The Solution Structure of Heparan Sulfate Differs from That of Heparin

IMPLICATIONS FOR FUNCTION

Sanaullah Khan, Elizabeth Rodriguez, Rima Patel, Jayesh Gor, Barbara Mulloy, and Stephen J. Perkins

The highly sulfated polysaccharides heparin and heparan sulfate (HS) play key roles in the regulation of physiological and pathophysiologic processes. Despite its importance, no molecular structures of free HS have been reported to up to now. By combining analytical ultracentrifugation, small angle x-ray scattering, and constrained scattering modeling recently used for heparin, we have analyzed the solution structures for eight purified HS fragments degree of polymerization 6–18 (dp6–dp18) and dp24, corresponding to the predominantly unsulfated GlcA-GlcNAc domains of heparan sulfate. Unlike heparin, the sedimentation coefficient $s_{20,w}$ of HS dp6–dp24 showed small rotor speed dependence, where similar $s_{20,w}$ values of 0.82–1.26 S (absorbance optics) and 1.05–1.34 S (interference optics) were determined. The corresponding measurements of HS dp6–dp24 gave radius of gyration ($R_g$) values from 1.03 to 2.82 nm, cross-sectional radius of gyration ($R_{S3}$) values from 0.31 to 0.65 nm, and $T_2$ from 3.0 to 10.0 nm. These results indicate a more bent structure than the corresponding modeling starting from 5000–8000 conformationally randomized molecular structures for HS based on the combination of constraints scattering, and constrained scattering modeling recently used for heparin, which is an analog for HS but possesses a higher degree of sulfation, being predominantly S region-like in sequence, and for at least 19 heparin-protein co-crystal complexes. This abundance results from the ease with which heparin is obtained and its strong binding to many of the cell surface proteins whose physiological ligand is HS. An NMR structure is known for heparin (18). Solution structures are known for six heparin dp6–dp36 forms from constrained scattering modeling; these were shown to be similar in conformation to heparin when observed in heparin-protein crystal structures (19). In distinction, up to now, no molecular structures for free HS are known, and only one crystal structure at 0.21 nm resolution for a dp4 HS oligosaccharide complexed with heparinase II is available (20).

Given the importance of understanding the HS solution structure, we have used a multidisciplinary approach to determine molecular structures for HS based on the combination of three methods, namely analytical ultracentrifugation, small angle x-ray scattering, and constrained scattering modeling (21, 22). This approach is well established for solution structure

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§The abbreviations used are: HS, heparan sulfate; dp, degree of polymerization; GlcA, β-D-glucuronic acid; IdoA, α-L-iduronic acid; GlcNS, N-sulfated glucosamine; NAG, N-acetyl-D-glucosamine; GCU, D-glucuronic acid; GCD, 4,5-dehydro-D-glucuronic acid.

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determinations of large multidomain complement and antibody proteins and was recently applied to small heparin glycosaminoglycan fragments (19, 23). Here, we apply this approach for the second time for oligosaccharide solution structures, this time for eight HS fragments ranging in sizes from dp6 to dp24, thus permitting detailed comparisons with heparin. These fragments exhibited solution structures that were similar to those of the heparin fragments. In particular, the overall heights of the HS fragments, combined with a much reduced degree of sulfation compared with heparin. Only minor sequence differences in monosaccharide sequence between HS and heparin thus permitting detailed comparisons with heparin. The HS fragments were eluted using 2% ammonium bicarbonate at a flow rate of 0.2 ml/min in 2-ml fractions. The absorbance of the fractions was measured at 234 nm, and the top fractions corresponding to each individual resolved peak were pooled. The HS oligosaccharides larger than dp12 were not completely resolved (Fig. 2). The pooled fractions were evaporated under reduced pressure and lyophilized before assessment of their sizes by analytical gel permeation chromatography (25). Like previous heparin chromatography, gel permeation chromatography was carried out using two columns (TSK G3000 SW–XL (30 cm) and TSK G2000 SW–XL (30 cm), Anachem) connected in series. The eluant was 0.1 M ammonium acetate solution at a flow rate of 0.5 ml/min, and HS was detected with a refractive index detector (RI-1530, Jasco). The chromatography system was calibrated using the First International Reference Reagent Low Molecular Weight Heparin for Molecular Weight Calibration (National Institute for Biological Standards and Control 90/686). HS quantification was achieved by integration of the area under each refractometric peak and comparison with a standard curve prepared with known concentrations of low molecular weight heparin and a refractive index coefficient of 5500 m$^2$/g·cm$^{-1}$/s. The experiments (28).

Purification of HS Fragments—HS oligosaccharide fragments were analyzed by CZE (29) and SDS-PAGE (31). Each one according to the manufacturers' protocols. HS fragment (5 µg) was dissolved in a minimal volume of 0.1 M phosphate buffer (pH 7.4). The buffer density was measured at 20 °C using an Anton-Paar DMA5000 density meter to be 1.00480 g/ml. A discontinuous buffer system of Laemmli (30) consisted of 0.125 M Tris/HCl, pH 6.8, in the stacking gel and 0.375 M Tris/HCl, pH 8.8, in the resolving gel. The gel running buffer was 25 mM Tris/glycine, pH 8.3. The gel was stained with 0.08% aqueous Azure A for 10 min to visualize HS bands. The gel was then destained in water to remove excess dye and clear the gel background.

Analytical Ultracentrifugation of HS Fragments—Sedimentation velocity data for eight HS fragments (dp6, dp8, dp10, dp12, dp14, dp16, dp18, and dp24) were obtained on two Beckman XL-1 analytical ultracentrifuges (Beckman-Coulter Inc., Palo Alto, CA) using both absorbance and interference optics. Experiments with the dp6–dp24 fragments were performed at concentrations of 0.5 mg/ml in 10 mM HEPES and 137 mM NaCl (pH 7.4). The buffer density was measured at 20 °C using an Anton-Paar DMA5000 density meter to be 1.00480 g/ml. A partial specific volume of 0.467 ml/g determined for heparin (31) was used for HS. An alternative higher value of 0.55 ml/g for HS has been reported elsewhere and was used for data processing only when required to confirm that the partial specific volume has no effect on the outcome of this study (32). Analytical ultracentrifugation runs were carried out in an eight-hole AnTi50 rotor with standard double-sector cells with column heights of 12 mm at 20 °C using absorbance optics at 234 nm and interference optics. Sedimentation velocity data were col-
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lected at 40,000, 50,000, and 60,000 rpm using absorbance optics and interference optics. The continuous $c(s)$ analysis method was used to determine the sedimentation coefficients $s_{20,w}$ of the eight HS fragments using SEDFIT software (version 9.4) (33, 34). The $c(s)$ analysis directly fits the experimental sedimentation boundaries using the Lamm equation, the algorithm for which assumes that all species have the same frictional ratio $f/f_0$ in each fit. The final SEDFIT analyses used a fixed resolution of 200, and optimized the $c(s)$ fit by floating the meniscus and cell bottom when required, and holding the $f/f_0$ value, base line, and cell bottom fixed until the overall root mean square deviations and visual appearance of the fits were satisfactory (Fig. 3). The individual $f/f_0$ values calculated previously for the heparin fragments were used for the equivalent HS fragments (19).

Synchrotron X-ray Scattering of HS Fragments—X-ray solution scattering of the above eight HS fragments dp6–dp24 were performed on beamline ID02 at the European Synchrotron Radiation Facility in Grenoble, France in two sessions with a ring energy of 6.0 GeV (35). In the first session, data were collected for six HS fragments in 16-bunch mode using beam currents from 63 to 89 mA. In the second session, data were collected for all eight HS fragments in 16-bunch mode using beam currents from 65 to 78 mA. Data were acquired using an improved fiber optically coupled high sensitivity and dynamic range CCD detector (FReLoN) with a smaller beam stop. The sample-to-detector distance was 3.0 meters. Experiments collected for all eight HS fragments in 16-bunch mode using beam cur-...
41, 44). X-ray curves were calculated without instrumental corrections because these were considered to be negligible for the pinhole optics used in synchrotron x-ray instruments. First, the number of spheres N in the dry and hydrated models after grid transformation was used to assess steric overlap between the HS disaccharides, where models showing less than 95% of the optimal totals (Table 1) were discarded. This procedure was found to be insensitive to steric overlap in the case of oligosaccharides and was discontinued in favor of the DREIDING minimization procedure (above). Next, the models were assessed by calculation of the x-ray $R_g$ values from Guinier fits of the modeled curves using the same Q ranges used for the experimental Guinier fits in order to allow for any approximations inherent in the use of the $Q-R_g$ range up to 1.5. Models that passed the N and $R_g$ filters were then ranked using a goodness of fit $R$ factor in order to identify the best fit eight models for each HS fragment.

Sedimentation coefficients $s_{20,w}$ for each of the eight best fit HS scattering models were calculated directly from molecular structures using the HYDROPRO shell modeling program (45). The default value of 0.31 nm for the atomic element radius for structures using the HYDROPRO shell modeling program (45).

RESULTS

Sedimentation Velocity Data Analysis for Eight HS Fragments

The purification profile from the Bio-Gel column showed that the four smallest HS fragments revealed two as well resolved peaks, whereas dp14–dp18 and dp24 yielded one signal with high performance data. For the larger oligosaccharide fractions, fittings were performed to show that all of the observed size distributions as expected and were well represented by the preferred Proton NMR spectroscopy analyses of the GlcA-GlcNAc disaccharide with heparin-like S domains present with a minor content of NA and S domains. Signals typically observed for the HS domains were almost completely absent.

Analytical ultracentrifugation studies of macromolecular structures and sizes through quantitative measurements of sedimentation rates in a high centrifugal field (46). Sedimentation velocity experiments at three rotor speeds were performed for the eight HS fragments (dp6–dp18 and dp24) to determine their shapes and degree of polydispersity. The sedimentation coefficient distribution function $c(s)$ was calculated by direct fitting of the sedimentation boundaries using SEDFIT software. The absorbance optics analyses for each HS fragment reproducibly resulted in good boundary fits that resulted in single major peaks (Fig. 3A). The mean sedimentation coefficient $s_{20,w}$ values at three speeds ranged from 0.82 ± 0.05 S for dp6 to 1.26 ± 0.08 S for dp24. The corresponding interference optics analyses for dp6–dp24 also resulted in good boundary fits and single major $c(s)$ peaks with mean $s_{20,w}$ values that ranged from 1.05 ± 0.04 S for dp6 to 1.35 ± 0.04 S for dp24 (Fig. 3B). The

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The theoretical and heparin solution structures differ in their degree of elongation. The overall solution structures are more compact through bending of the heparan sulfate (HS) when compared with their experimental values calculated from linear models, showing reduced values instead (Fig. 6A). Thus, these HS fragments show bending in solution. In addition, the experimental HS $R_G$ values are larger for the dp18–dp36 fragments than those seen for the heparin dp18 and dp24 fragments (Fig. 6A). This shows that HS has a more elongated structure than that of heparin.

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**FIGURE 3. Sedimentation velocity size distribution analyses $c(s)$ of six HS dp$n$ fragments.** Absorbance and interference boundary scans were fitted using SEDFIT software for the HS fragments, each at 0.5 mg/ml. The absorbance data using a wavelength of 234 nm and a rotor speed of 50,000 rpm gave $s_{20,w}$ peaks at 0.84 S for dp6, 0.95 S for dp8, 0.98 S for dp10, 1.08 S for dp12, 1.11 S for dp18, and 1.23 S for dp24. Below these panels, representative boundary fits are shown for clarity. B, the interference data using a rotor speed of 50,000 rpm gave $s_{20,w}$ peaks at 1.04 S for dp6, 1.12 S for dp8, 1.11 S for dp10, 1.19 S for dp12, 1.25 S for dp18, and 1.34 S for dp24. Below these panels, representative boundary fits are shown for dp6 and dp24.
Macromolecules that are sufficiently elongated in shape will show a cross-sectional radius of gyration ($R_{XS}$) value. The $R_{XS}$ value monitors the degree of bending within the macromolecular length. As for the $R_G$ analyses, different $Q$ ranges were used for the $R_{XS}$ fits for the different HS fragments, depending on the size of the HS fragment, all of which were larger than the $Q$ ranges used for the $R_G$ analyses (Fig. 5B). Despite the worsened signal/noise ratios of the $I(Q)$ data, linear fit ranges were identified in the plots of $\ln I(Q)/H$ versus $Q^2$. These gave experimental $R_{XS}$ values of 0.31 nm for dp6 that increased up to 0.65 nm for dp24 (Fig. 6B and Table 1). This increase in the $R_{XS}$ values correlated with the deviation of the $R_G$ values from linearity (Fig. 6A). They were larger than the calculated $R_{XS}$ values from 0.31 to 0.40 nm for the linear HS dp6 – dp30 models, thus supporting the conclusion that the HS fragments become progressively more bent with increase in size. Combination of the $R_G$ and $R_{XS}$ values according to the relationship $L^2 = 12 (R_G^2 - R_{XS}^2)$ for an elliptical cylinder (36) showed that HS dp6, dp8, dp10, dp12, dp14, dp16, dp18, and dp24 have approximate lengths of 3.4, 3.9, 4.6, 5.5, 5.8, 6.8, 7.8, and 9.5 nm in that order. Similar lengths of 7.0, 9.1, 9.6, and 10.7 nm were observed for the heparin dp18, dp24, dp30, and dp36 fragments (19). In conclusion, the comparison of the dp18 and dp24 lengths showed that HS has a longer structure than that of heparin, in addition to being more bent than heparin.

The distance distribution function $P(r)$ is calculated from the full $Q$ range of the $I(Q)$ scattering curve (see “Experimental Procedures”). This provides $R_G$ values and model-independent determinations of the overall length $L$ following an assumption of the maximum dimension $D_{max}$ (Fig. 6C); note that $L$ is not a contour length. The mean $R_G$ values obtained from the $P(r)$ curves increase from 0.98 ± 0.05 nm for dp6 to 3.0 ± 0.05 nm for dp24 (Table 1). These $P(r)$ $R_G$ values are in excellent accord with the corresponding Guinier $R_G$ values from the low $Q$ values and follow the same trends with size (Table 1 and Fig. 6A). Model-independent $L$ values are determined from the $r$ value where the $P(r)$ curve reaches zero at large $r$. These experimental $L$ values were 3.0 nm for dp6, 3.5 nm for dp8, 4.5 nm for dp10, 5.5 nm for dp12, 6.0 nm for dp14, 7.0 nm for dp16, 8.5 nm for dp18, and 10.0 nm for dp24. These values show increasing deviation with size from the longer lengths measured for the linear HS dp6 – dp24 models (i.e. 3.5 nm for dp6, 4.1 nm for dp8, 5.6 nm for dp10, 6.5 nm for dp12, 7.2 nm for dp14, 7.8 nm for dp16, 9.2 nm for dp18, and 11.2 nm for dp24), noting that a hydration shell of thickness 0.6 nm ($2 \times 0.3$ nm) is added to these linear model lengths (44). These experimental $L$ values from the $P(r)$
Table 1: X-ray scattering and sedimentation coefficient modeling fits for eight HS fragments

| HS fragment | Filter | Number of models | Hydrated spheres* | \( R_G \) nm | \( R_{XX} \) nm | \( R \) factor % | Length L nm | \( s_{20w} \) %
<table>
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</thead>
<tbody>
<tr>
<td>dp6</td>
<td>None</td>
<td>5000</td>
<td>9–23</td>
<td>0.82–1.19</td>
<td>0.04–0.43</td>
<td>4.4–7.8</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.01–1.03</td>
<td>0.30–0.51</td>
<td>4.4–4.5</td>
<td>3.0–3.5</td>
<td>0.52–0.79</td>
</tr>
<tr>
<td></td>
<td>Best fit</td>
<td>1</td>
<td>15–20</td>
<td>1.01</td>
<td>0.30</td>
<td>4.4</td>
<td>3.0</td>
<td>0.52</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.03 ± 0.08</td>
<td>0.31 ± 0.06</td>
<td>4.4–4.5</td>
<td>3.0</td>
<td>0.82 ± 0.05</td>
</tr>
<tr>
<td>dp8</td>
<td>None</td>
<td>5000</td>
<td>12–30</td>
<td>1.04–1.14</td>
<td>0.16–0.52</td>
<td>4.4–8.3</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Rps, Rps, R factor</td>
<td>8</td>
<td>20–23</td>
<td>1.17–1.18</td>
<td>0.39–0.40</td>
<td>4.4</td>
<td>3.7–3.8</td>
<td>0.45–0.80</td>
</tr>
<tr>
<td></td>
<td>Best fit</td>
<td>1</td>
<td>20</td>
<td>1.18</td>
<td>0.39</td>
<td>4.4</td>
<td>3.7</td>
<td>0.77</td>
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<tr>
<td></td>
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<td></td>
<td></td>
<td>1.19 ± 0.08</td>
<td>0.40 ± 0.03</td>
<td>4.4–4.5</td>
<td>3.5</td>
<td>0.94 ± 0.06</td>
</tr>
<tr>
<td>dp10</td>
<td>None</td>
<td>5000</td>
<td>15–37</td>
<td>1.17–1.63</td>
<td>0.24–0.63</td>
<td>4.3–8.0</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Rps, Rps, R factor</td>
<td>8</td>
<td>25–30</td>
<td>1.40–1.43</td>
<td>0.42–0.47</td>
<td>4.3–4.4</td>
<td>4.7–4.8</td>
<td>0.48–0.95</td>
</tr>
<tr>
<td></td>
<td>Best fit</td>
<td>1</td>
<td>26</td>
<td>1.42</td>
<td>0.42</td>
<td>4.3</td>
<td>4.8</td>
<td>0.91</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.41 ± 0.07</td>
<td>0.44 ± 0.04</td>
<td>4.5</td>
<td>4.5</td>
<td>0.95 ± 0.09</td>
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<tr>
<td>dp12</td>
<td>None</td>
<td>5000</td>
<td>16–43</td>
<td>1.33–1.84</td>
<td>0.23–0.72</td>
<td>4.1–9.9</td>
<td>NA</td>
<td>NA</td>
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<tr>
<td></td>
<td>Rps, Rps, R factor</td>
<td>8</td>
<td>30–37</td>
<td>1.64–1.65</td>
<td>0.49–0.50</td>
<td>4.2</td>
<td>5.3–5.8</td>
<td>0.47–0.98</td>
</tr>
<tr>
<td></td>
<td>Best fit</td>
<td>1</td>
<td>34</td>
<td>1.64</td>
<td>0.49</td>
<td>4.2</td>
<td>5.5</td>
<td>0.92</td>
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<tr>
<td></td>
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<td></td>
<td>1.65 ± 0.09</td>
<td>0.49 ± 0.04</td>
<td>4.5</td>
<td>5.5</td>
<td>1.08 ± 0.09</td>
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<td>dp14</td>
<td>None</td>
<td>8000</td>
<td>24–50</td>
<td>1.52–1.94</td>
<td>0.18–0.76</td>
<td>6.0–6.5</td>
<td>NA</td>
<td>NA</td>
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<td></td>
<td>Rps, Rps, R factor</td>
<td>8</td>
<td>35–40</td>
<td>1.75–1.78</td>
<td>0.48–0.62</td>
<td>6.2</td>
<td>6.0–6.5</td>
<td>0.74–1.05</td>
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<tr>
<td></td>
<td>Best fit</td>
<td>1</td>
<td>37</td>
<td>1.75</td>
<td>0.48</td>
<td>6.2</td>
<td>6.0</td>
<td>0.96</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td>1.76 ± 0.07</td>
<td>0.48± 0.06</td>
<td>6.0</td>
<td>6.0</td>
<td>1.07 ± 0.07</td>
</tr>
<tr>
<td>dp16</td>
<td>None</td>
<td>8000</td>
<td>28–54</td>
<td>1.57–2.01</td>
<td>0.18–0.76</td>
<td>6.0–6.5</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Rps, Rps, R factor</td>
<td>8</td>
<td>40–50</td>
<td>1.75–2.01</td>
<td>0.18–0.76</td>
<td>6.0–6.5</td>
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<tr>
<td></td>
<td>Best fit</td>
<td>1</td>
<td>43</td>
<td>1.75</td>
<td>0.48</td>
<td>6.2</td>
<td>6.0</td>
<td>0.96</td>
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<td></td>
<td>1.76 ± 0.07</td>
<td>0.48± 0.06</td>
<td>6.0</td>
<td>6.0</td>
<td>1.07 ± 0.07</td>
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<tr>
<td>dp18</td>
<td>Experimental</td>
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<td>1.65 ± 0.09</td>
<td>0.49 ± 0.04</td>
<td>4.5</td>
<td>5.5</td>
<td>1.08 ± 0.09</td>
</tr>
<tr>
<td>dp24</td>
<td>Experimental</td>
<td></td>
<td></td>
<td>1.62 ± 0.03</td>
<td>0.49 ± 0.04</td>
<td>4.5</td>
<td>5.5</td>
<td>1.08 ± 0.09</td>
</tr>
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</table>

* The optimal totals of hydrated spheres were 16 for dp6–dp12.
* The first experimental value is from the Guinier fits.
* The averaged experimental \( s_{20w} \) value is reported for absorbance and interference data recorded at rotor speeds of 40,000, 50,000, and 60,000 rpm.

NA, not applicable.

Constrained Modeling of Six HS Fragments—The experimental x-ray \( R_G \) and \( R_{XX} \) values showed that the solution structures for HS are longer and more bent than those of heparin. Here, constrained scattering modeling was performed with HS in order to provide a molecular explanation of these scattering data. The linear models created from the HS dp4 crystal structure were the starting constraint. All eight HS fragments dp6–dp24 were subjected to modeling. They were considered as belonging to a structurally homologous series. As illustrated in the previous modeling of heparin, the linkage connectivity between the oligosaccharide rings was maintained (Fig. 1A), whereas the \( \Phi \) and \( \Psi \) rotational angles at each glycosidic link-
what turned out to be comparatively flat distributions, unlike those in supplemental Fig. S1, which showed better agreement for the linear dp12 structure for dp12, unlike the cases of dp6 and dp24, unlike the cases of dp6–dp16.

For HS dp6, the modeling analyses showed slightly bent structures with slight bending, unlike the cases of dp6–dp16. The eight best fit models gave 0.52–0.79 S. These are lower but comparable with the experimental $s_{20,w}$ values of 0.82 ± 0.05 S and 1.05 ± 0.04 S, given that the typical accuracy of the $s_{20,w}$ calculation is ±0.21 S (22).

For HS dp8, the modeling analyses indicated slightly bent structures similar to those seen for dp6. The eight best fit models gave $R$ factors of 4.4%, $R_G$ values of 1.17–1.18 nm, $R_{XX}$ values of 0.39–0.49 nm, and $L$ values of 3.7–3.8 nm. These values agree well with the experimental $R_G$ value of 1.19 ± 0.08 nm, $R_{XX}$ value of 0.40 ± 0.03 nm, and the $P(r)$ length of 3.5 nm (Table 1). Contrary to these agreements, the linear model again gave a higher $R$ factor of 5.3%, an $R_G$ value of 1.10 nm, and an $L$ value of 3.5 nm. The visual agreement between the experimental and modeled $I(Q)$ curves and $P(r)$ curves was excellent (Fig. 7A). The calculated $s_{20,w}$ values from the eight best fit models gave 0.52–0.79 S. These are lower but comparable with the experimental $s_{20,w}$ values of 0.82 ± 0.05 S and 1.05 ± 0.04 S, given that the typical accuracy of the $s_{20,w}$ calculation is ±0.21 S (22).

For HS dp10, the modeling analyses showed good agreement with slightly bent structures, in which the deviation from the linear dp10 structure for dp10 was slightly increased. The eight best fit dp10 models gave $R$ factors of 4.3–4.4%, $R_G$ values of 1.40–1.43 nm, $R_{XX}$ values of 0.42–0.47 nm, and $L$ values of 4.7–4.8 nm. These correspond well with the experimental $R_G$ value of 1.41 ± 0.07 nm, $R_{XX}$ value of 0.44 ± 0.04 nm, and $L$ value of 4.5 nm (Table 1). The deviations from a linear dp10 model are larger, for which the $R$ factor is 4.5%, the $R_G$ value is 1.46 nm, the $R_{XX}$ value is 0.36 nm, and the $L$ value is 5.6 nm. The visual agreement of the experimental and modeled $I(Q)$ and $P(r)$ curves was again excellent (Fig. 7C). The eight modeled $s_{20,w}$ values of 0.48–0.95 S are similar to the experimental $s_{20,w}$ values of 0.95 ± 0.09 S and 1.09 ± 0.06 S, with the best fit model giving a modeled value of 0.91 S, within the typical accuracy of ±0.21 S (22).

For HS dp12, the modeling analyses were also successful, in which the deviation from a linear dp12 structure was greater. The eight best fit models gave $R$ factors of 4.2%, $R_G$ values of 1.64–1.65 nm, $R_{XX}$ values of 0.49–0.50 nm, and $L$ values of 5.3–5.8 nm. These agree well with the experimental $R_G$ value of 1.65 ± 0.09 nm, $R_{XX}$ value of 0.49 ± 0.04 nm, and $L$ value of 5.5

**FIGURE 5.** Experimental Guinier x-ray scattering analyses of eight HS dp6–dp24 at concentrations of 0.5 mg/ml. The filled circles represent the experimental analytical ultracentrifugation data. The eight best fit models gave 0.52–0.79 S. These are lower but comparable with the experimental $s_{20,w}$ values of 0.82 ± 0.05 S and 1.05 ± 0.04 S, given that the typical accuracy of the $s_{20,w}$ calculation is ±0.21 S (22).
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FIGURE 6. Experimental Guinier and P(r) x-ray data analyses of eight HS dp6 – dp24 fragments. A, Guinier plots (△) and P(r) curves (○) with the predicted \( R_G \) values calculated from Guinier analyses for each HS fragment. The \( R_F \) values from Guinier analyses for heparin from (19) are denoted by open and filled diamonds, respectively, and fitted to a dotted line. C, the distance distribution function \( s_{20,w} \) curves were fitted to a dotted line. D, comparison of the experimental \( P(N) \) curves for HS dp6 – dp24 with heparin dp6 – dp24. The curves corresponding to the four HS fragments dp6, dp12, dp18, and dp24 are denoted by continuous, dashed, and dotted lines in alternation. The eight modeled \( P(r) \) data are from Ref. 19.

For HS dp14, good agreement between the experimental and modeled data were obtained, whereas for HS dp16 from a linear dp14 structure was larger. The \( R_F \) values of 4.4%, \( R_G \) values of 1.75 ± 0.05 nm, \( R_{XX} \) values of 0.48–0.51 nm, and \( L \) values of 6.0–6.5 nm. These agree well with the experimental \( R_G \) value of 1.76 ± 0.07 nm and \( R_{XX} \) value of 0.51 ± 0.02 nm, and \( L \) value of 6.0 nm (Table 1). These values of 1.16 ± 0.05 S, with the best fit model giving a value of 1.10 S, again within the typical accuracy of ±0.21 S (22).

In conclusion, the best fit models for HS dp6 – dp16 show progressively more bent structures in solution with increase in HS size. This is visible from the superimposition of the eight best fit models for each HS fragment (Fig. 8). In terms of lengths, crystal structures containing HS and heparin showed that the glycosidic linkage in HS has a length similar to that in heparin (Fig. 9B). In HS, the separation between the C1 and C4 atoms of GlcA-GlcNAc is 0.237 ± 0.003 nm, and that between the C1 and C4 atoms of GlcNAc-GlcA is 0.235 ± 0.002 nm (dp6), 1.15 ± 0.06 nm (dp10), 1.43 nm (dp12), 1.44 nm (dp14), 1.61 nm (dp16), 1.87 nm (dp18), and 1.90 nm (dp24). The eight fragments are denoted by open and dotted lines, respectively, and fitted to a dotted line. The eight modeled \( P(r) \) data are from Ref. 19.

For HS dp16, the outcome of the modeling analyses was similar to that of dp14. The eight best fit models gave \( R_F \) factors of 6.3–6.4%, \( R_G \) values of 1.92–2.02 nm, \( R_{XX} \) values of 0.49–0.52 nm, and \( L \) values of 6.5–7.2 nm. These agree well with the doted lines.

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However, the comparison of the \(\text{H9021}\) and \(\text{H9023}\) angles of HS and heparin showed that these values differed (supplemental Fig. S2). The mean \(\text{H9021}\) and \(\text{H9023}\) angles for the GlcA-GlcNAc glycosidic linkage were 93 and 134° in HS and are similar to the corresponding IdoA-GlcNS values of 61 and 132° in heparin, although the S.D. values are large at 21–36° (Table 2). In distinction, the mean \(\text{H9021}\) and \(\text{H9023}\) angles for the GlcNAc-GlcA linker were 4 and 138° in HS, which are more different from the corresponding values of 98 and 86° for the GlcNS-IdoA linker in heparin, although the S.D. values are again large (Table 2). The distribution of the \(\text{H9021}\) and \(\text{H9023}\) values in supplemental Fig. S2B suggests that there is more rotational variability at this second glycosidic linkage in HS when compared with those for heparin in supplemental Fig. S2B. The experimental \(\text{H9021}\) and \(\text{H9023}\) scattering data are represented by black circles and lines, respectively; the red lines and models correspond to the best fit, the green lines and models correspond to the linear poor fit, and the inset lines and models correspond to the linear poor fit. The linear poor fit models are shown in the bottom left corner, together with their maximum lengths \(L\) in nm for comparison with the experimental \(L\) values in the \(\text{H9021}\) curves.

**DISCUSSION**

The size and spacing of S domains in heparan sulfate are proposed to be as important to its biologically significant interactions with proteins as are the detailed sequences of the S domains (48). Heparin, a commonly used model compound for heparan sulfate, consists of lengthy S domains, made up largely of the repeating trisulfated disaccharide shown in Fig. 1B, separated by much smaller, unsulfated NA domains. In heparan sulfate, the position is reversed, and long NA domain sequences (Fig. 1A) act as spacers to separate the short S domains. Although the S domain conformation, exemplified by heparin, has been the subject of numerous studies (49), the NA domain has not. It has been proposed that the NA domain repeating sequences are both less flexible (50) and more flexible (51) than the S domains.

By this study, we have determined molecular solution structures for small and medium sized HS fragments dp6 to dp16 and obtained insights into the structures of HS dp18 and dp24. The fragments we used were produced by extensive depolymerization using heparinase 1, an enzyme that cleaves only within the S domains (28) leaving NA domains untouched. It is therefore likely that some minor degree of sulfation remains at the reducing and non-reducing ends of our fragments but that internal disaccharides are unsulfated. Such fragments bear a closer resemblance to NA domains of intact HS than the most commonly used model compound for this sequence, the capsular polysaccharide from *Escherichia coli* K5 (50, 51). These HS...
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Figure 8. Superimposition of the eight best fit models for each of the six HS dp6–dp16 fragments (dp6 (A), dp8 (B), dp10 (C), dp12 (D), dp14 (E), and dp16 (F)). Each set of eight best fit models for the six HS fragments was superimposed globally using Discovery Studio VISUALISER software, and then their non-hydrogen atoms were displayed as shown.
for all six structures. This outcome suggests that the heparin structures show greater rigidity than the HS fragments.

The solution structures of the HS dp6–dp16 fragments exhibited a degree of bending (Figs. 8 and 9A). In addition, the HS structures are longer by reason of alterations in the glycosidic Φ and Ψ angles (supplemental Fig. S2). The physical basis of these changes in HS is likely to arise from the GlcA-GlcNAc sequences, together with less repulsion between sulfate and sulfate, between sulfate and carboxylate, and between carboxylate and carboxylate groups in HS.

This study has provided fresh insight into potential HS-protein interactions and heparin-protein interactions. The outcome of 19 protein-heparin crystal structures has been discussed previously (19). In distinction, only one protein–HS crystal structure is known (20). It was of interest that the Φ and

| Table 2 Summary of the Φ and Ψ angle in the solution and crystal structures of HS and heparin |
|---------------------------------|-------------------------------------------------|-------------------------------------------------|
|                                  | GlcA-GlcNAc                                  | GlcNAc-GlcA                                    |
|                                  | Φ      | Ψ      | Φ      | Ψ      |
| HS crystal structure*           | −90 ± 1 | 127 ± 4 | 83 ± 3 | 94 ± 1 |
| HS dp6                          | −101 ± 38 | 140 ± 19 | −16 ± 27 | 129 ± 48 |
| HS dp8                          | −93 ± 26 | 121 ± 29 | 34 ± 55 | 152 ± 15 |
| HS dp10                         | −121 ± 26 | 126 ± 17 | 18 ± 41 | 143 ± 23 |
| HS dp12                         | −78 ± 30 | 114 ± 26 | 24 ± 35 | 121 ± 30 |
| HS dp14                         | −64 ± 32 | 149 ± 21 | −12 ± 53 | 139 ± 42 |
| HS dp16                         | −112 ± 38 | 136 ± 45 | −11 ± 88 | 143 ± 52 |
| Average (27 of 35 values)       | −93 ± 36 | 134 ± 32 | 4 ± 58  | 138 ± 37 |
| IdoA-GlcNS                      | Φ      | Ψ      | Φ      | Ψ      |
| Heparin dp18                    | −79 ± 20 | 132 ± 19 | 84 ± 22 | 100 ± 19 |
| Heparin dp24                    | −52 ± 22 | 135 ± 15 | 104 ± 27 | 96 ± 25 |
| Heparin dp30                    | −61 ± 17 | 127 ± 20 | 100 ± 26 | 78 ± 17 |
| Heparin dp36                    | −64 ± 36 | 136 ± 21 | 87 ± 25  | 82 ± 22 |
| Average (50 of 54 values)       | −98 ± 25 | 186 ± 22 | 98 ± 25  | 86 ± 22 |

* The mean values are taken from those seen in the crystal structure (Protein Data Bank entries 1H90 and 1H91).

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The solution structure of heparan sulfate differs from that of heparin. IMPLICATIONS FOR FUNCTION.

Sanaullah Khan, Elizabeth Rodriguez, Rima Patel, Jayesh Gor, Barbara Mulloy, and Stephen J. Perkins

This article has been withdrawn by the authors.

We determined solution structures for six forms of heparan sulfate dp6–dp16 in this article. Following publication of our 2011 study, we regretfully discovered an error in the anomeric configuration of our heparan sulfate structural models. Correction of this error requires substantial reinterpretation of our experimental data. We wish to withdraw the manuscript and will submit a corrected report. We apologize for any inconvenience caused by this error.

Authors are urged to introduce these corrections into any reprints they distribute. Secondary (abstract) services are urged to carry notice of these corrections as prominently as they carried the original abstracts.