Endogenous Antithrombin Associated with Microvascular Endothelium. Quantitative Analysis in Perfused Rat Hearts†

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ABSTRACT: A recirculating Langendorff heart preparation is used to characterize the endogenous antithrombin associated reversibly with murine vascular endothelium. Rat hearts are perfused clear of blood and then recirculated with a physiological salt solution. Addition of heparin educes antithrombin activity continuously into the perfusate during 6 min of recirculation. This process contrasts with a more rapid equilibration of the system as assessed by displacement of [^{125}I]thrombin with hirudin or with a heparin–antithrombin mixture. Perfusion of washed hearts with [^{125}I]factor Xa, which evidences no significant binding to the coronary endothelium, identifies a minor fraction of the endogenous antithrombin that reacts immediately with factor Xa, i.e., at a rate indicative of heparin enhancement. This rapid-reacting antithrombin is not reproducibly detected with [^{125}I]thrombin, which binds preferentially to thrombomodulin in this system. The amount of antithrombin reacting rapidly with factor Xa is too low to detect as a burst of antithrombin activity eluted into the perfusate when the hearts are perfused with heparin. It is concluded that the murine myocardial microvasculature harbors at least two pools of antithrombin, the minor of which is in an activated configuration characteristic of association with heparin. The major pool is in a more slowly accessible compartment or configuration.

A vascular heparan sulfate–antithrombin anticoagulant system has been invoked as a mechanism to impart thromboresistance to vascular endothelium (Damus et al., 1973). Support for this hypothesis came with observations that thrombin *in vivo* is inhibited rapidly (Lollar & Owen, 1978) and that heparan sulfate proteoglycans capable of activating blood antithrombin were indeed constituents of the endothelium (Busch & Owen, 1982; Marcum et al., 1984; Frebelius et al., 1990). However, subsequent investigations indicated that proteoheparan sulfate enhancement of thrombin inhibition may not be a significant activity at the luminal surface of endothelium (Lollar et al., 1984; Hatton et al., 1986; Delvos et al., 1987). The character and hemostatic significance of the interaction between antithrombin and endothelial-cell-associated proteoglycans has thus remained poorly understood [reviewed by Preissner (1988)]. Some function for proteoglycans in regulation of procoagulants is indicated by the occurrence of thrombotic disease in humans bearing mutations affecting only the heparin binding properties of antithrombin (Ueyama et al., 1990).

In intact aortic segments the primary interaction between antithrombin and heparan sulfate proteoglycans has been localized to the subendothelial matrix (de Agostini et al., 1990), with relatively little antithrombin detected on the luminal surface. Although semiquantitative, this finding has qualified interpretation of experiments using cultured endothelial cell monolayers, which expose much more extracellular matrix to culture media, and invites reevaluation of the role of vascular proteoglycans in hemostasis. Quantitative assessment of the endothelial antithrombin *in vivo* is complicated by an endogenous antithrombin concentration of 2–3 μmol/L (Barrowcliffe et al., 1978; Murano et al., 1980) as well as by protease binding proteins such as thrombomodulin (Esmon & Owen, 1981) and protease nexin I (Baker et al., 1980). The Langendorff heart preparation is a opportune medium for this purpose. A closed, *ex vivo* system, it enables the quantitative use of purified reagents, control over fluid flow parameters, access to the high endothelium-surface-to-blood-volume ratio, and a cryptic abluminal extracellular matrix. Because the heparin–antithrombin interaction, maintained at equilibrium *in vivo* under saturating antithrombin concentrations, is rapidly reversible (Rosenberg & Damus, 1973), the freshly excised coronary vascular bed should retain and release antithrombin much in the manner of an affinity chromatographic medium. In the present study a recirculating Langendorff heart preparation was used to measure and characterize endogenous, active antithrombin associated with microvascular endothelium.

EXPERIMENTAL PROCEDURES

Materials. Bovine albumin, dextran (av mol wt 9400), sodium dodecyl sulfate, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES),[1] and lyophilized *Vipera russelli* venom were purchased from Sigma Chemical Company, St. Louis, MO. Hanks' balanced salt solution (HBSS) powder was purchased from Gibco Laboratories, Grand Island, NY. The peptide nitroanilide substrates Spectrozyme FXa and Spectrozyme FIIa were products of American Diagnostica, New York, NY. Heparin (Schein Pharmaceuticals, Inc., Port Washington, NY) had an average molecular weight of 16 000 (Owen & Owen, 1990). Iodo-gen (1,3,4,6-tetrachloro-3*a*,6*a*-diphenylglycouril) was obtained from Pierce Chemical Company, Rockford, IL.

Proteins. Published methods were used to prepare bovine thrombin (Owen, 1975), antithrombin (Owen & Owen, 1990),

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[1] Abbreviations: HBSS, Hanks' balanced salt solution; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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factor X (Owen & Owen, 1990), and the factor X clotting protein (X-CP) from *Vipera russelli* venom (Kisiel et al., 1976). Recombinant hirudin was a gift from Dr. Robert Wallace of Ciba-Geigy, Bern, Switzerland. All proteins were judged homogeneous by gel electrophoresis in sodium dodecyl sulfate. Thrombin and factor X were labeled with ^{125}I by the Iodo-gen method (Salacinski et al., 1979). Factor X (2 μg/mL) was activated by incubation with X-CP (120 ng/mL) in buffer containing 0.1 mol/L Tris-HCl, 50 mmol/L NaCl, and 10 mmol/L $CaCl_2$, pH 7.4, for 20 min. The reaction was stopped with EDTA (10 mmol/L), and the [^{125}I]factor Xa was purified on a Pharmacia Mono-Q column developed with a gradient of 0.1–0.6 mol/L NaCl (20 mmol/L Tris-HCl, pH 7.5). The [^{125}I]thrombin and [^{125}I]factor Xa were judged homogeneous by sodium dodecyl sulfate–polyacrylamide gel radioelectrophoresis and were ≥95% active with peptide nitroanilide substrates.

Myocardium Perfusion. A modified Langendorff heart preparation (Langendorff, 1897) was used to perfuse rat myocardial microvasculature. The perfusion solution (HBSS, 1 g/L glucose, and 10 mmol/L HEPES, pH 7.4, saturated with O_2) entering the heart was maintained at 37 ± 2 °C. Hearts were warmed with a 40-W incandescent lamp placed 25 cm away, to maintain a perfusate temperature of approximately 32 °C. Perfusion pressure was 50 Torr. Endogenous blood was perfused from the myocardium for 6 min, and then the system was switched to a recirculating mode by a three-way valve so that the perfusate, collected in a polyethylene sump, was pumped back through the heart. The perfusate was not supplemented with O_2 during recirculation. The total recirculating volume (tubing, sump, and heart) was 7.1 ± 0.2 mL as estimated by pumping the heart dry into a graduated cylinder. Reagents were added and samples were withdrawn at the sump. Heart rates for the 6 min preceding recirculation ranged from 120 to 180 beats per minute. Preparations were considered inviable when the heart rate halved or when the hearts became appreciably dilated.

Assays. Antithrombin activity in perfusates was measured as factor Xa inhibitory capacity. Perfusate samples (50 μL) were incubated at room temperature with 1.2 nmol/L factor Xa and 1 μmol/L heparin in buffer containing 0.1 mol/L Tris-HCl, 0.1 mg/mL albumin, and 50 mmol/L NaCl, pH 7.5 (total volume = 100 μL). After 12 min, 200 μL of 0.15 mmol/L Spectrozyme FXa was added to the incubating mixture, and the change of absorbance at 405 nm with time was determined with a Molecular Devices V_{max} plate reader. Rates were compared to a standard curve obtained with authentic antithrombin of known concentration.

Antithrombin was assayed directly by reaction with radiolabeled thrombin or factor Xa. Samples (90 μL) from hearts perfused with radioiodinated enzymes were combined with 10% sodium dodecyl sulfate (10 μL), heated for 1 min at 90 °C, and subjected to $NaDodSO_4$–gel radioelectrophoresis (10–15% gradient). Radioautographs were obtained with a Kodak X-Omat film and developing process. A standard on each radioautograph consisted of the radiolabeled protease diluted to approximate the initial concentration of the protease recirculating in the perfusion. Optical densities of bands, measured with a video densitometer (Harmon & Owen, 1986), were compared to a standard curve derived from radioautographs bearing excess radiolabeled protease complexed with known concentrations of antithrombin (100 fmol/L to 10 μmol/L). Radioautographs were computer-enhanced to highlight faint bands with the Enhance graphics program of Micro Frontier, Des Moines, IA.

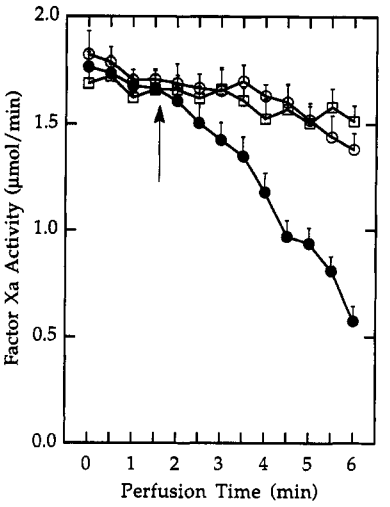

FIGURE 1: Elution of antithrombin activity from the perfused heart. Beginning at the switch to the recirculating mode (0 min), perfusate samples were withdrawn from the sump every 30 s for 6 min. Immediately after the fourth sample (1.5 min) was taken, 20 μL of a solution of 9 mmol/L heparin (●), 9 mmol/L dextran (□), or HBSS (○) was added to the perfusate in the sump.

RESULTS

When the Langendorff preparations were switched to the recirculating mode, antithrombin activity in the perfusate remained undetectable or increased to barely detectable concentrations during the 6 min perfusion (Figure 1). Adding dextran to the system did not alter this behavior. Addition of heparin to the system, however, elicited significant additional antithrombin activity. Comparison to standards of known antithrombin concentrations indicates that the perfusate antithrombin concentrations at the end of 6 min in the buffer and dextran control perfusates were 0.046 ± 0.004 and 0.055 ± 0.003 nmol/L antithrombin, respectively. Addition of heparin to the hearts eluted 1.1 ± 0.1 nmol/L antithrombin by the end of the perfusion. With the volume of the recirculating system at 7.1 ± 0.2 mL, the maximal amount of endogenous active antithrombin displaced from the murine myocardium after 6 min with 25 μmol/L heparin is 0.077 ± 0.01 nmol.

Radioelectrophoretic analysis of perfusions with radioiodinated proteases yielded values similar to those of the enzyme inhibition assays and in addition revealed a minor pool of fast-reacting antithrombin not resolved by the data in Figure 1. The radioautographs for hearts equilibrated with [^{125}I]factor Xa (Figure 2) show the band at 45 kDa corresponding to factor Xa plus an additional band appearing immediately and having a mobility corresponding to that of factor Xa–antithrombin. Neither the factor Xa band nor the factor Xa–antithrombin band changed in intensity during the course of control perfusions (Figure 2A, lanes 2–7). On addition of heparin (Figure 2B, lane 3) the band corresponding to complex intensified at the expense of that of factor Xa, to yield a density corresponding to that obtained with 0.80 nmol/L purified antithrombin. Because the concentration of recirculating factor Xa (1.0 nmol/L) was close to that of endogenous antithrombin, the factor Xa–inhibitor complex band has significant density relative to that of the factor Xa band.

In the gels from the [^{125}I]thrombin-equilibrated hearts (Figure 3), bands at 37 kDa are present initially in all perfusions and, in contrast to factor Xa, decrease in intensity as thrombin binds in the myocardium. Bands of complex at 92 kDa, faint

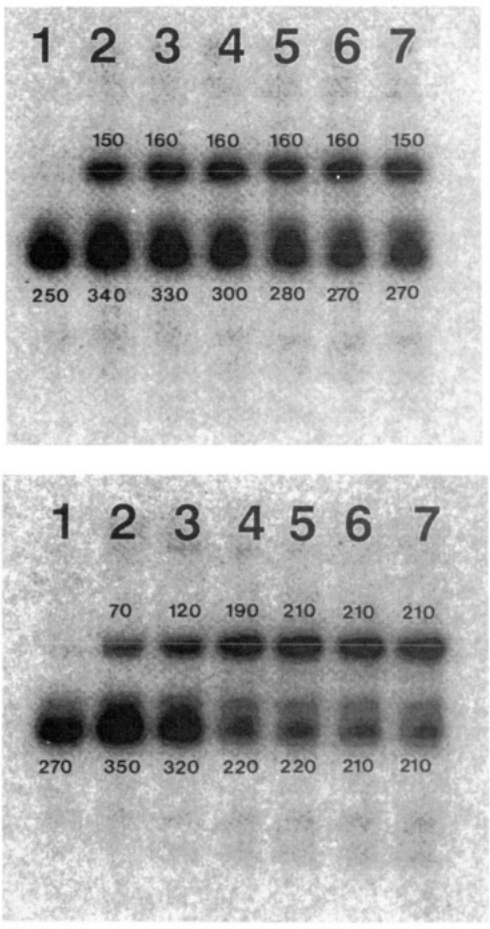

FIGURE 2: Perfusion of hearts with [^{125}I]factor Xa. In each SDS-PAGE radioautograph, the first lane shows a sample containing 1.0 nmol/L [^{125}I]factor Xa. Immediately upon recirculation, [^{125}I]factor Xa was added to the sump at a dilution calculated to yield 1 nmol/L. After 0.5 min and then at 1-min intervals thereafter, samples withdrawn from the sump were added to 1/10 vol of 10% NaDodSO$_4$. Immediately after withdrawal of the second sample (lane 3), 20 μL of HBSS (A, top) or heparin (B, bottom) was added to the sump. The numbers just above and below the upper and lower bands, respectively, are the digitized densitometries in arbitrary units.

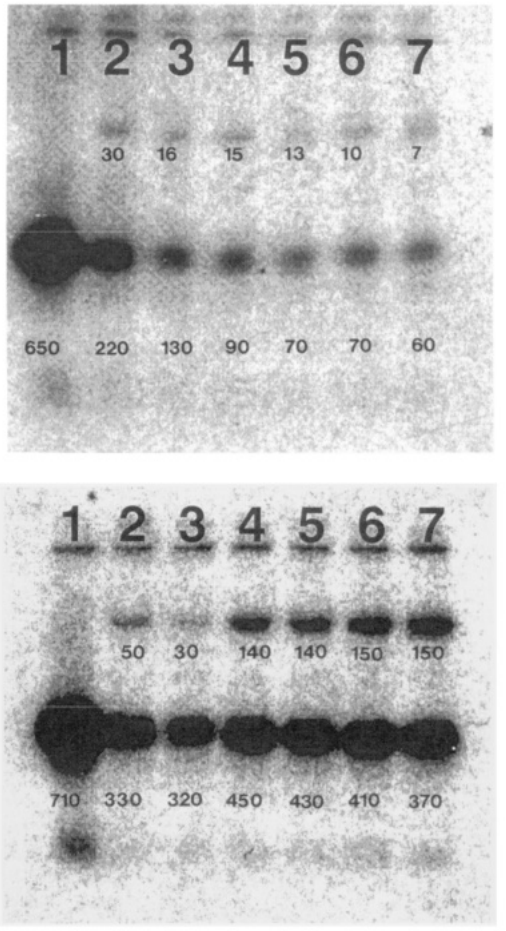

FIGURE 3: Perfusion of hearts with [^{125}I]thrombin. In each radioautograph, the first lane shows a sample containing 6 nmol/L of [^{125}I]thrombin. Immediately upon recirculation, [^{125}I]thrombin was added to the sump at a dilution calculated to yield 6 nmol/L. Sampling intervals and additions of [^{125}I]thrombin (diluted to 6 nmol/L) and HBSS (A, top) or heparin (B, bottom) were carried out as described for Figure 2. The numbers just below the bands are the digitized densitometries in arbitrary units.

relative to those obtained with [^{125}I]factor Xa, appear prior to any reagent addition and remain relatively constant throughout the perfusion (Figure 3A) until addition of heparin, which elicits an increase in intensity of the band of the protease–inhibitor complex in parallel with an increase in free thrombin (Figure 3B). By comparison to standards, the concentration of the protease–inhibitor complex prior to addition of exogenous heparin in the perfusates of the [^{125}I]thrombin ranged from barely detectable levels to 0.02 nmol/L (n = 12), substantially less than the yield obtained with [^{125}I]factor Xa. However, the concentration of heparin-induced radiolabeled complex averaged 0.40 ± 0.1 nmol/L (n = 12), or 0.003 nmol per heart, reproducibly less than that obtained with factor Xa; the relative dominance of the protease band on the gels reflects the higher (6 nmol/L) total thrombin concentration in the system, relative to that of factor Xa in Figure 2.

Sampling of perfusates over a time scale of minutes presupposes concomitantly rapid equilibration of reagents throughout the recirculating perfusion system. In hearts equilibrated with [^{125}I]thrombin, addition of hirudin (a noncovalent inhibitor) elicited the maximum increase in the 37-kDa thrombin band within 30 s of recirculation (Figure 4A). Likewise, the formation of [^{125}I]thrombin–antithrombin complex is essentially complete 30 s after addition of a heparin–antithrombin mixture, as evidenced by the protease–inhibitor complex band at 92 kDa (Figure 4B). The rapid equilibration evidenced by the immediate appearance of thrombin or complex apparent in Figure 4, and seen in Figures 2 and 3 as well, was verified by quantitative densitometry of the Figure 4 radioautographs (Figure 5). Relative to the continuous increase in antithrombin activity (extracted from Figure 1), thrombin was eluted quantitatively by either hirudin or antithrombin–heparin by the first sampling at 30 s.

DISCUSSION

The existence at endothelial surfaces of antithrombin cofactor activity conventionally ascribed to heparin has been a difficult issue to resolve (Preissner, 1988). Although proteoglycans bearing heparin-like moieties are associated with endothelial cell surfaces, their physiological role at these surfaces has not been established. Viewed radiohistochemically as antithrombin binding activity, most of the proteoglycan appears localized around the subendothelial cell basement membrane (de Agostini et al., 1990), with a smaller but indeterminate fraction at the luminal interface.

The approach to the ways that anticoagulant proteoglycans might regulate hemostasis at the interface between undamaged endothelium and blood hinges on how much, if any, activity is expressed at the interface. Quantitative analysis using

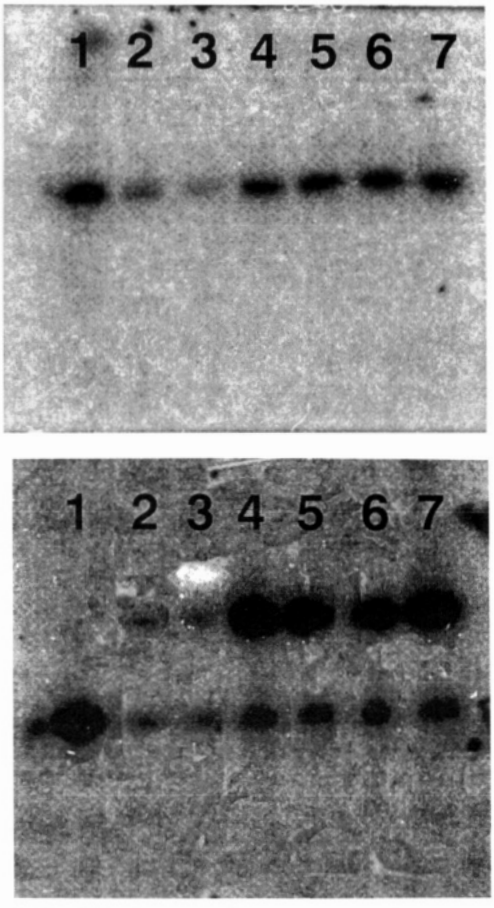

FIGURE 4: Equilibration rate of the recirculating perfusion system. Hearts were perfused (recirculation) with 6 nmol/L of [^{125}I]thrombin. Lanes 2 and 3 show samples taken after 0.5 and 1.5 min of recirculation. Immediately after the second sample was withdrawn (lane 3), 100 μL of a solution of 1 mg/mL hirudin (A, top) or 200 μL of a mixture of 1 μmol/L heparin and 1 μmol/L antithrombin (B, bottom) was added. The next sample was taken 30 s later (lane 4), and samples were then taken each minute thereafter (lanes 5–7).

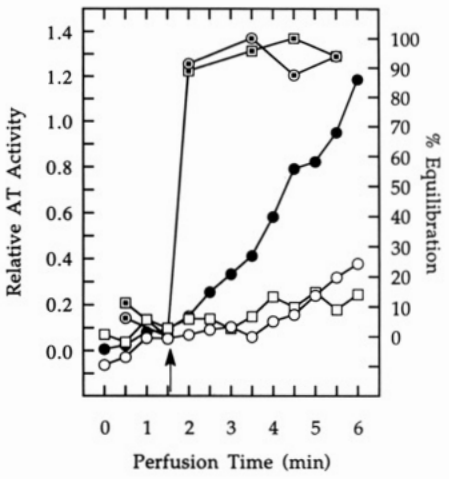

FIGURE 5: Comparison of rates of system equilibration with rate of endogenous antithrombin elution. Densitometry values for the gel radioautographs (Figure 5) showing the displacement of [^{125}I]-thrombin from the heart by hirudin (■) and heparin-antithrombin (■) normalized to a 100% scale. The data in Figure 1 were used to calculate the relative antithrombin activity eluted from the heart after addition of HBSS (○), dextran (□) and heparin (●).

thrombin as a reactant has proven difficult to interpret because of the impact of thrombomodulin on the measurements. Furthermore, the magnitude of antithrombin cofactor activity would reflect *net* antithrombin binding capacity of endothelium equilibrated physiologically with the full complement of plasma heparin binding proteins. We reasoned first that factor Xa, which binds neither to thrombomodulin (Esmon & Owen, 1981) nor (with significant affinity) to heparin (Jordan et al., 1980; Owen & Owen, 1990), might act as a reliable bulk-phase reactant. Second, if vascular heparin binds antithrombin with an affinity near that of isolated heparin (Nordenman & Bjork, 1978), a perfused microvascular bed should retain endogenous antithrombin with sufficient affinity (i.e., retention time) to enable its measurement. The amount retained should also reflect the capacity of the retained antithrombin to have competed *in vivo* with other plasma heparin-binding proteins. An analogous rationale is the basis for the use of heparin–agarose to affinity purify antithrombin, which can be eluted by mobile-phase heparin (Pomerantz & Owen, 1978) as well as salt.

Freshly obtained rat myocardium contains at least two pools of vascular antithrombin. Perfusion with heparin yielded about 0.008 nmol (or 1 nmol/L of recirculating perfusate) of antithrombin that was not eluted with physiological salt solutions. Unless exogenous heparin was added, no more than about 0.002 nmol of endogenous vascular antithrombin reacted rapidly with excess factor Xa, which itself showed no tendency to associate with the endothelium; at the reactant concentrations in the perfusates, bulk-phase reaction of factor Xa and antithrombin is negligible. On this basis, the intravascular concentration (moles of surface antithrombin per liter of perfusate) of antithrombin–proteoglycan complex could be no more than about 1/40 the thrombomodulin concentration in comparable preparations (Lollar et al., 1982). This pool of activated antithrombin is clearly visible in Figure 2B, but was not of sufficient magnitude to be resolved reproducibly as a burst after perfusion with heparin (Figure 1). Minor differences between the actual and nominal factor Xa concentrations would serve to obscure the burst phase in averaged perfusions. The yet smaller amount of antithrombin reacting rapidly with thrombin follows first from the association of most thrombin with thrombomodulin, which effectively partitions the thrombin from bound antithrombin, and then from the incomplete reaction of thrombin with antithrombin in solutions containing high concentrations of heparin.

The kinetics of elution of the major portion of antithrombin, slow relative to the equilibration rate of the system, suggest that most of the antithrombin arose from a compartment, such as the subendothelium, where diffusion through the endothelium would moderate egress. If subendothelial, which would follow from the findings of de Agostini et al. (1990), then elution must entail some penetration of the exogenous heparin into the subendothelial compartment to displace the antithrombin. Immediate reaction of a minor fraction of vascular antithrombin with factor Xa at the luminal surface suggests that the activated inhibitor has some capacity for catabolism of circulating procoagulants other than thrombin, which partitions to the more abundant thrombomodulin. Although the chemical nature and precise location of the site to which the rapid-reacting antithrombin is bound remain unknown, the reaction kinetics suggest a small amount, relative to the subendothelial pool, of heparan proteoglycan located on the luminal surface of the endothelium.

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