Structural Study of Hyaluronic Acid Oligomers and Their Complexes with Copper in Water by NMR and IR and Molecular Dynamics Calculations

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SUMMARY: The hexasaccharide (trimer) and tetrasaccharide (dimer) oligomers of hyaluronic acid were investigated by ^{1}H and ^{13}C high resolution Nuclear Magnetic Resonance. The dynamic behavior of the molecules and their complexation with copper(II) were analysed by ^{1}H NMR relaxation studies. A specific site for the complexation of the tetrasaccharide with Cu^{2+} was demonstrated by analysis of the paramagnetic effect of the metal on non-selective proton relaxation rates. A model for the complex involving two molecules of the tetrasaccharide is proposed.

Introduction

Hyaluronan (HA) belongs to a family of extracellular polymers, the glycosaminoglycuronans, found throughout the animal kingdom. It is a high molecular weight linear polysaccharide with the repeating disaccharide structure composed of β-D-glucopyranosyluronic acid and 2-acetamido-2-deoxy-β-D-pyranosyl residues linked (1→3) and (1→4), respectively. Its structure is shown in Fig. 1[1].

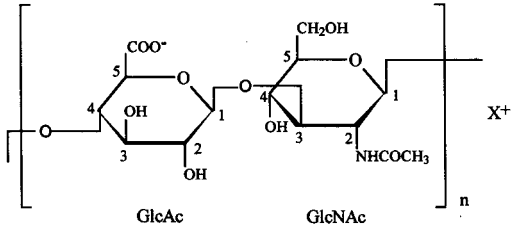

Fig.1: Repeating disaccharide units of hyaluronic acid: $[(1\rightarrow4)$-*O*-(β-D-glucopyranosyluronic)-$(1\rightarrow3)$-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)$]_n$ with X=Na, TBA.(GlcAc: β-D-glucuronate, GlcNAc: N-acetylglucosamine).

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Its biological functions include organization of cartilage proteoglycans into aggregates, providing a viscoelastic medium in the vitreous humur of the eye and synovial fluid, maintenance of tissue hydration, and control of cell activities such as adhesion, differentiation, migration and tissue morphogenesis by specific cell-surface receptor interactions[2].

Although HA has a simple disaccharide repeating unit, its conformation in aqueous solution is difficult to determine because of strong coupling and overlapping resonance. In this study we propose aqueous solution conformations of two oligosaccharides of HA obtained by experimental NMR measurements and molecular dynamics calculations. A model for Cu(II)-complex formation is also proposed.

Short HA oligosaccharides have been shown to retain the specific interactions and biological effects of high molecular weight HA polysaccharides[3].

Conformation and Dynamic Analysis

The 3D structure of hyaluronic acid tetra- (HAt) and hexa-saccharide (HAh) were investigated in water solution by high resolution ^{1}H and ^{13}C Nuclear Magnetic Resonance and FT-IR spectroscopy, combining experimental data and molecular dynamics calculations. The procedure and complete results are reported elsewhere[4]. HAh and HAt proton and carbon-13 assignment were performed using DQF-COSY, TOCSY, HMQC, and HETCOR spectra. These assignments are consistent with those published recently [5].

From two-dimensional experiments, NOESY and ROESY, the complete network of dipolar internuclear interactions was observed, and it was possible to define specific conformational constraints between nuclei within a radius of 5Å.

The dynamic behavior of the oligomers was studied in terms of ^{1}H and ^{13}C relaxation times. Because of low natural isotope abundance of ^{13}C, the internuclear relaxation vector ^{1}H-^{13}C can be regarded as isolated and it is possible to calculate accurate correlation time values using the following relation:

$$R_{1C} = \frac{1}{10}\frac{\gamma_H^2\gamma_C^2\hbar}{r^6}\left[\frac{3\tau_c}{1+\omega_C^2\tau_C^2} + \frac{\tau_c}{1+\left(\omega_H-\omega_C\right)^2\tau_c^2} + \frac{6\tau_c}{1+\left(\omega_H+\omega_C\right)^2\tau_c^2}\right] \qquad (1)$$

Table 1 shows the correlation time calculated for the carbons of the methyl groups that occur in two different parts of the dimer. Since the correlation time exhibits only slight differences isotropic motion can be assumed for the molecule with a lower degree of freedom for the

methyl on the GlcNAc3 residue, probably due to the hydrogen bond involving the acetyl group.

Tab.1. Correlation time of the methyl group of the dimer.

group	residue	[a] τ_c (10^{-10} s)	[a] τ_r (10^{-10} s)	[a] τ_j (10^{-10} s)
CH_3	GlcNAc-3	0.115	1.009	0.130
CH_3	GlcNAc-1[β]	0.096	1.009	0.106
CH_3	GlcNAc-1[α]	0.087	1.009	0.095

[a] τ_c, τ_r and τ_j are the actual correlation time, the overall reorientational correlation time and the internal rotation correlation time respectively, and are related by the formula $\tau_c^{-1} = \tau_r^{-1} + \tau_j^{-1}$

The molecular dynamics simulations confirmed these results[4]. The theoretical calculations were performed with the AMBER suite of programs, using Glycam-93 as the force field with explicit water as solvent [6].

The conformations of these short saccharides are characterized by an helicoidal shape with the sugar planes rotated approximately 60° degrees with respect to the adjacent sugar residue.

This structure is stabilized by a hydrogen-bond network linking residue; the complete set of four hydrogen bonds with the acetamido group located centrally, requires three sugar units (GlcAc1-3GlcNAc1-4GlcAc). This arrangement is compatible with inclusion of a water bridge between the donor (NH) and the acceptor (CO) groups [7].

Both the GlcAc and GlcNAc pyranosyl rings have been determined to be in the $^4C_1$ configuration, with no other detectable ring conformation [8].

FT-IR analysis confirms this proposal. The infrared spectrum of HA in aqueous solution shows the characteristic absorption bands of OH, NH e C=O groups. Their frequencies suggest the involvement in intra- and inter- H-bonding interactions.

Interaction with Copper(II)

A specific site for the complexation of the tetrasaccharide with Cu^{2+} was demonstrated by ^{1}H-NMR relaxation time analysis. Paramagnetic metal ions like copper(II) introduce an additional relaxation contribution to the observed relaxation rate of hyaluronate. As the paramagnetic dipolar term is a dominant contribution to the spin lattice relaxation rate, the relaxation parameters of nuclei situated at a short distance from the Cu(II) ion will be the most affected.

Table 2 shows the proton spin-lattice relaxation rates measured in the diamagnetic system (R_{1b}) and in the presence of the copper ion (R_{1exp}); R_{1p} is the paramagnetic contribution to the proton relaxation rate.

Tab. 2. Experimental proton spin-lattice relaxation rates of HAt in solution at 25°C in the absence (R_{1b}) and in the presence of 10-3 M Cu(II) paramagnetic metal ion (R_{1exp}).

nucleus	residue	δ (ppm)	R_{1b} (s^{-1})	R_{1exp} (s^{-1})	R_{1p} (s^{-1})
H1	GlcNAc-1[a]	4.9570	0.4209	0.5459	0.1250
H1	GlcNAc-1[b]	4.6230	0.6680	1.2011	0.5331
H1	GlcNAc-3	4.4700	0.8810	11.8371	10.9561
H1	GlcAc-2[a]	4.4230	0.8857	3.3311	2.4454
H1	GlcAc-4	4.3740	0.8969	5.0556	4.1587
H2	GlcNAc-1[a]	3.9460	0.6098	1.3280	0.7191
H2	GlcAc-4	3.2200	0.3520	4.2194	3.8674
Me	GlcNAc-3	1.9420	0.7320	4.7393	4.0073
Me	GlcNAc-1	1.9260	0.7230	1.6474	0.9244
H_2O		4.7000	0.0830	0.6100	0.5270

This data suggest that the H1 proton of the GlcNAc-3 residue appears the most affected nucleous. Selective paramagnetic effects are also observed for methyl protons of the acetyl group of the GlcNAc-3 residue. The observed selective paramagnetic effects define the ligand microenvironment around the Cu(II) ion and suggest bridging of Cu(II) ions between the carboxyl of iduronate and the amidic group of the glucosamine residue (Fig. 2).

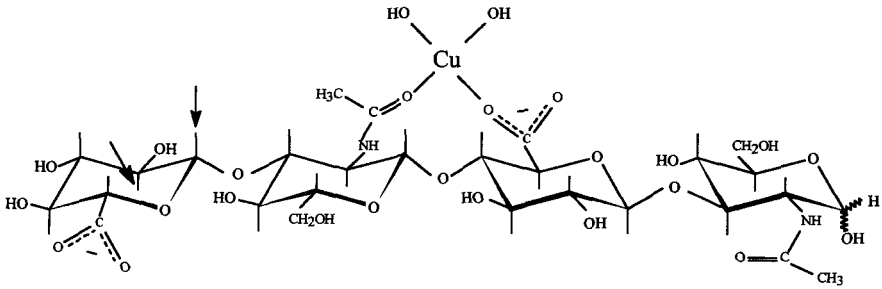

Fig. 2: Specific site for the complexation of Cu^{2+} with the tetrasaccharide. Arrows indicate the protons of the GlcAc-4 residue affected by strong paramagnetic relaxation.

In order to obtain more information about the nature of this complex, we carried out UV-VIS and IR analysis. UV-VIS spectra showed that this complex forms at pH=7.2. The IR spectra confirms the nature of the coordination site: we can deduce that one COO^- and one C=O group are involved in coordination of the paramagnetic ion.

Experimental relaxation rates also showed that the H1 and H2 protons of the GlcAc-4 residue are affected by the presence of the paramagnetic ion. This may be explained by the existence of intermolecular interactions which determine the formation of auto-associated structures. The proposed model is shown in Fig. 3.

For a two molecule model, we assume that the two chains are in an antiparallel arrangement. As stated before, the coordination site of the first chain is located between the GlcAc-2 and the GlcNAc-3 residues, and the site of the second, antiparallel chain is "shifted" between GlcNAc-3 and GlcAc-4, involving the carbonyl group of the latter residue.

This hypothesis could explain some of the biological properties of the copper(II) complexes of hyaluronic acid.

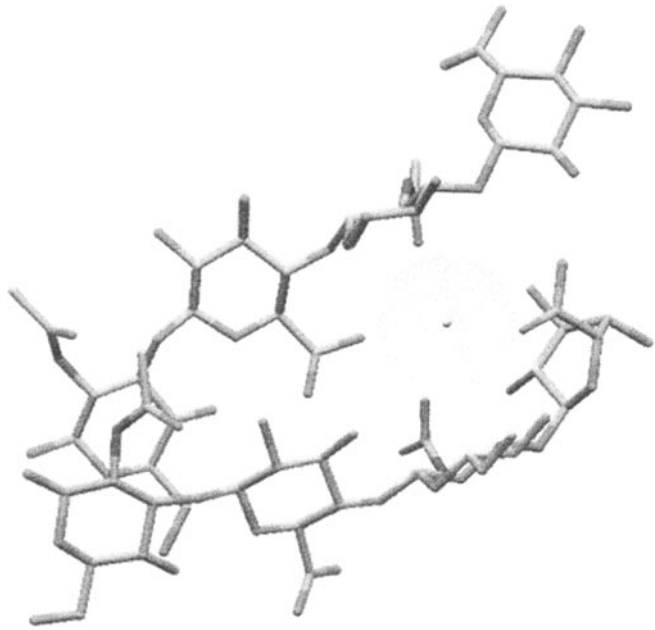

Fig. 3: Proposed model of the complex Cu(II)-HAt obtained by minimization of the potential energy with the Molecular Mechanics option of AMBER.

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