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CARBOXYLATE POLYANIONS ACCELERATE INHIBITION OF THROMBIN BY HEPARIN COFACTOR II

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The heparin cofactor II (HCII)/thrombin inhibition reaction is enhanced by various carboxylate polyanions. In the presence of polyaspartic acid, the HCII/thrombin reaction is accelerated more than 1000-fold with the second-order rate constant increasing from 3.2×10^4 M^{-1} min^{-1} (in the absence of polyAsp) to 3.6×10^7 M^{-1} min^{-1} as the polyAsp concentration is increased from 1 to 250 ug/ml. This accelerating effect was observed for HCII/thrombin, though to varying degrees, with other carboxylate polyanions. In contrast to HCII, the rate of antithrombin III inhibition of thrombin was decreased in the presence of polyAsp. The HCII/thrombin complex is rapidly formed in the presence of 10 ug/ml polyAsp when ^{125}I-labeled-thrombin is incubated with plasma. It is possible that at physiological sites rich in carboxylate polyanions, thrombin may be preferentially inhibited by HCII.

Heparin, which has been used as a therapeutic anticoagulant for over forty years, is a highly negatively charged (sulfated and carboxylated) mucopolysaccharide composed of alternating 1→4-linked glucosamine and uronic acid residues (1). Heparin greatly increases the rate of thrombin inhibition by the plasma glycoproteins antithrombin III (ATIII) and heparin cofactor II (HCII) (2-9). It is now generally thought that the mechanism of action for heparin-catalyzed thrombin inhibition by ATIII or HCII occurs through a ternary complex (or "template model") (10-14). A specific pentasaccharide sequence in heparin, containing four sulfate groups in invariant positions, is the high-affinity binding site for ATIII (15-18). Structure and activity relationships of HCII/glycosaminoglycan interactions have recently been described (19-23). The HCII/thrombin inhibition reaction, unlike the ATIII/thrombin reaction, is greatly enhanced either by heparin or by dermatan sulfate (20,23). The present investigation was undertaken to further characterize the specificity of the polyanion-binding site of HCII. In this study, we show that a number of carboxylate-containing polyanions can accelerate the HCII/thrombin inhibition reaction.

Abbreviations: ATIII, antithrombin III; HCII, heparin cofactor II; T, thrombin; TosGlyProArgNA, N^{α}-p-tosyl-Gly-Pro-Arg-p-nitroanilide; TNP, triethanolamine-acetate, NaCl, poly(ethyleneglycol)-containing buffer; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DEGR, dansyl-Glu-Gly-Arg-chloromethyl ketone.

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EXPERIMENTAL PROCEDURES

Materials

PolyAsp (M_r = 15,000 and 34,000), polyGlu (M_r = 14,000 and 46,000), polyGlu,Ala (M_r = 30,000; Glu:Ala of 6:4), colominic acid (polysialic acid), pyromellitic acid (1,2,4,5-benzenetetracarboxylic acid), trimesic acid (1,3,5-benzenetricarboxylic acid), heparin, β-lactoglobulin and citraconic anhydride were obtained from Sigma. Polybrene and mellitic acid (1,2,3,4,5,6-benzenehexacarboxylic acid) were purchased from Aldrich. N^{α}-p-tosyl-Gly-Pro-Arg-p-nitroanilide (TosGlyProArgNA) was from Boehringer-Mannheim. All other chemicals were of the highest commercial grade. HCII and ATIII were purified from human plasma as detailed previously (24). Protein concentrations of HCII [M_r = 65,600 (7)] and ATIII [M_r = 56,600 (25)] were determined at 280 nm with specific absorption coefficients of 0.593 (26) and 0.624 (26), respectively. Human α-thrombin (T) (3000 NIH u/mg; >98% active by active-site titration) was prepared as reported previously (27). Human plasma Factor Xa was prepared as described (24). Thrombin was ^{125}I-labeled with Iodobeads following the instructions provided by Pierce Chemical Co., resulting in approximately 3 x 10^{17} dpm per mol proteinase. Thrombin with a dansylated probe incorporated in its active site was prepared using dansyl-Glu-Gly-Arg-chloromethyl ketone (DEGR) (28). The lysines of β-lactoglobulin were modified with repeated additions of citraconic anhydride (29) to the protein solution on ice.

Assays

HCII activity was determined by measuring the rate of thrombin inhibition in the absence (antithrombin activity) and presence of either heparin (heparin cofactor activity) or the carboxylate polyanions essentially as detailed previously (30,31). Assays were started by adding thrombin (5 nM) to a solution at 25°C and pH 8.0 containing TNP buffer [50 mM triethanolamine-acetate, 100 mM NaCl, 0.1% poly(ethyleneglycol)], HCII (25-100 nM) and the polyanions (at various concentrations). At timed intervals, portions (0.1 ml) were removed and added to a substrate solution (0.8 ml) containing 0.3 mM TosGlyProArgNA in TNP buffer and 0.1 mg/ml Polybrene at 25°C. Substrate hydrolysis was terminated by addition of 0.1 ml acetic acid (some of the carboxylate polyanions precipitated after addition of the acid; this was removed by centrifugation), and the absorbance was measured at 404 nm. Assays for the inhibition of thrombin and Factor Xa by ATIII were performed in a similar manner. The antichymotrypsin activity of HCII was determined as detailed previously (32). Apparent pseudo-first-order (k_{obs}) and second-order (k_2) rate constants were calculated as described (30,31).

Methods

Intrinsic fluorescence measurements of ATIII were performed at 25°C and pH 8.0 with TNP buffer in 1.0-cm-pathlength cuvettes with excitation at 280 nm and emission scanned from 300 to 400 nm (30). Binding of polyanions to DEGR-thrombin was monitored by changes in fluorescence of the dansyl group essentially as described previously (28). SDS-PAGE used the Laemmli buffer system with 7.5% polyacrylamide gels (27) while plasma incubation with radiolabeled-thrombin and autoradiography were performed essentially as detailed (23).

RESULTS AND DISCUSSION

Effect of Carboxylate Polyanions on the HCII/Thrombin Reaction

Addition of polyAsp and polyGlu to HCII/thrombin at pH 8.0 and 25°C accelerated the rate of thrombin inhibition (Figure 1A). Apparent k_2 values for the HCII/thrombin reaction in the presence of 10 ug/ml polyAsp and 100 ug/ml polyGlu were 1.4 x 10^7 and 1.7 x 10^6 M^{-1} min^{-1}, respectively; this represents an enhancement of 440- (polyAsp) and 54- (polyGlu) fold over the HCII/thrombin

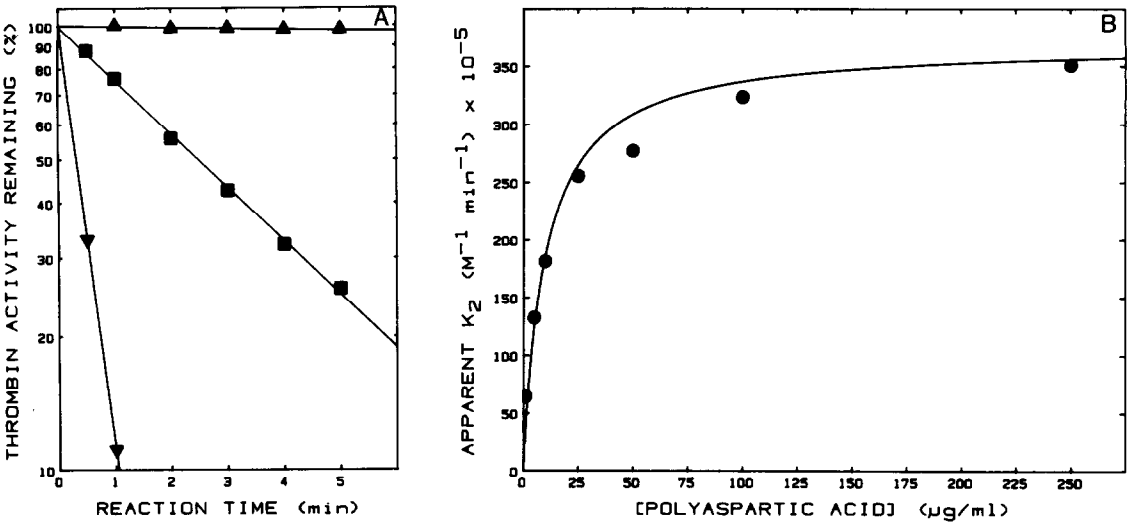

Figure 1. Effect of polyAsp/polyGlu on the heparin cofactor II/thrombin reaction. **(A)** Thrombin was incubated at 25°C and pH 8.0 in TNP buffer with heparin cofactor II (HCII) in the absence (▲) and presence of 10 ug/ml polyAsp (M_r = 15,000) (▼) and 100 ug/ml polyGlu (M_r = 14,000) (■). Residual thrombin activity was measured as described under "Experimental Procedures". **(B)** Thrombin and HCII were incubated with various amounts of polyAsp (M_r = 15,000) (●) and the apparent k_2 values determined (see under "Experimental Procedures").

reaction in the absence of added polyanion (k_2 = 3.2 x 10^4 M^{-1} min^{-1}). PolyAsp did not enhance the HCII/chymotrypsin reaction and this is similar to results described previously for glycosaminoglycans during the HCII/chymotrypsin (32) and HCII/cathepsin G (33) inhibition reactions.

The rate of HCII/thrombin inhibition reaction as a function of polyAsp concentration appeared to follow saturation kinetics, showing a rapid rate increase between 1 and 50 ug/ml and reaching a maximum rate as polyAsp approached 250 ug/ml (Figure 1B). Replotting the titration data by the Henri-Michaelis-Menten equation (a Hanes-Woolf plot with r = 0.999) showed that the HCII/thrombin reaction was increased more than 1000-fold by polyAsp with a maximum k_2 value of 3.6 x 10^7 M^{-1} min^{-1} (data not shown). These results indicate that heparin more effectively accelerates the HCII/thrombin inhibition reaction compared to polyAsp (the maximum k_2 value for the heparin-catalyzed HCII/thrombin reaction is about 1 x 10^8 M^{-1} min^{-1} with approximately 5-10 ug/ml heparin). The mechanism of acceleration of HCII/thrombin inhibition by polyAsp does not appear to involve the formation of a ternary complex (simultaneous binding of inhibitor and proteinase to polyAsp). As shown previously for the heparin-catalyzed ATIII (and HCII)/thrombin reaction (10-14), when a ternary complex is involved, the apparent k_2 value increases to a maximum value and subsequently decreases as the concentration of catalyst is increased.

Considering the differences found between polyAsp and polyGlu (M_r each of about 15,000) we examined the ability of a variety of carboxylated polyanions to

TABLE 1
Effect of Various Carboxylate Polyanions on the Inhibition of Thrombin by Heparin Cofactor II

Carboxylate polyanion[a]	Concentration (ug/ml)	Apparent k_2 value $(M^{-1} min^{-1}) \times 10^{-4}$	Enhancement (Fold)
None	--	3.2	1
PolyAsp (M_r = 15,000)	10	1400	440
PolyAsp (M_r = 34,000)	10	1883	580
PolyGlu (M_r = 14,000)	100	170	54
PolyGlu (M_r = 46,000)	100	234	72
PolyGlu,Ala (M_r = 30,000)	250	36	11
Mellitic acid	250	123	38

[a]Inhibition reactions were performed as described under "Experimental Procedures".

accelerate the HCII/thrombin reaction (Table 1). As expected, the higher M_r species of polyAsp and polyGlu were more effective at enhancing the HCII/thrombin reaction. Other carboxylate polyanions were able to accelerate this inhibition reaction to varying degrees (Table 1). Some carboxylate polyanions failed to enhance the HCII/thrombin reaction; these included citric acid, succinic acid, tartaric acid, colominic acid, pyromellitic acid, trimesic acid, succinylated-polyLys and citraconylated-β-lactoglobulin (data not included). These results indicate that a specific charge density ratio in combination with a unique structure is important for acceleration of thrombin inhibition by HCII.

Effect of PolyAsp on the ATIII-Catalyzed Thrombin and Factor Xa Reactions

The rate of inhibition of thrombin, but not Factor Xa, by ATIII was decreased in the presence of polyAsp (Table 2). To further evaluate the role of

TABLE 2
Effect of Polyaspartic Acid on the Inhibition of Thrombin and Factor Xa by Antithrombin III

	Apparent k_2 value $(M^{-1} min^{-1}) \times 10^{-5}$	
PolyAsp (ug/ml)[a]	Thrombin	Factor Xa
None	4.1 (0)[b]	1.3 (0)
1	3.2 (22)	1.3
10	3.1 (24)	1.3
50	2.6 (37)	1.3
100	2.4 (41)	1.3
250	2.3 (44)	1.3

[a]Inhibition reactions were started by adding proteinase (5 and 30 nM for thrombin and Factor Xa, respectively) to TNP buffer at pH 8.0 and 25°C containing 820 nM ATIII, polyAsp (M_r = 15,000) at various concentrations and the residual proteinase activity was determined as described under "Experimental Procedures". Values reported are the means of at least three separate experiments.

[b]Values reported in parentheses are percent changes compared to a control.

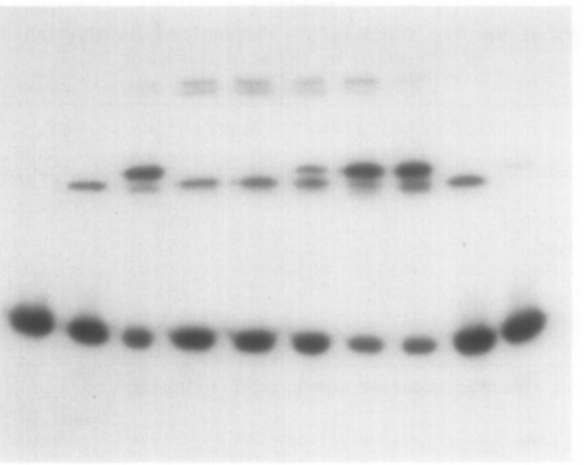

Figure 2. Activation of heparin cofactor II in human plasma by polyAsp. Citrated plasma (diluted 1:50) in TNP buffer or purified inhibitors (diluted to plasma concentration levels) were incubated with ^{125}I-labeled thrombin (5 nM) for 10 min at 25°C with various amounts of polyAsp (M_r = 15,000). The samples were then analyzed by SDS-PAGE. An autoradiogram of the gel shows the position of thrombin (T), purified ATIII/T and HCII/T complexes in the presence of 10 ug/ml polyAsp (lanes 1-3, respectively); the plasma system with 0, 0.1, 1 and 10 ug/ml polyAsp (lanes 4-7, respectively); purified HCII and ATIII mixed with T and 10 ug/ml polyAsp (lane 8); and purified ATIII and HCII with T in the absence of polyAsp (lanes 9 and 10, respectively).

polyAsp in these ATIII/proteinase reactions, we measured the effect of polyAsp on the intrinsic and extrinsic fluorescence of ATIII and DEGR-thrombin, respectively. Unlike the "heparin-induced" ATIII conformational change which results in an intrinsic fluorescence increase of about 40% (mean value of three separate experiments (also see Refs. 34-37)), there was no intrinsic fluorescence increase in ATIII following the addition of 250 ug/ml polyAsp (mean value of five separate experiments was a decrease of 1.5%). Additionally, polyAsp was not able to displace heparin from ATIII as assessed by intrinsic ATIII-heparin fluorescence measurements. There was a 3-fold dansyl fluorescence enhancement following the addition of heparin to DEGR-thrombin, in agreement with the results of Nesheim *et al.* (28). PolyAsp did not produce a fluorescent signal enhancement when added to DEGR-thrombin (data not included). These results demonstrate that polyAsp does not perturb protein conformation (measured by fluorescence spectroscopy) either of ATIII or of thrombin in the same manner as does heparin. Since there was no effect of polyAsp on the ATIII/Factor Xa inhibition rate, the decreased rate of the polyAsp-catalyzed ATIII/thrombin reaction implies that polyAsp binding to thrombin alters the ability of thrombin to interact with ATIII, possibly by steric hindrance.

Effect of PolyAsp on Thrombin Inhibition in Plasma

The inhibition of thrombin in human plasma in the presence of various amounts of polyAsp was examined (Figure 2). With this *ex vivo* system, ^{125}I-

labeled-thrombin was incubated with plasma or purified HCII or ATIII and the reaction products were subsequently analyzed by SDS-PAGE and autoradiography. As shown in Figure 2, increasing amounts of polyAsp, to 10 ug/ml, were correlated with incorporation of a majority of the radioactive thrombin into a complex with HCII. However, there was essentially no effect of polyAsp on formation of the ATIII/thrombin complex.

Concluding Remarks

Although HCII is a potent *in vitro* thrombin inhibitor in the presence of heparin or dermatan sulfate and, as described here, for polyAsp and other carboxylate polyanions, the physiological function of HCII is still unknown. We have recently found that the *in vivo* catabolism of the HCII/thrombin complex is mediated through the same receptor that recognizes ATIII/proteinase complexes (38). Intravenous dermatan sulfate prevents thrombus formation presumably by production of HCII/thrombin complexes (39) suggesting that thrombin may be inhibited by HCII at sites where dermatan sulfate is prevalent (20,21,23). McGuire and Tollefsen (40) recently proposed that a potential *in vivo* role of thrombin inhibition by HCII exists during vessel wall injury that exposes fibroblasts, smooth muscle cells and the extracellular matrix. It is also intriguing to speculate that within this exposed region of the blood vessel, an area potentially rich in carboxylate groups may participate in the *in vivo* HCII/thrombin reaction. Further details remain to be elucidated regarding the specificity of the carboxylate polyanion-catalyzed HCII/thrombin reaction.

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