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Anisotropic Orientation of Lactate in Skeletal Muscle Observed by Dipolar Coupling in ^{1}H NMR Spectroscopy

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Double quantum (DQ), *J*-resolved ^{1}H NMR spectra from rat and bovine skeletal muscle showed a splitting frequency (~24 Hz) for the lactate methyl protons that varied with the orientation of the muscle fibers relative to the magnetic field. In contrast, spectra of lactate in solution consist of a *J*-coupled methyl doublet and a *J*-coupled methine quartet (J_{HH} = 7 Hz) with no sensitivity to sample orientation. Spectra acquired in magnetic fields of 4.7, 7, and 11 T showed that the splitting was not due to inhomogeneities in magnetic susceptibility within the muscle, because the magnitude of the splitting did not scale with the strength of B_0 fields. Triple quantum coherence (TQC) spectra revealed two distinct transition frequencies on the methyl resonance. These frequencies resulted from intra-methyl and methine–methyl couplings in this four spin system (A_3X). Decoupling experiments on the triple quantum coherence showed that the observed frequency splitting was due mainly to the dipolar interactions between the methine and methyl protons of the lactate molecule. Thus, all the proton resonances of the lactate molecules in muscle behave anisotropically in the magnetic field. Adequate design and interpretation of spectroscopic experiments to measure lactate in muscle, and possibly in any cell and organ which contain asymmetric structures, require that both the dipolar coupling described here and the well-known scalar coupling be taken into account.

Key Words: **proton NMR spectroscopy; skeletal muscle; lactate; dipolar coupling; scalar coupling.**

INTRODUCTION

The observation of narrow linewidths in ^{31}P NMR spectra of metabolites in solution and from red cells and muscle (*1*) inaugurated the extraordinarily successful application of NMR spectroscopy in medicine and biology to quantify the relations between metabolism and function in intact biological systems. This application is commonly made under the assumption that the spectral resonances of metabolites from cells, tissues, and organs satisfy the requirements for rotational narrowing; that is, the molecules of interest tumble freely. Isotropic molecular motion has the effect of averaging to zero any orientation-dependent properties, such as dipolar coupling, which are common features in the NMR spectra of solids and liquid crystals.

However, recent findings (*2, 3*) suggest dipolar coupling interactions within the methylene group of creatine and phosphocreatine. The frequency difference due to dipolar coupling was shown to vary with the orientation of the muscle fibers relative to the magnetic field in accordance with the theoretical prediction for ordered magnetic dipole–dipole interactions (*4*). These observations are an indication of incomplete motional averaging of the creatine and/or phosphocreatine molecules in skeletal muscle. For these metabolites, the system of spins behaves like a liquid crystal where, on a time average, the spins have a preferred orientation.

The purpose of this study was to test whether an anisotropy due to dipolar interactions can be observed in the proton spectra of lactate in skeletal muscle. This was motivated by significant variations in lactate spectral intensity first noted in preliminary experiments with rat hindlimb muscle. Experiments were then designed to test for the presence of dipolar coupling using bovine abdominal muscle. This muscle was chosen for its homogeneously oriented fibers, which allow accurate measurement of the orientation dependence with respect to B_0.

The observation of dipolar interaction of lactate molecules in skeletal muscle poses a number of issues and difficulties for most spectroscopic investigations of lactate currently in use in biological samples. The results presented here show unambiguously that there is an orientation-dependent dipolar coupling in lactate resonances in muscle. The coupled spins within the lactate molecule responsible for the observed anisotropy have been identified.

METHODS

NMR Spectroscopy

Spectra were acquired on a Bruker (GE Omega) 4.7 T (200.1 MHz) CSI spectrometer, with 5 G/cm maximum gradient strength; a Bruker (GE Omega) 7 T (300.521 MHz) CSI spectrometer, with 134 G/cm maximum gradient strength; or a

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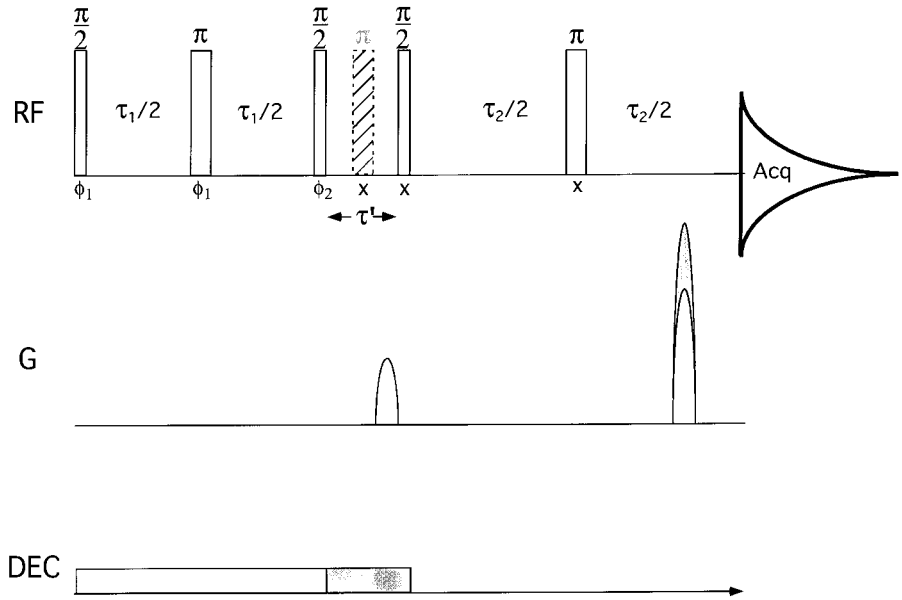

FIG. 1. Pulse sequences used for multiple quantum coherence (MQC) selection in lactate. In order to eliminate any unwanted coherences, the pulse phases ϕ_1 and ϕ_2 were cycled according to well defined schemes (*8*). To select for DQC the strength of the second gradient pulse (unshaded) was double that of the first gradient. To select for TQC the second gradient pulse (shaded) was triple the strength of the first gradient pulse. For both DQC and TQC, τ_1 was incremented from approximately zero to $1/J_{max}$, where J_{max} was the largest coupling expected to be observed. These spectra are referred to as "DQ, *J*-resolved" and "TQ, *J*-resolved," respectively. In these experiments τ' was set to slightly exceed the length of the encoding gradient pulse (~1 ms). In the TQC spectra acquired for the observation of the multiple quantum frequencies, τ_1 was fixed at approximately 20 ms and τ' was incremented to cover the expected MQ frequency range. For observing the splitting of the multiple quantum coherence, the π pulse in the middle of τ' was added and τ' was incremented through the range of expected MQ splittings. In all cases τ_2 was fixed at approximately 20 ms, and variation of this time did not qualitatively alter the results obtained. Decoupling experiments were performed applying a low level decoupling field ($\gamma B_2/2\pi$, approximately 200 Hz) selectively to the methine proton during either τ_1 (unshaded) or τ' (shaded).

Bruker DRX500 operating at 11.7 T (500.13 MHz) with approximately 70 G/cm maximum gradient strength. All spectra were acquired at ambient temperature, (21–23°C)

At 200.1 MHz, three coils were used for transmission and detection of proton signals: (a) a cylindrically symmetric saddle coil was used for phantom solutions verifying pulse sequences, (b) a single turn solenoid mounted above a stage rotatable through 360 degrees was used to test the effects of muscle fiber orientation on the proton spectra of bovine abdominal muscle, and (c) a single turn surface coil (1.5 cm in diameter) was used to study rat muscle samples. At 300.5 MHz, a single 16 element, linear driven, bird-cage, Bruker (GE) coil was used for both transmission and reception of the proton signals. At 500 MHz, a standard Bruker triple resonance, triple axis gradient TXI 5 mm probe was used. The muscle samples were oriented in the magnetic field parallel to the B_0 axis. The samples were rotated about the *y* axis in the *xz* plane for all of the experiments testing for the angular dependence with respect to the B_0 field. In one set of experiments, the samples were rotated about the *x* axis in the *yz* plane. In order to remove the effects of intermolecular multiple-quantum coherences (*5*), all spectra were acquired using magic angle gradients (*6*).

After shimming, double quantum sequences with a pulse width of 20 μs at 1 kW for 80 acquisitions of 1024 complex points (Fig. 1) were used to selectively excite the coupled spin system of lactate separately from lipid resonances (*7*) at 4.7 T; similar conditions were used at other field strengths. The echo time (DQ preparation time shown as τ_1 in Fig. 1) was incremented in 32 steps of $1/J_{max}$, common for *J*-resolved experiments. Phase cycling was utilized to further reduce spurious signals (*8*).

Triple quantum (TQ) spectra were acquired by changing the ratio of the gradient intensity from 2 to 3 (see Fig. 1). Both double and triple quantum coherences were modulated during the preparation time τ_1 as result of couplings that allow these signals to develop. When these couplings are measured by varying τ_1 in a two-dimensional manner, these spectra are referred to as "DQ, *J*-resolved" and "TQ, *J*-resolved," respectively.

TQ spectra were also acquired after selective decoupling of the methine proton with cw irradiation during τ_1 (Fig. 1) (in which case the influence of the methine proton on the development of the TQ coherence is removed), or during τ' (in which case the effect of the methine proton on the TQ splittings is removed). Decoupling will not occur during the gradient pulse which is contained in τ' (Fig. 1). However, any splittings in the f_1 dimension will not be observed since the gradient pulse length is not incremented with τ'. In these experiments phase cycling (*8*) was also used to reduce spurious signals.

Data Processing

Free induction decays acquired at 200 and 300 MHz from one-dimensional spectra were baseline corrected and apodized with a 1.5 Hz Gaussian function prior to Fourier transformation. Free induction decays of the two-dimensional spectra were apodized with a 10 Hz Gaussian function in the t_2 dimension and with a sine^2 function (10° phase shift and spanning 80% of the data set) in the t_1 dimension. Baseline correction was used before Fourier transformation in both t_1 and t_2 dimensions. Integration of the lactate peaks in both cases was done using a line integral from the acquisition software (GE Omega 6.0.2). Data acquired at 500 MHz were processed using a sine bell apodization and displayed in magnitude mode without any baseline correction.

Statistics. Non-linear curves for equations $\Delta f = \Delta f_{dc} \pm 7$, where $\Delta f_{dc} = 1.5\ D_0\ [1 - 3(\cos\theta\cos\alpha - \sin\theta\sin\phi\sin\alpha)^2]$ describes the angular dependence of dipolar coupling interactions (*2*), were fit to the data using Fig.P. for Windows (Biosoft, Cambridge, U.K.). The fitting values for D_0, ϕ, and θ are given as means ± SD. Error of fit is given as the square root of the mean of the squared residuals.

Sample Preparation

Phantom studies were made with solutions of lactate with and without liquid triglyceride (Crisco). The phantom was a 3 ml plastic syringe filled with a 0.5 *M* sodium lactate solution (pH = 7) with and without the insertion of a glass tube filled with liquid triglyceride (0.6 cm diameter by 4 cm length − 58% of observed volume). All muscle samples were placed in the coil covered with paraffin film to minimize dehydration during long spectral acquisition. All samples were at ambient temperature (20–23°C).

The dependence of the lactate ^{1}H NMR spectra on the orientation of the muscle fibers relative to the magnetic field was determined on samples from rat and bovine muscle. Bovine muscle was studied with lactate at its endogenous concentration or with the lactate concentration increased by injecting lactate solution into the sample. The muscles with the added exogenous lactate are referred to as spiked samples.

Rat muscle preparation. Male Sprague–Dawley rats (350 and 745 g) were sacrificed with an intraperitoneal injection of an overdose of ketamine/xylazine according to a protocol approved by our university's animal care committee. Muscle samples of irregular shapes, but sufficient to identify the major orientation of the muscle fibers (average 4.0 g wet weight (n = 5)), were dissected from the thigh. Samples were wrapped in paraffin film and left in the refrigerator (T = 6°C) for 15 h prior to spectral acquisition, by which time glycogenolysis and glycolysis produced a stable concentration of lactate.

Bovine muscle preparation. Bovine abdominal muscle, commonly referred to as flank steak, was obtained from a local meat market. Muscle fibers from this muscle are uniformly oriented and free of visible fat. Cylindrical samples (1.6 cm diameter by 1.7 cm length) were cut from the slab of meat to generate samples of reproducible size and shape. The cylindrical shape of the samples gave a radial symmetry which enhanced the spectroscopic resolution and allowed good control of angular orientation of the fiber bundles easily visible with the unaided eye. For the experiments at the 7 T cylindrical samples were cut to fit into the 5 mm tube; therefore some irregularities of the sample shape were unavoidable. The samples used in these experiments were cheap, readily available, and highly reproducible and required no additional animal sacrifice.

Proton NMR spectra were initially obtained from bovine muscle samples (n = 9) containing only endogenous lactate. To positively identify the lactate doublet, these samples (n = 6) were spiked by injecting 130 μl of 5 *M* sodium lactate solution into the muscle. The lactate was injected into multiple sites to minimize formation of liquid pockets inside the tissue and to facilitate its subsequent diffusion. Spectra were acquired within 40 min of the injection and after 15 h storage (T = 6°C).

Bovine Muscle Characterization

Because the history of the bovine muscle was unknown and likely differed from the rat muscle samples, bovine muscle was characterized as described below:

Extraction of tissue metabolites. Samples from the same package were frozen in liquid nitrogen, pulverized, and extracted with 0.59 *M* perchloric acid solution with a ratio of volume to tissue weight of 8:1 (*9*). The samples contained either endogenous metabolite concentrations (n = 4) or were spiked with exogenous lactate (40 min, n = 2 and 15 h, n = 2). Extracts were centrifuged (14,500*g*) and the supernatant was neutralized with a solution of KOH (0.59 *M*)/TES (40 m*M*). The insoluble $KClO_4$ was removed by centrifugation (3000*g*) and the supernatant was stored in the freezer (−77°C) for subsequent metabolite analysis.

Lactate assay. This assay was based on lactate dehydrogenase oxidation of lactate to pyruvate with concomitant formation of NADH (*10*); a Sigma kit (catalog No. 826-B) was used. The increased absorbency at 340 nm of NADH was linearly proportional to the amount of lactate present in the concentration range used, 0.3 to 13 m*M*. Aliquots of the neutralized perchloric acid extract were diluted as needed for linearity. Muscle content was expressed as micromoles per gram weight of muscle. This assay determined the reproducibility of lactate content from various batches of meat and the stability of the added exogenous lactate.

AA analysis. Extracts of bovine muscle samples (n = 4) were analyzed for amino acid composition by HPLC on an ion-exchange column (15 cm) using ninhydrin for post column derivatization (Pickering PCX 3100 reaction module, Pickering Laboratories, Mountain View, CA) and photodiode array detection (Water's). This analysis was developed in our hos-

pital laboratory using a gradient of lithium buffers, pH 2.8, 3.5, and 7.5 (*11*). This assay determined whether other metabolites, such as alanine, which might contribute to our spectra were present.

Histochemical analysis. Two pieces of bovine muscle tissue were fixed in 10% neutral-buffered formalin after the 15 h of spectra acquisition. Routine methods were used to obtain paraffin-embedded sections which were stained with hematoxylin and eosin (H & E). Photomicrographs were taken with a 40×/0.95 *planapo* Zeiss objective on a Zeiss microscope interfaced with a 35 mm camera.

Electrophoretic gels. Proteins of two different samples from bovine abdominal muscles were electrophoretically separated on a Tris–glycine gradient gel (4 to 20%, NOVAX) and stained with Coomassie Blue to assess the extent of proteolysis (*12*). The electrophoretic patterns were compared to control profiles obtained from freshly dissected EDL and soleus mouse muscles and rabbit psoas myofibrils. This analysis tested for proteolysis of the major proteins of the thick and thin filaments of muscle's nanostructure.

Water content. The water content of samples of rat thigh muscle ($n = 3$) and of bovine abdominal muscle from three packages was measured to check for any dehydration of the bovine muscles samples compared to the rat muscle. Weights of the muscle samples were measured before and after lyophilization; the difference in weight was taken as the water content. They were dried for an average of 72 h (when no change in the vacuum pressure was recorded for at least 4 h).

RESULTS

DQ Spectroscopy in Phantoms and Rat Muscle

The spin system for the lactate molecule gives rise to double and triple quantum coherences which can be chosen to edit and distinguish lactate resonances in skeletal muscle from those of water and lipid signals (*13*). Figure 2a shows a "one-pulse" spectrum of a 0.5 *M* lactate solution with added liquid triglyceride. Note the large water signal and also the presence of the triglyceride methylene resonances at ~1.2 ppm overlapping with the lactate doublet. Good suppression of both the water signal and the lipid methylene peaks accompanies DQ filtered spectra with selection of the lactate methyl resonance at 1.2 ppm (Fig. 2b). These results verify the expected performance of the pulse sequence.

The novel observation in this work is that the proton NMR spectrum of lactate in skeletal muscle is dependent on the orientation of the muscle fibers relative to the magnetic field, B_0. Typical DQ spectra of muscle from a rat hindlimb are shown in Fig. 3; these spectra are the same as those from bovine muscle (shown later.) When the muscle fibers were oriented predominantly parallel with B_0 ($\alpha = 0°$, Fig. 3a), there was a splitting of ~25 Hz for the lactate methyl (mean 25 Hz, $n = 5$, stdev = 0.8). In contrast, when the muscle fibers were

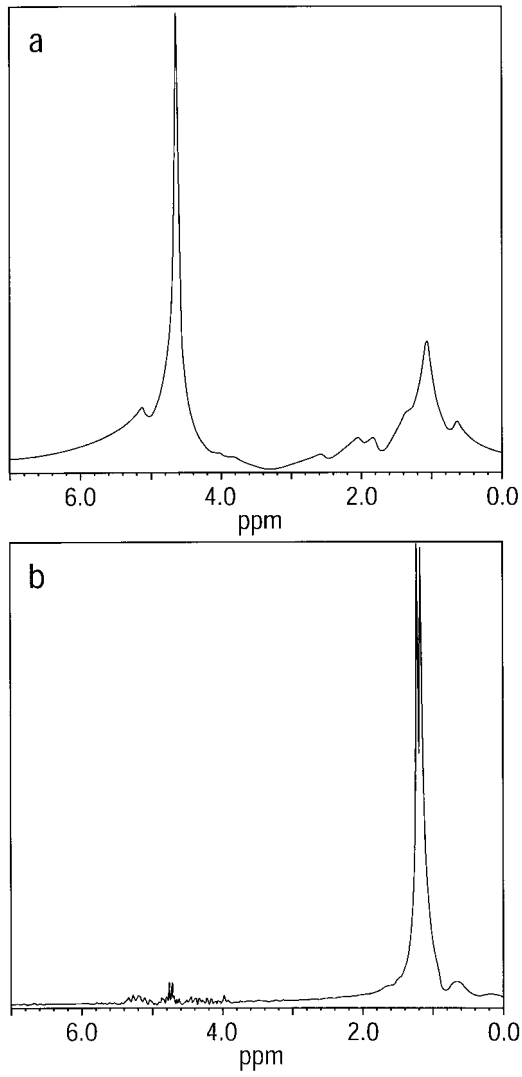

FIG. 2. Selectivity of DQ filtered spectrum for lactate editing. (a) One-pulse spectrum of lactate from a composite sample with 58% volume triglyceride (Crisco) and 42% volume 0.5 *M* lactate solution. (b) DQ filtered ^{1}H NMR spectrum of the same sample as that used in (a). The pulse sequence produced very good suppression of the water signal (at ~4.7 ppm) and also a good selection of the *J*-coupled protons of the lactate methyl group from the signal rising from triglyceride resonances at ~1.2 ppm. Spectra were acquired at 4.7 T. The gain in (b) was ~1000 times higher than the gain used in (a).

oriented perpendicular to B_0 ($\alpha = 90°$), the lactate methyl splitting became unresolvable (Fig. 3b, $n = 5$). These spectra prove that the lactate methyl splitting varies with the rotation of a sample from parallel to perpendicular orientation with respect to B_0. The 7 Hz *J*-coupled doublets are not resolved in these spectra due to inhomogeneity of the muscle samples (shimming on water gave a linewidth of ~40 Hz).

Characterization of Bovine Abdominal Muscle

The anatomical arrangement of the cells in the rat hindlimb muscle is not optimal for orienting the muscle fibers with

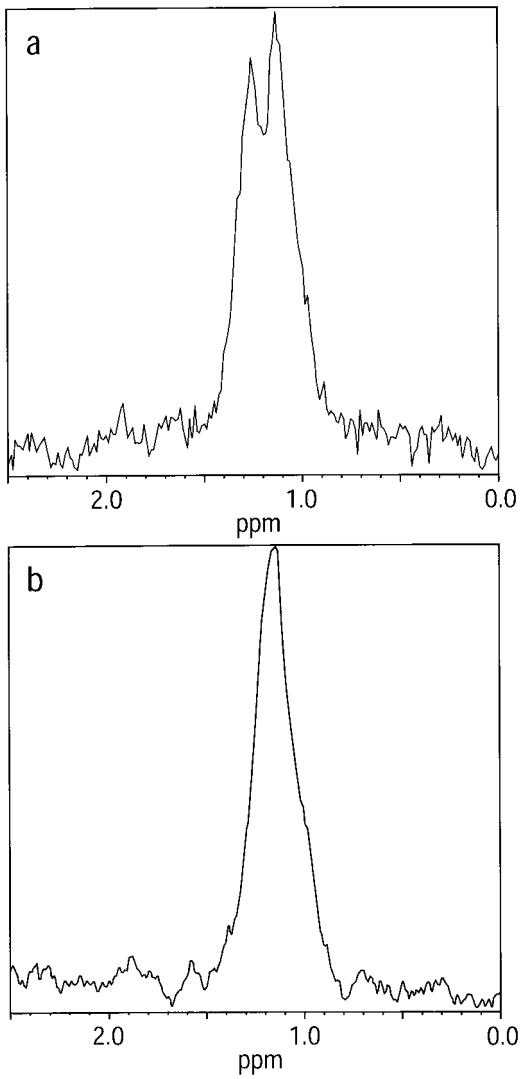

FIG. 3. DQ filtered ^{1}H NMR spectra of lactate in rat hindlimb muscles. (a) Muscle fibers are oriented parallel with B_0. (b) Muscle fibers are oriented perpendicular to B_0. The same sample was used in both cases. Note that in the case of parallel orientation a 25 Hz frequency splitting is observed. This frequency splitting becomes unresolvable when the muscle fibers are oriented perpendicular with respect to B_0. (Spectra were acquired at 4.7 T.)

certainty relative to the magnetic field. We chose the bovine muscle because it contains very homogeneously oriented fibers in bundles easily verified by eye. It also proved to be metabolically stable and reproducible.

The following evidence shows that the tissue obtained from the meat market retained the essential features of skeletal muscle. Figure 4 shows normal electrophoretic gel patterns from the bovine muscle (columns 3, 4, 7, 8) compared with patterns from fresh mouse EDL (columns 5, 9) and soleus (columns 2, 6), and rabbit psoas myofibrils (columns 1, 10). There was no evidence for proteolytic fragmentation of larger-molecular-weight proteins into smaller ones as indicated by the lack of low-molecular-weight protein fragments at the bottom of the gel and the presence of the normal amounts of high-molecular-weight proteins at the top of the gel. Figure 5 shows two photomicrographs demonstrating the normal sarcomeric pattern and inter-cellular connective tissue. Thus, the muscle fibers retain their essential histological structure. The water content in these samples was found to be an average of 74.6% mass ($n = 5$, stdev = 0.9), a typical value for mammalian muscle (*14*). There was no significant variation between different packages of meat ($n = 3$) or between bovine and rat muscle.

Metabolite Composition

Lactate content in samples cut from different areas of the same package averaged 43.2 μmol/g tissue ($n = 5$, stdev = 4.2). These measurements, repeated on muscle samples from three packages of flank steak, did not show substantial variation in lactate content. The spiked muscle samples were metabolically stable since lactate content did not change during the 15 h after the addition of exogenous lactate. Lactate assay of the spiked samples showed an 8-fold increase in the lactate content of the spiked samples compared to endogenous samples as expected from its endogenous content and the amounts of exogenous lactate added. This unphysiologically high concentration of lactate was used to saturate any binding sites which are present in muscle, both specific binding to lactate dehydrogenase and non-specific binding.

Amino acid content of bovine muscle was analyzed for the presence of additional metabolites which might have proton resonances in the region of interest. Taurine and glutamine

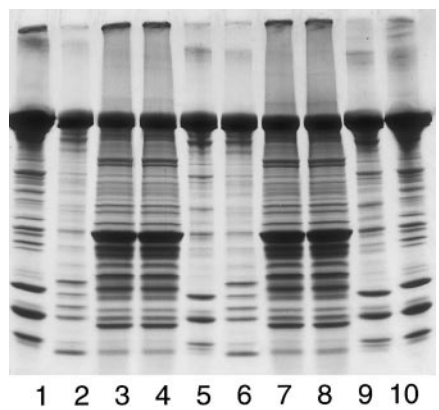

FIG. 4. Normal molecular weight distribution of proteins in postmortem muscle. Electrophoretic gel patterns of an endogenous sample from bovine muscle (columns 3, 4, 7, 8) compared with patterns from standard mouse EDL (columns 5, 9) and soleus (columns 2, 6). Rabbit psoas myofibrils were used as controls (columns 1, 10). Note that there is no evidence of accumulation of low-molecular-weight peptides at the bottom of the gel, which would be indicative of proteolytic fragmentation. Also, the presence of the major myofibrillar proteins (bands on the top of the gel), seen by comparison with proteins from fresh mouse muscle, indicates that any proteolysis was not sufficient to destroy the major structural proteins of the muscle even after a prolonged spectroscopic experiment.

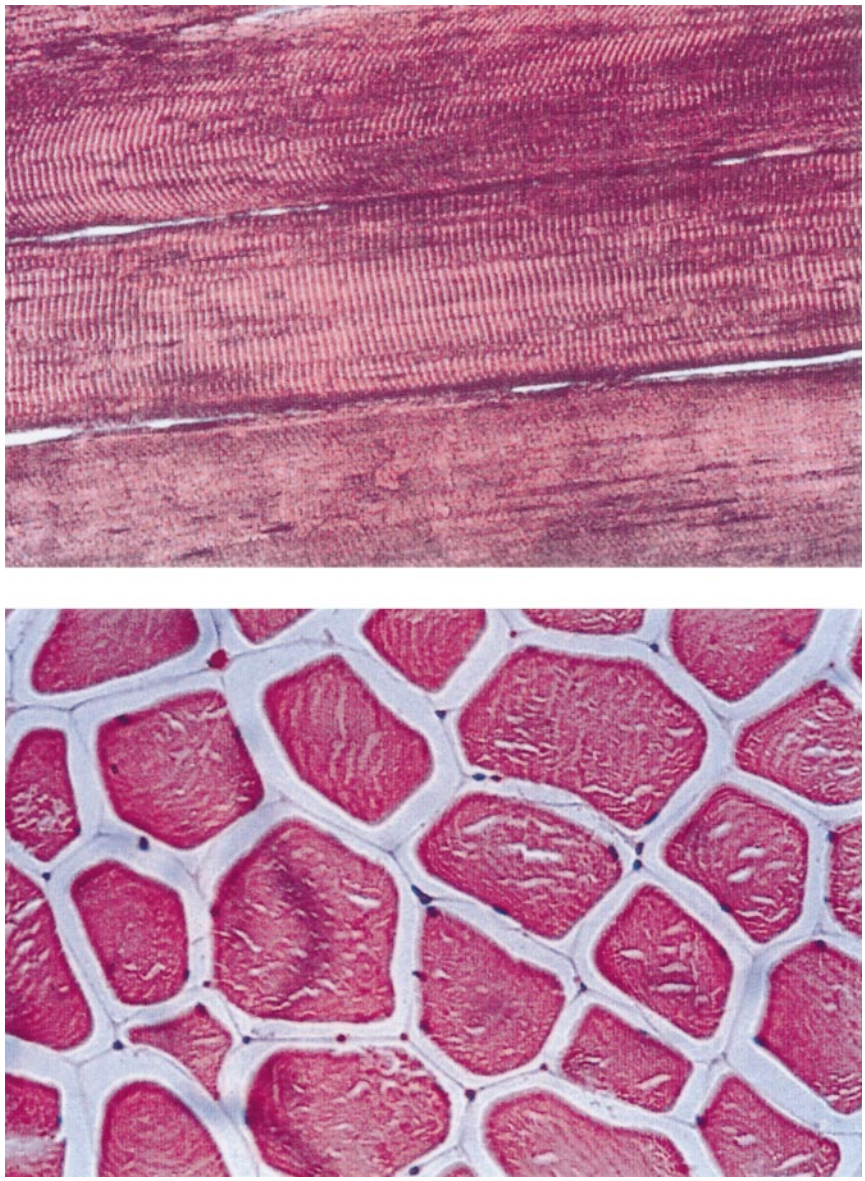

FIG. 5. Histology of postmortem muscle. Photomicrographs of H&E stained histological sections of bovine muscle, fixed after spectroscopic data were collected, show characteristic features in the light microscope of muscle fibers in longitudinal section (*top*) and in transverse section (*bottom*). The scale is given by the striation pattern at approximately 2 μm. Note that the muscle fibers retained their normal sarcomere striation pattern even after a 15 h spectroscopic experiment.

were present at 4 and 6 μmol/g, respectively. The only metabolite besides creatine with a content similar to endogenous lactate was carnosine, present at 15 μmol/g. None of these metabolites have resonances within the selected spectral window of 0–2.5 ppm. Alanine has a *J*-coupled doublet that appears at around 1.3 ppm in the proton spectra, but its content in bovine muscle was found to be 3.5 μmol/g muscle ($n = 4$, stdev = 0.8), that is, 8% of lactate in endogenous samples and only 2% of lactate in spiked samples. Because of its low concentration, the possible contributions of resonances from alanine were not studied.

Tests for Bulk Susceptibility Effects

The multiple quantum filtered acquisition (Fig. 1) should exclude any effect of resonance frequency inhomogeneity during τ_1, so magnetic susceptibility effects should not show up in the indirectly detected dimension of the multiple quantum, *J*-resolved experiments (*7*, *8*). However, as another test of the possibility that this orientation-dependent anisotropy of the lactate proton spectra might be due to an anisotropy in susceptibility, spectra of bovine muscle were acquired on three different magnets: 4.7, 7, and 11.7 T. If the additional splitting

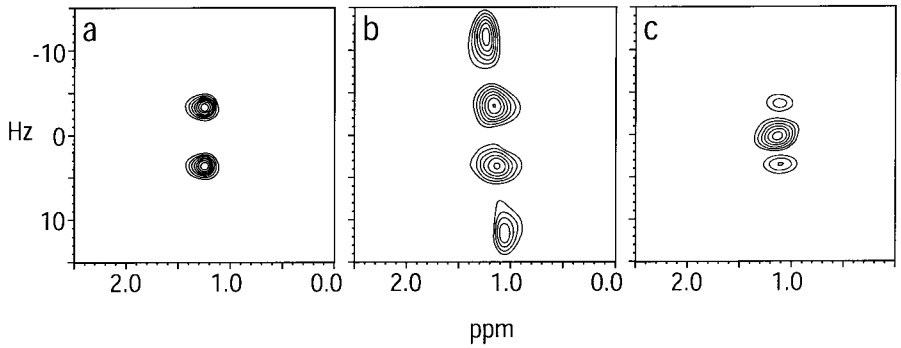

FIG. 6. DQ filtered ^{1}H NMR spectra demonstrating dependence of sample orientation on the magnetic field. Spectra were acquired at 4.7 T from: (a) a 0.3 *M* sodium lactate solution, (b) lactate in an endogenous sample from bovine abdominal muscles with fibers oriented parallel to the magnetic field, B_0, (c) lactate from the same sample as that in (b) but with the muscle fibers oriented perpendicular to B_0. Note that although the coupled contours due to *J*-coupling ($J_{HH} \sim$ 7 Hz) are present in all cases, an additional frequency splitting (~24 Hz) is present in the case of parallel orientation of the muscle fibers with respect to B_0. This additional 24 Hz splitting is not observed when the sample is oriented perpendicular to B_0. In this case dipolar and scalar couplings have canceled each other, thus collapsing into an apparent singlet.

that we observe was due to susceptibility inhomogeneities or experimental artifact, then its value would be expected to be proportional to the field strength. However, spectra showed the same splitting value (~24 Hz) at all three different fields when muscle fibers were oriented parallel to B_0. These results eliminate magnetic susceptibility variation within the sample as an explanation for the observed chemical shift differences in the lactate resonances, and assigns this orientation-dependent splitting to dipolar coupling interactions.

DQ Spectra of Bovine Muscle

Typical DQ, proton spectra from a cylindrical sample of bovine muscle are compared with lactate in solution in Fig. 6. Figure 6a shows a double quantum filtered, *J*-resolved spectrum of a 0.3 *M* sodium lactate solution with the expected contours at +/− 3.5 Hz. In Fig. 6b, contours are seen both for the *J*-coupled doublet, $J_{HH} = 6.9$ Hz, and for an additional doublet coupled by 23.4 Hz ($n = 15$, stdev = 2.1) when the sample was oriented parallel to B_0. When the sample was rotated by 90° around the *y* axis in the *xz* plane of the laboratory frame, only the familiar *J*-coupled doublet is obtained (Fig. 6c). In this case dipolar and scalar couplings cancel each other, resulting in an unresolved single peak. Note that there is a large difference in signal to noise between the spectrum in Fig. 6a (solution, 0.3 *M* lactate) and the spectra in Figs. 6b and 6c due to a lower concentration of lactate in the muscle than in the solution. Thus, spectra acquired on samples from bovine muscles showed splittings like those obtained from rat muscles for similar angles of orientation but at better resolution.

To validate the assignment of these peaks to lactate, spectra were also acquired at 4.7 T on spiked samples both immediately after exogenous lactate was added and 15 h later. In the case of parallel orientation, the peak intensities of the coupled systems (24 and 6.9 Hz) increased equally. Thus, both sets of resonances originated from lactate in the muscle samples. Spectra were also acquired on spiked samples every h after the lactate addition, for a total duration of 13 h. Initially, the intensity of the peaks with 7 Hz coupling was approximately 50% higher than the intensity of the peaks with 24 Hz coupling. Within an h, the intensities of the sets of coupled peaks became the same and the system appeared to have reached steady state. These results suggest that the exogenous lactate mixes with the endogenous molecules after a short time of diffusion and has an indistinguishable orientation-dependent splitting due to dipolar coupling. A related result was obtained by Kreis *et al.* for Cr/PCr pool in muscle (*2, 3*). However, lactate, unlike creatine, has a four-spin system (A_3X) with homonuclear *J*-coupling (methyl–methine) that contributes to the observed splitting in addition to the contribution from dipolar coupling (*15*).

TQ Spectra

It is important to identify the interactions that contribute to the observed splittings in the complex spin system of the lactate molecule since they could be due to intra-methyl and/or methine–methyl dipolar coupling. Figure 7 shows TQ, *J*-resolved spectra acquired on spiked samples from bovine muscles and from lactate solution (0.3 *M*). No TQ proton signal was seen from lactate in solution in the region of interest (0–2.5 ppm), as expected for the isotropic case where there is no intra-methyl coupling present. However, TQ spectra acquired on muscle samples did show a signal in this region. Figure 7a shows a typical TQ, *J*-resolved spectrum with the muscle fibers parallel to B_0, and Fig. 7b, a spectrum with fibers perpendicular to B_0. Thus, this signal was modulated with respect to τ_1 at the same frequency as that observed for the DQ spectra (Fig. 6) in both the parallel and the perpendicular orientations. These results prove that the proton spectra receive

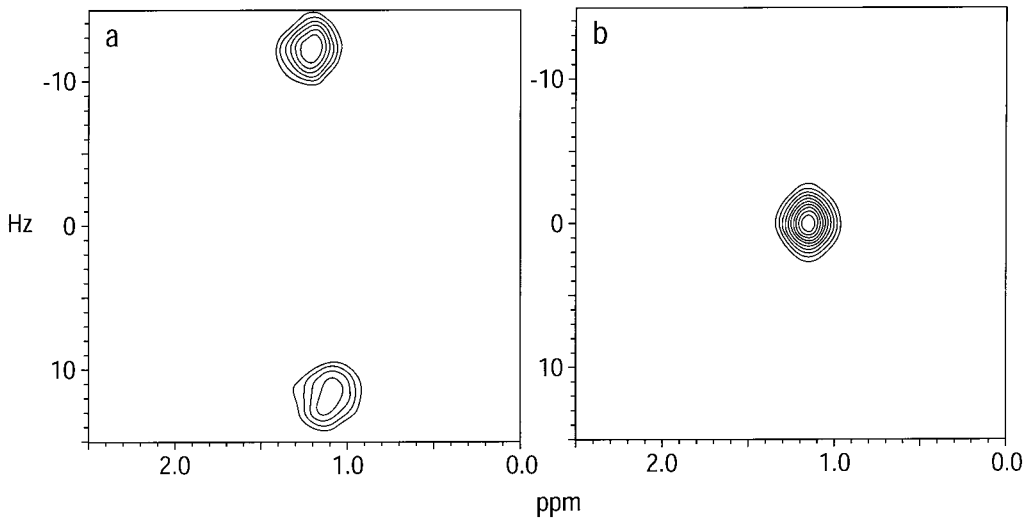

FIG. 7. Triple quantum coherence spectroscopy. TQ, *J*-resolved spectra from: (a) a spiked bovine abdominal muscle sample with the fibers oriented parallel to B_0, (b) the same sample as that in (a) but with the muscle fibers oriented perpendicular to B_0. Note that TQ, *J*-resolved spectra from lactate in solution show no signal in the region of interest (0–2.5 ppm). (Spectra were acquired at 4.7 T.)

contributions from at least one three-spin system involved in dipolar coupling interactions.

Figure 8 shows TQ coherence spectra from spiked, bovine muscle samples with the carrier frequency at 3.2 ppm. The existence of two peaks at two different frequencies (740 and 1280 Hz) indicates that two distinct transition frequencies are present. These frequencies correspond to two sets of dipolar coupled spins, A_3 and A_2X. This result shows that two three-spin systems are involved. The transition frequencies at ~1280 and ~740 Hz correspond to $\omega_1 = 3 \times \Omega_A = 3 \times 424 = 1272$ Hz and $\omega_2 = (2 \times \Omega_{A3}) - \Omega_X = (2 \times 424) - 160 = 688$ Hz. Observation of both frequencies at the methyl resonance shows that an intra-methyl coupling is present (*7*, *8*). This intra-methyl coupling could only be dipolar in origin. The intensities of these peaks depend on the magnitude of the intra-methyl and methine–methyl coupling.

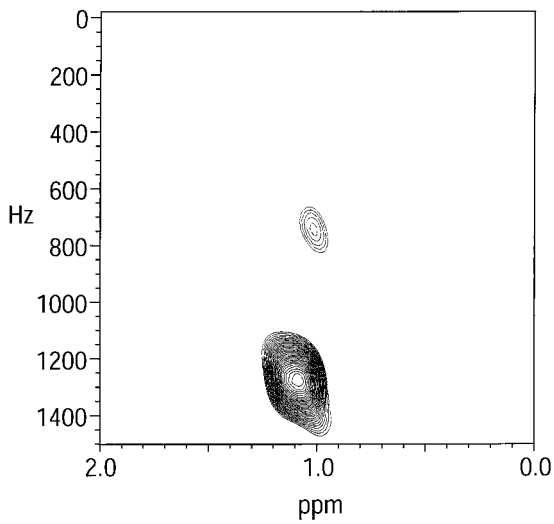

FIG. 8. TQ coherence spectra from spiked, bovine muscle samples with fibers oriented parallel to B_0. The carrier frequency was set at 3.2 ppm. The transition frequencies appear at ~740 and ~1280 Hz, corresponding closely to two sets of dipolar coupled spins, A_3 and A_2X (see text). Note that the peak corresponding to the transition frequency of ~1280 Hz is larger than the peak corresponding to the frequency of ~740 Hz. The existence of both frequencies at the methyl resonance shows that an intramethyl dipolar coupling is present.

Decoupling experiments were performed to ascertain whether the observed 24 Hz splitting is a result of intra-methyl or methyl–methine interactions. TQ *J*-resolved spectra were acquired on spiked bovine muscle samples while the methine proton was selectively decoupled during τ_1 or during τ' (Fig. 1). In the TQC experiments, the decoupling during τ_1 removed all peaks except for the peak at $3 \times \Omega_{A3}$ when it was applied to the methine proton, and it removed all the TQC signals when it was applied to the methyl protons. Decoupling during τ' collapsed the 24 Hz splitting observed in the indirectly detected dimension of the 2D, *J*-resolved experiment, and demonstrated its origin in the methine–methyl coupling. Without decoupling during τ', the TQC is observed to be split into a doublet by an approximately 80 Hz coupling (Fig. 9a). This is the expected multiplicity and splitting of an A_3 TQC in an A_3X spin system due to an approximately 26 Hz AX coupling (*8*). This signal collapsed to a singlet when the methine was decoupled selectively (Fig. 9b). Although methine decoupling in this experiment would also be expected to randomize any TQC containing methine coherence, the fact that the observed splitting without decoupling collapsed when decoupling was applied clearly shows that this splitting results from the methine–

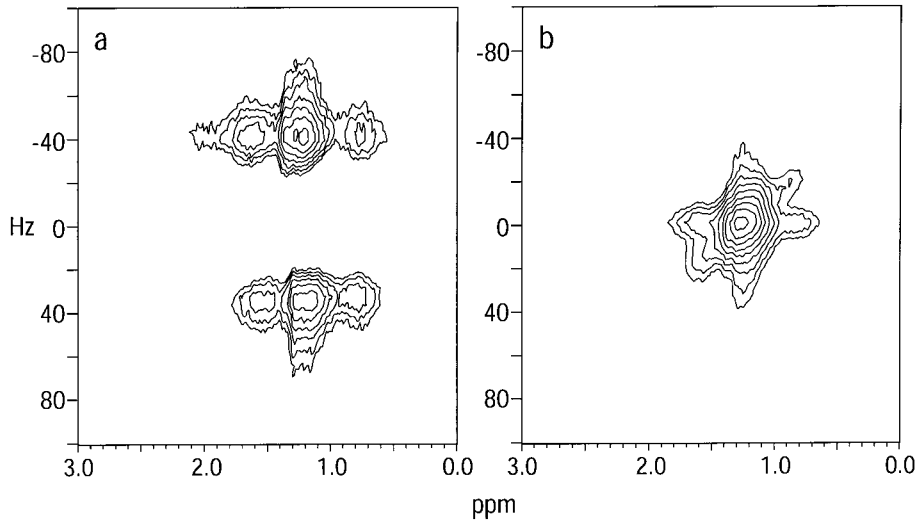

FIG. 9. Decoupling TQ spectra to identify the spin interactions. Splitting of the triple quantum coherence observed on the lactate methyl without (a) and with (b) selective decoupling of the methine proton. Spectra were acquired at 11.7 T with the muscle fibers approximately aligned with B_0. In (a) the decoupler is applied equidistant to the methine proton upfield of the methyl, while in (b) it is applied directly at the methine frequency. For these data τ_1 and τ_2 (Fig. 1) were set to 5 ms (the observed splitting is not sensitive to the value of these delays).

methyl coupling. The correlation of the value of this splitting with that observed in the *J*-resolved DQ spectra and the DQ filtered 1D spectra points to a common origin for all.

These results are explained by the presence of both intra-methyl and methine–methyl TQC couplings, with the observed 24 Hz splitting arising from methine–methyl coupling only. The magnitude of the intra-methyl coupling, even taking into account the 3-fold averaging from methyl group rotation, should be larger than that with the methine proton because shorter inter-proton distances are involved. The geometry of the lactate molecule would dictate that the intra-methyl coupling should also be observable if a methine–methyl coupling appears, which we have shown is the case. The intra-methyl coupling is not observed directly here, presumably due to a distribution in the value of its orientationally averaged coupling constant which yields only broad peaks.

Note that the lactate signal in Fig. 9 appears to show some structure in the f_2 dimension. Although couplings cannot be ruled out, the samples used at 11.7 T were much smaller than those used at 4.7 T. Therefore, irregularities of the sample shape relative to its size were larger, resulting in multiplet-like structures possibly due to magnetic susceptibility effects and shimming. The important observation is that no susceptibility effects were observed in the f_1 dimension.

Quantifying the Orientational Dependence of the Observed Spectra

To quantify the angular dependence of the dipolar coupling, three data sets were obtained (Fig. 10). For each experiment, the orientation angle was varied about the *y* axis with α defined as zero when fibers were parallel relative to the magnetic field, B_0. The angle α was varied in a controlled way to measure the change in the splitting frequency for the lactate methyl doublet in the indirectly detected dimension of the DQ, *J*-resolved spectra. In the first data set (Fig. 10, empty circles), an endogenous bovine muscle sample was rotated clockwise from 0 to 90° with respect to B_0 and the splitting was measured for each recorded α. The second data set (Fig. 10, full circles), was obtained from a spiked sample within 40 min after addition of lactate. The third data set (Fig. 10, empty triangles) was obtained from a spiked sample 15 h after lactate injection to test the effects of long diffusion times. To test for any asymmetry in the apparatus the experiment was repeated for a spiked sample rotated in the counterclockwise direction (Fig. 10, full triangles).

The dipolar coupling contribution, Δf_{dc}, is described by (*2*),

$$\Delta f_{dc} = 1.5 D_0[1 - 3(\cos\theta\cos\alpha - \sin\theta\sin\phi\sin\alpha)^2], \quad [1]$$

where θ and ϕ are the Euler angles defining the transformation from the laboratory reference system to the molecular one, α is defined above, and D_0 is the dipolar coupling constant. Because the TQ experiments showed that the methine–methyl dipolar coupling is the main contributor to the observed dipolar coupling effects, the contribution from the scalar coupling needs to be taken into consideration as well (*15*). Therefore, the data were fit with two functions that account for both dipolar coupling and *J*-coupling: $\Delta f = \Delta f_{dc} + 7$ and $\Delta f = \Delta f_{dc} - 7$. The fitting of the data with $\Delta f = \Delta f_{dc} - 7$ gave a best fit (fitting error, 0.64) for $D_0 = (19.9 \pm 1.65)$ Hz, $\theta = (92 \pm$

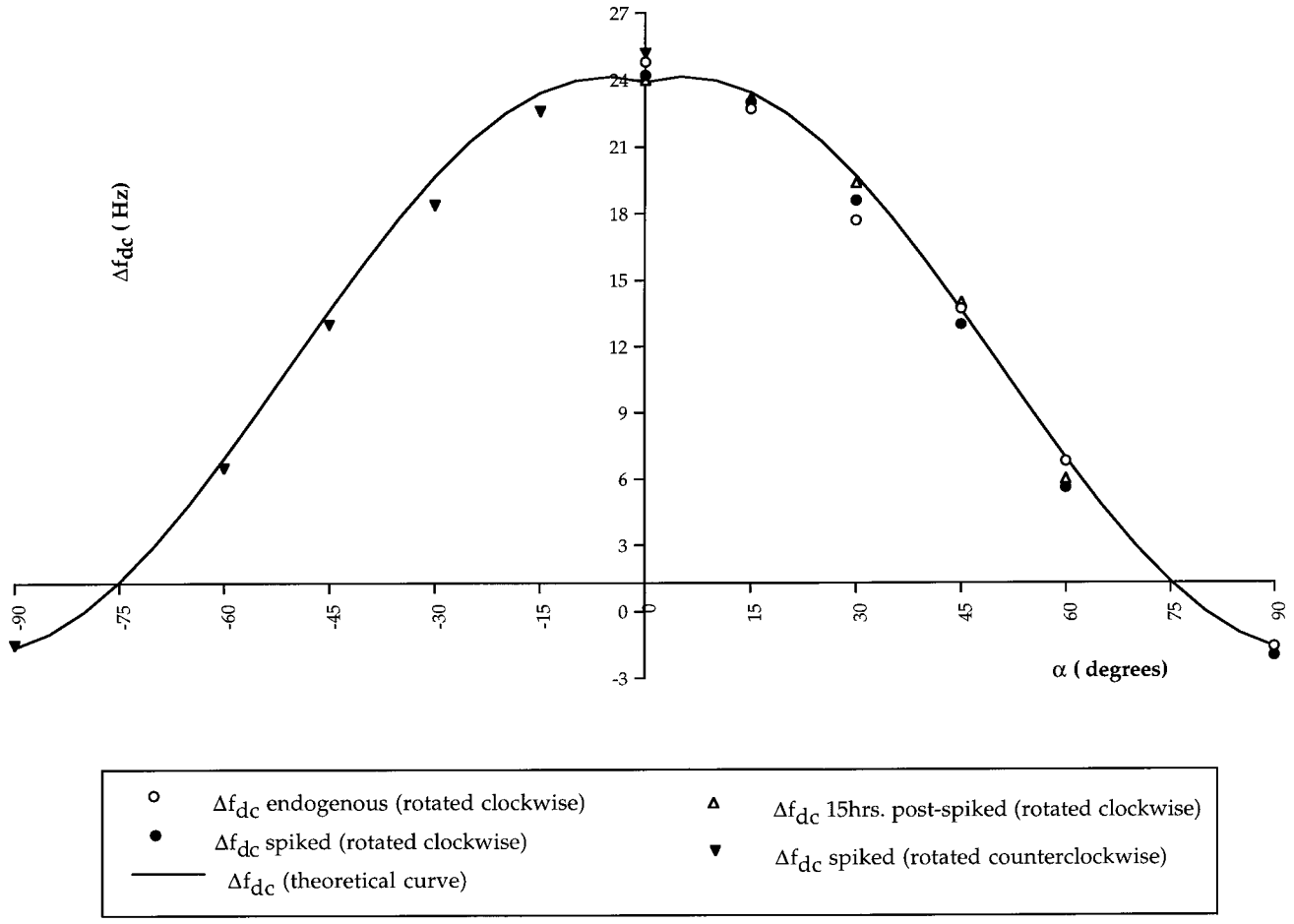

FIG. 10. Orientational dependence of the observed frequency splitting, (Δf) with the angle (α) between muscle fibers and B_0. In the first data set (empty circles), a muscle sample containing only endogenous lactate was rotated clockwise, 90° around B_0, and Δf was measured for each corresponding α. The second data set (full circles), belongs to a spiked sample. The third data set (triangles), belongs to a spiked sample 15 h after exogenous lactate is added. The solid line is the best fit to the theoretical curve $\Delta f = \Delta f_{dc} + 7$, where Δf_{dc} is given in Eq. [1]. There was no difference in the measured Δf for counterclockwise rotations around B_0 (full triangles). (Note that from Eq. [1] it is apparent that there is a term that would depend on the sign of α; however, for $\theta = 10°$ and $\phi = 26°$ that term has a negligible effect on the value for Δf_{dc}.)

0.89)°, and ϕ =(31 ± 2.4)°. Two sets of values for D_0, θ, and ϕ yielded indistinguishable fits for $\Delta f = \Delta f_{dc} + 7$. D_0 = (−5.9 ± 1.2) Hz, θ = (10 ± 2.2)°, ϕ =(26 ± 2.8)° gave a fitting error of 0.81, whereas D_0 = (10.6 ± 0.66) Hz, θ = (88 ± 4.6)°, ϕ = (44 ± 5.3)° gave a fitting error of 0.46.

The only solution that is in accordance with muscle axial symmetry is the solution that results from fitting the data with the function $\Delta f = (\Delta f_{dc} + 7)$ where $D_0 = -5.9$ Hz, $\theta = 10°$, $\phi = 26°$. This solution indicates that the preferred orientation for the time averaged methine-methyl vector in lactate molecules is nearly parallel to the muscle fiber axis. Interestingly, this angular orientation is very similar to that obtained by Kreis *et al.* (*2*) for the Cr/PCr pool ($\theta = 5.1°$, $\phi = 90°$, $D_0 = 4.92$ Hz). All the other solutions would sugest that muscle fibers lack cylindrical symmetry. In order to verify that these are indeed improbable solutions, a control experiment was done. DQ spectra were acquired with the muscle fiber axis perpendicular to the *xy* axis of the laboratory frame. Rotation of the sample around B_0 in the *xy* plane showed no change in the splitting frequency of 24 Hz.

DISCUSSION

The experiments described here show that the proton NMR spectra of lactate in skeletal muscle depend on the orientation of the muscle fibers relative to the magnetic field. We have shown that this result is not due to susceptibility effects because spectra acquired on different magnetic fields showed the same splitting frequency. Dawson *et al.* have observed two peaks, typically 15–20 Hz apart, for the lactate methyl of frog muscles (*16*). The presence of this chemical shift difference has been explained as due to susceptibility effects that arise from the presence of paramagnetic myoglobin in oxidative

fibers. As such this mechanism is different from the results that we present. The spectra presented in this paper are highly dependent on the orientation of the fibers with the magnetic field but they do not depend on the field strength. This result is a direct indication that the splitting frequency that we observe is not a magnetic susceptibility effect, thereby excluding paramagnetic effects like those presented by Dawson *et al.* as the mechanism for the dipolar coupling effects reported here.

This orientational effect observed in the proton NMR spectra of lactate in skeletal muscle is due to dipolar coupling interactions. The DQ filtered spectra of rat (Fig. 3) and bovine muscle (Fig. 6) showed a split lactate methyl resonance only when the muscle fibers were oriented parallel to B_0. A combination of TQ *J*-resolved and TQC spectra with and without decoupling proved that there were two three-spin systems involved; that is, all of the protons in the lactate A_3X spin system are involved in the dipolar coupling. Further, the TQC experiments suggest that methyl–methine interaction is the main contributor in the observed frequency splitting. Intramethyl dipolar coupling, which the decoupling experiments demonstrate to be present, is not observed, probably because a distribution in coupling constants gives broad peaks. For all of these interactions to exist, there must be anisotropic motion of lactate molecules in muscle.

This anisotropy is observable as an additional coupling in lactate (A_3X) proton spectra of muscle with and without added exogenous lactate. As expected, this anisotropy is not seen for lactate in solution. Neither endogenous lactate nor injected exogenous lactate satisfies the conditions required for isotropic motional averaging. Thus, lactate in muscle, on a time average, has a preferred orientation within the structure of muscle. This anisotropy is clearly present in post-mortem muscle, and thus contrasts the phenomena described by Kreis *et al.* (*2, 3*) for the dipolar coupling of methylene protons in the creatine/phosphocreatine resonance, which they report is observable only in fresh living muscle. We have not studied the reasons for this apparently different result.

The presence of both the 7 and 24 Hz splittings in the same sample means that both scalar and dipolar coupling are present. This can only occur if there are separate pools of lactate in muscle. For a spin system with both dipolar (Δ_{dc}) and scalar (J_{HH}) coupling, the observed splitting corresponds to the algebraic sum $J_{HH} + \Delta_{dc}$ (*15*). Lactate in one pool gives only the expected 7 Hz *J*-coupling; that is, these molecules are not subject to dipolar coupling. Lactate in the other pool gives rise to a frequency splitting which depends on the angular orientation of the fibers in the magnet. The observed signal is thus the sum of both scalar and dipolar coupling (*15*). When lactate was added exogenously into the sample, the intensities of both scalar and dipolar signals increased, suggesting a uniform distribution of the added lactate between the two pools, neither of which is naturally close to saturation. One hypothesis would be that lactate inside the muscle fiber structure *per se* is subjected to order effects whereas lactate in the interstitial space behaves as lactate in solution, i.e., tumbles freely, and only the 7 Hz scalar coupling is observed. The volume of interstitial space in normal muscle in intact animals and humans (*14*) is approximately 7% of the total. Thus, our present results may form a basis for distinguishing intracellular and extracellular lactate in living systems because it is clear that the complex MQ pulse sequences can be used successfully in skeletal muscle.

Several hypotheses for the *orientational* force involved, which have been proposed for the case of creatine and phosphocreatine dipolar coupling (*2*), may also apply to the restricted movement of lactate molecules in skeletal muscle. The movement of the observed molecules may be diffusionally restricted due to the small spacing between actin and myosin filaments. However, for lactate, comparison of this spacing (around 20 nm) (*17*) to the width of a hydrated lactate molecule (around 0.6 nm, using simple molecular calculations from given bond lengths and bond angles) suggests that steric restriction is unlikely. However, recent studies of the diffusivity of ATP and phosphocreatine in muscle by ^{31}P spectroscopy implicated this structural space as the cause of the approximately 2-fold reduction in diffusivity of molecules (*12*) in muscle. Specific lactate binding to the lactate dehydrogenase and non-selective binding to other macromolecules present in muscle cells is another possibility. This possibility appears to be excluded by a similar increase in both 24 and 7 Hz couplings when exogenous lactate is added several-fold in excess of the physiological range; at this high concentration specific binding sites would be saturated. Another class of explanations arises from the highly structured organization of muscle, from the nanostructure of the intracellular myofilaments at the macromolecular level to connective tissue matrix at the multicellular level. Regular protein structures would order charges on the surface of the proteins and set up an electrical field. Such a field within the myofilaments is well described in (*18*). Thus, as an anion at normal physiological pH's, lactate could be subject to an oriented electrostatic force in addition to the random Brownian motion. Obviously, this hypothesis would imply that other small and charged metabolites, like most metabolites and signaling molecules, would show partial orientation and thus dipolar coupling effects on the NMR spectra in skeletal muscle.

The observation of dipolar coupling interactions in the proton NMR spectra of muscle has far-reaching implications for the use of NMR spectroscopy in biological systems. Quantification of resonances will be affected both by the orientation of structurally anisotropic samples and by the choice of mixing times used to develop the coherences used for editing spectra. In the case of lactate, these effects have not yet been taken into account except in this work. Our results demonstrate how these effects can be observed in the application of proton NMR spectroscopy of highly structured tissues, such as connective tissue, peripheral nerves, and tracts within the brain. Analysis of the mechanisms yielding orientation-dependent spectra

could make NMR a valuable tool for studying functional aspects of structure at the cellular, tissue, and organ levels.

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