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ORIGINAL PAPER

Determination of the primary structure and carboxyl pK_As of heparin-derived oligosaccharides by band-selective homonuclear-decoupled two-dimensional ^{1}H NMR

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Abstract Determination of the structure of heparin-derived oligosaccharides by ^{1}H NMR is challenging because resonances for all but the anomeric protons cover less than 2 ppm. By taking advantage of increased dispersion of resonances for the anomeric H^1 protons at low pD and the superior resolution of band-selective, homonuclear-decoupled (BASHD) two-dimensional ^{1}H NMR, the primary structure of the heparin-derived octasaccharide ΔUA(2S)-[(1→4)-GlcNS(6S)-(1→4)-IdoA(2S)-]$_3$-(1→4)-GlcNS(6S) has been determined, where ΔUA(2S) is 2-*O*-sulfated $\Delta^{4,5}$-unsaturated uronic acid, GlcNS(6S) is 6-*O*-sulfated, *N*-sulfated β-D-glucosamine and IdoA(2S) is 2-*O*-sulfated α-L-iduronic acid. The spectrum was assigned, and the sites of *N*- and *O*-sulfation and the conformation of each uronic acid residue were established, with chemical shift data obtained from BASHD-TOCSY spectra, while the sequence of the monosaccharide residues in the octasaccharide was determined from inter-residue NOEs in BASHD-NOESY spectra. Acid dissociation constants were determined for each carboxylic acid group of the octasaccharide, as well as for related tetra- and hexasaccharides, from chemical shift–pD titration curves. Chemical shift–pD titration curves were obtained for each carboxylic acid group from sub-spectra taken from BASHD-TOCSY spectra that were measured as a function of pD. The pK_As of the carboxylic acid groups of the ΔUA(2S) residues are less than those of the IdoA(2S) residues, and the pK_As of the carboxylic acid groups of the IdoA(2S) residues for a given oligosaccharide are similar in magnitude. Relative acidities of the carboxylic acid groups of each oligosaccharide were calculated from chemical shift data by a pH-independent method.

Keywords Heparin · Heparin octasaccharide · Oligosaccharides · Acid dissociation constants · Carboxylate pK_A · BASHD-NMR · NMR

Introduction

Heparin is expressed in mast cells as a proteoglycan (M_r, 750,000–1,000,000) comprised of linear copolymers of alternating 1→4 linked D-glucuronic acid (GlcA) and *N*-acetyl-D-glucosamine (GlcNAc) monosaccharides covalently attached to a protein core [1–4]. Post synthesis of the polysaccharide chains, the monosaccharide residues are variously modified by *N*-deacetylation followed by *N*-sulfation, epimerization of D-glucuronic acid to L-iduronic acid (IdoA), 2-*O*-sulfation of uronic residues, and 6-*O*-sulfation of glucosamine residues. The heparin chains are then cleaved at random points to give polydisperse mixtures of smaller heparin polysaccharides (M_r 5000–25000) that are stored in the cytoplasmic secretory granules of mast cells. The polydispersity together with the microheterogeneity that results from variable patterns of substitution of the disaccharide units with *N*-sulfate, *O*-sulfate and *N*-acetyl groups and epimerization of GlcA to IdoA gives rise to a large number of complex sequences (Fig. 1).

A complete sequence analysis of heparin would involve identification and quantitation of the uronic acid-(1,4)-D-glucosamine disaccharide building blocks and determination of their sequence along the polysaccharide chain [3, 4]. We know the disaccharides that might be in a heparin chain,

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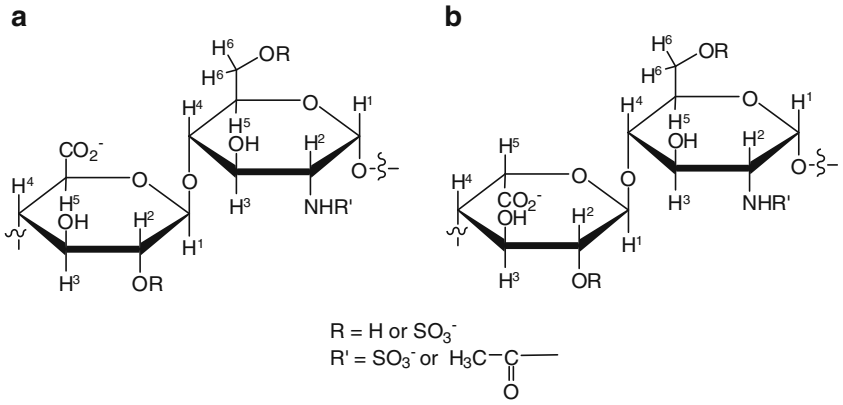

Fig. 1 Structural formulas of the **a** minor (β-D-glucuronic acid-(1⟦4)-α-D-glucosamine) and **b** major (α-L-iduronic acid-(1⟦4)-α-D-glucosamine) heparin repeating disaccharides

but the polydispersity and the different disaccharide sequences of the polymer chains in a heparin preparation, even in highly purified preparations, preclude the isolation of chemically discrete heparin polymers. Thus sequence analysis is limited to chemically discrete oligosaccharides obtained by depolymerization of heparin [5–15].

Among the methods that have been used to determine the sequence of heparin-derived oligosaccharides, ^{1}H NMR is particularly useful [6–8, 12, 14–20]. The monosaccharide residues present in the oligosaccharide can be established using scalar connectivities determined by two-dimensional correlation spectroscopy (COSY) or total correlation spectroscopy (TOCSY), followed by determination of the sequence of the monosaccharides in the oligosaccharide using inter-residue dipolar connectivities determined by nuclear Overhauser enhancement spectroscopy (NOESY) and rotating frame Overhauser enhancement spectroscopy (ROESY) [20]. The presence of *N*-sulfate or *N*-acetyl groups on the glucosamine residues, *O*-sulfate groups on the uronic acid and D-glucosamine residues and the conformation of the uronic acid residues are then inferred from chemical shift data [20].

Key to success of the ^{1}H NMR method is that resonances for the anomeric (H^1) protons are in a region of the spectrum free from resonances for the other carbon-bonded protons and that there is a resolved resonance for the H^1 proton of each monosaccharide residue [20]. However, for heparin-derived oligosaccharides larger than tetrasaccharides, there can be extensive overlap of resonances for the H^1 protons. We have shown previously that the resonance overlap issue can be solved in some cases with the superior resolution that can be achieved with the band-selective, homonuclear-decoupled (BASHD) versions of the TOCSY, ROESY, and NOESY experiments [20].

In this manuscript, we report that dispersion of the H^1 proton resonances is increased when the uronic acid carboxylic acid groups are protonated, and use this increased dispersion together with the superior resolution of BASHD experiments to assign the sequence and determine the chemical shifts of all the carbon-bonded protons of a heparin-derived octasaccharide.

With these resonance assignments in hand, we have used the resolving power of the BASHD TOCSY experiment to determine the pK_A of the carboxylic acid group of each uronic acid of the heparin-derived octasaccharide. Knowledge of the pK_As of the carboxylic acid groups is of interest in heparin-peptide and heparin-protein binding studies. Heparin binds to and modulates the activity of peptides and proteins by electrostatic interactions between anionic sites on heparin and cationic sites on the peptide or protein [2–4, 21]. The anionic sites on heparin are *N*- and *O*-sulfate groups and, depending on pH, the carboxylate groups of the uronic acid residues. Thus, the nature and extent of binding of peptides and proteins, particularly histidine-containing peptides and proteins, is pH-dependent [22–26]. Because acid dissociation constants for the multiple carboxylic acid groups are similar, it is not possible to determine residue-specific acid dissociation constants by standard pH titration methods.

We report acid dissociation constants for each of the four carboxylic acid groups of the octasaccharide, and for the two and three carboxylic acid groups of sequence-related heparin-derived tetra- and hexasaccharides. The residue-specific acid dissociation constants were determined from chemical shift–pD titration curves for ^{1}H reporter nuclei located near the acidic groups. Chemical shifts of the reporter nuclei were obtained from BASHD-TOCSY spectra that were measured as a function of pD. We also report relative acidities of the carboxylic acid groups of each oligosaccharide; relative acidities were calculated from the chemical shift data by a pH-independent method.

Experimental section

Materials

Porcine intestinal mucosal heparin (sodium salt), sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS), ammonium

chloride and heparinase I (EC 4.2.2.7) were purchased from Sigma Chemical Co. (St. Louis, MO 63103, USA). NaOD (40%), DCl (35%) and D_2O were obtained from Cambridge Isotope Laboratories Inc. (Andover, MA 01810, USA). A semi-preparative scale (9×250 mm) CarboPac PA1 strong anion exchange (SAX) column was purchased from Dionex Co. (Sunnyvale, CA 94086, USA). A pH microelectrode for measuring pH in NMR tubes was obtained from Microelectrodes Inc. (Bedford, NH 03110, USA). Shigemi NMR tubes were obtained from Shigemi Co., Inc. (Allison Park, PA 15101, USA).

Heparin-derived oligosaccharides

The tetrasaccharide ΔUA(2S)-(1→4)-GlcNS(6S)-(1→4)-IdoA(2S)-(1→4)-GlcNS(6S), the hexasaccharide ΔUA(2S)-[(1→4)-GlcNS(6S)-(1→4)-IdoA(2S)]$_2$-(1→4)-GlcNS(6S), and the octasaccharide ΔUA(2S)-[(1→4)-GlcNS(6S)-(1→4)-IdoA(2S)]$_3$-(1→4)- GlcNS(6S) were prepared by depolymerization of heparin with heparinase I [20]. ΔUA(2S) represents 2-*O* sulfated uronic acid with an unsaturated 4,5 carbon bond, GlcNS(6S) represents *N*-sulfated, 6-*O*-sulfated β-D-glucosamine, and IdoA(2S) represents 2-*O*-sulfated α-L-iduronic acid. One gram of porcine intestinal mucosal heparin was dissolved in 50 mL pH 7 solution containing 100 mM sodium acetate, 30 mM calcium acetate, and 0.02% sodium azide. 250 units of heparinase I were added, and depolymerization was monitored by measuring the absorbance at 232 nm. When the absorbance reached a constant value, the oligosaccharide mixture was concentrated by lyophilization. The mixture was separated by gravity flow size exclusion chromatography on a 3×200 cm Bio-Gel P6 column using a 0.5 M NH_4HCO_3 eluent to obtain size-uniform heparin fragments. The tetra-, hexa-, and octasaccharides studied in this work were isolated from the size-uniform tetra-, hexa-, and octasaccharide fractions by strong anion exchange HPLC with a Dionex 500 ion chromatography system equipped with a GP40 gradient pump, an AD20 UV/visible detector and a Dionex semi-preparative scale CarboPac PA1 column. The oligosaccharides were eluted with a linear gradient of 70 mM pH 3 phosphate buffer (solvent A) and 70 mM pH 3 phosphate buffer containing 2 M NaCl (solvent B) at a flow rate of 3.3 mL/min.

NMR samples

Oligosaccharide solutions of 1–7 mM were prepared in 99.98% D_2O. DSS was added for a chemical shift reference and a trace amount of EDTA-D16 was added to the samples to reduce line broadening caused by paramagnetic cations. To reduce the intensity of the residual HOD resonance, oligosaccharides were lyophilized from D_2O three times to exchange labile oligosaccharide OH protons with deuterium. Because the H^5 resonances for the IdoA(2S) residues of the tetra- and hexasaccharide are overlapped by the residual HOD resonance at greater than pD 6, NMR samples at pD 6.06 and 6.96 for the tetrasaccharide and pD 6.06 and 6.91 for the hexasaccharide were prepared with ~0.5 M NH_4Cl to eliminate the residual HOD resonance by the WATR method [27]. Shigemi NMR tubes were used to reduce the sample volumes (~320 μL for structural characterization and ~500 μL for titration experiments) and to improve suppression of the HOD resonance. The pD was measured directly in the Shigemi tube using an Orion Research EA 920 pH meter equipped with a pH microelectrode. Solution pD was adjusted with 0.1 M DCl and 0.1 M NaOD. The pH meter was calibrated with Fisher Scientific certified pH 4.00, 7.00 and 10.00 pH standard solutions. pD values were obtained by using the equation $pD = pH_{meter\ reading} + 0.40$ to correct for the deuterium isotope effect.

NMR measurements

One- and two-dimensional ^1H NMR spectra were measured at 499.795 MHz and 25 °C with a Varian Unity INOVA spectrometer (Palo Alto, CA 94304, USA) equipped with waveform generators, a Performa *X, Y, Z* gradient module, and a $^1H\{^{13}C, ^{15}N\}$ triple-resonance, *X, Y, Z* triple-axis pulsed field gradient probe. Chemical shifts are reported relative to the methyl resonance of DSS at 0.000 ppm. The residual HOD resonance was suppressed with a presaturation pulse. The residual HOD resonance for the samples that contained NH_4Cl was completely and selectively eliminated by measuring the spectrum with the Carr–Purcell–Meiboom–Gill pulse sequence $(90^o_x-(\tau-180^o_y-\tau)_n$-acquisition, using τ=0.0003 s with the length of the period $2\tau n$=300 ms. Two-dimensional TOCSY, ROESY, and NOESY spectra and band-selective, homonuclear-decoupled (BASHD) TOCSY, BASHD ROESY, and BASHD NOESY spectra were measured with standard pulse sequences [28, 29]. TOCSY, ROESY, and NOESY spectra were measured with the following parameters: spectral width of 5,500 Hz in both dimensions, 8,196 data points in the directly detected (F2) dimension, 64 transients, and 128 t1 increments. Mixing times of 120 ms were used for TOCSY experiments and 200 ms for ROESY and NOESY experiments. Shifted sine bell and Gaussian apodization functions were applied in the F1 and F2 dimensions, respectively. BASHD TOCSY, BASHD ROESY, and BASHD NOESY spectra were measured with the same parameters, with the exception that the spectral width in the F1 dimension was less, as needed to cover a specific band of resonances. One- and two-dimensional spectra were processed with Varian VNMR software.

Results and discussion

Strategy for determination of the primary structure of heparin-derived oligosaccharides Key to determination of the primary structure of heparin-derived oligosaccharides is that resonances for the anomeric (H^1) protons fall in a region free from other resonances. In the first step of structure determination, the monosaccharides that comprise the oligosaccharide are identified by cross peaks between the anomeric H^1 resonances and other carbon-bonded protons of the monosaccharide residue [20]. The pattern of cross-peaks is a fingerprint for identification of each of the possible monosaccharide residues. The chemical shifts of the carbon-bonded protons can be determined from subspectra obtained by taking traces through the TOCSY spectrum at the chemical shifts of the H^1 resonances. The next step is to determine the position of each residue in the sequence by using cross-peaks between the H^1 resonance of the *i*th residue and the H^4 and/or H^3 resonances of the $i+1$ residue in ROESY and NOESY spectra. In the last step, the presence of *O*- and *N*-sulfate groups is inferred from the chemical shifts of the carbon-bonded protons of each monosaccharide residue using reference ^{1}H chemical shift data [20].

Determination of the sequence of a heparin-derived octasaccharide Spectrum a in Fig. 2 is the ^{1}H NMR spectrum of the octasaccharide at pD 5.32. Determination of the sequence of heparin-derived oligosaccharides by ^{1}H NMR is a challenge because resonances for all but the anomeric H^1 protons cover a relatively narrow spectral region. However, it has been possible in this study to

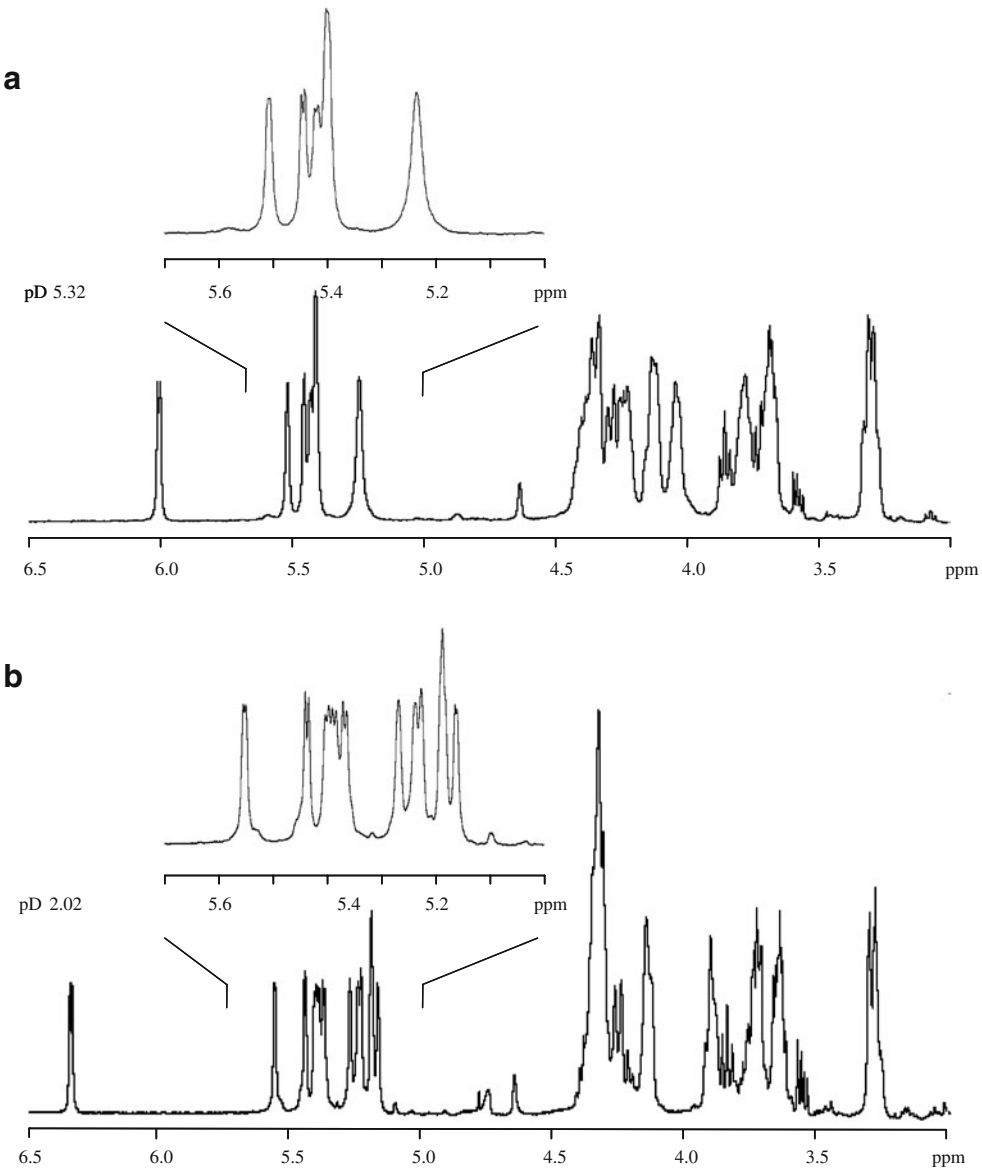

Fig. 2 ^{1}H NMR spectra of **a** pD 5.32 and **b** pD 2.02 solutions of the heparin-derived octasaccharide ΔUA(2S)-[(1→4)-GlcNS(6S)-(1→4)-IdoA2S]$_3$-(1→4)-GlcNS(6S) in D_2O at 25 °C

determine unequivocally the sequence of a heparin-derived octasaccharide by ^{1}H NMR, including determination of the sites of *N*- and *O*-sulfation, by using a combination of information from BASHD TOCSY and BASHD NOESY spectra together with the increased dispersion of the anomeric H1 resonances at lower pD.

Spectrum a in Fig. 2 can be divided into three regions: from 5.4 to 5.6 ppm for the eight anomeric H^1 protons, from 3.2 to 4.7 ppm for 38 of the other 39 carbon-bonded protons, and the resonance at ~6 ppm for the H^4 proton of the ΔUA residue that results from heparinase-catalyzed depolymerization of heparin [3]. While resonances for the eight H^1 protons are more resolved than those in the 3.2 to 4.7 ppm region, there is still extensive overlap. For example, H^1 resonances for three of the four IdoA residues are overlapped at ~5.23 ppm, which precludes unequivocal determination of the primary structure of the octasaccharide at pD 5.32 by the strategy described above, even with the increased resolution of the two-dimensional BASHD experiments.

The pD 2.02 spectrum in Fig. 2b shows significantly increased dispersion of the H^1 resonances, particularly those for the IdoA H^1 protons. In addition, H^5 resonances for three of the four IdoA residues are shifted into the 5.1–5.6 ppm region. Thus, not only is resolution of the H^1 resonances increased when the IdoA carboxylate groups are protonated, but the resolved H^5 resonances also provide additional "entry-points" through which to access subspectra for the IdoA residues from BASHD-TOCSY spectra.

Portions of the BASHD TOCSY spectrum of the pD 2.02 solution are shown in Fig. 3a and b. Figure 3a shows clearly resolved cross peaks to six resonances in the 5.15–5.30 ppm region (the F1 axis in Fig. 3a), while Fig. 3b shows resolved cross peaks to three resonances in the 5.355–5.405 ppm region.

The two overlapped resonances at ~5.18 ppm and the two at ~5.23 ppm in Fig. 2b are resolved in Fig. 3a, and the overlapped resonances at ~5.38 ppm are resolved in Fig. 3b. The corresponding regions of normal TOCSY spectra are shown in Fig. 3c and d, to illustrate the significant increase in resolution of the BASHD TOCSY spectra in Fig. 3a and b. It is difficult or impossible to identify cross peaks to the H^1 resonances at ~5.18, ~5.23 ppm and ~5.38 in Fig. 3c and d due to the extensive overlap of cross peaks. The superior resolution in the BASHD TOCSY spectra results from the finer digital resolution in the F1 dimension because data is collected for a smaller spectral window and, most importantly, ^{1}H–^{1}H spin-coupled multiplets are collapsed to singlets in the F1 dimension [28]. Resonances that have chemical shift differences so small they are overlapped in the 1D and normal 2D spectra are resolved in the BASHD TOCSY

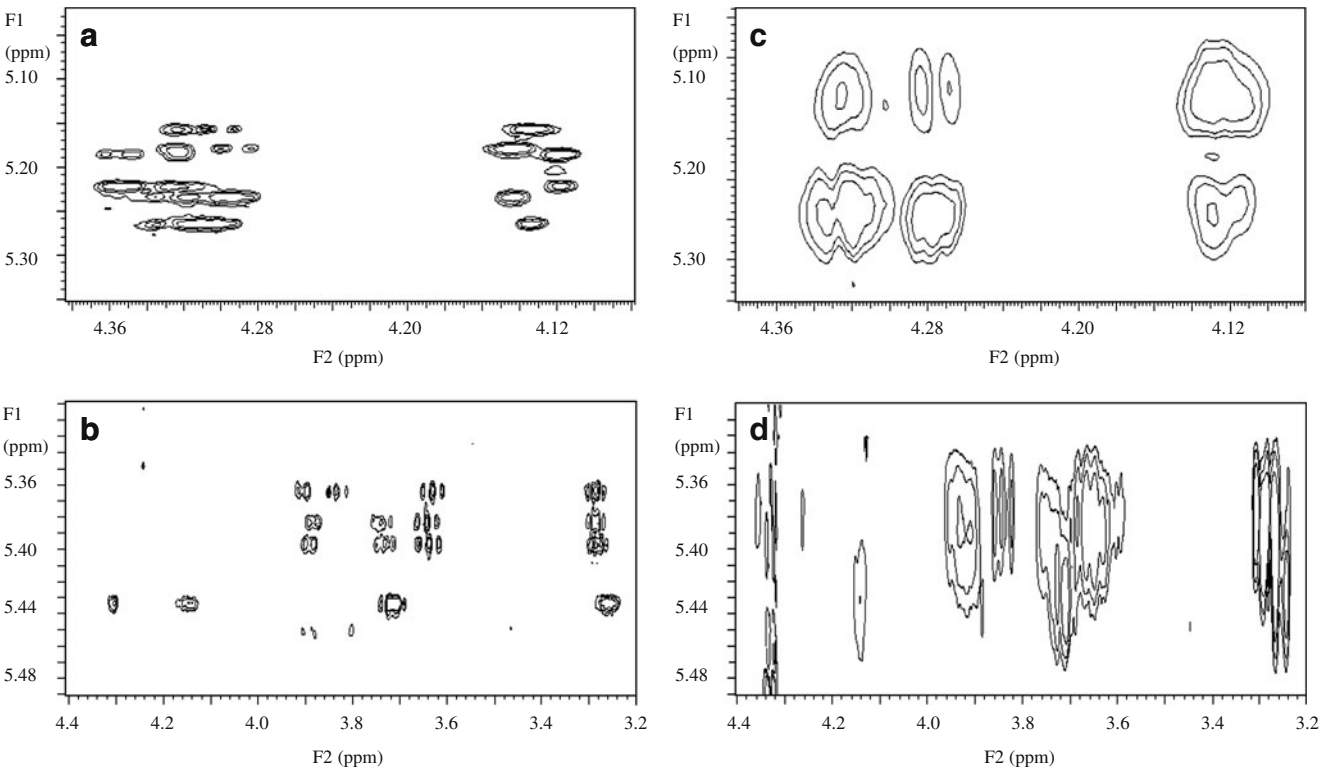

Fig. 3 **a** and **b** Portions of the BASHD TOCSY spectrum of a pD 2.02 solution of the octasaccharide measured with band selection of the 5.10 to 5.60 ppm region and **c** and **d** portions of the regular TOCSY spectrum of a pD 3.59 solution of the octasaccharide

spectra. For example, the overlapped resonances for the H^5 protons of IdoA(2S)^3 and IdoA(2S)^5 at ~5.18 ppm are resolved in Fig. 3a, even though their chemical shifts differ by only 1 Hz (the position of a monosaccharide residue in the sequence is indicated by a superscript, starting from the non-reducing end).

The chemical shifts of cross peaks in Fig. 3a establish that resonances in the 5.15–5.30 ppm region are for the H^1 and H^5 protons of the three IdoA residues, while the cross peaks in Fig. 3b establish that resonances in the 5.355–5.405 ppm region are for the H^1 protons of three of the 4 GlcN residues. Cross peaks to the resonances at 5.43 and 5.54 ppm establish that these resonances are for the H^1 protons of the fourth GlcN residue and the ΔUA residue, respectively.

The ^{1}H NMR spectrum of the octasaccharide was assigned using scalar and dipolar connectivities in BASHD TOCSY and BASHD NOESY spectra, respectively, for a pD 2.95 solution of the octasaccharide, using as a starting point the H^1 resonance of ΔUA at 5.539 ppm, which was assigned based on its scalar connectivity to the unique ΔUA H^4 resonance at 6.264 ppm. Using the strategy described above, the H^1 resonance of the i+1 residue in the sequence was identified by first observing cross peaks between H^1 of the ith residue and H^4 and/or H^3 of the i+1 residue in the BASHD-NOESY spectrum and then identifying the H^1 resonance of the i+1 residue by the presence of these H^4 and/or H^3 resonances in sub-spectra obtained from the BASHD TOCSY spectrum. In this way, the anomeric resonances were assigned to specific residues in the octasaccharide sequence (a BASHD-NOESY spectrum is presented in the Electronic Supplementary Material).

The chemical shifts of the carbon-bonded protons for each monosaccharide residue were determined from the sub-spectra obtained by taking traces through the BASHD TOCSY spectra at the chemical shifts of the H^1 and, for the IdoA residues, H^5 resonances. For some GlcN residues, resonances for the 2 H^6 protons are very weak in BASHD TOCSY sub-spectra taken at the H^1 chemical shift. In these cases, the chemical shifts of the H^6 protons of the i+1 GlcN residue were obtained from resonances due to dipolar interactions between the IdoA H^1 proton of the ith residue and the 2 H^6 protons of the i+1 GlcN residue in BASHD NOESY sub-spectra. Chemical shift data are reported in Table 1 for all the carbon-bonded protons of the octasaccharide.

The sites of *N*-sulfation and *O*-sulfation were then established using reference chemical shift data for heparin monosaccharides [20], with the result that the octasaccharide was determined to have the sequence ΔUA(2S)-[(1→4)-GlcNS(6S)-(1→4)-IdoA(2S)]$_3$-(1→4)-GlcNS(6S) (Fig. 4). Chemical shift data have been reported previously for this heparin-derived octasaccharide in neutral pD solution [6]. The reported chemical shifts are in reasonable agreement with those in Table 1, with the exceptions due to different pD values noted below.

The reference chemical shift data is for neutral pH, whereas the data in Table 1 was obtained at pD 2.95. However, within experimental error, the chemical shifts of all but four of the carbon-bonded protons of the fully sulfated octasaccharide agree with the reference chemical shift data at neutral pH, which suggests that the reference chemical shift data can be used when taking advantage of the increased dispersion of the anomeric H^1 resonances at low pD to sequence heparin-derived oligosaccharides. The exceptions are resonances for H^3 and H^5 of the IdoA(2S) residues (which are shifted downfield by 0.06 and 0.34 ppm, respectively, at pD 2.95), resonances for H^5 of the GlcNS (6S) residues (which are shifted upfield by 0.14 ppm), and the resonance for H^4 of the ΔUA(2S) residue (which is shifted downfield by 0.25 ppm at pD 2.95).

Determination of acid dissociation constants The acid dissociation constant for each carboxylic acid group of the tetra-, hexa-, and octasaccharides was determined directly from chemical shift–pD titration curves for the H^4 and H^5 reporter protons of the ΔUA(2S) and IdoA(2S) residues,

Table 1 ^{1}H chemical shift data for the octasaccharide

Proton	ΔUA(2S)^1	GlcNS(6S)^2	IdoA(2S)^3	GlcNS(6S)^4	IdoA(2S)^5	GlcNS(6S)^6	IdoA(2S)^7	GlcNS(6S)^8
H^1	5.539	5.367	5.226	5.393	5.234	5.377	5.262	5.430
H^2	4.627	3.286	4.326	3.275	4.328	3.280	4.323	3.250
H^3	4.334	3.641	4.285	3.638	4.288	3.643	4.297	3.702
H^4	6.264	3.834	4.116	3.730	4.139	3.738	4.129	3.717
H^5		3.915	5.162	3.896	5.160	3.888	5.136	4.140
H^{6a}		4.227		4.259		4.255		4.300
H^{6b}		4.335		4.335		4.363		ND

Chemical shifts in ppm vs DSS; 25 °C; uncertainty of chemical shifts: ± 0.001 to ±0.003 ppm

ND Not determined

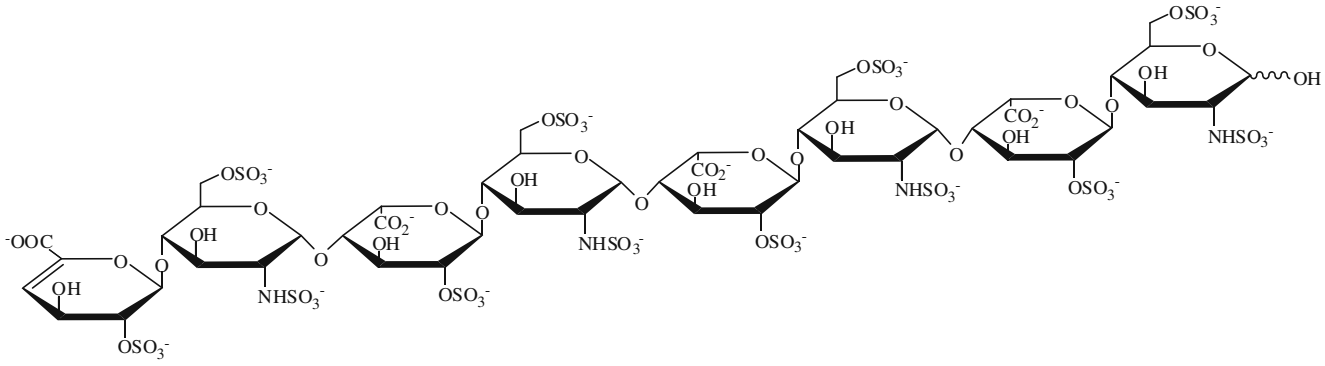

Fig. 4 The structural formula of the heparin-derived octasaccharide

respectively. The initial pD of each oligosaccharide solution was adjusted to ~2. The pD was then increased to ~6 in increments of 0.5 pD unit by addition of 0.1 M NaOD.

The observed chemical shift of the H^4 proton of ΔUA (2S) or the H^5 proton of an IdoA(2S) residue (δ_{Obs}) is given by Eq. 1, where f_A and f_{HA} are the fractional concentrations and δ_A and δ_{HA} the chemical shift of the H^4 or H^5 protons of the carboxylate and carboxylic

$$\delta_{Obs} = f_{HA}\delta_{HA} + f_A\delta_A \quad (1)$$

acid forms. pK_A values were obtained by fitting chemical shift–pD titration data to Eq. 2 using the computer program Scientist from Micromath Scientific software (Salt Lake City, Utah 84121, USA). Equation 2 is obtained from Eq. 1 by expressing f_A and f_{HA} in terms of K_a.

$$\delta_{Obs} = \left(\frac{[D^+]}{[D^+]+K_A}\right)\delta_{HA} + \left(\frac{K_A}{[D^+]+K_A}\right)\delta_A \quad (2)$$

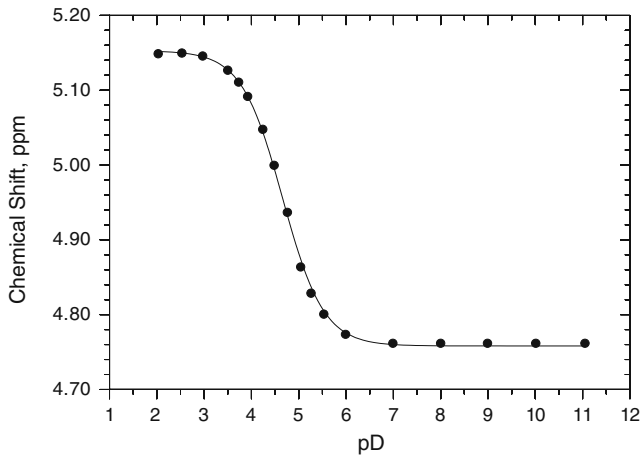

Fig. 5 The chemical shift–pD titration curve for the carboxylic acid group of the IdoA(2S)^3 residue of the tetrasaccharide. The chemical shift of the H^5 reporter proton is plotted vs pD; the smooth curve through the points is the non-linear least squares best-fit curve

Determination of acid dissociation constants for the carboxylic acid groups of the heparin-derived tetrasaccharide Chemical shift–pD data for the tetrasaccharide was obtained from 1D spectra. A representative chemical shift–pD titration curve for the carboxylic acid group of IdoA(2S)^3 is presented in Fig. 5. A pK_A of 4.69 was obtained by fitting the chemical shift–pD titration curve to Eq. 2. The pK_A values determined for the two carboxylic acid groups of the tetrasaccharide are reported in Table 2.

The chemical shift of the H^3 proton of GlcN$(SO_3)^2$ also changes as the carboxylic acid groups of the ΔUA(2S)^1 and IdoA(2S)^3 residues are titrated (Fig. 6). pK_A values were determined for the two carboxylic acid groups by fitting chemical shift–pD titration data for the H^3 proton of GlcN $(SO_3)^2$ to Eq. 3:

$$\delta_{Obs} = f_{H2A}\delta_{H2A} + f_{HA}\delta_{HA} + f_A\delta_A \quad (3)$$

where f_{H2A}, f_{HA}, and f_A are the fractional concentrations and δ_{H2A}, δ_{HA}, and δ_A the chemical shift of the H^3 proton of the diprotonated, monoprotonated and deprotonated forms of the tetrasaccharide. The fractional concentrations are expressed in terms of pD and the two acid dissociation constants as follows: $f_{H2A} = [D^+]^2/X, f_{HA} = K_{A1}[D^+]/X$ and $f_A = K_{A1}K_{A2}/X$, where $X = [D^+]^2 + K_{A1}[D^+] + K_{A1}K_{A2}$. The values obtained from the nonlinear least squares fit are pK_{A1}=4.17 and pK_{A2}=4.69. Residue-specific pK_As for the

Table 2 pK_A values of the carboxylic acid groups of the heparin-derived tetra-, hexa- and octasaccharides

Oligosaccharide	ΔUA(2S)^1	IdoA(2S)^3	IdoA(2S)^5	IdoA(2S)^7
Tetrasaccharide	4.17	4.69		
Hexasaccharide	4.02	4.54	4.48	
Octasaccharide	3.85	4.33	4.35	4.40

25 °C; ionic strength ~0.01 M; uncertainties are ±0.02 pK_A units

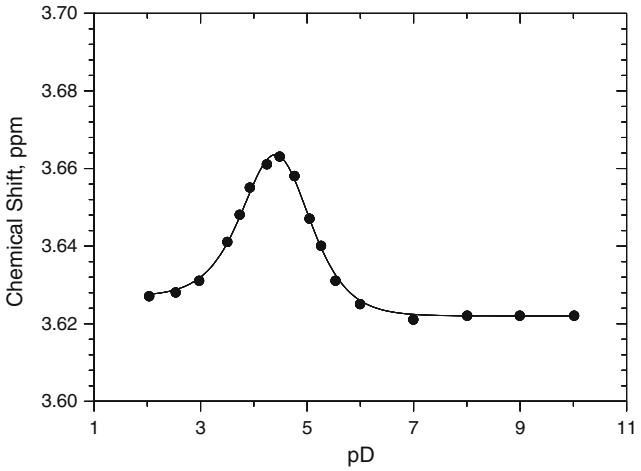

Fig. 6 The chemical shift–pD titration curve for the H^3 proton of the GlcNS(6S)^2 residue of the tetrasaccharide; the smooth curve through the points is the non-linear least squares best-fit curve

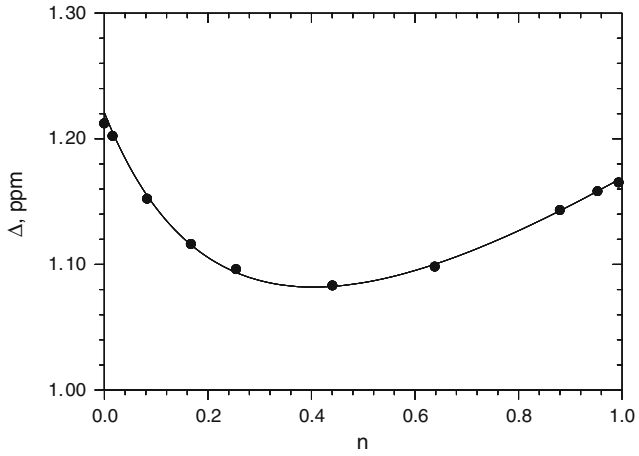

Fig. 7 Difference between the chemical shifts of the ΔUA(2S) H^4 and IdoA(2S)^7 H^5 resonances of the octasaccharide as a function of the fractional protonation of the ΔUA(2S) carboxylic acid group. The smooth curve through the points is the non-linear least squares best-fit to the data. A value of $R_{1,7}$=3.48±0.07 was obtained from the non-linear least squares fit

same heparin-derived tetrasaccharide were determined previously from ^{13}C chemical shift–pH titration curves [30]. The reported pK_As are similar to the values determined in the present study, but a direct comparison is not possible as the pK_As were converted to H_2O solution values.

Determination of acid dissociation constants for the carboxylic acid groups of the heparin-derived hexa- and octasaccharide Chemical shift–pD titration curves were measured for H^4 of the ΔUA(2S) residues and H^5 of each of the IdoA(2S) residues. Chemical shift data for H^4 of the ΔUA(2S) residues was measured from 1D spectra and for H^5 of each of the IdoA(2S) residues from subspectra obtained by taking traces at the chemical shifts of the H^1 resonances of the IdoA(2S) residues in BASHD TOCSY spectra measured as a function of pD. The pK_As determined for the three and four carboxylic acid groups of the hexa- and octasaccharide, respectively, are reported in Table 2. The excellent fit of the chemical shift–pD titration curves for each of the carboxylic acid groups to a monoprotic titration model indicates no anti-cooperativity effects in their titration, which is as might be expected since they are quite separated from each other in the helical heparin structure.

Relative acidities of the carboxylic acid groups Relative acidities of the carboxylic acid groups are of interest in determining the effect of sulfation patterns and location in the oligosaccharide sequence on pK_As. Relative acidities calculated using the pK_As in Table 2 for a given oligosaccharide are reported in Table 3. The relative acidities are reported as $R_{1,x}$, the ratio of the acid dissociation constants of the ΔUA(2S)^1 and IdoA(2S)^x carboxylic acids ($R_{1,x}=K_A{}^1/K_A{}^x$). The accuracy and precision of each pK_A, and thus the $R_{1,x}$ values calculated using the pK_A values, are determined, in part, by the accuracy and precision of the pD measurement.

Because the chemical shift–pD titration curves for all the carboxylic acid groups of a given oligosaccharide were obtained from the same experiment, the chemical shift for each reporter proton was measured at the same pD values, which makes it possible to also calculate relative acidities, with greater accuracy and precision, by a pD-independent

Table 3 Relative acid dissociation constants for the carboxylic acid groups of the tetra-, hexa- and octasaccharide

Oligosaccharide	$R_{1,x}$	Calculated using pK_A values	Calculated by pD-independent method
Tetrasaccharide	$R_{1,3}$	3.31±0.09	3.25±0.08
Hexasaccharide	$R_{1,3}$	3.31±0.25	3.25±0.10
	$R_{1,5}$	2.88±0.20	2.83±0.03
Octasaccharide	$R_{1,3}$	3.02±0.20	2.99±0.08
	$R_{1,5}$	3.16±0.21	3.09±0.09
	$R_{1,7}$	3.59±0.26	3.48±0.07

25 °C; ionic strength ~0.01 M

method described previously to determine ^{15}N isotope effects on the acid–base equilibria of amino groups in amino acids [31]. Since pD is not used in the calculation, errors associated with the pD measurement are eliminated. Relative acidities calculated using the pD-independent method are also reported in Table 3. The pD-independent values were calculated with Eq. 4, where Δ is the difference between the chemical shifts of the H^4 resonance of ΔUA $(2S)^1$ and the H^5 resonance of $IdoA(2S)^x$,

$$\Delta = \Delta_A + \frac{R_{1,X} n \Delta^X}{R_{1,X} n - n + 1} - n\Delta^1 \quad (4)$$

$\Delta_A = \delta_A{}^1 - \delta_A{}^x, \Delta^1 = \delta_A{}^1 - \delta_{HA}{}^1, \Delta^x = \delta_A{}^x - \delta_{HA}{}^x, R_{1,x} = K_A{}^1/K_A{}^x$, where 1 and x identify the residue in the oligosaccharide sequence, and n is the fractional concentration of the HA form of the ΔUA(2S) carboxylic acid. n is calculated from the H^4 chemical shift data for ΔUA(2S) using the relationship $n = (\delta_{obs} - \delta_A)/(\delta_{HA} - \delta_A)$. To illustrate, the difference Δ between the chemical shifts of H^4 of ΔUA(2S) and H^5 of $IdoA(2S)^7$ is plotted as a function of n in Fig. 7. A value of $R_{1,7}$=3.48±0.07 was obtained from a nonlinear least-squares fit of the data to Eq. 4. The results in Table 3 show smaller uncertainties associated with relative acidities calculated by the pD-independent method as a result of elimination of pD from the calculation.

Conclusions

The combination of low pD to increase dispersion of the anomeric proton resonances together with the superior resolution of the BASHD TOCSY, BASHD ROESY, and BASHD NOESY experiments is a powerful method for sequencing heparin-derived oligosaccharides, and for determining residue-specific pK_As for the carboxylic acid groups of the uronic acid residues.

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