The solution conformation of the homogeneous, heparin-derived tetrasaccharide ΔUA2S(1 → 4)-α-L-GlcNPs6S(1 → 4)α-L-IdoAp2S(1 → 4)-α-D-GlcNPs6S (residues A, B, C and D respectively, where IdoA is iduronic acid) has been investigated by using 1H- and 13C-NMR. Ring conformations have been defined by J-coupling constants and inter-proton nuclear Overhauser effects (NOEs), and the orientation of one ring with respect to the other has been defined by inter-ring NOEs. NOE-based conformational modelling has been done by using the iterative relaxation matrix approach (IRMA), restrained molecular dynamics simulations and energy minimization to refine structures and to distinguish between minor structural differences and equilibria between various ring forms. Both glucosamine residues B and D are in the 4C1 chair conformation. The 6-O-sulphate group is oriented in the gauche-trans configuration in the D ring, whereas in the B ring the gauche-gauche rotomer predominates. Uronate (A) and iduronate (C) residues are mostly represented by 1H and 2Sa twisted boat forms, respectively, with small deviations in expected coupling constants and NOEs suggesting minor contributions from other A and C ring conformations.

INTRODUCTION

Heparin is mainly a polydisperse sulphated copolymer of 1 → 4-linked glucosamine and uronic acid residues. Most of the heparin molecule is accounted for by this repeating disaccharide unit that consists primarily of 2-O-sulpho-α-L-idopyranosyluronic acid (α-L-IdoAp2S) and 2-amino-2-deoxy-di-2,6-sulpho-α-D-glucopyranose (α-D-GlcNPs6S) [1,2], although both α-D-glucopyranosyluronic acid and α-L-idopyranosyluronic acid residues are also found. The major repeating sequence, (1 → 4)-α-L-IdoAp2S(1 → 4)-α-D-GlcNPs6S(1 → 4)-α-L-IdoAp2S(1 → 4)-α-D-GlcNPs6S (residues A, B, C and D respectively, where IdoA is iduronic acid) has been investigated by using 1H- and 13C-NMR. Ring conformations have been defined by J-coupling constants and inter-proton nuclear Overhauser effects (NOEs), and the orientation of one ring with respect to the other has been defined by inter-ring NOEs. NOE-based conformational modelling has been done by using the iterative relaxation matrix approach (IRMA), restrained molecular dynamics simulations and energy minimization to refine structures and to distinguish between minor structural differences and equilibria between various ring forms. Both glucosamine residues B and D are in the 4C1 chair conformation. The 6-O-sulphate group is oriented in the gauche-trans configuration in the D ring, whereas in the B ring the gauche-gauche rotomer predominates. Uronate (A) and iduronate (C) residues are mostly represented by 1H and 2Sa twisted boat forms, respectively, with small deviations in expected coupling constants and NOEs suggesting minor contributions from other A and C ring conformations.

Previous findings suggest that different growth factors (and other proteins) might bind to heparin or heparan sulphate in a differential and specific manner. Fibroblast growth factor 1 (FGF-1) binds to two consecutive 6-O-sulphate are redundant [14,16–18]. In fact, a search for a more rigid β-glucopyranosyluronic acid residues show considerably less biological activity.

Abbreviations used: 1D and 2D, one- and two-dimensional; ESFF, electrostatic force field; FGF, fibroblast growth factor; g, gauche; HETCOR, heteronuclear correlated two-dimensional NMR; IdoA, iduronic acid; IRMA, iterative relaxation matrix approach; MD, molecular dynamics; NOE, nuclear Overhauser effect; RMS, root mean square; t, trans.

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to basic residues in FGF-2. Thus most of the O-sulphate groups in native heparin neither contribute to nor interfere with FGF-2 binding. In contrast, binding of hepatocyte growth factor to heparin/heparan sulphate seems to depend primarily on glucosamine 6-O-sulphate groups [20]. The conformation of methylene-O-sulphate groups is therefore of interest in understanding heparin–protein interactions involving the sulphate group at this position.

The present study is aimed at determining the solution conformation of the heparin derived tetrasaccharide by using 1H-NMR NOEs and coupling constants, 13C spin–lattice ($T_1$) relaxation rates, the iterative relaxation matrix approach (IRMA) and restrained molecular dynamics (MD) and energy minimization.

MATERIALS AND METHODS

Heparin tetrasaccharide isolation

Approx. 200 mg of heparin was dissolved in 10 ml of distilled water and dialysed exhaustively, freeze-dried, and prepared at exactly 20 mg/ml in distilled water [21]. To 10 ml of this 20 mg/ml heparin was added 1 ml of sodium phosphate buffer (100 mM sodium phosphate, pH 7, with 200 mM sodium chloride) containing 3 m-i.u. of heparin lyase 1 (EC 4.2.2.7). The reaction mixture was incubated at 30 °C for 8 h. At reaction completion, the depolymerization mixture was separated and individual low-molecular-mass heparin fractions were isolated by HPLC as described previously [6,22].

NMR measurements

For NMR measurements, freeze-dried heparin tetrasaccharide was dissolved to a concentration of 8 mM in 2H$_2$O. The pH was adjusted to the desired value by adding microlitre quantities of NaO$_4$H or HCl to the sample. For most experiments the temperature was controlled at 5 °C to effect shorter relaxation rates and to minimize the contribution from high-energy conformations. 13C-NMR spectra were acquired on Bruker AMX-600 and AM-250 NMR spectrometers operating at 13C frequencies of 150 MHz and 62.5 MHz respectively. 1H-NMR spectra were acquired on a Bruker AMX-600 NMR spectrometer operating at a 1H frequency of 600 MHz.

For 1H resonance assignments, two dimensional (2D) NMR homonuclear Hartman–Hahn spectroscopy spectra, obtained by spin-locking with an MLEV-17 sequence [23] with a mixing time of 60 ms, were used to identify spin systems. NOESY experiments [24,25] were performed for mixing times of 50, 100, 200, 300 and 500 ms to sequentially connect saccharide ring-spin systems and to define NOE build-up curves for use in IRMA calculations described below. 1H–1H coupling constants were derived either from high-resolution 32k one-dimensional (1D) 1H-NMR spectra or from a high-resolution double-quantum-filtered COSY experiment [26,27]. 13C resonance assignments were made via analysis of a 13C–1H heteronuclear correlated 2D NMR (HETCOR) experiment [28] and were consistent with those assignments reported by Merchant et al. [4]. 2D NMR data were acquired in the phase-sensitive mode by using time proportional phase incrementation (TPPI) or States-TPPI [29–31]. The residual water resonance was suppressed by direct irradiation (0.6 s) during the relaxation delay between scans. Spectra were normally collected as 256–512 $t_1$ experiments each with 2k complex data points and 32–64 scans over a spectral width of 5 kHz in both dimensions with the carrier placed on the water resonance. 2D NMR spectra were processed off-line on an SGI Indigo Extreme workstation with the program Felix® (Biosym/MSI, San Diego, CA, U.S.A.). Free induction decays were generally zero-filled to 2k in the $t_1$ dimension and the squared sine function apodized before Fourier transformation.

13C spin–lattice relaxation times ($T_1$) were measured with broadband proton decoupling by using the inversion-recovery method. The number of 13C acquisitions was varied from 10000 to maintain a signal-to-noise ratio in the equilibrium spectrum greater than 10. Ten partly relaxed spectra were acquired for each relaxation experiment. To reduce errors from radio-frequency field inhomogeneities, a composite 180° pulse [$90^\circ$–$180^\circ$–$90^\circ$] was used. $T_1$ values were calculated from the initial slope of relaxation rate curves by using the method described by Daragan and Mayo [32]. To minimize the error in determining these rates, a least-squares method with weighted functions, e.g. $A(t) = \exp(-2W_1t)$, was used [32]. $W_1$ was calculated by minimizing the function $\Sigma(I_n-I_e-A\exp(-W_1t))^2$, where $I_n$ and $I_e$ are equilibrium and transient values respectively of resonance intensities. To calculate the relaxation rate, $W_1$, the function:

$$S = S_0\exp(-2W_1t)(I_n-I_e-A\exp(-W_1t))^2$$

was minimized. This method reduces errors arising from inaccuracies normally present at the tail of relaxation curves plotted on a semilogarithmic scale. Statistical errors in determining spin–lattice relaxation rates were less than 5%.

For this heparin-derived tetrasaccharide, we are away from the extreme narrowing limit; therefore, 13C $T_1$ values acquired at two 13C-NMR frequencies were used to calculate a rotational correlation time by using a rigid isotropic model. The root mean square (RMS) value between experimental and theoretical $T_1$ values was 0.11 for a $\tau_0$ of 400 ps. To account for internal mobility, the Lipari and Szabo [33] model free approach was used and gave a smaller RMS value of 0.05, indicating the

Figure 1 Chemical structure of heparin-derived tetrasaccharide

Residues are labelled as A, B, C and D for uronate, glucosamine, iduronate and glucosamine rings respectively, as discussed in the text. Note that ring A uronate has an unnatural double bond resulting from heparinase digestion.
Suppression by using the inversion recovery method. Where \( A \) and \( R \) model structures, were used. The definition of the NMR which provide a comparison between experimental data and relaxation matrix calculations. After calculating a set of constraints for each structure, restricted molecular dynamics simulations were run with the same software. After calculating a set of constraints for each structure, restricted molecular dynamics simulations were run for 5000 ps at 300 K with the dielectric constant, \( \epsilon \), set to 6. This was then followed by energy minimization. Final structures were used for subsequent relaxation matrix calculations.

To measure the quality of IRMA calculations, \( R \)-factors, which provide a comparison between experimental data and model structures, were used. The definition of the NMR \( R \)-factor used here is analogous with that used by Gonzales et al. [36]:

\[
R = \frac{\sum \sum \omega_i (\tau_m) |A_i^{\text{obs}}(\tau_m) - A_i^{\text{cari}}(\tau_m)|}{\sum \sum \omega_i (\tau_m) |A_i^{\text{cari}}(\tau_m)|}
\]

where \( A_i \) are cross-peak intensities and \( \omega_i \) are weighting functions that were chosen to be equal to the mixing time.

**RESULTS**

\(^1\)H and \(^3\)C-NMR spectra for heparin-derived tetrasaccharide have been previously assigned [6] based primarily on chemical shifts taken from COSY [7] and HETCOR [8] experiments. Of the four sugar rings, namely A, B, C and D from the non-reducing to reducing ends respectively (see Figure 1), glucosamine B and D rings could not be assigned unambiguously. In the present study, sequence-specific resonance assignments have been made by identifying inter-residue H1–H4 NOE connectivities in NOESY spectra (results not shown). Various ring and side-chain conformations discussed in this text are depicted in Figure 2 for clarity.

**Ring conformation of glucosamine residues**

Coupling constants obtained from an analysis of 1D \(^1\)H-NMR and 2D double-quantum-filtered COSY spectra of the tetrasaccharide indicate that both 2-amino-deoxy-di,2,6-sulpho-\( \alpha \)-glucosamine (referred to here simply as glucosamine) residues B and D are in the \(^1\)C\(_1\) conformation (see Figure 2) as suggested for glucosamine residues found in heparin and in other heparin-derived oligosaccharides [11,12,38,39]. The small H1 and H2

**Figure 2. Possible conformations of both uronate (ring A) and iduronate (ring C) residues in the tetrasaccharide**

The uronate residue at the non-reducing terminus in the half-chair \(^2\)H, conformer, despite having all substituents equatorial, is less favoured than the \(^1\)H conformer owing to anomeric, intramolecular hydrogen bonding and allylic effects. The internal iduronate residue can adopt a number of conformations including \(^1\)C\(_4\), \(^3\)C\(_4\), \(^2\)B and \(^4\)S. The \(^2\)S conformer is observed in solution, in which the substituents are either equatorial or bowsprit and the H2–H5 flagpole interaction is reduced in comparison with that observed in the \(^2\)B conformer.
coupling constants ($^1J_{H_3} = 3.3 \text{ Hz}$) indicate an axial-equatorial relationship between $H1$ and $H2$ protons, and larger $^3J_{H_1}$ values (more than 8 Hz) for $H3$, $H4$ and $H5$ indicate a trans-diaxial relationship between these protons. Use of these $J$ values in the Karplus equation [40] yields dihedral angles listed in Table 1. These experimentally determined angles agree well with those calculated by energy minimization of the tetrasaccharide without using NOE-derived distance constraints (Table 1). Furthermore the $^4C_1$ chair form is supported by NOE-based conformational modelling discussed below. These data indicate that neither the unsaturated uronate nor the iduronate residue substantially modifies either glucosamine ring conformation. This conclusion supports a previous observation that adjacent $\beta$-glucuronate and 2-O-sulphated $\alpha$-iduronate residues in a pentasaccharide that corresponds to the ATIII binding site in heparin and contains a 2-amino-2-deoxy-ribo-2,3,6-sulpho-$\alpha$-D-glucopyranose ring [39] do not perturb that glucosamine conformation either.

### Ring conformation of uronate residues

The conformation and flexibility of uronate residues in heparin have been the subject of a number of studies [11, 13, 39, 41, 42]. The heparin-derived tetrasaccharide under study here contains two different types of uronate residue, a presumably more rigid, 4,5-saturated uronate ring (ring $A$) and a non-reducing end and a more flexible, 2-O-sulphated $\alpha$-l-iduronopyranosyluronic acid (ring $C$). In solution, the unsaturated uronate ring $A$ with co-planar $C3$, $C4$, $C5$ and $O5$ atoms can exist in two different half-chair conformations, $^2H_1$ and $^2H_2$ (Figure 2), differentiated by above- and below-plane positions for $C1$ and $C2$ atoms. The $^2H_2$ conformation is generally favoured owing to an anomic effect with ring B glucosamine, a potential hydrogen bond between $O1$ and $H03$, and an allylic effect at $C3$ (see Figure 2). The $^2H_1$ conformation, with $C1$ and $C2$ lying below and above this plane respectively, shows $^3J_{H_1}$ and $^3J_{H_2}$ coupling constants greater than 6 Hz, whereas the $^1H_1$ conformation, with $C2$ and $C1$ lying below and above this plane respectively, shows smaller and near-equal $^3J_{H1,2}$ and $^3J_{H2,3}$ coupling constants of less than 4 Hz. Initial heparin tetrasaccharide structures were taken from the heparin dodecasaccharide structure given by Mulloy et al. [37]; a carbon–carbon double bond was added at $C4$–$C5$, and with the $A$ ring in the $^2H_1$ form the tetrasaccharide structure was energy minimized as described in the Materials and methods section. The resulting dihedral angles for both forms are listed in Table 1. Experimentally, $^3J_{H1,2}$ and $^3J_{H2,3}$ values for ring $A$ are 2.0 and 1.0 Hz respectively, and by using the Karplus equation [40], $H1$–$C1$–$C2$–$H2$ and $H2$–$C2$–$C3$–$H3$ torsion angles are estimated to be $+59^\circ$ and $-89^\circ$ respectively (Table 1), consistent with a preferred $^1H_1$ half-chair conformation.

For the 2-O-sulpho-$\alpha$-l-idopyranosyluronate (called simply iduronate) ring $C$, $^3J_{H1,2}$ and $^3J_{H2,3}$ coupling constants are 6.6 and 3.8 Hz respectively, with assessable dihedral angles $H2$–$C2$–$C3$–$H3$ and $H3$–$C3$–$C4$–$H4$ [40] listed in Table 1. The $H2$–$C2$–$C3$–$H3$ dihedral angle deviates considerably from that expected for a $^4C_1$ chair form, which should give $^3J_{H1,2}$ and $^3J_{H2,3}$ both less than 5 Hz. For the most part, the calculated dihedral angles in fact suggest that the average solution conformation for the iduronate ring $C$ is a slightly twisted boat form, $^2S_0$ (Table 1). Four possible conformers for ring $C$, i.e. $^4C_1$, $^4C_2$, $^4S_2$ and $^4S_3$ forms, are shown in equilibrium in Figure 2.) This conclusion is further supported by variable-mixing-time NOESY data and IRMA NOE-build-up curve simulations (see below). Furthermore NOESY spectra show intra-residue NOE cross-peaks between $H2$ and $H5$ and between $H1$ and $H3$ in ring $C$, which would not be observed in a true $^4C_1$ chair form. However, in a $^2S_3$ twisted boat form, these NOEs might be observed. Either of these conformations tends to minimize unfavourable 1,3 diaxial non-bonded interactions that are expected in the $^4C_1$ chair form where four of the substituents occupy axial positions and only the carboxy group is equatorial. In this respect, the conformation of ring $C$ becomes a boat, bringing three of the substituents into equatorial (e) positions and the 2-O-sulphate and carboxy group in the bowsprit (s) orientation. The $H2$ and $H5$ flagpole (fP) protons are pulled apart resulting in a $^2S_3$ twist-boat in which the fP–fP interaction is minimized and the conformation becomes more stable (see Figure 2). One exception to this reasoning can be found in the difference between calculated and experimentally determined $H1$–$C1$–$C2$–$H2$ dihedral angles (Table 1). The two possible ranges for this angle derived from the coupling constant by using the Karplus equation are far from the calculated value given in Table 1 for the $^2S_3$ form. By the IRMA procedure, however, this dihedral angle in the modelled conformation is considerably closer to the experimentally determined value.

### Orientation of 6-O-sulphate side-chains

The orientation of both hydroxymethyl and 6-O-sulphate is determined by rotation about the C5–C6 bond with dihedral angles $\phi$ (O5–C5–C6–O6). Normally, this orientation is described by three staggered conformations [gauche $(g)$ or trans $(t)$]: $gg$, $gt$, $tt$.
predominance of either the gt or tg rotomer. For ring B, however, approximately equal coupling constants were found for both 6H resonances, suggesting a major contribution from the gg rotomer.

Stereospecific H6 methylene proton assignments have been made in monosaccharides by using partly deuterated hydroxymethyl groups [45]. For longer-chain heparin molecules, Mulloy et al. [37] accepted these same relative hydroxymethyl R and S proton chemical shifts and concluded a preferred gg conformation for 6-O-sulphated side-chains. Because it is unclear whether or not this assumption is correct for polysulphated oligosaccharides, we calculated the internal energy of the tetrasaccharide in different conformations by using an electrostatic force field (ESFF in DISCOVER) and partial charges (see the Materials and methods section). Electrostatic interactions were not explicitly considered by Mulloy et al. [37]. During our calculations the terminal A ring and non-terminal C ring were fixed in their preferred conformations 1H$_6$ and 2S$_6$ respectively, with B and D rings having all nine possible 6-O-sulphate group conformations: gg,gg; gg,gt; gg,tg; etc. Each overall conformation was minimized by using different values for the dielectric constant, $\epsilon$, to provide more reliable results. The dependence of relative rotomer populations on $\epsilon$ is shown in Figure 3. For $\epsilon$ in the range 4–10, gg and gt (or tg) 6-O-sulphate side-chain orientations in the B ring and a gt orientation in the D ring contribute most to the ensemble of sampled conformations. For ring D, this result agrees qualitatively with the ratio for hydroxymethyl rotomer populations mentioned above and is consistent with our qualitative J values. On the basis of these coupling constants and internal energy calculations, we have assigned for ring D the more downfield H6 signal to the H6$\beta$ proton and the more upfield resonance to the H6$\alpha$ proton. This is just the opposite of glucose hydroxymethyl assignments [45].

Motional dynamics

$^{13}$C spin–lattice ($T_1$) relaxation rates for carbons in the tetrasaccharide were measured at two resonance frequencies (62.5 and 150 MHz) to determine the overall rotational correlation time and motional order parameters, S\(^2\). Only small differences in $T_1$ values were observed among different carbon positions in all four rings. At 62.5 MHz, $T_1$ values ranged from 0.13 to 0.18 s. These differences most probably originate from variations in internal motions. Ring-averaged motional order parameters [33], S\(^2\), all fell in the range 0.83 ± 0.05 to 0.98 ± 0.02. S\(^2\) values are largest for residues B (0.98 ± 0.02) and C (0.97 ± 0.03) and are smallest for residues A (0.87 ± 0.03) and D (0.83 ± 0.05) (all values given ± S.E.M.). As expected, these order parameters indicate decreased internal motions in rings B and C relative to the terminal rings A and D. Given the generally large S\(^2\) values, however, overall inter-ring mobility is highly constrained. In this respect a simple rigid isotropic rotational model was used in subsequent IRMA calculations. This isotropic hard-sphere rotational model with a rotational correlational time of 400 ps can account fairly accurately for observed $T_1$ values. Alternatively, anisotropic tumbling over a restricted range of angles between CH vectors and the main symmetrical top axis (as was found for longer oligosaccharides [37]) can also account for observed $T_1$ values. This alternative explanation is less likely for the tetrasaccharide studied here because components of the moment of inertia tensor are nearly the same and multiple charged groups promote greater isotropic tumbling.

NOE-based conformational modelling

Four different initial structures were used in IRMA calculations. Because the non-terminal iduronate residue C can exist in two
forms, \( ^1C_1 \) and \( ^3S_\alpha \), both forms were used as initial structures and, for reasons outlined above, both \( gg \) and \( gt \) 6-O-sulphate side-chain rotomers in residue B were used in separate calculations with the glucosamine D ring 6-O-sulphate group being initially set in the \( gt \) rotomer state. Furthermore owing to the absence of a \( J_{NH,CH} \) coupling constant (NH proton exchanged with deuteron \(^2H\)), the H2–C2–N–H dihedral angle was fixed in the trans state according to Mulloy et al. [37].

Symmetry-related NOESY cross-peaks were volume integrated and averaged. Some NOESY cross-peaks were not used owing to NOE build-up curve anomalies arising from cross-peaks close to the diagonal. A total of 37 NOEs measured at five mixing times were used as input for IRMA calculations. Four NOEs, B2–B4, B3–B5, D2–D4 and D3–D5, were taken as references for calculating relaxation matrix elements. With rings B and D in a \(^4C_1 \) conformation, these inter-proton NOEs provide good distance calibrations. The resulting structures were then used in a 5000 ps NOE-restrained molecular dynamics simulation at 300 K with a force constant of 20 kcal/mol/Å² and \( \epsilon = 6 \). Dihedral angular constraints obtained from experimental \( J \) coupling constants were not included in these simulations. Structures were then energy minimized and used as initial structures in subsequent IRMA calculations.

R-factors given in Table 2 indicate that three iterations of this protocol are sufficient for convergence of the IRMA procedure. Figure 4 compares an actual and a calculated NOESY spectrum. Apart from the residual water resonance observed in the experimental NOESY data set, the calculated NOESY spectrum yields very similar cross-peak patterns. For a better comparison of NOE amplitudes, Figure 5 shows the respective stacked plots.

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**Figure 4** Tetrasaccharide NOESY spectra

Two NOESY spectra are shown for the tetrasaccharide. Left panel, experimental \(^1H\) NOESY data accumulated at 600 MHz with a mixing time of 500 ms. Right panel, an IRMA-calculated \(^1H\) NOESY spectrum of the same spectral region. The residual water resonance is absent from the calculated spectrum.

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**Figure 5** Tetrasaccharide NOESY spectra in stacked-plot format

Regions from two NOESY spectra (experimental at the left and IRMA calculated at the right) are shown in stacked-plot format.
from the same experimental and calculated NOESY data sets. Although amplitudes vary somewhat, it should be emphasized that NOEs are heavily weighted by an inverse sixth power of the inter-nuclear distance; therefore even a factor 2 change in amplitude (assuming that motional effects have been treated equally) will affect the inter-nuclear distance by only a few tenths of an Ångström. Figure 6 presents some typical NOE build-up curves: triangles indicate experimental data and lines indicate IRMA-calculated curves for four final tetrasaccharide structures, which have been superimposed in Figure 7. Although the fits do not always seem to account for observed NOE intensities accurately, the resulting distance variations are minimal, being on the order of 0.1–0.2 Å. The set of glycosidic bond dihedral angles given in Table 3 that define the overall conformation of the tetrasaccharide (Figure 7) results from computational analysis of experimentally determined intra- and inter-ring NOE build-up curves, IRMA calculations and restrained molecular dynamics simulations with an ESFF. Experimental data always bias conformational modelling results when strong force constants are used for NOE-derived distance constraints. For these tetrasaccharide structures, however, it was found that glycosidic bond rotational energy profiles are rather steep (results not shown) and the dependence of dihedral angles on the force constant is minimal. RMS deviation (RMSD) values for these tetrasaccharide structures are less than 0.2 Å for all ring atoms in residues B, C and D. RMSD values for A ring and side-chain atoms are slightly higher, at about 1 Å.

The absence of significant distance violations supports our initial use of the chair conformation for both glucosamine rings (Table 4) and the D ring 6-O-sulphate group being predominantly in the gt state (Table 5). NOE distance violations for the B ring 6-O-sulphate group in the gg conformation (Table 5) indicate the presence of mostly the gt form with some gg character. In agreement with our previous conclusions, iduronate residue C has a slightly twisted boat form due to the presence of an H2–H5 NOE that provides a relatively strong non-bonded potential to overcome the chair–boat energy barrier. Furthermore minor variations in ring NOEs (Table 4) might indicate some contribution from the C-3 form in ring C. On the basis of coupling constants, \(^1\)H, is the major ring conformation for uronate residue A. After restrained MD runs, however, the \(^1\)H, form became distorted (Table 4) owing to an NOE constraint between A1 and A3. Because the A1–A3 inter-proton distance is one-sixteenth as long in the \(^2\)H, form, a minor fraction of \(^2\)H, is most probably

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**Figure 6** NOE build-up curves

Experimental and IRMA-calculated NOE build-up curves are shown for several inter-proton NOEs as indicated in the figure. Triangles represent experimentally determined NOEs and lines give calculated build-up curves for four final structures calculated with the modelling protocol described in the text.

**Figure 7** Tetrasaccharide structures

Four final IRMA-calculated structures with the modelling protocol described in the text have had their rings B, C, and D superimposed. Three of the four structures have the B ring 6-O-sulphate side chain as the gt conformer, whereas one shows the gg conformer. The D ring side chain has all four in the gt conformer state.

**Table 3** Glycosidic bond dihedral angles

<table>
<thead>
<tr>
<th>Glycosidic linkage</th>
<th>(\phi) (deg)</th>
<th>(\psi) (deg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A–B</td>
<td>45 ± 6</td>
<td>42</td>
</tr>
<tr>
<td>B–C</td>
<td>–43 ± 2</td>
<td>–18</td>
</tr>
<tr>
<td>C–D</td>
<td>45 ± 1</td>
<td>57</td>
</tr>
</tbody>
</table>

* Average over four IRMA structures; values given ± S.E.M.
† \(\phi,\psi\) angles taken from [19].
present in equilibrium with the major $^1H_2$ form. IRMA calculations do not discriminate well between these two conformations. Lastly, no 6-O-sulphate oxygens were found within 4 Å of any potential hydrogen-bond donor (2.5 Å being the standard O–H H-bond distance), thereby ruling out the possible influence of hydrogen-bonding in determining the observed conformations.

**DISCUSSION**

The use of a homogeneous fraction of heparin-derived tetrasaccharide allows one to determine a number of reliable experimental conformational parameters essential for understanding and modelling the structure of longer heparin chains. Tetrasaccharide ring conformations show both glucosamine residues B and D in the $^{4}C_1$ conformation as proposed for this residue in parent heparin and in other heparin-derived oligosaccharides [11, 12, 38, 39]. Moreover neither uronate (ring A) nor iduronate (ring C) substantially modifies this glucosamine ring conformation, supporting a previous observation that adjacent β-D-glucuronate and 2-O-sulphated α-L-iduronate residues in the synthetic ATIII binding-site pentasaccharide do not perturb its glucosamine ring conformation [39]. The orientation of the 6-O-sulphate group in the D ring was determined as being primarily $gt$. For the B ring the orientation of the 6-O-sulphate group is not as clear. Rotational energy profiles and coupling constants suggest mostly $gg$ character; however, some NOEs are more consistent with the presence of a $gg$–$gt$ rotomer equilibrium. The $gg$ character in the orientation of the B ring 6-O-sulphate group might result from the presence of the unsaturated uronate ring A instead of the normal iduronate ring as in position C. The existence of $gt$ character in the glucosamine ring D is consistent with the results of Nishida et al. [44] for hydroxymethyl rotomer populations in monosaccharides, but runs contrary to the structure given by Mulloy et al. [37] for a heparin dodecasaccharide where the side chains were fixed in the $gg$ conformation. In contrast our glucosamine B ring does show the $gg$ side-chain orientation. Because the heparin tetrasaccharide is short and the glucosamine D ring is at a terminal position, the $gt$ side-chain state might be preferred for a terminal ring.

For residues A and C, the most probable ring conformations are the $^1H_2$ and twisted boat, $^5S_8$, respectively. The twist of the boat in ring C brings three substituents (except for the C2 sulphate and the carboxy group) into equatorial positions, thereby minimizing the unfavourable 1,3 steric interactions, which would be expected for axial positions in the $^1C_1$ chair form. The conformation of iduronate in parent heparin and in dermatan sulphate has been suggested to exist in a slightly distorted (C2 and C3 positions) $^1C_1$ chair conformation [13, 39, 46]. Huckerby et al. [42], who analysed saccharides obtained after chemical depolymerization of heparin, also concluded that the iduronate residue is not a true $^1C_1$ chair form. Small deviations in expected coupling constants and NOEs in the tetrasaccharide, however, might indicate a minor contribution from other ring forms that coexist in equilibrium. Conformational equilibria of the α-L-idopyranosyluronate have been found to depend on its substituents as well as on those from adjacent residues [11, 13, 39, 41, 42].

Ferro et al. [13] investigated the conformation of α-L-iduronate in various mono- and oligosaccharides and suggested that when α-L-idopyranosyluronate and 2-O-sulpho-α-L-idopyranosyluronate residues are present within a saccharide sequence only the $^1C_1$ and $^5S_8$ conformers are present in equilibrium. When 2-O-sulphated α-L-idopyranosyluronate is at the non-reducing terminus it exists mainly in the $^1C_4$ form but when it is present at non-terminal positions the $^5S_8$ form is the major contributor to the equilibrium. This equilibrium is shifted heavily to the $^1C_4$ form (more than 90%) for the iduronate monomer (methylglycoside), whereas for iduronate preceded by a 3-O-sulphated glucosamine in heparin’s ATIII binding site it is shifted more towards the $^5S_8$ form [11, 13, 39]. This ring distortion to an $^5S_8$

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**Table 4** Distance violations for IRMA structures: rings, no side chains

<table>
<thead>
<tr>
<th>A ring</th>
<th>B ring</th>
<th>C ring</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proton pair</td>
<td>$^{1}H_2$</td>
<td>$^{2}H_4$</td>
</tr>
<tr>
<td>1–2</td>
<td>2.52</td>
<td>3.07</td>
</tr>
<tr>
<td>2–3</td>
<td>2.63</td>
<td>3.08</td>
</tr>
<tr>
<td>3–4</td>
<td>2.45</td>
<td>2.56</td>
</tr>
<tr>
<td>1–3</td>
<td>4.3</td>
<td>2.7</td>
</tr>
</tbody>
</table>

* From structures energy-minimized without constraints.

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**Table 5** Distance violations for IRMA structures: side chains

<table>
<thead>
<tr>
<th>B ring</th>
<th>D ring</th>
</tr>
</thead>
<tbody>
<tr>
<td>$gg$</td>
<td>$gt$</td>
</tr>
<tr>
<td>$^1H_2$</td>
<td>2.45</td>
</tr>
<tr>
<td>$^5S_8$</td>
<td>3.02</td>
</tr>
<tr>
<td>$gt$</td>
<td>2.90</td>
</tr>
<tr>
<td>$^5S_8$</td>
<td>2.90</td>
</tr>
</tbody>
</table>
form is considered essential for the high-affinity interaction between heparin and ATIII [11,39] and is thought to be the result of the presence of an adjacent trisulphated glucosamine residue with a 3-O-sulphate group. In the absence of structural data it is impossible to establish whether the 3-O-sulphate group does indeed distort the conformation of the adjacent iduronate. The tetrasaccharide under investigation here does not contain a trisulphated glucosamine adjacent to a 2-O-sulphated α-L-iduronate.

The overall conformation of the tetrasaccharide has all sulphates extending equatorially from each ring. The derived glycosidic bond angles give a right-handed sense to the inter-ring conformation. In general this tetrasaccharide structure agrees with the heparin dodecamer structure of Mulloy et al. [37]. However, the dihedral angles in the tetrasaccharide B–C linkage do differ from those calculated for the glucosamine–iduronate disaccharide unit in the longer heparin chain [37] but are close to those values reported for the glucosamine–iduronate disaccharide when the iduronate ring is in the alternative $C_1$ chair form as opposed to the $S_0$ form found to be preferred in this tetrasaccharide. In the present work, the tetrasaccharide conformation results from experimental NOE build-up curves, IRMA and restrained MD calculations and energy minimization with an electrostatic force field. Although one can bias an experimentally based conformation by using strong force constants for NOE-derived distance constraints, the dependence of dihedral angles on the force constant for these heparin tetrasaccharide structures was found to be minimal. This is supported by the observation of high motional order parameters and rather steep rotational energy profiles for glycosidic bond dihedral angles, which indicate highly constrained inter-ring mobilities.

When this heparin-derived tetrasaccharide was co-crystallized in the presence of FGF-2, the X-ray structure [19] showed that the interacting iduronate residue (ring C) is in a true $C_1$ chair form, with all of its substituents, except the C5 carboxylate, axially oriented. The $C_1$ chair form of iduronate is apparently stabilized by interactions with basic amino acid residues in FGF-2 because the non-interacting 2-O-sulphated α-L-iduronate is not similarly distorted. For the tetrasaccharide in solution, iduronate (ring C) exists primarily in the $S_0$ twisted boat form even in the absence of an adjacent 3-O-sulphated glucosamine residue. Here the iduronate is flanked by two 2-amino-2-deoxy-di-2,6-sulpho-α-D-glucopyranose residues. A trisaccharide prepared from the tetrasaccharide by removing the unsaturated uronate residue with mercuric acetate [47] shows the same $J_{1,2}$ coupling constant (8.5 Hz), indicating that the iduronate residue is indeed twisted at the C2 and C3 bond. The need to consider the $S_0$ conformation in order to interpret the unusual iduronate coupling constant was first suggested by Auge and David [48]. The larger iduronate $J_{1,2}$ coupling constants observed for both the tetrasaccharide and the trisaccharide suggest that the $S_0$ twisted boat form is an important contributor to the conformational equilibrium of all 2-O-sulphated α-L-idopyranosyluronate residues in heparin. Furthermore the variability of coupling constants for different α-L-iduronate derivatives described by Ferro et al. [13] may be due to the flexibility of the ring around the skewed boat form rather than to the existence of an equilibrium between two distinct conformations.

Further structural comparison of the solution free and crystal FGF-2 bound tetrasaccharide shows essentially the same B and D ring conformations (average ring atomic RMSD values of 0.07 and 0.11 Å respectively) with both side chains in the gg orientation. The slightly higher RMSD value for the D ring might reflect increased internal mobility owing to its terminal position. The crystal structure D ring side chain is distorted ($-12°$ instead of $-60°$). Moreover because the orientation is a gg form in the FGF-2 bound structure, this supports the idea that non-terminal, or in this case structure-bound, glucosamine 6-O-sulphate side-chain orientations prefer the gg state. The average atomic RMSD value for ring A comes out higher at 0.24 Å, most probably because of the equilibrium between $H_1^1$ and $H_2^1$ forms in solution. Glycosidic bond angles are compared in Table 3. The A–B and C–D ring orientations are essentially the same in solution and in the FGF-2 bound state. The B–C ring φ, θ angles, in contrast, each deviate by about $25°$–$30°$. This is apparently due to the C ring conformational equilibrium.

**Conclusions**

The NMR solution conformation of a heparin-derived tetrasaccharide has been derived. As in an FGF-2-bound crystal state, both glucosamine rings exist in the $C_1$ chair conformation, and unlike the terminal ring D, the internal ring B 6-O-sulphate preferred orientation is gauche–gauche. In solution, both uronate and iduronate rings interconvert between twisted boat forms. When the tetrasaccharide is bound to FGF-2, the uronate (ring A) remains in the twisted boat conformation, whereas the energy barrier to the iduronate (ring C) $C_1$ chair form is overcome, most probably by electrostatic interactions with basic amino acid residues. Although glycosidic bond angles between the free and bound states do vary between $10°$ and $30°$ for rings B–C and C–D (but not A–B), the overall tetrasaccharide conformation is maintained.

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