Evidence of Random Structural Features in the Heparin Polymer†

Robert J. Linhardt,*,‡ Zohar M. Merchant,‡ Kevin G. Rice,‡ Yeong S. Kim,‡ Gerald L. Fitzgerald,§ Arthur C. Grant,‡,§ and Robert Langer§

College of Pharmacy, Division of Medicinal Chemistry and Natural Products, The University of Iowa, Iowa City, Iowa 52242, and Department of Applied Biological Sciences and the Whitaker College of Health Sciences, Management and Technology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

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ABSTRACT: The first use of computer-simulation studies to examine heparin's structure has been reported. The product distributions obtained when porcine mucosal heparins were depolymerized with heparinase have been compared to computer-simulated distributions. The modeled distribution was relatively unaffected by the polydispersity and molecular weight of heparin. However, the percent of heparinase-cleavable glycosidic linkages and their distribution throughout the polymer resulted in a marked change in the simulated product distribution. The similarity between experimentally observed and computer-simulated product distributions is consistent with the random distribution of heparinase-cleavable sites in porcine mucosal heparin. Finally, a random distribution of *N*-acetyl residues with respect to heparinase-cleavable sites was experimentally observed.

Heparin, a polydisperse sulfated polysaccharide, is the most frequently used clinical anticoagulant (Jaques, 1979). Despite its importance, heparin's precise chemical structure remains unknown. Heparin (I) is a copolymer comprised of (1→4) linked glucosamine and uronic acid residues. The major repeating unit (Comper, 1981) in the heparin polymer is →4)-2-deoxy-2-sulfamino-α-D-glucopyranose 6-sulfate-(1→4)-α-L-idopyranosyluronic acid 2-sulfate-(1→. Heparinase (heparin lyase, EC 4.2.2.7) is an eliminase (Linker & Hovingh, 1972) cleaving heparin only at this linkage (Linker & Hovingh, 1979; Merchant et al., 1985), resulting in a disaccharide, tetrasaccharides, hexasaccharides, and higher oligosaccharides (Figure 1).

With the help of computer-simulation studies we have recently shown that heparinase acts on heparin in a random endolytic fashion (Linhardt et al., 1982a). The computer-simulation studies have now been extended to determine how these heparinase-cleavable linkages are distributed throughout the heparin polymer. Although the use of mathematical modeling to examine heparin's structure has recently been introduced (Radoff & Danishefsky, 1984), this represents the first use of computer simulation to study structural features of the heparin polymer.

Theory

General Assumptions. The computer-simulation model for heparin degradation assumes that heparinase acts as an eliminase (Linker & Hovingh, 1972) in a random endolytic fashion (Linhardt et al., 1982a) to cleave certain specific α-glycosidic linkages (Linker & Hovingh, 1979; Merchant et al., 1985) present in the heparin polymer. To accurately represent heparin both its polydispersity and its microheterogenicity must be taken into account. The experimentally observed polydispersity (Figure 2) is used to calculate the number of chains built at each length. Heparin's microheterogenicity is represented by the presence, in each disaccharide unit within a given chain, of either a heparinase-cleavable (designated 1) or uncleavable (designated 0) α-glycosidic linkage. A total of 10 000 number chains (i.e., ...10110...) were constructed, each comprised of from 7 to 84 disaccharide units representing a molecular weight distribution of 4000–46 000 (90 mol % of the chains fell between 4000 and 16 500). The experimentally measured ultraviolet absorbance of the product mixture, associated with their unsaturation, was used to calculate the fraction of "heparinase-cleavable" α-glycosidic linkages (P_{CLV}) in porcine mucosal heparin. This method of determining the percent of heparinase-cleavable linkages is independent of the particular product distribution and depends only on the final ultraviolet absorbance and the average molecular weight of the heparin.

Model with Random Site Distribution. In this model the cleavable α-glycosidic linkages are distributed randomly throughout the heparin chains. First, the total number of chains of each length to be built is calculated. As a chain is built, one α linkage is added at a time, each one independent of all the others. The probability that any given α linkage is cleavable is P_{CLV}. After a chain is constructed it is completely degraded in a random endolytic fashion before the next chain is built. The algorithm for degradation proceeds as follows: The chain is inspected by the program to determine the number of cleavable sites it contains. One of these sites is selected at random, and the chain is split into two pieces, eliminating the selected cleavable linkage. The process is then repeated until all cleavable glycosidic linkages have been split.

An interesting point emerges from recent experimental data (Linker & Hovingh, 1984; Merchant et al., 1985) which indicates that tetrasaccharides containing the appropriate α-glycosidic linkage for enzymatic cleavage cannot be cleaved by heparinase. If all of the α-glycosidic linkages between 2,6-disulfated glucosamine and 2-sulfated iduronic acid (including those in the tetrasaccharide) were cleaved by heparinase, the product distribution after exhaustive degradation would be independent of the action pattern (e.g., random endolytic, nonrandom exolytic, etc.) of heparinase. Such a distribution could be calculated as the model built each chain simply by counting the number of disaccharide units between

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* Author to whom correspondence should be addressed.
‡ The University of Iowa.
§ Massachusetts Institute of Technology.

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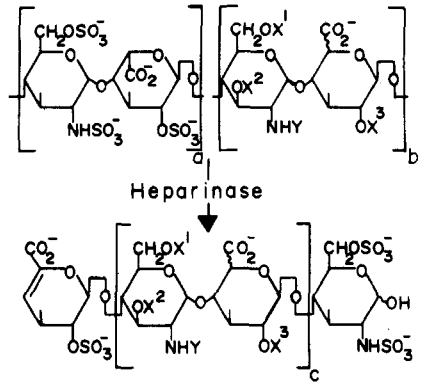

FIGURE 1: Heparinase depolymerization of heparin. Heparin is comprised of heparinase-cleavable sites (a) and uncleavable sites (b) where X^1, X^2, and X^3 are SO_3^- or H, and Y is SO_3^- or CH_3CO. The products formed are a disaccharide ($c = 0$), tetrasaccharides ($c = 1$), hexasaccharides ($c = 2$), etc.

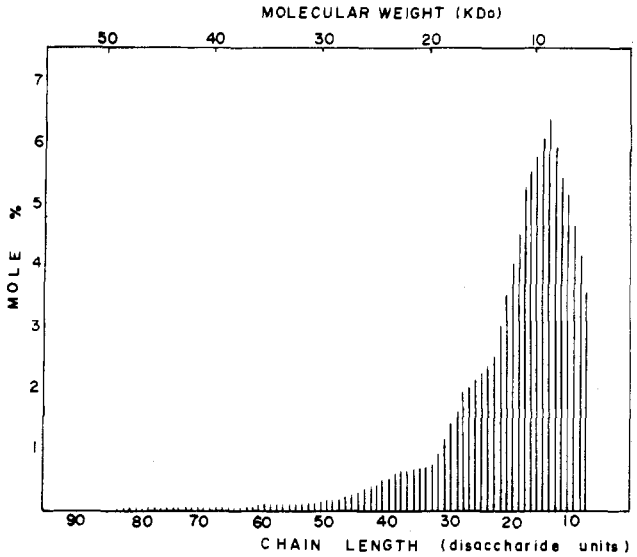

FIGURE 2: Polydispersity of porcine mucosal heparin sized by GPC shown as a plot of mole percent vs. molecular weight and chain length in disaccharide units.

each cleavable site. Such a program would be able to process a large number of chains (10^9) in a reasonable time. However, to accurately predict the final product distribution the enzyme's substrate-size requirements must be taken into account. Accordingly, the computer simulates heparin's random endolytic action (Linhardt et al., 1982a) on each substrate chain. The model can be made to act on one substrate chain at a time, or after each cleavage it can return the chain to the substrate pool and select a new chain at random on which to act. Obviously, it is necessary to return a chain to the pool only when simulating kinetic enzymatic degradation. This approach results in added computational time and added storage burdens and is therefore limited to ensembles of about 10^3 chains. The final distributions, presented in this paper, are modeled by acting completely on one chain at a time, easily permitting the use of ensembles of up to 10^6 chains. The final product distributions obtained with both models have been compared and are identical.

The program records the length of each chain and whether it possesses one of the two original end groups. The products resulting from the nonreducing end of the original heparin chain are not unsaturated and therefore are not detected empirically. The computer model compensates for this experimental limitation. For each size product, only half of those derived from an original end group are included in the calculated final product distribution.

Model with Nonrandom Site Distribution. In this model, heparin chains are built in one of two nonrandom ways: cleavable-site clustered or cleavable-site distributed. The probability that the first site is cleavable is P_{CLV}. In the clustered case the probability that the nth site is cleavable ($n > 1$) is $P_{CLV} + \delta$ if the $(n-1)$th site is cleavable or $P_{CLV} - \delta$ if the $(n-1)$th site is uncleavable. The offset δ is a variable input to the program. In the limit (when $P_{CLV} = \delta = 0.5$), the clustered distribution results in a chain consisting of entirely cleavable or uncleavable sites determined by the cleavability of the first site. In the distributed case the probability that the nth site ($n > 1$) is cleavable is $P_{CLV} + \delta$ if the $(n-1)$th site is not cleavable or $P_{CLV} - \delta$ if the $(n-1)$th site is cleavable. In the limit (when $P_{CLV} = \delta = 0.5$) this produces alternating cleavable and uncleavable sites giving only tetrasaccharides. Consider the following specific example of how nonrandom chains are built. Suppose $P_{CLV} = 0.53$, δ 0.05, and the first site in the chain is cleavable. Then the probability that the second site is cleavable will be 0.58 or 0.48 for the clustered or distributed cases, respectively. If the first site had been uncleavable, then these values would have been reversed. Chains built by either of these nonrandom methods are split in the same way as the random chains.

Experimental Procedures

Chemicals. Heparin sodium salt from porcine mucosa was obtained from Hepar Industries (157 units/mg) and from Sigma Chemical Co. (grade II, 159 units/mg). Sephadex G-10, *N*-acetylglucosamine, and Blue dextran were from Sigma Chemical Co. Fractogel TSK HW40 and Fractogel TSK HW55(S) were obtained from MCB Manufacturing Chemists. Hydroxylapatite and Bradford reagent were obtained from Bio-Rad. A chromatofocusing kit was purchased from Pharmacia. 3-(Trimethylsilyl)-1-propanesulfonic acid sodium salt (DSS) and 99.996 atom % D_2O were purchased from Aldrich Chemical Co. Standard heparin was generously provided by Drs. Mathews and Cifonelli of the University of Chicago.

Equipment. High-pressure liquid chromatography (HPLC) was performed on an LDC Constametric III pump, which used a Rheodyne 7125 injector, and an ISCO Model 1840 variable wavelength UV detector. The detector output was read directly through an analog to digital interface from Interactive Microware, and the data were processed and peaks were integrated on an Apple IIe microcomputer. Freeze-drying was performed on a Labconco Model 8 lyophilizer. Spectrophotometric measurements were made with Pye Unicam SP8-100 UV and Bruker WM 360-MHz nuclear magnetic resonance spectrophotometers. Computer modeling was performed on a Digital Equipment Corp. VAX 11/750 running under VMS and Fortran versions 4.1.

Methods. (A) Heparinase Preparation and Assays. Heparinase was prepared fermentatively from *Flavobacterium heparinum* (Galliher et al., 1981) and purified by batch hydroxylapatite chromatography (Linhardt et al., 1984), followed by chromatofocusing and gel permeation chromatography (Yang et al., 1985). This purified enzyme preparation was assayed for heparinase activity (Linhardt et al., 1982a) and contaminating glycuronidase (Linhardt et al., 1982a; Warnick & Linker, 1972), sulfamidase (Ototani et al., 1981; Linhardt et al., 1982a), and sulfatase (Warnick & Linker, 1972; Ototani & Yosizawa, 1979) activities. Protein concentration was measured with Bradford reagent (Bradford, 1976). Purified heparinase had a specific activity of 5 units/mg of protein (1

unit = 1 μmol of bonds cleaved/min) and contained no contaminating glycuronidase, sulfamidase, or sulfoesterase (sulfatase) activities (Linhardt et al., 1982a).

(*B*) *Heparin Sizing.* Heparin's polydispersity was measured by HPLC gel permeation chromatography (GPC) by loading 10 μg of heparin in 10 μL on TSK gel G-3000SW and G-2000SW columns (in series) (two 7.5 mm × 50 cm columns with a 10-cm guard column) and eluting at 25 °C with 0.5 M sodium chloride at a flow rate of 1.0 mL/min. Heparin elution was measured directly at 206 nm (Irimura et al., 1983). Molecular weight and polydispersity were determined by comparison to heparin-derived di-, tetra-, hexa-, octa-, and decasaccharide standards of known molecular weight (Sharath et al., 1985; Rice et al., 1985). Standard heparins of average M_r 5000 and 14 000 (as determined by viscosity) were used to confirm the accuracy of this method. Low-pressure GPC on Fractogel TSK HW55(S) took longer but resulted in identical molecular weight determinations (Sharath et al., 1985).

(*C*) *Enzymatic Depolymerization of Heparin.* A solution containing heparin (25 mg/mL) and acetate (sodium acetate, 0.025 M, and calcium acetate, 0.25 mM, adjusted to pH 7.0 with acetic acid) was prepared. To 1 volume of this solution was added 2 volumes of purified heparinase (0.1 mg/mL), and the reaction was run at 30 °C until complete (Linhardt et al., 1982b). The product solution was then immediately frozen and stored at −40 °C.

The depolymerized heparin (10 μg in 10 μL) was sized by HPLC–GPC on TSK gel G-3000SW and G-2000SW (both 7.5 × 50 cm with a 10-cm guard column) columns run in series and eluted with 0.5 M sodium chloride. The separation was performed at 25 °C with a flow rate of 1 mL/min, and the eluent was measured directly at 232 nm, the absorbance maxima of heparinase-cleaved heparin products (Linker & Hovingh, 1972).

The depolymerized heparin was fractionated on a preparative scale for characterization with a Fractogel TSK HW40 column (1.5 cm × 150 cm) equilibrated with 1.0 M ammonium bicarbonate. (Because of its volatility, NH_4HCO_3 is removed by freeze-drying.) Depolymerized heparin (250 mg in a 2.5-mL volume) was added to the column and eluted at a flow rate of 0.1 mL/min. The eluent was measured after a 1/50 dilution in distilled water at 232 nm. Each peak was collected and freeze-dried, and the procedure was repeated.

(*D*) *Nuclear Magnetic Resonance (NMR) Spectroscopy.* The GPC-fractionated oligosaccharides were freeze-dried 3 times from D_2O. The NMR was performed at 0.03 M in D_2O at 25 °C with DSS as an internal standard, and data were collected for ^1H NMR at 360 MHz.

Results and Discussion

The heparins used in this study were polydisperse commercial porcine mucosal heparins with an average molecular weight of 13 000 as measured against standards with HPLC–GPC (Figure 2). Five heparin subfractions were prepared having average molecular weights of 8600, 11 000, 15 000, 16 700, and 20 000 (Sharath et al., 1985).

Heparinase cleaves heparin specifically (Linker & Hovingh, 1979; Silverberg et al., 1985; Merchant et al., 1985) at the α-glycosidic linkage between N-sulfated, 6-sulfated glucosamine and 2-sulfated iduronic acid (Figure 1). Heparinase has recently been shown to have minimum substrate size requirements (Linker & Hovingh, 1984; Merchant et al., 1985). A tetrasaccharide with this linkage (Figure 1) can be isolated from the heparinase-derived product mixture, and this tetrasaccharide has been shown to be resistant to heparinase cleavage (Linker & Hovingh, 1984; Merchant et al., 1985).

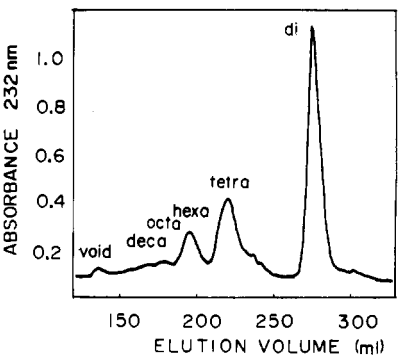

FIGURE 3: Gel permeation chromatography of heparinase-depolymerized heparin on a low-pressure Fractogel TSK column shown as a plot of absorbance (232 nm) vs. elution volume (milliliters).

Table I: Dependence of Simulated Product Distribution on Percent of Cleavable Sites

	mathematical model mol %	computer simulated mol %			obsd mol %
cleavable sites (%)	53	52	53	54	53 ± 1[a]
fragment					
disaccharide	53.0	53.4	54.4	55.2	55.6
tetrasaccharide	24.9	26.7	26.6	26.7	25.8
hexasaccharide	11.7	11.2	10.9	10.5	8.8
octasaccharide	5.5	4.9	4.7	4.4	3.5
decasaccharide	2.6	2.2	2.0	1.8	2.1
oligosaccharide (dp >10)	2.3	1.6	1.4	1.4	nd[b]

[a] Calculated from the final ultraviolet absorbance and the average molecular weight of the heparin being depolymerized. [b] Value not determined.

Heparin, which has been depolymerized with heparinase, affords a disaccharide, tetrasaccharides, hexasaccharides, octasaccharides, decasaccharides, and higher oligosaccharides (Merchant et al., 1985). The proportion in which products of different sizes are found is dependent on the way heparinase-cleavable linkages are distributed throughout the heparin polymer. Heparinase is an α-eliminase, and its action produces a $\Delta^{4,5}$ site of unsaturation in the nonreducing-end uronic acid residue of each resulting product (Linker & Hovingh, 1972). The presence of this chromophore [$\epsilon_{232} = 5.2 \times 10^3$ M^{-1} cm^{-1} (Linker & Hovingh, 1972)] permits the determination of the percentage of heparin's α-glycosidic linkages that are cleaved by heparinase. The commercial heparin used in this study was found to contain 53 ± 1% α-glycosidic linkages that were cleaved by heparinase. Even in a complete degradation the fraction of α-glycosidic linkages actually cleaved is somewhat less than the total number of cleavable linkages because a few will be hidden within the tetrasaccharides, which are not suitable substrates for the enzyme (Linker & Hovingh, 1984; Merchant et al., 1985). The input to the simulation program is the total percent of cleavable sites, and from the output one can calculate the observed percent of cleaved sites. For example, 53% of observed cleaved sites represents 54.6% of cleavable sites in the original heparin. To avoid confusion, in the remainder of this paper all references to cleaved or cleavable sites refer to the empirically observed value of percent of cleaved sites, the input to the model being adjusted appropriately. The products obtained from this heparin were fractionated on the basis of size by GPC (Figure 3). The mole percent of each sized product (Table I) was calculated by measuring its absorbance at 232 nm (Figure 3).

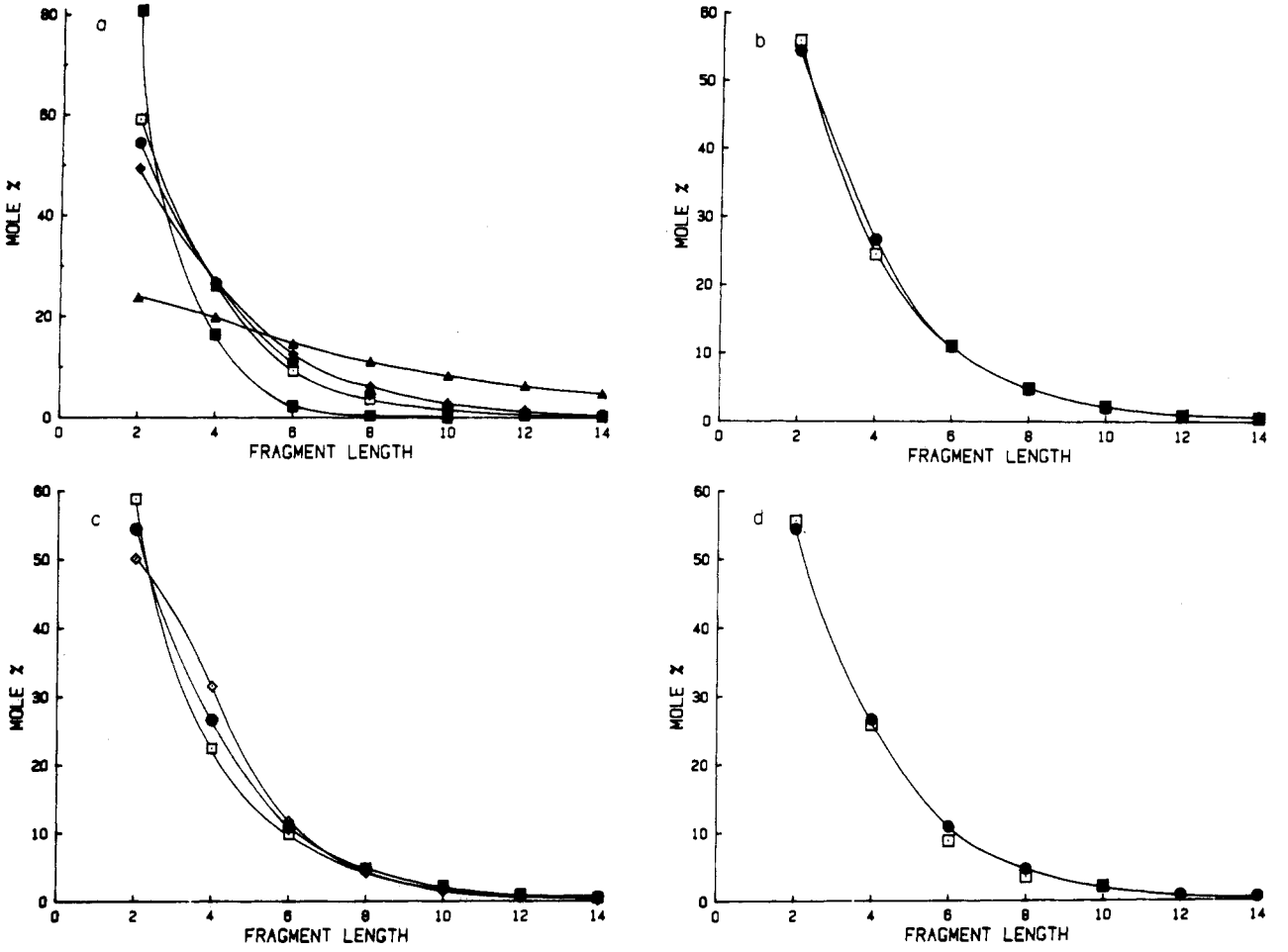

FIGURE 4: (a) Modeled effect of the percent of cleavable sites on the product distribution for 80% (■), 58% (□), 53% (●), 48% (◆), and 20% (▲) randomly distributed cleavable sites. (b) Modeled effect of the presence of an uncleavable tetrasaccharide (●) compared to a cleavable tetrasaccharide (□) on the product distribution. Both simulations have 53% randomly distributed cleavable sites. (c) Modeled effect of cleavable site positioning on the product distribution given for clustered (□) and homogeneous (◇) nonrandomly distributed sites where $\delta = 0.05$ and randomly distributed sites (●). All are 53% cleavable. (d) Simulated product distribution (●) compared to experimentally observed product distribution (□) for 53% randomly distributed cleavable sites and an uncleavable tetrasaccharide.

The computer-simulated product distributions are shown in Figure 4. For a simulation to have value, its sensitivity to variability in both arbitrarily selected and experimentally measured parameters must be tested.

The number of heparin chains used each time the simulation was run was selected as 10^4. The product distribution obtained with 10^4 chains is the same (differing by no more than 0.2% for any product size) as that obtained with 10^5 or 10^6 chains, yet the computer time required to complete one run on 10^4 chains was short enough to permit the adequate testing of all other variables.

The computer-generated ensemble of heparin chains requires the experimental determination of heparin's polydispersity. The fraction of heparin chains of each length has been estimated by GPC. The mole percent of each chain used in the computer simulation is shown in Figure 2. Because of the error inherent in an estimation, the relationship between heparin's polydispersity and the predicted product distribution was examined. Monodisperse heparin [M_r 13 000; polydispersity (M_n/M_w) = 1.0] and polydisperse heparin (average M_r 13 000; polydispersity, from Figure 2) give simulated product distributions that differ by <0.3% for any product size. This suggests that precise determination of heparin's polydispersity is not necessary in order to model its enzymatic degradation. This will be true as long as the heparin chains are long with respect to the average product chain length (i.e., as long as P_{CLV} is reasonably high).

The percentage of cleavable α linkages within the heparin polymer is determined from the 232-nm absorbance of a known quantity of exhaustively depolymerized heparin. Heparins from various sources (i.e., porcine, bovine, etc.) contain varying amounts of cleavable linkages (Linker & Hovingh, 1979). We have observed from 40% to 60% cleavable α-glycosidic linkages in porcine mucosal heparin from different suppliers. The heparin used in this study had 53 ± 1% cleavable α-glycosidic linkages. The change in product distribution as the percent of cleavable sites present is shown in Figure 4a. A closer examination of the variation introduced by the experimental error present in measuring the percent of cleavable sites is shown by the results of the simulated product distribution presented in Table I.

The isolation of a hexasulfated tetrasaccharide having a linkage that should have been cleaved, but remains stable with respect to heparinase even after repeated exposure, requires an additional level of complexity in the model. This hexasulfated tetrasaccharide does not compete with heparin for heparinase (Merchant et al., 1985), suggesting its stability is probably the result of its failure to bind to the enzyme. This inability to bind might be due to either the shortness of the chain or a conformational difference between it and the longer parent chains. This restriction, the cleavability of tetrasaccharides, shown in Figure 4b, results in an increase in the percentage of tetrasaccharide products (24.7–27%) with a concomitant decrease in the percent of disaccharide

Table II: Distribution of N-Acetylated Glucosamine Residues in Sized Product Mixtures

mixed fragment	% *N*-acetate/mol[a]	% *N*-acetate per disaccharide repeating unit
tetrasaccharides	0.43	0.22
hexasaccharides	0.61	0.21
octasaccharides	0.89	0.23
decasaccharides	1.25	0.25

[a] Determined by integration of *N*-acetate in the ^{1}H NMR.

(55.7–53%). This 2.3% increase in the tetrasaccharide components corresponds well with the isolation of 3 mol % hexasulfated tetrasaccharide from the oligosaccharide mixture (Merchant et al., 1985).

The predicted product distribution as a function of the distribution of cleavable sites throughout the heparin chains in the substrate ensemble has been examined (Figure 4c). A random distribution of cleavable sites is obtained by making the decision of whether a site is cleavable or uncleavable, based only on the measured parameter P_{CLV}. Cleavable sites can be clustered or distributed by making their placement dependent on whether the previous site was cleavable or uncleavable.

The simulated product distribution generated with 10^4 chains of polydisperse heparin (Figure 1) with 53% randomly distributed cleavable sites is shown in Figure 4d. Also plotted in Figure 4d is the experimentally observed product distribution. The agreement between experimental and simulated data is extremely close, and even a 3% variation from random site distribution could be detected. A second polydisperse porcine heparin from an alternate source (Sigma) having 44% cleavable sites gave similar results. This heparin gave the following experimental and simulated product distributions: disaccharide, 46.8 and 45.6; tetrasaccharide, 25.2 and 27.1; hexasaccharide, 14.0 and 13.2; oligosaccharide [degree of polymerization (dp) $\geq$8], 14.0 and 14.1. The experimentally observed product distributions from each of the sized heparins that we examined were nearly identical. Simulated product distributions based on each sized subfraction of heparin were also identical. The cleavable sites appear to be randomly distributed irrespective of heparin's average molecular weight.

The product distribution can be estimated by using a simple mathematical model (Table I). This model assumes that the substrate chain is infinitely long and that the hexasulfated tetrasaccharide is cleaved. The probability of a site being cleavable is ρ, that of a site being uncleavable is q, and $\rho + q = 1$. Given that the nth site is cleavable, the probability that (1) the $(n + 1)$th site is cleavable is ρ, resulting in a disaccharide; (2) the $(n + 1)$th site is uncleavable and the $(n + 2)$th site is cleavable is $q\rho$, resulting in a tetrasaccharide; (3) and so on. In general the probability of obtaining a polysaccharide containing d disaccharide units is $q^{d-1}\rho$.

The apparent random distribution of heparinase-cleavable sites, the major α-glycosidic linkage in the heparin polymer, has certain implications. First, it suggests that the biosynthesis of heparin is random with respect to the placement of these sites. This prediction is consistent with the proposed mechanism of heparin biosynthesis (Lindahl, 1979). A less likely possibility is that the cleavable site distribution actually represents a unique nonrandom sequence of sites whose statistics happen to match those of the random distribution. There is currently no evidence supporting such a unique sequence for heparin. Second, it has been shown that certain domains within the heparin chain are responsible for many of heparin's biological activities (Lindahl et al., 1980; Oosta et al., 1981). The results of these simulation studies suggest that the biologically important sequences either are randomly distributed or occur nonrandomly at a frequency too low to be detected by our model.

A random distribution of antihrombin III (ATIII) binding sites is consistent with the reported relationship between high ATIII affinity and molecular weight in commercial heparin (Laurent et al., 1978). Recent studies suggest these sites are nonrandomly distributed in both proteoglycan (Horner & Young, 1982) and commercial heparin (Radoff & Danishefsky, 1984). Our inability to detect this order may be because the heparinase cleavability is not a sensitive probe for the ATIII binding site. Although the octasaccharide ATIII binding site is cleaved by heparinase (Atha et al., 1984), so are many other portions of the heparin polymer.

We have, therefore, begun to examine the distribution of other functionality including some more closely tied to heparin's anticoagulant activity. The presence of an N-acetylated glucosamine residue in the ATIII binding sequence is well-known (Atha et al., 1984; Lindahl et al., 1983). The sized mixtures of heparin products obtained by GPC (Figure 3) have been examined by ^{1}H NMR, and the percentage of N-acetylated glucosamine present in these mixtures has been measured (Table II). From these data it is clear that the amount of these residues contained in a given sized mixture is proportional to the number of internal glucosamine residues [the external glucosamine is defined by the specificity of heparinase (Merchant et al., 1985)]. This is consistent with the random distribution of N-acetylation with respect to the disposition of heparinase-cleavable sites.

Further refinements of the computer-simulation studies including the modeling of residues peculiar to the ATIII binding site such as 3-O-sulfation in the glucosamine residue (Lindahl et al., 1983; Atha et al., 1984) are necessary. Finally, other heparins including raw proteoglycan heparin require study by these methods to develop the real biological significance of these findings.

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Registry No. Heparin, 9005-49-6; heparinase, 37290-85-0; N-acetylated glucosamine, 7512-17-6.

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Segmental Flexibility of Receptor-Bound Immunoglobulin E†

James Slattery, David Holowka, and Barbara Baird*
Department of Chemistry, Baker Laboratory, Cornell University, Ithaca, New York 14853
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ABSTRACT: The segmental flexibility of mouse immunoglobulin E (IgE) bound to its high-affinity receptor on membrane vesicles from rat basophilic leukemia cells was compared to that of IgE in solution by measuring the steady-state anisotropy as a function of temperature and viscosity. A monoclonal IgE was used to bind the fluorescent probe *N*-[5-(dimethylamino)naphthalene-1-sulfonyl]-L-lysine (DNS-Lys) rigidly and specifically in the antigen combining site at the tip of the Fab region. The average rotational correlation time, ϕ, of 74–89 ns for the receptor-bound IgE is only slightly longer than that for IgE in solution where ϕ = 54 ns. Another mouse monoclonal IgE was covalently labeled in the Fab region with *N*-(1-pyrenyl)maleimide. Anisotropy measurements with this derivative yielded results that are very similar to those found with anti-DNS IgE and DNS-Lys. These findings are strikingly different from that expected for a rigid IgE bound to its receptor since in this case ϕ is likely to be very much larger. Evidently, the segmental flexibility of IgE is not greatly altered upon binding to its receptor.

Immunoglobulin E (IgE)[1] is a Y-shaped molecule that binds tightly to its receptor on mast cells and basophils and mediates the triggering of cellular degranulation by multivalent antigen. IgE is analogous to other classes of immunoglobulins, being composed of two heavy (ϵ) chains and two light polypeptide chains that are connected by disulfide bonds and noncovalent interactions. The amino acid sequence of human ϵ (Bennich & von Bahr-Lindstrom, 1974) and DNA sequences of the murine ϵ gene (Ishida et al., 1982; Liu et al., 1982) have shown IgE to have a domain structure similar to other immunoglobulins. IgE is like IgM in that these both exhibit an extra domain, $C_\epsilon 2$ or $C_\mu 2$, in the region corresponding to the hinge of IgG (Dorrington & Bennich, 1978).

A controlling factor in initiating the transmembrane signal is the ability of receptor-bound IgE to be cross-linked by multivalent antigen, and this probably depends on several factors including the flexibility of IgE. In similar situations, the relative abilities of IgG subclasses and IgE to bind antigen and fix complement in solution (Oi et al., 1984) and of IgE to precipitate protein covalently conjugated with hapten (Dudich et al., 1978) have been correlated with their segmental flexibility. Previous studies (Nezlin et al., 1973; Cathou, 1978; Oi et al., 1984) have examined the segmental flexibility of IgE in solution by measuring fluorescence anisotropy. These studies have indicated that IgE is probably less flexible than

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[1] Abbreviations: DNP, 2,4-dinitrophenyl; DNS, 5-(dimethylamino)-naphthalene-1-sulfonyl; DNS-Lys, DNS-L-lysine; DNP-Lys, DNP-L-lysine; EDTA, ethylenediaminetetraacetate; FWHM, full width at half-maximum; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HBS, HEPES-buffered saline; Ig, immunoglobulin; Na-DodSO$_4$-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PM, *N*-(1-pyrenyl)maleimide; PM–IgE, anti-DNP-IgE covalently modified with PM; RBL, rat basophilic leukemia; TNP-Cap-Tyr, [[(2,4,6-trinitrophenyl)amino]caproyl]-L-tyrosine; Tris, tris(hydroxymethyl)aminomethane.

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