^1H Nuclear-Magnetic-Resonance Spectra of the Methyl Group of the Acetamido Moiety and the Structure of Acid Glycosaminoglycans in Solution

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The ^1H resonances of the methyl group in the acetamido moiety of several types of glycosaminoglycans are reported at 300 MHz in $^2\text{H}_2\text{O}$. Dermatan sulphates with various L-iduronate/D-glucuronate ratios are compared with chondroitin sulphates with various contents and positions of substitution of sulphate esters. Hyaluronate oligomers are compared with 2-acetamido-2-deoxy-D-glucose, and with heparan sulphate and keratan sulphate. The major determinant of the chemical shift of the acetamido methyl resonance is the closeness of approach between carboxy groups and the acetamido group, in agreement with chemical evidence based on periodate-oxidation kinetics.

X-ray-crystallographic studies on the glycosaminoglycuronans (Atkins *et al.*, 1972; Rees, 1975; Winter *et al.*, 1975) have shown that there are regular and characteristic configurations in the solid state. The situation in solution is not so clear, but chemical investigations (Scott & Tigwell, 1978) suggest that the acetamido group is involved in hydrogen-bonded sequences that are possible in some of the glycosaminoglycuronans. Such interactions would influence the n.m.r. spectrum, and studies in $[^2\text{H}]$ -dimethyl sulphoxide solution (J. E. Scott & F. Heatley, unpublished work) have shown that the NH signal has two components in hyaluronate oligomers. One peak, at low field, is considerably distant from the normal NH proton resonance observed in, e.g. 2-acetamido-2-deoxy-D-glucose, as would be expected of a hydrogen-bonded NH proton (Scott, 1979). We also noticed that the resonance of the methyl group of the acetamido moiety shows characteristic changes. We therefore examined the ^1H n.m.r. spectrum of this group in $^2\text{H}_2\text{O}$, and have correlated it with the behaviour in periodate-oxidation systems (Scott & Tigwell, 1978) and with chemical features of the polymer, particularly the presence of an axial or equatorial carboxy group on the neighbouring uronic acids.

Materials and Methods

The glycosaminoglycans were those used in a previous investigation (Scott & Tigwell, 1978), with the addition of keratan sulphate, which was a gift from Dr. H. Greiling, University of Aachen, Aachen, Germany, and several dermatan sulphates, which were gifts from Dr. L. A. Fransson, University of Lund, Lund, Sweden. These were made by hyaluronidase digestion of dermatan sulphates, followed by

fractionation with alcohol, copper salts etc., by the method of Fransson & Roden (1967). The carbazole/orcinol ratios of these compounds were determined by Dr. L. A. Fransson. Oligomers from hyaluronidase digests of hyaluronate were gifts from Dr. E. A. Balazs, Columbia University, New York, NY, U.S.A., and Dr. A. Olavsen, University of Wales, Cardiff, Wales, U.K. All polyanions were used as sodium salts.

Methods

Periodate oxidation and computation of second-order rate constants was carried out by the method of Scott & Tigwell (1978). N.m.r. spectra were recorded with a 300 MHz Varian SC300 spectrometer. Exchangeable protons on the polysaccharides were converted into the deuterated form by dissolving in $^2\text{H}_2\text{O}$, and freeze-drying. This cycle was repeated three times, and the solutions were finally prepared in $^2\text{H}_2\text{O}$ at concentrations of 6–10 mg/ml. Sodium 2,2-dimethyl-2-silapentane-5-sulphonate was used as reference compound.

Results

The second-order rate constants of the series of dermatan sulphates, which vary in their ratio of iduronic acid to glucuronic acid are shown in Fig. 1, plotted against the carbazole/orcinol ratios. Specimens of chondroitin 4-sulphate and chondroitin 6-sulphate are included in the graph, representing 100% glucuronic acid-containing glycosaminoglycuronans. The carbazole/orcinol ratio for these compounds is 2.2 (Fransson *et al.*, 1968). There is an approximately monotonic relationship between the carbazole/orcinol ratio and the second-order rate constant for periodate oxidation, confirming and extending the

Fig. 1. Second-order rate constant of periodate oxidation at 29°C of chondroitin sulphates (1) and dermatan sulphates (2–6) plotted against their carbazole/orcinol ratio. The values of k_2 for point (1) are taken from Scott & Tigwell (1978). Symbols: Δ , chondroitin 6-sulphate; $\bullet$, chondroitin 4-sulphate; $\blacksquare$, dermatan sulphate.

previous findings that iduronate in dermatan sulphate is much more rapidly oxidized than glucuronate in chondroitin sulphates.

Figs. 2–5 show the acetamido methyl region of the ^1H n.m.r. spectra of a number of polymers and oligomers. Fig. 2 shows the spectra of the compounds presented in Fig. 1, except for chondroitin 6-sulphate. There is a consistent displacement of the methyl peak to low field with increasing values of the periodate-oxidation rate constant. This shift in the methyl peak correlates with the structural change from glucuronate to iduronate, i.e. with change in position of the carboxy group. Fig. 3 compares the spectra of polymers differing in the amounts and positions of the sulphate ester group. The spectra of chondroitin 4- and 6-sulphates (Fig. 3) each show a main sharp peak and a shoulder, the peak in the chondroitin 4-sulphate spectrum being at the same frequency as the shoulder of the chondroitin 6-sulphate spectrum, and vice versa. Over-sulphated chondroitin sulphate (Type D) shows both resonances at about equal intensities. Teichuronic acid, an isomer of chondroitin, has only

Fig. 2. ^1H n.m.r. spectra of the acetamido methyl group of chondroitin 4-sulphate (1) and dermatan sulphates (2–6) at 300 MHz in $^2\text{H}_2\text{O}$ at 22°C, with sodium 2,2-dimethyl-2-silapentane-5-sulphonate as reference.

The polyanions were at 6–10 mg/ml. The dermatan sulphates and chondroitin 4-sulphate are the preparations shown on Fig. 1, identified by the same numbers.

one clear resonance, slightly down-field from chondroitin 4-sulphate.

The CH_3 resonances in the series of hyaluronate oligomers are all up-field of that of 2-acetamido-2-deoxy-D-glucose (Fig. 4), whereas those of keratan sulphate and heparan sulphate are almost coincident with it (Fig. 5).

Further samples of chondroitin 4-sulphate and dermatan sulphate showed exactly the same peak position and shape as those used in Fig. 1 (dermatan sulphates represented by points 5 and 6 and chondroitin sulphate A). The chemical shift of their CH_3 protons was studied as a function of temperature (22–60°C) and concentration (0.125–0.5%, w/v). The extremes of chemical shift differed by less than 0.005 p.p.m. Both samples, plus chondroitin sulphates C and D (used in Fig. 3), in salt solutions (0.2 M-NaCl, KCl, potassium methyl sulphate and NaClO_4) showed similar peak positions and shapes to those obtained in salt-free $^2\text{H}_2\text{O}$ solution. Extremes of chemical shift differed by 0.005 p.p.m.

Fig. 3. ^1H n.m.r. spectra of the acetamido methyl group of chondroitin 4- and 6-sulphates (A and C), chondroitin sulphate D and teichuronic acid (TeA)

The experimental conditions were as described for Fig. 2.

Fig. 4. ^1H n.m.r. spectra of the acetamido methyl group of hyaluronate oligomers and 2-acetamido-2-deoxy-D-glucose (N-acetylglucosamine)

Abbreviations used: Di, hyalobiuronate; Tet, tetrasaccharide; Hex, hexasaccharide. Experimental conditions were as for Fig. 2.

Fig. 5. ^1H n.m.r. spectra of the acetamido methyl group of heparan sulphate (HS2) and keratan sulphate (KS). Experimental conditions were as for Fig. 2.

Discussion

The insensitivity of the chemical shift and peak shape of the methyl resonance to variations in temperature, polymer concentration and ambient salts implies that solute-solute or solute-solvent interactions are unimportant in this context. The observed characteristic patterns must therefore arise from screening differences caused by the chemical structure. The effect on the methyl resonance of considerable changes in the amounts and positions of sulphate ester groups is much less marked than that of the much smaller change in the position of the carboxy group, from L-iduronate to D-glucuronate. Inductive effects can be discounted because of the number of atoms through which they would have to be relayed, especially in the case of the uronates, because the carboxy group remains on the same carbon atom. Fairly gross changes in the location and total amount of negative charge within the acetamido sugar are less important than similar but smaller changes in the neighbouring sugars. The simplest explanation is that the carboxy group can be closer to the acetamido group than any of the sulphate esters, and fluctuations in the carboxy group-acetamido-group interactions are the main determinants of the chemical shift of the acetamido methyl resonance. A similar conclusion emerged from X-ray-crystallographic (Atkins *et al.*, 1972) and periodate-oxidation studies (Scott & Tigwell, 1978). It seems likely that a closer approach of the carboxy and acetamido groups is associated with an up-field shift of the methyl resonance, since those compounds in which carboxy-group-acetamido-group interactions are not possible (2-acetamido-2-deoxy-D-glucose and keratan sulphate) are down-field of those in which interactions are probable. The up-field movement of the methyl resonance in the ascending series of hyaluronate oligomers (Fig. 4) is compatible with this possibility. The behaviour of the disaccharide is at

first sight anomalous, in that the new up-field peak, which is absent from the 2-acetamido-2-deoxy-D-glucose spectrum, can hardly be due to carboxy-group-acetamido-group interactions, since the distance across the glucuronate-(1→3)-acetamidoglucose link is much greater than that across the acetamidoglucose-(1→4)-glucuronate bond. However, examination of the proton resonances on C-1 in the region of δ 4–5 p.p.m. shows that there are two anomeric forms (α and β) present. The up-field peak is due to the α -form, whereas the low-field peak is due to the β -form. The influence of the anomeric configuration must decrease as the oligomers increase in size, and the up-field shift in the tetra- and hexasaccharides is attributable to other factors, e.g. carboxy-group-acetamido-group interactions.

There are four different arrangements within the hybrid polymers in which the acetamido sugar is linked to glucuronic acid and/or iduronic acid. Glucuronic acid at both 1 and 3 positions of the 2-acetamido-2-deoxy-D-galactose gives rise to a chondroitin 4-sulphate 'block'. If both substituents are iduronic acid, the structure is typical of dermatan sulphate. The hybrid polymers contain both arrangements and clearly resolved resonances at positions characteristic of chondroitin 4-sulphate (δ 2.06 p.p.m.) and dermatan sulphate (δ 2.12 p.p.m.) are present in their spectra. The two remaining structures have glucuronic acid at either position 1 or 3 of the acetamido sugar with iduronic acid filling the other position. Presumably the intermediate peak, at δ 2.09 p.p.m. is due to one or other of these arrangements.

Assuming that the different resonance frequencies correspond to different distances between the carboxy group and the acetamido group, the closest distance resulting in hydrogen-bonding, there are four situations that might be observable: (i) there is no hydrogen bonding to the acetamido group; (ii) the acetamido NH group is hydrogen-bonded as a donor; (iii) both carbonyl and NH groups are involved in hydrogen-bonds; and (iv) the carbonyl oxygen atom is hydrogen-bonded as an acceptor. Assuming further that the sugars are all in the C1 conformation, and that the acetamido and carboxy groups are *cis*, as indicated by X-ray crystallography (Atkins *et al.*, 1972) and inferred from periodate-oxidation data (Scott & Tigwell (1978), situation (iii) is possible if glucuronate is at position 1 of the acetamido sugar. Provided that the glycol hydroxy groups of the uronates are equatorial, situation (iii) does not differ from that in chondroitin 4-sulphate, even if the uronate is iduronic acid. The resonance would not be expected to be visible as a third peak.

However, it seems likely that L-iduronate in dermatan sulphate is in the 1C conformation (Gatti *et al.*, 1979), at least in aqueous solution from which NaClO₄ or NaIO₄ are absent (B. Levine, J. E. Scott & R. J. P. Williams, unpublished

work). The hydroxy groups of iduronate are then not equatorial and cannot participate in the linear hydrogen-bonded array of situation (iii), with glucuronate at position 1 of the acetamido sugar and iduronate at position 3. Only situation (ii) is possible. If the uronate substituents are reversed, a $\text{>C=O} \leftarrow \text{H—}$ bond (situation iv) would probably be the only interaction. On the grounds that an $\text{—OH} \rightarrow \text{O=C<}$ bond is likely to be less strong and persistent than an $\text{—NH} \rightarrow \text{COO—}$ bond, the third peak is tentatively attributed to an L-iduronate-(1→3)-acetamido-2-deoxy-D-galactose-(1→4)-D-glucuronate structure.

It is noteworthy that if e.g., hydrogen-bonding of situations (ii) and (iii) is not postulated, and only close approach of the carboxy group and the acetamido group is assumed, there is no reason for more than two resonances, in contrast with the three or more resonances observed.

The chemical shifts of the methyl group with change in structure are very small in magnitude, and it would be unwise to attempt to explain their origin in terms of magnetic anisotropy or electric-field effects, without an exact knowledge of chain configurations. For firmer assignments and better understanding of the effects of hydrogen-bonding on the methyl resonance of acetamido groups, work on suitable model compounds is required. An estimate of the magnitude and direction of chemical-shift changes due to hydrogen-bonding in amido groups can be obtained from an n.m.r. study of self-association in *N*-methylpropionamide (Hinton & Westerman, 1970). Hydrogen-bonded dimers in the neat liquid are disrupted by dilution with water, resulting in an up-field shift of the NH signal of approx. 0.3 p.p.m. and a down-field shift of the COCH₂ signal of approx. 0.03 p.p.m. This latter shift is consistent with the observations described above for the methyl signals in glycosaminoglycans that probably do not contain hydrogen-bonded acetamido groups, as compared with those that do.

These present results indicate that the acetamido group occurs in well-defined and discretely different environments in different glycosaminoglycans. They strongly suggest that some polymers have preferred conformations in solution. In general, the n.m.r. results support the conclusions drawn from periodate-oxidation studies, and extend into solution the findings of X-ray-crystallographic studies.

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References

- Atkins, E. D. T., Phelps, C. F. & Sheehan, J. K. (1972) *Biochem. J.* **128**, 1255–1263
- Fansson, L. A. & Roden, L. (1967) *J. Biol. Chem.* **242**, 4161–4169

- Fransson, L. A., Roden, L. & Spach, M. L. (1968) *Anal. Biochem.* **21**, 317-330
- Gatti, G., Casu, B., Torri, G. & Vercellotti, J. R. (1979) *Carbohydr. Res.* in the press
- Hinton, J. F. & Westerman, C. E. (1970) *Spectrochim. Acta* **26A**, 1387-1398
- Rees, D. J. (1975) IRS Biochemistry Series One, Vol. 5, Butterworths London pl-42
- Scott, J. E. (1979) *Chem. Br.* **15**, 13-18
- Scott, J. E. & Tigwell, M. J. (1978) *Biochem. J.* **173**, 103-114
- Winter, W. T., Smith, P. J. C. & Arnott, S. (1975) *J. Mol. Biol.* **99**, 219-235