Antithrombin Activation by Nonsulfated, Non-Polysaccharide Organic Polymer

Bernhard H. Monien and Umesh R. Desai*

Department of Medicinal Chemistry and Institute for Structural Biology and Drug Discovery, Virginia Commonwealth University, Richmond, Virginia 23298-0540

Received August 27, 2004

Accelerated antithrombin inhibition of procoagulant enzymes has been exclusively achieved with polysulfated polysaccharides. We reasoned that antithrombin activation should be possible with *nonsulfated* activators based only on carboxylic acid groups. As a proof of the principle, linear poly(acrylic acid)s were found to bind to antithrombin and accelerate inhibition of factor Xa and thrombin. Our work demonstrates that molecules completely devoid of sulfate groups can activate antithrombin effectively and, more importantly, suggests that it may be possible to develop orally bioavailable, carboxylate-based antithrombin activators.

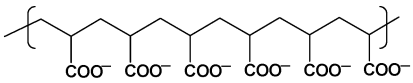
Introduction

Antithrombin, a plasma glycoprotein and member of the serpin family of proteins, is an inhibitor of factor Xa and thrombin, enzymes of the blood coagulation cascade.[1,2] Although antithrombin is an inhibitor, its reaction with both the enzymes is very slow under physiological conditions. The inhibitor requires an activator to effectively nullify the pro-coagulant enzymes.

Numerous molecules have been found to activate antithrombin. Heparin, a clinically used antithrombin-based anticoagulant for the past 7 decades,[3,4] has served as a prototypic activator on which all subsequent molecular designs have been based. These designs include the chemically modified heparins and low-molecular weight heparins.[5] In addition, a specific five-residue sulfated saccharide sequence DEFGH and its derivatives have been studied as potent antithrombin activators.[5−9] Recently, major advances on chemoenzymatic front have been made to synthesize these small sulfated saccharides.[10−14] Likewise, some non-heparin molecules have also been investigated as antithrombotics.[15−17] These possess sulfated or phosphorylated thrombin binding domain that are non-heparin structures, but retain the highly sulfated DEFGH structure as the critical antithrombin binding domain.

The principal reason proposed for retaining a sulfated polysaccharide structure in the design of antithrombin activators is the nature of antithrombin−heparin interaction. A specific sequence of five saccharide residues in the heparin chain is found essential for tight binding,[18−20] which allows heparin to exert its effect on antithrombin at low concentrations. Yet, low-affinity heparin, with a low antithrombin binding affinity of ∼25−30 kJ/mol, activates the inhibitor some 60-fold. This activation arises from the bridging of the inhibitor and the enzyme on the sulfated polysaccharide.[21]

Sulfated polysaccharides are known to bind many proteins,[22−24] thus explaining their severe side-effects.[25,26] In contrast, fondaparinux, a pentasaccharide DEFGH derivative, appears to have reduced side-effects, yet the risk for enhanced bleeding is not eliminated.[27−30] Thus, new heparin-like mimics are desirable.



	M_R	Polydispersity	Monomers	Length
PAA1500	1,500 Da	1.12	~21-mer	~40 Å
PAA2280	2,280 Da	1.67	~32-mer	~61 Å
PAA3450	3,450 Da	1.64	~48-mer	~91 Å
PAA6200	6,200 Da	1.34	~86-mer	~164 Å

Figure 1. Structure of poly(acrylic acid)s (PAA) studied for antithrombin activation. M_R = average molecular weight; Length refers to the predicted length of an extended linear chain.

Engineering new antithrombin activators without side-effects, and possibly with oral activity, would represent a major advance in anticoagulation therapy. The fundamental assumptions made to-date in designing antithrombin activators are that the designs (i) have to be polysaccharide-based and (ii) should possess multiple sulfate (OSO_3^-) groups. Previously we demonstrated that antithrombin activation is possible without a saccharide scaffold.[31−33] As a further challenge, we reasoned that this activation should also be possible with *nonsulfated* molecules based only on carboxylic acid groups.

The work described here demonstrates the proof of this principle wherein poly(acrylic acid)s are found to bind and activate antithrombin. This suggests that one may be able to develop appropriate organic scaffold(s) containing only carboxylic acid groups, without any sulfate groups, as antithrombin activator(s). An advantage with carboxylate-based activators is that they may be potentially converted into prodrugs that possess oral bioavailability, a concept deemed impossible with heparin.

Results

Equilibrium Binding to Antithrombin. To test whether linear poly(acrylic acid)s (PAAs) interact with plasma antithrombin, we followed the change in fluorescence of an external probe, TNS, as function of PAA concentration at pH 6.0 and pH 7.4 for four polymers of differing chain length (Figure 1). We and others have previously exploited this technique to study the interac-

* To whom correspondence should be addressed. Tel: (804) 828-7328. Fax: (804) 828-7625. E-mail: urdesai@vcu.edu.

10.1021/jm0492960 CCC: $30.25
Published on Web 01/27/2005

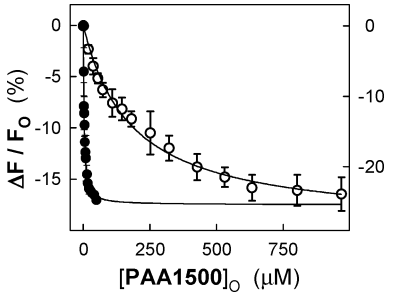


Figure 2. Representative fluorescence titration of antithrombin-TNS complex as a function of PAA1500 at pH 6.0 (●) and pH 7.4 (○). The K_D values for the interactions were determined by nonlinear least-squares fittings of two or three averaged measurements to the quadratic binding equation (solid lines).[35,36] See Experimental Section for details.

Table 1. Equilibrium Dissociation Constants (K_D) of PAA−Antithrombin Complexes at pH 6.0 and 7.4[a]

	pH 6.0, *I* 0.05, 25 °C, μM	pH 7.4, *I* 0.075, 25 °C, μM
PAA1500	2.3 ± 0.2	180 ± 15
PAA2280	2.3 ± 0.2	96 ± 14
PAA3450	1.3 ± 0.2	91 ± 15
PAA6200	1.0 ± 0.1	34 ± 2[b]
DEF[c,d]	2.0 ± 0.6	66 ± 4
DEFGH[c,d]	~5 × 10^{-5}	~1.4 × 10^{-3}
(+)-CS[c,e]	11.6	18 ± 2

[a] See Experimental Section for determination of K_D values. [b] Determined by factor Xa inhibition method[31] because the ΔF_{max} observed in direct fluorescence titrations was <5%. [c] DEF, DEFGH, and (+)-CS are small sulfated molecules known to activate antithrombin. DEF and DEFGH correspond to specific trisaccharide and pentasaccharide,[38] while (+)-CS is a synthetic sulfated flavanoid.[32] [d] K_D values reported here are taken from ref 20 and not corrected for the small difference in ionic strength at both pHs. [e] (+)-CS and DEFGH K_D values were calculated from the salt dependence of their binding affinity profile.[32,38]

tions of antithrombin with sulfated organic activators and heparin pentasaccharide derivatives.[31,32,34] Binding of each PAA studied to antithrombin led to a saturable decrease (ΔF_{max} = −14 to −35%) in TNS fluorescence (Figure 2) from which the equilibrium dissociation constant (K_D) was determined (Table 1).[35,36]

The antithrombin binding affinity ranged from 1.0 μM for the longest PAA to 2.3 μM for the shortest PAA tested at pH 6.0, while the range was 34−180 μM at pH 7.4. This corresponds to a free energy of binding range of 32−35 kJ/mol and 21−26 kJ/mol at pH 6.0 and 7.4, respectively. Similar values have been observed for trisaccharide DEF[37,38] (33 and 24 kJ/mol) and a rationally designed organic activator, catechin sulfate ((+)-CS)[32] (28 and 25 kJ/mol) (Table 1), whereas the values are 59 and 51 kJ/mol at pH 6.0 and 7.4, respectively, for pentasaccharide DEFGH.[37,38] Further, the binding energies of PAAs at pH 7.4 are also comparable to low-affinity heparin (~25 kJ/mol).[21]

These results suggest that multiple carboxylate groups with appropriate charge density provide sufficient energy to bind antithrombin. It is interesting to note that the binding energies of PAAs are essentially invariant although the chain length increases ~4-fold, suggesting almost identical binding. The small increase in affinity with the chain length is most likely a statistical phenomenon rather than an engagement of additional interaction sites. This suggests that the affinities being

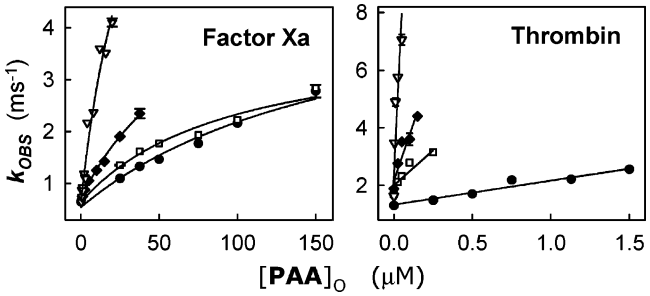


Figure 3. Dependence of the observed pseudo-first-order rate constant k_{obs} for the inhibition of factor Xa and thrombin at pH 7.4 as a function of PAA concentration. (PAA1500 (●), PAA2280 (□), PAA3450 (◆), and PAA6200 (▽)) See Experimental Section for details on measurement of k_{obs}.

measured are apparent values and that the intrinsic affinity of antithrombin for a unique PAA site is likely to be different. It is difficult to quantitate this intrinsic site affinity because of conformational flexibility of PAA chains as well as the possibility of overlapping binding sites.

The crystal structure of antithrombin−pentasaccharide complex shows the presence of an extended electropositive domain consisting of the so-called pentasaccharide-binding site and the extended heparin-binding site.[5,32,39] The combined length of both these sites is ~30 Å, which is equivalent to the extended length of the smallest PAA studied (~40 Å). It is likely that the PAAs engage both these sites, although this remains to be conclusively demonstrated.

Acceleration of Factor Xa Inhibition by PAA. To test whether PAA binding to antithrombin results in accelerated inhibition of procoagulant enzymes, we measured the second-order rate constant of antithrombin inhibition of thrombin and factor Xa in the presence and absence of PAA at pH 6.0 and pH 7.4 (Figure 3).[35] Table 2 lists the second-order rate constants and the acceleration in inhibition achieved for the four PAAs studied.

The second-order rate constant for antithrombin inhibition of factor Xa at pH 6.0 increased from 3200 M^{-1} s^{-1} for PAA1500 to 59 300 M^{-1} s^{-1} for PAA6200. Thus, the acceleration (k_{cat}/k_{uncat}) in factor Xa inhibition increased from about 15-fold to 284-fold as the length of the chain increased. Further, smaller chains PAA1070 and PAA830 retained the 15-fold acceleration shown by PAA1500 (not shown). Thus, acceleration shows chain length dependence beyond ~21 monomers. These accelerations compare favorably with heparin saccharides DEF and DEFGH (~300-fold)[37,38] and are significantly better than the rationally designed molecule (+)-CS (21-fold).[32,33] In contrast, at pH 7.4 the k_{cat} for PAAs ranged from 11 400 M^{-1} s^{-1} to 23 100 M^{-1} s^{-1}, suggesting considerably weak acceleration. Under similar conditions, heparin saccharides show ~300-fold acceleration.[37,38]

Although the mechanism of heparin (and pentasaccharide DEFGH) acceleration of antithrombin inhibition of factor Xa involves the conformational activation of the inhibitor,[5,40,41] recent work suggests a significant bridging component, especially with longer heparin chains, wherein the activator bridges the inhibitor and the enzyme in a ternary complex in the presence of Ca^{2+} ions.[42,43] The increase in acceleration with PAA chain

Table 2. Second-Order Rate Constants (k_{cat}) for Antithrombin−PAA Inhibition of Factor Xa and Thrombin at pH 6.0 and 7.4

	factor Xa				thrombin			
	pH 6.0, *I* 0.05, 25 °C		pH 7.4, *I* 0.075, 25 °C		pH 6.0, *I* 0.05, 25 °C		pH 7.4, *I* 0.075, 25 °C	
	k_{CAT} (mM^{-1} s^{-1})	k_{CAT}/k_{UNCAT}	k_{CAT} (mM^{-1} s^{-1})	k_{CAT}/k_{UNCAT}	k_{CAT} (mM^{-1} s^{-1})	k_{CAT}/k_{UNCAT}	k_{CAT} (mM^{-1} s^{-1})	k_{CAT}/k_{UNCAT}
PAA1500	3.2 ± 0.4	15[a]	11.4 ± 0.5	9.5[a]	13 ± 1	24[a]	1030 ± 120	114[a]
PAA2280	13.1 ± 0.8	63	8.8 ± 0.4	6.2	71 ± 2	125	3390 ± 810	259
PAA3450	32.0 ± 2.2	153	13.9 ± 0.3	8.7	196 ± 3	345	9000 ± 1900	597
PAA6200	59.3 ± 3.3	284	23.1 ± 1.2	17	793 ± 80	1392	19100 ± 5300	1109

[a] The uncatalyzed second-order rate constant k_{uncat} under each condition was determined from the intercept of the pseudo-first-order rate constant k_{obs}−$[PAA]_O$ profile at pH 7.4. At pH 6.0, independently measured k_{uncat} was used because of the small value of the intercept, which tended to give inaccurate k_{uncat}. The k_{uncat} values were found to be 2100 ± 50 M^{-1} s^{-1} and 210 ± 10 M^{-1} s^{-1} for factor Xa at pH 7.4 and pH 6.0, respectively, while the values were 13 840 ± 125 M^{-1} s^{-1} and 570 ± 27 M^{-1} s^{-1} for thrombin.

length at pH 6.0 suggest such a bridging mechanism, while this effect is abolished at pH 7.4.

A plausible explanation for this differential behavior can be obtained from heparin acceleration studies. Studies with factor Xa derivatives suggest that the anionic Gla domain, when not neutralized by Ca^{2+} ions, interferes with heparin binding, preventing bridging and acceleration.[44] Thus, at pH 7.4 in the absence of Ca^{2+} ions, acceleration due to bridging is not expected for PAA molecules, while partial or complete neutralization of the Gla residues at pH 6.0 likely induces ternary complex formation resulting in significant acceleration. Finally, it is interesting to note that the acceleration achieved (∼20-fold) at pH 7.4 is similar to that achieved with (+)-CS, a small sulfated molecule that cannot form the ternary complex.[32,33]

Acceleration of Thrombin Inhibition by PAA. The second-order rate constant for PAA-catalyzed thrombin inhibition increased from 13 400 M^{-1} s^{-1} to 793 000 M^{-1} s^{-1} at pH 6.0 and from 1 030 000 M^{-1} s^{-1} to 19 100 000 M^{-1} s^{-1} at pH 7.4 as the chain length increased (Table 2). These correspond to a dramatic increase in acceleration of thrombin inhibition from 24-fold to 1392-fold at pH 6.0 and from 114-fold to 1109-fold at pH 7.4. For full-length heparin, the second-order rate constant for thrombin inhibition was measured to be 37 000 000 M^{-1} s^{-1} corresponding to an acceleration of ∼4000-fold.[40,41] In contrast, low-affinity heparin accelerates antithrombin-dependent thrombin inhibition 60-fold.[21]

The results demonstrate that PAAs are significantly better than low-affinity heparin in accelerating thrombin inhibition. Yet, it is important to recognize that the second-order rate constants derived in this study are obtained at low antithrombin saturations and therefore represent values obtained after significant extrapolations. Detailed mechanistic studies on the order of formation and affinities involved in antithrombin−PAA−thrombin ternary complex are needed to ascertain the accelerations reported here. However, the results demonstrate that PAAs accelerate thrombin inhibition by antithrombin in a chain length-dependent manner at both pHs.

Discussion

Several points about PAA are attractive. Assuming full chain extension and ionization of all carboxylic acid moieties, PAAs carry a charge density of ∼-0.46 charges per Å, which is similar to the average charge density of −0.4−0.5 for heparin. (The average disaccharide in heparin contains 2.5 sulfate groups and a carboxylate, while typical end-to-end distance between C-1 of reducing-end residue and C-4 of nonreducing end residue is in the range of 7-8 Å. Thus the average charge density for heparin turns out to be approximately -0.44-0.5 charges per Å.) The primarily alkyl backbone is flexible; thus, the probability of inducing interaction with antithrombin is high. Finally, modeling shows that the carboxylate groups of PAA can organize to form a linear negatively charged "face", which may engage the heparin-binding site of the inhibitor.[39]

Overall, the results suggest that carboxylate-based polymers, poly(acrylic acid)s, bind and activate antithrombin for accelerated inhibition of factor Xa and thrombin. This principle is critical to establish because it has been widely believed, but not rigorously demonstrated, that replacing sulfates in the antithrombin-binding domain with any other anions nullifies the anticoagulant activity. This understanding arose following the observation that replacing a specific sulfate group of the sequence-specific pentasaccharide DEFGH with a phosphate group dramatically reduces antithrombin binding.[45] The reduced binding affinity could have arisen from the loss of a critical sulfate, or from the introduction of phosphate, or from a subtle conformational change in the activator.

The understanding that sulfated polymers are necessary was also furthered by the observation that whereas polyaspartic acid, a carboxylic acid polymer, was an effective accelerator of heparin cofactor II−proteinase reactions, it failed to enhance antithrombin reactions with thrombin.[46] Modeling reveals that polyaspartate polymer has a charge density of −0.31 charges per Å, approximately 33% lower than PAA. Thus, our results with PAAs are extremely interesting because they demonstrate some structural requirements, specifically the need for appropriate charge density.

Despite this interesting property, one must exercise caution in overinterpreting the results described here. We do not expect PAA to function as anticoagulants in vivo, although an activated partial thromboplastin test of at least one PAA indicated a dose-dependent lengthening of clotting time (not shown). This is because the binding affinities at pH 7.4 are fairly weak (Table 1). In addition, the potentially high charge density of these polymers would suggest that they interact with available cationic proteins, including platelet factor 4. However, the work highlights the need to investigate PAA−antithrombin−thrombin interaction for elucidating design principles.

On the basis of this work, one can predict that antithrombin activators based primarily on carboxylic acid groups may be rationally designed in three steps. These steps include designing carboxylate-based organic

scaffolds that interact with heparin-binding sites in antithrombin and in thrombin, followed by designing a neutral linker that could connect these two domains. Such carboxylic acid-based two-domain organic molecules may provide us with orally bioavailable antithrombin activators.

Conclusions

Our work demonstrates a proof-of-principle that *organic, nonsulfated, nonsaccharidic* molecules, containing only carboxylic acid groups, can bind and activate antithrombin for accelerated inhibition of two critical enzymes, thrombin and factor Xa, of the coagulation cascade.

Experimental Section

Proteins and Chemicals. Human antithrombin and α-thrombin were generous gifts from Professor Steven Olson of the University of Illinois (Chicago). Human factor Xa was purchased from Hematologic Technology (Essex Junction, VT) and 2-(*p*-toluidino)naphthalene-6-sulfonic acid (TNS) from Sigma-Aldrich (Milwaukee, WI). Poly(acrylic acid)s PAA2280, PAA3450, and PAA6200 were acquired from American Polymer Standards (Mentor, OH); PAA1500 was from Polymer Source (Dorval, Quebec).

Fluorescence Spectroscopy and Equilibrium Binding Studies. Fluorescence experiments were performed with a PCI Spectrofluorometer (ISS Instruments, Champaign, IL) at room temperature. Equilibrium dissociation constants (K_D) for the interaction of PAA with plasma antithrombin were determined by titrating the polymer into a solution of antithrombin−TNS complex and monitoring the decrease in the fluorescence at 432 nm ($\lambda_{ex} = 330$ nm). Titrations of 0.95−1.9 μM antithrombin and 5.5 μM TNS were carried out in 20 mM sodium phosphate buffer containing 20 mM NaCl, 0.1 mM EDTA, and 0.1% PEG8000, adjusted to pH 6.0 or 7.4. Two or three averaged measurements were fit to the quadratic equilibrium binding equation to determine the K_D of interaction,[30,31] assuming a 1:1 binding model for the interaction of antithrombin with PAA1500, PAA2280, and PAA3450, and a 2:1 model for PAA6200. Activity of antithrombin was checked before and after titrations to ensure absence of aggregation.

Inhibition of Factor Xa and Thrombin. The rate of PAA-catalyzed antithrombin inhibition of factor Xa or thrombin was determined by monitoring the pseudo first-order rate constant (k_{obs}) as a function of concentration of PAA with 150−450 nM antithrombin and 20−30 nM factor Xa or 150 nM antithrombin and 15 nM thrombin.[34,39] The reactions were carried out in PEG20K-coated cuvettes at 25 °C in 20 mM sodium phosphate containing 20 mM NaCl, 0.1 mM EDTA, and 0.1% PEG8000 at either pH 6.0 or pH 7.4. The k_{obs} at each $[PAA]_O$ was determined by monitoring the residual enzyme activity as a function of time using either Spectrozyme fXa (factor Xa) or S2238 (thrombin) as chromogenic substrates.[30,31,37] The $[PAA]_O$ chosen resulted in antithrombin saturation of 0.5−15% for thrombin and 25−50% for factor Xa. The second-order rate constant k_{cat} of the enzyme inhibition was then determined by fitting k_{obs} dependence on $[PAA]_O$ with equation $k_{obs} = k_{uncat}[AT] + k_{cat}[AT:PAA]$, where the second-order uncatalyzed rate constant k_{uncat} was determined independently and [AT:PAA] was obtained using the quadratic equilibrium binding equation.[34,37]

Acknowledgment. We thank Professor Steven T. Olson (University of Illinois—Chicago) for his critical comments and his generous gift of human factor Xa and thrombin. This work was supportedby grants from the National Heart, Lung and Blood Institute (RO1 HL 069975), the American Heart Association (0256286U and 9960250U), and the A. D. Williams Foundation.

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JM0492960