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Multiple Quantum Filtered NMR Studies of the Interaction between Collagen and Water in the Tendon

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Abstract: We studied the physical processes and the chemical reactions involved in magnetization transfer between water and large proteins, such as collagen, in bovine Achilles tendon. Since the NMR spectrum for such proteins is broadened by very large dipolar interactions, the NMR peaks of the various functional groups on the protein cannot be separated from one another on the basis of their different chemical shifts. A further complication in observing the protein spectrum is the intense narrow peak of the abundant water. Thus, magnetization transfer (MT) within the protein or between water and the protein cannot rely on differences in the chemical shifts, as is commonly possible in liquids. We present a method that separates the protein spectrum from that of the water spectrum on the basis of their different intramolecular dipolar interactions, enabling exclusive excitation of either the protein or water. As a result, the protein spectrum as well as the effect of spin diffusion within the protein can be measured. In addition, the MT rates from the protein to water and vice versa can be measured. Two types of mechanisms were considered for the MT: chemical exchange- and dipolar interaction-related processes (such as NOE). They were distinguished by examining the effects of the following experimental conditions: (a) temperature; (b) pH; (c) ratio of D_2O to H_2O in the bathing liquid; (d) interaction of the protein with small molecules other than water, such as DMSO and methanol. Our results lead us to the conclusion that the MT is dominated below the freezing point by the dipolar interaction between the protein and water, while an exchange of protons between the protein and the water molecules is the most significant process above the freezing point. On the basis of the fact that the spin temperature is established for the protein on a time scale much shorter than that of the MT, we could measure protein spectra that are distinguished by the contributions made to them by the various functional groups; i.e., contributions of methylenes were distinguished from those of methyls.

Introduction

The role of proton exchange between water and proteins has been the subject of extensive research over the past few years. The interaction between collagen and its water of hydration is of special importance since the mechanical properties of connective tissues such as cartilage, tendons, and ligaments are dependent upon this interaction.

Selective excitation of either the protein or water is required in order to measure the rate of magnetization transfer (MT) between water and the protein in tissues, such as a bovine Achilles tendon. For solutions containing small proteins, the methods implemented for selective excitation had been based upon techniques used to study proton exchange in liquids wherein the NMR peaks of various functional groups and water could be distinguished by their different chemical shifts, enabling measurement of exchange rates between the various functional groups and water. For large proteins, such as collagen, however, the NMR spectrum is broadened by very large dipolar interactions while the abundant water signal is narrowed by motion. Thus, the NMR peaks of the various functional groups on the protein cannot be separated from one another and are masked by the intense water peak. Consequently, MT within the protein or between water and protein cannot rely on differences in the chemical shifts, as is commonly possible in liquids. The problem of measuring MT in systems where the spectrum is broadened by dipolar interactions is usually dealt with by measuring saturation transfer.[1−8] In this method, rf power is applied off the water resonance, causing the broad protein spectrum to become saturated. An attenuation of the water peak is observed as a result of MT to water. Analysis of the results of this method requires the subtraction of the effect of the rf on the water peak and relies on a priori knowledge of the line shapes of water and of the protein.[6−8] In the current

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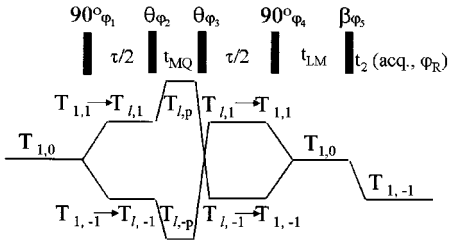

Figure 1. MQF-MT pulse sequence where MQF is ZQF, DQF, or TQF for $p = 0$, 2, and 3, respectively. The coherence pathways used were $p = 0$ or 2 for $l = 2$ and $p = 3$ for $l = 3$. $\theta = 45°$, $\beta = 90°$ for $p = 0$; $\theta = \beta = 90°$ for $p = 2$; $\theta = 90°$, $\beta = 63.4°$ for $p = 3$. A phase cycling of $16*l^2$ steps was used: $\varphi_1 = j \times 180°/l$, $\varphi_2 = j \times 180°/l + k \times 90° + \varphi_c$, $\varphi_3 = k \times 90° + m \times 180°/l + \varphi_c$, $\varphi_4 = m \times 180°/l$, $\varphi_5 = 0°$, $\varphi_R = (j + k + m) \times 180°$ for $p = 2$, 3 and $\varphi_R = k \times 180°$ for $p = 0$ (j, $m = 0$, 1, 2, .., $2l - 1$, $k = 0$, 1, 2, 3), $\varphi_c = 90°$ for $p = 0$, 3 and 0° for $p = 2$.

work, we present a method that avoids these difficulties. Our method separates the protein spectrum from that of the water spectrum on the basis of their different intramolecular dipolar interactions, thereby making it possible to excite either the protein or the water independently. This enables MT from the protein to water and vice versa to be detected directly without any need for modeling. The question of spin diffusion and the establishment of the spin temperature within the protein are also studied, and the application of the latter phenomenon to the measurement of the protein spectrum is demonstrated.

Theoretical Background

For large proteins in which the overall and the internal motions are slow or anisotropic, dipolar interactions between protons are not scaled down to a significant degree and high rank spherical tensors that involve two or more protons can be formed on a very short time scale. In water, these tensors evolve over a much longer time scale due to the reduction of the intramolecular dipolar interaction, which results from the reorientational motion in the free and bound states as well as from the chemical exchange between these two states. Furthermore, proton exchange among water molecules causes an additional reduction of the proton−proton intramolecular dipolar interaction. The different time scales of the evolution of the high rank tensors in the protein and in water make it possible to retain the longitudinal magnetization of either the protein or water using the multiple quantum filtered MT (MQF-MT[9]) pulse sequence shown in Figure 1. Retaining the magnetization of only one of the two species enables the detection of the MT between them.

The single rank single quantum coherences, $T_{1,\pm1}$, obtained after the application of the first 90° pulse evolve during the period $\tau/2$ and become second rank tensors, $T_{2,\pm1}$, that are filtered through a double quantum coherence (DQF), $T_{2,\pm2}$. The filtered tensors reconvert to $T_{2,\pm1}$ by the application of the third pulse and then the latter tensors evolve into $T_{1,\pm1}$, during the second $\tau/2$ period. For short creation time, $\tau/2$ (Figure 1), the amplitude of the observed signal was found[9] to be proportional to ω_D^2, where ω_D is the first-order (residual) dipolar interaction. Thus, since ω_D of the protein is much larger than that of water, the intensity of the protein signal can become either comparable to or larger than that of the water signal.

From the above discussion it is clear that all the relaxation times of the single quantum coherence tensors of the protein are expected to be much shorter than those of water and they also relax on a time scale that is much shorter than the chemical exchange between water and exchangeable protons on the protein.[10−12] Thus, it is impossible to observe the formation of single quantum coherences of the protein by chemical exchange with water and vice versa. However, given that the relaxation times of the longitudinal magnetization can be long for both water and the protein, the reconversion of the DQF signal to its initial state (aligned along the magnetic field) enables the detection of chemical exchange between water and protein. It is important to notice that in the above discussion it is implicitly assumed that only a single proton is exchanged between a single water molecule and the protein. For the longitudinal component of the magnetization, it was shown[10,11] that the following evolution matrix describes quantitatively the MT process:

$$\begin{pmatrix} a_+e^{-t/T_{1+}} + a_-e^{-t/T_{1-}} & b(e^{-t/T_{1-}} - e^{-t/T_{1+}}) \\ \frac{p_w}{p_p} b(e^{-t/T_{1-}} - e^{-t/T_{1+}}) & a_-e^{-t/T_{1+}} + a_+e^{-t/T_{1-}} \end{pmatrix}$$

$$M_{zp} = m_{zp}(t=0)(a_+e^{-t/T_{1+}} + a_-e^{-t/T_{1-}}) + m_{zw}(t=0)b(e^{-t/T_{1-}} - e^{-t/T_{1+}})$$

$$M_{zw} = m_{zp}(t=0)\frac{p_w}{p_p} b(e^{-t/T_{1-}} - e^{-t/T_{1+}}) + m_{zw}(t=0)(a_-e^{-t/T_{1+}} + a_+e^{-t/T_{1-}})$$

$$a_\pm = \frac{1}{2}\left[1 \pm \frac{R_{1p} - R_{1w} + k - 2p_pk}{\sqrt{(R_{1p} - R_{1w} + k)^2 - 4p_pk(R_{1p} - R_{1w})}}\right]$$

$$b = \frac{p_pk}{\sqrt{(R_{1p} - R_{1w} + k)^2 - 4p_pk(R_{1p} - R_{1w})}}$$

$$\frac{1}{T_{1\pm}} = \frac{1}{2}[R_{1p} + R_{1w} + k \pm \sqrt{(R_{1p} - R_{1w} + k)^2 - 4p_pk(R_{1p} - R_{1w})}] \quad (1)$$

where M_{zp}, M_{zw}, $m_{zp}(t=0)$, and $m_{zw}(t=0)$ are the longitudinal magnetizations and their values at $t = 0$ for the protein and the water, respectively. $R_{1p} = 1/T_{1p}$, $R_{1w} = 1/T_{1w}$, with T_{1p} and T_{1w} being the longitudinal relaxation rates of the protein and water, respectively. The fractions of the protons residing on the protein and in water are given by p_p and p_w, respectively. The MT rate, k, is the sum of the forward and backward reactions rates of the MT process. From eq 1, it is clear that if either the protein or water is exclusively excited, the other is formed in a process described by a difference of two exponents. Under fast MT, i.e., $k \gg R_{1p}$, R_{1w}, the expressions in eq 1 simplify to

$$\begin{pmatrix} p_we^{-t/T_{1+}} + p_pe^{-t/T_{1-}} & p_p(e^{-t/T_{1-}} - e^{-t/T_{1+}}) \\ p_w(e^{-t/T_{1-}} - e^{-t/T_{1+}}) & p_pe^{-t/T_{1+}} + p_we^{-t/T_{1-}} \end{pmatrix}$$

For the protein selective excitation $m_{zw}(t=0) = 0$, and

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$$M_{zp} = m_{zp}(t=0)(p_w e^{-t/T_{1+}} + p_p e^{-t/T_{1-}})$$

$$M_{zw} = m_{zp}(t=0)p_w(e^{-t/T_{1-}} - e^{-t/T_{1+}})$$

$$\frac{1}{T_{1+}} = k, \quad \frac{1}{T_{1-}} = p_p R_{1p} + p_w R_{1w} \qquad (2)$$

That is, the formation rate of the signal is given by the MT rate, and its decay rate is a weighted average of the longitudinal relaxation rates of the protein and water, respectively. As can be seen from eq 2, if one selectively excites the protein or water under the condition of fast exchange, on a time scale shorter than that of the longitudinal relaxation, the sum of the magnetizations of water and the protein equals that of the initially excited magnetization. Clearly, the protein and water magnetizations vary considerably on this time scale; i.e., magnetization transfer dominates the dynamics on a time scale shorter than that of the longitudinal relaxation. So we conclude that under fast exchange we can divide the time scale into a short one when exchange takes place and a long one when relaxation dominates.

Experimental Section

The experiments were conducted on a bovine Achilles tendon under various conditions of temperature, pH, deuteration levels, and concentrations of DMSO and methanol. Measurements were carried out on a Bruker ARX 500 NMR spectrometer using a 5-mm probe with double resonance capabilities for deuterium and proton. For this probe, the 90° pulse lengths were 9 μs for protons. For experiments where shorter pulses were needed, a Bruker 360 WB Avance spectrometer with a 5-mm polarization enhancement (PE) probe was used, giving 90° pulses of 2.5 μs.

Results and Discussion

a. Selective Excitation and the Establishment of Spin Temperature for the Protein. As explained in Theoretical Background, selective excitation of either the protein or water is required in order to measure MT rate between water and the protein in tissues, such as a bovine Achilles tendon. This goal was achieved by the pulse sequence (MQF-MT) shown in Figure 1. The selective excitation is demonstrated in Figure 2, where spectra obtained for five values of $\tau/2$ and very short t_{LM} are shown. For very short $\tau/2$ ($\leq$50 μs), the broad (tens of kilohertz) protein spectrum is the dominant component. On the other hand, for $\tau/2 = 90$ μs, this component is very small while the water signal with a line width of less than 1 kHz makes the more significant contribution.

The chain of processes following the selective excitation of the protein with a short value of $\tau/2$ (7 μs) is shown in Figure 3. For very short t_{LM} ($\leq$1 ms), where the whole spectrum consists mainly of that of the protein, the line shape changes significantly with t_{LM} while the integral remains practically constant. These results indicate spin diffusion, i.e., establishing spin temperature for the protein at t_{LM} of $\sim$1 ms, after which the protein line shape does not change. At longer t_{LM} values, the water signal builds up as a result of MT processes from the protein to water.

A demonstration of the spin diffusion process within the protein in the absence of MT to water is obtained by measuring the signal intensity of the tendon immersed in D_2O solution (Figure 4) as a function of t_{LM}. The figure clearly demonstrates

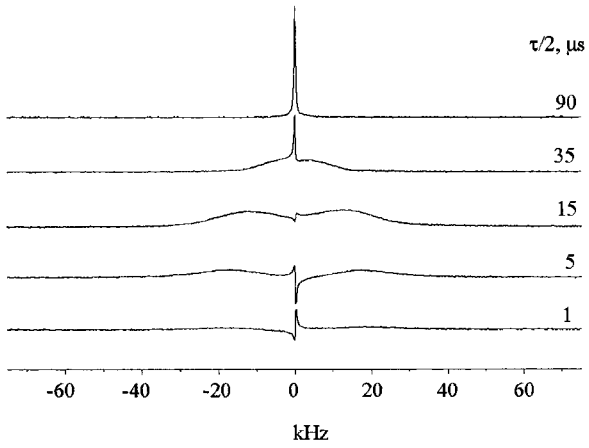

Figure 2. Spectra obtained by the DQF-MT pulse sequence given as a function of $\tau/2$ with $t_{LM} = t_{DQ} = 2$ μs. The measurements were carried at frequency of 360 MHz using a high-power probe with a 90° pulse of 2.3 μs. The number of accumulations was 64. The sample temperature was 37 °C.

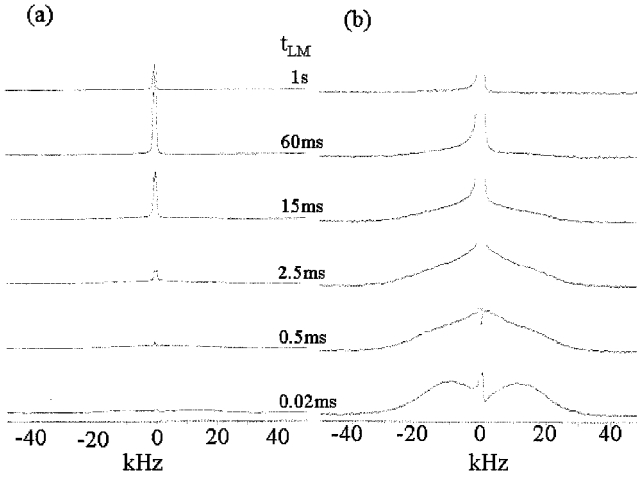

Figure 3. Spectra obtained by the DQF-MT pulse sequence for various values of t_{LM} with $\tau/2 = 7$ μs and $t_{DQ} = 5$ μs. The spectra given in (a) and (b) are the same but those in (b) are magnified by a factor of 6 relative to those in (a). The measurements were carried out at a frequency of 500 MHz using a 90° pulse of 9 μs. The number of accumulations was 64. The sample temperature was 1.5 °C.

that for $t_{LM} \leq 0.5$ ms the line shape of the protein changes significantly, while the changes are very small for $t_{LM} = 0.5-5.0$ ms, indicating the establishment of spin temperature. A similar conclusion is obtained from studying the dependence of the MQF-MT spectral line shapes as a function of the period τ for the above two ranges of t_{LM}. While for $t_{LM} \leq 0.5$ ms the shape changes very significantly (Figure 2), for $t_{LM} = 0.5-5$ ms the line shape hardly changes and only the spectral intensity varies with τ (figure not shown).

b. Direct Evidence of Magnetization Transfer between Collagen and Water. As can be seen from Figure 3, water signal is formed as a result of MT from the protein to water after the establishment of spin temperature. To quantitatively study the MT from the protein to water, their spectral integrals were separated using a cubic spline procedure. The resulting integrals as a function of t_{LM} are given in Figure 5. For periods much shorter than the longitudinal relaxation time, the integral over the range of frequencies that covers both the protein and the water spectra remains almost unchanged (Figure 5). As mentioned above, these results indicate a transfer of magnetiza-

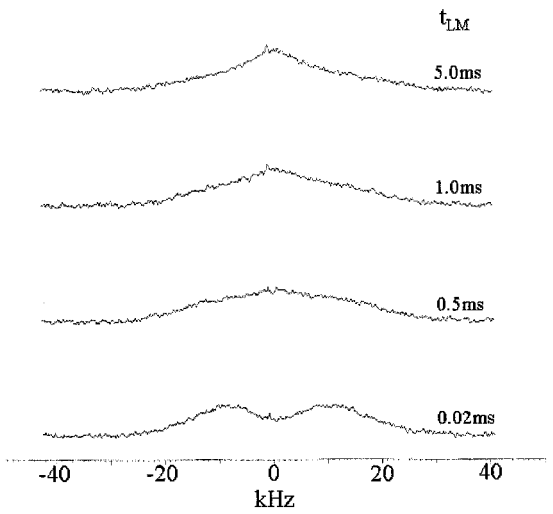

Figure 4. Spectra of bovine Achilles tendon immersed in D_2O as obtained by the DQF-MT pulse sequence for various values of t_{LM} with $\tau/2 = 7$ μs and $t_{DQ} = 5$ μs. The measurements were carried out at a frequency of 500 MHz using a 90° pulse of 9 μs. The number of accumulations was 64. The sample temperature was 1.5 °C.

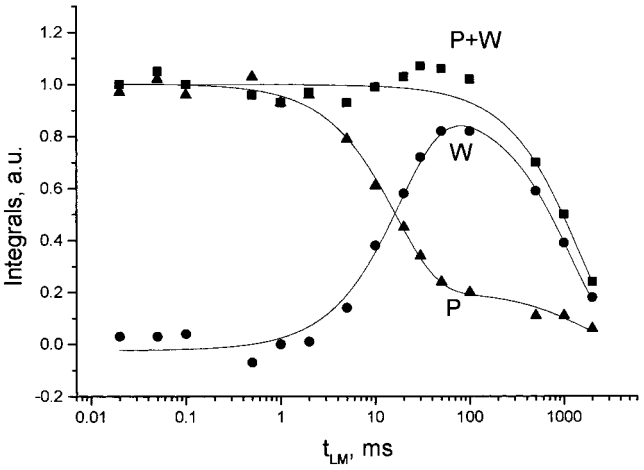

Figure 5. Integrals of water (W, ●) and the protein (P, ▲) spectra as measured by the DQF-MT pulse sequence shown as a function of t_{LM}. P + W (■) is the sum of the two integrals. The pulse sequence parameters are given in the caption to Figure 3. The sample temperature was 38 °C. The data fitted biexponential expressions (solid lines; see also text) with the parameters given in Table 2.

tion from the protein to water that occurs on a time scale shorter than T_1. This MT process is described by eq 2, which predicts a biexponential decay of the protein and a concomitant biexponential rise and decay of the water signal. The results of fitting the amplitudes of both the protein and the water spectra at $\tau/2 = 7$ μs and at two temperatures to eq 2 are given in Table 1. As can be seen from the table, the rates obtained for the decay of the protein signal k(p) are similar to the rates of the water signal formation k(w) within the experimental error.

Another measurement that verifies that the water signal in the DQF-MT experiment with a short creation time ($\tau/2 = 7$ μs) originates from the protein magnetization is the dependence of the intensity of the water signal on the double quantum period, t_{DQ}. A very fast decay was observed. Fitting the decay curve to exponential or Gaussian decay showed a better fit to the latter with a decay time of $T_{DQ} = 13$ μs (Table 1). This is typical of solids and is similar to the T_{DQ} decay time found for the protein

Table 1. Magnetization Transfer Rates (k), Longitudinal Relaxation Times (T_1), and Double Quantum Relaxation Times (T_{DQ}) for Water (w) and the Protein (p) at Two Different Temperatures[1]

creation time, τ, μs	k(p), s^{-1}	T_1(p), s	T_{DQ}(p), μs	k(w), s^{-1}	T_1(w), s	T_{DQ}(w), μs
			Temperature 1.5 °C			
14	25	0.9	14	26	0.81	14
1360	19	0.9	400	32	0.8	600
			Temperature 26 °C			
7	40	1.4	13	36	1.2	14
680	50	1.1		50	1.2	450

[1] The experimental errors for k(w), T_1(w) and T_{DQ}(w) are ±9% and for k(p), T_1(p) and T_{DQ}(p) are ±30%.

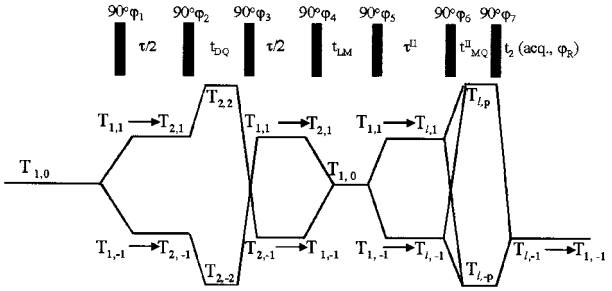

Figure 6. DQF-MT pulse sequence appended by a pair of 90° pulses that act as DQ ($l = p = 2$) or TQ ($l = p = 3$) filters. $\varphi_5 = n \times 180°/l$ ($n = 0$, 1, .., $2l - 1$), $\varphi_6 = \varphi_5 + \varphi_c$, $\varphi_c = 0°$ for $p = 2$ and 90° for $p = 3$, $\varphi_1 - \varphi_4$ are given in the caption to Figure 1.

that was measured when the exchange period (t_{LM}) was selected to be very short (Table 1).

A reverse MT from water to the protein was observed in the DQF-MT experiment with long $\tau/2$ (680 μs). The kinetics of the system was similar to that of the experiment with short τ, with the role of the protein and water interchanged. Indeed, the decay of the water signal gives a biexponential decay with exchange rate constants (the sum of forward and backward reactions involved in the MT process) similar to those measured for a short creation time (Table 1). A biexponential formation of the protein magnetization was measured with rates similar to those obtained from the formation of the water signal in the experiment with the short creation time. However, due to the presence of the intense water signal, the accuracy of the measurement was limited. To overcome this difficulty, the pulse sequence in Figure 1 was modified to include an additional pair of pulses that act as double quantum filter (Figure 6, $l = p = 2$). The formation of the protein signal is clearly evident (Figure 7). Thus, on the basis of the above results, we conclude that two types of dynamic processes occur in the tendon tissue. The first one occurs on a time scale of tens to hundreds of microseconds and involves spin diffusion within the protein, and the second one involves MT between the protein and water occurring on a time scale of hundreds of microseconds to tens of milliseconds.

c. Examining the Mechanism of MT. Two types of mechanisms can account for the observed MT. One is an exchange of exchangeable protons on the protein and on the water molecules, and the other one is mediated by a dipolar interaction. The latter mechanism can be either of the first order, where the dipolar interaction between the protein and water protons is not averaged out by motion (a situation commonly found in solids), or of the second order, commonly found in

Table 2. Temperature Dependence of the Magnetization Transfer Rate, Longitudinal Relaxation (T_1), Line Width ($\Delta\omega_{1/2}$), Observed Dipolar Splitting ($2\nu_D$), and Ratio between the Amounts of Protein and Water (m_p/m_w)[a]

	temperature, °C								
	−72	−60	−47	−27.5	−14	−6.7	1.5	13	38
k(w), s^{-1}	2100	770	480	140	52	20	26	33	65
T_1(w), s	2.4	1.15	0.95	0.63	0.48	0.73	0.81	0.93	1.1
$\Delta\omega_{1/2}$ (w), Hz	6800	2500	700	420	400	200	230	−	280
$2\nu_D$ (w), Hz	−	2780	2900	2800	2600	1000	950	890	−
m_p/m_w	−	−	1.1	0.92	0.77	0.25	0.25	0.25	0.22

[a] The experimental errors of k, T_1, $2\nu_D$, m_p/m_w, and $\Delta\omega_{1/2}$ are ±8% for temperatures above −60 °C and ±15% for −60 and −72 °C.

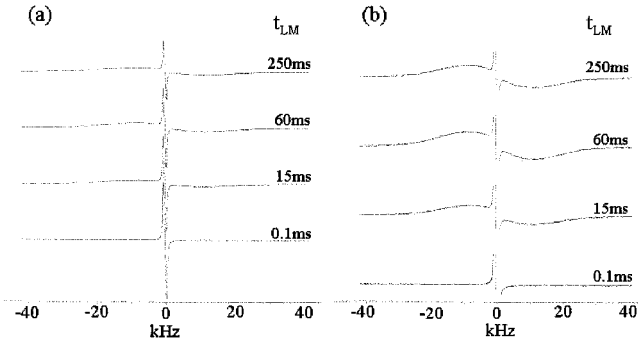

Figure 7. Spectra obtained by the pulse sequence shown in Figure 6 (for $p = 2$) given as a function of t_{LM} with $\tau/2 = 680$ μs and $t_{DQ} = \tau^{II} = t^{II}_{DQ} = 6$ μs. The spectra given in (a) and (b) are the same, but those in (b) are magnified by a factor of 3 relative to those in (a). The measurements were carried out at a frequency of 500 MHz using a 90° pulse of 9 μs. Number of accumulations was 1024. Sample temperature was 26 °C.

Table 3. Effect of pH on the Magnetization Transfer Rate from the Protein to Water at Two Different Temperatures[1]

	Temperature, 1.5 °C					
pH	4.78	5.13	5.62	6.3	7.02	7.55
k(w), s^{-1}	24	20	18	17	23	27
	Temperature, 38 °C					
pH	4.78	5.13	5.62	6.3	7.02	7.55
k(w), s^{-1}	54	47	52	50	65	72

[a] The experimental error for k is ±8%.

Table 4. Magnetization Transfer Rate from the Protein to Water Protons as a Function of Temperature and Percentage of Water Protons in the Bathing Saline H_2O/D_2O Mixtures[a]

temperature, °C	−27.5		26		
H_2O, %	100	15	100	50	3
k(w), s^{-1}	130	110	40	18	7

[a] The experimental error for k is ±8%.

liquids (NOE). The two MT mechanisms are expected to depend differently on the following experimental conditions: (a) temperature; (b) pH; (c) ratio of D_2O to H_2O in the solvent; (d) the interaction of the protein with small molecules other than water, such as DMSO and methanol. The effects of these conditions on the MT rate, the longitudinal relaxation time, the line width, the spectral splitting, and the integrals of the various components in the system are given in Tables 2−5. The value of the MT rate (Table 2, Figure 8) at room temperature agrees with the values measured by Edzes and Samulski[10,11] and by Renou et al.[13,14] Upon lowering the temperature, the MT rate decreases, but near the freezing point, which occurs around −7 °C,[15−17] there are abrupt increases in the MT rate, in the water line width, and in the splitting of the water signal. These changes around the freezing point are mostly due to the decrease in the amount of the free water molecules, a condition that leads to an increase of the fraction of bound water. This is reflected in the ratio of the spectral integrals of the protein (m_p) and the water (m_w) (m_p/m_w in Table 2). Upon lowering the temperature below the freezing point, there is a sharp increase in both the MT rate and the line width. This is consistent with an increase in the reorientation and residence times of the water molecules at the protein surface, which is expected to cause an increase in the MT rate due to NOE as well as an increase in the transverse relaxation rate due to the spectral density $J_o(0)$. Above the freezing point, increasing the temperature causes the MT rate to increase. This is consistent with proton exchange between the protein and water molecules.[12−14,18−21] The transverse

Table 5. Magnetization Transfer Rate (s^{-1}) from Protein To Water, DMSO and Methanol as a Function of Temperature[a]

temperature, °C	−19	37
H_2O	70	85
DMSO	200	0.8
methanol	50	1.7

[a] The experimental errors in the MT rates for water, DMSO, and methanol are ±8%, ±35%, and ±20%, respectively. See text for the composition of the bathing liquids.

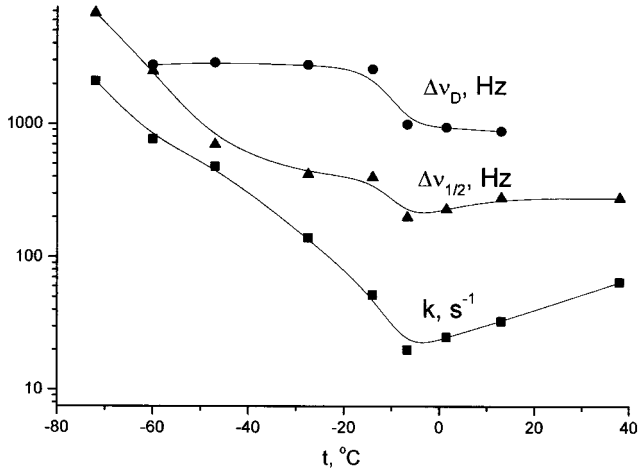

Figure 8. Temperature dependence of the MT rate (k), the spectral width at half-height ($\Delta\nu_{1/2}$), and the dipolar splitting ($\Delta\nu_D$) measured in bovine Achilles tendon.

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relaxation rate decreases at this temperature range due to the decreased correlation time and the larger proton exchange rate among the water molecules.[9]

The MT rate is given as a function of the pH for two temperatures in Table 3. It is evident that a minimum is obtained for pH ~6 above the freezing point. Such a minimum was observed previously for amino acids.[19−21] However, the depth of this minimum is much shallower in the tendon—a fact that we attribute to the heterogeneity of the system. Lowering the temperature below the freezing point causes the MT rate to increase and to become pH independent, which is consistent with the MT process becoming dipolar interaction dependent.

The dependence of the MT rate on the volume percent of H_2O in the H_2O/D_2O solutions is given in Table 4. The MT rate is strongly dependent on this percentage at temperatures above the freezing point, while at lower temperatures it is almost independent of it. These results also support the two mechanisms, discussed previously, for the MT between the water molecules and the protein. It is clear that below the freezing point where motion is restricted and chemical exchange is slow, the NOE is expected to be the dominant MT process and the rate of the MT, k, should not depend on the H_2O/D_2O ratio. On the other hand, above the freezing point, the NOE is expected to decrease while the chemical exchange increases and becomes the dominant MT process. The strong dependence of this mechanism on the H_2O/D_2O ratio can be understood on the basis of the following: k is the sum of the forward (k_{off}) and backward (k_{on}) MT rates; i.e., $k = k_{off} + k_{on}$. Both k_{off} and k_{on} are expected to decline with increasing percentage of D_2O. The replacement of exchangeable protons on the protein with deuterons decreases the number of sites available for magnetization transfer and thus causes a decrease of k_{off}. Also, k_{on}, which comprises proton exchange from the bulk with either proton or deuteron on the protein, is expected to decrease with an increase in the fraction of D_2O due to the isotope effect. Replacing water in the tendon by a mixture of 5% DMSO and 95% water enabled the detection of MT processes between the protein and a small molecule that does not have exchangeable protons. It is interesting to note that, in the anisotropic environment of the tendon, the spectrum of the methyl group of the DMSO appears as three peaks (Figure 9) separated by 270 Hz. While the separation is not well-resolved in the single pulse experiment (Figure 9, spectrum on the left), it is clearly resolved in the antiphase TQF spectrum (Figure 9, spectrum on the right), obtained by applying only the first three pulses in Figure 1 with $l = p = 3$. Upon freezing, both the peak separation and the line width increase by a factor of 3.5, a trend similar to that found for the water signal. The MT rate between the protein and the DMSO was measured by the DQF-MT (Figure 1, $\tau/2 = 7$ μs) pulse sequence above the freezing point. Below the freezing point, where water and the DMSO peaks overlap, the DQF-MT pulse sequence was appended by TQF (Figure 6 with $l = p = 3$ and $\tau^{II} = 2.4$ ms) in order to remove the water's peak, thus making it possible to observe MT between the protein and the methyl group of the DMSO. The results are shown in Table 5, where the MT rate for the DMSO can be seen to be of the same order of magnitude as that for the water below the freezing point, while it is smaller by 2 orders of magnitude at 37 °C. The origin of the DMSO magnetization, observed in the DQF-MT experiment, was verified by measuring T_{DQ} relaxation times. The results are compatible with those measured for water by the DQF-MT pulse sequence and are typical for solid-state; i.e., the protein magnetization is the source of the small molecules magnetizations. The above experiments for DMSO were repeated for methanol using solutions of 12% methanol and 88% H_2O and obtaining results that show similar trends (Table 5). Since the methyl groups of DMSO and methanol do not exchange protons with the protein, the rate of their MT from the protein gives an estimate of dipolar-mediated MT at all temperatures, which, above the freezing point, is most likely to be NOE. Since this rate for DMSO is smaller than the MT rate for water by 2 orders of magnitude, it is reasonable to assume that the contribution of a dipolar-mediated process to MT from and to water is negligible, leaving chemical exchange as the most important mechanism of MT in aqueous solutions.

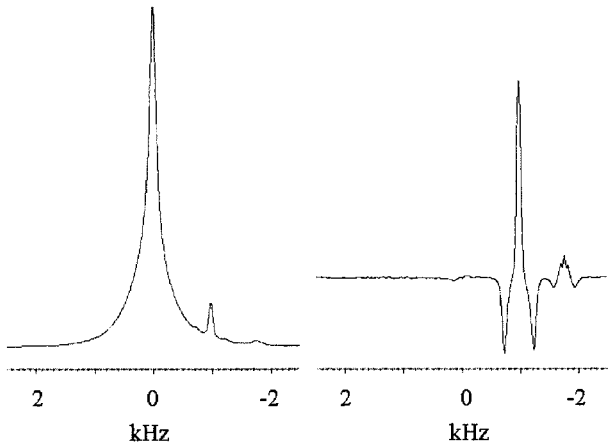

Figure 9. Spectra of bovine Achilles tendon immersed in solution of 95% H_2O and 5% DMSO. (a) Spectrum obtained after a single 90° pulse. The peaks are those of water (left) and the DMSO methyl group (right), (b) An antiphase TQF spectrum (see text) of the DMSO methyl group. Note that the water peak is removed by this method. The measurements were carried out at a frequency of 500 MHz using a 90° pulse of 9 μs and $\tau = 2.4$ ms for (b). The sample temperature was 38 °C.

All the experiments described thus far indicate that two types of processes dominate the MT between the protein and the water molecules. The first type is governed by a dipolar interaction and is most significant at temperatures below the freezing point, while the second type is dominated by chemical exchange and is more pronounced at temperatures above the freezing point. These conclusions can be understood by the interplay of the following factors: the water residence time at the protein, the rate of the chemical exchange of protons between the protein and water, and the spin diffusion rate within the protein. For molecules immersed in water, the residence time of water molecules at the surface of the protein is considered to be on a time scale of nano- and picoseconds,[20,22] making the NOE time scale significantly longer than that of the chemical exchange.[19] In systems where spin diffusion due to intramolecular dipolar interaction is very fast, quasi-equilibrium is achieved within the whole protein on a very short time scale. Thus, a transfer of magnetization from one particular functional group to the water either by chemical exchange or by NOE merely causes redistribution of the magnetization within the protein to obtain a new quasi-equilibrium condition; i.e., the various functional

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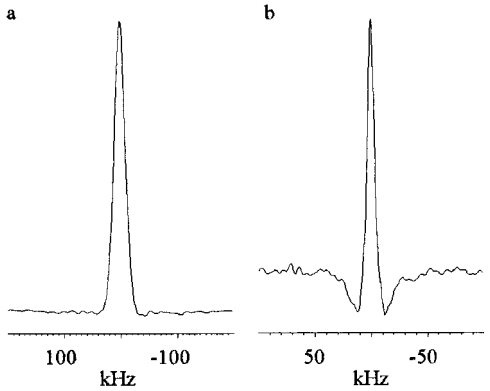

Figure 10. Projections of 2D spectra obtained from 2D version (see text) of the DQF-MT pulse sequence. For quadrature detection in the indirect dimension, the States method was implemented. The spectra shown in the figure are obtained by Fourier transformation with respect to the indirect dimensions t_{DQ} (a) and τ (b), $t_{LM} = 90$ ms. The measurements were carried out at a frequency of 360 MHz using a 90° pulse of 2.3 μs.The sample temperature was 38 °C.

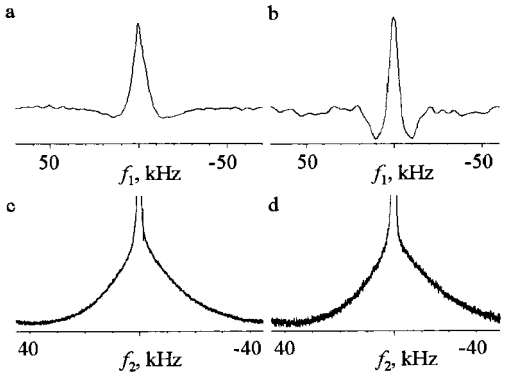

Figure 11. Projections of 2D spectra obtained from 2D version of the MQF-MT pulse sequence (see text). For quadrature detection in the indirect dimension, the States method was implemented. The two dimensions were obtained by Fourier transformation with respect to the indirect dimension τ (a, b) and the acquisition time, t_2 (c, d). The spectra shown in the figure are the results of filtering either through DQ (a, c) or TQ (b, d) coherences. The period t_{LM} was set to 90 ms. The measurements were carried out at a frequency of 360 MHz using a 90° pulse of 2.3 μs. The sample temperature was 38 °C.

groups cannot be considered to be isolated and the protein magnetization and the line shape of the protein spectrum is hardly changing as a result of the MT process. We may conclude that under the quasi-equilibrium condition the transfer of the magnetization of the whole protein to water can be considered to proceed, simultaneously, via two channels. This situation is quite different from that found for molecules much smaller than collagen, such as globular proteins with molecular weight smaller than 100 000, where the reorientation motion is fast enough to average out the dipolar interaction and thus reduce the intramolecular spin diffusion rate. Therefore, for those smaller molecules, it is possible for functional groups that are remote from the exchangeable protons to transfer magnetization to the water via a NOE mechanism. Below the freezing point of water, the exchange rates decrease and the residence times increase, causing the MT between the protein and water to be dominated by dipolar interaction.

d. Measurements of Protein Spectra via a Water Signal with an Improved S/N. The establishment of spin temperature within the protein and the subsequent MT to water causes the line shape of the observed spectrum to be independent of the tensors' ranks and of coherences that exist during the periods τ and t_{MQ}. Indeed, the spin dynamics during these periods affects only the intensity of the observed spectrum. Thus, by varying either τ or t_{MQ}, it is possible to obtain a second, indirect, spectral dimension characterized by the protein's spin dynamics. The spectra of the DQ and SQ coherences of the protein, obtained by the Fourier transformation of the variations of the FID as a function of t_{DQ} (Figure 1, $p = 2$) and τ, respectively, are shown in parts a and b of Figure 10, respectively. It is important to note that the widths of the spectra that dominate each of the two dimensions are very different. For the indirect dimensions (f_1) of either the DQ or the SQ, the widths are typical for large proteins (tens of kilohertz, Figure 10), while the dominant component in the direct dimension (f_2) is water with a line width typical for partially ordered media (hundreds of hertz), causing an improvement of the S/N in the indirect dimension. This enables us to measure the protein spectrum via the much narrower water signal. To compare the spectrum obtained in this way with the one obtained without the MT step, we measured the DQ spectrum using an antiphase DQF (DQF-AP) method[9] (only the first three pulses in Figure 1 are applied), where the FID of the protein is modulated by its decay during the period t_{DQ}. The spectrum obtained in this way (not shown) is almost identical with that shown in Figure 10a but with a reduced S/N. Upon setting optimal conditions for both experiments, i.e., selecting spectral width and applying exponential apodizing windows that are comparable to those of the observed spectra, this reduction of the S/N is 12. Multiple quantum filtering provides a method to distinguish between various functional groups on the basis of different numbers of protons within each group as well as on the differences in the internuclei distances. Those differences lead to the formation of different spherical tensors as well as to different spectral splitting,ω_D, caused by the dipolar interaction. More specifically, groups with two and three protons will make a major contribution to experiments designed to detect tensors of ranks two and three, respectively. The detection methods are shown in Figure 1, with the selection of the pathways $l = p = 2$ and $l = p = 3$ for the second (DQF-MT) and third rank (TQF-MT) tensors, respectively. Although higher tensors may also contribute to both experiments, their contribution is not expected to be very significant.

For single valued ω_D systems and under the condition of negligible relaxation, the spectra obtained in the indirect dimension, in which τ is varied, for DQF-MT and TQF-MT experiments are given by the Fourier transformations of $\sin^2\omega_D\tau/2$ and $\sin^4\omega_D\tau/2$, respectively. The spectra of the former experiment consists of three peaks located at ω_D, 0, and $-\omega_D$ with intensities ratios of $-1{:}2{:}-1$ while spectra of the latter experiment have five peaks whose positions are $2\omega_D$, ω_D, 0, $-\omega_D$, and $-2\omega_D$ and the intensity ratios are $1{:}-4{:}6{:}-4{:}1$. For proteins such as collagen, it is reasonable to assume that methyl groups will make a major contribution to the TQF-MT spectra. This is distinct from the DQF-MT experiment, in which a dipolar interaction within pairs of protons will make a very significant contribution. The above differences between the two experiments (DQF-MT and TQF-MT) are reflected in the spectra shown in Figure 11a and b (obtained by Fourier transformation of the variation of signal intensities with respect to the creation time τ), for t_{LM} longer than the time required for the establish-

ment of the spin temperature. Figure 11 demonstrates that the splitting as well as the intensity ratios in the two experiments are different, as expected from the above discussion. The deviations of the spectrum intensities in Figure 11a from the theoretical ratios of −1:2:−1 are probably a result of broadening of the two satellites due to the variety of the functional groups as well as to the distribution of their orientations with respect to the magnetic field. As explained above, the direct dimension in the MQF-MT experiments reflects the spectrum of the tensor $T_{1,-1}$, after the spin temperature has been established for the protein. Thus, the spectrum line shape in this dimension ($f2$, FT with respect to the FID acquisition time) is expected to be independent of the type of MQ filter used in the experiment (Figure 11c and d). The signal intensity of the TQF-MT was smaller by a factor of 6 than that of DQF-MT. In addition to factors such as different transfer functions that are inherent to the two experiments, the intensity difference reflects the smaller number of protons (mostly methyl groups) that contribute to the TQF-MT experiment. In summary, The DQF-MT and TQF-MT 2D experiments enabled us to obtain two different kinds of spectra for the protein in an indirect dimension, reflecting proton groups that are distinguished by the number of interacting protons.

The current investigation has clearly shown that the water signal decays on a time scale of milliseconds much longer than that of the protein (tens of microseconds). Thus, imaging that is based directly on the protein signal is impractical for conventional imagers in which magnetic field gradients pulses are typically longer than 200 μs. This limitation does not prevail for the water signal, however, and thus the detection of the protein spectra via the water signal may make it possible to create images that are related directly to the amount of protein and its intramolecular dipolar interaction.

Conclusions

1. Two types of MT processes take place. One is most significant below the freezing point and is dominated by the dipolar interaction between the protein and water. The other one occurs above the freezing point when an exchange of protons between the protein and the water molecules dominates the MT process.

2. The process of establishing the spin temperature for the protein can be monitored and is found to occur on a time scale much shorter than the time needed for the MT process between the collagen and water.

3. The acquisition of water signal, which originates from the protein magnetization in the DQF-MT technique, give rise to the possibility of creating images that are related directly to the amount of protein and its intramolecular dipolar interaction.

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