^{13}C NMR Studies of Hyaluronan. 2. Dependence of Conformational Dynamics on Chain Length and Solvent†

Mary K. Cowman,* Joan Feder-Davis, and Daniel M. Hittner

Department of Chemistry and Herman F. Mark Polymer Research Institute, Polytechnic University, Six Metrotech Center, Brooklyn, New York 11201

Received June 22, 2000; Revised Manuscript Received September 27, 2000

ABSTRACT: The conformational dynamics of low molecular weight hyaluronan (HA) polymer and oligosaccharides have been assayed by ^{13}C NMR T_1 relaxation time measurements. For polymeric HA in aqueous solution, the average relaxation time for ring carbons was not significantly affected by changing the ionic strength or by changing counterion type. The average relaxation time of HA was found to be similar to that for chondroitin 4-sulfate or chondroitin 6-sulfate in aqueous solution. As has been observed previously for other polysaccharides, the segmental motions of HA chains studied at room temperature in aqueous solution appear to be dominated by viscous damping of the chain motions. Within a chain structure, there are variations in observed T_1 that correspond to differences in relative mobility. The hydroxymethyl substituent group shows a difference in relaxation rate relative to the ring to which it is attached, depending on the ring configuration (glucose vs galactose). These differences correlate with relative rotational isomerization rates measured by ultrasonic relaxation. In short HA chains, the nonreducing and reducing terminal residues show much greater mobility than penultimate residues, and these are in turn more mobile than interior residues. In contrast, the more rigid hydrogen-bonded conformation of an HA tetrasaccharide in dimethyl sulfoxide solution shows less position-dependent variation in T_1. These data are in accord with the dynamic nature of conformation-stabilizing hydrogen bonds for HA chains in aqueous solution.

Introduction

Hyaluronan (HA) is a linear polysaccharide with the repeating disaccharide structure[1] poly[(1→3)-β-D-GlcNAc−(1→4)-β-D-GlcA]. It has widespread occurrence in the extracellular matrix of animal tissues. It is a major contributor to the biomechanical properties of tissues, helps control tissue hydration and water transport, and participates in a number of physiologically important processes via interactions with extracellular and cell-surface proteins.[2] The rheological properties of HA solutions are of special interest with respect to the functions of liquid connective tissues such as the eye vitreus and joint synovial fluid[3,4] and have been exploited for the development of viscoelastic products for biomedical applications.[5] Thus, it is important to understand those aspects of HA structure which determine its contribution to the physical properties of its solutions.

Three characteristics of HA play major roles in determining its hydrodynamic properties in neutral physiological salt solution. The first of these is its naturally high molecular weight, which averages about 6 million in the normal vitreus or synovial fluid.[6] Statistical models for long polymer chains in dilute solution show that the molecular volume will be extremely large, with a correspondingly large volume of solvent contained within the polymeric domain. For HA, the hydrodynamic volume of the polymer coil increases as the molecular weight raised to the 1.8 power. The second important characteristic is the large size of the smallest independent unit of motion (conformer), which is the sugar ring. This limits the inherent flexibility of the chain. Finally, HA behaves hydrodynamically as if it has some slight additional stiffening influence. The plausible sources of such stiffening include steric restrictions to rotation about the glycosidic linkages between sugars,[7] dynamic formation and breakup of hydrogen bonds across one or both glycosidic linkages,[8−14] and intra- or intermolecular self-association.[15,16] It is possible that all of the above contribute to the chain expansion to some extent. The resulting structure of HA in dilute aqueous solution, as detected by its hydrodynamic properties, is well described as a slightly stiffened (wormlike) random coil.[17−20] Such a description represents an ensemble average of instantaneous conformations for many molecules or, equivalently, a time average of accessible conformations for a given molecule. We have undertaken the present study to shed light on the relative time frame of motions at various positions in short and long HA chains, in several solvent conditions affecting hydrogen bonding and/or self-association.

Our experimental approach to the investigation of HA mobility is measurement of the NMR relaxation times of the carbon (^{13}C) atoms in the backbone. For protonated carbons, the spin−lattice relaxation time, T_1, depends predominantly on dipolar interaction between the carbon and its attached hydrogens. That interaction depends on the motional dynamics of the molecule. Only that fraction of motions occurring at the ^{13}C Larmor frequency and its linear combinations with the ^{1}H Larmor frequency contribute to relaxation. For polysaccharides studied by high field NMR, T_1 values are thus strongly related to the fraction of local motions on the nanosecond time scale, corresponding to segmental motions of a few sugars in the chain, and by other hindered motions, such as hydroxymethyl group rota-

* To whom correspondence should be addressed. E-mail: mcowman@duke.poly.edu.

† Abbreviations: HA, hyaluronan; Ch 4-S, chondroitin 4-sulfate; Ch 6-S, chondroitin 6-sulfate; GlcA, D-glucuronic acid; GlcNAc, 2-acetamido-2-deoxy-D-glucose; GalNAc, 2-acetamido-2-deoxy-D-galactose; HexNAc, GlcNAc or GalNAc; NMR, nuclear magnetic resonance; DMSO, dimethyl sulfoxide.

10.1021/ma001082e CCC: $20.00
Published on Web 11/30/2000

tional isomerization.[21−23] Thus, T_1 is intrinsically a measure of local stiffness, such as might be conferred by hydrogen bonding.

Because our experiments cover only one field strength and are obtained at a single temperature, we are unable to subject our T_1 data to quantitative analysis to obtain distributions of correlation times. Such an approach is beyond the scope of the present investigation, but is addressed by the work of Cavalieri et al.[24] in this issue. Instead, we will use the relaxation data as an indication of relative motional frequencies of components within a given structure, as a function of structural and environmental variations.

Materials and Methods

Hyaluronan (HA) from human umbilical cord was obtained from Sigma Chemical Co. It was purified[25] to remove contaminating chondroitin sulfates by passage through a column of DEAE−Sephacel in 0.3 M NaCl. A low-polydispersity, low molecular weight HA polymer sample was prepared by enzymatic digestion with bovine testicular hyaluronidase (hyaluronate 4-glycanohydrolase, E.C. 3.2.1.35, specific activity 23 613 units/mg, from Cooper Biomedical) and chromatographically fractionated as described elsewhere.[25] The weight-average molecular weight was determined by polyacrylamide gel electrophoresis[26] and determined to be 1.6×10^4, corresponding to an average chain length of 40 disaccharides. Monodisperse HA oligosaccharides containing two, three, four, or seven disaccharide repeats were prepared by testicular hyaluronidase digestion of the purified umbilical cord HA, followed by chromatographic fractionation as described earlier.[10]

Chondroitin 4-sulfate (Ch 4-S) and chondroitin 6-sulfate (Ch 6-S) were obtained from Seikagaku Kogyo Co., Ltd., and used without further purification.

For NMR studies, samples were dissolved at a concentration of 4−15 mg/mL and studied in 5 mm NMR tubes. Unless otherwise noted, the solvent was 4/1 H_2O/D_2O buffered with 20 mM sodium phosphate, pH 7, containing 0.15 M NaCl. Other aqueous solvents were as above but with 0.15 M NaCl replaced with 0.15 M KCl, 0.05 M $CaCl_2$, or pure H_2O. For HA tetrasaccharide, DMSO-d_6 was used as a comparison solvent.

NMR spectra were collected at 22 °C on a General Electric GN-300 wide bore spectrometer operating at 75.57 MHz for ^{13}C, as previously described.[12,13] Pulsed broad-band proton decoupling was used. Observed resonances were assigned according to the schemes reported previously for HA oligosaccharides in water or DMSO.[13] For T_1 relaxation time measurements, the fast inversion recovery technique[27] was employed, with 8−10 delay times and a sequence delay of at least three times the maximum T_1. Approximately 30 000 scans were collected for each delay time, in 15 interleaved sets of 2000 scans for each of the delay time values. The GE software was used to extract the relaxation times using a three-parameter fit to the recovery kinetics. The standard deviation in T_1 reported by the GE software for any given curve was typically 2−8% of the T_1 value. Averages of relaxation times for carbons within the sugar rings are reported here as mean ± standard deviation.

Results and Discussion

Comparison of HA with Other Polysaccharides: Ring Carbon T_1 Averages. A preparation of enzymatically degraded HA with low polydispersity and a weight-average molecular weight of 1.6×10^4 (containing 40 disaccharides) was dissolved and studied in a buffered aqueous solution of 0.15 M NaCl at 22 °C. The T_1 relaxation times for all protonated carbons in HA were measured at 75 MHz. Individual resonance assignments were given previously.[12,13] The carbons of a given sugar ring show similar T_1 values, but there were greater uncertainties in the values for C5 than for other ring carbons, so that the relaxation behavior of the ring was best described by an average of the values for carbons 1−4. Table 1 shows the average T_1s for the GlcA residues and GlcNAc residues to be closely similar to each other, with average relaxation times of 230 and 220 ms, respectively. This result is in accord with the value of approximately 140 ms measured at 50 MHz by Hofmann et al.[28] for a lower molecular weight (5×10^3) HA. To investigate the possibility of subtle differences in the mobility of the two different linkages in HA, the relaxation of carbons directly participating in the glycosidic linkages were examined. Both carbons of the 1→4 linkage have T_1s of 210 ms; the C1 and C3 of the 1→3 linkage have T_1s of 230 and 220 ms, respectively. The differences are not great enough to be significant.

Table 1. Comparison of ^{13}C NMR Spin−Lattice Relaxation Times (NT_1) for HA and Chondroitin Sulfates

		NT_1 (ms)		
sample	salt added	glcA[a]	hexNAc[a,b]	hexNAc[b]C6
HA ($n = 40$)	0.15 M NaCl	230 ± 20	220 ± 10	220
	none	230 ± 20	240 ± 10	250
	0.15 M KCl	240 ± 10	250 ± 20	250
	0.05 M $CaCl_2$	240 ± 10	230 ± 10	240
Ch 4-S	0.15 M NaCl	240 ± 10	230 ± 20[c]	370 ± 20
Ch 6-S	0.15 M NaCl	240 ± 20	240 ± 10[d]	

[a] Average of T_1 values (mean ± standard deviation) for carbons 1−4 of ring, except where noted. Measured at 75 MHz in 4/1 H_2O/D_2O containing 0.02 M sodium phosphate, pH 7, containing salts as indicated. [b] HexNAc = GlcNAc in HA; HexNAc = GalNAc in Ch 4-S and Ch 6-S. [c] C1, C2, C3 only. [d] C2, C3 only

HA was also examined in aqueous solutions of different ionic content (Table 1). The relaxation times were not significantly different in either KCl or $CaCl_2$ solution, at the same ionic strength as the NaCl solution, and in salt-free solution. This result is also in agreement with previous studies on HA of very low molecular weight.[28] Because the expansion of the polyelectrolyte chain is sensitive to the ionic strength,[29−31] and the extent of dynamic self-association is sensitive to the ionic strength and counterion type,[15,16,32] it appears that the T_1 relaxation time does not provide information on those processes.

The relaxation times for carbons of the sulfated glycosaminoglycans chondroitin 4-sulfate (Ch4-S) and chondroitin 6-sulfate (Ch6-S) were similarly studied in physiological NaCl solution. The ring carbon T_1 averages were not significantly different in these polysaccharides from that for the HA sample (Table 1). Published reports for other polysaccharides studied at temperatures below about 35 °C, including heparin epoxide,[33] amylose,[34] dextran,[34] glycogen,[35] pullulan,[36] and cellulose triacetate[37] show remarkable similarity, after consideration of field strength differences. It has been suggested that the inability to quantitatively correlate differences in glycosidic linkage conformational freedom with differences in T_1 is a function of the domination by viscous damping effects of the solvent on segmental motions of the polysaccharide.[21,34] In studies performed at higher temperatures, the increased motional freedom of the polymer allows greater distinction between polymers according to their inherent flexibility.

Comparison of HA with Other Polysaccharides: Hydroxymethyl Group. The NT_1 for the carbon of the exocyclic hydroxymethyl group (220 ms) on the GlcNAc residue of HA in 0.15 m NaCl is closely similar to the average T_1 for the ring carbons (220 ms)

(Table 1). The same observation holds for HA in several other salt conditions (Table 1). In other polysaccharides containing glucose or glucose derivatives, the hydroxymethyl carbon also has the same or a slightly larger NT_1 than the ring to which it is attached.[33,34,36,38−40] Monomeric glucose and oligosaccharides containing glucose show the same effect.[41,42] In contrast, galactose and galactose-containing oligosaccharides and polysaccharides exhibit significantly longer relaxation times for the hydroxymethyl carbon than the ring carbons.[41−43] We have further examined this relationship for the galactosamine residues of Ch 4-S and find the hydroxymethyl group carbon to have an NT_1 of 370 ms, which is significantly longer than the average for the ring carbons (230 ms) (Table 1). The same comparison could not be made for Ch 6-S, because of overlap of the GalNAc C6 resonance with the GalNAc C4 resonance and the effect of sulfation on the group in question.

The difference in relaxation behavior of the hydroxymethyl carbon in glucose vs galactose has been suggested[41] to reflect formation of an intramolecular hydrogen bond to the ring oxygen, via a water bridge, in glucose. Thus, its motions would be tied to the ring motion. This proposal has not been supported by experimental evidence for that hydrogen bond in aqueous solution.

A much better case can be made that the rotational isomerism of the hydroxymethyl group, which adopts two predominate conformers (the GG and GT rotamers) in solution for glucose, and three (GG, GT, and TG) for galactose,[44,45] is reflected in a new contribution to the relaxation rate.[21,39,40] Relevant to this hypothesis, the kinetics of the hydroxymethyl group rotation in glucose and/or galactose have recently been investigated by ultrasonic absorption spectrometry,[46,47] modeling,[45] and NMR relaxation studies.[21,39,40] For glucose, there is an ultrasonic absorption process at a frequency of about 80 MHz, corresponding to a time constant of 2 ns (usually referred to as the ultrasonic relaxation time, but not to be confused with the NMR relaxation time), assigned to the interconversion of the GG and GT rotamers. The experimental[46] activation energy for this process was 19.2 kJ mol^{-1}, in good agreement with the calculated[45] value of 18.8 kJ mol^{-1}. A closely similar activation energy of 14−18 kJ mol^{-1} was obtained by NMR for three different glucans in DMSO solution, based on the temperature dependence of the correlation time calculated from the NT_1 data, using the restricted-amplitude internal diffusion model for motion of the hydroxymethyl group.[39,40] Thus, the relaxation of the hydroxymethyl group is now well understood in glucose or its polymers.

For galactose, ultrasonics detects two lower-amplitude processes, tentatively assigned to the hydroxymethyl group rotation: one at about 30 MHz (5.5 ns ultrasonic relaxation time), probably corresponding to the GG ↔ GT interconversion, and one at about 250 MHz (0.6 ns ultrasonic relaxation time), probably corresponding to the GT ↔ TG interconversion. The fast ultrasonic relaxation of the second process means a short correlation time will be associated with the components of the motion of the hydroxymethyl group, and should result in a longer NMR relaxation time NT_1 for C6 in galactose and the galactosamine-containing Ch 4-S. This is in good agreement with our observations and shows that the difference between HA and Ch 4-S is simply based on the different rotameric populations favored for their respective hexosamines. Thus, the NMR relaxation and ultrasonic absorption data both reflect the same process.

Table 2. Effect of Chain Length and Residue Position on Carbon NT_1 for HA Oligosaccharides and Polymer, (GlcA−GlcNAc)$_n$

	NT_1 (ms)[b]				
carbon[a]	$n = 2$	$n = 3$	$n = 4$	$n = 7$	$n = 40$
$A1_{i,p}$	284	226	210	244	208
$A1_{t\beta}$	300	249			
$A1_{t\alpha}$	227	256	154		
$A2_{i,p}$	257	209	252	236	223
$A2_{t\beta}$	289				
$A2_{t\alpha}$	267	300	303		
$A3_{i}$		181	197	232	217
$A3_{p}$	270	255	232	289	
$A3_{t\beta}$	296	350			
$A3_{t\alpha}$	288	233	226		
$A4_{i}$		208	206	218	220
$A4_{p,t\beta,t\alpha}$	291	253	245		
$A5_{i,p,t\beta}$	304	282	247	235	233
$A5_{t\alpha}$	360	228	205		
$A6_{i,p,t\alpha}$	296	282	226	229	218
$A6_{t\beta}$	278	306			
$U1_{i}$		209	196	210	225
$U1_{p\beta,t}$	319	276	269		
$U1_{p\alpha}$	273	258			
$U2_{i.p}$	296	236	227	225	250
$U2_{t}$	345	293	308		
$U3_{i,p}$	288	222	199	238	239
$U3_{t}$	304	282	247		
$U4_{i}$		223	226	218	209
$U4_{p}$	288	233	226		
$U4_{t}$	360	298	260		
$U5_{i}$		218	225	225	248
$U5_{p}$	272	233	225		
$U5_{t}$	366	309	313		

[a] Sugar residue designations refer to the general structure U_t−A_p−$(U_i$−$A_i)_{n-2}$−U_p−$A_{t\beta,t\alpha}$, where U = GlcA and A = GlcNAc. [b] Measured at 75 MHz in 4/1 H_2O/D_2O containing 0.15 M NaCl, 0.02 M sodium phosphate, pH 7. All values are approximately ± 10 ms.

Relative Conformational Dynamics of Sugar Residues within Short HA Chains in Physiological NaCl Solution. In an earlier study,[13] we showed that it is possible to differentiate the chemical shifts of many carbons of sugar residues at the nonreducing and reducing ends of HA oligosaccharides from penultimate and more interior residues. Thus, an oligosaccharide created by enzymatic cleavage at glucosaminidic linkages would have the general structure GlcA(t)−GlcNAc-(p)−(GlcA(i)−GlcNAc(i))$_{n-2}$−GlcA(p)−GlcNAc(t_α,t_β). Here t means terminal residue, p means penultimate residue, and i means interior residue. The number of repeating disaccharides in the oligosaccharide is n. In the present work, we have measured relaxation times for all of the observed resonances for protonated carbons, and assigned them to the appropriate structural unit(s) according to our previous scheme. The T_1 results are given in Table 2 for HA oligosaccharides in physiological NaCl solution. It can be seen that carbons in terminal residues can usually be differentiated from those in penultimate or interior residues, whereas the latter two groups more often give overlapping and indistinguishable resonances.

If the ring average T_1 values are calculated for each type of sugar residue in an oligosaccharide using C1−C4 resonances, there is a U-shaped pattern of dif-

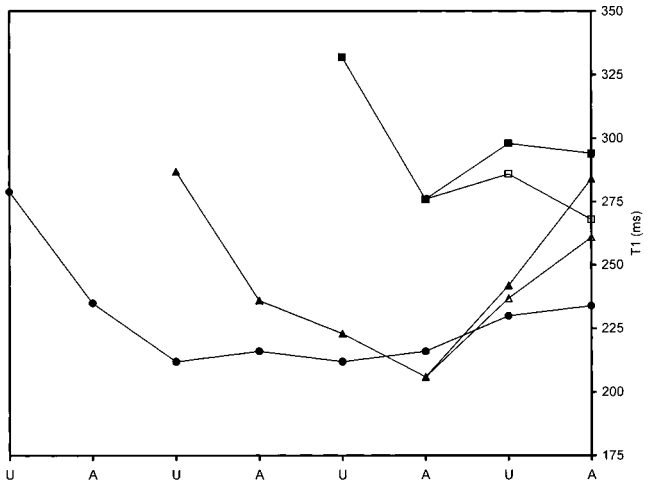

Figure 1. Dependence of sugar ring average ^{13}C NMR T_1 on HA oligosaccharide chain length and residue position. Chains are tethered to the right axis by their reducing ends: (■) tetrasaccharide, β anomer; (□) tetrasaccharide, α anomer; (▲) hexasaccharide, β anomer; (Δ) hexasaccharide, α anomer; (●) octasaccharide, α anomer.

Table 3. Effect of Chain Length and Residue Position on Average Ring Carbon T_1 for HA Oligosaccharides and Polymer, (GlcA−GlcNAc)$_n$

	T$_1$ (ms)[a,b]					
n	U$_t$	A$_p$	U$_i$	A$_i$	U$_p$ (β,α)	A$_t$ (β,α)
2	330	280			300, 290	290, 270
3	290	240	220	210	240, 240	280, 260
4	280	240	210	220	230, ND[c]	ND[c], 230
7			220	230		
40			230	220		

[a] Average of T_1 values for carbons 1−4 of ring. Measured at 75 MHz in 4/1 H_2O/D_2O containing 0.15 M NaCl, 0.02 M sodium phosphate, pH 7. All values are ±10−20 ms. [b] Sugar residue designations refer to the general structure U$_t$−A$_p$−(U$_i$−A$_i$)$_{n-2}$−U$_p$−A$_{t\beta,t\alpha}$, where U = GlcA and A = GlcNAc. [c] ND = not determined

ferential mobility across the chain, as seen in Figure 1 and reported in Table 3. The nonreducing terminal GlcA generally has the highest average T_1. The T_1 for the β anomer of the reducing terminal GlcNAc is equal to, or somewhat lower than, that of the nonreducing terminal GlcA. Penultimate residues and interior residues have progressively lower T_1s as the distances from the ends increase. Thus, both ends of the oligosaccharide chain are more mobile than the more interior residues. This result is similar to that for cellulose triacetate oligosaccharides.[37] In contrast, milk oligosaccharides[48] and maltose and isomaltose oligosaccharides[34,49] have significantly higher T_1 values at the reducing end than at the nonreducing end. The observation of greatest mobility at the ends of short chains is in accord with predictions for oligosaccharides analyzed using the ORZLD (optimized Rouse−Zimm local dynamics) framework.[23]

The α anomer of the reducing end residue has a lower average T_1 than the β anomer. This observation is common in other oligosaccharides and has been proposed to arise from the anisotropic motions of the chain, for which the equatorially oriented C−H at the C1 position of the α anomer lies close to the major axis of rotation, and C1 is thus more efficiently relaxed in the α anomer.[42]

The T_1 of an interior residue in a hexasaccharide is already approximately the same as that of the corresponding sugar in a longer HA polymer. In comparison, the critical length of an oligomer for which the interior sugars have the same characteristic T_1 as that of polymer in other polysaccharides varies from approximately 7 for cellulose triacetate,[37] to 8−10 for maltose and isomaltose,[34,49] to 12−15 for pullulan.[36] Thus, HA appears most similar to the relatively rigid polymer cellulose triacetate in this regard.

Table 4. Effect of Chain Length on Interior Linkage Carbon T_1 for HA Oligosaccharides and Polymer, (GlcA−GlcNAc)$_n$

		T_1 (ms)[b]			
linkage	carbon[a]	*n* = 3	*n* = 4	*n* = 7	*n* = 40
1→3	U1$_i$	209	196	210	225
	A3$_i$	181	196	232	217
	average	195	197	221	221
1→4	A1$_{i,p}$	226	210	244	208
	U4$_i$	223	226	218	209
	average	225	218	231	209

[a] Sugar residue designations refer to the general structure U$_t$−A$_p$−(U$_i$−A$_i$)$_{n-2}$−U$_p$−A$_{t\beta,t\alpha}$, where U = GlcA and A = GlcNAc. [b] Measured at 75 MHz in 4/1 H_2O/D_2O containing 0.15 M NaCl, 0.02 M sodium phosphate, pH 7. All values are approximately ±10 ms.

One possible complicating factor in determining the critical length of an HA oligosaccharide which can behave like polymer arises from the manner in which T_1 depends on correlation time. The minimum T_1 occurs when the motions of the sugar ring are at the Larmor frequency. For HA in aqueous solution, analyzed at 75 MHz, this point may be reached in the hexasaccharide interior. (Motions of the end residues of the short oligosaccharides lie on the high-frequency side of the minimum, and T_1 decreases with motional slowing due to increased chain length.) Interior residues in HA oligosaccharides and polymer may have somewhat slower motions, with little or only slight change in T_1. As an illustration of this effect, we compared the T_1s for specific carbons of the two glycosidic linkages in HA, as a function of chain length. Table 4 gives the results. The carbons of the interior 1→3 link in a hexasaccharide average a T_1 of 195 ms, whereas the same linkage in an HA polymer segment has an average T_1 of 221 ms. The T_1 appears to increase with chain length for interior sugars in structures longer than hexasaccharide. The 1→4 linkage shows a different picture. The average T_1 for this linkage in a hexasaccharide is higher than that for HA polymer. Over the same size range of HA oligosaccharides, Cavalieri et al.[24] have found the T_1 measured at 100 MHz to increase with chain length. They also found the heteronuclear NOE (nuclear Overhauser enhancement) measured at 100 MHz to be decreasing as chain length increases, indicating slowing motions. Thus, in short HA oligosaccharides, the motional frequencies appear to lie near the minimum in a T_1 vs correlation time plot for both 75 and 100 MHz.

Relative Conformational Dynamics of Sugar Residues in Short HA Chains. DMSO Solution. The influence of a rigidly hydrogen-bonded conformation on dynamics in HA has been investigated for the HA tetrasaccharide. In DMSO, HA adopts a structure with stable intramolecular hydrogen bonds across both glycosidic linkages.[11] In H_2O, the intramolecular hydrogen bonding is more dynamic, being in competition with hydrogen bonding to the solvent. Because the rigid structure adopted by HA in DMSO is poorly soluble, we have studied only the very low molecular weight tet-

Table 5. Effect of Solvent on Average Ring Carbon T_1 for HA Tetrasaccharide, (GlcA−GlcNAc)$_2$

	T_1 (ms)[a,b]			
solvent	U_t	A_p	U_p (β,α)	A_t (β,α)
H_2O/salt	330	280	300, 290	290, 270
DMSO	180	170	ND,[c] 160	ND,[c] 150

[a] Average of T_1 values for carbons 1−4 of ring. Measured at 75 MHz in either 4/1 H_2O/D_2O containing 0.15 M NaCl, 0.02 M sodium phosphate, pH 7, or d_6-DMSO. All values are ± 10−20 ms. [b] Sugar residue designations refer to the general structure U_t−A_p−$(U_i$−$A_i)_{n-2}$−U_p−$A_{t\beta,t\alpha}$, where U = GlcA and A = GlcNAc. [c] ND = not determined

rasaccharide in that solvent. We had previously reported the assignments of all carbon resonances for the HA tetrasaccharide in DMSO.[13] Table 5 gives the relaxation data for the HA tetrasaccharide in DMSO and in H_2O, reported as the ring carbon averages across the chain. In DMSO, the variation in average T_1 across the chain is small, with a trend toward decreasing from the nonreducing end. In H_2O, the variation in T_1 across the chain is more marked, with more significant differences between terminal and more interior sugars. This suggests a greater uniformity in dynamics at various positions along the chain for HA tetrasaccharide in DMSO than in H_2O.

It is also clear that the absolute values of T_1 are lower in DMSO than in H_2O. Similar observations have been reported for dextran and amylose,[40,50] where it reflects in part the greater viscosity of DMSO than H_2O. In the HA tetrasaccharide, the difference in hydrogen bonding in DMSO may also alter the distribution of correlation times, and lead to a more efficient relaxation process. These conclusions are tentative, and will require quantitative analysis to confirm.

Summary

The conformational dynamics of hyaluronan (HA) have been assayed by ^{13}C NMR T_1 relaxation time measurements. For HA in aqueous solution, the average relaxation time for ring carbons was not significantly affected by changing the ionic strength due to added salts (from 0 to 0.15) or by changing counterion type (sodium vs potassium vs calcium). Thus, changes in coil expansion due to electrostatic repulsion, or alteration in the tendency for self-association, are not reflected in significant changes in T_1. The behavior of HA was also not significantly different from that for chondroitin 4-sulfate or chondroitin 6-sulfate in aqueous solution. These results are in accord with the concept that the overall conformational dynamics of polysaccharide chains studied at room temperature in aqueous solution are dominated by viscous damping of the chain motions.

Within a chain structure, there are variations in observed T_1 that correspond to differences in relative mobility. The hydroxymethyl substituent group shows a difference in relaxation rate relative to the ring to which it is attached, depending on the ring configuration (glucose vs galactose). These differences correlate with relative rotational isomerization rates measured by ultrasonic relaxation.

In short HA chains, the nonreducing and reducing terminal residues show much greater mobility than penultimate residues, and these are in turn more mobile than interior residues. In contrast, the more rigid hydrogen-bonded conformation of an HA tetrasaccharide in dimethyl sulfoxide solution shows less position-dependent variation in T_1. These data suggest the more dynamic nature of conformation-stabilizing hydrogen bonds for HA chains in aqueous solution.

Acknowledgment. The authors wish to thank Prof. Gaio Paradossi (University of Rome "Tor Vergata") and Prof. Attilio Cesàro (University of Trieste) for helpful discussions.

References and Notes

(1) Meyer, K. *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **1958**, *17*, 1075−1077.
(2) Laurent, T. C.; Fraser, J. R. E. *FASEB J.* **1992**, *6*, 2397−2404.
(3) Balazs, E. A. *Univ. Mich. Med. Ctr. J.* **1968**, 255−259.
(4) Balazs, E. A.; Denlinger, J. L. *J. Equine Vet. Sci.* **1985**, *5*, 217−228.
(5) Denlinger, J. L. In *The Chemistry, Biology, and Medical Applications of Hyaluronan and its Derivatives*; Laurent, T. C.; Ed.; Portland Press: London, 1998; pp 235−242.
(6) Lee, H. G.; Cowman, M. K. *Anal. Biochem.* **1994**, *219*, 278−287.
(7) Potenzone, Jr., R.; Hopfinger, A. J. *Polym. J.* **1978**, *10*, 181−199.
(8) Morris, E. R.; Rees, D. A.; Welsh, E. J. *J. Mol. Biol.* **1980**, *138*, 383−400.
(9) Oberholtzer, J. C.; Englander, S. W.; Horwitz, A. F. *FEBS Lett.* **1983**, *158*, 305−309.
(10) Cowman, M. K.; Cozart, D.; Nakanishi, K.; Balazs, E. A. *Arch. Biochem. Biophys.* **1984**, *230*, 203-212.
(11) Scott, J. E. *The Biology of Hyaluronan*; Ciba Foundation Symposium 143; Wiley: Chichester, U.K., 1989; pp 6−20.
(12) Feder-Davis, J.; Hittner, D. M.; Cowman, M. K. In *Water-Soluble Polymers: Synthesis, Solution Properties, and Applications*; Shalaby, S. W., McCormick, C. L., Butler, G. B., Eds.; ACS Symposium Series 467; American Chemical Society: Washington, DC, 1991; pp 493−501.
(13) Cowman, M. K.; Hittner, D. M.; Feder-Davis, J. *Macromolecules* **1996**, *29*, 2894−2902.
(14) Almond, A.; Brass, A.; Sheehan, J. K. *Glycobiology* **1998**, *8*, 973−980.
(15) Sheehan, J. K.; Arundel, C.; Phelps, C. F. *Int. J. Biol. Macromol.* **1983**, *5*, 222−228.
(16) Turner, R. L.; Lin, P.; Cowman, M. K. *Arch. Biochem. Biophys.* **1988**, *265*, 484−495.
(17) Laurent, T. C.; Ryan, M.; Pietruskiewicz, A. *Biochim. Biophys. Acta* **1960**, *42*, 476−485.
(18) Cleland, R. L. *Biopolymers* **1984**, *23*, 647−666.
(19) Berriaud, N.; Milas, M.; Rinaudo, M. In *Polysaccharides in Medicine and Biotechnology*; Severian, D., Ed.; Marcel Dekker: New York, 1998; pp 313−334.
(20) Lapčík, L., Jr.; Lapčík, L.; De Smedt, S.; Demeester, J.; Chabreček, P. *Chem. Rev.* **1998**, *98*, 2663−2684.
(21) Dais, P. In *NMR of Biological Macromolecules*; Stassinopoulou, C. I., Ed.; NATO ASI Series H 87; Springer-Verlag: Berlin, 1994; pp 279−292.
(22) Bush, C. A.; Martin-Partor, M.; Imberty, A. *Annu. Rev. Biophys. Biomol. Struct.* **1999**, *28*, 269−293.
(23) Perico, A.; Mormino, M.; Urbani, R.; Cesàro, A.; Tylianakis, E.; Dais, P.; Brant, D. A. *J. Phys. Chem. B* **1999**, *103*, 8162−8171.
(24) Cavalieri, F.; Chiessi, E.; Paci, M.; Paradossi, G.; Flaibani, A.; Cesàro, A. *Macromolecules*, **2000**, *33*, 99.
(25) Hittner, D. M.; Cowman, M. K. *J. Chromatogr.* **1987**, *402*, 149−158.
(26) Min, H.; Cowman, M. K. *Anal. Biochem.* **1986**, *155*, 275−285.
(27) Canet, D.; Levy, G. C.; Peat, I. R. *J. Magn. Reson.* **1975**, *18*, 199−204.
(28) Hofmann, H.; Schmut, O.; Sterk, H.; Pölzler, H. *Int. J. Biol. Macromol.* **1983**, *5*, 229−232.
(29) Cleland, R. L.; Wang, J. L. *Biopolymers* **1970**, *9*, 799−810.
(30) Fouissac, E.; Milas, M.; Rinaudo, M.; Borsali, R. *Macromolecules* **1992**, *25*, 5613−5617.
(31) Hayashi, K.; Tsutsumi, K.; Norisuye, T.; Teramoto, A. *Polym. J.* **1996**, *28*, 922−928.
(32) Cowman, M. K.; Liu, J.; Li, M.; Hittner, D. M.; Kim, J. S. In *The Chemistry, Biology and Medical Applications of Hyalu-*

ronan and its Derivatives;Laurent, T. C.; Ed.; Wenner-Gren International Series 72; Portland Press: London, 1998; pp 17−24.
(33) Hricovíni, H.; Guerrini, M.; Torri, G.; Piani, S.; Ungarelli, F. *Carbohydr. Res.* **1995**, *277*, 11−23.
(34) Kadkhodaei, M.; Wu, H.; Brant, D. A. *Biopolymers* **1991**, *31*, 1581−1592.
(35) Zang, L.-H.; Laughlin, M. R.; Rothman, D. L.; Shulman, R. G. *Biochemistry* **1990**, *29*, 6815−6820.
(36) Benesi, A. J.; Brant, D. A. *Macromolecules* **1985**, *18*, 1109−1116.
(37) Buchanan, C. M.; Hyatt, J. A.; Kelley, S. S.; Little, J. L. *Macromolecules* **1990**, *23*, 3747−3755.
(38) Dais, P. *Macromolecules* **1985**, *18*, 1351−1354.
(39) Tylianakis, E.; Dais, P.; Andre, I.; Taravel, F. F. *Macromolecules* **1995**, *28*, 7962−7966.
(40) Tylianakis, E.; Spyros, A.; Dais, P.; Taravel, F. F.; Perico, A. *Carbohydr. Res.* **1999**, *315*, 16−34.
(41) Czarniecki, M. F.; Thornton, E. R. *J. Am. Chem. Soc.* **1977**, *99*, 8279−8282.
(42) Hayes, M. L.; Serianni, A. S.; Barker, R. *Carbohydr. Res.* **1982**, *100*, 87−101.
(43) Noble, O.; Taravel, F. R. *Carbohydr. Res.* **1987**, *166*, 1−11.
(44) Nishida, Y.; Hori, H.; Ohrui, H.; Meguro, H. *J. Carbohydr. Chem.* **1988**, *7*, 239−250.
(45) Rockwell, G. D.; Grindley, T. B. *J. Am. Chem. Soc.* **1998**, *120*, 10953−10963.
(46) Behrends, R.; Cowman, M. K.; Eggers, F.; Eyring, E. M.; Kaatze, U.; Majewski, J.; Petrucci, S.; Richmann, K.-H.; Riech, M. *J. Am. Chem. Soc.* **1997**, *119*, 2182−2186.
(47) Stenger, J.; Cowman, M. K.; Eggers, F.; Eyring, E. M.; Kaatze, U.; Petrucci, S. *J. Phys. Chem. B* **2000**, *104*, 4782−4790.
(48) Breg, J.; Romijn, D.; Vliegenthart, J. F. G.; Strecker, G.; Montreuil, J. *Carbohydr. Res.* **1988**, *183*, 19−34.
(49) Brant, D. A.; Liu, H.-S.; Zhu, Z. S. *Carbohydr. Res.* **1995**, *278*, 11−26.
(50) Matsuo, K. *Macromolecules* **1984**, *17*, 449−452.

MA001082E