Inhibition of Acrosin by Serpins. A Suicide Substrate Mechanism†

Josephine M. Hermans,‡,§ Denis Monard,|| Roy Jones,⊥ and Stuart R. Stone*,‡

Department of Haematology, University of Cambridge, MRC Centre, Hills Road, Cambridge CB2 2QH, U.K., Friedrich Miescher-Institut, P.O. Box 2543, CH-4002 Basel, Switzerland, and Department of Biochemistry, Institute of Animal Physiology and Genetics, AFRC, Babraham CB2 4AT, U.K.

Received July 29, 1994; Revised Manuscript Received December 6, 1994⊗

ABSTRACT: The serpins antithrombin, protease nexin 1, and α_1-antitrypsin with a reactive-center arginine (Arg-α_1-antitrypsin) were found to inhibit the sperm protease acrosin with varying efficiency. The serpins were titrated against acrosin to determine their specific activity with respect to this enzyme. While antithrombin was fully active against acrosin, more than one molecule of Arg-α_1-antitrypsin and protease nexin 1 was required to inhibit one molecule of acrosin. In particular, only 2.7% of protease nexin 1 molecules interacting with acrosin formed stable complexes with the enzyme at 37 °C and this value decreased to 0.03% at 12 °C. N-terminal sequence analysis indicated that acrosin had cleaved protease nexin 1 at its reactive-center Arg−Ser bond. The results could be interpreted in terms of protease nexin 1 acting as a suicide substrate for acrosin; after the formation of an initial complex, the serpin partitioned between pathways yielding either inactivated (cleaved) serpin or a stable serpin−enzyme complex. The association rate constant (k_{ass}) and inhibition constant (K_i) for the stable complexes were determined for each of the serpins by using slow-binding kinetics. The values of k_{ass} were 2×10^5, 4×10^4, and 5×10^3 M^{-1} s^{-1} for Arg-α_1-antitrypsin, antithrombin, and protease nexin 1, respectively. The K_i values for the serpins were 1 nM or less. Heparin markedly accelerated the inhibition of acrosin by antithrombin and protease nexin 1; at the optimal concentration, the degree of heparin acceleration of the inhibition rate was 250- and 500-fold for antithrombin and protease nexin 1, respectively. In the presence of heparin, a two-step mechanism could be demonstrated for the acrosin−antithrombin interaction, and the data indicated that the heparin-induced increase in the rate of the acrosin−antithrombin reaction was predominantly due to an increase in the tightness of the initial complex.

The acrosomal vesicle of mammalian spermatozoa contains large quantities of proacrosin, the zymogen form of a trypsin-like serine protease; it has been calculated that the concentration of proacrosin within the acrosome of boar spermatozoa is 1.4 mM (Müller-Esterl & Fritz, 1981). Acrosin is formed from its zymogen during the acrosome reaction in which the outer acrosomal membrane fuses with the overlying plasma membrane to release the contents of the acrosome (Rubinstein & Brietbart, 1991). The activity of the acrosin generated during the acrosome reaction is thought to be essential for penetration by the spermatozoa of the extracellular matrix (zona pellucida) surrounding the egg; acrosin is able to cleave a number of the components of this matrix (Nakano et al., 1989; Planchenault et al., 1991). Although acrosin's activity appears to be required for the penetration of zona pellucida, this activity also needs to be controlled so that other components of the male and female genital tract are not degraded by acrosin released from damaged or dead spermatozoa (Nicholson et al., 1983). Acrosin inhibitors from the Kazal family have been found in the seminal plasma of a wide range of mammalian species (Fink et al., 1990; Falase et al., 1991; Jonáková et al., 1992). In addition, we have recently shown that protein C inhibitor (PCI[1]), a member of the serpin family, is a rapid inhibitor of acrosin (Hermans et al., 1994). PCI is found in high concentrations (3−4 μM) in human seminal fluid (España et al., 1991; Laurell et al., 1992), and its inhibition of acrosin is markedly stimulated by the sulfated glycosaminoglycan heparin (Hermans et al., 1994). PCI is synthesized in the seminal vesicles and secreted into the lumen of the gland (Laurell et al., 1992).

Recent studies have shown that high levels of another serpin, protease nexin 1 (PN1), are also synthesized in the seminal vesicles and secreted into the seminal fluid (Vassalli et al., 1993). The synthesis of PN1 by the seminal vesicle was shown to be androgen dependent. PN1 levels were much lower in immature and castrated mice, and the level of PN1 mRNA in castrated mice was greatly increased by treatment of the animals with testosterone (Vassalli et al., 1993). The expression of PN1 is also regulated during development in other cell types (Mansuy et al., 1993; Reinhard et el., 1994). PN1 (also known as glia-derived nexin) was originally isolated from fibroblasts and glia cells (Baker et al., 1980; Guenther et al., 1985). It was isolated from glioma cell conditioned medium as a factor that promoted neurite elongation, and thus, it is thought to play

† This work was supported by the MRC.

* Address correspondence to this author at Department of Haematology, University of Cambridge, MRC Centre, Hills Road, Cambridge CB2 2QH, U. K.

‡ University of Cambridge.

§ Current address: Department of Molecular Sciences, James Cook University, Townsville, Qld 4811, Australia.

|| Friedrich Miescher-Institut.

⊥ Institute of Animal Physiology and Genetics.

⊗ Abstract published in *Advance ACS Abstracts,* March 1, 1995.

[1] Abbreviations: PN1, recombinant rat protease nexin 1; R-AT, recombinant human α_1-antitrypsin with a P_1 arginine residue [nomenclature of Schechter and Berger (1967)]; PCI, protein C inhibitor; pNA, *p*-nitroanilide.

0006-2960/95/0434-3678$09.00/0 © 1995 American Chemical Society

a role in neuronal development (Monard, 1988). PN1 has been shown to inhibit trypsin-like serine proteases such as thrombin, urokinase, trypsin, and plasmin (Scott et al., 1985; Stone et al., 1987). Thus, it seems possible that PN1 is involved in the control of acrosin activity *in vivo*.

In the present study, we have examined the interaction of acrosin with PN1 and two related serpins [antithrombin and α_1-antitrypsin with a P_1 arginine[2] (R-AT)]. While antithrombin and R-AT were found to be efficient inhibitors of acrosin, PN1 acted predominantly as substrate for acrosin. In titration experiments, less than 3% of the PN1 molecules formed a stable complex with acrosin at 37 °C, and only about 0.03% of the molecules inhibited acrosin at 12 °C. N-terminal sequence analysis showed that the low specific activity of PN1 with acrosin was due to cleavage of the PN1 at its P_1−P'_1 bond. The results could be interpreted in terms of a bifurcating mechanism for the interaction of PN1 with acrosin; after the formation of an initial complex, either a stable complex formed or PN1 was cleaved. Heparin was found to stimulate markedly the rate of inhibition of acrosin by both PN1 and antithrombin as well as to affect the partitioning of the serpins between the cleavage and inhibition pathways.

MATERIALS AND METHODS

Materials. D-Ile-Pro-Arg-pNA was purchased from Chromogenix (Mölndal, Sweden), and *p*-nitrophenyl *p'*-guanidinobenzoate was from Sigma (Poole, U.K). Porcine mucosal heparin was from Grampian Enzymes (Aberdeen, U.K.). Other chemicals were of the highest grade available commercially. Proacrosin was purified from ejaculated boar spermatozoa and activated to acrosin at pH 8.0 as described (Polakoski & Parrish, 1977; Lo Leggio et al., 1994). The reaction resulted in the formation of β-acrosin, which appeared as a single band of 37 kDa on an SDS−PAGE gel (Laemmli, 1970). Human α-thrombin was purified as described by Stone and Hofsteenge (1986). Recombinant rat PN1 was produced in yeast and purified as described by Sommer et al. (1989). Antithrombin was purified from human plasma as described by MacKay (1981) and was a gift of Dr. David Bruce (Department of Haematology, University of Cambridge). Recombinant R-AT from yeast was a gift from Delta Biotechnology (Nottingham, U.K.).

Determination of the Active Concentrations of Proteins. The concentrations of active acrosin and thrombin were determined by titration with *p*-nitrophenyl *p'*-guanidinobenzoate as described by Chase and Shaw (1970). The serpins antithrombin, PN1, and R-AT were titrated against thrombin and acrosin in 0.03 M sodium phosphate buffer, pH 7.4, containing 0.16 M NaCl, 0.1% (w/v) poly(ethylene glycol) 4000, and 0.2 mg/mL bovine serum albumin as described by Hermans et al. (1994). The total concentration of the serpin (active plus inactive molecules) was determined from its extinction coefficient for antithrombin (Nordenman et al., 1977) or by amino acid analysis for PN1 and R-AT. The preparations of antithrombin and PN1 were found to be, within experimental error, fully active against thrombin, while R-AT was about 70% active. For the purpose of determining their specific activity against acrosin, the specific activity of the serpins against thrombin was assumed to be 100%.

Slow-Binding Kinetics. Progress-curve kinetics were used to determine the kinetic constants for the inhibition of acrosin by the serpins. 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) poly(ethylene glycol) 4000, 0.2 mg/mL bovine serum albumin, and 200−750 μM D-Ile-Pro-Arg-pNA as previously described (Hermans et al., 1994). The polystyrene cuvettes were coated before use with a 0.5% (w/v) poly(ethylene glycol) 6000, 2% (w/v) bovine serum albumin, and 0.01% (v/v) Triton X-100 solution to prevent adsorption of proteins to the cuvettes. The release of *p*-nitroaniline from the substrate was followed by measuring the absorbance at 400−410 nm after the addition of acrosin (1−4 nM) to cuvettes containing the substrate and serpin. The activity of acrosin was shown to be stable over the time period used (Hermans et al., 1994). Only data points obtained at levels of substrate utilization less than 10% were used in the analyses.

The serpins antithrombin, PN1, and R-AT were found to be slow-binding inhibitors of acrosin. There is kinetic evidence that the formation of the stable protease−serpin complex involves two steps (Olson & Shore, 1982; Stone et al., 1987; Bruch & Bieth, 1989; Longstaff & Gaffney, 1991; Faller et al., 1993; Morgenstern et al., 1994), and thus, the mechanism presented in Scheme 1 is the most general inhibitory mechanism for serpins. In Scheme 1, E, S, P, and I represent acrosin, substrate (D-Ile-Pro-Arg-pNA), product (*p*-nitroaniline), and serpin, respectively. For this mechanism, the progress curve of formation of product (P) will be described by the following equation (Morrison, 1982):

$$P = v_s t + \frac{v_i - v_s}{k'}(1 - \exp(-k't)) \quad (1)$$

where P is the concentration of product at time t, k' is an apparent first-order rate constant, and v_i and v_s are the initial and steady-state velocities, respectively. The values of v_i, v_s, and k' will vary with the concentration of serpin according to the following equations (Cha, 1975; Morrison, 1982; Morrison & Walsh, 1988).

$$k' = \frac{k_2}{1 + K_{init}(1 + [S]/K_m)/[I]} + k_{-2} \quad (2)$$

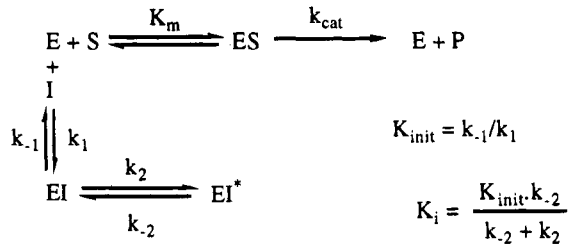
$$v_i = \frac{v_o}{1 + [I]/(K_{init}(1 + [S]/K_m))} \quad (3)$$

$$v_s = \frac{v_o}{1 + [I]/(K_i(1 + [S]/K_m))} \quad (4)$$

The parameter v_o is the enzyme's velocity in the absence of inhibitor. K_i is the overall inhibition constant derived from the amount of inhibited enzyme at infinite time, and K_{init} is the inhibition constant for the initial complex (EI). For all serpins in the absence of heparin and for PN1 and R-AT in the presence of heparin, v_i did not vary with the concentration of serpin, and k' displayed a linear dependence on serpin concentration. This will occur when significant concentra-

[2] The nomenclature for the numbering of the reactive-site loop of serpins is that of Schechter and Berger (1967). The numbering of residues is according to the following scheme: P_n−...−P_3−P_2−P_1−P'_1−P'_2−P'_3−...−P'_n where cleavage would occur at the P_1−P'_1 bond in substrates.

Scheme 1



tions of the initial (EI) complex are not formed at the concentrations of serpin used (Morrison & Walsh, 1988), and in this case, v_i equals v_o, and the dependence of k' on the concentration of serpin will be given by the following equation:

$$k' = \frac{k_2[I]}{K_{init}(1 + [S]/K_m)} + k_{-2} \quad (5a)$$

$$= k'_{ass}([I] + K'_i) \quad (5b)$$

where the effective association rate constant k_{ass} is defined as k_2/K_{init}; k'_{ass} and K'_i are apparent constants and equal to $k_{ass}/(1 + [S]/K_m)$ and $K_i(1 + [S]/K_m)$, respectively. For these interactions, eqs 4 and 5b were substituted into eq 1, and the data from a slow-binding inhibition experiment consisting of five or six progress curves with different concentrations of serpin were fitted to this overall equation by nonlinear regression to yield values of k_{ass} and K_i (Stone & Hofsteenge, 1986). In the presence of heparin, a significant concentration of initial complex (EI) formed with antithrombin; v_i decreased with the concentration of antithrombin. For analysis of these data, eqs 2−4 were substituted into eq 1 and the progress curve data obtained at five different concentrations of serpin were fitted to this overall equation to yield estimates for K_{init}, K_i, and the rate constants for the isomerization step (k_2 and k_{-2}). Previously determined K_m values for D-Ile-Pro-Arg-pNA were used in the calculation of the kinetic parameters (Hermans et al., 1994). Data from slow-binding inhibition experiments were also fitted to an equation that assumed that the inhibition was irreversible (v_s in eq 1 was set to zero); in all cases, a better fit was obtained when reversible inhibition was assumed (see Figure 1).

For cases where v_i did not vary with the inhibitor concentration, it was possible to calculate a lower limit for K_{init}. Assuming that a 20% decrease in velocity would have been detected, the following relationship for the lower limit for K_{init} can be derived:

$$K_{init} > 4I_{max}/(1 + S/K_m)$$

where I_{max} is the highest concentration of inhibitor used.

Determination of the Site of Cleavage in PN1 by Acrosin. PN1 (23 μM) was incubated with acrosin (2.5 μM) in volume of 0.1 mL for 1 min at 37 °C, after which the mixture was injected onto a 4.6 × 250 mm PLRP-S reverse-phase HLPC column (Polymer Laboratories, Church Stretton, U.K.). The flow rate for the HPLC separation of the cleavage products was 1.0 mL/min. After the column was washed for 10 min with 5% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid, the bound peptides were eluted with a linear gradient of 5−80% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid over 40 min. The absorbance of the effluent was monitored at 214 nm. Two absorbance peaks were observed. The initial peak eluted about 25 min after the start of the gradient, and this position corresponded to that previously observed for the C-terminal fragment of reactive-site cleaved serpins (Aulak et al., 1988). This peak was collected and its N-terminal sequence determined by Dr. L. Packman (Protein Sequence Facility, Department of Biochemistry, University of Cambridge).

Table 1: Specific Activities of Serpins with Acrosin[a]

serpin	temp (°C)	heparin	sp act.
antithrombin	37	−	1.0
	37	+	0.67
R-AT	37	−	0.37
	37	+	0.56
PN1	37	−	0.027
	37	+	0.059
	20	−	0.004
	12	−	0.0003

[a] Titrations of the serpins against acrosin were performed as described in Materials and Methods. Titrations against thrombin were performed at the same time, and the serpins were assumed to be fully active against thrombin. The serpins were also titrated against acrosin in the presence of a range of heparin concentrations. The titers did not vary significantly over the heparin concentration range tested (0.1−10 μM for R-AT, 7 nM−2 μM for PN1, and 2 nM−2 μM for antithrombin), and values given represent the averages of the titers determined at different heparin concentrations.

RESULTS

Specific Activity of the Serpins Antithrombin, PN1, and R-AT with Acrosin. The specific activities of the serpins against acrosin have been defined relative to their activity with thrombin. Antithrombin was equally active against thrombin and acrosin. In contrast, the titers of R-AT and PN1 were lower with acrosin (Table 1). Only 37% of the R-AT molecules capable of inhibiting thrombin formed stable complexes with acrosin. The specific activity of PN1 was surprisingly low; only 2.7% of PN1 molecules inhibited acrosin. Heparin affected the titers of the serpins against acrosin (Table 1). The titers did not vary significantly over the heparin concentration range tested (0.1−10 μM for R-AT, 7 nM−2 μM for PN1, and 2 nM−2 μM for antithrombin). Heparin increased the titer of PN1 and R-AT by 2.2- and 1.5-fold, respectively. In contrast, the specific activity of antithrombin was one-third lower in the presence of heparin (Table 1).

The above titrations were performed at 37 °C. As it has been reported that the ability of C1-inhibitor to form stable complexes with proteases varies with temperature (Patston et al., 1991), the titration of PN1 against acrosin was also performed at 20 and 12 °C. The specific activity of PN1 decreased markedly at lower temperatures; 0.4% of the PN1 inhibited acrosin at 20 °C, and only about 0.03% of the PN1 formed a stable complex with this protease at 12 °C. At least two time points were taken for the titrations at the lower temperatures (170 and 340 min at 20 °C; 280 and 460 min at 12 °C), and the degrees of inhibition at both time points were identical, indicating that the reaction was complete.

Acrosin Cleaves PN1 at the Reactive Center. The low specific activity of PN1 with acrosin suggested that most of the serpin may be acting as a substrate for this enzyme.

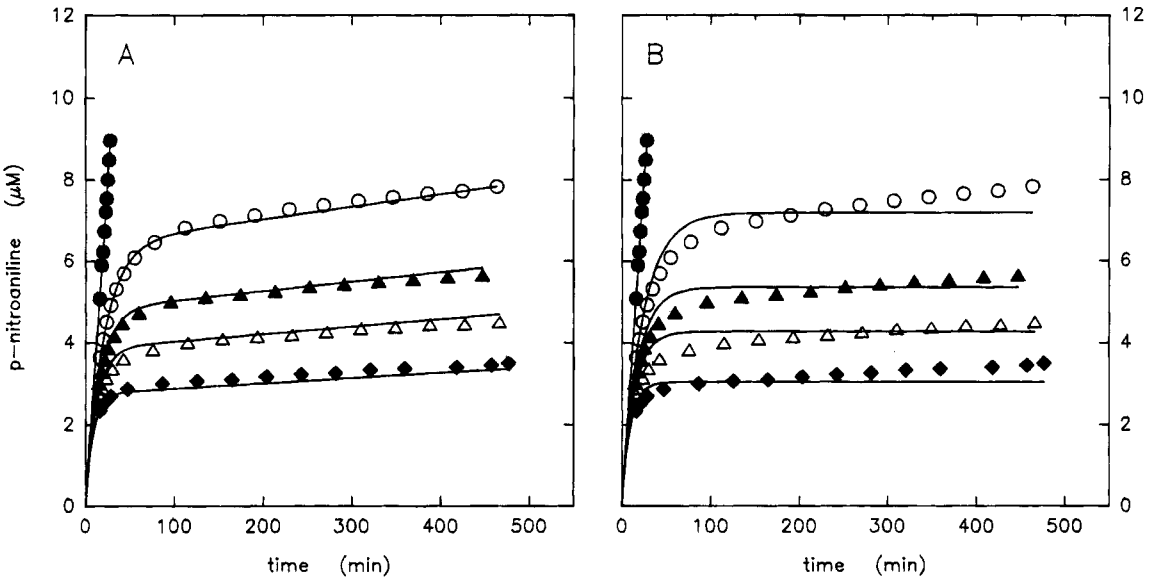


FIGURE 1: Inhibition of acrosin by R-AT. Assays were performed as described in Materials and Methods with 90 μM D-Ile-Pro-Arg-pNA, 1.1 nM acrosin, and the following concentrations of R-AT: 0 (●), 76 (○), 101 (▲), 128 (△), and 177 nM (◆). Data points at times less than 15 min are not plotted, and only each second data point thereafter is shown. The data of panel A were analyzed according to the equation for slow-binding inhibition to yield values for k_{ass} and K_i; the lines shown are the fits of the data to this equation. The data of panel B were analyzed according to the equation for irreversible inhibition (v_s in eq 1 was set to zero), and the lines show the significantly worse fit to this equation.

Analysis of the reaction by SDS−PAGE was consistent with this interpretation. The mobility of acrosin-treated PN1 was the same as that observed for PN1 that had been cleaved in the reactive-site loop by pancreatic elastase (Nick et al., 1990). Reverse-phase HPLC analysis of the reaction products confirmed that acrosin had cleaved PN1 at its reactive center, i.e., between the P_1 and P'_1 residues. Before treatment with acrosin, reverse-phase HPLC analysis of PN1 yielded a single absorbance peak. After incubation with acrosin, two peaks were observed, and the elution position of the first peak corresponded to that of the C-terminal peptide from reactive-site cleaved C1-inhibitor (Aulak et al., 1988). This peak was collected and subjected to N-terminal sequence analysis. The sequence obtained (Ser-Ser-Pro-Pro-Trp) was that of residues P'_1 to P'_5 of PN1 (Sommer et al., 1987). Thus, the results demonstrated that acrosin had cleaved PN1 at the P_1−P'_1 reactive-center bond.

Inhibition of Acrosin by Serpins. The serpins PN1, antithrombin, and R-AT were slow-binding inhibitors of acrosin. Analysis of progress-curve data similar to those shown in Figure 1 for R-AT yielded values of the association rate constants ($k_{ass} = k_2/K_{init}$) together with inhibition constants (K_i) for the stable (EI*) complexes (Scheme 1). R-AT yielded the highest k_{ass} value (2×10^5 M^{-1} s^{-1}) and the value obtained was equivalent to that previously observed with PCI (Table 2). R-AT has a P_2 proline which is the preferred P_2 residue for chromogenic substrates of acrosin (Skoog et al., 1989). The k_{ass} value for antithrombin with acrosin was 4-fold lower (4×10^4 M^{-1} s^{-1}), while PN1 exhibited the lowest association rate constant (5×10^3 M^{-1} s^{-1}; Table 2). The inhibition constants of the stable complexes (K_i) were 1 nM or less (Table 2), indicating extremely stable EI* complexes. The inhibition did, however, appear reversible. Analysis of the data according to the equation that assumed irreversible inhibition yielded a worse fit as shown in Figure 1 for R-AT.

There is kinetic evidence that the formation of the stable protease−serpin complex involves at least two steps (Olson & Shore, 1982; Stone et al., 1987; Bruch & Bieth, 1989; Longstaff & Gaffney, 1991; Faller et al., 1993; Morgenstern et al., 1994), and thus, the mechanism of Scheme 1 is the most general one for serpins. However, with the concentrations of serpins used, the initial EI complex between the serpins and acrosin did not form to a significant extent. Kinetic evidence for the initial complex will only be obtained when the serpin concentrations are in the range of the apparent dissociation constant for the initial complex (K'_{init}; Morrison & Walsh, 1988). With such concentrations, the initial velocity (v_i) will decrease with the inhibitor concentration (Morrison, 1982). At the concentrations of serpins used, no variation in v_i was observed. A minimum value for K_{init} can be calculated by assuming that a 20% decrease in v_i would have been discernible (see Materials and Methods), and this value is given for each serpin in Table 1.

Table 2: Kinetic Parameters of Serpins with Acrosin[a]

	K_{init} (nM)	k_{ass} (M^{-1} s^{-1})	K_i (nM)	partition ratio
R-AT	>30	$(1.82 \pm 0.03) \times 10^5$	0.038 ± 0.003	1.7
PN1	>220	$(4.81 \pm 0.05) \times 10^3$	1.44 ± 0.11	37.3
antithrombin	>50	$(4.35 \pm 0.06) \times 10^4$	0.27 ± 0.04	<0.1
PCI	>20	$(2.41 \pm 0.03) \times 10^5$	0.046 ± 0.005	<0.1

[a] Assays were performed and data were analyzed as described in Materials and Methods to yield estimates of k_{ass} ($=k_2/K_{init}$) and K_i. The value given for PCI is taken from Hermans et al. (1994). The maximum serpin concentrations used were 320 nM R-AT, 2.0 μM PN1, and 600 nM antithrombin. These values were used to calculate minimum values for K_{init} as described in Materials and Methods. The partition ratio was calculated as described in the text from the specific activity determined in titration experiments (Table 1).

Effects of Heparin on Acrosin−Serpin Interactions. Heparin altered the kinetic constants for the serpins with acrosin; the values observed in the presence of optimal concentrations of heparin are given in Table 3. The values of k_{ass} for the heparin-binding serpins PN1 and antithrombin increased markedly; the values were highest in the presence of 10^{-7} M heparin for both serpins (Figure 2). At this concentration,

Table 3: Kinetic Parameters of Acrosin and Serpins at Optimal Heparin Concentrations[a]

	K_{init} (nM)	k_2 (s^{-1})	k_{ass} (M^{-1} s^{-1})	x-fold increase in k_{ass}	K_i (pM)	partition ratio
R-AT	>12		$(4.4 \pm 0.1) \times 10^4$	0.41	227 ± 20	0.8
PN1	>3		$(2.4 \pm 0.2) \times 10^6$	500	1.5 ± 0.2	16
antithrombin	0.24 ± 0.03	$(2.6 \pm 0.4) \times 10^{-3}$	$(1.1 \pm 0.2) \times 10^7$	250	9.1 ± 0.2	0.5
PCI	>0.2		$(2.2 \pm 0.1) \times 10^7$	90	1.7 ± 0.2	1.5

[a] Assays were performed and data were analyzed as described in Materials and Methods. The values of the kinetic parameters were calculated from data obtained at the following concentrations of heparin: R-AT, 0.11 μM; PN1, 0.07 μM; antithrombin, 0.2 μM; and PCI, 2.0 μM. The concentrations of serpins in the assays were 320−630 nM R-AT, 50−100 nM PN1, 29−59 nM antithrombin, and 15 nM PCI. The values for PCI were determined previously (Hermans et al., 1994). An average molecular mass of 18 kDa was used to calculate the molarity of the heparin. The partition ratio was calculated as described in the text from the specific activity determined in titration experiments (Table 1).

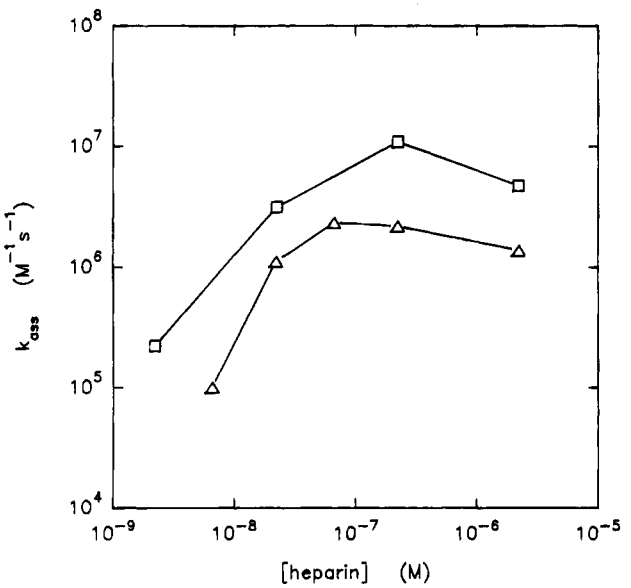


FIGURE 2: Variation with heparin concentration of the association rate of serpins with acrosin. Assays were performed and data were analyzed as described in Materials and Methods with 29−59 nM antithrombin (△) and 50−100 nM PN1 (□) in the presence of the indicated concentrations of unfractionated heparin (average M_r = 18 000).

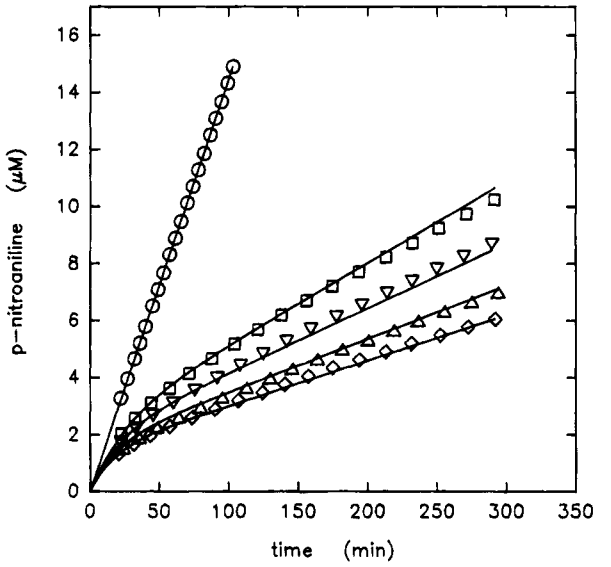


FIGURE 3: Inhibition of acrosin by antithrombin in the presence of heparin. Assays were performed as described in Materials and Methods with 752 μM D-Ile-Pro-Arg-pNA, 0.2 μM heparin and the following concentrations of antithrombin: 0 (○), 29 (□), 39 (▽), 49 (△), and 59 nM (◇). The concentration of acrosin was 1.6 nM. The data were analyzed according to the equation that describes the two-step mechanism of Scheme 1, and the lines show the fit of the data to this equation. Data points at times less than 20 min are not plotted, and only each second data point thereafter is shown.

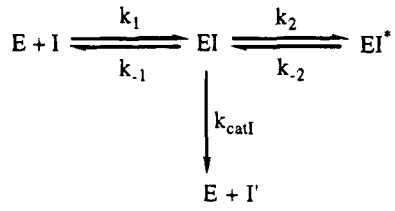
k_{ass} for antithrombin was 250-fold higher than that observed in the absence of heparin (Table 3). The degree to which heparin accelerated the PN1−acrosin interaction was even larger; a 500-fold increase in k_{ass} was observed for PN1. In contrast to PN1 and antithrombin, the k_{ass} value for R-AT was lower in the presence of heparin; the value did not change significantly over the heparin concentration range tested (0.1−11 μM) and was 2.5-fold lower than that observed in the absence of heparin (Table 3).

Although a significant concentration of the initial (EI) complex did not form with the concentrations of PN1 and R-AT used, kinetic evidence for the formation of this complex was obtained for the inhibition by antithrombin in the presence of heparin (Figure 3); v_i decreased with the concentration of antithrombin. Analysis of the antithrombin data yielded an estimate for the dissociation constant for the initial acrosin−antithrombin complex (K_{init}) and the rate constant for the conversion of this complex into the tighter complex (k_2); the estimates for K_{init} and k_2 were 0.24 nM and 2.6×10^{-3} s^{-1} (Table 3). Minimum values for K_{init} were calculated for the other serpins and are given in Table 3. In the presence of heparin, the inhibition of acrosin also appeared reversible (note the finite steady-state velocities in Figure 3) and K_i values for the stable complexes could be calculated. Heparin decreased the K_i values for the heparin-binding serpins with acrosin (Table 3). The decrease in the K_i value for PN1 was more marked (1000-fold) than that observed for antithrombin (20-fold).

DISCUSSION

Serpins Act as Suicide Substrates. The results of the present study demonstrate that whether a serpin acts as an inhibitor or a substrate is dependent on the conditions of the assay (temperature and the presence of heparin) and the target protease. The most dramatic illustration of this was PN1; only 2.7% of PN1 molecules inhibited acrosin at 37 °C, and the fraction of PN1 that formed a stable complex with acrosin decreased markedly at lower temperatures. HPLC analysis and N-terminal sequencing indicated that acrosin cleaved PN1 at the $P_1-P'_1$ bond. These results are consistent with a mechanism in which, after the formation of an initial complex, two alternative pathways exist for the reaction of PN1 as shown in Scheme 2, where I′ is the degraded inhibitor. In this bifurcating reaction pathway, the initial enzyme−inhibitor complex forms a stable complex with a rate constant k_2 or a cleavage reaction occurs with a rate constant k_{catI}. Such an inhibitor is termed a "suicide substrate". Enzyme inhibition and serpin cleavage are competing reactions, and the stable EI* complex does not lie *en route* to the production of degraded inhibitor. For

Scheme 2



this mechanism, Waley (1985) has defined k_{catI}/k_2 as the partition ratio (r). This partition ratio represents the number of catalytic turnovers per inactivation event. The relative amounts of cleaved serpin and inhibited complex are governed by the partition ratio (r) and the relative concentrations of enzyme and serpin. In the titration experiments to determine the specific activity of the serpins, the concentrations of serpin used in the analysis were less than that of acrosin. Under these conditions, a plot of the activity of acrosin against serpin concentration will yield a straight line with the intersection on the abscissa being $(1 + r)$ (Waley, 1985); i.e., the observed stoichiometry is $(1 + r)$. Apparent stoichiometries greater than 1.0 occur because inhibition does not take place each time the initial EI complex is formed. Values of r for each serpin in the absence and presence of heparin are given in Tables 2 and 3. For the slow-binding inhibition experiments, the initial concentrations of serpin ($[I]_0$) were always much greater than that of acrosin ($[E]_0$), and Waley (1985) has shown that, provided the relationship $(1 + r)[E]_0/[I]_0 << 1$ applies, the formation of the stable complex is the major pathway; i.e., cleavage of the serpin can be ignored if $[E]_0 << [I]_0/(1 + r)$. For the concentrations of serpins in the slow-binding inhibition experiments, the relationship $[E]_0 < 0.05[I]_0/(1 + r)$ always applied. Under these conditions, the data could be analyzed according to the equations for slow-binding inhibition (eqs 1−5). The mechanism presented in Scheme 2 differs for those previously proposed for suicide substrates in that the stable complex is reversible. In all cases, the equation assuming reversible inhibition yielded a better fit to the progress-curve data (see Figure 1). Previous studies have indicated that the stable protease−serpin complex is reversible (Jesty, 1979; Griffith & Lundblad, 1981; Stone et al., 1987; Shieh et al., 1989; Longstaff & Gaffney, 1991; Morgenstern et al., 1994). The observed reversibility of the complex could be due either to reversal of the association steps as shown in Scheme 1 or to the release of cleaved inhibitor; evidence for both pathways exists (Beatty et al., 1982; Danielsson & Björk, 1980, 1983; Griffith & Lundblad, 1981; Shieh et al., 1989). In any case, the complexes are extremely stable with K_i values of 1 nM or less. The dissociation rate constant for the release of free enzyme (k_{diss}) can be calculated using the formula $k_{diss} = k_{ass}K_i$. In the absence of heparin, the k_{diss} value was less than 10^{-5} s^{-1} for all the serpins, which corresponds to a half-life for dissociation of the complex of greater than 16 h.

The mechanism of suicide inhibition was first proposed for serpins in 1979 because a small amount of cleaved antithrombin was always produced when it was incubated with thrombin (Fish & Björk, 1979). It was later observed that antithrombin had a slightly lower titer against thrombin when heparin was present (Björk & Fish, 1982) as was also noted for antithrombin with acrosin (Table 1). Low ionic strength buffers together with heparin resulted in a partition ratio of 9 for antithrombin with thrombin (Olson, 1985). Different partition ratios (0.05−3.6) have also been observed for C1-inhibitor against kallikrein, C1s, and plasmin (Patston et al., 1991) and for recombinant α_1-antichymotrypsin with an arginine P_1 residue against thrombin and trypsin (Rubin et al., 1990).

Although the structure of the EI* complex is not known, there is evidence from studies utilizing antibodies that the serpin has a different conformation in the stable EI* complex than when free in solution and that its structure in the stable complex resembles that of the cleaved inhibitor (Björk et al., 1993; Zhu & Chan, 1987). In the cleaved serpin, the P_2−P_{14} residues are incorporated into the A β-sheet as the fourth strand (Loebermann et al., 1984). It is thought that the structural transition observed for the serpins in the stable complex is due to the insertion of several residues at the N-terminal end of the reactive-site loop into the A β-sheet (Engh et al., 1990; Carrell & Evans, 1992). When this partial insertion of the reactive-site loop is prevented due to prior insertion into the A β-sheet of a synthetic peptide with the sequence of the reactive-site loop, the serpin acts as a substrate, not as an inhibitor (Schulze et al., 1990; Björk et al., 1992). The P_{10} and P_{12} residues seem to be involved in this insertion. The variant C1-inhibitor Ma, which has the P_{12} residue mutated from an alanine to a glutamate, behaves as a substrate (Skriver et al., 1991). For antithrombin, replacement of the P_{12} alanine by a threonine or the P_{10} alanine by a proline converts the serpin into a substrate for thrombin (Ireland et al., 1991; Caso et al., 1991), and the P_{10} proline substitution in α_1-antitrypsin also leads to substrate-like behavior (Hopkins et al., 1993).

The above observations have led to the development of the induced conformational state model for serpin−protease interactions (Skriver et al., 1991; Potempa et al., 1994). In this model, the binding of the protease to the reactive-site loop of the serpin induces partial insertion of the region from P_{10} to P_{14} into the A β-sheet. This insertion arrests cleavage of the loop by the protease. In the absence of partial insertion, the reactive center of the serpin is cleaved. This model proposes that inhibition will only occur if the rate of insertion (k_2) is faster than the rate of cleavage (k_{catI}). The partition ratio (r) of the PN1−acrosin interaction increased at lower temperatures (70-fold between 37 and 12 °C); smaller increases in this ratio with decreasing temperature have been reported previously for C1-inhibitor with both kallikrein and plasmin (Patston et al., 1991). This increase in the partition ratio ($=k_{catI}/k_2$) could be due to an increase in k_{catI} or a decrease in k_2. An increase in k_{catI} at lower temperatures is unlikely. The rate of catalysis is expected to decrease with lower temperatures, and indeed the k_{cat} for acrosin with D-Ile-Pro-Arg-pNA was found to decrease 17-fold between 37 and 17 °C (data not shown). Thus, it seems probable that the increase in the partition ratio is due to a large decrease in k_2, and in this regard, it is interesting to note that the rate of insertion of synthetic reactive-site loop peptides into the A β-sheet decreases dramatically with lower temperatures (Schulze et al., 1990). The induced conformational state model proposes that interactions with the protease induce the partial insertion of the reactive-site loop into the A β-sheet; thus, the rate of insertion will not be an intrinsic property of the serpin, but it will be influenced by the protease as well. Data obtained with antithrombin support this proposal providing the magnitude k_2 is actually

determined by the rate of partial insertion. In the presence of heparin, the value of k_2 for antithrombin with thrombin was about 5 s^{-1} (Olson & Shore, 1982), while under similar conditions the value of k_2 for this serpin with acrosin was only 3×10^{-3} s^{-1} (Table 3). These results suggest that interactions between thrombin and the reactive-site loop of antithrombin induce a conformation that is more favorable for rapid insertion of residues into the A β-sheet. However, further experiments are required to determine whether partial insertion of the reactive-site loop into the A β-sheet is the rate-limiting step in the formation of the stable complex from the initial one.

Effect of Heparin on the Acrosin−Serpin Kinetics. Heparin-like molecules are present at high concentrations in the female reproductive tract (Bushmeyer et al., 1985), and previous studies have suggested that they may be involved in regulating the activity of acrosin during the fertilization process. Heparin modulates the catalytic activity of acrosin (Hermans et al., 1994) and its binding to the zona pellucida (Jones, 1990; Töpfer-Petersen, 1990). Heparin also accelerates the inactivation of acrosin by PCI (Hermans et al., 1994). The data of Figure 2 indicate that heparin markedly increased the k_{ass} values of two other serpins (antithrombin and PN1) with acrosin. At optimal heparin concentrations, the k_{ass} values for antithrombin and PN1 were respectively 250 and 500 times higher than in the absence of heparin (Table 3). The heparin effects on the kinetics of PN1 and antithrombin are probably due to the binding of heparin to both acrosin and the serpin. Such a mechanism has been shown to account for the heparin acceleration of the inhibition of thrombin by antithrombin and PN1 (Olson & Björk, 1992; Wallace et al., 1989). For the thrombin−antithrombin reaction, it has been shown that rate enhancement is due entirely to a decrease in the dissociation constant of the initial complex (K_{init}); i.e., the affinity of thrombin for the binary heparin−antithrombin complex is much greater than its affinity for antithrombin alone (Olson & Shore, 1982). Thrombin's increased affinity for the heparin-antithrombin binary complex is presumably due to additional binding sites for thrombin being created in this complex (Olson & Björk, 1992). Interestingly, the same mechanism is responsible for the heparin enhancement of the reaction of antithrombin with acrosin. In the absence of heparin, only a lower limit (50 nM) for the value of K_{init} could be obtained (Table 2). At the optimal heparin concentration, the value of K_{init} (0.24 nM) was at least 200-fold lower than that observed in the absence of heparin. This increase in affinity would entirely account for the 250-fold increase in the association rate constant (k_2/K_{init}) observed in the presence of heparin (Table 3).

Physiological Role of PN1 in the Reproductive Tract. PN1 was originally isolated from the culture supernatant of human foreskin fibroblasts (Baker et al., 1980). At about the same time, it was shown that a protein produced by glioma cells was able to stimulate neurite outgrowth from neuroblastoma cells (Schürch-Rathgeb & Monard, 1978), and this protein was later demonstrated to be PN1 (Gloor et al., 1986; Sommer et al., 1987). The neurite-promoting activity of PN1 has stimulated research into its role in the nervous system where its level of expression is regulated during development and increases following lesions (Reinhard et al., 1988, 1994; Meier et al., 1989; Hoffmann et al., 1992; Scotti et al., 1994). PN1 also shows a restricted distribution in other embryonic and adult tissues, suggesting that it has a role in organogenesis and tissue homeostasis (Mansuy et al., 1993). The highest level of expression of PN1 that has been observed is in the adult seminal vesicle where PN1 mRNA represents 0.2−0.4% of the total mRNA (Vassalli et al., 1993). Moreover, its expression in seminal vesicles is regulated by androgens, and it has been suggested that PN1 plays a role in the control of protease activity in the male and/or female reproductive tracts (Vassalli et al., 1993). The results obtained in the present study indicate, however, that the target enzyme of PN1 is unlikely to be acrosin, since it was degraded by this enzyme. Although the target enzyme for PN1 in the male and/or female reproductive tract remains to be definitively identified, urokinase-type plasminogen activator represents a possible target for PN1. This enzyme is synthesized in relatively high levels in the male genital tract of rodents (Larsson et al., 1984; Huarte et al., 1987) and is inhibited rapidly by PN1 (Scott et al., 1985; Stone et al., 1987). An interesting possibility is that PN1 controls the activity of urokinase, which is bound to the head of the spermatozoon (Huarte et al., 1987), until the acrosome reaction takes place. This step releases active acrosin which can then degrade PN1 with the consequence that urokinase activity is no longer restricted, and the urokinase can assist in the degradation of the zona pellucida that is necessary for fertilization to occur.

ACKNOWLEDGMENT

We thank Dr P. C. R. Hopkins for his helpful comments on the manuscript.

REFERENCES

Aulak, K. S., Pemberton, P. A., Rosen, F. S., Carrell, R. W., Lachmann, P. J., & Harrison, R. A. (1988) *Biochem. J. 253*, 615−618.

Baker, J. B., Low, D. A., Simmer, R. L., & Cunningham, D. D. (1980) *Cell 21*, 37−45.

Beatty, K., Travis, J., & Bieth, J. (1982) *Biochim. Biophys. Acta 704*, 221−226.

Björk, I., & Fish, W. W. (1982) *J. Biol. Chem. 257*, 9487−9493.

Björk, I., Nordling, K., Larsson, I., & Olson, S. T. (1992) *J. Biol. Chem. 267*, 19047−19050.

Björk, I., Nordling, K., & Olson, S. T. (1993) *Biochemistry 32*, 6501−6505.

Bruch, M., & Bieth, J. G. (1989) *Biochem. J. 259*, 929−930.

Bushmeyer, S. M., Bellin, M. E., Brantmeier, S. A., Boehm, S. K., Kubajak, C. L., & Ax, R. L. (1985) *Endocrinology 117*, 879−883.

Carrell, R. W., & Evans, D. Ll. (1992) *Curr. Opin. Struct. Biol. 2*, 438−446.

Caso, R., Lane, D. A., Thompson, E. A., Olds, R. J., Thein, S. L., Panico, M., Blench, I., Morris, H. R., Freysinnet, J. M., Aiach, M., Rodeghiero, F., & Finazzi, G. (1991) *Br. J. Haematol. 77*, 87−92.

Cha, S. (1975) *Biochem. Pharmacol. 24*, 2177−2185.

Chase, T., Jr., & Shaw, E. (1970) *Methods Enzymol. 19*, 20−27.

Danielsson, A., & Björk, I. (1980) *FEBS Lett. 119*, 241−244.

Danielsson, A., & Björk, I. (1983) *Biochem. J. 213*, 345−353.

Engh, R. A., Wright, H. T., & Huber, R. (1990) *Protein Eng. 3*, 469−477.

España, F., Gilabert, J., Estellés, A., Romeu, A., Aznar, J., & Cabo, A. (1991) *Thromb. Res. 64*, 309−320.

Falase, E. A. O., Storey, B. T., & Teuscher, C. (1991) *Mol. Reprod. Dev. 29*, 29−39.

Faller, B., Cadéne, M., & Bieth, J. G. (1993) *Biochemistry 32*, 9230−9235.

Fink, E., Hehlein-Fink, C., & Eulitz, M. (1990) *FEBS Lett. 270*, 222−224.

Fish, W. W., & Björk, I. (1979) *Eur. J. Biochem. 101*, 31−38.
Gloor, S., Odink, K., Guenther, J., Nick, H., & Monard, D. (1986) *Cell 47*, 687−693.
Griffith, M. J., & Lundblad, R. L. (1981) *Biochemistry 20*, 105−110.
Guenther, J., Nick, H., & Monard, D. (1985) *EMBO J. 4*, 1963−1966.
Hermans, J. M., Jones, R., & Stone, S. R. (1994) *Biochemistry 33*, 5440−5444.
Hoffmann, M. C., Nitsch, C., Scotti, A. L., Reinhard, E., & Monard, D. (1992) *Neuroscience 49*, 397−408.
Hopkins, P. C. R., Carrell, R. W., & Stone, S. R. (1993) *Biochemistry 32*, 7650−7657.
Huarte, J., Belin, D., Bosco, D., Sappino, A.-P., & Vassalli, J.-D. (1987) *J. Cell Biol. 104*, 1281−1289.
Ireland, H., Lane, D. A., Thompson, E. A., Walker, I. D., Blench, I., Morris, H. R., & Freyssinet, J. M. (1991) *Br. J. Haematol. 79*, 70−74.
Jesty, J. (1979) *J. Biol. Chem. 254*, 1044−1049.
Jonáková, V., Calvete, J. J., Mann, K., Schäfer, W., Schmid, E. R., & Töpfer-Petersen, E. (1992) *FEBS Lett. 297*, 147−150.
Jones, R. (1990) *J. Reprod. Fertil., Suppl. 42*, 89−105.
Laemmli, U. K. (1970) *Nature 227*, 680−685.
Larsson, L. I., Skriver, L., Nielsen, L. S., Grøndahl, J., Kristensen, P., & Danø, K. (1984) *J. Cell Biol. 98*, 894−903.
Laurell, M., Christensson, A., Abrahamson, P., Stenflo, J., & Lilja, H. (1992) *J. Clin. Invest. 89*, 1094−1101.
Loebermann, H., Tokuoka, R., Deisenhofer, J., & Huber, R. (1984) *J. Mol. Biol. 177*, 531−556.
Lo Leggio, L., Williamson, R. M., & Jones, R. (1994) *J. Reprod. Fertil. 100*, 177−185.
Longstaff, C., & Gaffney, P. J. (1991) *Biochemistry 30*, 979−986.
MacKay, E. J. (1981) *Thromb. Res. 21*, 375−382.
Mansuy, I. M., van der Putten, H., Schmid, P., Meins, M., Botteri, F. M., & Monard, D. (1993) *Development 119*, 1119−1134.
Meier, R., Spreyer, P., Ortmann, R., Harel, A., & Monard, D. (1989) *Nature 342*, 548−550.
Monard, D. (1988) *Trends Neurosci. 11*, 541−544.
Monard, D., Niday, E., Limat, A., & Solomon, F. (1983) *Prog. Brain Res. 58*, 359−364.
Morgenstern, K. A., Sprecher, C., Holth, L., Foster, D., Grant, F. J., Ching, A., & Kisiel, W. (1994) *Biochemistry 33*, 3432−3441.
Morrison, J. F. (1982) *Trends Biochem. Sci. 7*, 102−105.
Morrison, J. F., & Walsh, C. T. (1988) *Adv. Enzymol. Relat. Areas Mol. Biol. 61*, 201−301.
Müller-Esterl, W., & Fritz, H. (1981) *Methods Enzymol. 80*, 621−632.
Nakano, M., Tanaka, Y., Kimura, T., Hatanaka, Y., & Tobita, T. (1989) *J. Biochem. (Tokyo) 105*, 138−142.
Nicholson, N., Irwin, M., & Poirier, G. R. (1983) *J. Exp. Zool. 225*, 481−487.
Nick, H., Hofsteenge, J., Shaw, E., Rovelli, G., & Monard, D. (1990) *Biochemistry 29*, 2417−2421.
Nordenman, B., Nyström, C., & Björk, I. (1977) *Eur. J. Biochem. 78*, 195−203.
Olson, S. T. (1985) *J. Biol. Chem. 260*, 10153−10160.
Olson, S. T., & Shore, J. D. (1982) *J. Biol. Chem. 257*, 14891−14895.
Olson, S. T., & Björk, I. (1992) in *Thrombin Structure and Function* (Berliner, L. J., Ed.) pp 159−218, Plenum Press, New York.
Patston, P. A., Gettins, P., Beechem, J., & Schapira, M. (1991) *Biochemistry 30*, 8876−8882.
Planchenault, T., Cechová, D., & Keil-Dlouha, V. (1991) *FEBS Lett. 294*, 279−281.
Polakoski, K. L., & Parrish, R. F. (1977) *J. Biol. Chem. 252*, 1888−1894.
Potempa, J., Korzus, E., & Travis, J. (1994) *J. Biol. Chem. 269*, 15957−15960.
Reinhard, E., Meier, R., Halfter, W., Rovelli, G. F., & Monard, D. (1988) *Neuron 1*, 387−394.
Reinhard, E., Suidan, H. S., Pavlik, A., & Monard, D. (1994) *J. Neurosci. Res. 37*, 256−270.
Rubin, H., Wang, Z. M., Nickbarg, E. B., McLarney, S., Naidoo, N., Schoenberg, O. L., Johnson, J. L., & Cooperman, B. S. (1990) *J. Biol. Chem. 265*, 1199−1207.
Rubinstein, S., & Breitbart, H. (1991) *Biochem. J. 278*, 25−28.
Schechter, I., & Berger, A. (1967) *Biochem. Biophys. Res. Commun. 27*, 157−162.
Schulze, A. J., Baumann, U., Knof, S., Jaeger, E., Huber, R., & Laurell, C. B. (1990) *Eur. J. Biochem. 194*, 51−56.
Schürch-Rathgeb, Y., & Monard, D. (1978) *Nature 273*, 308−309.
Scott, R. W., Bergaman, B. L., Bajpai, A., Hersh, R. T., Rodriguez, H., Jones, B. N., Barreda, C., Watts, S., & Barker, J. B. (1985) *J. Biol. Chem. 260*, 7029−7034.
Scotti, A. L., Monard, D., & Nitsch, C. (1994) *J. Neurosci. Res. 37*, 155−168.
Shieh, B., Potempa, J., & Travis, J. (1989) *J. Biol. Chem. 264*, 13420−13423.
Skoog, M. T., Medhi, S., Wiseman, J. S., & Bey, P. (1989) *Biochim. Biophys. Acta 996*, 89−94.
Skriver, K., Wikoff, W. R., Patston, P. A., Tausk, F., Schapira, M., Kaplan, A. P., & Bock, S. C. (1991) *J. Biol. Chem. 266*, 9216−9221.
Sommer, J., Gloor, S. M., Rovelli, G., Hofsteenge, J., Nick, H., Meier, R., & Monard, D. (1987) *Biochemistry 26*, 6407−6410.
Sommer, J., Meyhack, B., Rovelli, G., Buergi, R., & Monard, D. (1989) *Gene 85*, 453−459.
Stone, S. R., & Hofsteenge, J. (1986) *Biochemistry 25*, 4622−4628.
Stone, S. R., Nick, H., Hofsteenge, J., & Monard, D. (1987) *Arch. Biochem. Biophys. 252*, 237−244.
Töpfer-Petersen, E., Steinberger, M., Ebner von Eschenbach, C., & Zucker, A. (1990) *FEBS Lett. 265*, 51−54.
Vassalli, J.-D., Huarte, J., Bosco, D., Sappino, A.-P., Sappino, N., Velardi, A., Wohlwend, A., Ernø, H., Monard, D., & Belin, D. (1993) *EMBO J. 12*, 1871−1878.
Waley, S. G. (1985) *Biochem. J. 227*, 843−849.
Wallace, A., Rovelli, G., Hofsteenge, J., & Stone, S. R. (1989) *Biochem. J. 257*, 191−196.
Zhu, X.-J., & Chan, S. K. (1987) *Biochem. J. 246*, 19−23.

BI9417426