Vol. 141, No. 1, 1986 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 26, 1986 Pages 73-77

HYDRATION OF SODIUM HYALURONATE, NMR STUDY OF AN ORIENTED SAMPLE

G. Lahajnar[1] and A. Rupprecht[2*]

[1]J. Stefan Institute, E. Kardelj University of Ljubljana,
Ljubljana, Yugoslavia

[2]University of Stockholm, Arrhenius Laboratory,
Division of Physical Chemistry, S-106 91 Stockholm, Sweden

Received September 22, 1986

SUMMARY. A sample of highly oriented Na-hyaluronate was prepared with a wet-spinning method and equilibrated to 75% relative humidity. Its proton NMR spectrum was recorded as a function of the angle Θ between the hyaluronate helix axis and the external magnetic field. It appeared as a singlet with an angle-dependent line width expressed by $\Delta\nu_0 + A(1-3\cos^2\Theta)^2$. This reveals a residual intramolecular dipolar interaction of anisotropically rotating water molecules. The merging of the expected doublet into a single line is assumed to be caused by proton exchange between water molecules. The result indicates a hydration similar to that of many other fibrous biopolymers at comparable relative humidities.

NMR studies of dynamically oriented water molecules at interfaces of macroscopically aligned systems are of continued interest. Examples include such diverse systems as oriented collagen (1,2) and rayon (3) fibers, oriented samples of DNA (4-6), zeolite crystals (7), layered silicates (8) and lamellar phases of lyotropic liquid crystals (9). A common observation is the existence of the intramolecular residual proton dipolar (H_2O) or deuteron electric quadrupolar (D_2O) couplings due to anisotropic tumbling of water molecules at interfaces, resulting in a doublet NMR spectrum. It was also observed already in the cases of oriented DNA (5) and collagen (2,10) that proton exchange between H_2O molecules can further modulate the residual intramolecular dipolar coupling, causing the NMR doublet to merge into a singlet whose line-width exhibits a strong angular dependence. Such a physical situation was treated in detail for dynamically oriented H_2O molecules in a magnetically oriented sample of the lamellar phase of a lyotropic liquid crystal (9).

Here we report for the first time on the proton NMR study of water sorbed in solid, oriented Na-hyaluronate. Hyaluronic acid is a linear poly-

---

[*] To whom reprint requests should be sent.

disaccharide, a glycosaminoglycan of the form $(\text{-G-N-})_n$ where G is glucuronic acid, N is N-acetylglucosamine and n can be as large as 10^4. This biopolymer is present in the intercellular matrix of most vertebrate connective tissue and is known to participate in a hydrated network between collagen fibers (11).

MATERIALS AND METHODS

A parallelepipedic sample of oriented Na-hyaluronate (Healon from Pharmacia) with the approximate dimensions 8x4.5x3 mm (helix orientation parallel to the 4.5 mm side) was prepared with a wet-spinning method as previously described for K-hyaluronate (12) and DNA (13,14). An X-ray diffraction pattern was taken from the wet-spun Na-hyaluronate at 75% relative humidity using a universal flat X-ray diffraction camera, Type PW 1030. The sample consisted of a square, concertina-like pack containing six layers of hyaluronate film (total thickness = 0.30 mm) which was mounted in a holder described earlier (15). Nickel-filtered CuK_α radiation was used, and a collimator of 500 µm. The specimen-to-film distance was 53.4 mm and the exposure time about 3 h. As is seen from Fig. 1, the material exhibits excellent crystallinity and a very high degree of molecular orientation.

The NMR measurements were performed at 27°C and at a fixed water content (about 22 g H_2O/100 g dry sample) obtained by equilibrating the sample in an atmosphere of 75% relative humidity in a desiccator over saturated $NaClO_3$ solution. A commercial NMR spectrometer (Bruker SXP 4-100/270) with a high resolution iron magnet was used. The magnetic field strength B_o = 2.1 T corresponded to the proton resonance frequency ν = 89.4 MHz. The duration of the excitation radiofrequency pulses was 10 µs. The free induction signals were acquired with a repetition time of 1 s and input to a

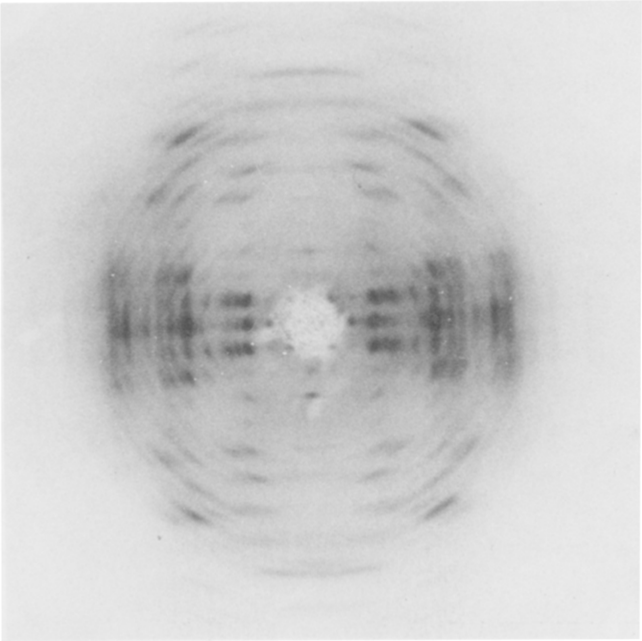

Fig. 1. X-ray diffraction pattern from wet-spun, oriented Na-hyaluronate at 75% relative humidity. The meridional direction is vertical. Magnification 1.13

Nicolet B-NC 12 computer. 36 signals were accumulated in one measurement and subsequently Fourier transformed. The resulting NMR spectra were displayed and plotted in the phase sensitive mode. They were measured as a function of the angle Θ between the hyaluronate helix axis and the direction of the external magnetic field B_o.

RESULTS AND DISCUSSION

In the whole range of Θ values between 0^o and 90^o the proton NMR spectrum appeared as a (nonresolvable) singlet, but its line-width showed a strong dependence on Θ (Fig. 2). This means that the residual intramolecular proton dipolar coupling responsible for a doublet spectrum of splitting $2\omega_d$ is averaged further by the intermolecular proton exchange (10). Hence the proton NMR doublet has collapsed to a singlet because the condition $\omega_d\tau_{ex} \ll 1$ is satisfied, where τ_{ex} is the lifetime of a proton in a water molecule. In such a case the corresponding transverse proton NMR relaxation time $T_{2,ex}$ is given by (9)

$$(1/T_2)_{ex} = \frac{9}{16}\frac{\gamma^4\hbar^2}{r^6}S^2(1-3\cos^2\Theta)^2\left(\frac{n_b}{n}\right)^2\tau_{ex} \qquad (1)$$

Here S is the component of the ordering matrix along the H_2O interproton vector r, Θ the angle between the B_o direction and the normal to ordered planar water-substrate interfaces (in our case the latter coincides with the hyaluronate helix axis), n_b the number of dynamically oriented water molecules at interfaces and n the total number of H_2O molecules in the system. γ is the proton magnetogyric ratio and $\hbar$ Planck's constant divided by 2π. It should be noted that an expression for the water proton T_2 - very similar to that of Eq. (1) - is derived if instead of proton exchange there

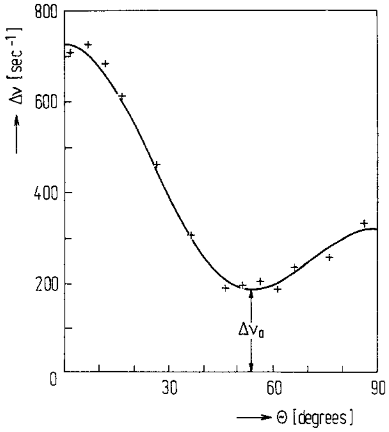

Fig. 2. Angular dependence of the proton NMR line-width of sorbed water in oriented Na-hyaluronate: Crosses=experimentally determined half-intensity line-widths. Solid line=computer fit according to Eq. (2) (see text for further details).

is efficient diffusion or exchange of dynamically oriented H_2O molecules at interfaces with those remote from interfaces (9).

It is seen from Fig. 2 that the observed proton NMR linewidth $\Delta\nu$ consists of two contributions: an angle-dependent part $\Delta\nu(\Theta)$ fitting the $(1-3\cos^2\Theta)^2$ dependence of Eq. (1), and a constant part $\Delta\nu_0$. Thus, as $\Delta\nu(\Theta)$ corresponds to $(1/T_2)_{ex}$ of Eq. (1), i.e. $\pi\Delta\nu(\Theta)\approx(1/T_2)_{ex}$, the data of Fig. 2 were analyzed in terms of the expression

$$\Delta\nu = \Delta\nu(\Theta) + \Delta\nu_o = A(1-3\cos^2\Theta)^2 + \Delta\nu_o \tag{2}$$

The constant πA is identified with the factor of the right hand side of Eq. (1). The computer analysis of the $\Delta\nu$ data in terms of Eq. (2) gave $A = 136\ s^{-1}$ and $\Delta\nu_o = 185\ s^{-1}$. Three parameters of interest are contained in A: the local "order parameter" of H_2O molecules, the relative number of "bound" H_2O molecules, and the exchange time of protons (or water molecules). The angle-independent part, $\Delta\nu_o$, is indicative of the fact that one fraction of water molecules in the Na-hyaluronate sample does not participate in the characteristic orientation mechanism in the interface region. The result obtained indicates that the hydration of Na-hyaluronate at 75% relative humidity is similar to that of many other fibrous biopolymers at comparable relative humidities (16,17). For a recent NMR study of solutions, see Ref. (18). They showed among other things that the ion-binding and hydration properties of hyaluronate and chondroitin are quite similar, except at high pH.

An earlier attempt (6) to observe an angle-dependent T_2 from hydrated, wet-spun, oriented K-hyaluronate failed, possibly due to the use of a less sensitive NMR method (pulse technique). Another reason might be that these wet-spun hyaluronate samples occur in different conformations, K-hyaluronate as 4-fold helices (12) and Na-hyaluronate as 3-fold helices (19); see also Ref. (20). Further work is needed to elucidate this point.

It is also of interest to extend the NMR study of oriented hyaluronic acid to other hydration conditions (varying water content and temperature, D_2O hydration) as well as to diffusion measurements and measurements of the ^{23}Na resonance; cf. Ref. (21).

ACKNOWLEDGEMENTS

The work has been supported in part by a grant from the Swedish Natural Science Research Council. One of us (A.R.) is indebted to Pharmacia AB and its representative Dr. R. Einarsson for a gift of Na-hyaluronate (Healon).

REFERENCES

1. Berendsen, H.J.C. (1962) J. Chem. Phys. 36, 3297-3305.
2. Dehl, R.E., and Hoeve, C.A.J. (1969) J. Chem. Phys. 50, 3245-3251.

3. Dehl, R.E. (1968) J. Chem. Phys. 48, 831-835.
4. Rupprecht, A. (1966) Acta Chem. Scand. 20, 582-583.
5. Migchelsen, C., Berendsen, H.J.C.,and Rupprecht, A. (1968) J. Mol. Biol. 37, 235-237.
6. Lahajnar, G., Zupancic, I.,and Rupprecht, A. (1982) Proton NMR relaxation and diffusion study of water sorbed in oriented DNA and hyaluronic acid samples. In: Franks, F., Mathias, S. (eds) Biophysics of water. Wiley, New York.
7. Ducros, P. (1960) Bull. Soc. Franç. Minér. Crist. 83, 85-112.
8. Graham, J., Walker, G.F., and West, G.W. (1964) J. Chem. Phys. 40, 540-550.
9. Ahmad, S.B., Packer, K.J., and Ramsden, J.M. (1977) Mol. Phys. 33, 857-874.
10. Migchelsen, C., and Berendsen,H.J.C. (1973) J. Chem. Phys. 59, 296-305.
11. Hopfinger, A.J. (1977) Intermolecular interactions and biomolecular organization. Wiley, New York.
12. Rupprecht, A. (1979) Acta Chem. Scand. B33, 779-780.
13. Rupprecht, A. (1963) Biochem. Biophys. Res. Commun. 12, 163-168.
14. Rupprecht, A. (1970) Biotechnol. Bioeng. 12, 93-121.
15. Rupprecht, A., and Forslind B. (1970) Biochim. Biophys. Acta 204, 304-316.
16. Berendsen, H.J.C. (1975) Specific interactions of water with biopolymers. In: Franks, F. (ed) Water, a comprehensive treatise. Vol. 5. Plenum Press, New York, pp. 293-330.
17. Derbyshire, W. (1982) The dynamics of water in heterogeneous systems with emphasis on subzero temperatures. In: Franks, F. (ed) Water, a comprehensive treatise. Vol. 7. Plenum Press, New York, pp. 339-430.
18. Piculell, L., Lindman, B., and Einarsson, R. (1984) Biopolymers 23, 1683-1699.
19. Sheehan, J.K., Atkins, E.D.T., and Nieduszynski, I.A. (1975) J. Mol. Biol. 91, 153-163.
20. Mitra, A.K., Raghunathan, S., Sheehan, J.K., and Arnott, S. (1983) J. Mol. Biol. 169, 829-859.
21. Edzes, H.T., Rupprecht, A., and Berendsen, H.J.C. (1972) Biochem. Biophys. Res. Commun. 46, 790-794.