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Mobility of Water in Biological Systems Studied by ^{17}O NMR via Multiple-Quantum Filtered Relaxation Analysis

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Multiple-quantum filtering NMR sequences were used to study the multiexponential relaxation behaviour of $H_2{}^{17}O$ in the presence of macromolecules. By this means, the fraction and the correlation time of water in slow motion, or 'bound' water, were determined, in addition to the relaxation time of bulk water in the extreme narrowing limit. Experiments were carried out on aqueous solutions of bovine serum albumin, hen egg white lysozyme and intact human red blood cells in which intracellular water properties were studied. The small fraction of bound water of less than 1% seems to correspond to 'strongly bound' water, whereas the behaviour of bulk water was different from that of pure water and may be due to weak (or transient) interactions with macromolecules. The experiments and the data analysis gave reproducible results which support the idea that diverse samples could be studied in this way in order to help understand their water properties. © **1997 John Wiley & Sons, Ltd.**

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INTRODUCTION

In spite of the extensive literature concerning water properties in biological systems,[1–4] these properties are still not well understood. With regard to the results obtained by different methods, it is generally assumed that water, in presence of macromolecules, is composed of three phases: free water, whose properties are similar to those of pure water; weakly bound water, also called vicinal water, whose physical properties are different from those of both the solid and liquid states; and water bound to macromolecules. The fractions of these different types of water may be of considerable importance for the macroscopic properties of a sample, and particularly those of food products. They have different dynamic properties which may be studied by NMR spectroscopy.

The fluctuations of water relaxation observed by ^1H NMR are difficult to interpret, as the effects of proton exchange, cross-relaxation and hindered water rotation tend to modify the water relaxation properties simultaneously. In the same way, relaxation measurements by ^2H NMR are often perturbed by deuteron exchange. Proton and deuterium NMR were used by Grad and Bryant[5] to study the interactions of water with BSA. They found that direct dipolar interactions between water and protein are the predominant relaxation mechanism. Hills and Favret[6] performed a similar study, but with a different viewpoint, and found that water molecules hydrating the protein surface have a residence time of less than 1 ns, which is too short for cross-relaxation to be efficient. They concluded that the proton-exchange mechanism dominates the cross-relaxation of water.

Studies of water relaxation by ^{17}O NMR permit the selective analysis of the effect of reorientationaly hindered water on the overall relaxation process. It is then complementary to ^1H and ^2H NMR. Moreover, as ^{17}O has a spin number of $\frac{5}{2}$, its magnetization evolves multiexponentially when the correlation time of water, which characterizes its reorientation speed, becomes large. This can be shown via multiple-quantum filtering NMR sequences.[7] It should be noted that $H_2{}^{17}O$ can also be studied indirectly by ^1H $T_{1\rho}$ measurements.[8]

In this work, multiple-quantum filtering pulse sequences were used to study the multiexponential relaxation behaviour of $H_2{}^{17}O$ in presence of macromolecules. This was done for aqueous solutions of bovine serum albumin, lysozyme and red blood cells in

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different metabolic states and in osmotically altered sizes.

THEORY

The natural way to describe spins $I > \frac{1}{2}$ quadrupolar relaxation is to use the irreducible tensor operator formalism.[9] In this basis set, and for uncoupled spins in an isotropic medium, the magnetization components of odd and even rank vary independently. Then, the magnetization time course of a spin $\frac{5}{2}$ after a 90° or a 180° pulse can be described by a 3×3 relaxation matrix, called $\mathbf{R}'^{(1)}$ for transverse relaxation, of $\mathbf{R}'^{(0)}$ for longitudinal relaxation. The matrices $\mathbf{R}'^{(0)}$ and $\mathbf{R}'^{(1)}$, whose components are given in Ref. 10, are diagonal only if the correlation time of water satisfies the inequality $\omega_0 \tau_c \ll 1$. Elsewhere, in the slow-motion limit, the longitudinal and transverse magnetizations evolve triexponentially. If the spins are in fast chemical exchange between two sites with different relaxation behaviours, then the relaxation matrix will be the weighted sum of the two relaxation matrices of the two sites.[11]

Water, in the presence of macromolecules, can be considered to be composed of two populations: water in the extreme narrowing limit, which we shall call bulk water, and water bound to macromolecules in the slow motion limit. It should be noted that this definition of bound and bulk water is given for the technique employed; it is not necessarily the same as in the literature. These two populations are in fast chemical exchange. The relaxation matrix of water is then

$$\mathbf{R}'^{(n)}_{\text{water}} = p_b \mathbf{R}'^{(n)}_{\text{bound}} + p_f \mathbf{R}'^{(n)}_{\text{bulk}} \quad (1)$$

with $n = 0$ or 1. The relaxation matrix of bound water is non-diagonal, and so is the relaxation matrix of water in exchange between the two states; then, the magnetization of water evolves triexponentially. This multiexponential behaviour of transverse and longitudinal magnetization can be studied by standard one-quantum pulse sequences but it is more readily studied with triple-quantum filtering sequences.[7]

For a spin $I = \frac{5}{2}$, transverse magnetization is detected after a triple-quantum filtration via the following pulse sequence:

$$90^\circ_\phi\text{–}\tau_e/2\text{–}180^\circ_\phi\text{–}\tau_e/2\text{–}70.5^\circ_{\phi+90}\text{–}\tau_m\text{–}90^\circ_0\text{–Acq}(t_2) \quad (2)$$

with ϕ chosen to select the desired multiple quantum order[10] and τ_m as small as possible. In the same way, longitudinal magnetization can be studied via the following pulse sequence:

$$180^\circ_0\text{–}\tau_e\text{–}70.5^\circ_\phi\text{–}\tau_m\text{–}90^\circ_0\text{–Acq}(t_2) \quad (3)$$

The signal detected after these pulse sequences are called $s^{(1)}(\tau_e)s^{(1)}(t_2)$ and $s^{(0)}(\tau_e)s^{(1)}(t_2)$, respectively. The functions $s^{(0)}(t)$ and $s^{(1)}(t)$ are differences of exponential functions and are zero at $t = 0$. They can be studied in either the time or frequency domain. The easiest and fastest way to determine the $s^{(1)}(t)$ time course is to study the 'transverse' triple-quantum filtered signal in the time domain for a given value of τ_e. The function $s^{(0)}(t)$ is more easily studied by comparing the amplitude of the 'longitudinal' and 'transverse' triple-quantum filtered signals, after Fourier transformation. These functions, which are characteristic of the relaxation behaviour, can be deduced from the relaxation matrices[12] $\mathbf{R}'^{(1)}$ and $\mathbf{R}'^{(0)}$.

EXPERIMENTAL

Preparation of samples

In all the samples, water was not oxygen-17 enriched. Bovine serum albumin (Sigma–Aldrich, Castle Hill, NSW, Australia) was dissolved in deuterated water. Different amounts of a fresh 10 M solution of urea (BDH Chemicals, Kilsyth, Vic., Australia) in D_2O, a 200 g l^{-1} BSA solution and pure D_2O were added to obtain different samples with a BSA concentration of 85 g l^{-1} and a urea concentration from 0 to 5.5 M. Different amounts of hen egg white lysozyme from Fluka (L'Isle d'Abeau, France) were diluted in deuterated water. Unless stated otherwise, cell suspensions were prepared as described below. Human erythrocytes were obtained fresh by venipuncture from a healthy donor (E.B. or P.W.K.). Cells were either prepared for the NMR experiment immediately or washed twice in isotonic saline and stored overnight at 4 °C with 10 mM glucose. Prior to the NMR experiments, cells were prepared in the following manner: they were centrifugally washed (twice) in three volumes of ice-cold isotonic saline, then resuspended in D_2O saline with 10 mM glucose prior to being bubbled with carbon monoxide for 5 min, and packed by centrifugation. The cells were then ready for the NMR experiments. The haematocrit rate (Ht) of the cell suspension was determined in duplicate and the number of cells per millilitre of suspension was measured using a particle counter (Sysmex Medical Electronics, Kobe, Japan). The osmolality of the supernatant was measured with a vapour pressure osmometer (Wescor, Logan, UT, USA).

NMR

The ^{17}O NMR experiments on BSA and red blood cells were performed on a Bruker AMX400 wide-bore spectrometer, whereas for lysozyme the experiments were performed on a Bruker DPX400 spectrometer. In all cases, 3 ml of the sample were studied in a 10 mm o.d. NMR tube with a broadband probe at 54.25 MHz. The probe temperature was set at 298 ± 1 K for BSA and RBC and at 301 ± 0.1 K for lysozyme. The 90° pulse length was 19 μs for BSA solutions, 20.5 μs for red blood cells and 24 μs for lysozyme solutions. For all the triple-quantum filtered experiments, the evolution delay, τ_m, was kept to a minimum at 4 or 5 μs. On the AMX400 spectrometer, the data acquisition time was 21.3 ms, the time domain data contained 256 points, the spectral width was 6.0 kHz and the recovery delay was set to 21 ms. On the DPX400 spectrometer, the acquisition time was 69 ms, the recovery delay 7 ms and the time domain data contained 512 points. The pulse sequences employed were those described in the Theoretical section. In all the pulse sequences, no proton

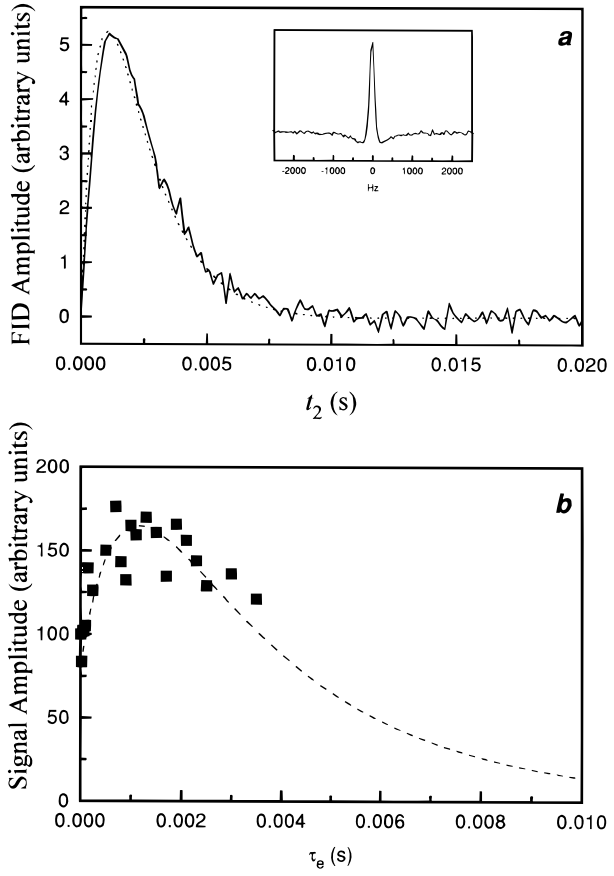

Figure 1. Simultaneous analysis of triple-quantum filtered longitudinal and transverse relaxation experiments. BSA concentration = 150 g l^{-1}; solvent D_2O; $T = 298$ K; $B_0 = 9.4$ T. The plotted theoretical curves correspond to a fraction of bound water of 0.0965% whose correlation time is 9 ns. (a) FID obtained after the transverse triple-quantum filtered sequence; $\tau_e = 1.4$ ms; 981 000 transients. The spectrum obtained after Fourier transformation of the FID is plotted in the inset. (b) Amplitude of the signal from the longitudinal triple-quantum filtered experiment plotted as a function of the delay τ_e; 65 400 transients per spectrum for each delay time.

decoupling was applied, since in preliminary experiments we observed that this did not modify the spectra. For the single-quantum experiments, the data acquisition time was set to 85 ms, the recovery delay to 1 ms and the number of points in the time domain data set was 1024. The three-quantum data analysed in the frequency domain were zero filled to 1024 points. Exponential line broadening of 20 Hz was applied prior to Fourier transformation. The peaks were characterized/quantified by their amplitudes. The data analysed in the time domain were transformed as ASCII files and studied on a power Macintosh. The longitudinal relaxation times were determined with the inversion–recovery pulse sequence. A one-pulse sequence permitted the determination of the water magnetization at thermodynamic equilibrium.

Data analysis

The relaxation rate R_{bulk} was deduced from the longitudinal relaxation rate measurement for various values of the parameters p_b and τ_c (the correlation time of bound water). The matrices $\mathbf{R}'^{(n)}_{water}$ were then calculated and diagonalized numerically, which permitted the determination of the functions $s^{(0)}(t)$ and $s^{(1)}(t)$ and then the signal detected after longitudinal and transverse triple-quantum filtered pulse sequences as a function of p_b and τ_c.

The comparison of theoretical simulations with experimental results permitted the determination of the fraction of bound water, its correlated time and the relaxation rate of bulk water (Fig. 1).

RESULTS

Solutions of BSA

BSA solutions of different concentrations were analysed. The number of bound water molecules per BSA was calculated for the different concentrations of BSA, deduced from the density of the solutions. The T_1 obtained for pure water is 5.7 ms, which is in good agreement with the value reported by Meiboom[13] (4 ms). The results are given in Table 1. The fraction of bound water and its correlation time increased when the BSA concentration was increased. However, the number of bound water molecules per BSA molecule decreased. At the same time, the relaxation rate constant of bulk water increased significantly for high BSA concentrations.

The effect of urea on water in a BSA solution was analysed in the same way, for a given concentration of BSA. The relaxation rate constant of bulk water and the correlation time of bound water did not change significantly for the different concentrations of urea. The fraction of bound water decreased significantly for urea concentrations higher than 4 M (Fig. 2).

Solutions of lysozyme

Lysozyme solutions of different concentrations were analysed in the same way as BSA solutions. The results are given in Table 2. As for BSA, R_{bulk} and τ_c increase at higher lysozyme concentrations. The fraction of bound water is of same order as for BSA. In fact, as the molecular weight of lysozyme is much smaller than that

Table 1. Water 'properties' for different BSA concentrations[a]

BSA concentration (g l^{-1})	R_{bulk} (s^{-1})	p_b (%)	τ_c (ns)	$n_{D2O/BSA}$
0	174	0		
50	226	0.062	5.7	40
85	248	0.076	7.6	30
150	289	0.096	9.0	21

[a] R_{bulk}, relaxation rate constant of bulk water; p_b, fraction of bound water; τ_c, correlation time of bound water; $n_{D2O/BSA}$, number of bound D_2O molecules per BSA molecule.

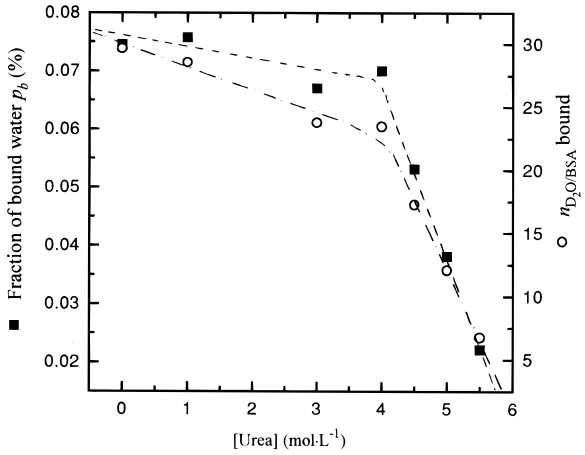

Figure 2. Effect of urea on bound water in BSA solutions of 85 g l^{-1} in deuterated water. (■) p_b, fraction of bound water (%); (○) number of water molecules bound per BSA molecule. For all the fitting curves, $R_{bulk} = 248$ s^{-1} and $\tau_c = 7.6$ ns. The lines are empirical fits to the data (to guide the eye).

Table 2. Water 'properties' for different lysozyme concentrations[a]

Lysozyme concentration [%(w/v)]	R_{bulk} (s^{-1})	p_b (%)	τ_c (ns)	$n_{D2O/lys}$
0	174	0		
20	200	0.098	3.9	3.8
30	249	0.096	5.4	2.5
40	300	0.14	8.3	2.7

[a] $n_{D_2O/lys}$, number of bound D_2O molecules per lysozyme molecule; other symbols as in Table 1.

Table 3. Intracellular-water properties of human erythrocytes with osmotically altered sizes[a]

[NaCl] (mM)	R_{bulk} (s^{-1})	p_b (%)	τ_c (ns)
110[b]	367	0.169	6.3
154[c]	407	0.221	6.3
257[d]	445	0.272	5.3

[a] Symbols as in Table 1.
[b] Corresponds to hypervolume erythrocytes.
[c] Corresponds to the physiologically normal red cell volume.
[d] Corresponds to hypovolume erythrocytes.

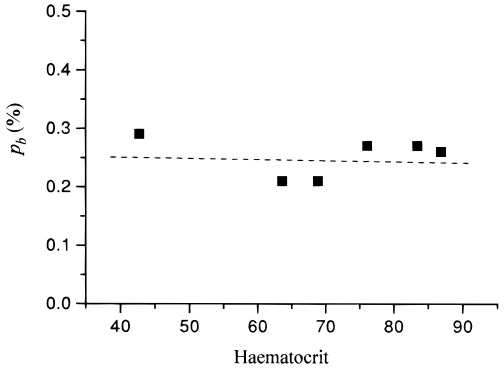

Figure 3. Fraction of intracellular bound water in human erythrocytes as a function of the Ht. The last wash of the cells was in isotonic D_2O saline, [NaCl] = 145 mM.

of BSA, this corresponds to a small number of bound water per lysozyme.

Red blood cells

The same analysis was performed on RBC of physiological volume for a very high haematocrit rate value. Extracellular water was then neglected in the measurements. A global analysis (study of both longitudinal and transverse relaxation) was done on red blood cells with osmotically altered sizes. Significant fluctuations of the bound-water fraction were observed (Table 3). The parameters p_b, R_{bulk} and τ_c obtained for red blood cells washed in an isotonic saline solution were close to those obtained for a haemoglobin solution of 28% (w/v).

The correlation time of intracellular bound water found in this way was supposed to be independent of Ht. It was also assumed that the intra- and extracellular bulk water were in fast chemical exchange and that the relaxation rate constant of extracellular bulk water, in the absence of water transport, would be the same as for pure water, R_{pure}. Then, the relaxation rate constant of intracellular bulk water is given by

$$R_{bulk} = p_{int} R^0_{bulk} + (1 - p_{int}) R_{pure}$$

where R^0_{bulk} is the relaxation rate that intracellular bulk water would have in the absence of any exchange with extracellular water and p_{int} is the fraction of intracellular water. The fraction of intracellular bound water was then deduced from the analysis of the signal obtained after a transverse-relaxation triple-quantum filtered experiment. The fraction did not vary significantly with Ht and was approximately 0.25% (Fig. 3).

DISCUSSION

Water mobility was studied in BSA and lysozyme solutions of different concentrations. The number of bound water molecules found in this way was between 20 and 40 for BSA and between 2 and 4 for lysozyme, i.e. very small in the latter case. This may correspond to structural water. This structural water has a correlation time of about 10 ns, which is much longer than the residence times previously found by Hills and Favret,[6] which were shorter than 1 ns. This is because the water whose magnetization evolves triexponentially necessarily has a correlation time long enough for it to be in the slow motion limit. Therefore, we studied selectively heavily bound water, whereas studies performed by proton and deuterium NMR concern both structural and vicinal water. The structural water has a correlation time long enough for dipolar couplings between water and the macromolecules to modify proton magnetization. It would be interesting to establish whether dipolar couplings may modify in an important manner the global proton relaxation, as Grad and Bryant[5] suggested. However, probably the structural water molecules are insufficient for that.

Urea added to BSA plays two roles, it unfolds the protein and replaces water bound to BSA. When the

urea concentration was higher than 4 M, the fraction of bound water decreased significantly. The signal measured after a triple-quantum filtered sequence was then very small. This result implies that at the magnetic field strength used for the studies (9.4 T), only 'strongly' bound water was detected.

The data from experiments on whole cells were more difficult to interpret. The chemical exchange between intra- and extracellular water could modify the results, as could the interaction of external water with the outer surface of the membranes. However, we have found that the water properties of red cells of physiological volume correspond to a solution of haemoglobin of 28% (w/v) and that the fraction of intracellular bound water did not vary significantly for different Ht values. These results appear to be consistent with the model employed for the data analysis.

For the different biological systems employed, the estimated fraction of bound water corresponds to the water which has been previously called 'strongly bound'. The relaxation rate constant of bulk water is different from that of pure water. This value can be interpreted as a characteristic of vicinal water, which interacts weakly with macromolecules. It can be interpreted as an average of the relaxation rate constants of vicinal water and pure water in fast exchange. The knowledge of the relaxation rate constant of vicinal water could permit the determination of its correlation time. It is not possible to determine simultaneously the fraction of vicinal water and its correlation time from the data obtained by our analysis. However, the knowledge of one of these two parameters would permit the deduction of the other.

CONCLUSION

The simultaneous analysis of data from transverse and longitudinal triple-quantum NMR experiments permits the determination of the fraction of the total water that is bound to macromolecules, and their correlation times, either in protein solutions or in intact cells. The experiments are complementary to those with ^{1}H NMR using more conventional relaxation analysis. The quantitative method employed here should be useful for characterizing the properties of water in solutions of synthetic and biological macromolecules and in cells. It can be employed on solutions of protein with various sizes (BSA, lysozyme) and in heterogeneous systems (red blood cells). It could then be used successfully to study water properties in food samples.

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