Interaction of activated protein C with serpins

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The inhibition of activated protein C by six different serine protease inhibitors (serpins) that have arginine residues in the P_1 position has been investigated. Micromolar concentrations of C1-inhibitor failed to inhibit the enzyme, and it was inhibited only slowly by antithrombin III with an association rate constant ($k_{ass.}$) of 0.15 $M^{-1} \cdot s^{-1}$. The $k_{ass.}$ values for the other serpins tested (protease nexin I, protein C inhibitor, and mutants of α_1-antichymotrypsin and α_1-antitrypsin with P_1 arginine residues) were at least 1000-fold higher, with P_1-Arg-α_1-antitrypsin ($k_{ass.} = 7 \times 10^4$ $M^{-1} \cdot s^{-1}$) being the most effective inhibitor. The inhibition with these four serpins appeared to be reversible, with inhibition constants in the nanomolar range. The relatively high value of $k_{ass.}$ for protease nexin I (5×10^3 $M^{-1} \cdot s^{-1}$) suggested that it may be involved in the control of activated protein C on the surface of platelets where protein nexin I is present at relatively high concentrations. The value of $k_{ass.}$ for protease nexin I, protein C inhibitor and antithrombin III showed a bell-shaped dependence on heparin concentration. At optimal concentrations, heparin accelerated the rate of inhibition by protease nexin I, protein C inhibitor and antithrombin III by 44-, 18- and 13-fold respectively. The kinetic constants for the inhibition of thrombin were also determined, and in all cases the serpins were more effective inhibitors of thrombin. Comparison of the sequences of the active-site regions of activated protein C and thrombin suggested that the more hydrophobic active site of thrombin may be more favourable for interactions with serpins.

INTRODUCTION

Protein C is a vitamin K-dependent serine protease zymogen that is important in haemostasis. Thrombin is formed from prothrombin on the surface of activated platelets by a complex of factors Xa and Va. Thrombin is then able to cleave fibrinogen to yield fibrin, which forms the basis of the blood clot. The activity of thrombin is controlled by various anticoagulant mechanisms. On one hand, thrombin is inactivated by the serpin antithrombin III. Another anticoagulant pathway is initiated by thrombin itself. When bound to the endothelial cell-surface protein thrombomodulin, thrombin becomes an efficient activator of protein C. Activated protein C in a complex with its cofactor protein S is then able to cleave and inactivate cofactors Va and VIIIa, which are essential for the production of thrombin [1]. The physiological importance of protein C is indicated by observations that protein C deficiencies are associated with thrombotic disorders. Homozygous protein C-deficient infants [2,3] suffer potentially fatal *purpura fulminans*, and in families with heterozygous deficiency [4] there is an association with venous thrombotic disease.

Activated protein C (APC) is inhibited in plasma by a combination of inhibitors, the major ones being protein C inhibitor (PCI) and α_1-antitrypsin [5], and the minor ones being α_2-macroglobulin and α_2-antiplasmin [6]. All of these except α_2-macroglobulin are members of the serpin superfamily [7], and they all inhibit APC slowly in comparison with the inhibition of other coagulation enzymes by serpins. Slow inhibition of APC may be advantageous from a physiological point of view, in that it would allow APC time to inactivate factors Va and VIIIa before inhibition occurs. However, the mechanistic reason for the slow inhibition of APC by serpins is unclear. In order to investigate this problem, the kinetics of APC with serpins containing an arginine residue at the P_1 position (nomenclature of Schechter and Berger [8]) have been determined to examine whether there are inherent features in either APC or the serpins that are responsible for the slowness of inhibition.

MATERIALS AND METHODS

Chemicals

The chromogenic substrates S-2222 (benzoyl-Ile-Glu-Gly-Arg-*p*-nitroanilide), S-2266 (D-Val-Leu-Arg-*p*-nitroanilide), S-2238 (D-Phe-pipecolyl-Arg-*p*-nitroanilide), S-2288 (D-Ile-Pro-Arg-*p*-nitroanilide) and S-2366 (<Glu-Pro-Arg-*p*-nitroanilide) were purchased from KabiPharmacia (Mölndal, Sweden). The trypsin substrate benzyl-Arg-*p*-nitroanilide, the chymotrypsin substrate *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide, *p*-nitrophenyl *p'*-guanidinobenzoate and *p*-nitrophenyl acetate were from Sigma (Poole, Dorset, U.K.). Pig mucosal heparin was from Grampian Enzymes (Aberdeen, U.K.). The heparin used for the protease nexin I (PNI) kinetics with APC was 15 kDa heparin that had a high affinity for PNI–Sepharose [9].

Serpins

Recombinant α_1-antichymotrypsin with arginine instead of leucine at the P_1 position (residue 416) was prepared and purified from *Escherichia coli* as described by Rubin et al. [10]. Recombinant α_1-antitrypsin with arginine instead of methionine at position 358 (the P_1 residue) was a gift from Delta Biotechnology (Nottingham, U.K.) and was prepared and purified from *Saccharomyces cerevisiae*. Recombinant PNI was a gift from Professor D. Monard (Friedrich Miescher-Institut, Basel, Switzerland) and was prepared and purified as described by Sommer et al. [11]. PCI was purified by using a monoclonal-antibody (M11-15) column, followed by two passes through a Pharmacia Superose 12 column [12]. Antithrombin III (ATIII) was purified as described by McKay [13]. C1-inhibitor was purified from plasma by a modification of the method of Harrison [14]. The

Abbreviations used: APC, activated protein C; R-rAT, recombinant antitrypsin with an arginine in the P_1 position; R-rACT, recombinant antichymotrypsin with an arginine in the P_1 position; ATIII, antithrombin III; PCI, protein C inhibitor; PNI, protease nexin I.

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eluate from the lysine–Sepharose column was applied to a dextran sulphate–Sepharose column equilibrated with TCE buffer (0.05 M Tris/HCl, 0.02 M sodium citrate, 0.005 M EDTA, pH 7.4) and was eluted with a linear salt gradient of 0.1–0.5 M NaCl. The immunogenically positive fractions were then dialysed against 0.02 M KH_2PO_4, pH 7.0, containing 0.15 M KCl before loading on to a hydroxyapatite column as described by Harrison [14].

Proteases

Human thrombin was purified from plasma as previously described [15]. Purified human APC was a gift from Dr. J. Stenflo and Dr. A. Öhlin. Bovine chymotrypsin and pig pancreatic trypsin were purchased from Sigma.

Kinetics

All kinetics assays were performed at 37 °C in 0.03 M sodium phosphate buffer, pH 7.4, containing 0.16 M NaCl, 0.1 % poly(ethylene glycol) (M_r 4000) and 0.2 mg/ml BSA. Slow binding kinetics were performed with a Hewlett–Packard diode-array spectrophotometer, and a Kontron 940 spectrophotometer was used for other kinetics. The production of *p*-nitroaniline was measured at 402 nm with the Kontron spectrophotometer and between 400 and 410 nm with the Hewlett–Packard. Plastic 1.5 ml cuvettes were coated before use with a solution of 0.5 % poly(ethylene glycol) (M_r 6000), 2 % BSA and 0.01 % Triton X-100 to prevent adsorption of proteins in the reaction mixtures to the cuvettes. The addition of BSA to the buffer was found to be of utmost importance for the kinetics of PCI. In the absence of BSA added freshly to the reaction mixture anomalous kinetics were observed, presumably due to the adsorption of the inhibitor to the cuvette. This observation may explain the large variation in reported kinetic constants for PCI [16,17].

Titrations

Trypsin and thrombin were titrated with *p*-nitrophenyl *p'*-guanidinobenzoate in 0.1 M sodium barbitone/0.02 M $CaCl_2$, pH 8.3 at 25 °C [18]. Chymotrypsin was titrated with *p*-nitrophenyl acetate [19]. With a known concentration of enzyme, samples of different concentrations of serpin were incubated for a period of time sufficient to ensure that the formation of complex was complete. The residual enzyme concentration was then determined by the rate of change in A_{402} after the addition of the appropriate chromogenic substrate. R-rAT (recombinant antitrypsin with arginine in P_1 position) and ATIII were titrated against trypsin; R-rACT (recombinant antichymotrypsin with arginine in P_1 position), PNI and PCI were titrated against thrombin; and C1-inhibitor was titrated against chymotrypsin. APC was titrated against R-rAT. It should be noted that the titration of the serpins only estimates that fraction of the serpin that is capable of forming a stable complex with the enzyme used. The concentration of serpin capable of interacting with the enzyme may be greater if a portion of the serpin is cleaved without forming a stable complex.

Determination of K_m values

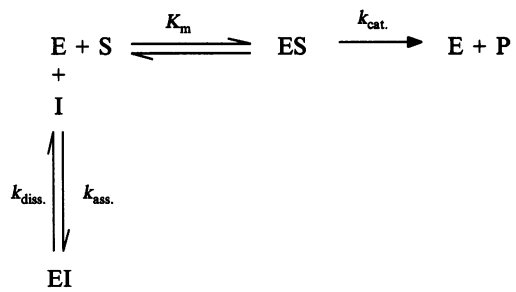
Initial rates for the hydrolysis of chromogenic substrates by APC and thrombin were determined with a range of different substrate concentrations (at least nine different substrate concentrations were used). The data were fitted to the Michaelis–Menten equation by non-linear regression. The K_m values of APC for S-2366, S-2266 and S-2288 were determined as 2.17 ± 0.03 mM, 654 ± 62 μM and 504 ± 58 μM, and the values of thrombin for S-2238, S-2266 and S-2222 were 2.1 ± 0.3 μM, 262 ± 14 μM and 103 ± 10 μM. Substrate concentrations were determined from their A_{342} by using a molar absorption coefficient of 8270 [20].

Pseudo-first-order kinetics

Values of the association rate constant ($k_{ass.}$) for ATIII and APC were obtained by using pseudo-first-order kinetics [21]. The enzyme (30 or 150 nM APC) and a vast excess of inhibitor (30 to 280 μM) were incubated in a small volume. Time courses of the reactions at different inhibitor concentrations were obtained by taking samples over a period of 12 h. These samples were made up to a volume of 1 ml in a cuvette, chromogenic substrate was added, and the initial velocity was measured. This initial velocity is proportional to the amount of uninhibited enzyme present, and the variation of the initial velocity with time obeys the equation $A_t = A_0 e^{-kt}$, where t is the time when the sample is taken, A_t and A_0 are the velocities at times t and zero respectively, and k is the apparent first-order rate constant. Non-linear regression was used to obtain estimates of k. Variation of k with inhibitor concentration was then analysed by linear regression (Figure 2), and the resultant slope yielded an estimate of $k_{ass.}$ [21].

Slow-binding kinetics

For slow-binding kinetics the reaction was started by adding enzyme to the cuvettes. Five to six assays were performed together: one cuvette without inhibitor, and the rest with different inhibitor concentrations. The absorbance was measured every 15 or 30 s for between 1 and 8 h. Control assays in the absence of inhibitor indicated that the enzymes were stable for the time course of the experiment in the buffer used. In general, data were utilized in the analyses only if the level of substrate utilization was less than 5 %. For thrombin with the substrate S-2238, higher levels of substrate utilization (10 %) could be accommodated, due to the low K_m value of this substrate. For all interactions investigated in detail (R-rAT, R-rACT, PNI and PCI against APC and thrombin), the mechanism of inhibition could be described by Scheme 1:



Scheme 1

where E, S, P and I represent the enzyme, substrate (peptidyl *p*-nitroanilide), product (*p*-nitroaniline) and inhibitor (serpin) respectively; K_m and $k_{cat.}$ are the Michaelis and catalytic constants for the enzyme–substrate reaction, and $k_{ass.}$ and $k_{diss.}$ are the association and dissociation rate constants for the enzyme–inhibitor complex. The inhibition constant (K_i) is equal to

$k_{diss.}/k_{ass.}$. For this mechanism, the progress curve of product formation will be given by eqn. (1) [22]:

$$P = v_s t + \frac{(v_0 - v_s)}{k'}(1 - e^{-k't}) \quad (1)$$

where P is the amount of product at time t, k' is an apparent first-order rate constant, and v_0 and v_s are the initial and steady-state velocities respectively. For the mechanism shown in Scheme 1, v_0 will be independent of the inhibitor concentration, and v_s and k' will vary with the inhibitor concentration according to eqns. (2) and (3) [23,24]:

$$v_s = \frac{v_0}{1 + I/K_i'} \quad (2)$$

$$k' = k_{diss.} + k'_{ass.} \cdot I \quad (3a)$$

$$= k'_{ass.}(K_i' + I) \quad (3b)$$

where K_i' and $k'_{ass.}$ are apparent constants that are related to the true constants by the expressions [23,24]:

$$K_i = K_i'/(1 + S/K_m) \quad (4)$$

$$k_{ass.} = k'_{ass.}(1 + S/K_m) \quad (5)$$

The expressions for v_s and k' given by eqns. (2) and (3b) were substituted into eqn. (1), and the data from a progress-curve experiment consisting of 5–6 curves were fitted to this equation by non-linear regression [15]. This analysis yielded estimates for $k'_{ass.}$ and K_i', and values for the true constants were calculated by using eqns. (4) and (5). For an irreversible inhibitor, v_s in eqn. (1) will be equal to zero and k' will equal $k'_{ass.} \cdot I$. For all the serpins tested, v_s was significant at all concentrations, and analysis of progress-curve experiments assuming irreversible inhibition ($v_s = 0$, $k' = k'_{ass.} \cdot I$) yielded a worse fit to the data. Different chromogenic substrates were used for different protease–serpin reactions such that optimal conditions for the determination of $k_{ass.}$ and K_i were obtained. Substrates with high K_m values were used for inhibitors with lower $k_{ass.}$ values. At least two progress-curve experiments were performed for most of the different enzyme–inhibitor combinations, and the weighted mean values of the estimates of the parameters are given. It should be noted that the large number of points used in progress experiments leads to an underestimation of the standard errors of the estimated parameters.

For experiments examining the effect of heparin on the $k_{ass.}$ value, one or two serpin concentrations were used and the data were fitted to the equation for slow-binding inhibition (eqn. 1). This analysis yielded values for the initial (v_0) and steady-state (v_s) velocities as well as the apparent first-order rate constant (k'). For the mechanism in Scheme 1, $k_{ass.}$ can be calculated by using the following relationship [23]:

$$k_{ass.} = k'(1 - v_s/v_0)(1 + S/K_m)/I \quad (6)$$

RESULTS

Inhibition of APC and thrombin

The serpins PNI, PCI, R-rAT and R-rACT were found to be slow-binding inhibitors of APC and thrombin. A typical set of data for the inhibition of APC by PCI is shown in Figure 1. Analysis of the data indicated that the inhibition could be described by the mechanism presented in Scheme 1. In this mechanism, the inhibitor slowly combines with the enzyme to form an inhibited complex. Analysis of data similar to those shown in Figure 1 yielded estimates of the association rate constants ($k_{ass.}$) for the formation of the enzyme–inhibitor complexes for each of the serpins, and these are given in Table 1. The values of $k_{ass.}$ ranged from 8.0×10^2 $M^{-1} \cdot s^{-1}$ and 7.3×10^2 $M^{-1} \cdot s^{-1}$ for PCI and R-rACT respectively, through 5.2×10^3 $M^{-1} \cdot s^{-1}$ for PNI, to 7.0×10^4 $M^{-1} \cdot s^{-1}$ for R-rAT (Table 1). The value obtained for R-rAT compares well with that of 4.9×10^4 $M^{-1} \cdot s^{-1}$ previously obtained by Heeb et al. [21] using a different method.

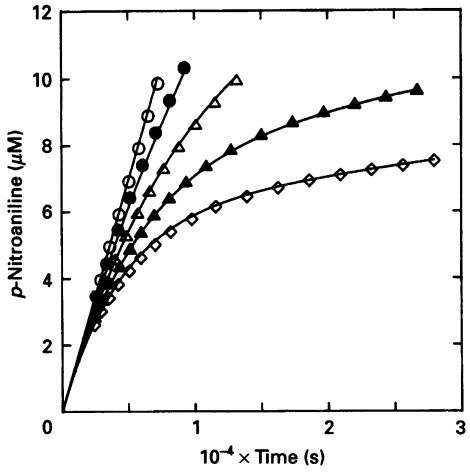


Figure 1 Inhibition of APC by PCI

Assays were performed as described in the Materials and methods section with 300 pM APC and 200 μM S-2266. The assays contained 0 (○), 90 (●), 175 (△), 260 (▲) or 350 (◇) nM PCI. Data time points at less than 4 min are not shown; only each second point thereafter is shown. The curves drawn in the Figure represent the fit of the data to the equation describing the slow-binding inhibition of Scheme 1.

The values of $k_{ass.}$ for ATIII and C1-inhibitor with APC were too low to be measured under conditions of the slow-binding assay. The presence of competing substrate slows down the reaction, and a significant degree of inhibition must be observed before the enzyme has utilized a significant proportion (5–10%) of the substrate [24]. The $k_{ass.}$ values for ATIII and C1-inhibitor were determined by incubating high concentrations of inhibitor with APC and taking samples over several hours to determine the residual enzyme activity. By using this method, pseudo-first-order rate constants were obtained for various concentrations of ATIII with APC, and analysis of these data (Figure 2) yielded a $k_{ass.}$ value of 0.15 $M^{-1} \cdot s^{-1}$. Incubation of APC with 6.8 μM C1-inhibitor for 3.5 h did not result in any decrease in APC activity. Therefore, it can be concluded that the $k_{ass.}$ is less than 1 $M^{-1} \cdot s^{-1}$. C1-inhibitor has also been shown to have $k_{ass.}$ values less than 10 $M^{-1} \cdot s^{-1}$ with tissue plasminogen activator [25] and urokinase [26].

The inhibition of thrombin by the serpins was found to follow the same mechanism (Scheme 1). The values of $k_{ass.}$ for the serpins with thrombin are compared with those obtained for the inhibition of APC in Table 1. In all cases, the serpins inhibited thrombin more rapidly than APC. The ratio of $k_{ass.}$ for thrombin to that for APC ranged from 15 for R-rAT to 7.3×10^5 for ATIII (Table 1). The $k_{ass.}$ ratios for PCI and R-rACT (41 and 24 respectively) were of the same order as that obtained for R-rAT, whereas the ratio for PNI was an order of magnitude higher (290; Table 1).

Analysis of progress-curve data also yielded values for the dissociation constant (K_i) of the serpin–protease complexes. The K_i values are a measure of the stability of the complexes, with higher K_i values corresponding to less stable complexes. The complexes with APC were less stable than those with thrombin (Table 1). The complexes of R-rACT and PCI with both APC

Table 1 Comparison of serpin interactions with APC and thrombin

Assays were performed and data were analysed as described in the Materials and methods section.

	Activated protein C		Thrombin		
	$k_{ass.}$ ($M^{-1} \cdot s^{-1}$)	K_i (nM)	$k_{ass.}$ ($M^{-1} \cdot s^{-1}$)	K_i (pM)	$k_{ass.}$ ratio thrombin:APC
ATIII	0.148 ± 0.003		1.1×10^{4}*		7.3×10^{5}
PNI	$(5.2 \pm 0.1) \times 10^{3}$	0.80 ± 0.04	$(1.48 \pm 0.01) \times 10^{6}$†	2.7 ± 1.1	290
PCI	$(8.0 \pm 0.1) \times 10^{2}$	11.9 ± 0.6	$(3.31 \pm 0.02) \times 10^{4}$	1100 ± 100	41
R-rACT	$(7.3 \pm 0.2) \times 10^{2}$	40.5 ± 9.1	$(1.74 \pm 0.02) \times 10^{4}$†	360 ± 40	24
R-rAT	$(7.0 \pm 0.1) \times 10^{4}$	1.0 ± 0.1	$(1.08 \pm 0.01) \times 10^{6}$	2.0 ± 1.5	15

* This value was taken from Wallace et al. [44].
† These estimates are from a single determination, whereas the others represent the weighted means of at least two determinations.

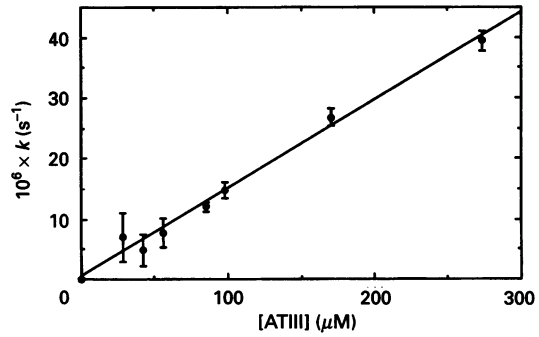


Figure 2 Determination of $k_{ass.}$ for ATIII and APC using pseudo-first-order kinetics

The reaction of APC (30 or 150 nM) with various concentrations of ATIII was monitored in the absence of heparin (for up to 12 h) and the apparent rate constants (k) were determined; these are plotted against the ATIII concentration. Error bars represent the standard errors of the estimates of k obtained from the non-linear regression analysis. The gradient of the line gives the $k_{ass.}$ value.

and thrombin were less stable than those formed with R-rAT and PNI. For example, the K_i value of the APC–PCI complex was 12 nM, whereas the APC–PNI complex had a K_i of 0.8 nM (Table 1).

Effect of heparin on the interaction of APC with serpins

The $k_{ass.}$ values of PNI, PCI, ATIII and R-rACT with both APC and thrombin displayed a bell-shaped dependence on the concentration of heparin. In contrast, the value of $k_{ass.}$ for the interaction of R-rAT (30 nM) with APC and thrombin was not affected by heparin concentrations between 30 nM and 3 μM. The data for PNI and PCI with APC and thrombin as well as those for ATIII with APC are shown in Figure 3; previous studies have demonstrated that the $k_{ass.}$ for the thrombin–ATIII reaction shows a bell-shaped dependence on the concentration of heparin [27]. The data for R-rACT are not shown; this inhibitor showed a small increase in $k_{ass.}$ (2–3-fold) with both APC and thrombin at the optimal heparin concentration of 3–6 μM. The concentrations of inhibitors used in the heparin-stimulation experiments were adjusted such that accurate determination of $k_{ass.}$ values was possible. The concentration of ATIII used (86 μM) was much higher than those used for PCI (37 or 74 nM) and PNI (10 or 11 nM), and thus the results obtained with ATIII cannot be easily compared with those obtained for the other two inhibitors. For the ATIII–APC interaction, the optimal heparin concentration was at a concentration roughly equivalent to the ATIII concentration (0.1–1 mM). The heparin concentration required for optimal stimulation of inhibition of APC and thrombin by PNI was lower than that required for the inhibition of these two enzymes by PCI (10–100 nM versus 1–10 μM). In addition, with both PCI and PNI, the optimal concentration of heparin was 10-fold lower with thrombin than with APC (Figure 3).

The $k_{ass.}$ and K_i values for each inhibitor at its optimal heparin concentration are given in Table 2. Only a small stimulation

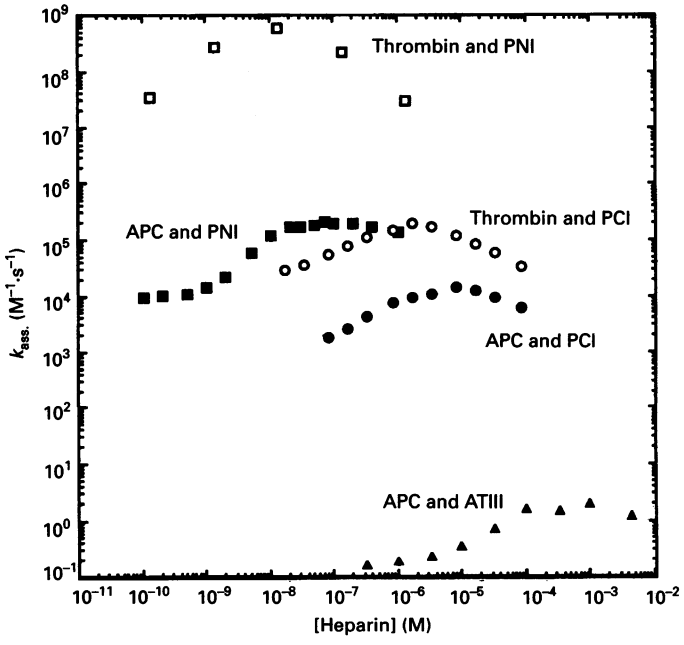


Figure 3 Variation of association constants with heparin concentration

The association constants at different heparin concentrations were determined by using slow-binding kinetics. The inhibitor concentrations used were 10 or 11 nM PNI, 37 and 74 nM PCI, and 86 μM ATIII. Unfractionated heparin was used for ATIII and PCI, and an average molecular mass of 18 kDa was used to calculate its molar concentration. The heparin used for PNI was 15 kDa and had a high affinity for PNI–Sepharose (0.35–1 M NaCl fraction [9]).

Table 2 Effect of heparin on the inhibition of APC and thrombin

Assays were performed and data were analysed as described in the Materials and methods section. The optimal heparin concentration was 1 mM for ATIII (86 μM) against APC; 100 nM for PNI (10 or 11 nM) against APC and 10 nM against thrombin; 8 μM for PCI (37 and 74 nM) against APC and 2 μM against thrombin; and 3 μM for R-rACT (400 nM) against APC and 6 μM against thrombin.

	Activated protein C			Thrombin			
	$k_{ass.}$ ($M^{-1} \cdot s^{-1}$)	K_i (nM)	Increase in $k_{ass.}$ (fold)	$k_{ass.}$ ($M^{-1} \cdot s^{-1}$)	K_i (pM)	Increase in $k_{ass.}$ (fold)	$k_{ass.}$ ratio thrombin:APC
ATIII	1.9 ± 0.1		13	4×10^7*		3600	2.1×10^7
PNI	$(2.26 \pm 0.04) \times 10^5$	0.28 ± 0.01	44	$(4.8 \pm 0.2) \times 10^8$†	0.25 ± 0.03	320	2.1×10^3
PCI	$(1.47 \pm 0.03) \times 10^4$	4.1 ± 0.3	18	$(2.05 \pm 0.01) \times 10^5$	430 ± 130	6	14
R-rACT	$(1.99 \pm 0.02) \times 10^3$†	15.3 ± 0.5	2.7	$(3.18 \pm 0.02) \times 10^4$†	270 ± 30	1.8	16

* This value was taken from Wallace et al. [44].

† These estimates are from a single determination, whereas the others represent the weighted means of at least two determinations.

(2–3-fold) was observed for the interaction of R-rACT with both APC and thrombin. For APC, the extent to which heparin accelerated the reaction of ATIII, PCI and PNI was similar (13-, 18- and 44-fold respectively). In contrast, heparin accelerated the reaction of ATIII and PNI with thrombin (3600- and 320-fold respectively) to a much greater extent than the thrombin–PCI association (6-fold). In all cases where it was possible to measure a K_i value, the heparin-stimulated increase in $k_{ass.}$ was accompanied by a decrease in K_i.

DISCUSSION

Each of the interactions investigated in detail (R-rAT, R-rACT, PNI and PCI against APC and thrombin) was found to conform to the slow-binding inhibition mechanism presented in Scheme 1. This mechanism assumes that a reversible complex is formed between the proteinase and the serpin and, in this respect, it is at variance with the commonly held view that the serpin–proteinase complex is irreversible [28]. There is, however, evidence that a reversible complex is formed. Kinetic studies indicate that serpins can be treated as slow-binding inhibitors [29,30]. In addition, it has been shown that the complex can break down to yield both uncleaved and cleaved serpin [31–34]. A mechanism that incorporates all these observations is given in Scheme 2:

$$E + I \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} EI \xrightarrow{k_{+2}} E + I^*$$

Scheme 2

	$P_3 P_2 P_1 P_1'$	
C1 inhibitor	S AISVA**R**TTLV	FEVQQP
ATIII	TAVVIAG**R**SLNPNRVT	FKANRP
PNI	TTAILIA**R**SSPPW	FIVDRP
PCI	TGTIFTF**R**SARLNSQR	LVFNRP
R-rACT	TAVKITL**R**SALVETRTIVRFNKP	
R-rAT	MFLEAIP**R**SIPPE	VKFNKP

Figure 4 Comparison of serpin reactive-loop sequences

The sequences start with the semi-conserved threonine residue and end with the conserved proline residue. The P_1 residue is in **bold** type. Sequences were taken from Huber and Carrell [61].

where I* represents cleaved inhibitor and k_{+2} is the dissociation rate constant for its release. Once this mechanism has reached steady-state equilibrium, the K_i value will be given by $(k_{-1}+k_{+2})/k_{+1}$. Steady-state equilibrium will only be achieved if k_{+2} is small, such that there is not significant depletion of the inhibitor during the course of the experiment. Depletion of the inhibitor would result in an upper curvature of the progress curves due to relief of the inhibition; such curves were not observed. The amount of depletion of the inhibitor that is required before relief of the inhibition is observed will depend on the concentration of the inhibitor relative to the K_i' value. When $I \gg K_i'$ (as in all the experiments in the present study), a large depletion of the inhibitor will be required for a significant decrease in the degree of inhibition. It should be noted that the K_i value for the mechanism of Scheme 2 is not an equilibrium dissociation constant; it takes the same form as a Michaelis constant. Moreover, the $k_{diss.}$ values given in Tables 1 and 2 will represent the sum of the dissociation rate constant for the steps to release cleaved and uncleaved inhibitor ($k_{-1}+k_{+2}$). The value of $k_{ass.}$, however, still represents the true association rate constant ($k_{ass.} = k_{+1}$).

Each of the serpins examined associated more rapidly with thrombin than with APC (see $k_{ass.}$ ratios in Table 1). In addition, the serpin–thrombin complexes were more stable as a result of the increase in $k_{ass.}$ (Table 1). These results indicate that the active site of thrombin encourages a more rapid formation of the inhibited complex. An interpretation of these results in structural terms is difficult, due to the lack of structures for serpin–protease complexes, uncleaved serpins and APC. However, alignment of the reactive-site loops of serpins indicates that the region expected to bind the active site of the protease (P_4–P_4') is rich in hydrophobic amino acids (Figure 4). Although the P_1 arginine residue will be accommodated in the primary specificity pocket of the target protease, the remaining residues will be better bound to hydrophobic binding sites in the active-site cleft. Thrombin is particularly rich in such sites. In the crystal structure of thrombin complexed to the methyl ketone of D-Phe-Pro-Arg [35], the specificity pocket for the P_1 arginine residue is geometrically similar to that of bovine trypsin. The S_2 subsite is quite different in that the pyrrolidine ring of the P_2 proline is encapsulated in an hydrophobic cage formed by two side chains of the B-insertion loop, Tyr-60A and Trp-60D, as well as His-57, Trp-215 and Leu-99 (this numbering system is based on topological equivalence to chymotrypsinogen [35]). The D-Phe residue is bound to another hydrophobic pocket that is also utilized by

thrombin to bind fibrinopeptide A [36,37]. This pocket is formed by Tyr-60A, Leu-99 and Ile-174. Comparison of the sequences of thrombin and APC demonstrates that many of these interactions will not take place in the APC active site. The B-insertion loop that partially occludes the active site of thrombin and makes it more hydrophobic is not found in APC; Tyr-60A and Trp-60D, which make hydrophobic interactions with the S_2 site of thrombin, are found in this loop. The hydrophobicity of the APC active site is also less because Leu-99 and Ile-174 are replaced by threonine and asparagine respectively. Another insertion in thrombin contains Trp-148, and this residue could also be involved in hydrophobic interactions at the rim of the active-site cleft; in APC, Trp-148 can be aligned with a lysine. Thus, on the basis of this sequence comparison, it is tempting to speculate that the poorer reactivity of APC with serpins is due to the lower hydrophobicity of its active site. This proposal is supported by observations made with a mutant of thrombin in which a segment of the B-insertion loop (Pro-60B–Pro-60C-Trp-60D) was deleted. This mutation, which would decrease the hydrophobicity of thrombin's active site, led to a much poorer interaction with antithrombin III; over a 100-fold decrease in the $k_{ass.}$ value was observed [38].

The difference between the inhibitory potency with respect to thrombin and APC was particularly marked in the case of ATIII. Like ATIII, APC has anticoagulant activity. Therefore it could be expected that there would be some evolutionary pressure to prevent rapid inhibition of APC by ATIII. By using the $k_{ass.}$ value determined (0.15 $M^{-1} \cdot s^{-1}$) and the known ATIII plasma concentration (2.4 μM) [39], a rate constant of 3.5×10^{-7} s^{-1} can be calculated for the inhibition of APC by ATIII, which corresponds to a half-life of 23 days for the reaction. Even in the presence of heparin, the half-life of the reaction would still be 2 days. The $k_{ass.}$ value of 0.15 $M^{-1} \cdot s^{-1}$ for APC–ATIII appears to be the lowest that has been reported for a protease–serpin interaction. Indeed, the lowest previously reported $k_{ass.}$ for ATIII was 53 $M^{-1} \cdot s^{-1}$ with Factor XIIf [40]. Comparison of the reactive-site loops of the serpins tested (Figure 4) does not reveal any marked differences between ATIII and the other serpins, except that ATIII, unlike the APC-inhibiting serpins, has glycine in the P_2 position. A series of peptidylchloromethanes with glycine in the P_2 position failed to inhibit APC [41], whereas inhibition was observed with hydrophobic residues at this position. This supports the hypothesis that the residue in the P_2 position is important for interaction with APC. C1-inhibitor is another serpin which inhibits APC with a $k_{ass.}$ value < 1 $M^{-1} \cdot s^{-1}$. Sequence comparisons indicate that C1-inhibitor has a threonine in place of the usual serine in the P_1' position. Future studies will examine the importance of a P_1' threonine and a P_2 glycine to the poor inhibition of APC by using site-directed mutagenesis.

The $k_{ass.}$ values for ATIII, PNI, PCI and R-rACT with APC displayed a bell-shaped dependence on the concentration of heparin (Figure 3), and these data are consistent with the ternary-complex mechanism that was originally proposed for the thrombin–ATIII interaction [27,42,43] and subsequently shown to apply to reactions with PNI [44], heparin cofactor II [45,46] and PCI [45]. In this mechanism heparin binds to both the serpin and the protease; this increases the effective concentration of the two proteins and thereby accelerates the interaction. However, when the heparin concentration is increased above a certain value, the protease and the serpin are more likely to be bound to different heparin molecules, and so the degree of acceleration decreases. This mechanism requires both the protease and the inhibitor to bind heparin, so the absence of heparin stimulation with R-rAT can be explained on the basis that R-rAT does not bind to heparin [47]. The ternary-complex mechanism predicts that the optimal concentration of heparin will be determined by both the protease and serpin concentrations, and their affinity for heparin [48]. Thus the lower concentrations of heparin required by PNI compared with those required by PCI for maximal acceleration of the inhibition rate are consistent with the higher affinity of PNI for heparin [49,50]. Similarly, the lower concentrations required to stimulate the inhibition of thrombin compared with APC are consistent with the higher affinity of thrombin for heparin [50].

According to the ternary-complex mechanism, the maximal stimulation of the reaction will depend on the orientation of the protease and the serpin when they are bound to heparin. The orientations of the protease thrombin and the serpins ATIII and PNI when bound to heparin seem to facilitate productive complex formation; the reaction of thrombin with ATIII and PNI is stimulated 3600- and 320-fold respectively. In contrast, the orientation of PCI when bound to heparin appears to be less optimal for thrombin inhibition; heparin only stimulates this reaction 6-fold. Structural data support this conclusion. The heparin-binding sites of ATIII and PNI consist of positively charged residues located on or around the D-helix [9,51], whereas the putative heparin-binding site of thrombin consists of a positively charged area on the surface flanking the active-site cleft. Arg-93, Arg-126, Lys-236 and Lys-240 form the nucleus of thrombin's heparin-binding site [52]. Modelling studies indicate that in a productive complex between thrombin and ATIII the heparin-binding sites of the two molecules would form a contiguous positively charged patch that could be effectively bridged by heparin [52]. In contrast, the heparin-binding site of PCI is located in a different region of the serpin, namely the H-helix [50] and probably the N-terminal region [53], such that a less optimal orientation of PCI with respect to thrombin would be expected. The reaction of thrombin with R-rACT was only accelerated to a small extent (2-fold) by heparin. A possible site for the binding of heparin on R-rACT is the 'DNA binding site', which consists of three lysine residues located on the turn s4C-s3C near the reactive-site loop of the serpin [54]. Thus the beneficial effect that heparin has in bringing the protease and the serpin together could be largely offset for R-rACT by a poor orientation of the two molecules with respect to each other, and possibly by obstruction of the enzyme-binding region of the serpin by the heparin. For ATIII and PNI, the degree to which heparin accelerated the reaction with APC was much less than that observed for thrombin (Table 2). These results suggest that the heparin-bound orientation of APC relative to these serpins is less optimal than that of thrombin. Comparison of the sequences of APC and thrombin indicates that the region of APC corresponding to the heparin-binding site of thrombin is not rich in positively charged residues, and is therefore unlikely to constitute the heparin-binding site of APC. It seems likely that the region of APC that binds heparin is found on the opposite side of the molecule. A positively charged surface patch would be formed by the surface loops consisting of residues 35–40, 65–80 and 144–151 (chymotrypsin numbering).

Kinetic parameters for the inhibition of APC by PNI have not previously been reported. The values of $k_{ass.}$ for PNI of 5.2×10^3 $M^{-1} \cdot s^{-1}$ in the absence of heparin and 2.3×10^5 $M^{-1} \cdot s^{-1}$ in its presence suggest that PNI may be of some importance as a physiological inhibitor of APC. Although no APC–PNI complexes have been detected in plasma [6], complexes of proteases with PNI bind avidly to cell surfaces, where they are internalized and degraded [55], so it may not be possible to detect PNI complexes in plasma. Although the plasma concentration of PNI is only about 20 pM (1 ng/ml) [56], PNI has been found on the surface of platelets at a concentration of about 200 molecules

per platelet [57]. The substrates of APC (factors Va and VIIIa) are also found on the platelet surface. For efficient cleavage of factors Va and VIIIa, APC requires Protein S as a cofactor. Protein S binds to APC and localizes it on the surface of platelets, and there are about 200 binding sites for Protein S per platelet [58]. Thus it is expected that APC and PNI will be present on a platelet in roughly equal quantities. Platelets are roughly 2 μm in diameter [59], and if it is assumed that the PNI bound to the platelet surface is confined to a layer 0.1 μm (about 10 times the diameter of a serpin) from the surface, it can be calculated that the concentration of PNI will be about 300 nM. At this concentration, the half-life of APC will be 6 min. If, however, the glycosaminoglycans on the platelet surface are able to stimulate the reaction, the half-life may be as little as 10 s. For comparison, the plasma concentration of PCI is about 100 nM [60] and the half-life of APC in plasma will be about 3 h in the absence of heparin and 10 min in its presence. From these calculations, it is apparent that PNI may be important for protecting platelet-bound factors Va and VIIIa from cleavage by APC. It should be noted, however, that thrombin interacts more rapidly with PNI than APC does, so that any thrombin on the platelet surface will be inhibited in preference to APC.

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