for binding of a range of active site-specific inhibitors of thrombin are shown in Figure 2. In contrast with the behaviour of rhir-(52–65), binding of MD805 ($5 \mu\text{M}$) to free thrombin results in a decrease in intrinsic fluorescence ($\Delta F = -0.27 \pm 0.01$). The stopped-flow trace could be fitted to an exponential curve with a k_{obs} of $284 \pm 4 \text{ s}^{-1}$. The value of k_{obs} was found to be linearly dependent on MD805 concentration in the range 1 – $6 \mu\text{M}$ indicating that a bimolecular step with a second-order rate constant of $(4.90 \pm 0.14) \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ is rate-limiting under these conditions. When thrombin was incubated with a saturating concentration of rhir-(52–65) ($27 \mu\text{M}$) before mixing with MD805 two changes were observed. Firstly, the magnitude of ΔF was -0.41 ± 0.02 , an increase of 0.14 ± 0.03 compared to its magnitude in the absence of rhir-(52–65). Secondly, although an exponential trace

Figure 2 Changes in the intrinsic fluorescence of thrombin on reaction with active site-specific inhibitors

(a) Stopped-flow traces for the reaction of MD805 (MD) with free thrombin (T) or thrombin pre-incubated with rhir-(52–65) (T[CP]). In the presence of rhir-(52–65), a larger decrease in equilibrium fluorescence was observed and the exponential decay rate constant was enhanced 1.6-fold. Reactant concentrations are the same as for Figure 1(a). (b) Comparison of ΔF_{eq} values for reaction of a range of active site-specific inhibitors with free thrombin (solid bars) or thrombin pre-incubated with rhir-(52–65) (open bars). ΔF_{eq} values are reported relative to the fluorescence of free thrombin. Conditions are the same as for Figure 1(b).

was still observed, the value of k_{obs} had increased 1.6-fold to $441 \pm 8 \text{ s}^{-1}$. Figure 2(b) summarizes the fluorescence changes obtained when this experiment was repeated using other active site-specific thrombin inhibitors. Interestingly, the magnitude of the intrinsic fluorescence change was found to be strongly dependent on the identity of the inhibitor and therefore on the nature of the individual thrombin–inhibitor interactions. However, premixing of thrombin with rhir-(52–65) always resulted in a negative shift in ΔF of 0.10 – 0.16 . This ΔF shift corresponds in magnitude to the ΔF for the exponential curve observed for binding of free thrombin to rhir-(52–65) (see previously).

Proteolytic digestions

In order to try and relate the common fluorescence change described above to a structural event, we examined the susceptibility of three thrombin–inhibitor complexes to limited proteolysis. As illustrated in Figure 3, each of the three proteinases employed has a unique site specificity within human α -thrombin [33–35]. Digestion of α -thrombin by elastase and chymotrypsin occurs C-terminally of Trp¹⁴⁸ and Ala^{149A}, respectively. Both these cleavage sites are situated in the γ -loop of thrombin. In contrast, under the conditions employed here, trypsin cleaves C-terminally of Arg^{77A}, which constitutes part of the anion-binding exosite of thrombin [19].

Proteolytic digestion mixes were subjected to gel filtration under denaturing, non-reducing conditions. Good separation of

Figure 3 Schematic diagram of human α -thrombin showing the amino acid sequences at the N-terminus and the sites of proteolysis by trypsin (tr), chymotrypsin (ct) and elastase (el)

The location of the disulphide bridges is indicated.

Figure 4 H.p.l.c. gel filtration of α -thrombin and partial proteolysis mixtures

Non-reducing, denaturing conditions were used as described in the Experimental section. The identity of the small fragment (shaded peaks) was confirmed by N-terminal amino acid sequencing. The large peak eluting at 40 min in the chromatograph of the chymotrypsin digest was due to the quenching reagent Eglin C.

the small fragment from the large fragment and uncleaved thrombin was obtained in each case, as shown in Figure 4. The increase in elution peak area of the small fragment as a function of time was used to quantify the initial proteolysis rate. The effect of two active site-specific thrombin inhibitors on the rate of proteolysis was examined. The thrombin-inhibitor complexes

Figure 5 Effect of inhibitor binding on the initial rate of partial proteolysis of thrombin

Conditions were as described in the experimental section. Abbreviations: T, free thrombin; T-CK, FPRCK-thrombin; T[CP] and T[MD], thrombin incubated with rhir-(52-65) or MD805, respectively. The extent of the reaction was deduced from the peak area of the small fragment (Figure 4, shaded peaks). AU, absorbance units.

were compared with rhir-(52-65)-bound thrombin and with the free enzyme. The results are shown in Figure 5. Control experiments indicated that, in the absence of extrinsic proteinase, α -thrombin was stable over the time course of these experiments. Furthermore, the amidolytic activity of the extrinsic proteinases was unchanged ($\pm 5\%$) in the presence of the thrombin inhibitors at the concentrations used in the proteolysis experiments (results not shown). Tryptic digestions were not performed in the presence of MD805, which is a relatively good trypsin inhibitor with a dissociation constant of $5\ \mu\text{M}$ [22].

The susceptibility of free and bound thrombin to proteolysis is summarized in Table 1. Significant inhibition of proteolytic cleavage at the γ -loop of thrombin was observed in the presence of MD805, FPRCK and rhir-(52-65). The pattern of inhibition was remarkably similar, irrespective of the bond cleaved. The best protection (a 95-fold decrease in initial chymotrypsin cleavage rate) was afforded by active site binding of FPRCK. The poorest protection (6-fold) was observed for the rhir-(52-65)-thrombin complex, while MD805 afforded an intermediate degree of protection. A very different situation was found for cleavage of the Arg^{77A}-Asn⁷⁸ bond located in the anion-binding exosite of thrombin. The initial proteolysis rates

Table 1 Comparison of the relative initial cleavage rates of free and complexed thrombin

The conditions were as described in the Experimental section. The values represent linear least-squares fits of the data presented in Figure 5. Standard errors are shown in parentheses.

Ligand	Relative initial cleavage rate		
	Elastase (2%)	Chymotrypsin (0.5%)	Trypsin (0.2%)
None	1.000 (0.029)	1.000 (0.037)	1.000 (0.067)
rhir-(52–65)	0.153 (0.004)	0.176 (0.007)	0.778 (0.152)
MD805	0.077 (0.0051)	0.0571 (0.0028)	–
FPRCK	0.0334 (0.0022)	0.0105 (0.0004)	0.0929 (0.0092)

for FPRCK-inhibited thrombin (0.110 ± 0.022 mAU·min) and for free thrombin (0.149 ± 0.019 mAU·min) were almost indistinguishable (AU, absorbance units). Rhir-(52–65) was a much more effective inhibitor of trypsinolysis than FPRCK. Since the C-terminal domain of hirudin is known to interact directly with Arg^{77A} via Glu⁵⁸ of rhir [24], rhir-(52–65) would be expected to act as a competitive inhibitor of trypsinolysis. Similar findings were reported by Dennis et al. using rhir-(53–65) [36]. Although quantitative experiments were not performed for all the active site-specific inhibitors used in the fluorescence studies, a qualitative estimate of their effect on proteolysis was made using SDS/PAGE (results not shown) as described previously [36]. The trend in inhibition observed using MD805 and FPRCK was followed by all the active site-specific inhibitors listed in Figure 1(b); cleavage by elastase and chymotrypsin was inhibited, while trypsinolysis was unaffected.

DISCUSSION

It was suggested initially from circular dichroism studies that the binding of C-terminal peptides of hirudin induces a change in thrombin structure [25]. It has been proposed that this conformational change occurs at the γ -loop, which is believed to be the most flexible region of the molecule [17–19,21,24]. The results of limited proteolysis experiments with elastase and chymotrypsin presented in Figure 5 support this hypothesis. It had been shown previously using SDS/PAGE that the C-terminal region of hirudin could partially protect the γ -loop of thrombin from cleavage of Ala^{149A} by elastase [36,37]. In the present study, a 6-fold reduction in the rate of partial proteolysis of thrombin by either elastase or chymotrypsin was observed in the presence of a saturating concentration of rhir-(52–65). The binding of rhir-(52–65) to the anion-binding exosite appears to have induced a change in the conformation of the γ -loop, such that it is less susceptible to proteolytic cleavage at Ala^{149A} by elastase and at Trp¹⁴⁸ by chymotrypsin. Unexpectedly, similar results were also observed with inhibitors that bind only at the active site (Figure 5). A marked reduction in the susceptibility of the γ -loop to proteolytic cleavage was observed with these inhibitors (Table 1). The N-terminal core of hirudin was reported previously to protect thrombin against cleavage at Ala^{149A} by elastase [36,37]. The N-terminal domain of rhir is a relatively bulky ligand and, given the flexibility of the γ -loop, direct molecular contacts between rhir-(1–51) and the γ -loop cannot be ruled out. In contrast, the other inhibitors employed in the present study appear to be too small to make direct contacts with the γ -loop once they are bound to the active site. The binding of MD805 or

FPRCK to the active site of thrombin seems to induce a conformational change in the γ -loop that reduces its sensitivity to cleavage by elastase and chymotrypsin. However the mechanism by which the effect of active site-binding is communicated to the γ -loop remains to be elucidated. The different degrees of protection afforded by saturating concentrations of rhir-(52–65), MD805 and FPRCK towards cleavage of the same bond suggests that their binding may induce different mean conformations of the γ -loop. A similar protection pattern was observed with both elastase and chymotrypsin (Table 1), which is consistent with the close proximity of these cleavage sites. In contrast with the protective effect of the active site-directed inhibitors against cleavages in the γ -loop, their effect on trypsin cleavage in the anion-binding exosite was negligible. Thus, the effects of these inhibitors seem to be at least to some extent specific to the γ -loop.

Further evidence that the γ -loop undergoes a structural change comes from fluorescence measurements. Premixing experiments (Figures 1 and 2) showed that a fluorescence change with a magnitude of +0.13 to +0.16 was induced by the binding of either rhir-(52–65) or any of the active site-specific inhibitors tested. This change has about the same magnitude as for the exponential curve observed for the reaction of rhir-(52–65) with thrombin [26] (Figure 1). Furthermore, this decay could no longer be detected when thrombin was pre-incubated with the inhibitors (Figure 1). Thus, protection against proteolytic cleavage at the γ -loop by a ligand is correlated with a fluorescence enhancement of 0.10–0.16 observed on binding of the ligand. Unfortunately, the crystal structures of thrombin in complexes with different ligands that protect against cleavage do not reveal a common structure for the γ -loop. The fluorescence enhancement could be caused by a decrease in solvent exposure of one or more tryptophans [38]. In the crystal structures of thrombin with rhir [17–19] the side-chain of Trp¹⁴⁸ is well shielded from solvent by the N-terminal core of rhir, but the bonds Trp¹⁴⁸–Thr¹⁴⁹ and Ala^{149A}–Thr^{149B} appear to be solvent-exposed. In the crystal structures of FPRCK-inactivated thrombin [20,21], in contrast, both the side-chain of Trp¹⁴⁸ and the cleavage sites are fully solvent-exposed. A different conformation of the γ -loop is seen in the MD805–thrombin complex, but Trp¹⁴⁸ and the cleavage sites are also solvent-exposed in this structure [21]. In the structure of a complex of thrombin with a C-terminal fragment of hirudin (hirugen), the γ -loop is disordered [24]. The variety of conformations seen for the γ -loop in crystal structures has led to the conclusion that a single conformation of the loop is stabilized only by crystal contacts [19,21,24]. Thus, while data presented in this paper suggest that active site-directed inhibitors and rhir-(52–65) induce a structural change in the γ -loop resulting in a fluorescence-enhancement and a protection against proteolysis, X-ray crystallographic studies do not indicate the nature of this conformational change.

One interesting aspect of these studies was the fluorescence changes observed for the reaction of free thrombin with the various small, active-site specific inhibitors (Figure 2). The intrinsic fluorescence change on complex formation was found to be very dependent on the nature of the inhibitor. An intrinsic fluorescence increase was found for the inhibitors FPRCK and SDZ 115–358, whose design is based on tripeptidyl thrombin substrates. In contrast, a fluorescence decrease was found for the inhibitors MD805, NAPAP and 3-TAPAP, which also induce the fluorescence-enhancement caused by binding of rhir-(52–65). Thus, their binding must involve a fluorescence increase caused by their effect on one region of thrombin (presumably the γ -loop), which is offset by a larger fluorescence decrease caused by their effect on another region. The three inhibitors that induced

a fluorescence decrease are believed to bind thrombin in an 'inhibitor-like' manner [21]. In this binding mode the apolar pocket of thrombin is occupied by the N- and C-terminal aromatic residues of the inhibitor, but the oxyanion hole is vacant. In FPRCK-inhibited thrombin, however, the apolar binding pocket is filled by D-Phe and Pro of the inhibitor and the oxyanion hole is occupied [20,21]. Interactions of the inhibitors with tryptophans which line the active-site hydrophobic binding pockets (namely Trp^{60D} and Trp²¹⁵) [20,21], could contribute to the observed fluorescence changes. One factor that may modulate the intrinsic fluorescence in these complexes is the proximity of a charged group on the inhibitor to Trp²¹⁵ and Trp^{60D} of thrombin. MD805 and NAPAP possess a negatively-charged sulphonyl group that is situated close to these residues [21], whereas in FPRCK- and rhir-inactivated thrombin this position is occupied by the positively-charged primary amino group of the N-terminal D-Phe and Ile, respectively [17,18,20,21]. Modulation of tryptophan fluorescence by an adjacent charged group has been reported for the ribonuclease barnase [39].

The binding of rhir-(52-65) affected the kinetics of the interaction of MD805 with the active site of thrombin: the k_{obs} for the reaction of MD805 was 1.6-fold greater in the presence of rhir-(52-65). C-Terminal fragments of hirudin have been shown to cause similar alterations in the kinetic parameters of tripeptidyl substrates and of N-terminal fragments of rhir [26,32,36,40,41]. Other ligands (fragments of heparin cofactor II, fibrinogen and the thrombin receptor) that bind to the anion-binding exosite of thrombin have also been shown to cause similar changes in the kinetics of substrate hydrolysis [8,40]. It is not clear whether this communication between the anion-binding exosite and the active site is mediated by changes in the γ -loop structure or whether another mechanism is involved. In this context it is interesting to note that the binding of thrombomodulin to thrombin causes similar alterations in the kinetic parameters for hydrolysis of tripeptidyl substrates and for activation by peptidyl chloromethyl ketones [10]. When thrombomodulin binds to thrombin it greatly enhances the efficiency with which the enzyme cleaves protein C [2]. The mechanism is not yet known, although a recent mutagenesis study [42] indicated that Glu¹⁹² may be involved. Thrombomodulin appears to interact with both the anion-binding exosite [10-12] and the γ -loop [15,16]. While previous studies have indicated that binding at the anion-binding exosite may cause alterations in the active site, the present study is the first to report an allosteric link between the active site and the γ -loop. Thus thrombomodulin may influence the reactivity of thrombin towards protein C by interactions both at the anion-binding exosite and at the γ -loop.

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