Antithrombin Activation by Nonsulfated, Non-Polysaccharide Organic Polymer

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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.6–9 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.

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 (OSO₃⁻) 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 differing chain length (Figure 1). We and others have previously exploited this technique to study the interac-
Figure 2. Representative fluorescence titration of antithrombin-TNS complex as a function of PAA1500 at pH 6.0 (●) and pH 7.4 (○). The K₀ 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 (K₀) of PAA−Antithrombin Complexes at pH 6.0 and 7.4

Table 2 lists the second-order rate constants and the acceleration 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

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. The crystal structure of antithrombin–pentasaccharide complex shows the presence of an extended electrostatic 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.

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 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.

Table 2. Second-Order Rate Constants (kcat) and the Acceleration (kcat/kuncat) for Antithrombin Inhibition of Thrombin and Factor Xa in the Presence and Absence of PAA at pH 6.0 and pH 7.4

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 Ca²⁺ ions.42,43 The increase in acceleration with PAA chain...
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 19 100 000 M\(^{-1}\)s\(^{-1}\) at pH 6.0 and from 1 030 000 M\(^{-1}\)s\(^{-1}\) to 210 000 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 at 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 reduc-
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) 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 PCl Spectrofluorometer (ISS Instruments, Champaign, IL) at room temperature. Equilibrium dissociation constants (Kd) 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 (λex = 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 Kd 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 (k1obs) 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.34,35 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 kobs at each [PAA]0 was determined by monitoring the residual enzyme activity as a function of time using either Spectrozyme Ixa (factor Xa) or S2238 (thrombin) as chromogenic substrates.30,31,33 The [PAA]0 chosen resulted in antithrombin saturation of 0.5–15% for thrombin and 25–50% for factor Xa. The second-order rate constant (kcat) of the enzyme inhibition was then determined by fitting kobs dependence on [PAA]0 with equation kobs = kuncat [AT] + kcat [AT·PAA], where the second-order uncatalyzed rate constant kuncat was determined independently and [AT·PAA] was obtained using the quadratic equilibrium binding equation.34,35

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References
(10) Kast, N.; A. Linhardt, R. Critical enzymes, thrombin and factor Xa, of the coagulation cascade.


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