Heparin enhances the catalytic activity of des-ETW-thrombin

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The thrombin mutant, des-ETW-thrombin, lacking Glu¹⁴⁶, Thr¹⁴⁷ and Trp¹⁴⁸ within a unique insertion loop located at the extreme end of the primary specificity pocket, has been shown previously to exhibit reduced catalytic activity with respect to macromolecular and synthetic thrombin substrates and reduced or enhanced susceptibility to inhibition. Investigation of the hydrolysis of peptidyl *p*-nitroanilide substrates by des-ETW-thrombin showed increased activity in the presence of heparin and other sulphated glycosaminoglycans. No effect was observed upon the activity of wild-type thrombin. Heparin was found to decrease the K_m for cleavage of four thrombin-specific substrates by des-ETW-thrombin, by 3–4-fold. Similarly, pentosan polysulphate (PPS) decreased the K_m with these substrates by 8–10-fold. Heparin also increased the rate of inhibition of des-ETW-thrombin by antithrombin III and D-phenylalanyl-prolyl-arginyrchloromethane (PPACK). The inhibition of des-ETW-thrombin by a number of thrombin-specific peptide boronic acids also showed significant reduction in the final K_i in the presence of heparin, due to reduction in the off-rate. A peptide analogue of a sequence of hirudin which binds thrombin tightly to exosite 1 (fibrinogen recognition site) potentiated the activity

of des-ETW-thrombin against peptide *p*-nitroanilide substrates in a manner similar to heparin. The K_i for the inhibition of des-ETW-thrombin by *p*-aminobenzamidine was decreased by these ligands from 9.7 mM to 7.5 mM, 5.1 mM and 2.5 mM in the presence of heparin, hirudin peptide and PPS respectively, suggesting the increased catalytic activity is due to enhanced access to the primary specificity pocket. The positive influence of these ligands on des-ETW-thrombin was reversed in the presence of ATP or ADP; the latter has previously been shown to inhibit thrombin activity by blocking initial interaction with fibrinogen at exosite 1. Because the effect of heparin and PPS is similar to that of hirudin peptide, it is proposed that the most likely mechanism is that binding at the heparin-binding site (thrombin exosite 2) facilitates interaction at exosite 1 causing a conformational change which partially corrects the defective ground-state binding of the mutant thrombin. Although no effect was observed upon the activity of wild-type thrombin, our findings do provide further evidence of an allosteric property of thrombin which may control the geometry of, and access to, the primary specificity pocket.

INTRODUCTION

Thrombin, the final enzyme of the blood clotting cascade, is a multifunctional serine proteinase whose primary role is to convert fibrinogen into fibrin monomer, thereby promoting formation of the clot. In addition, thrombin activates other members of the coagulation cascade and also cleaves a specific receptor on the surface of platelets, promoting their aggregation (reviewed in [1,2]). Aside from this central role in haemostasis, thrombin also has mitogenic and chemotactic effects upon other cells implicating it in wound repair and inflammatory responses.

In these reactions, thrombin acts as a constrained protease in that it cleaves a limited number of peptide bonds although possessing the full catalytic apparatus of the serine protease family and having the ability to cleave Arg-Xaa (principally) and Lys-Xaa according to the primary specificity of trypsin [3]. This constraint is due to a number of unique structural features on the surface of the thrombin molecule which control the interaction of thrombin with substrates, including accessibility to the specificity pocket [4,5]. The features involved are as follows. First, the fibrinogen recognition site (thrombin exosite 1), an anionic region (shown by crystallography to be on the westerly aspect of

the primary specificity pocket), known to be essential for the initial interaction between the enzyme and fibrinogen, the thrombin cell receptor and hirudin [6]. Secondly, an apolar binding site on the easterly aspect which provides subsites for binding of substrate on the N-terminal side of the cleaved peptide bond. Lastly, two unique insertion loops which form a canyon-like structure limiting access to the primary specificity pocket.

Another unique site on thrombin is constituted by a highly electropositive surface near the C-terminal helix of the serine protease domain [6,7], which in the precursor of thrombin, prothrombin, is ligated by salt bridges to an anionic motif in the second kringle. This site becomes exposed on the loss of the kringle structures, during the formation of thrombin, and is thought to constitute the heparin-binding region [8] (also termed thrombin exosite 2 [9]) which plays a crucial role in the potentiation by heparin of the inhibition of thrombin by antithrombin III [10,11]. This site is also involved in the enhancement by thrombomodulin of protein C activation by thrombin [12]. Thrombomodulin primarily binds to exosite 1 of thrombin while the interaction with exosite 2 appears limited to the chondroitin sulphate that the membrane-bound thrombomodulin carries.

Recently a thrombin mutant, des-ETW-thrombin, has been

Abbreviations used: GAGS, sulphated glycosaminoglycans; HPA, peptide analogue to hirudin sequence (54–65); PPS, pentosan polysulphate; PPACK, D-phenylalanyl-prolyl-arginyrchloromethane; Irg, isothiuronium; SBTI, soybean trypsin inhibitor (Kunitz); Pip, pipercoline; CHG, cyclohexylglycine; But, L- α -aminobutyryl; pNA, *p*-nitroanilide.

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characterized lacking Glu¹⁴⁶, Thr¹⁴⁷ and Trp¹⁴⁸ of the insertion loop on the southerly aspect of the entrance to the specificity pocket [13]. The ETW sequence is highly conserved between species. Interestingly, although crystallographic studies of thrombin in complex with various inhibitors show the orientation of this loop to be highly variable, the loss of these residues is associated with a severe change to the catalytic activity of the mutant particularly in respect to binding of specific substrates and inhibitors. This is due, it is considered, to alteration of the conformation of the specificity pocket including the catalytic triad with some effect also on the S2 binding site. In this paper we report that pre-incubation with heparin and sulphated glycosaminoglycans (GAGS) and other effectors can considerably enhance the activity of the mutant due, perhaps, to allosteric effects induced by binding at exosites 1 and 2. Although these GAGS have no effect on the catalytic activity of wild-type thrombin under these experimental conditions, our findings indicate that heparin binding may be of significance in considering the known allosteric properties of thrombin [14].

MATERIALS AND METHODS

Proteins and reagents

The des-ETW-thrombin mutant was prepared as previously reported [13] and plasma-derived human α -thrombin was obtained from Dr. Freyssinet, Strasbourg. Heparin (Leo Pharmaceuticals) and pentosan polysulphate (PPS; Hemoclar, Clin-Midi, France) were fractionated by gel filtration as described previously [15,16]. Molecular masses were estimated by reference to the elution volume of heparin fractions of known molecular mass as described previously [15,17]. Low-molecular-mass heparin fractions were obtained from Sandoz (Mono-Embolex, average 8 kDa), Kabi-Pharmacia (Fragmin, 6 kDa) and Choay (CY216, 4 kDa and CY222, 2.5 kDa). Dermatan sulphate was obtained from Mediolanum Pharmaceuticals and a heparin pentasaccharide with high affinity for antithrombin III from Choay Pharmaceuticals, France [18]. Oversulphated dermatan sulphate and chondroitin sulphate K were obtained from Dr. S. Seno [19]. Chondroitin sulphates A and C were purchased from Sigma.

Peptide *p*-nitroanilide (pNA) substrates: H-D-Val-Leu-Arg-pNA, H-D-Phe-Pip-Arg-pNA, Bz-Ile-Glu-Gly (OR) pNA (where 50 % of R is H and 50 % is CH₃), H-D-Val-Leu-Lys-pNA, Pyro-Glu-Gly-Arg-pNA, H-D-Phe-Phe-Arg-pNA, Meo-Suc-Arg-Pro-Tyr-pNA, CH₃CO-Ile-Glu-Gly-Arg-pNA, Pyro-Glu-Pro-Arg-pNA were obtained from Quadrantech (U.K.), H-D-CHG-But-Arg-pNA and H-D-CHG-Ala-Arg-pNA from Diagnostica Stago (France), and Tos-Gly-Pro-Arg-pNA from Boehringer Mannheim (Germany). (The following abbreviations were used above and throughout: Pip, pipercoline; CHG, cyclohexylglycine; But, 1- α -aminobutyryl.) An analogue (HPA) of the hirudin sequence (54–65) (G₄DFEPIPL) [20] was prepared using fluoren- α -ylmethoxycarbonyl (Fmoc) chemistry on a Milligen peptide synthesizer. Peptide boronic acid inhibitors of thrombin were prepared and characterized (J. J. Deadman, S. Elgendy, C. A. Goodwin, N. Chino, V. V. Kakkar, G. Claeson and M. F. Scully, unpublished work).

Kinetic analysis of the properties of the des-ETW mutant

All measurements were made in 0.1 M triethanolamine/HCl, 0.1 M NaCl, 0.5 % (w/v) poly(ethylene glycol) 6000, 0.02 % sodium azide, pH 7.6, (buffer A) and at 25 °C unless otherwise stated. In all experiments, des-ETW-thrombin was equilibrated for 5 min with heparin or other sulphated polysaccharides before

initiating each enzymic reaction. The activity towards a range of concentrations of various peptide pNA substrates was measured in microwell plates using a Molecular Devices plate reader with a 405 nm filter. Initial rates were fitted to the Michaelis equation using the Enzfitter program to give a value for K_m and k_{cat} . In experiments to determine whether the enhancement of K_m observed could be blocked, the des-ETW-thrombin was incubated for 5 min with ADP, ATP or *p*-aminobenzamidine at room temperature. The heparin or other ligand was then added and after a further 5 min incubation the K_m was determined using a range of concentrations of H-D-Phe-Pip-Arg-pNA and the Michaelis equation as explained above.

Potential by heparin of the antithrombin III–des-ETW-thrombin interaction at 37 °C was measured by subsampling a mixture of heparin, antithrombin III (50 nM) and des-ETW mutant (5 nM) at timed intervals into buffer containing Polybrene (0.2 %, w/w) and H-D-Phe-Pip-Arg-pNA (0.2 mM). Residual des-ETW-thrombin activity was measured as the change in absorbance at 405 nm.

The potentiation by heparin of the inhibition of des-ETW-thrombin by D-phenylalanyl-prolyl-arginylchloromethane (PPACK) was measured by pre-incubation at 32 °C of heparin with des-ETW-thrombin (final concentration 10 nM) before the addition of PPACK at $t = 0$; achieving a concentration of 25 nM. Samples were taken at timed intervals and added to substrate, H-D-Phe-Pip-Arg-pNA (final concentration, 0.4 mM) for measurement of residual des-ETW-thrombin activity.

The inhibition of des-ETW-thrombin by peptide boronic acids was measured by the addition of increasing concentrations of inhibitor to a mixture of enzyme (final concentration 0.6 nM) and heparin, with H-D-Phe-Pip-Arg-pNA as substrate maintained at 25 °C in microwell plates. The change in absorbance was measured over a 2 h period and fitted to eqn. (1) [22] using the program Enzfitter.

$$[P] = \frac{vk_4t}{k_{obs}} + \frac{vk_3[I]}{(1 + [S]/K_m)k_{obs}^2} \cdot (1 - e^{k_{obs}t}) \quad (1)$$

where v is the initial velocity in the absence of inhibitor, k_{obs} is the apparent first-order rate constant characterizing the logarithmic phase, $[S]$ is the substrate concentration, $[I]$ is the inhibitor concentration, t is the time and k_3 and k_4 are the on- and off-rates respectively as defined by the simplest mechanism of slow binding.

The final K_i (K_i^*) is calculated as the ratio, k_4/k_3 . Certain peptide boronic acids acted as simple competitive inhibitors and K_i values were determined from Dixon plots.

The inhibition of des-ETW-thrombin by soybean trypsin inhibitor (SBTI) was measured in a similar fashion. Des-ETW-thrombin (final concentration 3 nM) was incubated with PPS (final concentration 3 nM) in the presence of increasing concentrations of SBTI. Analysis of progress curves was carried out as described above.

RESULTS

Influence of GAGS, PPS and hirudin peptide (HPA) upon catalytic activity

Previously, Le Bonniec et al. have shown that, in general, all the catalytic activities of des-ETW-thrombin are diminished when compared with those of thrombin [13]. With regard to the cleavage of peptidyl pNA substrates, the deletion resulted in

Figure 1 The influence of increasing concentrations of various sulphated glycosaminoglycans upon the cleavage of H-D-Phe-Pip-Arg-pNA by des-ETW-thrombin

Des-ETW-thrombin (fluid concentration 2.5 nM) in buffer A (see the Materials and methods section) was pre-incubated with increasing concentrations of GAGS for 3 min at 37 °C before the addition of substrate, H-D-Phe-Pip-Arg-pNA (final concentration 0.2 mM). Results are expressed as a percentage of the activity in control incubations with no added GAGS. ■, Heparin; ▼, dermatan polysulphate; ◆, chondroitin sulphate K; ▲, dermatan sulphate; △, chondroitin sulphate A; □, chondroitin sulphate C. The results shown are the means of three separate determinations.

changes in both the Michaelis constant (K_m) and/or the catalytic rate (k_{cat}) by up to two orders of magnitude. We confirmed these observations but noted a considerable enhancement of catalytic activity in the presence of heparin and a number of other GAGS. Under the conditions of assay used in Figure 1, a maximal 2.7-fold enhancement of the rate was observed at concentrations of heparin greater than about 100 µg/ml. The effect of the GAGS appeared to be related partially to the charge density since the effective order was heparin > dermatan polysulphate > chondroitin sulphate K > dermatan sulphate > chondroitin sulphate A = chondroitin sulphate C. Furthermore the effect was lost when the concentration of NaCl was raised to 0.2 M.

Heparin, which had caused a marked enhancement of rate,

was chosen for further study. Screening of a range of chromogenic substrates (listed in the Materials and methods section) in the presence and absence of heparin showed that only the activity towards those substrates designed as being specific for thrombin was enhanced by heparin. No effect was seen upon the activity of wild-type thrombin under these experimental conditions, showing that a specific influence upon the catalytic activity of des-ETW-thrombin is occurring rather than a non-specific effect on the substrate as has been shown previously to occur with the substrate Bz-Phe-Val-Arg-pNA, which was not used herein (discussed in [23]). Furthermore, a peptide (HPA) based on the hirudin sequence (residues 54–65) which has been shown previously to act as an allosteric effector of wild-type thrombin [24–26] and, also, a high-molecular-mass fraction of PPS (a synthetically sulphated polysaccharide which was shown previously to bind to thrombin [16]) were observed to cause an increase in the amidolytic activity of des-ETW-thrombin.

The study was then extended to a kinetic analysis of the influence of a single concentration of heparin, PPS and HPA upon the activity of des-ETW-thrombin towards the thrombin-specific substrates. The results showed that the enhancement was principally due to an increase in the binding affinity for each substrate (reduced K_m) (Table 1). The influence of increasing concentrations of heparin- and PPS-fractions of varying molecular mass and also HPA were measured too (Figure 2). A molecular-mass dependence was observed. Higher concentrations of low-molecular-mass fractions of heparin and PPS were required to reduce the K_m . Furthermore, heparin of lower molecular mass did not decrease the K_m to the same extent as high-molecular-mass heparins were able to, even at high concentrations. A heparin pentasaccharide with high affinity for anti-thrombin III [18] showed no ability to reduce the K_m (even at high concentrations > 10 µM). A high concentration of HPA, similar to those used previously to enhance thrombin amidolytic activity of wild-type thrombin [25] by hirudin peptide (54–65), was required to enhance des-ETW-thrombin activity. These observations also support the conclusion that each agent is having a specific effect on the catalytic activity of the des-ETW-thrombin since the EC_{50} values for heparin and PPS are between two and four orders of magnitude lower than the concentration of substrate (Figure 2).

Table 1 Observed kinetic constants for the hydrolysis of peptidyl p-NA substrates by des-ETW-thrombin

Des-ETW-thrombin (final concentration 1 nM) in buffer A was pre-incubated with heparin (H; 10 nM), PPS (3 nM) or HPA (130 µM) for 5 min at 25 °C before the addition of various concentrations (5–500 µM) of each substrate. A, absence of any effector. Progress curves were analysed according to the Michaelis equation. Results are the mean values of three separate experiments.

Substrate	Effector	k_{cat} (s ⁻¹)	K_m (µM)	k_{cat}/K_m (µM ⁻¹ ·s ⁻¹)	Fold enhancement
H-D-Phe-Pip-Arg-pNA	A	45 ± 0.6	194 ± 5	0.23	
	H	54 ± 1.53	53 ± 1.53	1.02	4.43
	PPS	45 ± 2.5	14.9 ± 2.7	3.02	13.1
	HPA	56 ± 1.0	83 ± 4.4	0.67	2.91
H-D-CHG-But-Arg-pNA	A	32 ± 1.4	273 ± 19	0.12	
	H	38 ± 1.2	129 ± 8	0.29	2.42
	PPS	35 ± 0.92	18.3 ± 1.5	1.91	6.59
	HPA	38 ± 1.4	120 ± 8	0.32	2.67
2AcOH H-D-CHG-Ala-Arg-pNA	A	38 ± 8.2	314 ± 94	0.12	
	H	59 ± 4.4	188 ± 21	0.31	2.58
	PPS	43 ± 1.4	45.7 ± 2.4	0.94	7.83
	HPA	54 ± 3.4	175 ± 9.3	0.31	2.58
Tos-Gly-Pro-Arg-pNA	A	15 ± 5.5	589 ± 253	0.025	
	H	NT	—	NT	
	PPS	13 ± 2.3	90 ± 40	0.14	5.60
	HPA	9 ± 1.6	152 ± 33	0.059	2.37

Figure 2 The influence of increasing concentrations of heparin, HPA, PPS and various molecular-mass fractions of heparin and PPS upon the observed K_m values for cleavage of H-D-Phe-Pip-Arg-pNA by des-ETW-thrombin

Des-ETW-thrombin (final concentration 5 nM) was pre-incubated in buffer A at 25 °C for 5 min before the addition of increasing concentrations of substrate. The K_m was calculated by curve fitting and is shown according to increasing concentration of each effector. ■, Unfractionated heparin; ▽, HPA; □, heparin 8 kDa (Mono Embolux); △, heparin 6 kDa (Fragmin); ○, heparin 4 kDa (CY 216); ▲, heparin 2.5 kDa (CY 222); ◇, heparin pentasaccharide (Choay); ●, PPS (1.5 kDa); ▼, PPS (3 kDa); ▴, PPS (10 kDa). The results shown are means of 3 to 4 separate determinations.

Figure 3 The influence of increasing concentrations of heparin upon the inhibition of des-ETW-thrombin by antithrombin III

Des-ETW-thrombin (final concentration 5 nM) in buffer A was incubated with antithrombin III (50 nM) at 37 °C and increasing concentrations of heparin. At timed intervals, samples were removed to buffer containing substrate, H-D-Phe-Pip-Arg-pNA (0.2 nM) and Polybrene (0.2%, w/v) and residual activity was measured. ▲, 10 nM heparin; ■, 20 nM heparin; ◆, 40 nM heparin; ●, absence of heparin. Results of three separate determinations are shown $\pm$ S.E.M.

Heparin enhancement of the inhibition of des-ETW-thrombin

Le Bonniec et al. have shown previously that the association rate for inhibition of des-ETW-thrombin by a number of inhibitors is considerably reduced with respect to wild-type thrombin [13]. For example, a 360-fold reduction in the rate of association with antithrombin III was reported. When the inhibition by antithrombin III was measured in the presence of heparin, a concentration-dependent acceleration of the rate was observed (Figure 3). At 20 nM heparin, an association rate of $1.26 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$ was calculated, representing a 126-fold acceleration with respect to the basal rate of $1.0 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$ [13]. This compares

Figure 4 The influence of increasing concentrations of heparin upon the inhibition of des-ETW-thrombin by PPACK

Des-ETW-thrombin (final concentration 10 nM) in buffer A was incubated with PPACK (25 nM) at 32 °C. At timed intervals samples were removed to buffer containing substrate, H-D-Phe-Pip-Arg-pNA (final concentration 0.2 mM) and residual activity was measured. Concentration of heparin: ■, 330 nM; △, 100 nM; ▲, 33 nM; ▽, 10 nM; ▼, 0. Results of three separate determinations are shown $\pm$ S.E.M.

with an estimated 1000-fold acceleration with thrombin measured previously by us under similar conditions [27].

Similarly, an enhancement by heparin of the rate of inhibition of des-ETW-thrombin by PPACK was observed. Experiments were set up in which PPACK was present in a 2.5-fold molar excess over des-ETW-thrombin. The rate of inhibition increased according to the concentration of heparin from 3- to 15-fold within the range of 10 to 330 nM heparin (Figure 4).

It has been shown previously that the interaction between SBTI and bovine pancreatic trypsin inhibitor (BPTI) with des-ETW-thrombin is enhanced compared with wild-type thrombin [13]. In this case, we observed that PPS increased the association rate (k_a) from $174 \pm 30 \text{ M}^{-1} \cdot \text{s}^{-1}$ to $(2.41 \pm 0.39) \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$ and hence lowered the final K_i (K_i^*) from $2.01 \pm 0.48 \text{ nM}$ to $205 \pm 42 \text{ pM}$ while the dissociation rate (k_d) remained largely unchanged, going from $(2.27 \pm 0.2) \times 10^{-4} \text{ s}^{-1}$ in the absence of PPS to $(3.62 \pm 0.22) \times 10^{-4} \text{ s}^{-1}$ in its presence.

The same magnitude of enhancement was also observed in the inhibition of des-ETW-thrombin by a series of peptide boronic acids (general formula, $\text{P}_4\text{-P}_3\text{-Pro-boro-Irg}$) which have been designed as specific inhibitors of thrombin [18]. Peptide boronic acids are able to form transition-state adducts with the catalytic site of serine proteinases of which they can simply act as competitive (Type 1) or slow tight binding inhibitors (Type 2) [28–31]. Type-2 inhibitors are considered to arise when binding of the peptide to subsites is favourable, permitting decay of the initial complex with the formation of a tetrahedral adduct with serine. In the absence of heparin all the compounds tested acted as type-2 inhibitors of wild-type thrombin and des-ETW-thrombin. Addition of heparin (10 nM) (unfractionated) caused a 3–4-fold increase in the affinity of the inhibitors for des-ETW-thrombin due to a significant decrease in the off-rate with no effect upon the on-rate (Table 2). Interestingly, neither heparin, nor PPS had any effect on the inhibition of des-ETW-thrombin by the thrombin-specific inhibitor, Z-D-Phe-Pro-boromethoxyglycine [32,33]. This compound acts as a type-1 inhibitor of thrombin (K_i , 7 nM) and des-ETW-thrombin (K_i , 700 nM).

Table 2 Inhibition of wild-type and des-ETW-thrombin by peptide boronic acids of the type P₄-P₃-L-Pro-L-boro-Irg

Wild-type thrombin (WT; final concentration 0.18 nM) or des-ETW-thrombin (ETW final concentration 0.6 nM) were pre-incubated in buffer A in the absence or presence of heparin (H; final concentration 10 nM). The progress curves were analysed according to the method of Baici and Gyger-Marazzi [22] to calculate on (k_3) and off (k_4) rates and final K_i (K_i^*). Abbreviations: D-Phe, D-β-naphthylalanyl; D-Fgl, D-fluorenylglycyl; Ac-D-Dpa, D-β,β-diphenylalanyl.

Inhibitor No.	P ₄ -P ₃	10 ⁻⁵ × k_3 (M ⁻¹ · s ⁻¹)			10 ⁻⁵ × k_4 (s ⁻¹)			K_i^* (pM)		
		WT	ETW	ETW (H)	WT	ETW	ETW (H)	WT	ETW	ETW (H)
1.	Z-D-Phe	14.5 ± 0.03	1.2 ± 0.12	1.42 ± 0.27	1.28 ± 0.11	13.9 ± 0.6	3.39 ± 0.09 ^b	8.83 ± 0.63	1218 ± 130	226 ± 40
2.	Z-D-βNal	9.38 ± 0.17	1.63 ± 0.20	1.04 ± 0.14	3.05 ± 0.28	35 ± 3.8	6.04 ± 0.6 ^b	32.5 ± 3.3	2211 ± 184	608 ± 81
3.	Z-D-Fgl	4.98 ± 0.1	1.02 ± 0.054	0.60 ± 0.152	4.57 ± 0.57	89.3 ± 1.4	13.6 ± 2.3 ^b	91.7 ± 7.3	8759 ± 493	2733 ± 545
4.	Ac-D-Dpa	12.3 ± 0.05	1.00 ± 0.18	1.94 ± 0.45	1.03 ± 0.036	6.91 ± 0.6	3.01 ± 0.43 ^b	8.37 ± 0.51	751 ± 117	177 ± 33

^a Certain of the inhibitors acted as competitive inhibitors of the enzyme and the K_i is shown as calculated from Dixon plots.

^b Significant at $P < 0.001$ versus des-ETW-thrombin in the absence of heparin.

Figure 5 The influence of increasing concentrations of ADP and ATP upon the enhancement of the catalytic rate of des-ETW-thrombin by heparin, PPS and HPA

Des-ETW-thrombin (final concentration 2 nM) in buffer A was pre-incubated in the presence of increasing concentrations of ADP or ATP at 25 °C before the addition of heparin, PPS (10 kDa) or HPA. Incubation was continued for a further 5 min before the addition of varying concentrations of substrate H-D-Phe-Pip-pNA (4.0 to 500 μM). Symbols: ■, □, heparin (100 nM); ▼, ▽, PPS (3 nM); or ▲, △, HPA (260 μM). The enhancement in catalytic rate over control incubations in the absence of effector is shown in the presence of increasing concentration of ADP (open symbols) and ATP (closed symbols).

Noting that binding of hirudin peptide (55–64) to the fibrinogen recognition site had been shown previously to have an allosteric effect on the activity of wild-type thrombin we investigated whether binding of heparin, PPS and HPA also occurred to this site. Previously, Berliner et al. have shown that the nucleotides ATP and ADP inhibit both the clotting of fibrinogen [34] and the binding of fibrin monomer to thrombin by binding to an anionic binding site which has since been identified as being closely allied to thrombin exosite 1 [35]. We used these nucleotides to determine whether increasing the concentration could block the enhanced activity observed with heparin and PPS. We observed that the potentiation of des-ETW-thrombin activity towards substrate, H-D-Phe-Pip-pNA by heparin, PPS and HPA were each reversed by increasing concentrations of ADP and ATP (Figure 5) according to IC₅₀ values which were comparable with those previously reported by Berliner et al. for inhibition of fibrinogen clotting by thrombin [34].

The affinity of des-ETW-thrombin for *p*-aminobenzamidine, which binds to the specificity pocket, is considerably reduced with

respect to wild-type thrombin [13]. The catalytic rate for cleavage of H-D-Phe-Pip-pNA was measured in the presence of increasing concentrations of *p*-aminobenzamidine. Inclusion of heparin (100 nM), HPA (100 μM) and PPS (3.5 nM) caused a decrease in the apparent K_i values from 9.7 mM to 7.5, 5.1 and 2.5 mM respectively measured by Lineweaver–Burk plot. These findings suggest that binding of these ligands corrects defects in the specificity pocket permitting easier access of *p*-aminobenzamidine.

DISCUSSION

Previous comparison of the enzymic properties of des-ETW-thrombin and wild-type recombinant thrombin has shown that the deletion causes a marked impairment of most of thrombin's catalytic properties, including the cleavage of fibrinogen and the activation of protein C. Comparison of the kinetic parameters with a series of peptide pNA substrates showed a reduction in both the Michaelis (K_m) and/or catalytic rate (k_{cat}) constants by up to two orders of magnitude. The deletion lowered the rate of association with antithrombin III and reduced the affinity for hirudin, a highly specific inhibitor of thrombin, while enhancing the susceptibility to inhibition by SBTI. It has been concluded that the mutation alters the conformation of the charge-stabilizing system of the catalytic triad, the primary specificity pocket and the S2 binding site [36].

In this study, we show clearly that at least part of this catalytic impairment can be reversed by binding of heparin, other GAGSs, PPS and HPA. Enhancement of activity towards peptide pNA substrates was observed only with thrombin-specific substrates which have been designed to form favourable interactions with the apolar binding site. In agreement with the findings of others, heparin had only a slight inhibitory effect upon the amidolytic activity of wild-type thrombin. HPA, which has been shown to be an allosteric effector of amidolytic activity of wild-type thrombin, also caused a decrease in the K_m of des-ETW-thrombin with each of the thrombin substrates (Table 1). Heparin enhanced the rate of inhibition of des-ETW-thrombin by antithrombin III, PPack, and a series of peptide boronic acids which are specific for thrombin. Slow tight binding inhibition of des-ETW-thrombin by the peptide boronic acids tested (compounds 1, 2, 3 and 4 in Table 2) was on average 30-fold less potent compared with wild-type thrombin measured with respect to the final K_i values. This reduction in inhibitory potency was due to both a decrease in the rates of association (k_3) and an increase in the dissociation rate (k_4). In the presence of heparin a decrease in the final K_i for inhibition by the peptide boronic acids was observed due to a

significant decrease in the off-rate (k_4) rather than an increase in the on-rate (k_3). This would accord with the previous findings from a study of the interaction of des-ETW-thrombin with recombinant hirudin mutants [36] where the reduced affinity was attributed principally to an increase in the off-rate. Previously, De Cristofaro et al. have showed that the allosteric enhancement of thrombin amidolytic activity by the hirudin peptide is due to an increased rate of acylation [37]. A corresponding part of the mechanism of the formation of tight-binding complexes between peptide boronic acids and serine proteinases is the attainment of a transition-state-like adduct with active-site serine, the stability of this being reflected in the off-rate. The inhibition of des-ETW-thrombin by SBTI was also enhanced in the presence of PPS but interestingly in this case the principal effect was upon the association rate (k_3). Although the ETW deletion favours interaction with SBTI, a putative model of the complex showed a number of unfavourable contacts still remained. Thus the access of Arg⁶³ of the inhibitor to the primary binding pocket may be restricted. Such restriction is abrogated to some degree in the presence of PPS. It is probable that the enhanced inhibition of des-ETW-thrombin by *p*-aminobenzamidine in the presence of heparin, PPS and HPA occurs for similar reasons.

These observations, together with the lack of effect of these ligands on the inhibition of des-ETW-thrombin by a peptide boronic acid containing a neutral side chain at the P₁ site, which may not occupy the S₁ site when complexed to thrombin, led us to propose that the principal cause of the enhanced activity is a conformational change correcting defects in the structure of the specificity pocket, the integrity of which is crucial to the specificity and catalytic mechanism of the serine proteinase family of enzymes [38].

The enhancement of the amidolytic activity of des-ETW-thrombin by heparin and PPS was related to concentration and molecular mass (with decreasing molecular mass, higher concentrations of polysaccharides were required). This is of interest in considering the means by which these ligands may induce such change. The heparin-binding site occurs in a highly electropositive region of thrombin and is thought to be a groove composed of a C-terminal helix containing Arg²³³–Lys²³⁶ which includes a structural motif that resembles sequences found in other heparin-binding proteins [39]. Mutation studies have shown that these two residues together with Arg⁹³, Arg¹⁷⁵ and Lys²⁴⁰ are important in mediating heparin-dependent inhibition by antithrombin III [10,11]. In this area of the molecule Arg⁹³, Arg⁹⁷ and Arg¹⁰¹ appear to be important in the enhancement of activation of protein C by the chondroitin sulphate moiety of thrombomodulin [40]. Unlike antithrombin III, the binding of thrombin to heparin is not dependent upon sequence-specific sites within heparin but occurs non-specifically to the region via electrostatic interaction [41]. This electrostatic association has been estimated from binding studies to be due to five or six anionic residues [42] which would be contained within a two-disaccharide length of the polysaccharide chain. The heparin pentasaccharide fulfils these structural requirements and yet has no effect upon the amidolytic activity of des-ETW-thrombin, suggesting that additional binding to the enzyme is required. This binding may be to exosite 1 which has a known allosteric influence upon the activity of thrombin [24–26,43]. Heparin has been shown to be capable of binding to the electropositive exosite 1, since it protects thrombin from a chemical modification which destroys the ability of the enzyme to bind to hirudin peptide 54–65 and glycoprotein 1B [44]. Further evidence to support this conclusion is that potentiation by heparin, PPS and HPA is inhibited by the nucleotides ATP and ADP which have been used previously as probes of exosite 1 [34]. The similar effect of heparin, PPS and the hirudin

peptide (HPA) upon des-ETW-thrombin tends to argue against an alternative explanation in which the polysaccharide serves as a 'surface' simultaneously binding both enzyme and inhibitor. Such an approximation mechanism would cause a decrease in K_m but would not explain how heparin decreases the off-rate rather than increasing the on-rate during inhibition of des-ETW-thrombin by the boronic acids (Table 2).

Our findings contribute to increasing evidence of the existence of a degree of conformational flexibility of the thrombin molecule which may play an essential part in its catalytic mechanism and specificity. Thermodynamic evidence supports such changes during the binding of substrates to the apolar binding site [45]. The influence of peptides binding to exosite 1 on the activity of thrombin and the contribution of this mechanism to the tight binding of hirudin [46], mediated possibly by site-specific ion-binding interactions with Na⁺ and Cl[−] [47,48], demonstrate thrombin to be an allosteric enzyme. Early reports of an allosteric-type influence of heparin on the cleavage of synthetic substrates by wild-type thrombin generated conflicting reports and it is now generally accepted that heparin has little or no effect, as we also observed [23]. However, binding of heparin and chondroitin sulphate have been shown to modulate the fluorescence of exosite probes [49,50]. This property of chondroitin sulphate may contribute to its ability to enhance the activation of protein C by thrombin [51]. Another ligand of exosite 2, prothrombin fragment 2, enhances esterolytic activity [52] and affects the emission of fluorescence probes bound close to the apolar binding site [53].

Any effect of heparin binding to exosite 2 on thrombin activity may, therefore, be apparent only for certain reactions, when heparin binding dominates other allosteric contributions. We have shown here that the activity of des-ETW-thrombin, lacking an insertion loop situated on the exterior lip of an intact specificity pocket, is liable to marked enhancement by binding of HPA. Heparin may act in a similar fashion to this peptide, the initial binding to the exosite 2 permitting a perhaps less favourable interaction with exosite 1 to occur. Similar coincidental binding of exosite 1 and exosite 2 have been shown recently to occur between two adjacent molecules in the crystal structure of a thrombin/DNA aptamer [54]. The molecular-mass dependence of heparin enhancement of des-ETW-thrombin activity indicates that binding needs to occur to at least two sites on the same molecule. Such binding may be more favoured in the case of heparin and dermatan sulphate than chondroitin sulphate A and C because of the greater conformational flexibility of iduronic acid. PPS which can potentiate the antithrombin III–thrombin and antithrombin III–factor Xa interactions [56], consists of fully sulphated chains of β -(1→4)-linked D-xyloses with α -(1→2) lateral branching of 4-*O*-methyl-D-glucuronic acids [56]. The regularly branched nature of the PPS molecule may contribute in a similar way to the higher potency of this sulphated polysaccharide.

We are grateful to Barbara Lawless for expert assistance in the preparation of this manuscript.

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