Carbohydrate Research, 145 (1985) 141–144
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

Note

Polarographic investigations of the binding of copper(II) by hyaluronic acid*

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(Received May 22nd, 1985; accepted for publication, July 2nd, 1985)

Hyaluronic acid is a major component of connective tissue and body fluids in vertebrates. This glycosaminoglycan, which consists of repeating dimeric units of 2-acetamido-2-deoxy-D-glucose and D-glucuronic acid, behaves in solution as a typical polyelectrolyte and its properties are sensitive to pH, ionic strength, and the type of counter-ions. Several structures for this biopolymer have been described and, under certain conditions, viscoelastic putties or gels can be prepared from solutions of hyaluronic acid. We have reported[1] on a new type of hyaluronic acid gel prepared from a mixture of hyaluronic acid and Cu^{2+}. We now report on the use of polarography and amperometric titration to investigate the complexing of Cu^{2+} with native hyaluronic acid of high molecular weight, a hyaluronidase-degraded species of low molecular weight, and a partially methylated derivative. The stoichiometries of these complexes give further insight into the binding sites of this glycosaminoglycan.

The polarographic measurements of the Cu^{2+}–hyaluronate system were based on the difference between the diffusion coefficients of Cu^{2+} when complexed by water or phosphate groups and when bound to the functional groups of the hyaluronic acid. The reduction of Cu^{2+} to Cu^{0} at the polarographic electrode is shown as a peak in the potential–current diagram measured in the differential pulse polarography (d.p.p.) mode, and the peak height refers to the diffusion-limited current. As diffusion to the electrode is not dependent only on the concentration of the oxidised species, the shape of the metal ion–polymeric system plays an important role in the amount of measured current. The numerical values for the binding constants cannot be derived when only the concentrations are known and not the particular diffusion constants. The classical method of determining the binding constant, in which the shift in half-wave potential is related to the concentration of ligand, is not suitable for the hyaluronate system, because the values of the poten-

*Dedicated to Professor Dr J. Schurz on the occasion of his 60th birthday.

0008-6215/85/$ 03.30

tials for the Cu^{2+} buffer solution alone are of the same order of magnitude. When adding the stock solution of higher concentration of hyaluronic acid or its methylated derivative to the copper-ion solution, a time-dependent reaction is observed, where the diffusion-limited current fluctuates during several periods. A steady-state equilibrium is reached within about 60 min. This time-dependency is being studied further, but is discussed here qualitatively. The interchain linkage of the hyaluronic acid depends strongly on the concentration of the solution. The strong hydrogen bond between the acetamido and carboxylate group, from unit to unit on alternating sides of the chain[2,3], results in a very stiff chain that appears in solution as a random coil or, at a higher order, as helical structures[3,4]. When such a high-molecular-linked system is diluted and added to Cu^{2+}, loss of hydrogen bonding between different strands, unwinding, *etc.*, occur.

At the molecular level, Cu^{2+} has a high affinity for the nitrogen of 2-acetamido-2-deoxy-D-glucose, which results in the release of a proton. The increased acidity of hyaluronic acid in the presence of Cu^{2+} was found by titrations[5]. This effect is similar to that observed in Cu^{2+}–protein complexes where a similar release of protons takes place[6]. Thus, the addition of solutions of hyaluronic acid in a highly linked structure to Cu^{2+} leads to several changes in conformation, with different kinetic parameters, which results in a time-dependent current during polarography caused by changes in the shape and therefore in the diffusion coefficient.

Repeated polarography with increasing concentrations of ligand leads to amperometric titration curves, where Cu^{2+} is the reducible and hyaluronic acid the non-reducible species. These curves for the three different species of hyaluronic acid are illustrated in Fig. 1. Each curve exhibits a distinct region for increasing ratios of copper to glycosaminoglycan disaccharide unit, and shows well-separated steps in complex formation when certain stoichiometries are reached. Regions with potential-like curves rather than amperometric lines suggest drastic changes in the arrangement of the complexes initially formed.

The enzymically depolymerised sodium hyaluronate, which is a mixture of segments of different length, forms a strong complex when the ratio of Cu^{2+} to disaccharide unit is 1:1. This result can be explained by coordination of Cu^{2+} at the acetamido nitrogen, resulting in substitution of the proton. This attack may be facilitated by the fact that the 2-acetamido-2-deoxy-D-glucose residue is significantly less hydrated than the glucuronate[7]. The carboxylate group may act as the second ligand on the same side. The addition of more hyaluronate leads to a 1:2 complex, which suggests an arrangement of two nitrogens around one Cu^{2+}, originating from two different strands. This suggestion is supported by the weak decrease of diffusion-limited current in this region, as two strongly bound strands may not differ significantly in the diffusion coefficient from one isolated strand. The region behind the 1:2 ratio may be attributed to the influence of the fraction having a considerably longer chain-length.

The formation of copper complexes with intact hyaluronate or its partially

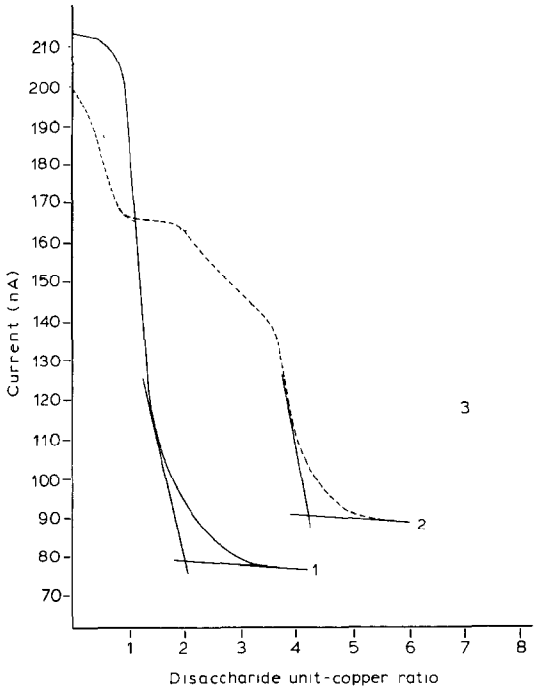

Fig. 1. Amperometric titration of Cu^{2+} with degraded (1), native (2), and partially methylated (3) hyaluronic acid.

methylated derivative is characterised by the first sharp decrease in current, reached at a 1:1 metal–hyaluronate ratio; on the addition of more ligand, there were no changes up to a ratio of 1:2, demonstrating that the complex formed initially must be stereochemically favoured. Intact hyaluronate formed a new type of complex which is completed at a 1:4 ratio. The most striking feature of the amperometric curve is the part just before the 1:4 ratio is reached, where there is a sharp decrease in current. This finding might be attributed to a positive co-operative effect where the binding of the remaining 10% of Cu^{2+} is facilitated by a drastic rearrangement of the 1:1 complex. The decrease of current for the partially methylated hyaluronate parallels the percentage of unmethylated carboxyl groups.

Higher concentrations of the copper hyaluronate complex lead to viscoelastic gels, and the dominant role of Cu^{2+} is the suppression of the electrostatic repulsion of different strands resulting in the formation of a network structure. Such behaviour is similar to the familiar putties and gels of hyaluronate, which are formed under certain conditions by strong intermolecular hydrogen-bridges. With Cu^{2+}, there is much stronger bonding to the functional groups and therefore the forced geometry leads to viscoelastic properties. The alteration of acetamido and carboxyl groups relative to their respective sugar rings favours junctions between different strands, resulting in a highly regular packing scheme.

Since metal ion–hyaluronate complexes may play a role in rheumatoid arthritis[8], the binding of copper to hyaluronic acid could be of physiological importance.

EXPERIMENTAL

Hyaluronic acid with a mol. wt. of 200,000–250,000 and a protein content of <2% was prepared[9] from the vitreous body of bovine eyes. Oligosaccharides with an average mol. wt. of 5,000 ±400 were isolated by partially digesting hyaluronic acid with hyaluronidase[10].

After the hyaluronate was converted into the free acid form by passage through a column of Dowex 50W-X8 (H^+) resin (50–100 mesh), the methyl ester was prepared by incubation with diazomethane[11]. The degree of methylation was ~60%, as shown by quantitative n.m.r. spectroscopy.

Polarography. — A Princeton Applied Research polarographic analyser Model 384, with an electrode 303, was used in the differential pulse mode with a static dropping-mercury electrode; drop time, 1 s; scan increment, 2 mV; pulse height, 10 mV. Potentials were measured against an AgCl electrode. A solution (50 μL) of hyaluronate containing 2×10^{-2}M of disaccharide units was added to a solution (20 mL) of 10^{-4}M Cu^{2+} in the polarographic cell so that the added volume was not taken into account. The supporting electrolyte was a 1:5-diluted, standard phosphate buffer pH 7. Mixing was performed by purging the cell with nitrogen.

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