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Characterization of the Heparin-Binding Site of Glia-Derived Nexin/Protease Nexin-1

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ABSTRACT: The interaction of heparin with glia-derived nexin (GDN) has been characterized and compared to that observed between heparin and antithrombin III (ATIII). Heparin was fractionated according to its affinity for immobilized GDN, and the ability of various fractions to accelerate the inhibition rate of thrombin by either GDN or ATIII was examined. Fractions with different affinities for GDN accelerated the thrombin–GDN reaction to a similar extent; heparin with a high affinity for immobilized GDN stimulated the reaction only about 30% more than the fraction that did not bind to immobilized GDN. Slightly greater differences were observed for the effect of these fractions on the thrombin–ATIII reaction; heparin that did not bind to the GDN affinity column was about 60% more effective than heparin with a high affinity for GDN in accelerating the inhibition of thrombin by ATIII. The CNBr fragment of GDN between residues 63 and 144 was able to reduce the heparin-accelerated rate of inhibition of thrombin by GDN indicating that this region of GDN was able to bind the heparin molecules responsible for the acceleration. Shorter synthetic peptides within this sequence did not significantly reduce the rate, suggesting that the heparin-binding activity of fragment 63–144 depends on a specific conformation of the polypeptide chain. Fragment 63–144 was less effective in decreasing the heparin-accelerated rate of inhibition of thrombin by ATIII. The results are discussed in terms of the heparin species that are responsible for the acceleration of the GDN– and ATIII–thrombin reactions and the heparin-binding sites of GDN and ATIII.

Glia-derived nexin (GDN)[1] is a 43-kDa secreted glycoprotein that can promote neurite elongation in neuroblastoma cells (Guenther et al., 1985) and in neuronal primary cultures (Zurn et al., 1988; Farmer et al., 1989). In vitro, GDN is secreted by primary cultures of rat brain origin (Schürch-Rathgeb & Monard, 1978; Rosenblat et al., 1987). In vivo, it has been shown to be expressed at high levels in the rat olfactory system (Reinhard et al., 1988) and lesions of the sciatic nerve were also found to induce a transient overexpression of GDN (Meier et al., 1989). GDN was first isolated from the conditioned medium of rat C6 glioma cells (Guenther et al., 1985). cDNA cloning and sequencing have shown that GDN and protease nexin-1[2] are identical proteins (Gloor et al., 1986; McGrogan et al., 1988). GDN is a member of the serpin (serine protease inhibitor) superfamily. It is a fast-acting inhibitor of thrombin and it also efficiently inhibits plasminogen activators and other trypsin-like proteases at a slower rate (Scott et al., 1985; Stone et al., 1987). Since

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[1] Abbreviations: GDN, glia-derived nexin; ATIII, antithrombin III; pNA, *p*-nitroaniline; Pip, pipecolyl; TFA, trifluoroacetic acid.
[2] Since glia-derived nexin and protease nexin-1 are identical proteins, the abbreviation GDN is used to indicate both molecules.

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Table I: Amino Acid Compositions of the Purified CNBr Fragments 63–144, 146–199, and 298–379 and of the Two Synthetic Peptides 70–87 and 98–112[a]

	63–144		146–199		298–379		70–87		98–112	
Ala	8	8.3	3	3.2	8	7.6	1	0.9	2	1.9
Arg	4	3.8	2	2.2	4	4.1			2	2.0
Asx	13	11.3	7	6.9	5	4.6	3	3.1	2	2.0
Cys	2	ND[b]			1	ND				
Glx	7	6.6	3	3.0	6	5.9			1	1.0
Gly	4	4.1	3	3.4	3	3.1	1	1.2	1	1.0
His					3	2.7				
Ile	4	3.4	2	1.9	8	7.8	2	1.9		
Leu	1	1.3	7	6.5	6	6.4	1	1.1		
Lys	9	9.3	6	5.1	6	5.8	7	7.2	2	1.9
Phe	6	6.4	3	2.9	6	6.0			2	2.0
Pro	2	2.3	3	3.4	6	6.3			1	1.1
Ser	3	2.4	4	4.5	7	7.1	1	1.1		
Thr	2	2.1	3	2.8	5	5.5				
Trp	1	ND	1	ND						
Tyr	1	0.9	2	2.1						
Val	14	12.9	5	4.0	6	5.6	2	1.8	2	2.0

[a] Amino acid compositions of peptides expected from the rat cDNA sequence (Sommer et al., 1987) (left columns) are compared with those determined by the amino acid analysis (right columns). [b] ND = not determined.

thrombin causes neurite retraction in neuroblastoma cells (Monard et al., 1983; Gurwitz & Cunningham, 1988) and neuronal primary cultures (Hawkins & Seeds, 1989; Grand et al., 1989), it seems probable that the neurite-promoting activity of GDN is a result of its inhibition of this protease (Monard, 1988).

Heparin stimulates markedly the rate of inhibition of thrombin by GDN (Scott et al., 1985; Stone et al., 1987; Wallace et al., 1989), and heparin-like molecules could also be responsible for GDN binding to the outer cell surface or to the extracellular matrix (Farrell & Cunningham, 1986; Halfter et al., 1989). It has previously been demonstrated that heparin accelerates the interaction of GDN with thrombin by a mechanism similar to that observed for its catalysis of the ATIII–thrombin interaction (Wallace et al., 1989). In this mechanism, heparin appears to act as a template to which both the protease and ATIII bind (Pomerantz & Owen, 1978; Griffith, 1982; Nesheim, 1983). Concurrently, heparin induces a conformational change in ATIII which might also contribute to the acceleration of the inhibition (Einarsson & Andersson, 1977; Peterson & Blackburn, 1987).

The present paper examines the nature of the interaction of heparin with GDN. Heparin was fractionated on the basis of its affinity for GDN, and a wide range of heparin fractions were shown to accelerate the thrombin–GDN reaction to a similar extent. In addition, peptide fragments of GDN prepared by either synthesis or chemical cleavage were used to localize and characterize the heparin-binding site on the GDN.

Experimental Procedures

Materials. Yeast recombinant rat GDN was purified to homogeneity as previously described (Sommer et al., 1989). ATIII was isolated according to the protocol of Miller-Andersson et al. (1974). Prothrombin was purified from human plasma and activated to thrombin as previously described (Stone & Hofsteenge, 1986). The concentration of thrombin was determined by active site titration (Jameson et al., 1973). The concentrations of active GDN and ATIII were measured by titration against thrombin as previously described (Stone et al., 1987). Poly-L-lysine (approximately 4000 M_r) and heparin (No. H-3125) were obtained from Sigma (St. Louis, MO). The thrombin synthetic substrates D-Phe-Pip-Arg-pNA (S-2238) and D-Ile-Pro-Arg-pNA (S-2288) were from Kabi AB (Molndal, Sweden). All other chemicals were of the highest purity available commercially.

Heparin Fractionation.[3] Portions of 50 mg of heparin were fractionated on a Sephadex G-100 column (1.6 × 100 cm) which was equilibrated with 50 mM Tris-HCl, pH 8.0, containing 50 mM NaCl at a flow rate of 0.15 mL/min. The void volume and total volume of the column were determined by using Blue Dextran (Pharmacia) and acetone, respectively. Heparin fractions with molecular masses of 4–6 kDa (Sigma) and 15 kDa [isolated as described by Nordenmann and Björk (1978)] were also used to calibrate the column. Fractions corresponding to M_r = 15 000 were pooled. Heparin of M_r 15 000 was selected because with this size the average number of inhibitor- and thrombin-binding sites per molecule is about 1 (Nesheim et al., 1986). GDN was coupled to activated CH-Sepharose 4B (30 mg/4 mL of Sepharose) according to the specifications of the supplier (Pharmacia). The GDN–Sepharose column (1 × 5 cm) was equilibrated with 50 mM Tris-HCl, pH 8.0, containing 50 mM NaCl at a flow rate of 0.5 mL/min. About 12 mg of the 15-kDa heparin was applied to the column. After the nonbound material was washed out with equilibration buffer, the bound heparin was eluted stepwise with 0.2, 0.35, and 1 M NaCl in Tris-HCl buffer, and the material obtained was termed low-, medium-, and high-GDN-affinity heparin, respectively. Heparin concentrations were determined using the azure A reaction with unfractionated heparin as a standard (Jacques et al., 1949; Nesheim, 1983).

Isolation of the Cyanogen Bromide Fragments of GDN. GDN (10 mg) was reduced and carboxylmethylated (Rüegg & Rudinger, 1977) and cleaved with CNBr (Gross, 1967). The digested GDN was chromatographed on a Bio-Gel P-10 column (1.5 × 95 cm) equilibrated with 0.1% TFA at a flow rate of 0.10 mL/min. The five resulting peaks (A–E) were collected and lyophilized. Pool A consisted of partially digested GDN (M_r higher than 20 000), whereas pool B contained the GDN fragments 63–144 and 298–379, and pool C mainly contained the GDN fragment 146–199. Calibration of the column using known M_r standards showed that pool D contained fragments of about 4000 and pool E consisted of peptides having a M_r less than 2000. Fragments 63–144, 146–199, and 298–379 were isolated by reverse-phase HPLC. The identity and purity of each fragment was determined by N-

[3] All chromatography steps were performed at room temperature if not otherwise stated.

terminal sequence and amino acid analysis (Figure 3A; Table I).

GDN Synthetic Peptides 70–87 and 98–112. The peptides corresponding to the GDN sequences from Gly^{70} to Asp^{87} (^{NH_2}G-K-V-L-K-K-I-N-K-A-I-V-S-K-K-N-K-D^{COOH}) and from Arg^{98} to Lys^{112} (^{NH_2}R-N-G-F-K-V-E-V-P-F-A-A-R-N-K^{COOH}) were made using a MilliGen 9050 peptide synthesizer. The peptides were purified using a reverse-phase HPLC column, and the identity of the peptides was verified by N-terminal sequence and amino acid analysis (Table I). Peptide concentrations were determined by amino acid analysis (Knecht & Chang, 1986).

Enzyme Assays. Amidolytic assays were performed at 37 °C as described previously (Wallace et al., 1989) in 50 mM Tris-HCl, pH 7.8/0.1 M NaCl/0.1% poly(ethylene glycol) (M_r 6000). Either GDN (2.0 nM) or ATIII (26.7 nM) was incubated for 15 min at 37 °C with a known amount of heparin (0.02–8.6 μg/mL) and chromogenic substrate (50–450 μM). Thrombin (0.2 nM) was then added, and substrate hydrolysis was immediately recorded by measuring the absorbance increase at 400–410 nm due to *p*-nitroaniline release with a Hewlett-Packard 8452A diode array spectrophotometer.

Cyanogen Bromide Fragments of GDN as Competitors of the Heparin-Accelerated Inhibition of Thrombin. The enzyme assay described above was used with the following modifications: before the addition of thrombin (0.2 or 0.05 nM), GDN (2.0 or 20 nM) was incubated for 15 min at 37 °C with a fixed amount of high-GDN-affinity heparin (1.6 nM) and increasing amounts (0–10 nM) of different GDN fragments or synthetic peptides of GDN. Alternatively, ATIII (2 or 26.7 nM) was incubated under the same conditions with a fixed amount (2.0 nM) of high-GDN-affinity heparin and increasing amounts of GDN fragments (0–10 nM).

Theory and Data Analysis. In the presence of heparin, low concentrations (nanomolar) of either GDN or ATIII are required to efficiently inhibit subnanomolar concentrations of thrombin. Under such conditions, the inhibition by GDN and ATIII appears to be reversible (Jesty, 1979; Stone et al., 1987) and follows the slow binding inhibition mechanism A (Morrison, 1982). Thus, if the inhibition reaction is followed by monitoring the hydrolysis of the chromogenic substrate, the amount of product P at time t is given by

$$P = v_s t + \frac{(v_0 - v_s)}{k'}[1 - \exp(-k't)] \quad (1)$$

where k' is an apparent first-order rate constant and v_0 and v_s are the initial and steady-state velocities, respectively. Pseudo-first-order conditions were maintained by using a concentration of inhibitor, I, at least 1 order of magnitude greater than the concentration of enzyme. Estimates of k', v_s, and v_0 were obtained by fitting the data to eq 1 using nonlinear regression. The value of the second-order rate constant, k_1, can be related to I, k', v_0, v_s, the Michaelis constants (K_m), and the substrate concentration (S) by eq 2

$$k_1 = k'(1 - v_s/v_0)\frac{1 + S/K_m}{I} \quad (2)$$

which is derived from equations given by Morrison & Stone (1985). Equation 2 was used to calculate estimates for k_1. The K_m values for the chromogenic substrates S-2238 and S-2288 under the assay conditions are 3.6 and 5.1 μM, respectively (Stone & Hofsteenge, 1986).

Plotting of the observed second-order rate constant k_1 against heparin concentration results in bell-shaped curves (Wallace et al., 1989). At low levels of heparin, the value of k_1 is proportional to the concentration, whereas high concentrations of heparin are inhibitory. In order to obtain an estimate of the active concentration of heparin, it was assumed that GDN only reacts rapidly with thrombin when it is bound to an active molecule of heparin. By using this assumption, the following equation can be derived for the dependence of k_1 on noninhibitory concentrations of heparin:

$$k_1 = \frac{k_{1(opt.)}}{2}(I + K_d + \alpha H' - [(I + K_d + \alpha H')^2 - 4I\alpha H']^{1/2} \quad (3)$$

where I is the concentration of GDN, H' is the concentration of heparin calculated on the basis of the colorimetric assay, $k_{1(opt.)}$ is the theoretical maximum value of k_1, K_d is the dissociation constant for the GDN–heparin complex, and α is the fraction of active heparin molecules in the preparation. The concentration of active heparin molecules (H) is equal to $\alpha H'$. The concentrations of active heparin molecules in the high- and low-GDN-affinity preparations were determined by fitting the observed dependence of k_1 on H' to eq 3 by nonlinear regression.

The bell-shaped curves for the dependence of k_1 on the heparin concentration (H) are empirically described by the following equation, which can be derived from the template model for the action of heparin (Nesheim, 1983):

$$k_1 = \frac{k_{1(opt.)}}{1 + \frac{H}{K_1} + \frac{K_2}{H}} \quad (4)$$

where K_1 and K_2 are empirical constants that represent the concentrations of heparin at which the half-maximal value of k_1 is observed. Values of k_1 obtained at different heparin concentrations were weighted according to the inverse of their variance and fitted to eq 4 using nonlinear regression.

Results

Effect of the Affinity-Fractionated Heparin on the Inhibition Rate of Thrombin. Heparin of M_r = 15 000 was fractionated according to its affinity for immobilized GDN. When heparin was applied to a column of immobilized GDN, the majority of the material passed through the column. Elution of the bound heparin with a gradient of 0–1.0 M NaCl yielded a broad peak of heparin that eluted between 0.1 and 0.6 M NaCl (data not shown). These results contrast with those obtained for the elution of heparin from immobilized ATIII where discrete peaks are obtained (Nordenman & Björk, 1978). Pools of heparin, eluted stepwise from immobilized GDN at 0.2, 0.35, and 1.0 M NaCl, were defined as low-, medium-, and high-GDN-affinity heparin, respectively. These heparin fractions together with the nonbound fraction were tested for their ability to accelerate the inhibition of thrombin by either GDN or ATIII (Table II). A fixed concentration of 0.5 μg/mL heparin was chosen, since preliminary assays showed that this concentration was within the range that induced an optimal acceleration of the inhibition rate. All forms of heparin produced about the same stimulation of the rate of association of GDN with thrombin; the second-order rate constant (k_1) was slightly (25–36%) higher with high-GDN-affinity heparin (Table II). In contrast, the fraction that did not bind to the GDN affinity column caused the greatest stimulation of the rate of inhibition of thrombin by ATIII. For the ATIII–thrombin reaction, the rate observed with nonbound heparin was 1.6-fold higher than that observed with high-GDN-affinity heparin (Table II). The ability of heparin fractionated according to its affinity for ATIII has been previously tested for its ability to stimulate the inhibition of

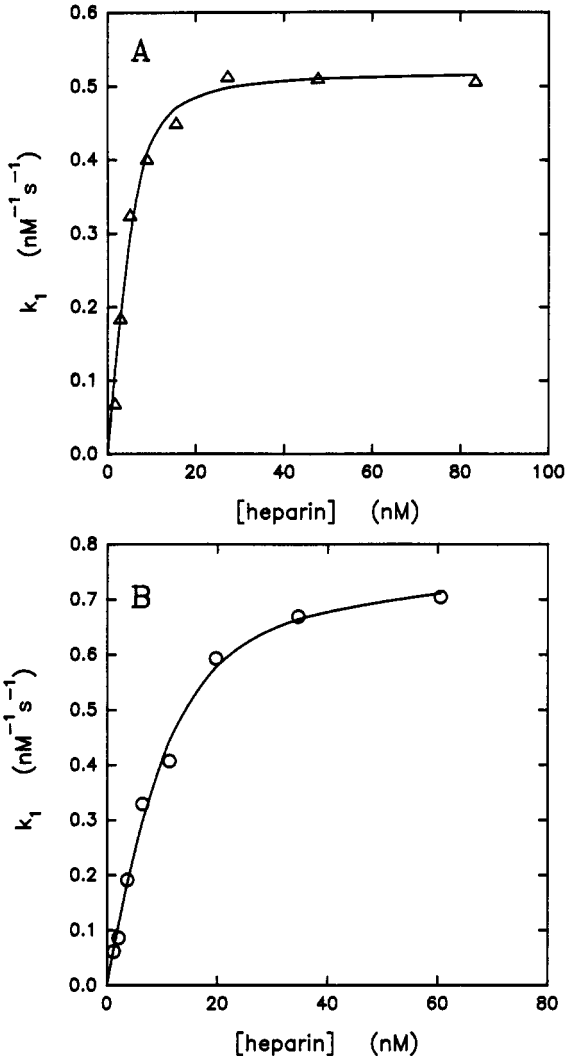

FIGURE 1: Titration of low- and high-GDN-affinity heparin. The dependence of the k_1 on the concentration of low- (A) and high-GDN-affinity heparin (B) is shown. Assays were performed as described under Experimental Procedures with a concentration of 2.0 nM GDN. The concentrations of heparin given were determined using the colorimetric assay, and a molecular mass of 15 kDa was used in calculating the molar concentration. The data were analyzed according to eq 3, and the lines drawn in the figures represent the fit of the data to this equation. The estimates of the parameters obtained with low-GDN-affinity heparin were 0.522 ± 0.014 $nM^{-1}s^{-1}$, 0.32 ± 0.22 nM, and 0.304 ± 0.056 for $k_{1(opt.)}$, K_d, and α, respectively, whereas the corresponding values for high-GDN-affinity heparin were 0.764 ± 0.042 $nM^{-1}s^{-1}$, 0.79 ± 0.59 nM, and 0.204 ± 0.086.

thrombin by GDN and ATIII (Wallace et al., 1989), and this data is given for comparison in Table II. Low- and high-ATIII-affinity heparin [as defined by Nordenman and Björk (1978)] stimulated the thrombin–GDN reaction to an equal extent (Table II). For the thrombin–ATIII reaction, the rate of inhibition was 3.5-fold faster in the presence of high-ATIII-affinity heparin (Table II).

The concentration of active heparin molecules capable of accelerating the thrombin–GDN reaction was determined by fitting data obtained at low heparin concentrations to eq 3. Data were obtained for low- and high-GDN-affinity heparin as shown in Figure 1. The results of the analyses indicated that the active concentrations of high- and low-GDN-affinity heparin were respectively 0.204 ± 0.086 and 0.301 ± 0.054 times those determined by the colorimetric assay.

Higher concentrations of low- and high-GDN-affinity heparin were found to inhibit the thrombin-GDN reaction as shown in Figure 2A. Similar bell-shaped curves for the plot

Table II: Inhibition Rates of Thrombin by Either GDN or ATIII: Effect of Heparin Fractionated According to Its Affinity for GDN or ATIII[a]

heparin fraction	k_1 ($M^{-1}s^{-1}$) GDN	ATIII
none[b]	1.41×10^6	0.011×10^6
nonbound	$(0.57 \pm 0.07) \times 10^9$	$(0.046 \pm 0.003) \times 10^9$
low-GDN-affinity	$(0.55 \pm 0.03) \times 10^9$	$(0.033 \pm 0.003) \times 10^9$
medium-GDN-affinity	$(0.60 \pm 0.05) \times 10^9$	$(0.027 \pm 0.002) \times 10^9$
high-GDN-affinity	$(0.75 \pm 0.06) \times 10^9$	$(0.028 \pm 0.004) \times 10^9$
low-ATIII-affinity[c]	$(0.43 \pm 0.06) \times 10^9$	$(0.022 \pm 0.006) \times 10^9$
high-ATIII-affinity[c]	$(0.41 \pm 0.04) \times 10^9$	$(0.077 \pm 0.006) \times 10^9$

[a] The observed second-order rate constant (k_1) for the association of thrombin with either GDN (2 nM) or ATIII (26.7 nM) was determined as described under Experimental Procedures in the presence of four different fractions of heparin at a fixed concentration of 0.5 μg/mL. Errors correspond to standard deviations of three measurements. [b] The k_1 for the GDN- and ATIII–thrombin reactions in the absence of heparin were taken from Wallace et al. (1989) and Hofsteenge et al. (1986), respectively. [c] These values were calculated from the data of Wallace et al. (1989).

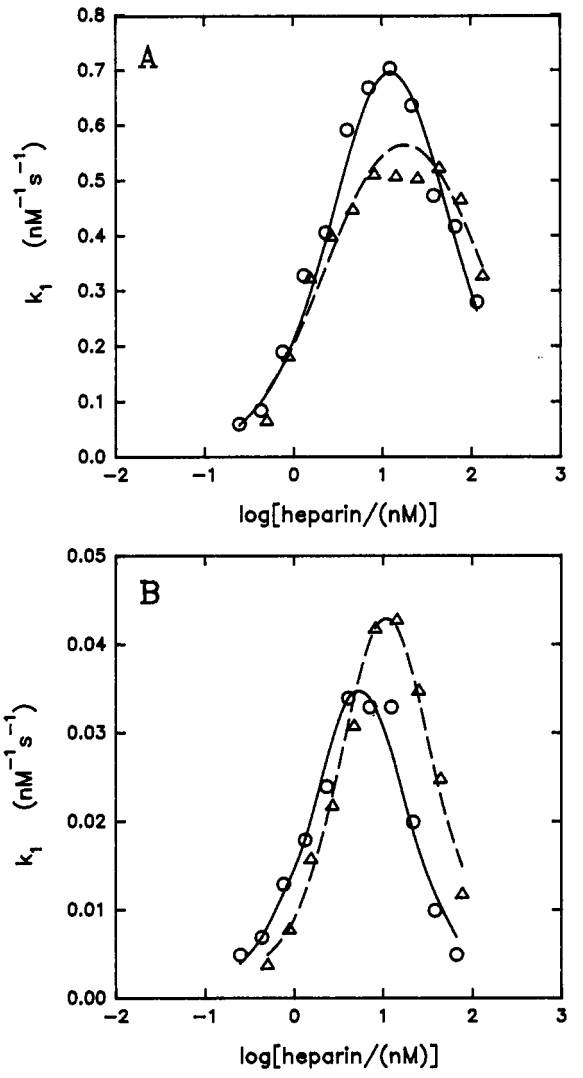

FIGURE 2: Effect of low- and high-GDN-affinity heparin on the inhibition rate of thrombin. The observed second-order rate constant (k_1) is plotted against the logarithm of the concentration of heparin. The points represent averages of three experiments. Kinetic data were obtained and analyzed as described under Experimental Procedures. The lines drawn show the fit of the data to eq 4. (A) Acceleration of the inhibition rate of thrombin by GDN as a function of low-GDN-affinity heparin (△) or high-GDN-affinity heparin (○). (B) Inhibition of thrombin by ATIII as a function of low-GDN-affinity heparin (△) or high-GDN-affinity heparin (○).

Table III: Kinetic Parameters for the Dependence of the Inhibition Rate of Thrombin by Either GDN or ATIII on the Concentration of Low- and High-GDN-Affinity Heparin[a]

heparin fraction	parameter	GDN	ATIII
low-GDN-affinity	$k_{1(opt.)}$	0.73 ± 0.07	0.19 ± 0.01
	K_1	123 ± 32	19 ± 7
	K_2	2.6 ± 0.6	6.4 ± 2.5
high-GDN-affinity	$k_{1(opt.)}$	1.25 ± 0.17	0.14 ± 0.01
	K_1	32 ± 8	8.1 ± 3.5
	K_2	4.9 ± 0.9	3.6 ± 1.7

[a] Units for $k_{1(opt.)}$ are $nM^{-1}\cdot s^{-1}$; those for K_1 and K_2 are nM. The data of Figure 2 were analyzed according to eq 4 as described under Experimental Procedures to yield the estimates of the parameters. The standard errors obtained from the analyses are also given.

of k_1 versus heparin concentration were previously obtained when heparin fractionated on the basis of its affinity for ATIII was used (Wallace et al., 1990). The data of Figure 2A were analyzed according to eq 4, and the estimates obtained for $k_{1(opt.)}$, K_1, and K_2 are given in Table III. The parameter $k_{1(opt.)}$ is the theoretical maximal value of k_1. The value of $k_{1(opt.)}$ for GDN of 0.73–1.25 $nM^{-1}\cdot s^{-1}$ suggests that the rate of association of GDN with thrombin, in the presence of heparin, is diffusion-controlled. Values of K_1 and K_2 define the heparin concentrations at which the half-maximal values of k_1 are observed and, thus, concentrations between K_1 and K_2 can be considered as the optimal range for the acceleration of the inhibition at the concentration of GDN used (2.0 nM). For high-GDN-affinity heparin with GDN, this range was between 5 and 30 nM, whereas the range was between 3 and 120 nM for the low-affinity type of heparin.

The dependence of k_1 for the ATIII–thrombin reaction on the concentrations of low- and high-GDN-affinity heparin is shown in Figure 2B. Values of k_1 of about 0.04 $nM^{-1}\cdot s^{-1}$ were obtained with optimal concentrations of low-GDN-affinity heparin, whereas the rate with the high-GDN-affinity heparin type was lower (ca. 0.03 $nM^{-1}\cdot s^{-1}$). With the concentration of ATIII used (26.7 nM), the optimal acceleration was achieved by the low- and high-GDN-affinity heparin at concentrations of 6–20 and 4–8 nM, respectively. The values of $k_{1(opt.)}$ of ATIII with thrombin (0.14 and 0.19 $nM^{-1}\cdot s^{-1}$) were nearly an order of magnitude lower than those observed with GDN (Table III).

Effects of Fragments of GDN on the Heparin-Accelerated Rate of Inhibition of Thrombin by GDN and ATIII. In order to localize specific areas of interaction between GDN and heparin, GDN was digested with CNBr. The three longest fragments, 63–144, 146–199, and 298–379 (Figure 3A), were isolated and tested together with a pool of shorter fragments (pools D and E, see Experimental Procedures) for their effect on the heparin-accelerated inhibition of thrombin by GDN. Increasing concentrations of the CNBr fragments were incubated with fixed amounts of GDN (2.0 nM) and high-GDN-affinity heparin (1.6 nM). The concentration of heparin was chosen such that k_1 showed a linear dependence on heparin concentration (the ascending limb of Figure 1A). Under these conditions, the binding of heparin to a competing fragment should prevent it from associating with GDN and a decrease in k_1 should result. Figure 4A shows that a 1 nM concentration of the unfractionated GDN/CNBr digest caused a 77% decrease in k_1, and a 1 nM concentration of the fragment 63–144 resulted in a 36% reduction in k_1. In contrast, equivalent amounts of either fragment 146–199 or 298–379 produced only a 8% decrease in k_1. In addition, the pool of the seven shorter fragments originating from the digestion of GDN with CNBr did not affect k_1 (data not shown). The observation that the CNBr digest and fragment 63–144 caused

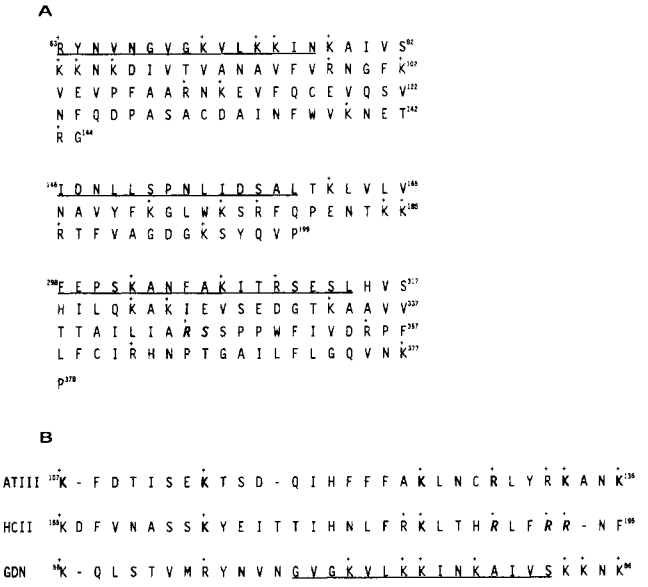

FIGURE 3: Peptide sequences of fragments of GDN. (A) Amino acid sequences of the CNBr fragments 63–144, 146–199, and 298–379. The sequence of rat GDN was derived from the cDNA sequence with the numbering starting from the putative NH_2 terminus of the mature protein (Gloor et al., 1986; Sommer et al., 1987). The C-terminal homoserine of fragments 63–144 and 146–199 is not shown. The distribution of positively charged residues is shown by the use of a plus sign above arginines and lysines. The identity of the fragments was established by N-terminal sequence and amino acid analysis. The partial N-terminal sequences that were determined are underlined. The putative reactive center of GDN, i.e., amino acid residues Arg^{346}–Ser^{347} (Gloor et al., 1986; Nick et al., 1990), is in bold italics. (B) Comparison of the proposed heparin-binding domains of GDN, ATIII, and heparin cofactor II (HCII). Positively charged amino acids are denoted by plus signs. Residues that have been shown to be involved in the binding of heparin to ATIII and heparin cofactor II are shown in bold, and those involved in dermatan sulfate binding to heparin cofactor II are in bold italics. The region of GDN corresponding to the α-helix D in α_1-antitrypsin is underlined (Loebermann et al., 1984).

a significant reduction in the value of k_1 when they were present at a concentration equivalent to that of GDN (2.0 nM) suggests that the affinities of GDN, its CNBr digest, and fragment 63–144 for high-GDN-affinity heparin are similar.

Higher concentrations of fragment 63–144 were required to inhibit the thrombin–GDN reaction if a higher concentration of GDN was used in the assays. When GDN was present at 20 nM, less than 5% inhibition was observed with 1 nM fragment 63–144 and a 10 nM concentration of fragment 63–144 was required to achieve a 30% reduction in the rate (data not shown). These results suggest that fragment 63–144 competes with GDN for binding to heparin. In the absence of heparin, the fragment 63–144 did not affect the value of k_1 observed for the association of thrombin with GDN and it did not inhibit the hydrolysis of chromogenic substrates by thrombin.

The GDN regions between residues 71–86 and 98–112 contain seven and four positively charged residues, respectively (Figure 3A). Synthetic peptides corresponding to these two regions of the GDN were synthesized and tested for their ability to interfere with the heparin stimulation of the GDN–thrombin reaction. In Figure 4B, the abilities of the synthetic peptides 70–87 and 98–112, the fragment 63–144, and a polymer of lysine residues (poly-L-lysine) to decrease the inhibition rate of thrombin are compared. Fragment 63–144 (1 nM) caused a 31% decrease in k_1, whereas an equivalent concentration of poly-L-lysine (M_r 4000) decreased k_1 by only 20%. It should be noted that at equimolar concentrations the

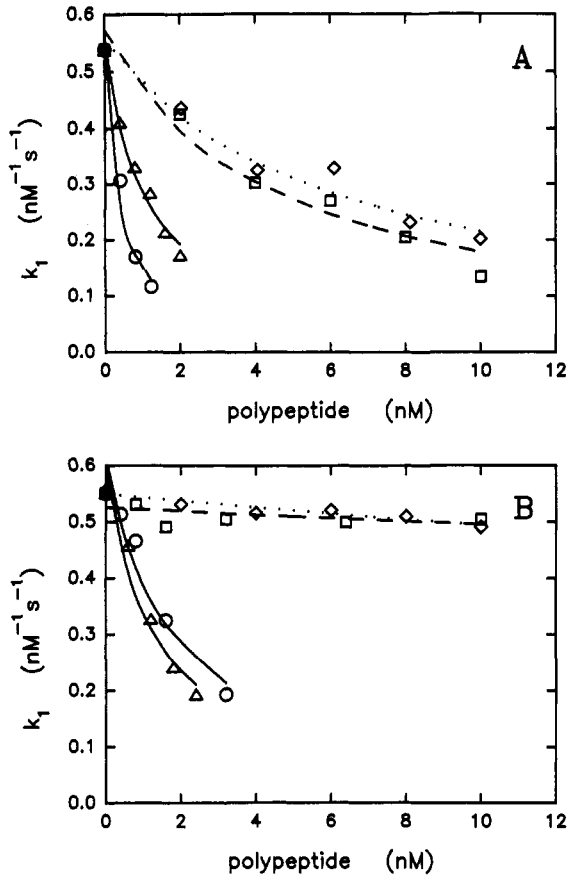

FIGURE 4: Effect of the fragments of GDN on the heparin-accelerated inhibition rate of thrombin by GDN. The observed k_1 for the association of thrombin with GDN is plotted against the concentration of CNBr fragments. (A) Native GDN (2.0 nM) and high-GDN-affinity heparin (1.6 nM) were preincubated with increasing concentrations of either unfractionated GDN/CNBr digest (○), fragment 63–144 (△), fragment 146–199 (◇), or fragment 298–379 (□). (B) Native GDN and high-GDN-affinity heparin were preincubated with increasing concentrations of fragment 63–144 (△), poly-L-lysine (○), or the synthetic peptides 70–87 (□) and 98–112 (◇). Assays were performed and kinetic data were analyzed as described under Experimental Procedures.

poly-L-lysine used contains 3 times more positively charged residues than fragment 63–144. On the other hand, a 10 nM concentration of either synthetic peptide caused only a small decrease (5–10%) in k_1. In addition, no synergy was seen when the two synthetic peptides were assayed together (data not shown). These results suggest that the effect of fragment 63–144 on k_1 is dependent on a specific conformation of the polypeptide chain.

The effect of fragment 63–144 was further analyzed by testing its ability to compete for the heparin-catalyzed inhibition of thrombin by ATIII. Assay conditions were similar to those used in the above competition assay with GDN; increasing concentrations of the CNBr fragments were incubated with fixed amounts of ATIII (2.0 nM) and high-GDN-affinity heparin (2.0 nM). In comparison with the results obtained for the heparin-accelerated inhibition of thrombin by GDN (Figure 4), the unfractionated GDN/CNBr digest and the isolated fragment 63–144 were considerably less effective in causing a decrease in k_1 (Figure 5). At a concentration of 1 nM, both fragment 63–144 and the CNBr digest did not cause a significant decrease in the value of k_1 for the thrombin–ATIII reaction. At a concentration of 10 nM, both the CNBr digest and fragment 63–144 caused a decrease in k_1 of slightly greater than 10%. In contrast to the effects seen on the thrombin–GDN reaction, poly-L-lysine was a more

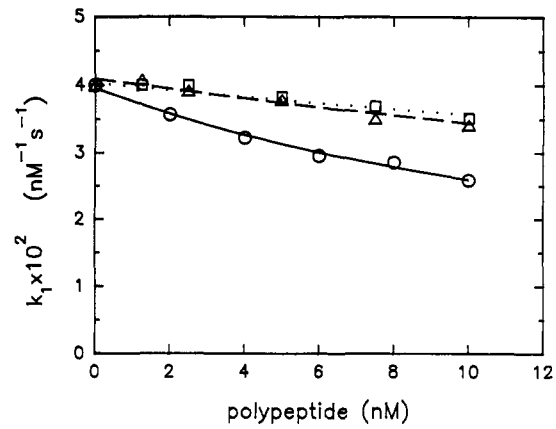

FIGURE 5: Effect of poly-L-lysine, fragment 63–144, and the total GDN/CNBr digest on the heparin-accelerated rate of inhibition of thrombin by ATIII. The observed k_1 for the association of thrombin with ATIII (2 nM) in the presence of 2 nM high-GDN-affinity heparin is plotted against an increasing concentration of poly-L-lysine (○), unfractionated GDN/CNBr digest (□), or fragment 63–144 (△). Assays were performed and kinetic data were analyzed as described under Experimental Procedures.

effective inhibitor of the thrombin–ATIII reaction than fragment 63–144 and the CNBr digest; a decrease of 35% in k_1 was seen with 10 nM poly-L-lysine (Figure 5). At higher concentrations of ATIII, fragment 63–144 was a less effective inhibitor. Increasing the concentration of ATIII from 2.0 to 26.7 nM decreased the degree of inhibition observed with 10 nM fragment 63–144 from 14% to about 3% (data not shown). These results suggest that fragment 63–144 and ATIII are competing at least to some degree for the same heparin species.

Discussion

In the extracellular space, GDN is thought to be mainly associated with molecules in the extracellular matrix and on the outer cell surface (Farrell et al., 1988; Halfter et al., 1989; Wagner et al., 1989; Rovelli et al., 1990) and its binding to heparin-like molecules could regulate its activity (Farrell & Cunningham, 1986). The aim of the present study was to characterize the binding of heparin to GDN. Heparins with different affinities for GDN and ATIII were found to differ only slightly in their ability to accelerate the inhibition of thrombin by GDN (Table II). The inhibition rate constant observed with the nonbound and low-GDN-affinity fractions was only about 25% less than that observed with high-GDN-affinity heparin (Table II). These results suggest that the affinity of heparin for GDN is not of primary importance for the acceleration of the inhibition of thrombin by GDN. In this respect, the thrombin–GDN reaction can be contrasted with that of ATIII with thrombin which requires a specific heparin with high affinity for ATIII for an optimal acceleration of the reaction (Lam et al., 1976; Höök et al., 1976; Andersson et al., 1976).

Competition studies with the GDN fragment 63–144 indicate that the heparin-binding site of GDN is located in this region. Fragment 63–144 decreased the rate of inhibition of thrombin by GDN in the presence of high-GDN-affinity heparin. The effect of k_1 was specific and was due to the competition between GDN and fragment 63–144 for binding to heparin. Increasing the concentration of GDN decreased the inhibitory effects of fragment 63–144. Both fragment 298–379, which contains the putative reactive center of GDN (Figure 3A), and fragment 146–199 showed a smaller effect on k_1. Furthermore, fragment 63–144 was also more effective than a polymer of lysine residues in decreasing k_1, in spite of the fact that the ratio of positively charged residues was about

3:1 in favor of poly-L-lysine. The inhibitory effects of fragment 63–144 and the CNBr digest of GDN were seen at concentrations in the range of GDN in the assay (Figure 4A). These results suggest that the CNBr digest and fragment 63–144 have an affinity for high-GDN-affinity heparin that is similar to that of native GDN. These results support those of Evans et al. (1991) that indicate that the native conformation of GDN is not required for high heparin affinity. In contrast, the native conformation of ATIII is required for high-affinity binding to heparin (Evans et al., 1991). In addition, the similar affinities of fragment 63–144 and native GDN for heparin suggest that much of the structural information required for the binding to heparin is contained in the fragment 63–144.

In comparison with its potent effect on the heparin-stimulated thrombin–GDN reaction, fragment 63–144 was less effective in reducing the inhibition rate of thrombin by ATIII in the presence of high-GDN-affinity heparin. This result is consistent with the observed modes of binding of GDN and ATIII to heparin. Although ATIII is known to bind to a specific site on heparin (Choay et al., 1981; Thunberg et al., 1982), the binding site for GDN seems to be less specific. In contrast to the discrete peaks seen for salt gradient elution of heparin from an ATIII affinity matrix (Nordenman & Björk, 1978), elution of heparin from immobilized GDN results in a broad peak suggesting multiple interactions. Thus, GDN and its heparin-binding fragments would compete only partially with the binding of ATIII to heparin because the specific ATIII-binding sites would represent only a subset of the GDN-binding sites. Moreover, the affinity of ATIII for its specific binding site would be expected to be greater than that of GDN (and its fragments) for this site such that high concentrations of fragment 63–144 would be required to compete effectively with ATIII. However, the fact that the degree of inhibition by fragment 63–144 of the heparin-catalyzed thrombin–ATIII reaction was decreased by increasing the concentration of ATIII indicates that the binding sites on heparin for fragment 63–144 and ATIII overlap to some extent.

Although the interaction of fragment 63–144 with heparin is not as specific as that of ATIII, there appears to be more involved than a completely nonspecific charge–charge interaction. If the binding of fragment 63–144 to heparin were a nonspecific electrostatic interaction, poly-L-lysine would be expected to be a more effective competitor than fragment 63–144 in both the ATIII and GDN reactions because of its greater charge. The fact that fragment 63–144 was more effective than poly-L-lysine in competing with GDN suggests some specificity in the interaction of fragment 63–144 (and GDN) with heparin.

Chemical modification studies and investigations of natural mutants have identified two regions of ATIII, Gly^{35}–Glu^{50} and Lys^{107}–Lys^{136}, that are important for heparin binding (Blackburn et al., 1984; Koide et al., 1984; Chang & Tran, 1986; Brennan et al., 1987; Lui & Chang, 1987; Peterson & Blackburn, 1987; Peterson et al., 1987; Owen et al., 1987; Chang, 1989). Both GDN (Lys^{56}–Lys^{86}) and heparin cofactor II (Lys^{164}–Phe^{195}) contain regions that are homologous to that between residues 107 and 136 of ATIII. The positively charged residues in this region are well conserved between the three molecules as shown Figure 3B. Moreover, a number of these residues have been implicated in the binding of heparin and/or dermatan sulfate to ATIII or heparin cofactor II (Blinder et al., 1989, 1990; Ragg et al., 1990; Whinna et al., 1991). The GDN region between residues 71 and 86 contains seven lysine residues, which are organized in two clusters (71–78 and 83–86), and the sequence in this region conforms to the consensus sequence for a heparin-binding site as defined by Cardin and Weintraub (1989). Homologous residues to four of these lysines have been shown to be involved in heparin binding to ATIII (Figure 3B; Peterson & Blackburn, 1987; Peterson et al., 1987; Liu & Chang, 1987; Chang, 1989). In addition, four positively charged residues in homologous positions have been implicated in heparin and/or dermatan sulfate binding to heparin cofactor II (Figure 3B; Blinder et al., 1989; Blinder & Tollefsen, 1990; Ragg et al., 1990; Whinna et al., 1991). Thus, the proposal that residues 71–86 of GDN constitute at least part of the heparin-binding site is supported by data obtained with ATIII and heparin cofactor II. Alignment of the primary structures of GDN and α_1-antitrypsin (Sommer et al., 1987) and examination of the three-dimensional structure of α_1-antitrypsin (Loebermann et al., 1984) indicate that the side chains of Lys^{71}, Lys^{74}, Lys^{75}, and Lys^{78} would be located along the same side of the α-helix D, facing toward the solvent (Huber & Carrell, 1989). The residues Lys^{83}, Lys^{84}, and Lys^{85} are located in the loop following the α-helix D, and the side chain of Lys^{84} could be well positioned with respect to the previous four lysine residues in the α-helix D. The poor heparin-binding activity of the peptide 71–86 could be explained by the failure of this peptide to form a helical structure. However, it cannot be excluded that other positively charged residues besides these clusters of lysyl residues play a role in heparin binding to fragment 63–144 and GDN.

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