Hydrogen-bonded conformation of hyaluronate oligosaccharide fragments in aqueous solution

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The hydrogen bonding in hyaluronate oligosaccharide fragments was studied in aqueous solution using hydrogen–tritium exchange techniques. The data reveal an acetamido hydrogen exchange rate that is 5–6-fold slower than that seen in model compounds. The magnitude of the slowing is interpreted as reflecting the participation of an acetamido hydrogen in a relatively labile intramolecular hydrogen bond.

Hydrogen exchange *Carbohydrate conformation* *Conformational equilibria*

1. INTRODUCTION

Hyaluronic acid is a linear glycosaminoglycan consisting of alternating residues of glucuronic acid and *N*-acetyl-β-D-glucosamine. Its solution conformation appears to exhibit several interesting properties not expected of random coils. One of these properties, an unusual degree of chain stiffness, has been characterized by rheological studies of hyaluronate solutions [1] as well as by carbon-13 [2] and proton [3] nuclear magnetic resonance studies. Another property is the low reactivity of the uronic acid C-2–C-3 glycol group towards periodate oxidation [4]. An intrachain hydrogen-bonding network has been proposed as a possible explanation for both of these properties. One of the proposed hydrogen bonds composed of an acetamido donor and carboxylate acceptor, bridges the β1–4 glycosidic linkage joining the reducing end of each *N*-acetylglucosamine residue with the adjacent glucuronic acid residue. Proton nuclear magnetic resonance studies of the acetamido methyl protons [5] and circular dichroism studies [6] have provided indirect evidence for this interaction. Here, we present direct evidence, based on the slowing of its exchange with solvent hydrogens, for the participation of the acetamido hydrogen in a hydrogen bond. The magnitude of the slowing is interpreted in terms of the stability of the hydrogen bond.

2. MATERIALS AND METHODS

Powdered chitin (practical grade) and testicular hyaluronidase (type IV) were purchased from Sigma. Hyaluronic acid (K^+ salt) was obtained from Miles.

The preparation of the β1–4-linked trimer of *N*-acetylglucosamine (triNAG) was detailed in [7]. The method used for the preparation of the hyaluronic acid tetrasaccharide and hexasaccharide fragments was based on procedures in [6,8–10]: 100 mg purified hyaluronic acid were dissolved in 8 ml digestion buffer (0.1 M sodium acetate, 0.15 M NaCl; pH 5.0) to which was added 0.25 ml hyaluronidase (10 mg/ml in the same buffer) and three drops of toluene. After 16 h at 37°C an additional 100 l hyaluronidase solution was added and the digestion was continued for a total of 42.5 h. Digestion was terminated by heating the sample in a boiling water bath for 5 min and a small amount of insoluble material was removed by low-speed centrifugation. The sample was then applied to a Sephadex G-25 (Fine) column (2.5 ×

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82 cm) which was eluted with 0.1 M ammonium acetate in 20% ethanol at a rate of 25 ml/h. Aliquots from each fraction were assayed for reducing groups by the Park-Johnson method [11]. Fractions from the two major peaks (i.e., those containing the tetrasaccharide and hexasaccharide) were pooled separately and lyophilized. The purified fragments were stored dry at −20°C until used. Hyaluronic acid octasaccharide fragments were prepared in a similar manner except that 300 mg hyaluronic acid were digested for 19 h to obtain increased quantities of the larger oligosaccharides. The resolution of the fragments upon gel filtration was somewhat poorer than that obtained in the previous preparation. Therefore the fractions containing the octasaccharide were pooled and rechromatographed on the same column, using 0.2 M NaCl rather than 0.1 M ammonium acetate, at 10–15 ml/h. The octasaccharide peak was well-separated from contaminating species and the appropriate fractions were pooled, lyophilized, and then desalted on a small Sephadex G-10 column.

Hydrogen–tritium exchange experiments using the Sephadex column method [12] were carried out as in [7]. All exchange experiments were performed at 0°C and pH 5.50. Hyaluronate fragment exchange-in mixtures were prepared at 6–7 mg/ml while the mixture for triNAG was prepared at 5 mg/ml. Exchange buffer consisted of 0.01 M sodium acetate, 0.1 M NaCl, 5 mM $CaCl_2$, 3.5 mM KCl, 0.5 mM $MgCl_2$ (pH 5.50). The number of hydrogens (H)/molecule (M) remaining after the desired exchange-out times was calculated according to:

$$H/M = [(111\epsilon/C_o)(C/A)]/1.19$$

where:

C_o = the number of counts in the exchange-in mixture;

A/ϵ and C = the molar concentration of oligosaccharide and counts, respectively;

111 = the gram–atom concentration of hydrogen in water.

The factor of 1.19 corrects for the tritium–hydrogen equilibrium isotope effect for secondary amides [13]. ϵ_{220}-Values for the hyaluronate fragments were determined by summing the contributions of the constituent chromophores (carboxylate, 103 M^{-1}; internal acetamido, 88 M^{-1}, terminal acetamido, 171 M^{-1}) yielding 465 M^{-1}, 656 M^{-1}, and 847 M^{-1} for the tetrasaccharide, the hexasaccharide and the octasaccharide, respectively.

3. RESULTS AND DISCUSSION

3.1. *Oligosaccharide preparations*

The preparation of the β1–4 linked trimer of N-acetyl-D-glucosamine (triNAG) was described previously. The product was homogeneous by paper chromatography. A limit digestion of hyaluronic acid (HA) by testicular hyaluronidase gives the gel filtration profile shown in fig.1. This profile is virtually identical to that in [10]. The products of testicular hyaluronidase digestion of HA are an homologous series of oligosaccharides containing integrals of the disaccharide repeating unit glucuronic acid-β1–3-*N*-acetyl-D-glucosamine [6]. Digestion leaves a free reducing group on the terminal amino sugar while oligosaccharides larger than the disaccharide contain the β1–4 glycosidic linkage joining non-terminal *N*-acetylglucosamine (GlcNAc) reducing ends to glucuronic acid (GlcUA) residues. As expected for the extensive digestion employed in the present studies, the ma-

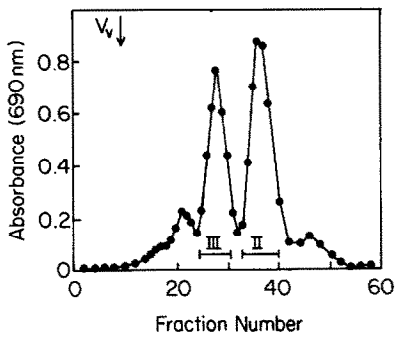

Fig.1. Separation of hyaluronate oligosaccharide fragments by gel filtration. The digestion mixture of 7 ml was added to a Sephadex G-25 (fine) column (2.5 × 82 cm) which was eluted with 0.1 M ammonium acetate in 20% ethanol at 25 ml/h. Aliquots of 15 μl were removed from each 4.25 ml fraction and assayed for reducing groups by the Park-Johnson method. V_v marks the column void volume and the bars indicate the fractions pooled to obtain the tetrasaccharide (II, 33–40) and hexasaccharide (III, 25–31).

jor product was the tetrasaccharide (II). Appreciable amounts of the hexasaccharide (III) were also obtained, and the indicated fractions were pooled to give the isolated tetrasaccharide and hexasaccharide fragments. A less-extensive digestion of HA was performed as in section 2 to preserve more of the octasaccharide. The resulting gel filtration profile was very similar to that in fig.1 except that the peak eluting immediately before peak III was much increased and the overall resolution was somewhat decreased. Fractions from this larger peak (IV, octasaccharide) were pooled and rechromatographed in higher salt resulting in the improved isolation of the octasaccharide fragments from contaminating oligosaccharide species.

3.2. *Hydrogen exchange*

The hydrogen–tritium exchange data from the various oligosaccharides are shown in fig.2. Among the exchangeable hydrogens present on these molecules (acetamido and hydroxyl hydrogens), one expects only the acetamido hydrogens to be measurably slow. At pH 5.50 and 0°C the hydroxyl hydrogens exchange in <1 s, too fast to be measured by the tritium–Sephadex method. Therefore, the number of exchangeable hydrogens measured for each molecule matches the number of acetamido groups present on that molecule. It is clear from the straight lines drawn on the semilogarithmic plot (fig.2) and the computer curvefitting parameters listed in table 1 that the hydrogen exchange rates of the HA fragments are consistently 4–6-fold slower than the triNAG-acetamido exchange rates.

A detailed discussion of hydrogen exchange rates can be found in [14–16]. For the experiments described here all conditions were held constant in the exchange runs so that differences in observed rates must be explained on the basis of intrinsic structural differences between the molecules. We feel the best explanation for the slowing of the HA exchange rates involves the participation of the acetamido hydrogens in hydrogen bonds.

The fastest exchange rate for a particular hydrogen under given conditions of temperature and catalyst concentration is exhibited when that hydrogen and the active catalyst species can come into free contact with each other. This free, or exposed, chemical exchange rate is a characteristic of

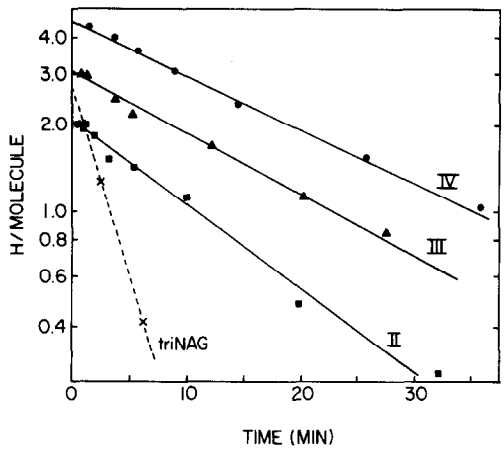

Fig.2. Hydrogen–tritium exchange on hyaluronate fragments and triNAG at 0°C and pH 5.50: II, tetrasaccharide; III, hexasaccharide; IV, octasaccharide; triNAG, β1–4-linked trimer of *N*-acetylglucosamine. The straight lines are drawn for clarity – the calculated exchange rates are listed in table 1.

hydrogens surrounded by a given primary structure and forms the standard with which to compare the rates observed for these hydrogens under conditions where they are suspected of being involved in some higher-order conformation. We take the rate observed for the acetamido hydrogens of triNAG as this exposed rate for the HA fragment acetamido hydrogens. The basis for this assumption rests on [7] in which we found that the exchange rates for triNAG and the monosaccharides GlcNAc and *N*-acetyl-neuraminic acid were all very similar. These molecules contain the acetamido group in a similar primary chemical structure. Furthermore, several of the amino sugar acetamido hydrogens on the complex N-linked fetuin glycopeptide also exhibited similar exchange rates.

Although the β1–4 linkage joining GlcNAc residues in triNAG has no measurable effect on the rate of acetamido hydrogen exchange (since triNAG and the monosaccharides display similar rates), an effect of the linkage in HA remains possible. In this case the GlcNAc residue is substituted with the *O*-glycosidic bond at the 3-position rather than at the 4-position as in triNAG. This change is 3 atoms removed from the amide hydrogen and replaces an hydroxyl group with an ether oxygen. The resulting relative de-

Table 1

Summary of acetamido–hydrogen exchange results for hyaluronate fragments and triNAG

Oligosaccharide fragment	Acetamido hydrogen exchange rate	Acetamido hydrogens/molecule	
		Calculated	Expected
HA tetrasaccharide	0.077	2.0	2
HA hexasaccharide	0.062	2.8	3
HA octasaccharide	0.053	4.3	4
triNAG[a]	0.3	3	3

[a] Exchange parameters for triNAG were estimated graphically. The result obtained here (0.3 min^{-1}) is very close to that measured more accurately (0.37 min^{-1}) in [7]

Exchange rates, given in units of $(min)^{-1}$, were measured at pH 5.50 and 0°C (HA, hyaluronic acid). The data points in fig.2 were computer-fit to a single exponential decay resulting in the rates and hydrogens/molecule calculated as listed. For the HA fragments the data were fit best above a 4.4% background (tetrasaccharide), a 10% background (hexasaccharide), and a 9% background (octasaccharide)

crease in the electron withdrawing inductive effect might decrease the acidity of the exchangeable hydrogen and thus slow its base-catalyzed exchange. (The exchange of secondary amide hydrogens is base catalyzed at pH 5.50.) We expect the magnitude of this effect, however, to be much less than the 4–6-fold slowing observed in these exchange measurements. This is because the similar but much larger decrease in electron-withdrawing inductive effect arising from the change from a serine to an alanine sidechain to the right of the amide hydrogen of the peptide group in a polypeptide slows the exchange rate only 3-fold [17]. In this example, the change is also 3 atoms removed from the amide hydrogen, but it replaces an hydroxyl group with a methyl group. The contribution of linkage effects in the slowing of exchange rates in HA, when compared with triNAG, must be much less than that in this example. Therefore triNAG appears to be an appropriate model for the free chemical exchange rate of the HA acetamido hydrogens, and some conformational feature is very likely responsible for the observed slowing.

The glucosaminidic linkage (GlcNAcβ1–4 GlcUA) of an hyaluronate oligosaccharide model can be arranged to allow the formation of an essentially linear hydrogen bond involving the GlcNAc acetamido group as the donor and the GlcUA carboxylate as the acceptor. No other interactions are apparent for the acetamido groups in the small oligosaccharide fragments. Thus the hydrogen exchange data most likely reflect the participation of the acetamido hydrogen in this hydrogen bond. This hydrogen is available for exchange only at times when the hydrogen bond is broken – resulting in the observed 4–6-fold slowing of the exchange rates.

Although each HA oligosaccharide contains 1 acetamido group/disaccharide unit, the terminal reducing GlcNAc acetamido is unable to participate in such an interaction since there is no adjacent GlcUA residue. Therefore only 1, 2 and 3 such hydrogen bonds are possible in the tetrasaccharide, hexasaccharide and octasaccharide, respectively. Yet each exchange curve appears to reveal only a single kinetic class of hydrogens. This apparent inconsistency can be resolved by proposing that the two forms of the terminal reducing GlcNAc do not exhibit equivalent acetamido hydrogen exchange rates. One of these anomeric forms (probably the more stable one and hence the more populated one) likely contains a hydrogen-bonded interaction between the anomeric hydroxyl and the C-2 acetamido group [18]. Our exchange experiments on the GlcNAc monosaccharide [7] which revealed two kinetic classes differing in rate by 4–5-fold support this conclusion. An interaction of this sort is also supported by the observation of distinct chemical shifts for the acetamido methyl protons of the two reducing GlcNAc anomeric forms in these HA oligosaccharides [5]. The magnitude of this effect on the hydrogen exchange rates (4–5-fold) is similar to the degree of slowing observed as a result of the acetamido–carboxylate hydrogen bond across the glucosaminidic linkage. These rates would be indistinguishable. The contribution of the faster exchange rate from the other anomeric form would be expected to be $< \frac{1}{2}$ of one hydrogen/molecule since this less-stable form should comprise $< \frac{1}{2}$ of the equilibrium anomeric mixture. This means that

perhaps < ⅓ of one hydrogen/molecule exchanges 4–5-fold faster than the remaining protons. The data shown in fig.2, would not reveal the presence of such a small, faster kinetic class. This explanation is supported further by our observation that the apparent first-order rates become progressively slower as the fragments increase in size – an expected result since the contribution of the faster class becomes a progressively decreasing fraction of the total.

The acetamido–carboxylate hydrogen bond has been proposed by others as at least part of the explanation for several aspects of the solution behavior of HA (see section 1). This hydrogen bond has been demonstrated in several solid state conformations suggested by fibre diffraction studies [1]. The present demonstration of the slowed HA acetamido hydrogen exchange rate is strong evidence for this hydrogen bond in solution and also provides an estimate of its stability. The 5–6-fold slowing corresponds to close to 1 kcal/mol of free energy (the equilibrium constant for H-bond breakage is about 1.15) and indicates that any particular hydrogen bond is present about 5/6 of the time. This relatively small equilibrium constant implies a fairly labile preferred conformation and is in accord with the 'bond making-and-breaking' character of the hydrogen-bonded network proposed in [1]. Thus the data support a model in which, at any point in time, the rotation about one of every 5 or 6 glucosaminidic bonds of an hyaluronate chain is relatively unrestricted by the acetamido–carboxylate interaction.

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