A water molecule participates in the secondary structure of hyaluronan

Frank HEATLEY* and John E. SCOTT†

*Department of Chemistry and †Department of Chemical Morphology, Cell and Structural Biology, Chemistry Building, University of Manchester, Manchester M13 9PL, U.K.

The structure of hyaluronan was investigated in water/dimethyl sulphoxide mixtures by using high-field n.m.r. and space-filling molecular models. The secondary structure previously established in detail in 'dry' dimethyl sulphoxide [Heatley, Scott & Hull (1984) *Biochem. J.* **220**, 197–205] undergoes changes on addition of water, compatible with the incorporation of a water bridge between the uronate carboxylate and acetamido NH groups. Molecular models show that such a configuration is highly probable, and saturation-transfer experiments yield rates of NH proton exchange that support this proposed structure. The existence of two distinct stable configurations for hyaluronan, in water-rich and water-poor conditions respectively, may have biological implications, e.g. during its biosynthesis in cell membranes. There are extensive hydrophobic regions in both forms, which may be important for interactions with, e.g., membranes, proteins and itself.

INTRODUCTION

Hyaluronan (HA) is one of the glycosaminoglycans (see [1] for a review), a family of polymers distributed extracellularly, for the most part, throughout the animal kingdom. HA is a ubiquitous constituent of connective tissue, and probably of great evolutionary antiquity, since it is also produced by streptococci. Alone among the glycosaminoglycans, it is not attached to

a polypeptide, and it is biosynthesized by a mechanism different from that of the others [2].

It is not surprising, in view of its long evolutionary history and very wide occurrence, that HA interacts with a variety of tissue and membrane structures [1] and that some, at least, of these interactions are specific. HA appears to play an important role in early development [3], and it may be one of the compounds that control the diameter of newly formed collagen fibrils [4]. HA is

Fig. 1. Secondary structure of HA in (a) Me₂SO and (b) Me₂SO containing water

Abbreviations used: HA, hyaluronan; Me₂SO, dimethyl sulphoxide.

found in high concentrations in certain connective tissues (vitreous humour of the eye, Wharton's jelly in the umbilical cord, rooster comb, sex skin of female baboons etc.), where its role seems to be 'mechanical', i.e. space-filling, as a kind of stuffing, serving to keep the tissue swollen, with a degree of rigidity. It also performs a completely different kind of mechanical service in the joints where, as a characteristic component of the synovial fluid, it is part of the lubricating system.

It seems unlikely that all the specific interactions of HA will turn out to be due to one site in the molecule, and even less likely that the properties that are desirable for its space-filling role will be attributable to the same structural features.

Whatever the requirements of the specific interactions, mechanical or lubricant properties of HA, the presence therein of a secondary structure would be of prime importance in determining their efficiency. The proof and detailed analysis of such a structure would be essential to an interpretation of the functions of HA in molecular terms. Secondary structures in HA might thus be of very wide significance throughout much of biology.

That HA is capable of forming a stable secondary structure, involving hydrogen bonds between adjacent sugar units, and present throughout the polymer, has been shown in complete detail by n.m.r. studies carried out in dimethyl sulphoxide (Me_2SO) [5]. This work provided a physical proof of the existence of a structure that had been predicted from computer simulations [6] and space-filling molecular models [7] (Fig. 1). Although this complete structure has so far been proven to exist only in Me_2SO , the first proposal for part of it (containing hydrogen-bonded acetamido, carboxylate and hydroxy groups [7]) was based on work in aqueous solutions, involving kinetic studies of periodate oxidation of the glucuronate glycol groups [7]. The extended hydrogen-bonded arrays, down both 'sides' of the HA, must, if present, result in considerable rigidity of the polymer, and it has long been known that HA behaves in aqueous solution as an unusually 'stiff' polymer [1,8]. This stiffness would be of advantage in its role as a space-filling material [5]. Moreover, the high viscosity of HA solutions decreases dramatically in strongly alkaline solutions, reverting to the original value on neutralization [9], and this phenomenon is interpretable as being due to disruption of some of the hydrogen bonds when participating protonated groups ionize and lose their H atoms.

The complete set of four hydrogen bonds in which the acetamido group is central requires three sugar units ($\text{GlcA1-3GlcNAc1-4GlcA}$), and it is relevant that the circular dichroism in aqueous solution of a set of HA fragments (mono-, di-, tri- and tetra-saccharides and higher oligomers, prepared by enzymic digestion) showed a change as the series ascended, levelling off at about the tetrasaccharide, which behaved much as the higher oligomers [10]. This was seen as being compatible with the secondary structure shown in Fig. 1 [5]. However, recent work has suggested that this need not be so, the change in circular dichroism could be due simply to changes in the environment of the acetamido group, caused by different patterns of substitution of C-1 and C-3 of the acetamido glucose moiety, as the oligomer series is ascended [11]. More importantly, it was found that the NH coupling constant, measurement of which is possible in water solutions since the NH proton exchanges slowly

with the water protons, strongly indicated that the acetamido group was oriented in the 'normal' conformation, with the NH bond *trans* to that of the C-2-H bond, rather than at right-angles to it, as required by the structure in Fig. 1(a) [11]. We have confirmed that the small coupling in Me_2SO consistent with the structure in Fig. 1(a), does indeed increase in water solution to values very close to that of the monosaccharide 2-deoxy-2-acetamidoglucose (F. Heatley & J. E. Scott, unpublished work). This change in orientation of the acetamido group would disrupt that part of the hydrogen-bonded array in which it participates.

These findings raise the following questions. (1) Are all H-bonds disrupted in water, as compared with Me_2SO ? (2) Are there hitherto unsuspected secondary structures of HA in water with features that would explain the periodate- and alkali-susceptibilities of HA, while embodying the c.d. and n.m.r. data bearing on the acetamido orientation? We have therefore re-examined the structure of HA as shown in space-filling models, to incorporate water interactions in the vicinity of the NH group, and extended our n.m.r. investigations from Me_2SO into mixtures containing up to 50% water, in which most of the relevant chemical shifts could still be accurately measured. We conclude that the answers to the questions above are 'no' and 'yes' respectively. The results are compatible with saturation-transfer measurements on the several NH protons of the hexasaccharide homologue of Fig. 1.

EXPERIMENTAL

Materials

The di-, tetra- and hexa-saccharides were the preparations used in previous investigations [5].

Methods

Chemical shifts and coupling constants for $[\text{}^2\text{H}_6]$ - Me_2SO /water mixtures were obtained from spectra obtained at 300 MHz by using a Varian Associates SC-300 spectrometer. Saturation transfer and relaxation time measurements on water solutions were obtained at 500 MHz by using a Bruker Spectrospin WM-500 spectrometer. In the latter case the Redfield 2-1-4 pulse [12] was used to diminish the water signal. Chemical shifts were measured by using the $[\text{}^2\text{H}_5]\text{Me}_2\text{SO}$ residual proton signal as reference ($\delta = 2.50$ p.p.m.). Spin-lattice relaxation rates were obtained by using the inversion recovery method [13] with Redfield pulses for both π and $\pi/2$ pulses. Saturation-transfer measurements were made with the use of gated irradiation.

Solutions contained approx. 1 mg of saccharide.

RESULTS

(a) $[\text{}^2\text{H}_6]\text{Me}_2\text{SO}$ /water mixtures

Spectra of the HA tetrasaccharide oligomer were recorded in $[\text{}^2\text{H}_6]\text{Me}_2\text{SO}$ mixtures containing up to 12% (v/v) water. Over this composition range at 25 °C all OH and NH protons except two gave distinct peaks. The exceptions were $\text{G}_{(2)}\text{HO-4}$, which disappears because of rapid exchange, and $\text{G}_{(2)}\text{HO-2}$, which is unobservable because of overlap by the H_2O signal. Both were observable only up to 3% (v/v) water.

As the amount of water increases, the OH and NH resonances shift to varying extents. The variation in

Fig. 2. Change in chemical shift of protons in HA tetrasaccharide oligomer with water content in $[^2\text{H}_6]\text{Me}_2\text{SO}$ /water mixtures at 25 °C

This Figure shows those peaks moving to lower resonance frequency with increasing water content.

Fig. 3. Change in chemical shift of protons in HA tetrasaccharide oligomer with water content in $[^2\text{H}_6]\text{Me}_2\text{SO}$ /water mixtures at 25 °C

This Figure is similar to Fig. 2 but shows peaks moving to higher resonance frequency with increasing water content.

chemical shift with water content is shown in Figs. 2 and 3 in the form of plots of the change in chemical shift with respect to neat $[^2\text{H}_6]\text{Me}_2\text{SO}$, $\Delta\delta$, against water concentration. By far the largest effect is shown by the $\text{N}_{(2)}\text{HN-2}$ proton (Fig. 2), which moves to lower frequency by almost 1 p.p.m. at 12% (v/v) water. The

movement of other protons is considerably less, some moving to lower frequency (Fig. 2) and some to higher (Fig. 3). For comparison, data for some CH peaks is also shown in Fig. 2; their concentration-dependence is small.

The chemical shift of the $\text{N}_{(2)}\text{NH-2}$ proton is non-linear in the water concentration used, decreasing rapidly on addition of relatively small amounts of water, suggesting a specific effect involving water and the acetamido group. That the $\text{N}_{(2)}\text{NH-2}$ chemical-shift variation was consistent with an association between water and acetamido groups was tested as follows. Assume the association is represented by:

where S and W represent saccharide and water. Let $[\text{X}_0]$ be the total concentration of free and associated X. For the condition $[\text{W}_0] \gg [\text{S}_0]$, which holds in the present case, the equilibrium constant is given by:

$$K = \frac{[\text{SW}_n]}{([\text{S}_0] - [\text{SW}_n]) \cdot [\text{W}_0]^n} \quad (1)$$

The chemical shift of a proton in S averaged over free (f) and associated (b) states is:

$$\hat{\delta} = P_f \delta_f + P_b \delta_b \quad (2)$$

where δ_x and P_x are the chemical shift and relative population of state x. Eqn. (2) can be re-written:

$$\begin{aligned} \hat{\delta} &= \delta_f + (\delta_b - \delta_f) \cdot P_b \\ &= \delta_f + (\delta_b - \delta_f) \cdot [\text{SW}_n] / [\text{S}_0] \end{aligned} \quad (3)$$

Using eqn. (1) in eqn. (3) and re-arranging gives

$$\frac{1}{\delta - \delta_f} = \frac{1}{(\delta_b - \delta_f) K \cdot [\text{W}_0]^n} + \frac{1}{(\delta_b - \delta_f)}$$

$\delta - \delta_f$ is simply $\Delta\delta$ as used in Figs. 1 and 2. A plot of $\Delta\delta^{-1}$ versus $[\text{W}_0]^{-n}$ should be linear for the correct value of n ; Fig. 4 shows that $n = 1$ is a good choice. From the slope and intercept we obtain $K = 0.14 \text{ dm}^3 \cdot \text{mol}^{-1}$ and $(\delta_b - \delta_f) = 1.7 \text{ p.p.m.}$ This value of $(\delta_b - \delta_f)$ should correspond to the difference in chemical shift on going from dry $[^2\text{H}_6]\text{Me}_2\text{SO}$ to water. The actual values are $\delta_f = 9.25 \text{ p.p.m.}$ [5] and $\delta_b = 8.1 \text{ p.p.m.}$ [10].

(b) Water solutions

Solutions of the hexasaccharide HA oligomer were investigated. At 25 °C resolved NH signals were observed, but the HO signals showed incipient coalescence with the solvent. The NH spectrum was identical with that reported by Cowman [11], consisting of two doublets ($J = 9.5 \text{ Hz}$) together representing unit intensity at $\delta = 8.3$ and 8.4 p.p.m. attributed to α and β forms of NH at the reducing end and a doublet ($J = 9.5 \text{ Hz}$) at $\delta = 8.2 \text{ p.p.m.}$, representing two units of intensity, attributed to the remaining (interior) NH protons.

On saturating the H_2O signal, a decrease in intensity of the NH signals was observed due to chemical exchange. The extent of saturation transfer is reported in Table 1 as the ratio S_i/S_0 , where S_i is the intensity with water saturation and S_0 the unperturbed intensity. The saturation transfer depends [14] on the NH spin-lattice relaxation rate, R_1 , as well as the chemical exchange rate k according to the equation:

$$\frac{S_i}{S_0} = \frac{R_1}{R_1 + k} \quad (4)$$

Table 1. Nuclear Overhauser effects (S_i/S_0), spin-lattice relaxation rates (R_1) and proton exchange rates (k) for NH protons in HA hexasaccharide oligomer in water at 500 MHz and 25 °C

Proton	S_i/S_0	R_1/S	k^* (s ⁻¹)
N _(i) -NH-2 β	0.10	2.0	18
N _(i) -NH-2 α	0.30	2.2	5.2
N _(n) -NH-2	0.56	2.9	2.2

* Uncertainty $\pm 20\%$.

[This equation assumes that there is no contribution to NH relaxation from dipolar interaction with water protons. If NH relaxation is determined entirely by this interaction, eqn. (4) becomes, for small molecules:

$$\frac{S_i}{S_0} = \frac{3R_1/2}{R_1 + k}$$

The derived values of k differ quantitatively but not qualitatively.]

The observed values of R_1 and the values of k calculated by using eqn. (4) are given in Table 1. The exchange rate for the interior NH protons is significantly less than for the terminal NH.

DISCUSSION

Previous work has proved in complete detail the presence of a secondary structure in HA in Me₂SO solution (Fig. 1). This structure had been predicted by molecular modelling, with space-filling models [7] and computer simulation [6], based on studies of oxidation rates of uronate glycol groups by periodate [7]. Although these studies were carried out in water, it was always envisaged that it would be necessary to examine HA by direct, i.e. spectroscopic, means in water [15]. Unfortunately, n.m.r. is unable to investigate the crucial OH groups in water because of rapid proton exchange. However, the NH signal is readily accessible, with appropriate software and experimental protocols, since it is some distance from the H₂O resonances and exchanges slowly with the water protons. It was found [11] that the observed NH-CH coupling constant was incompatible with the hydrogen-bonded acetamido carboxylate structure shown to be present in Me₂SO (Fig. 1a). This calls for revision of this feature, but not necessarily of the rest of the structure given in Fig. 1, the existence of some of which is not dependent on the acetamido $\rightarrow$ carboxylate bond.

The findings reported in this paper suggest that the direct -NH $\rightarrow$ -O₂C hydrogen bond in Me₂SO is replaced in water by a water bridge. Molecular models show that

$$\begin{array}{c} \text{H} \\ | \\ -\text{NH} \cdots \text{O}-\text{H} \cdots \text{O}_2\text{C} \end{array}$$

is a very favourable configuration (Fig. 1b). The N-H bond is then *trans* to the C2-H bond, as required by the coupling constant determined by Cowman [11] and by ourselves (F. Heatley & J. E. Scott, unpublished work). This *trans* orientation is energetically preferred in the 2-acetamidohexoses. Data of two kinds, namely (a) NH hydrogen exchange measured by saturation transfer, and (b) the demonstration that there is

Fig. 4. Plot of $\Delta\delta^{-1}$ versus $[W_0]^{-1}$ for the N₍₂₎ NH-2 proton in HA tetrasaccharide oligomer at 25 °C

only one water molecule bound to the NH group in a range of concentrations of Me₂SO/water, strongly suggest that this possible structure is very probable.

The rate of proton exchange of the 'polymer' NH groups is markedly less than that of the 'end-group' NH (see the Results section), which could not take part in the kind of structure proposed in Fig. 1(b). The comparison with the β -glycosidic NH seems most relevant, since the 'polymer' NH groups are also adjacent to β -glycosidic bonds, and models suggest that the α -glycosidic NH can take part in a water-bridged structure with the C-1-OH group, although with less facility than that proposed to occur throughout the polymer (Fig. 1b). The polymer NH protons are therefore hindered from exchanging with the solvent compared with the end-group NH, and this is compatible with the presence of a firm water bridge (Fig. 1b).

The analysis of the equilibrium between free NH and NH $\cdots$ OH₂ forms (see the Results section) indicates unequivocally that there is only one water molecule associated with the NH, since the results (Fig. 4) are incompatible with other stoichiometries. This does not in itself prove the water-bridge structure, but the hydrogen-exchange data indicate that this water molecule must be different from that of the bulk water and from that associated with the end-group NH. The close proximity of the -CO₂⁻ group provides the necessary anchoring point to give the water bridge greater stability. This -CO₂⁻ group must, in any case, be solvated H-O-H $\cdots$ O-C-O, and there is thus no need to invoke a second water molecule to fulfil this role.

The rest of the previously demonstrated structure, involving OH $\rightarrow$ ring O bonds, is unaffected by this new feature. There is a possibility of a second, not-so-favourable, water bridge between the acetamido -C=O group and the uronate C-2-OH group.

Water molecules were located by X-ray diffraction of HA fibres, as intra- and inter-molecular bridges [16]. One of them was similarly placed to that shown in Fig. 1(b) [17]. Other interactions may occur across stretches of HA chain longer than the hexasaccharide investigated here, but if so they must be much less frequent than the interaction we have demonstrated, since the properties of high-molecular-mass HA in aqueous solution are compatible with the structure in Fig. 1(b), repeated indefinitely throughout the polymer [7,10,14].

A feature of this structure, not previously noticed, is that it demonstrates considerable areas of hydrophobicity, consisting of patches of $-CH$ groups, over three neighbouring sugar units equivalent to an extended octane structure (cf. Fig. 4 of ref. [7]).

It seems likely that they would contribute to, e.g., interactions with hydrophobic peptide sequences or with membranes (see below) or even to self-associations.

We have thus shown that HA can form two stable secondary structures, depending on the environment. One (Fig. 1a) forms in conditions of 'water shortage', and the other in aqueous solutions. It would be surprising if this did not have biological implications. It may be particularly relevant that HA is thought to be synthesized at the cell membrane, and one scheme has the HA molecule actually passing through a membranous structure [18]. It would certainly be important to such a mechanism that a structure (Fig. 1a) specifically adapted to the water-poor environment of the membrane could be formed, perhaps changing to the structure shown in Fig. 1(b) after leaving the membrane.

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