Proc. Natl. Acad. Sci. USA
Vol. 78, No. 2, pp. 829–833, February 1981
Biochemistry

Multiple functional domains of the heparin molecule

(mucopolysaccharide/anticoagulant function)

GARY M. OOSTA, WILLIAM T. GARDNER, DAVID L. BEELER, AND ROBERT D. ROSENBERG

Sidney Farber Cancer Institute, Beth Israel Hospital, and Harvard Medical School, 44 Binney Street, Boston, Massachusetts 02115

Communicated by John F. Enders, October 27, 1980

ABSTRACT **Affinity-fractionated porcine heparin was randomly scissioned by chemical techniques to give hexasaccharides, octasaccharides, decasaccharides, and mucopolysaccharide fragments of ≈14 residues and ≈16 residues that were able to complex with the protease inhibitor. Direct measurements of the kinetic behavior of the hexasaccharides, octasaccharides, and decasaccharides showed that these fractions greatly enhanced the rate of Factor Xa inactivation by antithrombin. Indeed, these species exhibited specific molar activities that ranged from 6.9% (hexasaccharide) to 60.9% (decasaccharide) of that of the heparin fragment of ≈16 residues. However, these oligosaccharides exhibited essentially no ability to accelerate thrombin–antithrombin interactions. The avidity of the hexasaccharides, octasaccharides, and decasaccharides for the protease inhibitor increased as a function of size with the respective dissociation constants ranging from 5.5×10^{-6} M to 2.9×10^{-7} M. These data suggest that the region of the heparin molecule needed for catalyzing Factor Xa–antithrombin interaction is intimately related to the antithrombin binding domain. The smallest complex carbohydrate fragment that accelerated the inactivation of thrombin by antithrombin had ≈14 residues. This fraction had an avidity for the protease inhibitor of 2.8×10^{-7} M and specific molar activities of 140 units per μmol (thrombin neutralization) and 460 units per μmol (factor Xa inactivation). The largest heparin fragment examined contained ≈16 residues. This fraction had an avidity for antithrombin of 2.4×10^{-7} M and specific molar activities of 500 units per μmol (thrombin neutralization) and 560 units per μmol (Factor Xa inactivation). Detailed kinetic analyses showed that these two species are able to directly activate antithrombin to the same extent with respect to thrombin inhibition. However, the larger mucopolysaccharide fragment is also capable of approximating free enzyme with protease inhibitor.**

Only a small fraction of a given heparin preparation binds tightly to antithrombin, and this fraction is largely responsible for its anticoagulating activity (1, 2). Evidence has been provided that a unique tetrasaccharide sequence containing two nonsulfated uronic acid moieties is found almost exclusively in the highly active heparin, and it has been suggested that this portion of the mucopolysaccharide must represent a binding site that is recognized by antithrombin (3, 4). These findings have been confirmed by others (5). In this communication, we show that relatively small oligosaccharides can complex tightly with the protease inhibitor and dramatically accelerate Factor Xa–antithrombin but not thrombin–antithrombin interactions. We also show that larger mucopolysaccharide fragments are essential for approximating enzyme with protease inhibitor or activating antithrombin such that it can rapidly neutralize thrombin. Based on these data, we propose that heparin possesses multiple structural domains that modulate different functions of antithrombin.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

MATERIALS AND METHODS

The mucopolysaccharide concentrations of the samples were determined by uronic acid assay (6). The activity of the heparin fractions with respect to Factor Xa inactivation was measured by a two-stage assay. First, the procedure was initiated by adding a known amount of mucopolysaccharide to a solution containing Factor Xa and antithrombin, each at a final concentration of 0.227 μM. The environmental conditions were 0.136 M NaCl in 0.09 M Tris·HCl (pH 7.5) and 37°C. After the reaction mixture was incubated for 45 sec, Polybrene and S-2222 substrate (Ortho Diagnostics, Raritan, NJ) in water were added to final concentrations of 0.31 mg/ml and 3.75 mg/ml, respectively. Then, the solutions were incubated for 90 sec at 37°C and enzymatic activity was quenched by addition of glacial acetic acid to a final concentration of 6.7 M. The extent of substrate amidolysis was determined by measuring absorbance at 405 nm, using unfractionated preparation of heparin with a known USP potency as a reference standard. All assays were performed in triplicate.

The potency of the mucopolysaccharide samples with respect to thrombin inhibition was also determined by a two-stage assay. The first stage of this procedure was identical to that described above, except that thrombin and antithrombin were used at final concentrations of 0.127 μM and 0.272 μM, respectively, and the reaction mixture was incubated for only 12 sec. The second stage of this technique was similar to that described above, except that Polybrene and S-2160 substrate (Ortho Diagnostics) in water were added to final concentrations of 1.0 mg/ml and 26 μg/ml, respectively, and the reaction mixture was incubated for 96 sec before the action of the enzyme was quenched.

Human thrombin, human Factor Xa, human antithrombin, and bovine antithrombin were prepared in homogeneous form as described (7–9). The heparin preparations were porcine in origin and were provided by Riker Laboratories (Northridge, CA) (specific anticoagulating activity of 150 USP units/mg). The anticoagulant fraction was obtained by mixing complex carbohydrate and bovine antithrombin, each at a final concentration of 7.5 mg/ml. The environmental conditions were 0.15 M NaCl in 0.01 M Tris·HCl (pH 7.5). Heparin capable of binding to protease inhibitor was separated from free mucopolysaccharide and then from protein as described (2). The final product was a mixture of fragments having a variety of molecular sizes. Highly active heparin of M_r ≈6500 was also prepared from porcine mucopolysaccharide by affinity fractionation but with limiting amounts of human antithrombin (2).

The initial rates of thrombin inactivation by antithrombin were determined in the presence of mucopolysaccharide fragments at enzyme and inhibitor concentrations of 0.01 μM and oligosaccharide concentrations of 0.01–10 μM. The environ-

mental conditions were 0.15 M NaCl in 0.01 M Tris·HCl (pH 7.5). After the reaction mixture was incubated for 0–75 sec, the neutralization process was quenched and the residual amounts of thrombin were quantitated as described (8). The kinetic data were analyzed by fitting the initial level of thrombin, E_0, and all subsequent concentrations of residual enzyme, E, observed at times t to the equation

$$\frac{1}{E_0} - \frac{1}{E} = Kt.$$

The time points used included the first 30–50% of the reaction, with associated r values of 0.809–0.996. The initial velocity of the reaction at a given concentration of heparin or oligosaccharide is represented by $K[E_0]^2$ (2, 7, 8).

A series of determinations of this parameter at various levels of mucopolysaccharide allowed us to calculate the bimolecular rate constant for the interaction between free thrombin and antithrombin saturated with heparin or oligosaccharide (k_1), as well as the rate constant for the interaction between enzyme and protease inhibitor when both are saturated with heparin or oligosaccharide (k_2). Indeed, it has been shown that the maximal peak of this profile is $\approx k_1 E_0^2$ and the subsequent minimal plateau (if present) is $\approx k_2 E_0^2$. Absence of the latter phase of the plot indicates that $k_2 = k_1$ (2, 7, 8).

RESULTS

Approximately 300 mg of affinity-fractionated heparin were cleaved to a mixture of different molecular size fragments by treatment with limiting amounts of nitrous acid and then with dilute sulfuric acid. The mucopolysaccharide was dissolved in 0.05 M $NaNO_2$ in 0.2 M sodium citrate buffer (pH 1.5) to a final concentration of 50 mg/ml. After the reaction mixture was incubated for 30 min at 0°C, the pH of the solution was adjusted to ≈8, and the entire sample was chromatographed on a 1.8 × 200 cm Bio-Gel P-10 column equilibrated with buffer A (0.5 M NH_4HCO_3, pH 7.6). Under these conditions, the addition of nitrous acid to heparin results predominately in the formation of 2-aldehydo-D-pentofuranose residues (deaminative ring contracture) rather than of 2,5-anhydro-D-mannose moieties (deaminative ring cleavage). The mucopolysaccharide fragments recovered from the column were pooled and lyophilized to dryness. Then, the 2-aldehydo-D-pentofuranoside–uronic linkages were hydrolyzed by dissolving the lyophilate in 18 ml of distilled H_2O, adjusting the pH of the solution to 1.9, and incubating the reaction mixture for 30 min at 50°C. The sample was brought to pH ≈8, concentrated to ≈3 ml by rotary evaporation, and rechromatographed at ≈14 ml/hr on a 1.8 × 200 cm Bio-Gel P-10 column equilibrated with buffer A (Fig. 1). Comparison of the peak elution volumes with those of sulfated disaccharides and tetrasaccharides of known molecular structure permitted us to ascertain the dimensions of the two components of lowest molecular size. Additional assignments were based on the assumption that nitrous acid cleavage of mucopolysaccharide occurs in relatively random fashion and releases products having multiple numbers of disaccharide units.

Initially, oligosaccharides of a specific size were prepared by combining fractions from given regions of the above chromatogram, lyophilizing each of these pools, and individually rechromatographing the various species at flow rates of ≈3 ml/hr on 0.5 × 200 cm Bio-Gel P-10 columns equilibrated with buffer A. The recoveries of hexasaccharide, octasaccharide, and decasaccharide fragments averaged 12.6 mg, 12.3 mg, and 11.2 mg, respectively.

Subsequently, the oligosaccharides were affinity fractionated

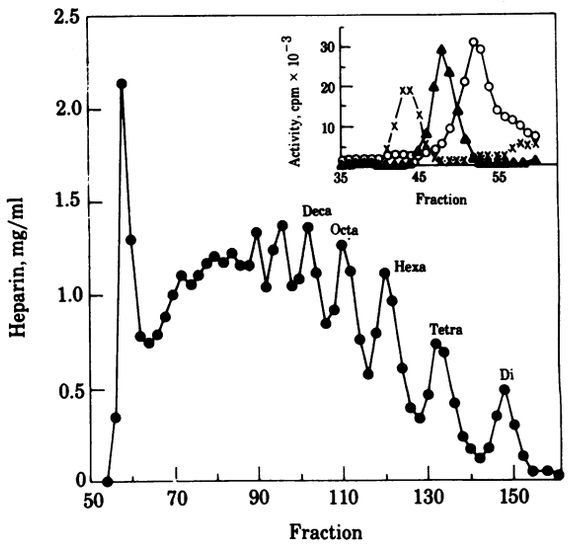

FIG. 1. Elution profile of oligosaccharide fractions. Porcine heparin was treated with nitrous acid at pH 1.5 and then sulfuric acid at pH 1.9, and the product was chromatographed on Bio-Gel P-10 (●). The volume of each fraction is 2.8 ml. (*Inset*) Activities after Bio-Gel P-10 chromatography of the hexasaccharide (○), octasaccharide (▲), and decasaccharide (×) fragments.

to obtain fractions that interacted strongly with antithrombin. These isolations were begun by mixing equimolar quantities of a given heparin fragment and bovine protease inhibitor. Environmental conditions were buffer B (0.15 M NaCl in 0.01 M Tris·HCl, pH 7.5) and 24°C. Species capable of binding to antithrombin were separated from free mucopolysaccharide and then from protease inhibitor as described (2). The recoveries of hexasaccharide, octasaccharide, and decasaccharide fractions averaged 0.71 mg, 1.27 mg, and 1.54 mg, respectively. The end groups of the affinity-fractionated heparin fragments were aldehyde reduced with 0.1 M NaB^3H_4 at pH 8.5, and the resultant labeled products were chromatographed at flow rates of ≈3 ml/hr on 0.5 × 200 cm Bio-Gel P-10 columns equilibrated with buffer A (see Fig. 1 *Inset*).

These three products were used to examine the mucopolysaccharide-dependent acceleration of Factor Xa–antithrombin and thrombin–antithrombin interactions. The avidities of the fragments for the protease inhibitor were determined by a solid-phase competitive binding assay using unlabeled oligosaccharides or mucopolysaccharides and ^{125}I-labeled highly active heparin. We found that the hexasaccharide, octasaccharide, and decasaccharide fragments possessed a significant capacity to accelerate Factor Xa neutralization by antithrombin compared with that of larger heparin fragments isolated from the same mucopolysaccharide hydrolysate (Table 1). The various forms of these fragments showed a substantial increase in this biologic activity as a function of size. In addition, the three oligosaccharides exhibited essentially no ability to enhance the rate of thrombin inactivation by antithrombin compared with that of the larger heparin fragments. Furthermore, they had a considerable avidity for the protease inhibitor that increased as a function of size. Indeed, the $K_d^{H\text{-}A}$ of the decasaccharide approaches that of the larger fragments.

We next attempted to define the smallest heparin fragment that is able to catalyze thrombin–antithrombin interactions. All components with an apparent molecular size greater than a dodecasaccharide (fractions 50–91 of Fig. 1) were combined, concentrated by rotary evaporation, and rechromatographed at a flow rate of ≈3 ml/hr on a 0.5 × 200 cm Sephadex G-50 column

Table 1. Kinetic behavior and binding constants of mucopolysaccharide fragments

	Factor Xa inhibitory activity		Thrombin inhibitory activity		
	Specific molar activity*	Final concentration, μM	Specific molar activity*	Final concentration, μM	$K_d^{H\text{-}A}$, M
Hexasaccharide	38.5	0.30	<0.1	15	5.5×10^{-6}
Octasaccharide	221	0.053	<0.1	21	8.0×10^{-7}
Decasaccharide	341	0.026	<0.1	13	2.9×10^{-7}
Heparin fragments of ≈14 residues	460	0.023	140	0.018	2.8×10^{-7}
Heparin fragments of ≈16 residues	560	0.021	500	0.017	2.4×10^{-7}

Highly active heparin has a Factor Xa-specific molar activity of 1840 units/μmol, a thrombin-specific molar activity of 2750 units/μmol, and a $K_d^{H\text{-}A}$ of 5.7×10^{-8} M. The larger affinity-purified mucopolysaccharide fragments possess similarly designated kinetic and binding parameters that are lower in magnitude (one-third to one-fifth). These phenomena may be secondary to the more selective separation schema used to isolate the highly active heparin. All kinetic and binding experiments were conducted with human antithrombin. The values cited represent the average of two separate determinations. The interactions of the various mucopolysaccharide fragments with human antithrombin were quantitated by a competitive solid-phase binding assay using ^{125}I-highly active heparin. The environmental conditions were 0.15 M NaCl in 0.01 M Tris·HCl (pH 7.5). The molar reaction stoichiometry and $K_d^{H\text{-}A}$ of the ^{125}I-highly active heparin were set at 8.3 and 83 nM, respectively. These parameters were obtained by performing direct binding determinations with radiolabeled, highly active heparin in the above assay system and are in excellent accord with those derived by other techniques (7). The molar stoichiometries of the unlabeled ligand were assumed to be one. However, small changes in this parameter would have little effect on our estimates of $K_d^{H\text{-}A}$. The experimental data were analyzed by a single-site, competitive binding model. The values of $K_d^{H\text{-}A}$ cited above represent an average of at least two separate determinations.

* Expressed as units/μmol. It should be noted that these fractionations were not conducted with limiting amounts of antithrombin; therefore, the values above represent minimal estimates of specific molar activity.

equilibrated with buffer A (Fig. 2). Examination of the various fractions to determine their abilities to accelerate thrombin neutralization by antithrombin showed that the mucopolysaccharide fragments of ≈14 monosaccharide units had this property but that the maximal specific activity was at 18–20 monosaccharide units, as judged by comparison with the elution position of highly active heparin of M_r ≈6500.

The kinetic properties of the fractions that catalyze thrombin-antithrombin interactions were examined in considerable detail. Fractions 40–41 (heparin fragments of ≈16 residues) and fraction 43 (heparin fragments of ≈14 residues) were affinity chromatographed by using bovine antithrombin as described above. The recoveries of mucopolysaccharide fragments from these two pools that were able to complex with protease inhibitor averaged 26% and 30%, respectively. As shown in Table 1 the two affinity-fractionated species were able to significantly accelerate both thrombin–antithrombin and Factor Xa–antithrombin interactions. However, heparin fragments of ≈16 residues possessed considerably greater activity than heparin fragments of ≈14 residues with respect to thrombin inactivation. Furthermore, the avidity of the larger species for antithrombin was slightly greater than that of the smaller species.

The detailed kinetics of the thrombin neutralization were next investigated. The results of a typical experiment in which the initial rates of thrombin inhibition by antithrombin were determined as a function of the concentration of heparin fragments of ≈16 residues are shown in Fig. 3. These species possess a kinetic profile that is similar to that obtained with highly active heparin (2, 7, 8). Indeed, the plot of reaction velocity as a function of the level of mucopolysaccharide exhibits an ascending limb, a pseudoplateau, a descending limb, and a final plateau. It has been shown that the initial phases of this process correlate with the formation of larger amounts of heparin–antithrombin complex, whereas the final phases are gen-

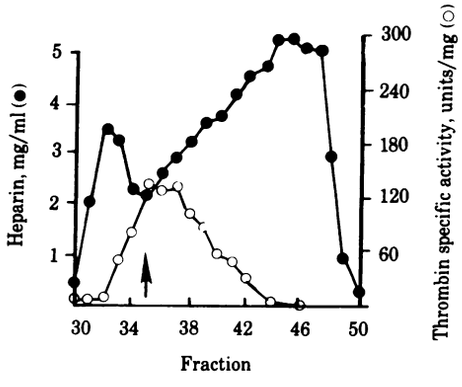

FIG. 2. Elution profile of fractions from Sephadex G-50 chromatography of fractions 54–91 of Fig. 1. The volume of each fraction is 0.72 ml. The arrow indicates the peak elution position of highly active heparin of 18–20 monosaccharide residues.

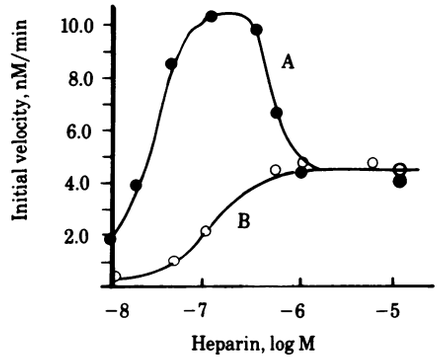

FIG. 3. Kinetic properties of heparin fragments that accelerate thrombin–antithrombin interactions. The initial rates of enzyme inactivation by protease inhibitor were examined in the presence of heparin fragments of ≈16 residues (curve A) or ≈14 residues (curve B), respectively. Thrombin adsorption to the vessels used in these experiments was evaluated in the presence of the above mucopolysaccharide fragments. The initial velocities of enzyme inactivation have been corrected to remove this artifactual effect. Each point in the kinetic profile represents either a single evaluation of initial velocity or the average of two separate determinations.

erated by increasing levels of mucopolysaccharide–thrombin interaction product (7, 8). Based on the above analyses, two characteristic parameters can be extracted from the kinetic data. On the one hand, the maximal value of initial velocity in the pseudoplateau region provides an estimate of the second-order rate constant (k_1) for the neutralization of thrombin by the heparin–antithrombin complex. On the other hand, the minimal initial velocity in the final plateau region allows us to compute the second-order rate constant (k_2) for the inactivation of the mucopolysaccharide–thrombin interaction product by the heparin–antithrombin complex. For heparin fragments of ≈16 residues, these two kinetic parameters are 1.0×10^8 M^{-1} min^{-1} and 4.6×10^7 M^{-1} min^{-1}, respectively.

The kinetic data obtained by quantitating the initial rates of thrombin neutralization by antithrombin as a function of the concentration of heparin fragments of ≈14 residues are also shown in Fig. 3. It is of particular interest that the initial velocity plot has only an ascending limb and a plateau region. Previous considerations of reaction mechanism have suggested that the descending limb and final plateau regions are absent when free enzyme and mucopolysaccharide–enzyme interaction product are neutralized at the same rate by heparin–antithrombin complex ($k_1 = k_2$). Under these conditions, the maximal value of initial velocity in the plateau region allows us to calculate k_1. For heparin fragments of ≈14 residues, this parameter is 4.6×10^7 M^{-1} min^{-1}.

DISCUSSION

We have randomly cleaved preparations of heparin having anticoagulating activity by using appropriate chemical techniques. The resultant species were separated by gel filtration chromatography. The smallest fragments that exhibited a capacity to bind to antithrombin were found in the hexasaccharide, octasaccharide, and decasaccharide regions. These fractions were further purified by affinity fractionation with equimolar amounts of the protease inhibitor.

Our studies showed that the hexasaccharide, octasaccharide, and decasaccharide species bind to antithrombin with progressively increasing avidity. Indeed, the latter oligosaccharide exhibits a $K_d^{H\text{-}A}$ that approaches that obtained when components of ≈16 residues are used. Our investigations also showed that the hexasaccharide, octasaccharide, and decasaccharide species are able to catalyze Factor Xa–antithrombin interactions at rates that range from 6.9% (smallest component) to 60.9% (largest component) of that obtained when heparin fragments of ≈16 residues are used. It should be emphasized that these components were assayed at virtually identical saturations of the protease inhibitor (≈5%). Thus, the specific molar biologic activities observed for the various oligosaccharides represent the effect of equivalent numbers of mucopolysaccharide molecules bound to protease inhibitor. The kinetic and binding parameters of the three mucopolysaccharide fragments suggest that the structures essential for accelerating Factor Xa–antithrombin interactions are closely linked to those required for complexing with antithrombin. Preliminary chemical data suggest that this critical region is represented in large measure by the tetrasaccharide sequence reported previously (4). Indeed, this unique monosaccharide sequence appears to be located on the nonreducing ends of the hexasaccharide, octasaccharide, and decasaccharide species. Our observations are strengthened by the recent findings of others concerning the partial structure of a heparin-derived oligosaccharide that binds tightly to antithrombin (10, 11).

Given the above, it was surprising to note that the three oligosaccharides had essentially no effect on the rate of inactivation of thrombin by antithrombin. These negative results are particularly impressive because sufficiently high concentrations of these components were used to saturate the protease inhibitor. Furthermore, heparin fragments of ≈14 monosaccharide residues or greater are capable of catalyzing thrombin inactivation by antithrombin (see below). These experimental observations suggest that carbohydrate sequences that lie outside the primary antithrombin binding domain must be essential for accelerating the neutralization of the above enzyme by the protease inhibitor.

Previous studies have shown that mucopolysaccharide-induced rate enhancement of the above reaction is dependent on the direct binding of heparin to antithrombin, as well as on the interaction between free enzyme and complex carbohydrate attached to protease inhibitor (approximation effect) (8). The latter phenomenon appears to be responsible for no more than 1–2% of the direct effect of heparin on antithrombin (7, 8). However, the approximation effect significantly augments the overall rate enhancement of enzyme neutralization because it acts in multiplicative fashion with the direct effect of the mucopolysaccharide (7, 8). On the basis of this information, we surmised that the heparin fragments of ≈14 monosaccharide units or greater must possess additional structural elements that approximate free enzyme with protease inhibitor and directly or indirectly activate antithrombin with respect to thrombin neutralization.

Initially, molecular species of the smallest possible dimensions (≈14 residues) that are able to catalyze thrombin inactivation by antithrombin were isolated. The resultant fragments had a k_1 of 4.6×10^7 M^{-1} min^{-1}. This kinetic parameter remains unchanged at high levels of mucopolysaccharide, at which potential approximation sites on the serine protease are saturated with complex carbohydrate (2, 7, 8). These findings imply that the above component must possess the ability to accelerate thrombin-antithrombin interactions because of a direct effect on the protease inhibitor. Furthermore, this mucopolysaccharide species appears to lack the structural regions needed to approximate free enzyme with antithrombin (see Fig. 3).

Subsequently, heparin fragments of somewhat larger size (≈16 residues) were obtained. The resultant species bind to the protease inhibitor with a slightly greater avidity than heparin fragments of ≈14 residues and have a k_1 of 1.0×10^8 M^{-1} min^{-1}. This kinetic parameter decreases significantly at high levels of complex carbohydrate but attains a constant value at heparin concentrations in excess of 1×10^{-6} M. Data obtained from the latter region allow us to calculate that the mucopolysaccharide has a k_2 of 4.6×10^7 M^{-1} min^{-1}, an estimate that represents the rate of inactivation of heparin–thrombin interaction product by heparin–antithrombin complex. Given that potential approximation sites on the serine protease must be saturated with mucopolysaccharide under these conditions, k_2 provides a quantitative measure of the direct kinetic effect of heparin on antithrombin. This parameter is identical in magnitude to that of k_1 obtained for the smaller complex carbohydrate fragment. These experimental findings suggest that the two forms of the mucopolysaccharide possess similar capacities to directly activate antithrombin with respect to thrombin neutralization. However, the larger species of heparin contains an additional structural element that is required for approximating free enzyme with protease inhibitor. Therefore, this component is able to more rapidly accelerate the interaction of thrombin with antithrombin. The results of experiments in which heparin fragments of ≈14 and ≈16 residues were degraded by using a purified endoglucuronidase derived from human platelets suggest that the monosaccharide sequences responsible for activating antithrombin with respect to thrombin neutralization

and for approximating free enzyme with protease inhibitor must lie on the nonreducing end of the critical tetrasaccharide.

In summary, our results suggest that heparin possesses multiple discrete structural domains that are responsible for its ability to complex with antithrombin and to modulate the biologic properties of the protease inhibitor. The absence or improper alignment of one or more of these regions within a heparin population would explain the previously reported differential actions of mucopolysaccharide preparations with regard to the neutralization of various hemostatic enzymes (11, 12).

Note Added in Proof. We have also analyzed the ability of the various mucopolysaccharide fragments to accelerate Factor IXa–antithrombin as well as Factor XIa–antithrombin interactions. The assay systems used to obtain these data monitor the release of ^{3}H-labeled activation peptides from Factor IX or Factor X. These methods are similar to those developed by Nemerson and coworkers and have been previously utilized in our laboratory (8). Our results indicate that hexasaccharide, octasaccharide, and decasaccharide species exhibit minimal biologic activities only slightly greater than those observed with thrombin. Furthermore, oligosaccharide fragments of $\approx$16 monosaccharides or greater are required to accelerate neutralization of Factor IXa or Factor XIa by antithrombin. These observations considerably strengthen our model of multiple functional domains within the heparin molecule and further suggest the existence of functional heterogeneity within the activation region of the mucopolysaccharide that lie on the nonreducing side of the tetrasaccharide-binding domain.

This work was supported by National Institutes of Health Grant HLB-19131.

1. Lam, L. H., Silbert, J. E. & Rosenberg, R. D. (1976) *Biochem. Biophys. Res. Commun.* **69**, 570–577.
2. Jordan, R., Beeler, D. & Rosenberg, R. D. (1979) *J. Biol. Chem.* **254**, 2902–2914.
3. Rosenberg, R. D., Armand, G. & Lam, L. H. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 3065–3069.
4. Rosenberg, R. D. & Lam, L. H. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 1218–1222.
5. Lindahl, U., Bäckström, G., Höök, M., Thunberg, L., Fransson, L. Å. & Linker, A. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 3198–3202.
6. Bitter, T. & Muir, H. M. (1962) *Anal. Biochem.* **4**, 330–334.
7. Jordan, R., Oosta, G. M., Gardner, W. T. & Rosenberg, R. D. (1980) *J. Biol. Chem.* **255**, 73–80.
8. Jordan, R., Oosta, G. M., Gardner, W. T. & Rosenberg, R. D. (1980) *J. Biol. Chem.* **255**, 81–90.
9. Nordenman, B., Nyström, C. & Björk, I. (1977) *Eur. J. Biochem.* **78**, 195–203.
10. Thunberg, L., Bäckström, G., Grundberg, H., Riesenfeld, J. & Lindahl, U. (1980) *FEBS Lett.* **117**, 203–206.
11. Choay, J., Lormeau, J. C., Petitou, M., Sinay, P., Casu, B., Oreste, P., Torri, G. & Gatti, G. (1980) *Thromb. Res.* **18**, 573–578.
12. Thunberg, L., Lindahl, U., Tengblad, A., Laurent, T. C. & Jackson, C. M. (1979) *Biochem. J.* **181**, 241–243.