NUCLEAR MAGNETIC RESONANCE MEASUREMENT OF SKELETAL MUSCLE

ANISOTROPY OF THE DIFFUSION COEFFICIENT OF THE INTRACELLULAR WATER

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ABSTRACT The anisotropy of the spin-diffusion coefficient D_s of water protons in skeletal muscle has been studied by pulsed NMR methods. The mid-portion of the tibialis anterior muscle of mature male rats was placed in a special sample holder by means of which the muscle fiber orientation θ relative to the diffusion direction could be varied over the range $0° \leq \theta \leq 90°$. The value of $D_s(\theta)$ was determined for $\theta = 0°$, 45°, and 90°. The measured anisotropy $D_s(0)/D_s(90)$ was 1.39, and the value of $D_s(0)$ was 1.39×10^{-5} cm^2/s. These results are interpreted within the framework of a model calculation in which the diffusion equation is solved for a regular hexagonal network similar to the actin-myosin filament network. The large anisotropy, and the large reduction in the value of D_s measured parallel to the filament axes lead to two major conclusions: (*a*) interpretations in which the reduction in D_s is ascribed to the effect of geometrical obstructions on the diffusion of "free" water are ruled out; and, (*b*) there is a large fraction of the cellular water associated with the proteins in such a way that its diffusion coefficient is substantially reduced.

INTRODUCTION

The understanding of the physicochemical state of the ions and water in the protoplasm of living cells is presently the subject of considerable controversy in biology. The most widely accepted theories of the functional cell assume that the interior can be characterized as a dilute aqueous solution, containing ions, small molecules, and biopolymers bounded by a cell membrane serving as the primary permeability barrier limiting accumulation or exclusion of substances (1–9). Several theories have been advanced which view the cell interior as something vastly different from a simple solution (10–15). According to these alternative theories, the permeability of cells for any substance is not determined solely by the ability of the substance to penetrate the cell membrane, but primarily by the solubility of the substance in the protoplasmic water

and its adsorption or chemical association by the intracellular biopolymers. Changes in the solvent properties of the cytoplasmic water are attributed to "ordering" of the water structure caused by interaction between the water and the biopolymers, where "ordering" implies the reduction of mobility (increased correlation time) for some degree of freedom (not necessarily translational). This "ordering" may include longer rotational or translational correlation times or preferential orientation of water dipoles due to the influence of macromolecular surfaces.

Some of the most widely cited evidence for physical changes in the intracellular water is itself subject to considerable controversy. Over the past decade, extensive nuclear magnetic resonance (NMR) data have been accumulated which demonstrate that the fundamental NMR parameters (T_2, the spin-spin relaxation time; T_1, the spin-lattice relaxation time; and D_s, the self-diffusion coefficient) of intracellular water hydrogens are significantly altered from those of pure bulk water. These parameters have been measured on a variety of biological tissues including nerve, muscle, brain, liver, spleen, kidney, lens, tendon, lung, and heart, as well as in various benign and malignant tumors (16–43). Allowing for the wide range of sample types, the studies have all produced values of T_2 reduced by a factor of 30–60, T_1 reduced by a factor of 4–10, and D_s reduced by a factor of about 2 when compared with the values of these parameters obtained for pure water or dilute electrolyte solutions.

These data have been interpreted by some investigators as being consistent with the concept of "ordering" of the intracellular water (i.e. shorter relaxation times imply longer correlation times and thus indicate a more ordered state) (20–30). Other investigators have proposed alternative explanations of the change in these parameters (32–43). The dominate hypothesis among opponents to the idea of changes in the physical properties of intracellular water is that the reduction in the NMR relaxation times can be explained by a two-phase fast-exchange model (44). In this model the vast majority of the water is assumed to behave as if it were an ideal solution, and the short relaxation times are attributed to the averaging effects of fast exchange with a small fraction of water molecules tightly bound to intracellular macromolecules (17, 32, 34, 35). The observation by many investigators of relaxation data fit by a single exponential has been cited as evidence in support of this interpretation. It is also pointed out that the water-ice transition reduces D by about four orders of magnitude (45), so that a reduction of 40–60% in the diffusion coefficient does not indicate much "ordering."

Since the fast-exchange hypothesis assumes that the vast majority of the intracellular water is "free," the observed diffusion coefficient should be essentially that of pure water. The observed reduction in D_s is therefore attributed to compartmentalization by the membrane systems of the cell, or to geometrical obstruction effects due to interaction of the water with biopolymers or other diffusion barriers within the cell (32, 34, 37, 46). Chang et al. have presented experimental evidence that the compartmentalization effects cannot account for the reduction of the diffusion coefficient in skeletal muscle (28). A preliminary report by Rorschach et al. on the self-diffusion

coefficient of water protons in skeletal muscle suggested that geometrical obstruction effects are also inadequate for this purpose (47).

In this paper, we will report a study of the obstruction effects of cellular biopolymers not in the form of compartment walls (48). It is well established that muscle fibers are filled with protein filaments arranged in a well-known geometry. These filaments act as barriers to diffusion. Also there are nonfilament proteins dissolved in the cytoplasmic water which must also be considered as potential barriers to the diffusion of water. The purpose of this paper is, therefore, twofold: (*a*) to evaluate the obstruction effect of the protein filaments and, (*b*) to determine if the geometrical interaction between water molecules and cellular biopolymers (filament and nonfilament) can account for the observed reduction in the diffusion coefficient of muscle water.

The obstruction by the actin-myosin filaments in skeletal muscle is expected to be anisotropic. (The obstruction is maximum for diffusion perpendicular to the filaments and zero parallel to the filaments.) We have measured the spin-diffusion coefficient $D_s(\theta)$ of muscle water for diffusion at various angles θ with respect to the muscle fiber. A significant anisotropy in the spin diffusion is found. The experimental results give useful information which may be important to the interpretation of other NMR data.

METHODS

NMR Measurements

Diffusion coefficients were determined using standard techniques of pulsed NMR (49, 50). The diffusion of water proton spins is measured along the direction of the static magnetic field by observing the echo amplitude following a 90°-τ-180° pulse sequence as a function of the applied field gradient, which is parallel to the static field. The amplitude of this echo is given by the expression:

$$A(\tau, G)/A(\tau,0) = \exp[-2/3\,\gamma^2 G^2 \tau^3 D_s],$$

where $A(\tau, G)$ is the echo amplitude for a 90°–180° pulse separation τ with an applied field gradient G, and γ is the gyromagnetic ratio. A τ of 25 ms was used for all data reported here. This value is large in comparison to the diffusion time t_D over the distance d between actin and myosin filaments (i.e. $t_D = d^2/D \cong 3.0 \times 10^{-7}$ s). Each water molecule can be assumed to have encountered many obstructions during the time of measurement.

Measurements were made on the pulsed NMR spectrometer in the Field Research Laboratory of Mobil Research and Development Corporation in Dallas. This spectrometer, which has previously been described in the literature (51, 52), accommodates a 12 mm (OD) sample tube. Measurements were made at 25 ± 1°C with the spectrometer operating at 25 MHz. The applied field gradient was achieved with a standard Maxwell coil pair. The gradient current was measured by observing the voltage drop across two oil-immersed 1 Ω resistors in series with the coils. The signal amplitude was measured by averaging over 10 pulses with a Biomation 610 Transient Recorder (Biomation, Cupertino, Calif.) and a boxcar signal averager. The voltages thus measured were recalibrated to correct for nonlinearities in the detector. The recalibration of the data and the calculation of the diffusion coefficients were accomplished by a CDC 6400 digital computer.

Sample Preparation

Samples in this study were prepared from the tibialis anterior of mature male rats (Texas Inbred) weighing between 350 and 500 g. Animals were killed by cervical fracture and the muscle (free of excess connective tissue) was excised with minimum delay. A thread was tied to the distal tendon while a broad Allis clamp was used to secure the proximal end. A reliable method for insuring muscle fiber orientation was developed as follows. A piece of 6 mm (ID) glass tubing was cut and beveled such that it could be inserted perpendicular to the axis of a 12 mm (OD) sample tube via an attached glass rod. The excised muscle could then be drawn through the 6 mm tube by the thread tied to the distal tendon, until a linear portion of the muscle was centered. This was a fairly tight fit. The excess muscle was then trimmed from both ends and the sample was inserted into the 12 mm tube. The sample tube was then placed in the probe and the orientation of the fiber axis, determined within ±3°, was noted on the top of the sample tube. The orientation of the fiber axis, relative to the magnetic field and its gradient, was adjusted by rotating the 12 mm sample tube manually. The actual time between sample preparation and the determination of the diffusion coefficient ranged between 10 min and 24 h with no dependence on "sample age" observed. Samples were stored in sealed sample tubes placed on ice.

RESULTS AND DISCUSSION

The results of these experiments are shown in Table I and plottted in Fig. 1. The error in angle measurement is ±3° and the combined spread in the data due to biological variations and physical measurement is adequately represented by the scatter in the data points at each angle. Results of measurements of the self-diffusion coefficient for pure bulk water and for randomized tibialis anterior are also included in Table I. The value of the spin-diffusion coefficient D_s for randomized muscle was obtained from two successive measurements on the same piece of muscle for which the fiber orientation had been thoroughly disordered by cutting the muscle into several pieces. This furnishes a point of reference as to what values might be expected for typical samples for which no care is taken to preserve fiber orientation.

The average value of $D_s(90)/D_s(0)$ in this study was 0.72, which represents an anisotropy of 28%. The value of 2.28×10^{-5} cm^2/s for the self-diffusion coefficient of pure bulk water compares favorably with other values in the literature (53–55).

TABLE I

MEASUREMENTS OF THE DIFFUSION COEFFICIENT VS. MUSCLE FIBER ORIENTATION

Sample	Diffusion coefficients ($\times 10^{-5}$ cm^2/s)			$D_s(90)/D_s(0)$
	$\theta = 0°$	$\theta = 45°$	$\theta = 90°$	
1	1.37	1.18	1.02	0.75
2	1.39	1.18	1.02	0.74
3	1.40	1.23	1.03	0.74
4	1.41	1.22	0.99	0.70
5	1.40	1.19	0.97	0.69
Average	1.39	1.20	1.01	0.72

Diffusion coefficient for pure bulk water: 2.28×10^{-5} cm^2/s; diffusion coefficient for randomized muscle: 1.19×10^{-5} cm^2/s.

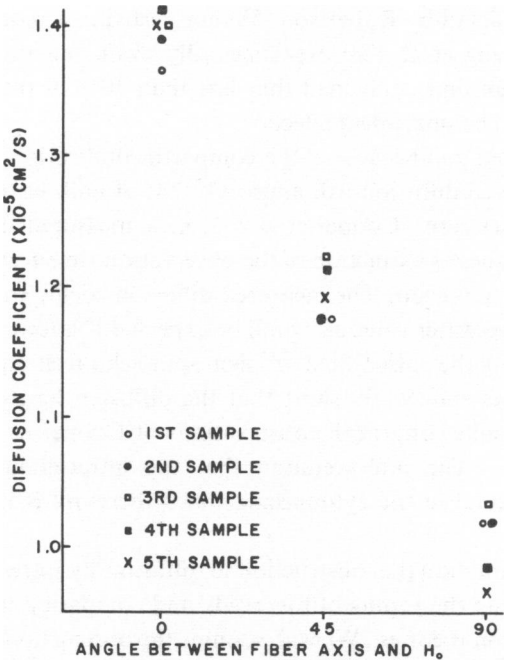

FIGURE 1 The spin-diffusion coefficient for oriented muscle fibers as a function of the angle between the fiber axis and the external magnetic field, H_0. The spin-echo measurement gives the value of D_s along H_0.

Comparing the average diffusion coefficient for each fiber orientation to the value of D_s for pure bulk water (D_0), one finds

$$D_s(90)/D_0 = 0.44,\ D_s(45)/D_0 = 0.53, \text{ and } D_s(0)/D_0 = 0.61.$$

Thus, the average diffusion coefficient measured parallel to the fiber axis, is still about 40% lower than D_0.

Several laboratories have reported that the diffusion coefficient of water molecules in living tissue is reduced by a factor of approximately two compared with pure water (28, 31–34, 56, 57). Three explanations for the observed reduction in the diffusion coefficient have been offered.

(*i*) The intracellular membrane systems serve to compartmentalize the cytoplasmic water.

(*ii*) Intracellular protein structures serve as obstructions to diffusion of cytoplasmic water.

(*iii*) The water-protein interaction induces long-range changes in the water-water interaction in a substantial fraction of the intracellular water.

The possibility that the various membrane systems might serve as impenetrable barriers to diffusion was one of the first explanations offered to explain the NMR observations. The reduction of D_s due to compartment effects has been examined in con-

siderable theoretical detail by Robertson (58) and tested in a model system by Wayne and Cotts (59). Chang et al. (28) experimentally evaluated this mechanism in the skeletal muscle system and concluded that less than 10% of the observed reduction could be attributed to compartment effects.

It has been proposed that because of the compartmentalization effect (interpretation (*i*) above), the observed diffusion will approach that of bulk water when the observation time t approaches zero. Cooper et al. (31) have measured the spin-diffusion coefficient in several tissues as a function of the observation time t using the technique of pulsed field gradient spin-echo. The measured diffusion coefficients at very short t did not approach the pure-water value as would be expected if interpretation (*i*) is correct. Tanner (60), also using the pulsed-field-gradient spin-echo technique, has extended the measurement to times sufficiently short that the diffusion length is on the order of 1 μm or less. His results confirm the observations of Cooper et al. and give a value $D_s/D_0 \simeq 0.4$ to 0.7. The interpretation that the intracellular membrane system serves to compartmentalize the cytoplasmic water therefore is not supported by experimental evidence.

The second interpretation (i.e. obstruction to diffusion by intracellular proteins) can be tested by examining the results of this study and comparing them with the predictions of the obstruction theories. We will examine the obstruction effect in two aspects: (*a*) Can the obstruction of the nonfilament-like proteins account for the reduction of the spin-diffusion coefficient along the direction parallel to the muscle fiber? (*b*) Can the actin-myosin filaments account for the observed anisotropy of the spin-diffusion of cellular water?

A model often considered to explain the reduction of D due to the obstruction effect in biological systems is that proposed by Wang (46). This model was intended to apply to capillary flow measurements of D in dilute protein solutions. Wang's model utilizes the steady state solution of the diffusion equation and assumes that the protein molecules are stationary impenetrable ellipsoids. The general expression for the effective self-diffusion coefficient D in a given direction is given by

$$D_i = (1 - \alpha_i \varphi) D_0, \qquad i = a, b, c$$

where D_0 is the self-diffusion coefficient of the "free" solution, φ is the volume fraction occupied by the hydrated protein molecules, and α_i is a dimensionless numerical coefficient for diffusion parallel to the ith axis of the ellipsoid. The value of α_i is determined by the dimensions of the principal semi-axes a, b, and c of the ellipsoids. The equation shows that the measured diffusion coefficient D should be less than D_0 if φ is not negligibly small. In addition, the diffusion of cell water is further reduced if the water molecules are in rapid exchange with a hydration fraction (the "direct hydration" effect). Wang shows that this effect introduces an additional factor $D' = D(1 - f)$, where f is the fraction of the water bound to the proteins. The final result for Wang's theory is

$$D_i' = (1 - \alpha_i \varphi)(1 - f) D_0. \tag{1}$$

A second approach to the problem of reduction of the measured diffusion coefficient perpendicular to the actin-myosin filament network was recently presented by Rorschach et al. (47). A more detailed calculation for the spin-echo case, with some corrections, will be submitted as a separate publication. In this model, the filaments are approximated by a hexagonal array of impenetrable rods of uniform radius a and lattice spacing $2R$ (see Fig. 2). The effective self-diffusion coefficient D_s is derived from an approximate solution to the steady-state diffusion equation with the appropriate boundary conditions within a hexagonal unit cell. This calculation yields the following expressions for the spin-diffusion coefficient D_s:

$$\text{Parallel to fiber axes:} \quad D_s(0) = D_0, \tag{2}$$

$$\text{Perpendicular to fiber axes:} \quad D_s(90) = D_0/(1 + 0.80\varphi), \tag{3}$$

where D_0 is the self-diffusion coefficient of the solution in the absence of obstructions, and φ is the volume fraction of the protein.

The reduction in D_s in a cubical array of spheres has also been calculated. The results show that the measured spin-diffusion coefficient is:

$$D_s = D_0/(1 + 0.63\varphi). \tag{4}$$

These results differ from those given by Wang for two reasons:

(*i*) The boundary conditions for Wang's calculation are imposed at infinity, and the flow pattern for a random array is determined by superposition. In the present calculation, the influence of the neighbors on the flow is taken into account by imposing the boundary conditions at the surface of the hexagonal cells.

(*ii*) The diffusion in a protein solution in a capillary is influenced by the distortion of the flow and also by a geometrical factor that would reduce the transport cross section, even if all the protein were to be congealed on the walls of the capillary. This geometrical factor does not enter in the spin-echo case.

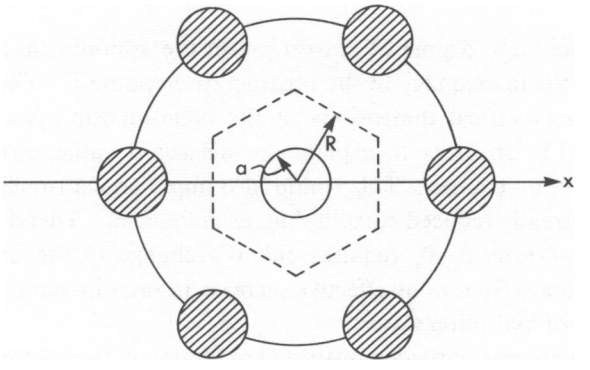

FIGURE 2 A cross-sectional view of the hexagonal lattice of protein filaments. The basic hexagonal cell is shown dotted. For the purposes of the diffusion calculation, the actin and myosin filaments are taken to be cylinders of equal radius a.

Both of these effects lead to corrections to the relation between D_s and D_0 which are of order φ.

Our experimental results clearly show that the measured spin-diffusion coefficient perpendicular to the muscle fiber is less than that parallel to the muscle fiber. This proves that the array of the actin-myosin filaments is an effective diffusion barrier; however, the spin-diffusion coefficient parallel to the muscle fiber is still significantly reduced from that of pure bulk water. According to Rorschach's calculation, this 40% reduction of diffusion coefficient cannot be accounted for by the protein filaments, since D_s should equal to D_0 for diffusion parallel to the muscle fiber. The question then is: Can the obstruction effect of the nonfilament proteins account for this reduction of diffusion?

The obstruction effect of the nonfilament proteins can also be estimated from Rorschach's calculation. If we assume that these proteins are spherical, the obstruction effect can be estimated from Eq. 4. In order to make $D_s(0)/D_0 = 0.61$, a value for φ of approximately 1 is required, which obviously is too large. The cytoplasm cannot be completely filled with proteins. The alternative is to consider the "direct hydration" effect. The "direct hydration" effect introduces a reduction factor $(1 - f)$ to the measured diffusion coefficient. (This is true in both Wang's calculation and Rorschach's calculation.) Adding this factor to Eq. 4, the obstruction effect may be more realistically evaluated. If one assumes the nonfilament proteins are dilute ($\varphi \simeq 0$), then $f \simeq 0.39$, i.e. 39% of the cytoplasmic water would be hydration water. If one assumes a more reasonable value for φ (e.g. the nonfilament proteins occupy $\simeq$ 5% of the volume of cytoplasm, and $\varphi = 0.05$), f will be about 0.37. The hydration water would have to constitute 37% of the cellular water.

It becomes clear that the obstruction effect of protein filaments and nonfilament proteins is not sufficient to account for the reduction in the spin-diffusion coefficient. The assumption that the cell water diffuses like free water and that the observed reduction in diffusion coefficient is caused by the "direct hydration" effect requires a large fraction of the cytoplasmic water to be tightly associated with the macromolecules.

Furthermore, the large degree of anisotropy in the spin-diffusion coefficient also strongly indicates the inadequacy of the obstruction hypothesis. The anisotropy predicted from the geometrical dimensions of the actin-myosin system ($\varphi = 0.16$) is $D_s(0)/D_s(90) = 1.13$. In order to explain the measured value $D_s(0)/D_s(90 = 1.39$, a value of $\varphi = 0.49$ is required. This would also imply an enormous sheath of water whose mobility is greatly reduced surrounding each filament. Therefore, the observed reduction and anisotropy in D_s requires either a change in the bulk diffusion coefficient of the cellular water, or an effective increase in myosin-actin filament size by a substantial sheath of hydration water.

In conclusion, we have clearly demonstrated in this study that there is an anisotropy in the spin-diffusion coefficient of water in muscle. This anisotropy is on the order of 25–30%, and this indicates that the actin-myosin filaments system is an effective bar-

rier. Thus, for a randomly oriented sample, only about 15% of the average reduction in D_s can be attributed to obstruction by the filament lattice. The fact that the value measured for $D_s(0)$ was 40% less than the diffusion coefficient for pure bulk water is significant. If one analyzes the data with a calculation valid for the spin-echo measurements, it is found that the obstruction effect of the biopolymers cannot explain the reduction of the spin diffusion of water in biological systems. A large fraction of hydration water must be assumed to account for the observed data.

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