Dynamic exchange between stabilized conformations predicted for hyaluronan tetrasaccharides: comparison of molecular dynamics simulations with available NMR data

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Studies of the hyaluronan (HA) tetrasaccharides are important for understanding hydrogen-bonding in the HA polymer, as they are probably the smallest oligomers in which characteristics of the constituent monosaccharides and the polymer are simultaneously exhibited. Here we present extensive molecular dynamics simulations of the two tetrasaccharides of HA in dilute aqueous solution. These simulations have confirmed the existence of intramolecular hydrogen-bonds between the neighboring sugar residues of HA in solution, as proposed by Scott (1989). However, our simulations predict that these intramolecular hydrogen-bonds are not static as previously proposed, but are in constant dynamic exchange on the sub-nanosecond time-scale. This process results in discrete internal motion of the HA tetrasaccharides where they rapidly move between low energy conformations. Specific interactions between water and intramolecular hydrogen-bonds involving the hydroxymethyl group were found to result in differing conformations and dynamics for the two alternative tetrasaccharides of HA. This new observation suggests that this residue may play a key role in the entropy and stability of HA in solution, allowing it to stay soluble up to high concentration. The vicinal coupling constants \( J_{\text{NHCH}} \) of the acetamido groups have been calculated from our aqueous simulations of HA. We found that high values of \( J_{\text{NHCH}} = 8 \text{ Hz} \), as experimentally measured for HA, are consistent with mixtures of both trans and cis conformations, and thus \( J_{\text{NHCH}} \) cannot be used to imply a purely trans conformation of the acetamido. The rapid exchange of intramolecular hydrogen-bonds indicates that although the structure is at any moment stabilized by these hydrogen-bonds, no one hydrogen-bond exists for an extended period of time. This could explain why NMR often fails to provide evidence for intramolecular hydrogen-bonds in HA and other aqueous carbohydrate structures.

Key words: hyaluronan/water/hydrogen-bonds/molecular dynamics

Introduction

Many of the important roles of carbohydrates are directly linked to their interaction with water, such as modifying solution viscosity or acting as cryoprotectants (Kawai et al., 1992). Despite this, little is known about the complex interaction of water and carbohydrate, since water is usually treated as an inert filler (Franks, 1990). To gain insight into the three-dimensional interplay between carbohydrate and water requires a technique which can provide detailed molecular information on the sub-picosecond time-scale, such as molecular dynamics (MD) simulation. Many illuminating simulations of polysaccharide sub-units have appeared in the literature, attempting to model cellulose (Hardy and Sarko, 1993), amylose (Brady and Schmidt, 1993), and \( \beta \)-carageenan (Ueda and Brady, 1996). Here we report MD simulations of the two tetrasaccharides of hyaluronan (HA) in aqueous solution.

HA is a mucopolysaccharide, a member of the glycosaminoglycan family. All glycosaminoglycans, except keratan sulfate, contain tandem repeats of a carboxyl-group-containing sugar monomer, and an amino-sugar monomer. Studies on HA have failed to identify sugars and linkages other than those of its disaccharide repeat (-GlcNAc-\( \beta \)-1,4-GlcA-\( \beta \)-1,3-). Where GlcNAc represents N-acetyl-D-glucosamine and GlcA represents D-glucuronic acid. HA is not postsynthetically sulfated, as are the other glycosaminoglycans, and this may be because it is extruded directly from cell surfaces. HA is found throughout the mammalian extracellular matrix, but particularly in the soft connective tissues, such as vitreous humor and synovial fluid. It has many roles both as a gel forming molecule in its own right and also as an organizer of proteoglycans in the extracellular matrix. HA is known to interact with a number of cell-surface receptors (e.g., CD44 and RHAMM), and is known to modulate cell adhesion, proliferation and migration (Aruffo et al., 1990; Pilarski et al., 1994). The oligosaccharides have also been implicated in angiogenesis (West et al., 1985).

The HA polymer has been characterized as a highly-hydrated stiffened worm-like coil in solution (Cleland, 1984). It has been postulated that the presence of intramolecular hydrogen-bonds in aqueous solution leads to stiffening of the HA polymer, and this gives rise to the rheological properties of HA which underlies its biological function. The basis for these claims has come from many sources, in particular alkali-induced conformational changes (Welti et al., 1979), periodate oxidation kinetics (Scott and Tigwell, 1978), and NMR studies performed in DMSO (Scott, 1989).

The observation that HA is oxidized by periodate more slowly than predicted led to the proposal that HA secondary structure can be stabilized by intramolecular hydrogen-bonded arrays (Scott and Tigwell, 1975, 1978). It was proven that they can, in principle, be formed by studying computer refinements of x-ray diffraction patterns from HA fibers (Atkins et al., 1980). This model of HA incorporates four intramolecular hydrogen-bonds in each disaccharide. The planar acetamido group found in HA plays a crucial role in this theory since it can simultaneously act as both an acceptor and donor of hydrogen-bonds. Conversion of
HA to its quaternary ammonium salt allows it to be dissolved in dimethyl sulfoxide. The advantage of using this solvent is that it allows all structural hydrogen atoms to be observed in NMR spectra (Scott and Heatley, 1982). Initial studies of the HA disaccharides assigned one resonance to the amide proton (Heatley et al., 1982). However, comparison with the NMR spectra from HA tetra-, hexa-, and octasaccharides revealed a new resonance for the amide proton, downfield from that observed in the disaccharide. This new resonance increases in magnitude as the oligomeric series is ascended (Scott et al., 1984). The two resonances were found to correspond to the external and internal linkages of the polymer, respectively. Thus, we expect the internal linkages of tetrasaccharides to resemble to polymeric linkages, and the external linkages to be similar to disaccharides.

Circular dichroism (CD) data for a series of HA oligosaccharides produced by testicular hyaluronidase digestion allowed the conformation to be assessed as a function of length (Cowman et al., 1981). It was concluded that the structural features of high molecular weight HA, which resulted in enhancement of the 209 nm CD band, were present in the interior residues of tetrasaccharides and larger oligosaccharides. Thus, analysis of the tetrasaccharides is important for understanding the role of hydrogen-bonding in the HA polymer, as they are the smallest oligomers in which characteristics of the constituent monosaccharides and the polymer are simultaneously exhibited.

Our approach involves performing MD simulations of solvated molecules, and comparing the results with x-ray scattering and hydrodynamic experiments. Here we extend our solvated MD studies of the HA disaccharides (Almond et al., 1997) to the more polymer-like HA tetrasaccharides. New hydrogen-bonding analysis tools, which we have developed, has allowed the dynamic interaction between intramolecular hydrogen-bonds and water molecules surrounding the HA tetrasaccharides to be investigated.

Results

By calculation of the Cremer-Pople ring puckering parameters (Cremer and Pople, 1975) and averaging them over the simulation, we determined that all sugar rings are stable in the $^3C_1$ conformation, see Table I. We determined the overall flexibility of the two different tetrasaccharides in solution using iso-energy contour maps obtained as described in our previous paper (Almond et al., 1997). These maps show the $\phi$ torsion as abscissa ($H_1-C_1-O_\times-C_\times$), and the $\psi$ torsion as ordinate ($C_1-O_\times-C_\times-H_\times$); $\times$ depends on linkage type, and all angles are in degrees. Figures 1 and 2 show these maps, and the linkages to which they refer for the two alternate tetrasaccharides of HA. For all linkages a highly populated glycosidic minimum corresponds to $\phi = 50^\circ$, and $\psi = 0^\circ$, equivalent to that found in our simulations the disaccharides of HA, and that predicted by the exo-anomeric effect (Tvaroska and Tomas, 1989). In both tetrasaccharides, we noticed that the central linkage ($\phi_2,\psi_2$) is tightly constrained within this single minimum. However, when we investigated the terminal linkages of each tetrasaccharide, it was clear that other distinct minima for the glycosidic torsional angles are also favored. This flexibility leads to specific conformations of the two tetrasaccharides which exist for subsections of their respective simulations. We have labeled these conformations with the letters A to C on Figure 1 and D–H on Figure 2, showing the glycosidic torsional angles at the terminal linkages. It should be noted that these observed minima are not seen in equivalent vacuum simulations, and thus we have to assume that they are induced by the presence of water.
Dynamic exchange between HA tetrasaccharide conformations

![Image of a tetrasaccharide structure]

**Fig. 2.** Persistent hydrogen-bonds found in a solvated HA tetrasaccharide with GlcA at the reducing terminus (top). Energy contour plots for each of the glycosidic linkages extracted from molecular dynamics simulation (bottom). Contours are marked at regular intervals between minimum and maximum energy.

<table>
<thead>
<tr>
<th>Tetrasaccharide</th>
<th>Distance from reducing sugar</th>
<th>Q (Å)</th>
<th>θ (degrees)</th>
<th>J\textsubscript{COCH} (Hz)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GlcNAc at reducing terminus</td>
<td>0</td>
<td>0.583</td>
<td>8.6</td>
<td>3.77</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.587</td>
<td>8.4</td>
<td>4.70</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.578</td>
<td>8.8</td>
<td>4.70</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.586</td>
<td>9.0</td>
<td>4.92</td>
</tr>
<tr>
<td>GlcA at reducing terminus</td>
<td>0</td>
<td>0.589</td>
<td>9.0</td>
<td>3.77</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.585</td>
<td>8.2</td>
<td>4.70</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.581</td>
<td>9.9</td>
<td>4.92</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.590</td>
<td>7.9</td>
<td>4.92</td>
</tr>
</tbody>
</table>

All are indicative of the 4C\textsubscript{1} conformation. Bottom shows a coupling constant across β1,3 linkages (see text).

Table I. Cremer-Pople puckering parameters for the pyranose rings of the two HA tetrasaccharides

We extracted the key torsional angles from each of the tetrasaccharides which describe internal molecular motion. For a HA tetrasaccharide with a GlcNAc residue at the reducing terminus glycosidic torsions \( ϕ_1 \) and \( ψ_3 \) were suitable (numbered from non-reducing terminus), and these are plotted against time in Figure 3. Conversely for a tetrasaccharide with a GlcA residue at the reducing terminus \( ψ_1 \) and \( ϕ_3 \) were suitable, and these are plotted in Figure 4. For comparison Figures 3 and 4 also show the presence of each of the intramolecular hydrogen-bonds, defined in Figures 1 and 2, over the whole 500 ps. Specific sections of the plot relate to conformations of the terminal glycosidic linkages are marked A–H, and refer to regions of Figures 1 and 2. This indicates that specific sets of hydrogen-bonds uniquely identify molecular conformations. Many distinct hydrogen-bonded states are visited, even in this short time window of 500 ps. These are stabilized for extended periods in some cases. However, hydrogen-bonds can be broken, and then they may stay broken for an extended period. This results in a discrete molecular motion, where the HA molecule rapidly exchanges between stabilized
conformations. Some hydrogen-bonds were found to be cooperative and some are mutually exclusive, and this relates to the three dimensional organization of substituents. Cooperative hydrogen-bonds indicate donor pairs that move together, for example, hydrogen-bonds 7 and 9 of the HA tetrasaccharide with a GlcNAc residue at the reducing terminus (Figures 1 and 3). Mutually exclusive hydrogen-bonds indicate the same donor, donating to different acceptors at different times during the simulation, for example, hydrogen-bonds 2 and 3 of the HA tetrasaccharide with a GlcA residue at the reducing terminus (Figures 2 and 4).

Therefore, questions to be answered regarding HA in solution are as follows. (1) Why are intramolecular hydrogen-bonds stabilized for periods lasting many picoseconds? (2) Why are they eventually broken? We chose a hydrogen-bond which had distinctly stabilized bonded and non-bonded states, for example hydrogen-bond number 9 defined in Figure 1, which involves GlcNAc OH4 … O5 GlcA. We investigated the nature of the stabilized bonded and broken states, and the transition boundaries. We found that the stabilized bonded state was a consequence of water caging around the particular intramolecular hydrogen-bond, for extended periods of time, and a dynamic equilibrium was found to exist between intramolecular hydrogen-
Dynamic exchange between HA tetrasaccharide conformations

Fig. 5. Stereo images describing the interaction between water and intramolecular hydrogen-bonds. The top diagram shows a stable hydrogen bond between GlcNAc OH4 and GlcA O5 (hydrogen-bond number 9 in Figures 1 and 3) and the two adjacent caging water molecules. In the middle diagram this intramolecular hydrogen-bond has been broken by a bridging water molecule, and other water molecules are providing solvation of the free carbohydrate groups. The bottom diagram shows the same linkage some time after the breaking of this hydrogen-bond, and the dramatic molecular rearrangement that has taken place. This conformation was found to be stable for many tens of picoseconds (see Figures 1 and 3). A key to atom shading is displayed on Figure 6.

bonded and water-bridged states. In the nonintramolecular hydrogen-bonded state, the GlcNAc acetamido group made a water-bridged complex with the carboxylate of the adjacent GlcA residue, resulting in a dramatic conformational shift. This is the transition from conformation A to conformation B, as marked on Figures 1 and 3. On the transition boundary a specifically arranged set of water molecules catalyzed the breaking by bridging the intramolecular hydrogen-bond, and providing solvation of both donor and acceptor. Figure 5 shows a the time evolution of this process as a series of stereo images. Effectively, water caging increases the energy barrier between the intramolecular hydrogen-bonded and nonbonded states, but in particular circumstances water can catalyze the transition across this energy barrier. Once an intramolecular hydrogen-bond has been broken, molecular motion can more readily occur due to the loss of constraint, and the nonbonded state will be unstable unless another intramolecular hydrogen-bond can be made quickly. Hydrogen-bond number 1 on the HA tetrasaccharide with a GlcNAc residue at the reducing terminus (Figure 1), GlcA OH2 … GlcNAc O7 (acetamido oxygen), is an example of this. The OH2 group has no other acceptor than the acetamido oxygen, and so this stable situation exists virtually constantly throughout the simulation (Figure 3). However, this is not the case for the terminal hydroxymethyl groups in the tetrasaccharide with a GlcA residue at the reducing terminus. Each has two different acceptors, and they are stable in both donor-acceptor configurations, for periods of greater than 50 ps (Figures 2 and 4). This led to the dramatic changes in conformation observed at the terminal glycosidic linkages. Figure 6 shows two different conformations of the HA tetrasaccharide with GlcA at the reducing terminus, the hydroxymethyl group taking place in different intramolecular hydrogen-bonds. Frames were extracted at time 20 ps (conformation D) and 270 ps (conformation G) into the simulation.

To compare simulation with published NMR data, we extracted all acetamido torsions $\nu$ (NH-N-C-H) over the whole 500 ps. Although there is no definitive Karplus relation for this acetamido torsion, a similar relation for model peptides (Bystrov et al., 1973) can be used. We used this Karplus relation to calculate the respective instantaneous $^{3}J_{COCH}$ coupling constants, and then averaged each $J$ over the whole simulation. Our calculated coupling constants based on this assumption are shown in Table II. In all but the tetrasaccharide with a GlcNAc at the reducing terminus, we observed a trans conformation of the acetamido group. However, at the non-reducing terminus the acetamido stabilized into a cis conformation for the whole simulation. We also noticed that a value for $^{3}J_{COCH}$ has previously been measured by NMR on the fragment C1-O3-C3-H3, across the $\beta_{1,3}$ linkage, at 4.8 Hz. Using a suggested Karplus equation for this linkage (Tvaroska et al., 1989) we obtained a value of between 3.77 and 4.90 Hz, depending on linkage (Table I). We calculated the rotamer populations of the hydroxymethyl groups, with torsions $\omega$ (O5-C5-C6-O6). Using a standard nomenclature (Sundaralingham, 1968), we observed typically 10% gg, 90% gt, and 0% tg rotamers; see Table II. Transitions to the gg rotamer were
short-lived, and corresponded to intramolecular reorganization. According to a study of α-dolichopyranoses only two rotamers are populated in monosaccharides in the ratio gg:gt of 3:2 (Marchessault and Pérez, 1979). The discrepancy between this data and our simulations is due to the presence of intramolecular hydrogen-bonds between adjacent sugar residues involving the hydroxymethyl groups.

Table II. Calculated coupling constants for GlcNAc acetamido, and rotamer population for the hydroxymethyl at the positions stated above

<table>
<thead>
<tr>
<th>Residues from reducing sugar</th>
<th>Calc. 1J[NHCH]</th>
<th>NH CH conformation</th>
<th>Hydroxymethyl % gg</th>
<th>% gt</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>7.95</td>
<td>trans</td>
<td>12</td>
<td>88</td>
</tr>
<tr>
<td>1</td>
<td>7.21</td>
<td>trans</td>
<td>12</td>
<td>88</td>
</tr>
<tr>
<td>2</td>
<td>7.18</td>
<td>trans</td>
<td>4</td>
<td>96</td>
</tr>
<tr>
<td>3</td>
<td>7.90</td>
<td>cis</td>
<td>7</td>
<td>93</td>
</tr>
</tbody>
</table>

Based on MD simulation for the two tetrasaccharides of HA in aqueous solution.

Discussion

By measuring the ring proton vicinal coupling constants, it has been confirmed that the pyranose sugars of HA exist in the 4C1 conformation in aqueous solution (Sicinska, 1993). We have measured the six membered ring puckering parameters for all pyranose rings in our simulations (Table I). Low average values for θ correspond to the stable 4C1 conformation, as experimentally measured. Although, the calculated puckering parameters Q suggest that the chains are slightly flattened. NMR studies using NOESY cross-peak analysis has suggested that the geometry of the HA linkages are in accord with the anomeric effect (Livant et al., 1992). This also agrees with our simulations, since we observed the minimum value of our φ glycosidic torsional-angles at approximately +60°, which is the value predicted by exo-anomeric effect theories for β-linkages (Tvaroska and Tomas, 1989).

It is well known that participation of a proton in a stable hydrogen-bond leads to a downfield shift in its resonance, which is unusually invariant to changes in temperature. The downfield shift of the amide proton in internal linkages relative to the external linkages is highly consistent with the presence of a hydrogen-bond between acetamido and carboxylate substituents in dimethyl sulfoxide. Measurement of hydroxyl proton resonance temperature coefficients also suggest that GlcNAc OH4, GlcA OH2, and GlcA OH3 are also involved in hydrogen-bonds (Scott et al., 1984). Further, GlcNAc OH4 has been observed to form a particularly stable intramolecular hydrogen-bond. Of course, experimental studies in dimethyl sulfoxide are not necessarily indicative of aqueous solution structure. Our simulations of tetrasaccharides agree with these hypotheses in the following ways. We observe a strong intramolecular hydrogen-bond between acetamido and carboxyl groups, which is important in stabilizing the conformation of the β1,4 linkage for greater than 50% of the time. Similarly, GlcNAc OH4 is observed to form an important intramolecular hydrogen-bond, which stabilizes the β1,3 linkage. All the other hydrogen-bonds mentioned above have been found in our tetrasaccharide simulations (Figures 1 and 2). We also conclude that there are two linkage environments corresponding to external and internal linkages in our tetrasaccharides. The internal linkages are constrained because they are not subject to end-effects, and would thus be expected to be more polymer-like. External linkages are seen to have greater flexibility due to end-effects and solvent interactions.

Extending the results from our previous paper, we can now detail the nature of hydrogen-bonding directly across β1,4 and β1,3 linkages without end effects, and therefore make predictions which apply to the HA polymer. Considering the β1,4 linkage first, and always considering the residue to the reducing end of the linkage as primed, we now observe the following hydrogen-bonds O6β…O3′, O3′…O5, and NH…O5′ as stabilizing this linkage (Figure 1). Similarly we propose that OH2, O7β and OH4′…O5 are the corresponding hydrogen-bonds across a β1,3 linkage (Figure 2). We can also detail the nature of the end effects, and three general conclusions can be drawn from our results. External linkages are more flexible than internal linkages. Non-reducing sugars are more flexible than those at the reducing termini, and external β1,4 linkages are more flexible than external β1,3 linkage. The first conclusion is due to a true end-effect, since the external residue are less constrained because they are subject to fewer intramolecular hydrogen-bonds. The latter two conclusions are more difficult to explain, but appear to be due to the positioning of residues containing hydroxymethyl groups at either the terminal or penultimate positions in the chain. Although hydroxymethyl groups provide stability to individual conformations by providing a hydrogen-bond, they also introduce an enhanced flexibility. With a GlcA at the non-reducing termini, an end effect hydrogen-bond stabilizes the carboxyl group by back bonding to OH4. There is no apparent reason for the nonreducing being more flexible than the reducing. However, it is probably more to do with accessibility of other donors/acceptors than stability of hydrogen-bonds shown in Figures 1 and 2. Although some linkage conformations are only seen at the terminal sugars, it should be noted that they are probably valid minima for internal linkages. However, they will favored to a greater or lesser extent depending on the surrounding residues and end-effects.

NMR spectra from aqueous HA oligosaccharides usually lack the important hydroxyl resonances. However, the amide protons exchange with water more slowly, and are relatively easy to observe. By studying the amide proton it has been concluded, based on chemical shifts, that at least two distinct environments can be observed in aqueous solution, consistent with the dimethyl sulfoxide observations (Cowman et al., 1984). A recent study (Cowman et al., 1996) went further, and suggested three distinct environments for external, penultimate, and internal sugar residues. In particular, different chemical shifts have been observed for the amide protons in a HA hexasaccharide having GlcNAc at the reducing terminus, as compared with a GlcA residue at the reducing terminus. We predict, based on our tetrasaccharide simulations, that the terminal residues of alternate oligosaccharides can partake in quite different dynamics. This is dependent on whether or not the terminal sugar residues contain hydroxymethyl groups; see Figures 1–4.

The amide vicinal coupling constant 1J[NHCH] has been measured in both aqueous solution and dimethyl sulfoxide. Table III shows literature values of 1J[NHCH] for different HA fragment sizes and solution conditions. For oligosaccharides in aqueous solution a rather high value of about 9 Hz has been measured (Cowman et al., 1984). A slightly lower value of 6.2 Hz has been measured for oligosaccharides in dimethyl sulfoxide (Scott et al., 1984). High values (≈8 Hz) of this coupling constant are consistent with a near trans arrangement of the amide proton. However, simple model building studies have indicated that this conformation is inconsistent with a direct hydrogen-bond between acetamido and carboxylate. This subsequently led to the
proposition that the acetamido-carboxylate hydrogen-bond exists predominantly in the water-bridged state (Heatley and Scott, 1988).

We observed no firm water-bridge between any adjacent acetamido and carboxyl groups in our simulations. However, we have observed the acetamido and carboxylate groups to be in a dynamic equilibrium between the hydrogen-bonded and water-bridged states, on the picosecond time-scale. This allows the amide proton to maintain an almost trans arrangement without losing its capability to hydrogen-bond to the carboxyl moiety. A dynamic equilibrium between hydrogen-bonded and water-bridged state has previously been proposed, based on the temperature coefficient of chemical shifts (Kvam et al., 1992).

Our simulations revealed three amide protons to be in the trans arrangement and one in the cis arrangement. However, using the Karplus equation of Bystrov et al. (1973) $J_{\text{HNCH}}$ was calculated to be between 7 and 8 Hz (Table II), irrespective of the amide proton arrangement. Although this is slightly lower than the experimentally measured value of 9 Hz in aqueous solution (Table III), it is a reasonable agreement after taking into account the relatively short simulation time and the absence of an exact Karplus equation for the acetamido moiety. It would therefore appear that the experimental data is consistent with an equilibrium between directly hydrogen-bonded and water bridged acetamido and carboxyl groups, with the amide proton almost trans. However, the simulation also suggests that the cis conformation of the amide proton cannot be ruled out based purely on measurement of $J_{\text{HNCH}}$, since the Karplus equation of Bystrov et al. (1973) predicts $J_{\text{HNCH}}$ to be greater than 8 Hz in both trans and cis conformations. Indeed our simulations suggest that some amide protons may exist in mixtures of both trans and cis, and this requires further experimental investigation. We therefore propose that a value of $J_{\text{HNCH}}$ greater than 8 Hz should not be taken to indicate the absence of a hydrogen-bond between the acetamido and other substituents, nor should it be taken to necessarily indicate the trans conformation of the acetamido.

Similar MD simulations to those presented here of neocarrabiose and maltose showed that large conformational shifts are induced by the presence of water. These fluctuations were put down to water competition for stabilizing intramolecular hydrogen-bonds. In neocarrabiose a hydrogen-bond was observed between hydroxyls OH2 on different sugar residues, and its percentage hydrogen-bonding decreased dramatically when simulated in water (Ueda and Brady, 1996). In simulations of cellobiose in water it was concluded that water has a damping effect and fewer transitions were observed in water simulations (Hardy and Sarko, 1993). In this simulation they observed ratios of 3.2 for $tg$:$gt$ conformers of the hydroxymethyl groups, compared with 1.9 $gg$:$gt$ found in our simulations. In most cases the percentage hydrogen-bonding was found to decrease on moving from vacuum to water, but not in all. In aqueous simulations of maltose a hydrogen-bond was found to form between hydroxyls OH2 and OH3 on neighboring residues (Brady and Schmidt, 1993), as observed in crystal structures. In solution the directly hydrogen-bonded state was favored over the solvent-solute hydrogen-bonds that temporarily replaced it. In all cases the effects of solvation appear to directly effect the dynamics of intramolecular hydrogen-bonds, rather than simply providing dielectric screening. Such effects would not be included in vacuum simulations employing mean-field treatments of solvation, and thus these simulations would appear to have little use in studying solvated carbohydrates.

**Conclusions**

We have developed a hydrogen-bonding analysis tool, which allows conformational rearrangements in solution to be correlated to changes in intramolecular hydrogen-bonding patterns. Using this tool we have probed the dynamic nature of intramolecular hydrogen-bonding within the HA molecule. On average the secondary structure of HA appears to be stabilized by sets of hydrogen-bonds involving the acetamido group. However, competition for intramolecular hydrogen-bonds by the surrounding water results in a discrete motion of the HA tetrasaccharides where they move between highly organized states on the nanosecond time-scale. We suggest that these conformational rearrangements may be applicable to other polysaccharides, and perhaps to other carbohydrates, that are capable of forming intramolecular hydrogen-bonds.

We have predicted that linkages at least two residues from the end of the HA molecule are highly stabilized and are polymer-like. This agrees with the current experimental data obtained from NMR and CD studies. Our simulations have also indicated that the hydroxymethyl group could be particularly important in determining the dynamical behavior of HA. This group is capable of donating to multiple acceptors which increases the conformational flexibility of the terminal residues. However, these conformations though observed as end effects indicate the presence of distinct low-energy states for both $\phi$ and $\psi$ permitting flexibility. This could be a key feature of HA which allows it to possess an extended nature whilst still allowing it some specific conformational flexibility, thus making it entropically favorable for HA to stay soluble up to the high concentrations found in vivo.

We have also calculated the value of the $J_{\text{HNCH}}$ coupling constant of acetamido groups, and found an agreement with published data. We propose a nearly trans arrangement for this proton in an equilibrium state between hydrogen-bonding and water-bridging. However, a small proportion of the cis conformation that we have also observed is indiscernible based purely on considering this coupling constant.

**Materials and methods**

Molecular dynamics trajectories (500 ps) were calculated in water for both the tetrasaccharides of HA, using the CHARMM package (Brooks et al., 1983) with version 22 parameters and partial charges. Both tetrasaccharides were modeled with the

<table>
<thead>
<tr>
<th>$J_{\text{HNCH}}$ (Hz)</th>
<th>Solvent</th>
<th>Sample</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.3</td>
<td>Me$_2$SO</td>
<td>Disaccharide</td>
<td>Heatley et al., 1982</td>
</tr>
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<td>8.8</td>
<td>H$_2$O</td>
<td>Oligosaccharide</td>
<td>Cowman et al., 1984</td>
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<td>Me$_2$SO</td>
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<td>Scott et al., 1984</td>
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<tr>
<td>9.5</td>
<td>H$_2$O</td>
<td>Hexasaccharide</td>
<td>Heatley and Scott, 1988</td>
</tr>
<tr>
<td>9.4</td>
<td>H$_2$O/D$_2$O</td>
<td>Tetrasaccharide</td>
<td>Livant et al., 1992</td>
</tr>
<tr>
<td>8.1</td>
<td>Me$_2$SO</td>
<td>Oligosaccharides</td>
<td>Kvam et al., 1992</td>
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<td>9.6</td>
<td>H$_2$O/D$_2$O</td>
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<td>Sicinska et al., 1993</td>
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<td>H$_2$O/D$_2$O</td>
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<td>Holmbeck et al., 1994</td>
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<td>9.0</td>
<td>H$_2$O</td>
<td>Oligosaccharides</td>
<td>Cowman et al., 1996</td>
</tr>
</tbody>
</table>
carboxyl groups in the unprotonated state throughout. All simulation work was performed on a dedicated Silicon Graphics R4000. Calculations were carried out using the leap-frog formulation (Hockney, 1970) of the Verlet algorithm (Verlet, 1967), and hydrogen covalent bond lengths kept constant by applying the SHAKE procedure (van Gunsteren and Berendsen, 1977). This allowed an integration step size of 1 fs to be used with excellent precision. Although some force fields contain a specific term to represent hydrogen-bonding, this type of interaction can be modeled by appropriate atomic partial charges and van der Waals parameters for the hydrogen-bond donor and acceptor atoms (Brady and Schmidt, 1993).

Solvated simulations used 32 Å water boxes, filled with 1000 TIP3P previously equilibrated water molecules (Jorgensen et al., 1983), consistent with an initial molar concentration of 0.05 M. The initial configuration was produced by extensive minimization using the adopted basis Newton-Raphson method. Equilibration consisted of 3 ps of heating at rate 100K ps⁻¹, followed by 40 ps during which the system was coupled to a heat bath at 300K. Subsequently, 10 ps of free dynamics was performed and discarded before each simulation. Coordinates of all atoms were written every 50 integration steps, or 0.05 ps. Nonbonded lists were generated using the grid search cubing algorithm, and updated every 20 steps. Edge effects were overcome by implementing periodic boundary conditions, and the non bonded cut-off was set at 12 Å and reduced to zero by using the shifting function (Steinbach and Brooks, 1994). All analysis was performed using software developed by the authors.

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Abbreviations

CD, circular dichroism; DMSO, dimethyl sulfoxide; GlcA, β-d-glucuronic acid; GlcNAc, β-N-acetyl-D-glucosamine; HA, hyaluronan; MD, molecular dynamics; NMR, nuclear magnetic resonance.

References