Articles

Two-Dimensional Affinity Resolution Electrophoresis Demonstrates That Three Distinct Heparin Populations Interact with Antithrombin III†

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ABSTRACT: Heparin is a polydisperse, highly sulfated polysaccharide consisting of repeating 1 → 4 linked uronic acid and glucosamine sugar residues that binds to coagulation proteins, complement proteins, and growth factors to regulate a variety of biological activities. Heparin is best known as an anticoagulant, an activity that results largely from a specific pentasaccharide sequence in heparin that interacts with a unique site in antithrombin III. Little is known about additional structures within heparin that might interact with antithrombin III or the heparin structures that interact with the myriad of other heparin-binding proteins and peptides. Unfractionated glycosaminoglycan heparin that had been prepared from porcine intestinal mucosa was examined for its capacity to bind antithrombin III using a new technique developed to quantitate that interaction. Two-dimensional affinity resolution electrophoresis is a powerful method that allows assessment of unique species of heparin molecules that bind to protein, allowing determination of heparin molecular weight for each protein-binding heparin species as well as the dissociation constant of each interaction. This study provides the first definitive evidence that glycosaminoglycan heparin contains at least three populations of molecules with affinity for antithrombin III. Furthermore, the affinity of each heparin species for antithrombin III appears to vary inversely with the size of the heparin chain, with some smaller oligosaccharides having greater affinity for antithrombin III than larger oligosaccharides.

Heparin is synthesized by mast cells and basophils as a proteoglycan having a protein core to which approximately 10 polysaccharide chains of molecular weight 100 000 are attached (Horner & Young, 1982). The heparin that is released on degranulation of mast cells has been degraded through the action of *endo*-β-D-glucuronidase and proteinases into raw heparin. Pharmaceutical or glycosaminoglycan heparin has been widely used as an anticoagulant since 1939 (Böttinger, 1987) and is prepared from porcine intestine or bovine lung by bleaching and oxidizing the raw heparin to remove residual core peptide (Casu, 1985; Comper, 1981; Linhardt, 1991). Commercial processing of heparin (Coyne, 1977) results in a mixture of glycosaminoglycan chains with diverse structure (Lasker, 1977; Nader *et al.*, 1981) and varying lengths (Lasker, 1977) with an average M_r of 9000−15 000 (Comper, 1981), making it polydisperse and heterogeneous.

Heparin binds to a variety of proteins with a wide spectrum of affinities and specificities (Cardin & Weintraub, 1989; Casu, 1985; Jackson *et al.*, 1991; Linhardt & Loganathan, 1990; Steinbuch, 1982). These interactions often result in a modification of the activity of the protein, causing either inhibition or enhancement of activity. The best studied of these interactions has been the binding of heparin with antithrombin III (AT III)[1] (Atha *et al.*, 1984; Griffith, 1986; Jackson *et al.*, 1991; Lindahl *et al.*, 1983; Rosenberg, 1985) which has been reported to have a dissociation constant (k_d) between 11 nM (Lee & Lander, 1991) and 1 μM (Dawes, 1988; Olson *et al.*, 1981; Rosenberg, 1985). A pentasaccharide within heparin has clearly been shown to be the high-affinity binding site for interaction with AT III (Atha *et al.*, 1984; Lindahl *et al.*, 1983). However, only about 30% of the heparin chains in glycosaminoglycan heparin are thought to contain these high-affinity sites; the other 70% lack the critical 3-*O*-sulfate group (Jackson *et al.*, 1991) and are thought to interact with AT III through lower affinity sites. The long polysaccharide chains of proteoglycan heparin may contain as many as five AT III binding sites (Horner, 1989).

In this paper we report the use of a new affinity electrophoresis technique called two-dimensional affinity resolution electrophoresis (2DARE) to examine the interac-

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[1] Abbreviations: AT III, antithrombin III; k_d, dissociation constant; 2DARE, two-dimensional affinity resolution electrophoresis; Hep-NAc, *N*-desulfated, *N*-acetylated heparin; HAH, high-affinity heparin; LAH, low-affinity heparin; MAH, medium-affinity heparin.

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tion of heparin with AT III. Affinity electrophoresis is a general technique to examine the interaction of essentially any two molecules with affinity for each other provided one moves in an electrical field (Heegaard & Bøg-Hansen, 1990; Horejsi, 1979, 1981, 1986, Horejsi & Kocourek, 1974; Shimura, 1990). Affinity electrophoresis couples the specificity of affinity chromatography with the facility of gel electrophoresis. Lee and Lander (Lee & Lander, 1991) pioneered the use of affinity electrophoresis to study the interaction of heparin with a variety of proteins including basic fibroblastic growth factor, fibronectin, and AT III. Lee and Lander's (Lee & Lander, 1991) single-dimensional approach requires using radiolabeled heparin, is unable to examine chain length of heparin interacting with proteins, and is not able to show directly the presence of distinct populations of heparin with varied affinity for AT III. The 2DARE method, based on Lee and Lander's approach, was devised (1) to enable visualization without using modified or radiolabeled heparin, (2) to examine the impact of heparin chain length on its interaction with AT III, and (3) and to resolve heparin into populations with varied affinities for AT III. Thus, 2DARE permits both the determination of the k_d of the interaction and the simultaneous determination of the M_r of the heparin chains in each interaction with AT III, leading to an improved understanding of the importance of heparin chain length to the affinity of heparin for AT III.

MATERIALS AND METHODS

Materials. Capillary coagulation tubes [150 mm (internal diameter, <1 mm)], ammonium hydroxide, sodium acetate, and sodium citrate were from Fisher Scientific (Fair Lawn, NJ). Low melting point agarose and glacial acetic acid were from Mallinckrodt (Paris, KY). The first-dimensional electrophoresis unit was a Hoefer SuperSub horizontal gel electrophoresis unit, Model HE 100, from Hoefer Scientific (San Francisco, CA). Acrylamide, ammonium persulfate, boric acid, glycine, piperazine diacrylamide, tris(hydroxymethyl)aminomethane, a Protean II xi multigel casting chamber, and a Protean II xi multigel electrophoresis unit were from Bio-Rad (Hercules, CA). Porcine mucosal heparin (163 units/mg) and an anti-Xa-heparin assay kit were from Kabi Pharmacia (Franklin, OH). Alcian yellow, 1,4-dimethylpiperazine, silver nitrate, and sodium thiosulfate were from Aldrich Chemical (Milwaukee, WI). Bromophenol blue was from MCB Manufacturing Chemists (Cincinnati, OH). Bovine lung heparin (146 units/mg), 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate, 3-(*N*-morpholino)-2-hydroxypropanesulfonic acid (sodium salt), and agarase (2500 units/mg) were purchased from Sigma Chemical Co. (St. Louis, MO). Disodium ethylenediaminetetraacetate and sucrose were from Boehringer Mannheim Biochemicals (Indianapolis, IN). Methanol was from EM Science (Gibbstown, NJ). AT III was purified to homogeneity as described by McKay (McKay, 1981). Porcine mucosal and bovine lung heparins were partially depolymerized using heparin lyase I that had been isolated and purified as described previously (Lohse & Linhardt, 1992; Oosta *et al.*, 1981).

The 2DARE System (Figure 1). Capillary tubes were filled with protein in 1% agarose and 0.5% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate in 50 mM 3-(*N*-morpholino)-2-hydroxypropanesulfonic acid, sodium salt, and 125 mM sodium acetate, pH 7.4 (buffer A) (Lee & Lander, 1991) at 37 °C. The agarose was allowed to gel for 15 min,

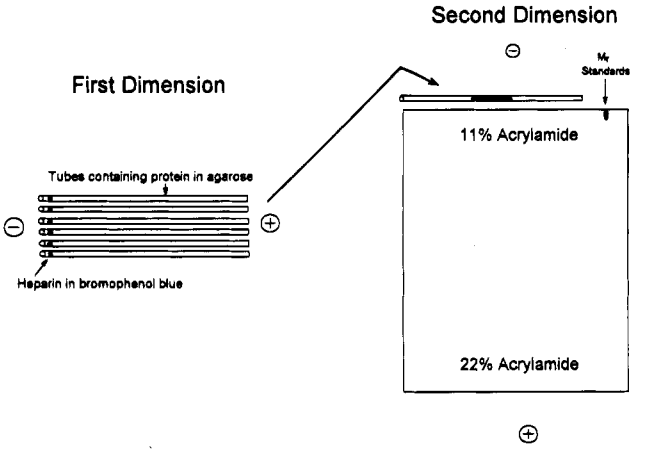

FIGURE 1: The 2DARE method. Capillary tubes were filled with protein in 1% agarose in buffer A [50 mM 3-(*N*-morpholino)-2-hydroxypropanesulfonic acid, sodium salt, and 125 mM sodium acetate, pH 7.4] at 37 °C. The agarose was allowed to gel, and heparin (in buffer A, 0.1% bromophenol blue, and 6% sucrose) was added to one end of the tube gel. The heparin was moved by electrophoresis through the protein-containing gel. The agarose tube gels were then laid on the stacking gels of gradient polyacrylamide gels (11% → 22%) so that the origin of each tube gel was exactly lined up with one edge of the polyacrylamide gel as shown. The tube gels were overlaid with more stacking gel to hold them in place, and the heparin was subjected to electrophoresis into the second-dimensional gel. Partially depolymerized heparin was used as a molecular weight standard (a "ladder"), to allow subsequent determination of the chain length of heparin. The polyacrylamide gels were stained with 0.1% alcian yellow followed by silver staining using ammoniacal silver. Photographic images were made of each gel and used during the analysis of each experiment.

and heparin (in buffer A that was 0.1% in bromophenol blue and 6% in sucrose) was added to one end of the tube gel (the tube gel origin). Bromophenol blue was used as tracking dye. Then, 3−5 μL 0.5% agarose in buffer A (60 °C) was layered over the heparin to prevent the heparin solution from spilling out of the end of the tube when laid horizontal. The remainder of the space between the agarose and the end of the tube was filled with buffer A. The tubes were held horizontal in the first-dimensional electrophoresis unit (a modified Hoefer SuperSub horizontal gel electrophoresis unit containing two Plexiglas blocks with holes to hold 12 parallel tubes) (Edens, 1994). The tube gels were submerged in Buffer A (4 °C), and electrophoresis was conducted at a constant voltage of ~75 V using an ISCO Model 453 power supply at ≤25 mA/tube. During electrophoresis, heparin (negatively charged) moved through the protein-containing gel toward the positive electrode.

Gradient polyacrylamide gels were prepared as described previously (Rice *et al.*, 1987) except that 11% and 22% polyacrylamide monomer solutions [made from 30% acrylamide/1% piperazine diacrylamide stock solution (Hochstrasser *et al.*, 1988), with the final monomer solutions containing 0.4 M tris(hydroxymethyl)aminomethane, 0.03% sodium thiosulfate, 34 mM 1,4-dimethylpiperazine (Hochstrasser & Merril, 1988), and 0.06% ammonium persulfate, pH 8.8] were used and 12 gels were simultaneously cast in the multigel casting chamber. A 5% polyacrylamide stacking gel was added to the top of each gradient polyacrylamide gel [5% polyacrylamide in 100 mM boric acid, 100 mM tris(hydroxymethyl)aminomethane, 10 mM disodium ethylenediaminetetraacetate, 27 mM 1,4-dimethylpiperazine, and 0.04% ammonium persulfate, pH 6.3, filling approximately

1 cm]. The tube gels were laid on the stacking gel so that the origin of the tube gel was exactly lined up with one edge of the polyacrylamide gel. The tube gels were overlaid with 1−2 mL of stacking gel to hold them in place during the second-dimensional electrophoresis. Partially depolymerized bovine lung heparin (500 ng dissolved in buffer A that was 0.1% in bromophenol blue and 6% in sucrose) was used as a molecular weight standard (a "ladder"), to allow subsequent determination of the molecular weight of heparin species (Edens *et al.*, 1992; Rice *et al.*, 1987). The polyacrylamide gels were fitted to the cooling cores of the Protean II xi unit and placed in the lower buffer chamber [100 mM boric acid, 100 mM tris(hydroxymethyl)aminomethane, and 10 mM disodium ethylenediaminetetraacetate, pH 8.3] in the Protean II xi at 4 °C. Buffer [0.2 M tris(hydroxymethyl)aminomethane and 1.25 M glycine, pH 8.3] was added to the upper chamber of each cooling core, and electrophoresis was conducted at a constant current of ≤30 mA/gel (typically at 400−500 V) until tracking dye moved to within 5−6 cm of the bottom of the gel (4−6 h). The polyacrylamide gels were stained with 0.1% alcian yellow in 1% acetic acid for 18−24 h. Excess alcian yellow was removed with washes of 5% acetic acid, then 50% methanol, and then distilled water. The alcian yellow stained heparin in the polyacrylamide gels was silver stained using ammoniacal silver for 1 h in a manner similar to that described by Hochstrasser *et al.* (Hochstrasser *et al.*, 1988). Development usually required 7−12 min. After development, the gels were placed in stop solution for 2−3 h and stored in distilled water until photographed. Photographic images (1:1) were printed for each gel and used during analysis of each experiment.

2DARE Analysis of Interaction of 1 μg of Heparin with AT III. To examine the interaction of heparin with AT III, 1 μg of heparin was subjected to horizontal electrophoresis through agarose tube gels containing varied concentrations of AT III (6.16, 4.93, 3.69, 3.07, 2.16, 1.85, 1.43, 1.22, 0.72, 0.36, or 0.14 μM or no AT III). Then, the contents of the tube gels were each placed on a gradient polyacrylamide gel and subjected to the second-dimensional (vertical) electrophoresis procedure. The gels were stained, and 1:1 photographic images were made. Borders were drawn around each heparin population, and measurements of the distance from the top of the polyacrylamide gel and from the side of the polyacrylamide gel corresponding to the origin of the agarose tube gel to various points in each of the heparin populations were recorded. The distance of the heparin from the side of the gel yielded information about the affinity of the heparin for the protein, and the distance that the heparin moved into the gradient polyacrylamide gel in the second dimension was inversely proportional to the molecular weight of the heparin in that population. The molecular weight markers contained on each gel were used to calculate the molecular weight of the heparin present at various points in each heparin population as previously described (Edens, 1994).

Derivation of Equations Used To Calculate k_d of the Interaction of Heparin with Protein during a 2DARE Analysis. The mobility of heparin through the gel matrix containing AT III has been described previously (Shimura, 1990) as

$$\frac{1}{m - m_0} = \left(\frac{k_d}{m_1 - m_0}\frac{1}{p_f}\right) + \frac{1}{m_1 - m_0} \quad (1)$$

where m is the measured mobility, m_0 is the mobility of the heparin in the absence of AT III, m_1 is the mobility of the heparin with one AT III molecule bound, p_f is the concentration of unbound AT III in the gel, and k_d is the dissociation constant for the binding of the AT III to the heparin. By the mass balance and equilibrium equations it can be shown that

$$\frac{k_d}{p_f} + 1 = \frac{1}{2p_t}[k_d + h_t + p_t + \sqrt{(k_d + h_t + p_t)^2 - 4p_t h_t}] \quad (2)$$

where p_t is the total concentration of AT III in the gel and h_t is the estimated concentration of the heparin in the gel as described above.

Let $y = 1/(m - m_0)$ and $A = 1/(m_1 - m_0)$. If $F(k_d;p_t,h_t)$ is defined to be the right-hand side of eq 2, then $y = AF(k_d;p_t,h_t)$ is the predicted value of $1/(m - m_0)$, given data p_t, h_t, and some assigned value of k_d. The optimal value of k_d is found by minimizing the sum of squares S of the deviation of the predicted values of y (namely, $AF(k_d;p_t^i, h_t^i)$ from the observed values of y (namely, y^i):

$$S = \sum_i |y^i - AF(k_d;p_t^i,h_t^i)|^2 \quad (3)$$

(The superscript i denotes the i^{th} data set.) This sum is minimized by first varying A (with k_d fixed) and then varying k_d. The minimum in A occurs at

$$A_{min} = \frac{\sum y^i F^i}{\sum (F^i)^2}, \quad \text{where } F^i = F(k_d;p_t^i,h_t^i) \quad (4)$$

Inserting the value $A = A_{min}$ into eq 3 (note that A_{min} is a function of k_d and the data) gives

$$S = \sum_i |y^i - A_{min}F(k_d;p_t^i,h_t^i)|^2 \quad (5)$$

The value of k_d that minimizes S is obtained by plotting k_d *vs* S using the software package MATLAB (The Mathworks, Natick, MA). Thus, knowing the protein and heparin concentrations and measuring the distance that the heparin migrated as it moved by electrophoresis through the protein, we were able to calculate the k_d of the heparin binding to the protein.

Heparin, Affinity Resolved by Electrophoresis through AT III and then Subjected to 2DARE. Experiments were conducted to demonstrate that the migration patterns of heparin through AT III−agarose resulted from interaction of the heparin with the AT III and were not simply an artifact of the electrophoresis. Heparin (1 μg) was subjected to electrophoresis in several identical tube gels prepared to contain 5.2 μM AT III. The gels were removed from the tubes, lined up evenly, cut into 12 1.2-cm sections, and placed in Eppendorf tubes labeled 1−12 corresponding to sections from origin to end of the tube gels, respectively. In each Eppendorf tube, the AT III was denatured by heating to 90 °C for 15 min and the agarose was digested with 250 units of agarase by incubating at 37 °C for 6 h. Agarose disaccharides were removed by dialysis against distilled water using 3500 molecular weight cutoff dialysis tubing, and the samples were lyophilized and dissolved in 5 μL of buffer A containing 0.1% bromophenol blue and 6% sucrose. A 2-μL

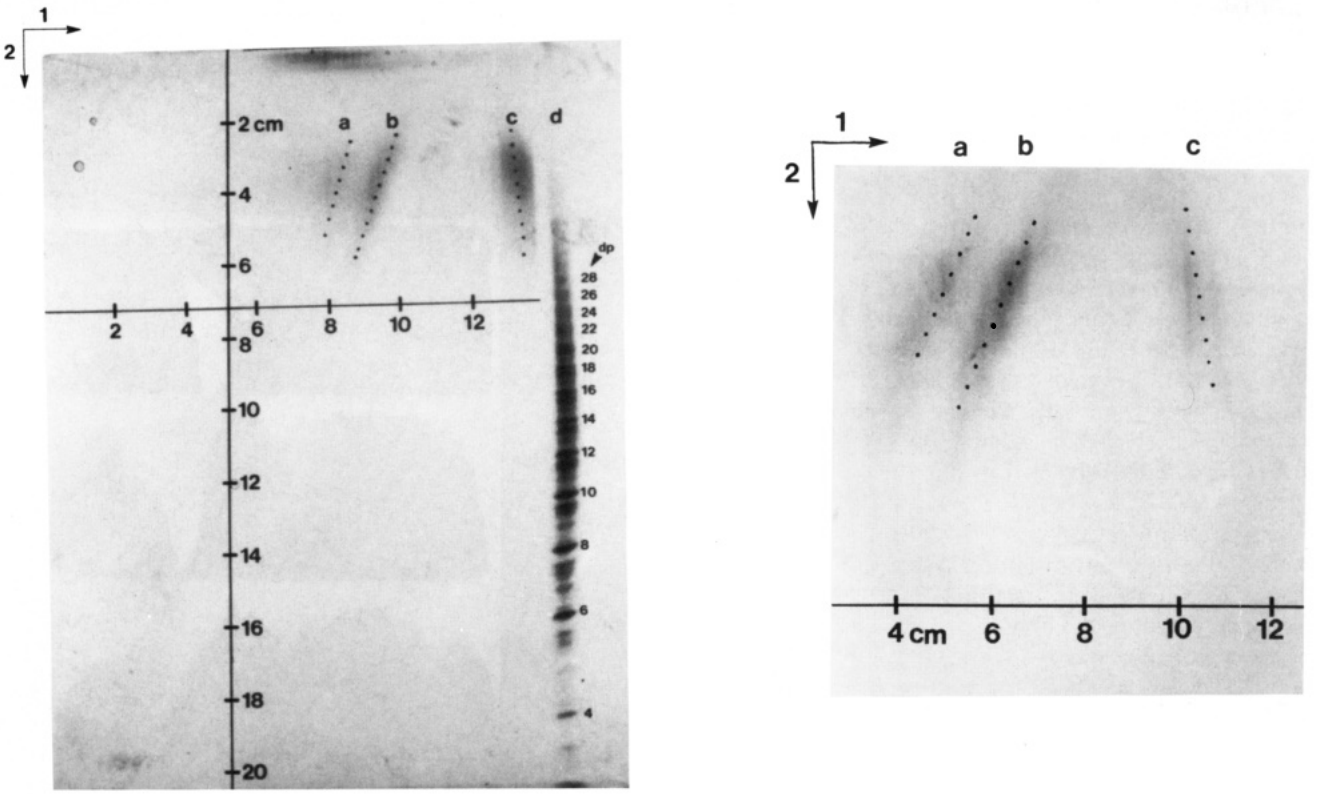

FIGURE 2: 2DARE analysis of interaction of 1 μg of heparin with AT III. Heparin (1 μg) was subjected to electrophoresis through agarose tube gels containing varied concentrations of AT III (6.16, 4.93, 3.69, 3.07, 2.16, 1.85, 1.43, 1.22, 0.72, 0.36, or 0.14 μM or no AT III). Then, the tube gels were placed on gradient polyacrylamide gels and subjected to the second-dimensional electrophoresis of the resolution electrophoresis procedure. The gels were stained, and 1:1 photographic images were made (panels A, left, and B, right). (A) The second-dimensional gel after heparin had moved through 1.43 μM AT III in the first dimension. (B) Close-up view of heparin populations from another second-dimensional gel in which heparin moved through 4.93 μM AT III in the first dimension. Borders were drawn around each heparin population, and measurements of distances from the top of the polyacrylamide gel and from the side of the polyacrylamide gel corresponding to the origin of the tube gel to various points in each of the heparin populations were recorded (see grid on panel A). The distance of the population from the side of the gel (horizontal grid on gel) yields information about the affinity of the heparin for the AT III: (a) the heparin chains that were the most retarded in their movement through the AT III are designated "high-affinity heparin" (HAH); (c) chains that were the least retarded are designated "low-affinity heparin" (LAH); (b) chains that had an intermediate affinity are designated "medium-affinity heparin" (MAH). The distance that the heparin moves into the gradient polyacrylamide gel in the second dimension was proportional to the M_r of the heparin in that population. The M_r markers (d) contained on each gel were used to calculate the M_r (Edens *et al.*, 1992) of the heparin present at various points in each population (a–c).

aliquot of each of the 12 samples was resubjected to 2DARE as described above (Figure 1). The anti-Xa anticoagulant activities of the heparin present in each of the 12 fractions were also determined (Linhardt *et al.*, 1986).

2DARE Analysis of Partially Depolymerized Heparin, Affinity-Fractionated Heparin, and Modified Heparin with Decreased Anticoagulant Activity. To further examine the effects of heparin size on its interaction with AT III, heparin lyase I (Lohse & Linhardt, 1992) was used to partially depolymerize porcine intestinal mucosal heparin (3 μg) (Rice *et al.*, 1987) and the oligosaccharide products were analyzed by 2DARE as described for glycosaminoglycan heparin using either 18 μM AT III or no AT III in the agarose tube gels. Heparin that had been fractionated by AT III affinity chromatography and chemically modified heparin were used to examine the interaction of AT III with heparins possessing varied anticoagulant activity. (1) Heparin was fractionated by affinity chromatography using Con-A–Sepharose to which AT III had been immobilized (noncovalently) as previously described (Bae *et al.*, 1994; Denton *et al.*, 1981), and then 1 μg of each heparin population was subjected to 2DARE analysis through 5.7 μM AT III in agarose tube gels. (2) Hep-NAc (*N*-desulfated, *N*-acetylated heparin) having greatly reduced anticoagulant activity was prepared as previously described (Weiler *et al.*, 1992). Hep-NAc (1 μg) was analyzed using 2DARE as described above with either 5.7 μM AT III or no AT III in the agarose tube gels.

RESULTS

2DARE Analysis of Heparin Interaction with AT III. To examine the interaction of heparin with AT III, 1 μg of porcine intestinal mucosal heparin was subjected to electrophoresis through agarose in tube gels containing varied concentrations of AT III (6.16, 4.93, 3.69, 3.07, 2.16, 1.85, 1.43, 1.22, 0.72, 0.36, or 0.14 μM or no AT III). The contents of the tube gels were then subjected to the second-dimensional gradient polyacrylamide gel electrophoresis (Figure 1). The gels were stained, and 1:1 photographic images were made. Figure 2A shows a representative second-dimension polyacrylamide gel in which 1 μg of heparin was applied to a capillary tube that contained 1.43 μM AT III. Figure 2B shows a close-up view of heparin populations from another second-dimension gel in which 1 μg of heparin was applied to a capillary tube that contained 4.93 μM AT III in the first dimension. The distance of the heparin from the side of the gel (horizontal grid on gel) yields information about the affinity of the heparin for the AT III: (a) the heparin chains that were the most retarded in their

movement through the AT III are designated "high affinity heparin" (HAH); (c) chains that were the least retarded are designated "low-affinity heparin" (LAH); (b) chains that appear to have an intermediate affinity are designated "medium-affinity heparin" (MAH).

When heparin was applied to capillary tubes in which there was no AT III, the heparin migrated as a single population and at a rate approximately equal to the LAH population (area c in Figure 2). The observation of three heparin populations as well as the angled pattern of the heparin populations was highly reproducible and was observed in more than 50 gels. Each time the lowest M_r chains in both HAH and MAH were most retarded in their movement through AT III, while the lowest M_r chains in the LAH population were the least retarded. When low concentrations of AT III were used (0.72 μM), only two populations of heparin were observed (not shown) because the higher affinity chains migrated almost as rapidly as the low-affinity chains. Likewise, when even lower concentrations of AT III were used in the first dimension (0.36 and 0.14 μM AT III), all resolution was lost in the first dimension and the heparin appeared as a broad smear (not shown) that moved almost as rapidly as the LAH population shown in Figure 2B, area c.

We drew borders around each heparin population and measured the distance from the side of the polyacrylamide gel corresponding to the origin of the agarose tube gel to various points in each of the heparin populations (see grid in Figure 2A). To determine the k_d of the interaction of heparin with AT III, 1 μg of heparin was subjected to electrophoresis through a series of tubes containing varied concentrations of AT III. These measurements were then used to calculate the k_d of the heparin−AT III interaction as described in the Materials and Methods section. These calculations of the k_d revealed that the MAH−AT III interaction had a k_d of 80 nM and the k_d of the LAH−AT III interaction was >1 μM. The k_d of the HAH−AT III interaction was not calculated using the above equations because these equations presuppose one binding site on heparin for AT III. However, these data suggest the possibility of more than one binding site on HAH molecules. Thus, to calculate accurately the k_d of the HAH−AT III interaction, new equations will be derived. This will require additional experiments which are in progress and are beyond the scope of the present report.

The distance that the heparin moved into the gradient polyacrylamide gel in the second dimension was inversely proportional to the M_r of the heparin chains in that population. The M_r markers (d in Figure 2A) run on each gel were used to calculate the M_r (Edens *et al.*, 1992) of the heparin chains present at various points in each heparin population (Figure 2, areas a−c). The M_r range of the heparin used in these experiments was ~7500−19 000, and we have extrapolated these M_r values from the markers that we used (Edens *et al.*, 1992). The ranges of the M_r for the three populations of heparin resolved during the 2DARE analysis were HAH = 11 700−19 300, MAH = 9100−19 100, and LAH = 7600−19 200.

The three populations of heparin with varied affinity for AT III are clearly delineated in the close-up view of a polyacrylamide gel in which 1 μg of heparin was visualized after having moved through 4.93 μM AT III (Figure 2B). The angle of the HAH and MAH populations, pointing toward the left lower corner of the gel, is clearly shown in this figure. Since the retardation of heparin movement through AT III in agarose was proportional to its affinity for AT III, this pattern was consistent with lower M_r chains in HAH and MAH populations having greater affinity for AT III than the chains with higher M_r in each population.

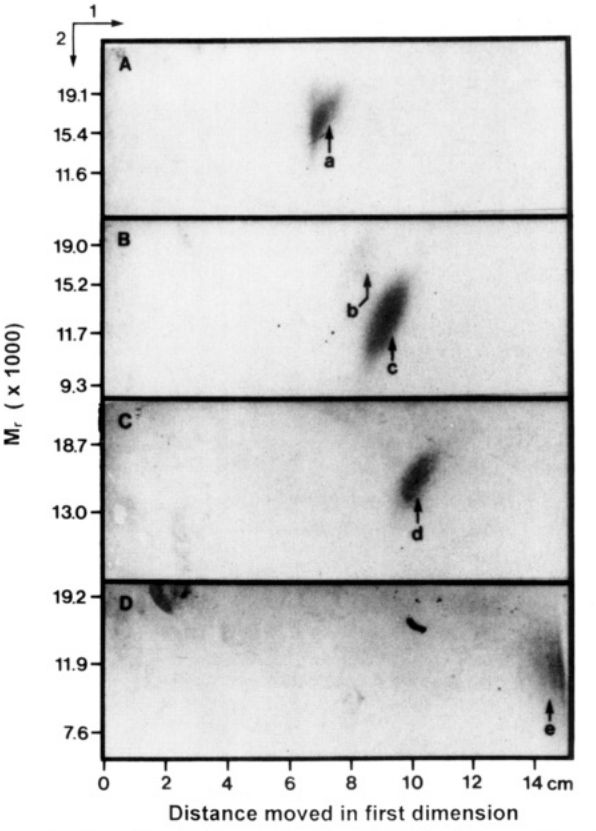

FIGURE 3: Heparin affinity resolved by AT III and then subjected to 2DARE against AT III again. Heparin (1 μg) was subjected to electrophoresis through tube gels containing 5.2 μM AT III. The gels were removed from the tubes, lined up evenly, and cut into 12 1.2-cm sections, and the sections were pooled in Eppendorf tubes. In each Eppendorf tube, the AT III was denatured by heating and the agarose was digested with agarase and then removed by dialysis against distilled water. The sections were dissolved in buffer A containing 0.1% bromophenol blue and 6% sucrose and were resubjected to 2DARE as described above. The four second-dimensional gels that contained heparin are shown with the left edges (corresponding to the first-dimensional tube gel origins) all aligned. The panels correspond to sections (A) 5, (B) 6, (C) 7, and (D) 10. Panels A, B, and D contained only the single populations marked with a, d, and e, respectively. Panel B contained two populations, b and c. The M_r values of the heparin in each population were also provided and reveal that the M_r of b was higher than the M_r of a, indicating that b was actually the least retarded portion of HAH that was cut in two pieces during the first portion of the experiment. Likewise d was actually the least retarded portion of the MAH which was also represented by c. Species e was the least retarded heparin, and it therefore represents the LAH.

The migration distances in both dimensions of the 2DARE gel were based on the center point of the grid corresponding to the center point of the stained elliptical band (Figure 2).

Heparin, Affinity Resolved by Electrophoresis through AT III and then Subjected to 2DARE. Heparin was subjected to the first (affinity) dimension of the 2DARE procedure, extracted from agarose tube gels, and then analyzed using 2DARE (Figure 3). Four second-dimension gels were found to contain heparin. These corresponded to sections 5 (A), 6 (B), 7 (C), and 10 (D) of the sectioned agarose tube gels. Panels A, B, and D in Figure 3 contain only the single

heparin populations marked with a, d, and e, respectively, while panel B contains two populations, b and c. The M_r of the heparin in each population in Figure 3 was calculated. Panel A contains the portion of the HAH population labeled a that was most retarded on preparative affinity electrophoresis. Panel B contains a portion of the HAH population labeled b and a portion of the MAH population labeled c. Interestingly, the M_r of b, corresponding to the least retarded portion of HAH, is higher than the M_r of a, the most retarded portion of the HAH population. This result is consistent with the angulation of HAH populations observed in Figure 2. Similarly, the M_r of the least retarded portion of the MAH population, labeled d in panel C, was higher than the most retarded MAH population, labeled c in panel B. The LAH population labeled e in panel D again shows a different behavior with the lowest M_r being the least retarded, similar to the pattern seen in Figure 2.

The anti-Xa anticoagulant activities of fractions 1−12 were also determined. The fractions corresponding to the HAH population had >7 times more anti-Xa anticoagulant activity than the LAH population on a weight basis (or ≥5 times on a molar basis). This difference in anti-Xa activity observed for high- and low-affinity populations is based upon extracted fractions whose concentrations were normalized using carbazole assays (Bitter & Muir, 1962).

2DARE Analysis of Partially Depolymerized Porcine Mucosal Heparin Interaction with AT III. Partially depolymerized heparin (3 μg) was subjected to 2DARE analysis using 18 μM AT III in the agarose tube gels (Figure 4). Both high- (a) and low-affinity (b) banding patterns were observed. While similar to those found using glycosaminoglycan heparin (Figure 2), no clear third population of bands having intermediate affinity was detected. When the partially depolymerized heparin was moved through agarose in the absence of AT III, the heparin was not fractionated and its movement through the gel was not retarded (not shown). M_r standards (labeled c in Figure 4) were used to determine the M_r values of various heparin populations. M_r analysis demonstrates that various lengths of heparin chains are present in each population. An octasaccharide (labeled 8 on the far left of the gel) was the smallest oligosaccharide possessing affinity for AT III. This octasaccharide also showed the greatest retardation on affinity electrophoresis through AT III−agarose.

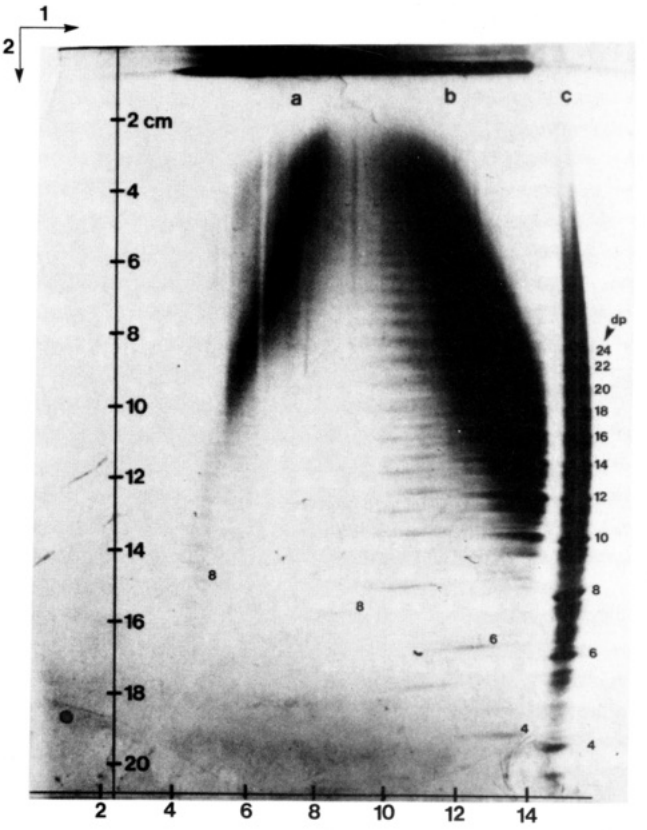

FIGURE 4: 2DARE analysis of partially depolymerized porcine intestinal mucosal heparin interaction with AT III. Partially depolymerized porcine heparin (3 μg) was subjected to 2DARE analysis though 18 μM AT III in the tube gels. High-affinity (a) and low-affinity (b) population patterns were observed, in contrast to the three populations found in glycosaminoglycan heparin (Figure 2). The pattern of angulation of the populations, with the lowest M_r in the high-affinity population having the highest affinity and the lowest M_r in the low-affinity population having the least affinity, was still present. (c) M_r standards were used to demonstrate that various lengths of heparin chains were present in each population, with an octasaccharide possessing the greatest affinity and a tetrasaccharide possessing the least affinity for AT III. The horizontal band at the origin of the second-dimensional gel (top of gel) is the stained AT III.

DISCUSSION

Heparin interaction with AT III (Atha *et al.*, 1984; Griffith, 1986; Jackson *et al.*, 1991; Lindahl *et al.*, 1983; Rosenberg, 1985) has been reported to have a k_d between 11 nM (Lee & Lander, 1991) and 1 μM (Dawes, 1988; Olson *et al.*, 1981; Rosenberg, 1985). A pentasaccharide within heparin has clearly been shown to be the high-affinity binding site for interaction with AT III (Atha *et al.*, 1984; Lindahl *et al.*, 1983), but the number of AT III binding sites per heparin chain is not known. Approximately 30% of heparin chains in glycosaminoglycan preparations are thought to contain the high-affinity pentasaccharide site; the other 70% lack the critical 3-*O*-sulfate group (Jackson *et al.*, 1991) and are thought to interact with AT III through lower affinity sites. Furthermore, because the primary structure of heparin remains largely undetermined, the existence of other binding sites for AT III cannot be excluded.

Lee and Lander (Lee & Lander, 1991) were the first to use affinity electrophoresis to study the interaction of heparin with AT III. We have modified the procedure substantially to allow examination of how heparin chain length impacts on its interaction with AT III; 2DARE allows us to determine simultaneously M_r and k_d. A two-dimensional approach, 2DARE, provides direct evidence that at least three populations of heparin chains with varied affinity for AT III exist in porcine intestinal mucosal heparin (Figure 2). Most literature suggests that only high- and low-affinity populations of heparin exist. The heparin populations defined in this paper as HAH and MAH together represent what previous authors referred to as high-affinity heparin.

In 1982, Horner and Young (Horner & Young, 1982) suggested that heparin with affinity for AT III could contain chains with higher affinity for AT III. The 2DARE analysis of heparin−AT III interaction described here confirms the observation of Horner and Young. The three populations in glycosaminoglycan heparin could represent multiplicity of the known pentasaccharide binding site for AT III. Specifically, LAH could be devoid of AT III binding sites, whereas MAH would have one and HAH would have two binding sites for AT III. Interestingly, partially depolymer-

ized heparin shows only two populations, one interacting with AT III and one not. This is not surprising because by a reduction of the molecular weights of the heparin chains the possibility of two AT III binding sites being present in a single chain was greatly reduced. For any given sized heparin chain, possession of two binding sites would decrease the "on−off" time of the interaction of heparin with AT III due to the closer proximity of the second binding site on a single heparin chain compared to chains with only one binding site for AT III. This theory is consistent with studies that show that the stoichiometry of some high-M_r heparin chains is 1 heparin to 2 AT III (Jordan *et al.*, 1982).

The calculated k_d values for the MAH (80 nM) and the LAH (>1 μM) compare well with literature values (Dawes, 1988; Lee & Lander, 1991; Olson *et al.*, 1981; Rosenberg, 1985). The calculation method required derivation of new equations due to the high concentration of heparin used in these experiments, necessitated by using silver stain to detect heparin movement. Silver staining of the heparin was used, as it possesses several advantages: (1) no radiolabel is required (safety), (2) no intrinsic labeling is required (this is impossible when studying pharmaceutical heparin), and (3) no modification of the glycosaminoglycan structure results from labeling. These represent distinct advantages over Lee and Lander's (Lee & Lander, 1991) method.

The 2DARE analysis has also enhanced our understanding of the importance of heparin chain length on the affinity of heparin for AT III. The distance that the heparin chain moved into the second gel dimension was proportional to the M_r of the heparin in that population. The M_r markers (Figure 2A, d) on each gel were used to calculate the M_r values of the heparin chains present at various points in each heparin population (Figure 2A, a−c). As shown, the lowest M_r species in both HAH and MAH possessed the greatest affinity for AT III, whereas the lowest M_r species in the LAH had the least affinity for AT III. This pattern was confirmed when heparin was run in the first dimension, extracted from the agarose, and then reanalyzed (Figure 3). The significance of this pattern was further illustrated by subjecting partially depolymerized heparin to 2DARE analysis for affinity to AT III (Figure 4). As shown in Figure 4, an octasaccharide possessed the greatest affinity for AT III of all the depolymerized heparin species. This result is supported by studies in our laboratory that show an octasaccharide to be the smallest product of heparin lyase I still able to exhibit significant affinity for AT III (Desai *et al.*, 1993; Linhardt *et al.*, 1992).

Nearly 20 years ago Andersson *et al.* (Andersson *et al.*, 1976) demonstrated that when heparin is separated by affinity for immobilized AT III and for size by gel permeation chromatography, the HAH with low M_r has the greatest anti-factor Xa activity. The 2DARE analysis conducted suggests that the increased anti-factor Xa activity they observed is due to the greater affinity of AT III for the low M_r heparin than for the higher M_r heparin. Studies of very high affinity heparins, prepared by AT III affinity chromatography and examined using fluorescence spectroscopy, found that the binding stoichiometry of the heparin to AT III is 1 heparin molecule to 2 AT III molecules (Jordan *et al.*, 1982). 2DARE analysis of heparin−AT III interaction confirms the existence of heterogeneity of binding of heparin to AT III. In fact, the ability of 2DARE to examine both the affinity and the M_r of all populations of heparin with varied affinity for AT III in a single experiment allowed us to demonstrate that heparin with both high and low M_r can have high, medium, or low affinity for AT III.

We have considered one likely mechanism to explain the observation that low M_r heparin has greater affinity for AT III than higher M_r heparin: Within a given heparin population (i.e., HAH or MAH), all chain lengths in that population possess an identical number of AT III binding sites, despite their M_r. Both AT III and heparin are negatively charged (Casu, 1985; Kim et al., 1988), and the difference in affinity may be due to increased charge−charge repulsion between residues flanking the pentasaccharide binding site on the heparin chain and AT III. This charge−charge repulsion increases as the length of the heparin chain increases.

Beyond demonstration that three distinct populations of heparin exist with varied affinity for AT III, the experiments described here also are highly relevant to current trends toward development of low molecular weight heparins as anticoagulants. Recently, Shimotori and Sakuragawa (Shimotori & Sakuragawa, 1990) demonstrated that two low-M_r heparins, PK 10169 and Fragmin, differed significantly in their affinity for AT III as well as their anticoagulant activity. This knowledge, combined with evidence of the heterogeneity of heparin with respect to affinity for AT III presented here, suggests that ideal methods of preparation of low molecular weight heparins would lead to enhanced levels of heparin chains with high affinity for AT III while eliminating chains with low affinity. Further, the techniques described here could be used to examine the relationship between M_r and affinity in low molecular weight heparin preparations.

As Salzman *et al.* (Salzman *et al.*, 1980) suggested more than a decade ago, "selection of heparin fractions of low molecular weight and high antithrombin affinity may improve anticoagulant therapy" by maintaining heparin−AT III interaction while decreasing the induction of platelet aggregation by high molecular weight heparin. The 2DARE analysis described here demonstrates that in glycosaminoglycan heparin preparations variations in affinity of heparin for AT III exist and are separable. Furthermore, in any given population of heparin with similar affinity (or similar numbers of binding sites), the heparin with the lowest M_r possesses the greatest affinity for AT III. Finally, it is apparent that 2DARE is a generic method of analysis capable of examining the interaction between any glycosaminoglycan and protein. This high-resolution technique should provide added insight into the interactions of glycosaminoglycans with a wide array of enzymes, receptors, extracellular matrix proteins, and growth factors (Cardin & Weintraub, 1989; Casu, 1985; Jackson *et al.*, 1991; Linhardt & Loganathan, 1990; Steinbuch, 1982).

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