Rapid Inhibition of the Sperm Protease Acrosin by Protein C Inhibitor

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ABSTRACT: Heparin was found to be an allosteric modulator of the amidolytic activity of the protease acrosin. In the presence of saturating concentrations of heparin, there was a 4.9-fold decrease in the value of the Michaelis constant for the substrate D-Ile-Pro-Arg-*p*-nitroanilide and the value of k_{cat} was 2.5-fold lower. Analysis of the data yielded a dissociation constant of 0.22 ± 0.04 μM for the heparin–acrosin complex. The presence of relatively high concentrations of protein C inhibitor in seminal plasma [Laurell, M., Christensson, A., Abrahamson, P., Stenflo, J., & Lilja, H. (1992) *J. Clin. Invest. 89*, 1094–1101] suggests that this serpin may be involved in the control of the activity of acrosin. Acrosin was found to be rapidly inhibited by protein C inhibitor with the association rate constant (k_{ass}) for the formation of the complex being (2.41 ± 0.03) × 10^5 M^{-1} s^{-1}. The value of k_{ass} showed a bell-shaped dependence on the concentration of heparin; it was maximal at concentrations of heparin between 0.08 and 3 μM and decreased at lower and higher concentrations. At the optimal heparin concentration, the value of k_{ass} for the acrosin–protein C inhibitor reaction was 230-fold higher ((5.6 ± 0.1) × 10^7 M^{-1} s^{-1}) than in the absence of heparin. The results suggest that protein C inhibitor may be important in the physiological control of acrosin activity, particularly where the presence of heparin-like glycosaminoglycans would stimulate the acrosin–protein C inhibitor reaction.

Acrosin (EC 3.4.21.10) is a trypsin-like serine protease that is found in large quantities within the acrosomal vesicle of mammalian spermatozoa; in the pig, it has been calculated that 0.09 pg of acrosin is present in each sperm, which is equivalent to a concentration of 1.6 mM in the acrosome (Müller-Esterl & Fritz, 1981). Release of the acrosomal contents (the so-called acrosome reaction) is a prequesite for successful fertilization and is defined as a Ca^{2+}-dependent exocytotic event in which the outer acrosomal membrane fuses with the overlying plasma membrane (Rubinstein & Brietbart, 1991). If the acrosome reaction does not take place, spermatazoa are unable to penetrate the zona pellucida (the extracellular matrix that surrounds all mammalian eggs). Coincident with the acrosome reaction, there is an increase in intracellular pH, and this is thought to be one of the factors that stimulates the conversion of proacrosin to acrosin. Unusual for a serine protease, this activation involves proteolysis at both the N- and C-terminal ends of proacrosin (Baba *et al.*, 1989).

Acrosin has long been thought to have a role in the penetration of the zona pellucida by digesting its component glycoproteins as well as the matrix proteins collagen type IV and fibronectin (Nakano *et al.*, 1989; Planchenault *et al.*, 1991). In addition, both acrosin and its zymogen are able to bind sulfated polysaccharides and zona glycoproteins, which are sulfated, and this may be involved in the binding of the acrosome-reacted sperm to the surface of the egg (Jones, 1990). The sulfate-binding domain of acrosin appears to be located in the N-terminal half of the molecule (Töpfer-Petersen *et al.*, 1990; Jansen & Jones, 1993).

Acrosin inhibitors of the Kazal family of small inhibitors have been found in the seminal plasma of every mammalian species so far investigated (Falase *et al.*, 1991), including boar (Jonáková *et al.*, 1992) and human (Fink *et al.*, 1990). A male accessory gland acrosin inhibitor from *Drosophila* of the Kunitz (pancreatic trypsin inhibitor) family has also been discovered (Schmidt *et al.*, 1989). These inhibitors may protect genital trace epithelia from proteolytic degradation by acrosin released from damaged spermatozoa (Nicholson *et al.*, 1983) or function as capacitation factors by binding to acceptor molecules on the sperm surface, thereby stabilizing receptors necessary for sperm-oocyte interaction (Jonáková *et al.*, 1992). Two acceptor molecules for boar acrosin inhibitors have also been characterized (Sanz *et al.*, 1992). Another serine protease inhibitor that is found in high levels in human seminal plasma is protein C inhibitor (PCI).[1] PCI was originally isolated from blood where it is thought to act as an inhibitor of activated protein C. However, while it is present in blood at a concentration of only 100 nM, its concentration in seminal fluid is 3–4 μM (España *et al.*, 1991; Laurell *et al.*, 1992). It is synthesized in the seminal vesicles (Laurell *et al.*, 1992), and seminal vesicle secretions obtained at autopsy retain PCI reactivity when incubated at 37 °C for a number of hours (España *et al.*, 1991). However, in seminal plasma, which also contains the prostatic secretion, almost all PCI activity is lost after 2 h at 37 °C. In whole semen (plasma plus sperm), the PCI remains active for twice as long. Thus, it appears that PCI is synthesized and secreted by the seminal vesicles, but after ejaculation and mixing with seminal plasma, it becomes degraded or complexed, and this interaction is prevented by association with sperm. In man, the prostatic secretion contributes most of the enzymes present in the ejaculate, including prostate-specific antigen, which lyses the

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[1] Abbreviations: PCI, protein C inhibitor; pNA, *p*-nitroanilide.

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seminal coagulum. This enzyme is a member of the glandular kallikrein family of serine proteases (Lilja, 1985) and is present in human seminal plasma at 2.5 mg/mL (10 times the molar concentration of PCI). The appearance of prostate-specific antigen–PCI complexes parallels the loss of PCI activity in seminal plasma, and so, prostate-specific antigen accounts for the observed inactivation of PCI (España *et al.*, 1991). However, the large excess of prostate-specific antigen over PCI suggests that the major function of PCI is not to inhibit this enzyme.

Results obtained in the present study indicate that acrosin is rapidly inhibited by PCI and that this reaction is stimulated markedly by heparin. Heparin was also found to be an allosteric modulator of the proteolytic activity of acrosin. Consideration of the association rate constants for the inhibition of activated protein C and acrosin by PCI together with the concentrations of PCI in human blood and seminal plasma suggests that the primary physiological function of PCI is the inhibition of acrosin rather than that of activated protein C.

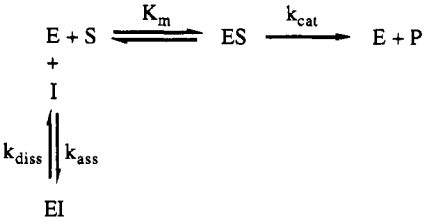
MATERIALS AND METHODS

Materials. The chromogenic substrate D-Ile-Pro-Arg-pNA was purchased from Chromogenix (Mölndal, Sweden). *p*-Nitrophenyl-*p'*-guanidinobenzoate was from Sigma (Poole, U.K.). Porcine mucosal heparin was from Grampian Enzymes (Aberdeen, U.K.). Bovine serum albumin was fraction V from Boeringher Mannheim (Mannheim, Germany). Other chemicals were of the highest grade available commercially. Human PCI was purified from fresh human plasma as described previously using a monoclonal antibody (M11-15) column (Laurell *et al.*, 1988; Hermans & Stone, 1993). Porcine proacrosin was purified from ejaculated sperm and activated to acrosin at pH 8.0 as described by Polakoski and Parrish (1977). The acrosin appeared as a single band of 37 kDa on an SDS–PAGE gel (Laemmli, 1970) and was assumed to be β-acrosin. Human α-thrombin was purified as described by Stone and Hofsteenge (1986).

Determination of the Active Concentrations of Proteins. The concentrations of acrosin and thrombin were determined by active-site titration with *p*-nitrophenyl-*p'*-guanidinobenzoate in 0.1 M sodium barbitone and 0.02 M $CaCl_2$, pH 8.3, at 25 °C (Chase & Shaw, 1970). Using a known concentration of thrombin or acrosin, aliquots of PCI were incubated with the enzyme at 37 °C in 0.03 M sodium phosphate buffer, pH 7.4, containing 0.16 M NaCl, 0.1% (w/v) polyethyleneglycol 4000, and 0.2 mg/mL bovine serum albumin. The period of incubation was sufficient to ensure that the formation of complex was complete. The residual enzyme concentration was then determined after addition of 100 μM D-Ile-Pro-Arg-pNA. The activity of PCI was determined from linear regression analysis of the dependence of the residual activity of the enzyme on the amount of PCI added (Hermans & Stone, 1993). The same estimate for the concentration of PCI was obtained from titration with thrombin and acrosin. For experiments in which the effect of heparin was examined, the active concentration of PCI was determined by titration against acrosin in the presence of the heparin. The active concentration of PCI did not vary significantly over the range of heparin concentrations used (0.8 nM–3 μM), but it was only 40% of the value obtained in the absence of heparin.

Kinetic assays were performed at 37 °C in 0.03 M sodium phosphate buffer, pH 7.4, containing 0.16 M NaCl, 0.1% (w/v) polyethyleneglycol 4000, and 0.2 mg/mL bovine serum albumin. The release of *p*-nitroaniline from the substrate

Scheme 1



D-Ile-Pro-Arg-pNA was monitored by measuring the absorbance between 400 and 410 nm with a Hewlett-Packard 8452A diode array spectrophotometer (Stone *et al.*, 1991). Reactions were performed in polystyrene cuvettes that were coated before use with 0.5% (w/v) polyethyleneglycol 6000, 2% (w/v) bovine serum albumin, and 0.01% (v/v) Triton X-100 solution to prevent adsorption of proteins in the reaction mixtures to the cuvettes.

Initial-Rate Kinetics. The kinetic parameters for the cleavage of D-Ile-Pro-Arg-pNA by acrosin were determined by measuring the initial rate of enzymatic hydrolysis with at least seven different substrate concentrations. The data were fitted to the Michaelis–Menten equation by nonlinear regression to yield estimates for the k_{cat} and K_m values. The concentration of D-Ile-Pro-Arg-pNA was determined from its absorbance at 342 nm (Lottenberg & Jackson, 1983).

Slow-Binding Kinetics. The inhibition of acrosin by PCI could be described in terms of slow-binding kinetics. The release of *p*-nitroaniline from the substrate was followed after the addition of acrosin to cuvettes containing the substrate and PCI. In the absence of heparin, five to six assays were performed in each slow-binding inhibition experiment: one without inhibitor and the others with different inhibitor concentrations. In the absence of inhibitor, the decrease in the activity of acrosin was less than 5% over the time period used. Data points were excluded from the analyses when the level of substrate utilization was greater than 10%. The mechanism of inhibition could be described by Scheme 1, where E, S, P, and I represent the enzyme, substrate (D-Ile-Pro-Arg-pNA), product (*p*-nitroaniline), and inhibitor (PCI), 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}. Analysis of progress-curve data by nonlinear regression according to the equation that describes this mechanism (Morrison & Walsh, 1988; Stone & Hofsteenge, 1986) yielded estimates for the apparent association rate (k'_{ass}) and inhibition constants (K'_i). The following relationships were used to calculate the values of the true constants (Morrison & Walsh, 1988):

$$K_i = K'_i/(1 + S/K_m)$$

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

For the experiment examining the effect of different heparin concentrations on the value of k_{ass}, data from two progress curves (0 and 6 nM PCI) were utilized for the determination of k_{ass} using the method of analysis outlined above.

All progress-curve data were also fitted to an equation describing irreversible inhibition ($k_{diss} = 0$; $K_i = 0$; Hermans & Stone, 1993), and in all cases, a better fit was obtained to the equation for reversible inhibition.

RESULTS

Effect of Heparin on the Kinetic Parameters for the Cleavage of the Substrate D-Ile-Pro-Arg-pNA by Acrosin.

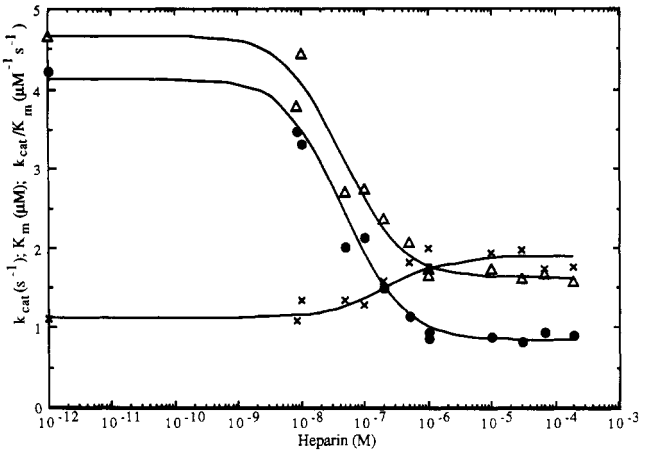
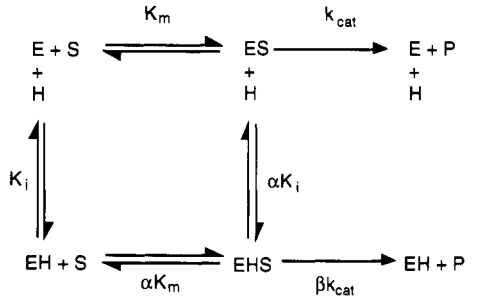


FIGURE 1: Variation of the kinetic parameters for D-Ile-Pro-Arg-pNA with the concentration of acrosin. Assays were performed with 0.34 nM acrosin and at least seven different concentrations of D-Ile-Pro-Arg-pNA as described in Materials and Methods. The data were analyzed by nonlinear regression according to the Michaelis–Menten equation to yield estimates of the apparent k_{cat} (△), K_m (●), and k_{cat}/K_m (×) values at different heparin concentrations. The estimates of the parameters obtained in the absence of heparin are plotted at 10^{-12} M heparin. The estimates obtained at different heparin concentrations were weighted according to the inverse of their variance and fitted to the appropriate equation (eqs 2–4) by nonlinear regression. The curves show the fit of the data to these equations. An average molecular mass of 18 kDa was used to calculate the molarity of the unfractionated heparin.

Scheme 2



The k_{cat} and K_m values for the substrate D-Ile-Pro-Arg-pNA were found to vary with the concentration of heparin; both values decreased as the heparin concentration increased (Figure 1). In the absence and presence of heparin, linear Lineweaver–Burk plots were obtained, but a hyperbolic dependence of k_{cat} and K_m on the concentration of heparin was observed. Both k_{cat} and K_m decreased as the concentration of heparin increased (Figure 1). The effect of heparin could be described by the mechanism presented in Scheme 2 (Segel, 1975), where E is the enzyme acrosin, S is the chromogenic substrate D-Ile-Pro-Arg-pNA, H is heparin, and P represents the products of the cleavage reaction (*p*-nitroaniline and D-Ile-Pro-Arg). An equation to describe this mechanism can be derived by using the rapid-equilibrium assumption:

$$\frac{v}{E_o} = \frac{k_{cat}S}{K_m\frac{(1 + H/K_i)}{(1 + \beta H/\alpha K_i)} + S\frac{(1 + H/\alpha K_i)}{(1 + \beta H/\alpha K_i)}} \quad (1)$$

where v is the initial velocity, E_o is the total enzyme concentration, and the other parameters are as defined in Scheme 2. The apparent K_m, k_{cat}, and k_{cat}/K_m values at a fixed heparin concentration are described by the following equations:

$$K_m(app) = K_m\frac{(1 + H/K_i)}{(1 + H/\alpha K_i)} \quad (2)$$

$$k_{cat}(app) = k_{cat}\frac{(1 + \beta H/\alpha K_i)}{(1 + H/\alpha K_i)} \quad (3)$$

$$\frac{k_{cat}(app)}{K_m(app)} = \frac{k_{cat}(1 + \beta H/\alpha K_i)}{K_m\ (1 + H/K_i)} \quad (4)$$

The apparent values of k_{cat} and K_m will decrease with increasing concentrations of heparin as was observed if $0 < \alpha < \beta < 1$. In order to estimate the values of the parameters of eq 1, initial-velocity data obtained at 14 different heparin concentrations (a total of 165 points) were fitted to eq 1 using nonlinear regression. Using this method of data analysis, each of the estimates displayed large standard errors. The accuracy of the estimation of these parameters was improved by fitting the variation of k_{cat}, K_m, and k_{cat}/K_m to eqs 2–4, respectively. For each heparin concentration, the apparent values of K_m, k_{cat}, and k_{cat}/K_m were obtained by fitting the data to the Michaelis–Menten equation using nonlinear regression. The estimates of these parameters obtained at different heparin concentrations were then weighted according to the inverse of their variance and fitted to the appropriate equation. Analysis of the variation of K_m(app) with the concentration of heparin according to eq 2 yielded estimates for K_m, α, and K_i of 4.13 ± 0.13 μM, 0.206 ± 0.008, and 0.208 ± 0.031 μM, respectively. A similar analysis of the dependence of the k_{cat}-(app) using eq 3 gave estimates for k_{cat}, β, and αK_i of 4.7 ± 0.1 s^{-1}, 0.349 ± 0.008, and 0.0657 ± 0.0089 μM. The data for k_{cat}(app)/K_m(app) were fitted to eq 4 to yield estimates of 1.13 ± 0.04 $\mu M^{-1} s^{-1}$, 1.68 ± 0.07, and 0.111 ± 0.057 μM for k_{cat}/K_m, β/α, and K_i. These estimates of k_{cat}/K_m and β/α agreed well with those derived from the analyses of k_{cat}(app) and K_m(app) data. Two estimates of K_i and one of αK_i were obtained. The weighted mean of these three determinations for K_i (αK_i was divided by α) was 0.22 ± 0.04 μM.

Inhibition of Acrosin by PCI. The inhibition of acrosin by PCI in the presence of substrate could be described by the slow-binding inhibition mechanism presented in Scheme 1 (Figure 2). Analysis of the data according the equation that describes this mechanism yielded estimates for the association rate constant (k_{ass}) and inhibition constant of (2.41 ± 0.03) × 10^5 M^{-1} s^{-1} and 46 ± 5 pM (Table 1).

The value of k_{ass} for PCI with acrosin showed a bell-shaped dependence on the concentration of heparin (Figure 3). The values of K_m required for the calculation of k_{ass} at different heparin concentrations (see Materials and Methods) were obtained by using eq 2 together with the values of K_i, α, and K_m determined above. It should be noted that these calculations for K_m assume that the concentration of the acrosin–heparin complex is not altered by the presence of PCI which will compete to some extent for the binding of heparin. However, the amount of heparin bound by PCI will be negligible in most cases, since the concentration of PCI (6 nM) was usually much lower than that of heparin. A broad optimum for k_{ass} between 0.1 and 3 μM heparin was observed, and the value of k_{ass} decreased at heparin concentrations above and below this optimal range. At optimal heparin concentrations, an increase of about 230-fold in the value of k_{ass} was observed (Figure 3). This increase in k_{ass} was accompanied by a marked decrease in K_i (Table 1).

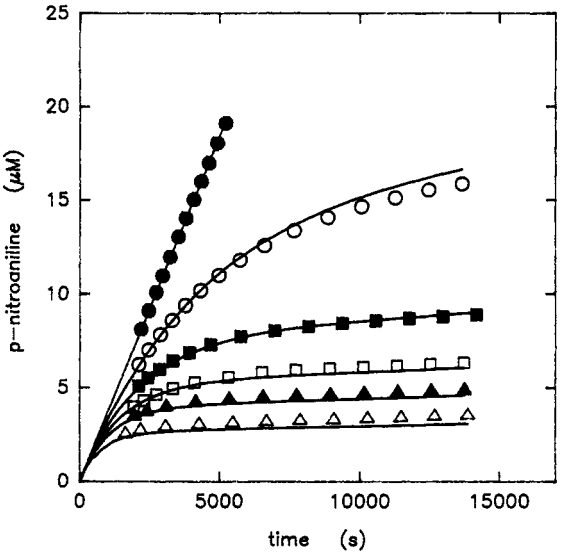


FIGURE 2: Inhibition of acrosin by PCI. Assays were performed as described in Materials and Methods with 1.1 nM acrosin, 200 μM D-Ile-Pro-Arg-pNA, and the following concentrations of PCI (nM): 0 (●), 43.5 (○), 87 (■), 130.5 (□), 174 (▲), and 261 (△). The data were fitted to the equation describing the mechanism presented in Scheme 1, and the lines drawn show the fit of the data to this equation. Data points at time less than about 1800 s are not plotted, and only each second point thereafter is shown.

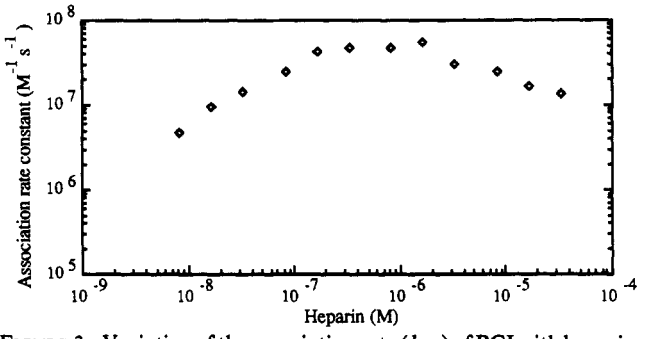


FIGURE 3: Variation of the association rate (k_{ass}) of PCI with heparin concentration. Assays were performed with 1.1 nM acrosin, 200 μM D-Ile-Pro-Arg-pNA, and 6 nM PCI in the presence of the indicated concentrations of unfractionated heparin (average M_r = 18 000), and data were analyzed as described in Materials and Methods.

Table 1: Kinetic Parameters for the Inhibition of Acrosin by PCI[a]

	k_{ass} (M^{-1} s^{-1})	K_i (pM)	increase in k_{ass} (fold)
PCI	(2.41 ± 0.03) × 10^5	46 ± 5	
PCI + heparin	(5.6 ± 0.1) × 10^7	0.67 ± 0.07	230

[a] Assays were performed and data were analyzed as described in Materials and Methods and the legends to Figures 2 and 3. The heparin concentration was 2 μM (an average molecular mass of 18 kDa was used to calculate the molarity of the unfractionated heparin).

DISCUSSION

The fast rate of inhibition of acrosin by PCI suggests that PCI may control acrosin activity *in vivo*. PCI is present at high concentrations (3–4 μM) in seminal fluid, while acrosin is found in the acrosome of the sperm. In the absence of heparin, the association rate constant for PCI with acrosin was 2.4 × 10^5 M^{-1} s^{-1} (Table 1). Thus, in seminal fluid, it can be calculated that the half-life for the formation of the acrosin–PCI complex would be about 1 s. Heparin-like molecules are present at high concentrations in the female reproductive tract (Bushmeyer *et al.*, 1985; Lee & Ax, 1984), and if these glycosaminoglycans are able to stimulate the acrosin–PCI reaction to an extent similar to that observed with heparin, the half-life for the formation of the acrosin–PCI complex would be greatly reduced and may be as low as 10 ms. The role of PCI in seminal fluid may be to control the activity of acrosin released from damaged sperm such that unwanted degradation of proteins in the female reproductive tract does not occur. Alternatively, PCI may function during fertilization to regulate dispersal of the acrosomal matrix and activation of proacrosin by acrosin. Moore *et al.* (1993) have reported that PCI is localized in the acrosomal region of human and hamster sperm and that synthetic peptides corresponding to the reactive-site region of PCI are able to block the binding of sperm to the zona pellucida *in vitro*. This suggests that the association of PCI with acrosin may represent a regulatory step in fertilization.

PCI was originally isolated as an inhibitor of activated protein C in blood. However, the k_{ass} value for PCI with activated protein C is an order of magnitude lower than that with acrosin. In addition, the degree of heparin stimulation of the reaction with activated protein C is much smaller than that obseved with acrosin (6- versus 230-fold; Hermans & Stone, 1993). Moreover, the concentration of PCI in blood is only about 100 nM (Laurell *et al.*, 1992), and it can be calculated that the half-life for the inhibition of activated protein C by PCI in blood will be about 3 h in the absence of heparin and 10 min in its presence. These calculations suggest that the role of PCI in regulating activated protein C may be secondary to its role in controlling the activity of acrosin in semen. Thus, PCI appears to have been misnamed, and just as α_1-antitrypsin would have been more appropriately named antielastase (Travis & Salvesen, 1983), acrosin inhibitor would be a more suitable designation for protein C inhibitor.

Heparin was found to affect both the activity of acrosin and the association rate constant of PCI with the enzyme. The heparin effects on the kinetics of the acrosin–PCI interaction are probably due to the binding of heparin to both acrosin and the serpin. The association rate constant for PCI displayed a bell-shaped dependence on the concentration of heparin (Figure 3), which suggests that heparin accelerated the inhibition of acrosin by a ternary complex mechanism (Olson & Björk, 1992). In this mechanism, heparin binds to both acrosin and the serpin and thereby increases the effective concentration of both molecules with a resultant increase in the inhibition rate constant. As the concentration of heparin increases, however, acrosin and the serpin are more likely to be bound to different heparin molecules and the observed inhibition rate constant decreases. The fact that PCI (Pratt & Church, 1992; Kuhn *et al.*, 1990) and acrosin (Williams & Jones, 1990) bind to heparin is consistent with the proposed mechanism. Heparin also appears to affect the rate at which antithrombin III inhibits proteases by causing a conformational change in this serpin (Olson & Björk, 1992). Given the fact that heparin is able to affect the proteolytic activity of acrosin, it seems possible that in addition to acting through a ternary complex mechanism, heparin also influences the rate of inhibition of acrosin by affecting its conformation.

In the ternary complex mechanism, the rate of inhibition of the protease by the serpin will depend not only on the binding of the two molecules to heparin but also on the orientation of the molecules when they are bound. A larger increase in k_{ass} will be observed if the two molecules are bound in such a way as to promote the formation of the inhibited complex. Heparin was able to increase the reaction rate of PCI with acrosin by

230-fold (Table 1), which is a larger increase than that which occurs for the reactions of PCI with thrombin and activated protein C (20- and 6-fold, respectively; Hermans & Stone, 1993). This comparison suggests that the orientations of PCI and acrosin when they are bound to heparin are more favorable for the formation of an inhibited complex than is the case for the participants in the PCI–thrombin and PCI–activated protein C reactions. The heparin-binding site of PCI is located in the H-helix (Pratt & Church, 1992) and probably the N-terminal region (Kuhn *et al.*, 1990), and it has been suggested that the orientation of PCI when bound to heparin would not be optimal for productive complex formation with thrombin when the protease is bound at its heparin-binding site (Hermans & Stone, 1993). The results obtained in the present study suggest that the heparin-binding site of acrosin is located in a different region of the molecule compared to the binding site in thrombin; the heparin-bound orientation of acrosin with respect to PCI is more favorable than that of thrombin. Although the tertiary structure of acrosin has not yet been determined, some information regarding the possible location of its heparin-binding site can be gained by aligning its sequence with those of other proteases.[2] In comparison to chymotrypsin, acrosin has two insertions in positions that would correspond to surface loops: one at residue 36 and the other at residue 60. Other proteases also have insertions at these positions. Thrombin has an insertion loop at position 60, and both tissue-type plasminogen activator and urokinase-type plasminogen have insertion loops at residue 36. Both of these acrosin loops, particularly the 60-loop, contain positively charged residues and, thus, constitute potential binding sites for the negatively charged heparin. There is evidence that the heparin-binding site of acrosin is found on the first 15 kDa of the heavy chain (Töpfer-Petersen *et al.*, 1990), which would be consistent with the involvement of one or both of the insertion loops at residues 36 and 60.

The proposal that the insertion after residue 60 is involved in binding heparin to acrosin would be consistent with the effect of heparin on the kinetics of cleavage of D-Ile-Pro-Arg-pNA (Figure 1). Heparin acted as an allosteric modulator of the cleavage reaction. In the presence of saturating heparin concentrations, both k_{cat} and K_m were decreased; K_m was reduced to a greater extent than k_{cat} (4.9- versus 2.9-fold) with the result that the specificity constant (k_{cat}/K_m) for D-Ile-Pro-Arg-pNA was 2-fold higher. In thrombin, the insertion after residue 60 forms part of the binding site for the P_2 residue of substrates (Bode *et al.*, 1989). Since the 60-loop should also form part of the S_2 site in acrosin, it is conceivable that the binding of heparin to the insertion in the 60-loop of acrosin could lead to the alteration in kinetic parameters observed in the presence of heparin. The effects observed, however, are more likely to be due to a conformational change affecting the active site rather than competition for binding, since the decrease in K_m would not be expected if heparin were merely competing for the binding to the S_2 site in acrosin. In any case, heparin appears to be an important modulator of the activity of acrosin, affecting its proteolytic activity and its rate of inactivation by PCI as well as its binding to the zona pellucida (Jones, 1990; Töpfer-Petersen *et al.*, 1990).

ACKNOWLEDGMENT

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[2] Sequences were obtained from the SWISSPROT databank for this alignment.

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