Molecular Basis of Water Proton Relaxation in Gels and Tissue

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An extensive set of water-¹H magnetic relaxation dispersion (MRD) data are presented for aqueous agarose and gelatin gels. It is demonstrated that the EMOR model, which was developed in a companion paper to this study (see Halle, this issue), accounts for the dependence of the water-¹H spin-lattice relaxation rate on resonance frequency over more than four decades and on pH. The parameter values deduced from analysis of the ¹H MRD data are consistent with values derived from ²H MRD profiles from the same gels and with small-molecule reference data. This agreement indicates that the water-¹H relaxation dispersion in aqueous biopolymer gels is produced directly by exchange-mediated orientational randomization of internal water molecules or labile biopolymer protons, with little or no role played by collective biopolymer vibrations or coherent spin diffusion. This ubiquitous mechanism is proposed to be the principal source of water-¹H spin-lattice relaxation at low magnetic fields in all aqueous systems with rotationally immobile biopolymers, including biological tissue. The same mechanism also contributes to transverse and rotating-frame relaxation and magnetization transfer at high fields. Magn Reson Med 56:73-81, 2006. © 2006 Wiley-Liss, Inc.

Key words: agarose; gelatin; proton exchange; internal water; magnetic relaxation dispersion

Intrinsic contrast in magnetic resonance images of soft tissue is generated largely by spatial variations in spin relaxation rates. Whereas the phenomenology of water-¹H relaxation in tissue is well documented (1–3), the underlying molecular mechanism remains controversial (4–9). The task of elucidating the relaxation mechanism is complicated by incomplete knowledge about the chemical composition and supramolecular structure of most tissues. In addition, it is usually not possible to vary biopolymer composition, pH, or temperature in a controlled way while maintaining the integrity of the tissue. For these reasons, most mechanistic studies have been carried out on model systems, such as aqueous biopolymer gels, with relaxation characteristics similar to those of tissue. Here we report an extensive set of water-¹H relaxation data from two widely used tissue models: aqueous gels of agarose and gelatin.

Detailed information about relaxation mechanisms in complex systems can be obtained from the magnetic relaxation dispersion (MRD) profile, that is, the field/frequency dependence of the spin-lattice relaxation rate, $R_1 = 1/T_1$. Ideally, the dispersion profile should be recorded from typical MRI frequencies of order 100 MHz down to the kHz range. The water-¹H MRD profiles reported here cover more than four frequency decades. The strong frequency dependence of R_1 at low fields, which is the focus of the present study, can be used to enhance image contrast in prepolarized MRI experiments (10). The low-field R_1 dispersion also yields information about the zero-frequency dipolar contributions to transverse relaxation and steadystate magnetization transfer and to the low-frequency dipolar contribution to rotating-frame spin-lattice relaxation.

Agarose is a linear polysaccharide with a disaccharide repeat (agarobiose) composed of 1,3-linked β-D-galactose and 1,4-linked 3,6-anhydro- α -L-galactose (11). In aqueous solution below \sim 35°C, agarose forms a double helix with two parallel, left-handed helices and three agarobiose units per turn (12). It has been proposed that this structure is stabilized by a string of internal water molecules in the narrow channel that extends along the helix axis (12). The agarose gel network is built from fibrils, formed by lateral aggregation of six or more double helices (13). The dramatic broadening of the water-¹H resonance upon gelation of agarose was originally attributed to extensive perturbations of bulk water (14), and similar ideas regarding longrange ordering and slowing down of water motions have been invoked to explain water-¹H and ²H relaxation enhancements in biological tissue (15,16). Subsequent more systematic studies attributed the relaxation enhancement to a small fraction of "bound" water molecules or exchanging hydroxyl protons (17,18). These studies were restricted to relatively high frequencies (>2 MHz) and could therefore not fully elucidate the molecular origin of the observed relaxation effects. Agarose gels have been widely used to model the ¹H relaxation behavior of biological tissue, and in this connection water-¹H MRD profiles from agarose gels have been reported (10,19,20), but without any molecular interpretation.

Aqueous solutions of gelatin, produced by partial hydrolysis of collagen, undergo a sol-gel transition at ~25°C (21). Gelatin gels are built from collagen-like triple-helical junction zones, 100-200 residues in length, connected by flexible single chains (22). The lateral aggregation of triple helices that gives rise to collagen fibrils in vivo does not occur in gelatin gels. The collagen triple helix contains three supercoiled polypeptide chains with the amino acid side-chains of all nonglycine residues exposed to solvent (23). Gelatin gels have been investigated by magnetic relaxation techniques since the early days of NMR (24). Water-¹H MRD profiles from gelatin gels were reported in 1970, but a quantitative interpretation was not attempted (25). More recently, several high-frequency (\geq 20 MHz) ¹H relaxation studies of water in gelatin gels focused on the

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Received 30 December 2005; revised 19 February 2006; accepted 2 March 2006.

DOI 10.1002/mrm.20912

Published online 26 May 2006 in Wiley InterScience (www.interscience.wiley. com).

CPMG T_2 dispersion caused by chemical shift fluctuations on the millisecond time scale (26,27).

To understand the mechanism of water-¹H relaxation in gels and tissue, we must identify the proton species involved as well as the molecular motions that modulate the magnetic dipole-dipole couplings of these protons. The proton species mediating the relaxation enhancement (relative to bulk water) was long thought to be hydration water at the surfaces of biopolymers (2,5,6). However, it is now clear that water molecules in the hydration layer are only marginally less mobile than bulk water (28). Water at biopolymer surfaces therefore cannot be responsible for the water-¹H dispersion observed with gels and tissue. The focus has now shifted to internal water molecules and labile biopolymer protons as the intermediary proton species, but opinions differ about the relative importance of the two. Whereas internal-water protons exchange with bulk-water protons through molecular exchange coupled to conformational fluctuations in the biomolecule, labile biopolymer protons exchange by an acid or base catalyzed process that involves covalent bond disruption. The two species can thus be distinguished on the basis of the strong pH dependence of the labile-proton exchange rate. Here we use this approach to show that the main dispersion is dominated by internal water molecules for agarose, and by labile biopolymer protons for gelatin.

The most challenging and controversial issue, about which radically different views are still held by different investigators, concerns the molecular motions that induce water-¹H relaxation in gels and tissue. These motions must be identified in order to develop a molecular-level relaxation model with quantitative prediction capability. Water-¹H relaxation in aqueous solutions of freely tumbling proteins is well understood (29), but relaxation in gels and tissue with rotationally inhibited biopolymers is fundamentally different. Nearly all previous treatments of water-¹H relaxation in tissue are based on a phenomenological so-called two-pool model (4), which, on account of its implicit assumption of fast exchange between intermediary and bulk-water protons, is not generally applicable to gels or tissue (Halle, this issue). The two-pool model has been merged with molecular models that attribute the relaxation enhancement either to water dynamics in the hydration layer (2,5,6) or to collective biopolymer vibrations (7–9), and sometimes a role for coherent spin diffusion is included as well. In the companion paper to this study (Halle, this issue), we developed a truly molecular model (not based on the phenomenological two-pool formalism) that recognizes that exchange of intermediary protons not only transfers magnetization to bulk-water protons but also drives relaxation by a mechanism of exchange-mediated orientational randomization (EMOR).

To unravel the mechanism of water-¹H relaxation in gels and tissue, it is helpful to record and analyze water-¹H and water-²H MRD profiles from the same system. Since crossrelaxation and spin diffusion can be neglected for the quadrupolar ²H nuclide, the analysis of the ²H MRD profiles is relatively straightforward. Although ²H relaxation is mediated by the electric quadrupole coupling (rather than by magnetic dipole-dipole couplings), the relaxationinducing molecular motions are the same as for ¹H and they are described by the EMOR model (30). As an important part of our strategy, we measured and analyzed the water-²H MRD profiles from the same agarose (31) and gelatin (Vaca Chávez et al., submitted) gels studied here by ¹H MRD. Molecular parameters such as intermediary proton populations and exchange rates and parameters relating to the gel structure can be carried over (after making minor corrections for H/D isotope effects) from the analysis of the ²H profiles, thus providing a stringent test of the EMOR model for ¹H relaxation.

MATERIALS AND METHODS

Gel Preparation

Agarose (ultrapure grade; Supra Sieve CPG) was used as supplied by American Bioanalytical (Natick, MA). Gel samples were prepared directly in 10 mm O.D. NMR tubes by dissolving agarose in H₂O (Fluka Biochimika). The samples were immersed in a water bath at 99°C for 30-40 min. They were then cooled to 45°C, and the pH was measured. The desired pH values were obtained by microliter additions of HCl or NaOH, without any buffers. The samples were then slowly (~ 2 hr) cooled to room temperature and stored at 16°C for 1-2 days before MRD measurements. The total number, $N_{\rm T}$, of water molecules in the sample per average monosaccharide of molar mass 153.2 g mol⁻¹ was calculated with a small correction for the moisture content (5% w/w, according the manufacturer) of the agarose preparation. For the acidic and neutral samples, $N_{\rm T}$ = 129.9 and 124.5, respectively.

Type B gelatin from bovine skin was used as supplied by Sigma (product no. G9382, lot no. 053H0271). A complete amino acid analysis was performed on this gelatin preparation (Vaca Chávez et al., submitted), and the results were in agreement with the expected amino acid composition. Gelatin was dissolved in H₂O (Fluka Biochimika) at 60°C and the pH was adjusted as for the agarose gels. Portions $(\sim 1 \text{ mL})$ of this stock solution were transferred to NMR tubes, the temperature was reduced to 40°C, and the pH was measured. The samples were then cooled to room temperature and stored at 15°C for at least 16 hr before the MRD measurements were performed. The total number, $N_{\rm T}$, of water molecules per average amino acid residue was calculated with a small correction for the moisture content (23% w/w, as inferred from the amino acid analysis) of the gelatin preparation. MRD samples at five pH values were made from the same gelatin stock solution with $N_{\rm T} = 60.5$.

To facilitate comparison of R_1 data from agarose and gelatin gel samples, the agarose and gelatin data were normalized to $N_{\rm T} = 100$ and 60, respectively. This corresponds, in both cases, to a biopolymer concentration of 7.8% (w/w). The normalization is based on the fact that $R_1 - R_1^{(0)}$ is inversely proportional to $N_{\rm T}$ (vide infra). Here, $R_1^{(0)}$ is the longitudinal ¹H relaxation rate measured (after O₂ purging) on a reference sample of pure H₂O at the same temperature as for the gel: 0.42 s⁻¹ at 10°C and 0.32 s⁻¹ at 20°C.

Water in equilibrium with air at atmospheric pressure and room temperature contains ~0.3 mM dissolved O_2 , which by virtue of the unpaired electrons produces a paramagnetic relaxation enhancement of ~0.07 s⁻¹ below and ~0.02 s⁻¹ above the dispersion frequency of ~40 MHz (32). The gelatin samples were purged from O_2 by slow argon bubbling for 4 hr at 40°C and then sealed with a septum. For the agarose samples, no attempt was made to remove dissolved oxygen. This has no significant consequences for the data analysis, since at low frequencies the O_2 contribution is less than the experimental uncertainty in R_1 , and at high frequencies the O_2 dispersion step is at most 6% of the smallest component (W1) of the observed dispersion profile.

MRD Experiments

The longitudinal relaxation rate, R_1 , of the water ¹H magnetization was measured over more than four frequency decades, from 8 or 10 kHz to 200 MHz. To cover this frequency range we used three types of NMR spectrometer: 1) a Stelar FC2000 fast field-cycling (FC) instrument (up to 10 MHz), 2) a field-variable iron-core magnet equipped with a Tecmag console (14-45 MHz for agarose, 14-74 MHz for gelatin), and 3) Bruker Avance DMX 100 and 200 spectrometers with conventional cryomagnets (100 and 200 MHz). The MRD measurements were carried out at 10°C for gelatin and 20°C for agarose, which in both cases is $\sim 15^{\circ}$ C below the gel setting temperature. The temperature was maintained to within ± 0.1 °C with a Stelar variable temperature control unit or a Bruker Eurotherm regulator. Temperatures were checked with a thermocouple referenced to an ice-water bath.

The FC technique overcomes the sensitivity problem of conventional fixed-field experiments in weak magnetic fields (33,34). The prepolarized sequence (PP/S) was used with polarization and detection at 20 and 9.1 MHz, respectively, and a 90° pulse length of 4.5 μ s. In the non-FC experiments (with variable detection field), R_1 was measured with the 180° – τ – 90° inversion recovery sequence. Single-exponential recovery curves were obtained throughout, from which R_1 was determined by a three-parameter fit.

Analysis of MRD Data

The water-¹H MRD profiles were analyzed with the EMOR model developed in the companion paper (Halle, this issue), to which we refer the reader for details. Here we only summarize the theoretical expressions used for the data analysis. In the Discussion section we justify the approximations invoked in the derivation of these expressions (Halle, this issue). The water-¹H spin-lattice relaxation rate is decomposed as

$$R_1(\omega_0) = \alpha + \sum_{I} f_I R_1^{I}(\omega_0).$$
 [1]

The α term includes all frequency-independent contributions to R_1 , notably from the bulk water phase and the mobile hydration layer at biopolymer surfaces. The sum in Eq. [1] runs over all intermediary proton classes: internal water molecules and labile biopolymer protons. The fraction, f_1 , of protons in class I is

$$f_{\rm I} = N_{\rm I}/N_{\rm T}.$$
 [2]

Here $N_{\rm I}$ is either the average number of internal water molecules in class I per monomer (average monosaccharide in agarose or average amino acid residue in gelatin) or the average number of labile biopolymer protons in class I divided by 2 (the number of water equivalents) per monomer. Further, $N_{\rm T}$ is the total number of water molecules per monomer (vide supra).

In Eq. [1] $R_1^{I}(\omega_0)$ is the apparent relaxation rate associated with intermediary protons in class I, defined as

$$R_{1}^{I}(\omega_{0}) = \{\tau_{I} + [\Re_{I}(\omega_{0})]^{-1}\}^{-1},$$
[3]

where $\tau_{\rm I}$ is the mean residence time of intermediary protons in class I, that is, the inverse of their exchange rate constant. In the fast-exchange regime, where $\tau_{\rm I} \Re_{\rm I}(\omega_0) \ll 1$, $R_{\rm I}^{\rm I}(\omega_0)$ becomes equal to the effective intrinsic relaxation rate, $\Re_{\rm I}(\omega_0)$, which includes the effect of cross-relaxation with nonlabile biopolymer protons. This rate is given by (Halle, this issue):

$$\Re_{I}(\omega_{0}) = \frac{3}{2} F_{I}(\omega_{0}) \left[\frac{0.2\tau_{I}}{1 + (\omega_{0}\tau_{I})^{2}} + \frac{0.8\tau_{I}}{1 + (2\omega_{0}\tau_{I})^{2}} \right], \qquad [4]$$

where

$$F_{\rm I}(\omega_0) = \begin{cases} (D_{\rm I}S_{\rm I})^2 H_{\rm I}(\omega_0), & \text{labile protons} \\ (D_{\rm I,intra}S_{\rm I,intra})^2 [1 + \kappa^2 H_{\rm I}(\omega_0)], & \text{internal water} \end{cases}$$

$$[5]$$

$$H_{\rm I}(\omega_0) = \frac{5 + 22(\omega_0 \tau_{\rm I})^2 + 8(\omega_0 \tau_{\rm I})^4}{10 + 23(\omega_0 \tau_{\rm I})^2 + 4(\omega_0 \tau_{\rm I})^4}, \qquad [6]$$

$$\kappa = \frac{D_{\text{I,inter}} S_{\text{I,inter}}}{D_{\text{I,intra}} S_{\text{I,intra}}}.$$
[7]

In Eq. [5] $D_{\rm I}$ is the total dipole coupling constant of labile proton I:

$$D_{\rm I} = \frac{\mu_0}{4\pi} \,\gamma^2 \hbar \left(\sum_k r_{\rm Ik}^{-6}\right)^{1/2},\tag{8}$$

where the sum runs over all nonlabile biopolymer protons, with separation $r_{\rm Ik}$ from labile proton I. Further, $S_{\rm I}$ is an effective, rank-2, orientational order parameter that describes the effect of partial averaging of these dipole couplings by internal motions on time scales shorter than $\tau_{\rm I}$. For internal water molecules, $D_{\rm I,intra}$ is the intramolecular dipole coupling between the two water protons, for which we use the value $2.36 \times 10^5 \, {\rm s}^{-1}$ obtained from the intramolecular second moment of ice Ih (35). The intermolecular internal-water dipole coupling constant $D_{\rm I,inter}$ is defined as in Eq. [8], except that each $r_{\rm Ik}^{-6}$ term is averaged over the two water protons.

In the so-called adiabatic regime, where $(\omega_0 \tau_I)^2 \gg 1$, the dispersion midpoint frequency is no longer inversely proportional to τ_I but is governed by the dipole frequency $\Omega_I = (6/5)^{1/2} D_I S_I$ (Halle, this issue). In this regime, Eqs. [3]–[6] yield

$$R_1^{\rm I}(\omega_0) = \frac{1}{\tau_{\rm I}} \frac{1}{1 + (\omega_0/\Omega_{\rm I})^2} \,. \eqno(9)$$

Fits to the ¹H MRD data were made with the Levenberg-Marquardt algorithm (36) with equal weighting of all data points and with the target function based on Eqs. [1]–[9]. Quoted uncertainties in parameter values (1 standard deviation) were calculated with an estimated 1% accuracy in all R_1 values. The number of parameters depends on the type of intermediary proton (labile or internal water) and the dynamic regime. For each internal-water class there are four parameters: $N_{\rm I}$, $S_{\rm I,intra}$, κ , and $\tau_{\rm I}$. However, internal water molecules with residence times much shorter than 1 µs are in the fast-exchange regime, where the first two parameters only occur in the combination $N_{\rm I}S_{\rm I,intra}^2$, thus reducing the number of independent parameters to 3. To further reduce the number of adjustable parameters, we constrain k to the same value for all (two or three) internalwater classes. For labile protons, $N_{\rm I}$ is known from the biopolymer structure, so there are only two parameters per class: $D_{I}S_{I}$ and τ_{I} .

RESULTS

Our aim here is to present water-¹H MRD profiles from agarose and gelatin gels, and show that they can be accounted for in terms of the EMOR model (Halle, this issue). We also measured water-²H MRD profiles from the corresponding agarose/D₂O and gelatin/D₂O gel samples. The ²H profiles, along with temperature-dependent ²H rates and water-¹⁷O MRD profiles, are fully described and analyzed with the quadrupolar version of the EMOR model (30) in separate publications on agarose (31) and gelatin (Vaca Chávez et al., submitted). To highlight similarities and differences between the ¹H and ²H profiles, we reproduce the ²H profiles in Fig. 1a and d. In the Discussion section we compare the parameter values derived from the ¹H and ²H profiles. This comparison demonstrates that the same molecular species and motions are responsible for ¹H and ²H relaxation, as suggested by the qualitative similarity between the ¹H and ²H profiles.

Agarose Gels

Water-¹H MRD profiles from agarose gels at neutral and acidic pH are shown in Fig. 1b, with the high-frequency region magnified in Fig. 1c. With exchange rate constants determined for hydroxyl protons in saccharides (37,38), the EMOR model predicts (see Materials and Methods) that the agarose hydroxyl contribution to $R_1(0)$ should be 1–2 orders of magnitude larger at pH 3.5 than at pH 7.2. Because the difference between the two profiles is small, we conclude that the pH 7.2 profile is produced by internal water molecules, with a negligible hydroxyl contribution. The internal-water contribution should not depend on pH and is therefore the same for the two profiles. The hydroxyl contribution at pH 3.5 can thus be identified with the difference between the two profiles.

Taking these considerations into account, we performed a simultaneous fit of the EMOR model to the two ¹H profiles in Fig. 1b. A satisfactory fit requires three internalwater classes (labeled by subscript I = W1, W2, and W3) with different residence times. As shown in Fig. 1b and c, the most long-lived internal-water class W3 dominates R_1 below \sim 0.2 MHz, but all three internal-water classes make comparable contributions around 1 MHz. At typical MRI fields only class W1 contributes significantly. Classes W1 and W2 are in the fast-exchange regime and together are modeled by five parameters: $N_{\rm W1}S_{\rm W1,intra}^2$, $N_{\rm W2}S_{\rm W2,intra}^2$, τ_{W1} , τ_{W2} and κ (see Materials and Methods). Internal-water class W3 has a residence time in the microsecond range and must therefore be modeled with the more general Eq. [3]. This introduces three additional parameters: N_{W3} , $S_{\rm W3,intra}$, and $\tau_{\rm W3}$. Because the ¹H dispersion could only be sampled down to 10 kHz (as compared to 1.5 kHz for the ²H dispersion), the low-frequency plateau is not accurately defined by our data. (The *existence* of the plateau is not in doubt. Our data above 10 kHz suggest, and other data at lower frequencies (10) show, that R_1 levels out between 1 and 10 kHz.) As a result, the parameters $N_{\rm W3}$ and $S_{\rm W3,intra}$ cannot both be determined with useful accuracy. We therefore fix N_{W3} to the value 0.070 deduced from the ²H fit (31).

For the hydroxyl protons in agarose, we write $N_{\rm I} = x_{\rm I}N_{\rm I}^0$, where $N_{\rm I}^0 = 1$ is the known structural number of hydroxyl protons per monosaccharide (11) divided by 2 (to convert it to water equivalents), and $x_{\rm I}$ is the unknown fraction of solvent-exposed hydroxyl protons in the agarose fibrils. Using proton exchange rate constants for hydroxyl groups in simple saccharides (37,38), we estimate that $\tau_{\rm I}$ is of order 10^{-4} s at pH 3.5. The labile hydroxyl protons in agarose are thus in the adiabatic regime, where Eq. [9] applies, and are modeled by the two parameters $D_{\rm OH} S_{\rm OH}$ and $x_{\rm OH} / \tau_{\rm OH}$.

Together with the frequency-independent contribution α in Eq. [1], there are 10 adjustable parameters in the model. Table 1 presents the values of these parameters resulting from the simultaneous fit to the 72 R_1 values in Fig. 1b. Fits of comparable quality can surely be obtained with other models that contain fewer parameters. However, our aim is not to represent the data with as few parameters as possible, but to identify the molecular species and motions responsible for the relaxation dispersion. The real test of the EMOR model is not the quality of the fit (which is excellent), but the values of the derived parameters. In the Discussion section we show that these values are consistent with the parameter values derived from the corresponding ²H profiles and with the known physicochemical properties of agarose gels.

Gelatin Gels

Water-¹H MRD profiles from gelatin gels at 5 pH values are shown in Fig. 1e, with the pH 3.0 profile magnified and decomposed in Fig. 1f. In contrast to the agarose profiles (Fig. 1b), the gelatin profiles show a strong pH dependence. For agarose the relaxation dispersion is dominated by the most long-lived internal water molecules, with a residence time of ~3 μ s (Table 1). For gelatin the longest water residence times are two orders of magnitude shorter (vide infra), so the internal water molecules produce only a small dispersion in the MHz range. Except at neutral pH, the gelatin profile is therefore dominated by labile protons in the amino acid side-chains.

FIG. 1. Water ²H (**a** and **d**) and ¹H (**b**, **c**, **e**, and **f**) MRD profiles from agarose gel at 20°C (a-c) and gelatin gel at 10°C (d-f) at the indicated pH values. The data are scaled to concentrations of 100 water molecules per monosaccharide unit (agarose) or 60 water molecules per amino acid residue (gelatin), corresponding to a biopolymer content of 7.8% (w/w) in both cases. To facilitate comparison between ¹H and ²H data, R_1 was divided by the bulk-water ¹H or ²H relaxation rate, R_{bulk}, at the respective temperatures. The dispersion curves resulted from fits according to the EMOR model. For agarose (a-c), the dash-dotted curves represent contributions from water components W1 (black), W2 (green), and W3 (blue), while the hydroxyl contribution corresponds to the difference between the red and blue solid curves. For gelatin (f), the dash-dotted curves represent the contributions from water (blue), hydroxyl protons (green), and an unidentified class of intermediary protons (black).



Table 1 Results of Simultaneous Fit to ¹H MRD Data from Agarose Gels at Two pH Values

Value
0.496 ± 0.003
0.14 ± 0.03
4.4 ± 0.2
0.026 ± 0.005
84 ± 6
0.79 ± 0.06
3.0 ± 0.4
1.2 ± 0.2
1.0 ± 0.2
1.3 ± 0.2

Because the ¹H dispersion could only be sampled down to 8 kHz, the low-frequency plateau is not directly evident in the ¹H data. This experimental limitation, together with the larger number of parameters associated with several labile-proton classes, precludes a global (multi-pH) analysis of the ¹H profiles. For the quantitative analysis, we focus on the pH 3.0 dispersion profile (Fig. 1f). At this pH, two labile-proton classes can contribute to the dispersion: carboxyl protons (in Asp and Glu side-chains) with $N_{\rm COOH} = 0.060$, and hydroxyl protons (mainly in Hyp, Ser, and Thr side-chains) with $N_{\rm OH}$ = 0.076. As compared to OH protons, the labile proton in COOH has a smaller dipole coupling constant and a much shorter residence time, of order 10^{-7} s (39). With these parameter values, the EMOR model predicts (see Materials and Methods) that the COOH contribution to $R_1(0)$ is ~0.1 s⁻¹, which is negligible. The only labile-proton contribution is thus modeled by the two parameters $D_{OH} S_{OH}$ and τ_{OH} .

Table 2 Results of Fit to ¹H MRD Data from Gelatin Gel at pH 3.0

Parameter (unit)	Value
α (s ⁻¹)	0.664 ± 0.007
$N_{W1}S_{W1,intra}^2$ (—)	$(2.7 \pm 1.8) imes 10^{-2}$
$N_{W2}S_{W2,intra}^2$ (—)	$(3.3 \pm 2.1) imes 10^{-3}$
к (—)	3.2 ± 1.3
$D_{\rm OH}S_{\rm OH}~(10^4~{ m s}^{-1})$	2.84 ± 0.04
τ _{ΟΗ} (μs)	13.1 ± 0.7
N _X (D _X S _X) ² (10 ⁸ s ⁻²)	1.1 ± 0.1
τ _X (μs)	0.80 ± 0.08

A satisfactory fit of the EMOR model to the pH 3.0 ¹H MRD data requires, in addition to the hydroxyl protons, two classes of internal water molecules (labeled by subscript I = W1 and W2). To reduce the number of parameters, we fix their residence times to the values deduced from the ²H profiles (disregarding any H/D isotope effect): $\tau_{W1} = 3.6$ ns and $\tau_{W2} = 39$ ns (Vaca Chávez et al., submitted). The two internal-water classes are thus described by three adjustable parameters: $N_{W1}S^2_{W1,intra}$, $N_{W2}S^2_{W2,intra}$ and κ .

Because the gelatin gel contains rigid triple-helical segments as well as flexible single-strand segments (22), each term in the sum of Eq. [1] should be decomposed into a population-weighted sum of helix and strand contributions. This introduces two additional parameters: the fraction, $x_{\rm strand} = 1 - x_{\rm helix}$, residues in single-strand segments, and the single-strand orientational order parameter, $S_{\rm strand}$. These parameters are the same for all intermediary protons and are fixed to the values deduced from the ²H profiles: $x_{\rm strand} = 0.23$ and $S_{\rm strand} = 0.11$ (Vaca Chávez et al., submitted). Since extended single-strand segments cannot trap water molecules, there should be no single-strand contribution for the two internal-water classes.

The fit (not shown) of the model defined above, with two internal-water classes and one hydroxyl class (six adjustable parameters), to the pH 3.0 1 H MRD data (36 R_{1} values) is not of the same high quality as the other fits to ¹H and ²H profiles from gelatin and agarose gels. Specifically, the model fails to account for a minor dispersion step with midpoint frequency near 200 kHz. We therefore incorporate an additional class of intermediary proton, labeled X and described by the two parameters $N_{\rm x} (D_{\rm x} S_{\rm x})^2$ and $\tau_{\rm x}$ (like the internal-water classes, this contribution is in the fast-exchange regime). The fit of this extended model is shown in Fig. 1f, along with the contributions from the different intermediary proton classes. The resulting parameter values are given in Table 2, and in the Discussion section we show that they are consistent with the corresponding ²H parameters and with other structural and kinetic data.

DISCUSSION

Validity of the EMOR Model

The simple expressions (Eqs. [1]–[9]) used to analyze the ¹H MRD data in Fig. 1 involve several approximations (Halle, this issue), which we shall now justify. The EMOR model is only valid in the dilute regime, where $f_I << 1$.

This condition may be checked with the aid of Eq. [2] and parameter values from Tables 1 and 2. The agarose dispersion is dominated by the most long-lived internal-water class (W3) with $f_{\rm W3} \approx 6 \times 10^{-4}$. The intramolecular ¹H order parameter for internal water molecules, $S_{\rm W,intra}$, is not likely to be much less than one (35), so the fractional populations of the other two internal-water classes are of order 10^{-3} (W1) and 10^{-4} (W2). For the hydroxyl contribution at pH 3.5, $f_{\rm OH} \approx 8 \times 10^{-3}$ if all hydroxyl groups are solvent-exposed (and less otherwise). For gelatin at pH 3.0, $f_{\rm OH} \approx 1 \times 10^{-3}$ and the internal-water populations are of order 10^{-3} (W1) and 10^{-4} (W2). All intermediary proton classes are thus in the dilute regime. This is also consistent with the exponential decay of the water-¹H magnetization observed in all cases.

Internal motions in the biopolymer or of internal water molecules have two effects. First, they modulate dipole couplings, giving rise to a relaxation dispersion with a correlation time τ_{int} . Second, they lead to partial orientational averaging of dipole couplings so that the slower, isotropic exchange randomizes the residual dipole coupling $D_{\rm I}S_{\rm I}$, rather than the full dipole coupling $D_{\rm I}$. In deriving the theoretical results used here, we neglected the first, direct relaxation effect (Halle, this issue). This approximation is valid if $(1 - S_{\rm I}^2) au_{
m int} \ll S_{\rm I}^2 au_{
m I}$ (Halle, this issue) (35), or if $\tau_{\rm int} \gg \tau_{\rm I}$ (since the dipole coupling is then already averaged to zero by exchange with bulk water). For the investigated gels, the main dispersion is associated with correlation times τ_{I} of order 10^{-6} – 10^{-4} s and S_{I} is of order unity. Hence, the approximation essentially requires that internal motions that modulate dipole couplings of intermediary protons are confined to time scales much shorter than 10^{-6} – 10^{-4} s. This is indeed expected to be the case, since most internal modes in structured biopolymers occur on subnanosecond time scales (40).

In the phenomenological two-pool model (4), all nonlabile biopolymer protons contribute equally to the water-¹H relaxation because magnetization is taken to be redistributed rapidly within the biopolymer proton pool by spin diffusion (i.e., coherent evolution under the static dipolar Hamiltonian). In the EMOR model, exchanging intermediary protons act as relaxation sinks for the water-¹H magnetization, and spin diffusion has little or no effect on the water-¹H relaxation (Halle, this issue). The nonlabile biopolymer protons play no role, except as dipole coupling partners if they happen to be located near an intermediary proton. Water-¹H magnetization will therefore not be transferred into the bulk of the nonlabile proton pool. For the gels investigated here, it is not even useful to speak about a nonlabile proton pool, since virtually all nonlabile biopolymer protons are close to an intermediary proton (Fig. 2). For the agarose double helix, all nonlabile protons have at least one intermediary proton within 3.7 Å, and for the collagen triple helix (the principal building block of the gelatin gel) 80% of the nonlabile protons have at least one labile proton within 5 Å.

Model Parameters Describing ¹H MRD Profiles From Agarose Gels

The parameter values resulting from the fit of the EMOR model to the ¹H MRD profiles from agarose gels (Fig. 1b



FIG. 2. Fraction nonlabile protons with at least one labile proton within a distance R in the agarose double helix (PDB file 1AGA with hydrogen atoms and internal water molecules added) and the collagen triple helix (PDB file 1QSU with hydrogen atoms added).

and c) are collected in Table 1. If the EMOR model describes the relaxation mechanism correctly, these values should be consistent with the physicochemical properties of the agarose gel. Moreover, these values should agree (except for modest H/D isotope effects) with the corresponding parameter values derived from the ²H MRD profiles shown in Fig. 1a and analyzed in detail elsewhere (31). The comparison of ¹H and ²H parameters is an important test of the EMOR model because ²H relaxation does not involve cross-relaxation or spin diffusion (30).

The dispersion profiles from agarose gels are strongly dominated by the most long-lived internal-water class (W3). For the residence time, τ_{W3} , of these water molecules, essentially the same value is deduced from the ¹H data (3.0 \pm 0.4 μs) and the 2H data (2.6 \pm 0.8 μs). In the 1H fit the population of this class was constrained to the value deduced from the ²H fit. The orientational order parameter, $S_{\rm W3,intra}$ = 0.79 ± 0.06, is slightly larger than the corresponding ²H value (0.64 \pm 0.03). This expected difference can be attributed to the different orientations in the water molecule of the quadrupole (along the O-H bond) and dipole (along the H-H line) coupling tensors (35). For the other two internal-water classes the residence times $\tau_{\rm W1}$ and $\tau_{\rm W2}$ and the quantities $\textit{N}_{\rm W1}\textit{S}_{\rm W1,intra}^2$ and $N_{\rm W2}S_{\rm W2,intra}^2$ agree to within a factor of 2.5 between the ¹H and ²H results. This small difference can be ascribed to H/D isotope effects and different coupling tensor orientations. These internal water molecules (classes W1-W3) are thought to reside in the central cavity of the agarose double helix (12) and/or may be trapped between double helices packed into agarose fibrils (13). The κ parameter, which is mainly determined by the W3 class, is 1.2 \pm 0.2. With the aid of Eq. [7] and Table 1, we thus infer that $D_{W3,inter}$ $S_{\rm W3,inter} = (2.2 \pm 0.4) \times 10^5 \text{ s}^{-1}$, which is also a lower bound for $D_{\rm W3,inter}$ (since $S_{\rm W3,inter} \leq 1$). If these internal water molecules occupy the central channel of the agarose double helix, as previously suggested (12), then there are several close proton neighbors and $D_{\rm W3,inter}$ is expected to be comparable to $D_{\rm W,intra} = 2.36 \times 10^5 \text{ s}^{-1}$, as indicated by our results.

Next, we consider the hydroxyl contribution at pH 3.5. The parameter $x_{\rm OH}$ / $\tau_{\rm OH}$ = 1.0 ± 0.2 ms⁻¹ is similar to the corresponding ²H value (2.4 \pm 0.1 ms⁻¹) and the small difference can be ascribed to H/D isotope effects. Most of the agarose fibrils in the gel contain six double helices (13), and if one of the four hydroxyl groups per agarobiose unit is solvent-inaccessible, as suggested by the structure (12), then $x_{\rm OH} = 0.25$ and we obtain $\tau_{\rm OH} = 0.25 \pm 0.05$ ms. This is not far from the value of 0.1 ms obtained with the rate constant (3 \times 10⁷ M⁻¹ s⁻¹) determined for acid-catalyzed proton exchange in glucose (37). The parameter $D_{\rm OH}$ $S_{\rm OH} = (1.3 \pm 0.2) \times 10^5 \, \rm s^{-1}$ provides a lower bound for the dipole coupling constant of the hydroxyl protons in agarose. From the structure of the agarose double helix (PDB file 1AGA with added hydrogen atoms and internal water molecules), we calculate D_{OH} values in the range of (0.7– 1.3) \times 10⁵ s⁻¹ for the four hydroxyl protons; however, these values would be somewhat larger in a fibril where there are also contributions from protons in adjacent double helices.

While the qualitative similarity between the ¹H and ²H profiles in Figs. 1a and 1b suggests a common molecular origin, there are substantial quantitative differences. These differences, notably the fivefold larger normalized dispersion amplitude, $R_1(0)/R_{\text{bulk}}$, and the twofold lower dispersion midpoint frequency for the ¹H profiles, are fully accounted for by the EMOR model. For both nuclides, the spin-lattice (dipole or quadrupole) coupling frequency is comparable to the exchange rate (the inverse residence time) of the dominant internal-water class (W3). This class is therefore outside the motional-narrowing regime of the conventional spin relaxation theory (Halle, this issue) (30). However, the ²H quadrupole coupling is somewhat stronger than the ¹H dipole coupling (by a factor 4.4 if we neglect differences in order parameters and intermolecular dipole couplings). The violation of the motional-narrowing (or fast-exchange) condition is therefore stronger for the ²H data, leading to a more pronounced attenuation of the dispersion amplitude and to a larger high-frequency shift of the dispersion profile (Halle, this issue) (30).

Model Parameters Describing ¹H MRD Profiles From Gelatin Gels

The parameter values resulting from the fit of the EMOR model to the ¹H MRD profile from gelatin gel at pH 3.0 (Fig. 1e and f) are collected in Table 2. As we have done for agarose, we now argue that these values are consistent with the properties of the gelatin gel and with the corresponding parameter values derived from the ²H MRD profiles shown in Fig. 1d and analyzed in detail elsewhere (Vaca Chávez et al., submitted).

At pH 3.0 the main ¹H dispersion (below ~100 kHz) is attributed to hydroxyl protons in the solvent-exposed amino acid side-chains (mainly Hyp, Ser, and Thr). The residence time deduced from the fit, $\tau_{OH} = 13.1 \pm 0.7 \mu$ s, corresponds to a rate constant, $k_1 = (7.6 \pm 0.4) \times 10^7 \text{ M}^{-1}$

s⁻¹, for acid catalyzed proton exchange. This is similar to the value of $k_1 = 4 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ reported for Ser and Thr in tetrapeptides at 4°C (41). The residual dipole coupling for the hydroxyl protons, $D_{\text{OH}} S_{\text{OH}} = (2.84 \pm 0.04) \times 10^4$ s⁻¹, is almost a factor of 5 smaller than for the agarose hydroxyl protons. From the structure of the collagen triple helix (PDB file 1QSU with hydrogen atoms added), we obtain $D_{\text{OH}} = 9 \times 10^4 \text{ s}^{-1}$ for the labile protons of hydroxyproline (which comprise 58% of the OH class), but D_{OH} should be smaller for the Ser and Thr side-chains. Furthermore, the order parameter, S_{OH} , is expected to be smaller in the relatively flexible gelatin side-chains than in the rigid pyranose rings of the agarose double helix.

Next, we consider the two classes of internal water molecules that are responsible for the high-frequency (1-100 MHz) dispersion (Fig. 1f). The parameters $N_{\rm W1}S^2_{\rm W1,intra}$ and $N_{\rm W2}S^2_{\rm W2,intra}$ are within 10% of the corresponding $^2{\rm H}$ values, but the uncertainties are sufficiently large to accommodate a kinetic isotope effect on the residence times τ_{W1} and τ_{W2} (which was not allowed for in the ¹H fit) and/or differences (due to anisotropic local water motions) between the ¹H and ²H order parameters (35). The κ parameter, 3.2 \pm 1.3, is larger than for the most long-lived internal waters in agarose (vide supra). This implies that the water contribution is dominated by intermolecular dipole couplings, which could be the case if the internal water molecules undergo large-amplitude internal motions that reduce S_{intra} more than S_{inter} (35). The internal water molecules responsible for the high-frequency ¹H and ²H dispersions are presumably associated with structural defects and branch points in the gel network (21,22).

Comparing Figs. 1d and 1e, we note significant differences between the corresponding ¹H and ²H profiles. As was the case for agarose, the ¹H data exhibit a larger normalized dispersion amplitude, $R_1(0)/R_{\text{bulk}}$, and a lower dispersion midpoint frequency. The origin of these differences is the same as for agarose: the larger spin-lattice coupling for ²H causes a stronger deviation from motionalnarrowing (fast-exchange) conditions, manifested in a more pronounced attenuation of the dispersion amplitude and a larger high-frequency shift of the dispersion midpoint (30). While the ¹H and ²H profiles vary in a similar way with pH, there are significant differences. In particular, the ¹H low-frequency dispersion is only slightly enhanced in going from pH 4.1 to 3.0 (due to faster acidcatalyzed hydroxyl proton exchange), whereas the ²H dispersion changes substantially between pH 4.2 and 3.1. The larger enhancement of the ²H profile reflects protonation of carboxyl groups (with pK_a 4.25), which contribute significantly to the ²H profile but not to the ¹H profile (vide supra). The origin of the relatively small contribution from intermediary proton class X (Fig. 1f) has not been identified, but it may be associated with a subgroup of the hydroxyl protons with faster proton exchange or in flexible single-chain segments. Class X cannot be internal water, since this contribution is not seen at pH 5.4 (Fig. 1e). The pronounced dispersions at pH 8.6 and pH 9.4 (Fig. 1e) are consistent with contributions from diffusion-controlled, base-catalyzed proton exchange in hydroxyl groups and NH groups of Arg and Lys. However, because of the necessity to model the internal motions in the flexible Arg and Lys side-chains, the model contains too many parameters to make meaningful fits to these profiles.

CONCLUSIONS

In the companion paper to this study (Halle, this issue) we developed a molecular theory for water-¹H spin-lattice relaxation in tissue and other aqueous systems with rotationally immobile macromolecules. This theory is based on the EMOR model, which links the relaxation dispersion to exchange-mediated orientational randomization of intermediary protons in internal water molecules or in labile biopolymer groups. In the present paper we demonstrate that the EMOR model accounts for an extensive set of ¹H MRD data from agarose and gelatin gels.

The ability to reproduce the MRD data is a necessary, but not sufficient, condition for accepting the model. The parameter values must also make physical sense. Our analysis shows that the parameter values deduced from the fit are fully consistent with the known properties of the gels. Moreover, the ¹H parameter values agree (when H/D isotope effects are taken into account) with the parameter values deduced from the ²H MRD profiles measured on the same gels. This agreement constitutes strong support for the EMOR model, since ¹H and ²H relaxation involve different nuclear couplings. In particular, this agreement supports theoretical arguments (Halle, this issue) indicating that collective biopolymer vibrations and coherent spin diffusion do not affect water-¹H relaxation from aqueous gels in a significant way.

The EMOR model provides a quantitative link between the observed water-¹H relaxation behavior and the specific chemical, structural, and dynamic properties of the investigated system. However, the relaxation mechanism physical exchange of trapped water molecules and chemical exchange of labile biopolymer protons—is ubiquitous. Here we have quantified the relative importance of these intermediary proton types for two widely used tissue models that produce MRD profiles similar to those observed in tissue. We believe that the EMOR model captures the essential mechanism of water-¹H relaxation in all aqueous systems with rotationally immobile biopolymers, including biological tissue.

ACKNOWLEDGMENT

We thank Hans Lilja for expert technical NMR support.

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