Journal of Magnetic Resonance **149,** 8–12 (2001)
doi:10.1006/jmre.2000.2266, available online at http://www.idealibrary.com on IDEAL®

# On the Importance of Exchangeable NH Protons in Creatine for the Magnetic Coupling of Creatine Methyl Protons in Skeletal Muscle

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Received May 25, 2000; revised November 6, 2000

**The methyl protons of creatine in skeletal muscle exhibit a strong off-resonance magnetization transfer effect. The mechanism of this process is unknown. We previously hypothesized that the exchangeable amide/amino protons of creatine might be involved. To test this the characteristics of the creatine magnetization transfer effect were investigated in excised rat hindleg skeletal muscle that was equilibrated in either $H_2O$ or $D_2O$ solutions containing creatine. The efficiency of off-resonance magnetization transfer to the protons of mobile creatine in excised muscle was similar to that previously reported in intact muscle *in vivo*. Equilibrating the isolated muscle in $D_2O$ solution had no effect on the magnetic coupling to the immobile protons. It is concluded that exchangeable protons play a negligible role in the magnetic coupling of creatine methyl protons in muscle.** 

*Key Words*: **creatine; magnetization transfer; deuterium; exchange; muscle.**

## INTRODUCTION

In the past decade, several *in vivo* $^1$H MR studies on brain and skeletal muscle demonstrated a magnetic coupling between the protons of certain low-molecular-weight metabolites and the immobile proton pool (*1–11*). Off-resonance saturation of the immobile proton pool results in a reduction of the $^1$H MR signal of the mobile protons of a number of different metabolites. For the methyl protons of creatine/phosphocreatine (tCr) this magnetization transfer (MT) effect was found in rat brain (*1–4*) and skeletal muscle (*5*) as well as in mouse skeletal muscle (6). Much smaller but measurable MT effects have also been reported for *N*-acetylaspartate (NAA) and choline-containing compounds (Cho) in rat brain (*2–4*), lactate in rat brain after induction of global ischemia (*3, 4*), and lactate in rat brain C6 glioma (*7*). The exchange of magnetization between the immobile proton pool and mobile metabolite protons can occur via two main routes: (i) chemical exchange in which the entire molecule exchanges between an immobile and mobile state; and (ii) cross-relaxation (exchange of magnetization through space).

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This study addresses the MT effects of the tCr protons in rat skeletal muscle. Figure 1 schematically depicts the mechanisms of magnetization transfer that are potentially relevant to tCr in muscle.

A number of studies have been carried out to gain a mechanistic understanding of the off-resonance MT effect of tCr in rodent skeletal muscle. Induction of ischemia of rat hindleg skeletal muscle had no effect on the magnitude of the off-resonance MT effect (*5*). This demonstrates that phosphocreatine (PCr) and creatine (Cr) contribute to the MT phenomenon to the same extent. Furthermore, comparative measurements in wild-type and transgenic mouse muscle (*6*) showed that creatine kinase deficiency hardly changes the tCr MT effect. This suggests that specific interactions of tCr with creatine kinase that is present up to 1 mM concentrations in skeletal muscle (*12*) are not responsible for the generation of the phenomenon.

It is also known that the creatine methyl protons in rat brain (*4*) and rat skeletal muscle (*5*) as well as human brain (*10*) and muscle (*10*) exhibit a magnetic coupling to the mobile water protons in these tissues. Selective inversion of the water magnetization causes a transient reduction in the intensity of the peak from tCr methyl protons. The mechanism of the inversion transfer process is also unknown. A number of different pathways that might magnetically couple the mobile creatine protons to the mobile water protons are depicted in Fig. 1.

In the present study, the possibility that exchangeable protons play a role in the transfer of magnetization from the immobile proton pool and the mobile water pool to the tCr methyl protons in rat skeletal muscle was tested (see Fig. 1). Exchangeable protons might be involved in the intermolecular transfer of magnetization to the tCr methyl protons. Transiently immobilized exchangeable protons can be saturated by the off-resonance RF field in the off-resonance MT experiment and become labeled by the selective water inversion pulse, followed by chemical exchange, in the inversion transfer experiment. Intermolecular cross-relaxation between these transiently immobile exchangeable protons and the tCr methyl protons would thus result in a reduced tCr signal intensity. Alternatively, or in parallel, an intramolecular MT via exchangeable amide/amino protons in tCr could contribute to the MT effect experienced by the tCr methyl protons. The spin states of the tCr amide/amino protons can be



1090-7807/01 $35.00

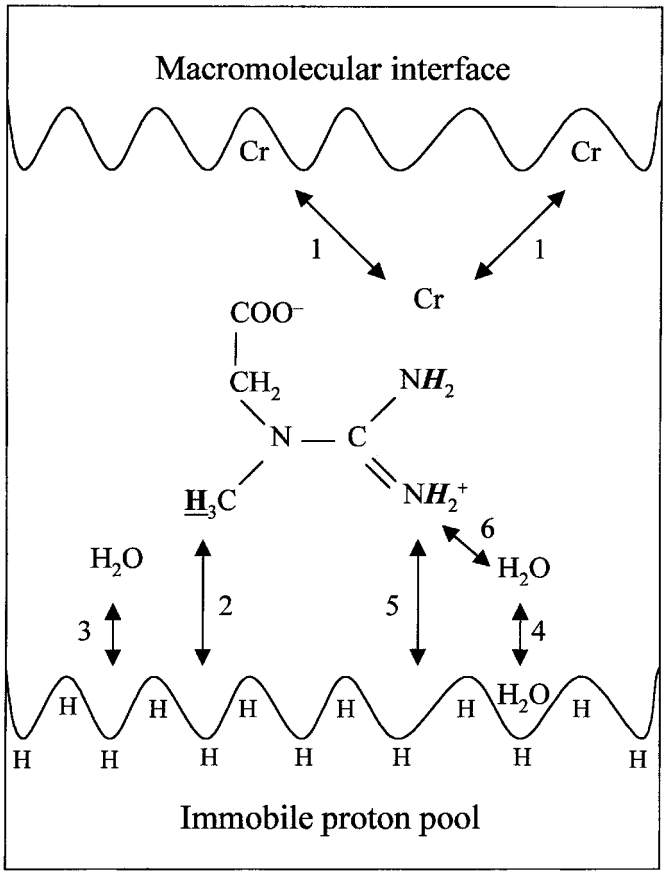


**FIG. 1.** Schematic representation of possible magnetization transfer pathways involved in the magnetic coupling of creatine protons in tissues. The creatine methyl protons are depicted in bold and underlined while the creatine amide and amino protons are indicated in bold and italic. Arrows: 1, chemical exchange of creatine between a mobile and a macromolecule bound state; 2, cross-relaxation between the immobile proton pool and the mobile creatine methyl protons; 3, cross-relaxation between the immobile proton pool and the protons of mobile water; 4, chemical exchange of water protons between a mobile and immobile state; 5, chemical exchange of the amide protons of Cr with the immobile proton pool; 6, chemical exchange of mobile protons between the Cr amide group and water. In this study the role of exchangeable protons in the magnetic coupling of Cr methyl protons was examined. Except for the processes as indicated by arrows 1 and 2, all the indicated magnetization transfer steps involve participation of exchangeable protons.

perturbed via chemical exchange and cross-relaxation from the immobile proton pool in the off-resonance MT experiment and, in a more direct manner, via chemical exchange with water protons in the inversion transfer experiment. Intramolecular transfer of magnetization from the amide/amino protons to the methyl protons of tCr could explain the *in vivo* observations. It should be noted that an efficient intramolecular MT requires transient immobilization of the tCr molecule. In *in vitro* solutions, water inversion has no effect on the signal intensity of the tCr methyl protons.

The role of exchangeable protons in the tCr MT effect was studied in excised rat skeletal muscle that had been fixed in paraformaldehyde. Following fixation, the muscle was equilibrated in an aqueous buffer containing creatine. Both of resonance and selective water inversion MT studies demonstrated that the characteristics of the magnetic coupling of creatine in excised muscle were essentially identical to those in intact muscle *in vivo*. This motivated us to assess the effects of equilibration of the muscle preparation in a $D_2O$-based solution of creatine.

## METHODS

An adult Wistar rat that had been used in a brain ischemia study was perfusion fixed with paraformaldehyde. One hindleg was excised and stored in fixative at 4°C. Prior to the NMR studies the muscle was stored in phosphate-buffered saline (PBS) solution (pH 7.4) containing 100 mM creatine and 6% $D_2O$ for 2 weeks (the buffer was refreshed three times). After the first series of $^1$H NMR studies (see below) the leg was stored in PBS buffer containing 100 mM creatine and 95% $D_2O$ for two weeks (the buffer was refreshed three times) and the NMR experiments were repeated. During the NMR studies, the muscle was covered with plastic foil to prevent dehydration.

$^1$H NMR experiments were performed at 200 MHz on a Varian spectrometer interfaced to a horizontal 40-cm-bore, 4.7-T Oxford magnet. During the MR experiments the temperature was maintained at 37°C by a flow of warm air. A volume coil was used for RF transmission and signal reception. To guide volume selection 21 consecutive slices of 1 mm thickness were imaged with a spin echo sequence (field of view, $5 \times 5$ cm; matrix, $256 \times 256$; echo time TE, 30 ms; repetition time TR, 2.1 s; two acquisitions). An external standard was used to estimate the proton density of the muscle and to determine the efficiency of $^1$H substitution by $^2$D. Using point resolved spectroscopy (PRESS) (*13*) a $7.5 \times 7.5 \times 5$-mm volume was selected inside the hindleg for $^1$H NMR measurements on Cr (using chemical shift selective (CHESS) water suppression prior to PRESS) and $H_2O$/HDO (CHESS was omitted).

Off-resonance $^1$H NMR MT experiments were performed by applying a 3-s saturation pulse ($\gamma B_2$, 300 Hz), prior to CHESS, of which the frequency offset was varied (from −100 to 100 kHz) around the offset of the Cr methyl (nt = 32) or $H_2O$/HDO protons (nt = 8, TR = 5 s, TE = 144 ms).

The magnetic coupling between the protons of water and the Cr methyl group was investigated using inversion transfer. The water (or Cr methyl) magnetization was selectively inverted, followed by a variable delay. Water-suppressed PRESS spectra were recorded (nt, 32; TR, 10 s; TE, 144 ms; 10 inversion delay times from 0 to 5 s).

To aid in the analysis of the off-resonance MT experiments, $T_1$ (TE, 144 ms; TR, 10 s; inversion delay times, from 0 to 5 s) and $T_2$ (seven TE times, from 30 to 150 ms; TR, 10 s) measurements of water (nt, 8) and Cr (nt, 32) were performed. The off-resonance MT curves were fitted according to a two-pool exchange model, as described in (*5*).

Spectra were quantified using iterative fitting of the time domain signals with the variable projection method (VARPRO) (*14*). A series of spectra was first fitted without prior knowledge to estimate the mean linewidths of the resonances ($H_2O$/HDO at 4.7 ppm and tCr at 3.03 ppm). The mean linewidths were next used as prior knowledge to fit the spectra once more. No prior knowledge about the chemical shift positions was included.

## RESULTS

In line with previous *in vivo* observations, the Cr methyl proton signal showed a pronounced off-resonance MT effect in excised muscle equilibrated in $H_2O$ (Fig. 2) as well as a strong coupling with the mobile water protons (Fig. 3). These observations made it possible to investigate the role of exchangeable protons in the magnetic coupling of Cr protons in excised muscle.

The pronounced off-resonance MT effect on the Cr methyl protons in muscle soaked in aqueous buffer is shown in Fig. 2A. A prominent off-resonance MT effect was also observed in $D_2O$-equilibrated muscle (Fig. 2B). Using $^1H$ NMR imaging it was estimated that equilibration in $D_2O$ solution reduced the proton density of the muscle by about 80% (data not shown). The graphical representation of the off-resonance MT experiments (Fig. 2C) demonstrates that the tCr MT effect remained unaltered when substituting $H_2O$ for $D_2O$. The MT data were fitted according to a two-pool exchange model. The immobile proton pool with which the Cr methyl protons exchange magnetization was estimated to amount to about 0.3% of the mobile creatine pool and its $T_2$ was found to be approximately 14 $\mu$s, in both $H_2O$- and $D_2O$-equilibrated muscle.

Off-resonance MT curves were also recorded for mobile water in both the $H_2O$- and the $D_2O$-soaked muscle (Figs. 2D and 2E, respectively). A more prominent MT effect was observed for water protons in $D_2O$-equilibrated muscle, as is also evident from Fig. 2F. The mathematical modeling indicated that the immobile proton pool with which mobile water exchanges magnetization had increased from 0.4 to 0.6% of the mobile water pool. Taking into account that the mobile water pool had decreased

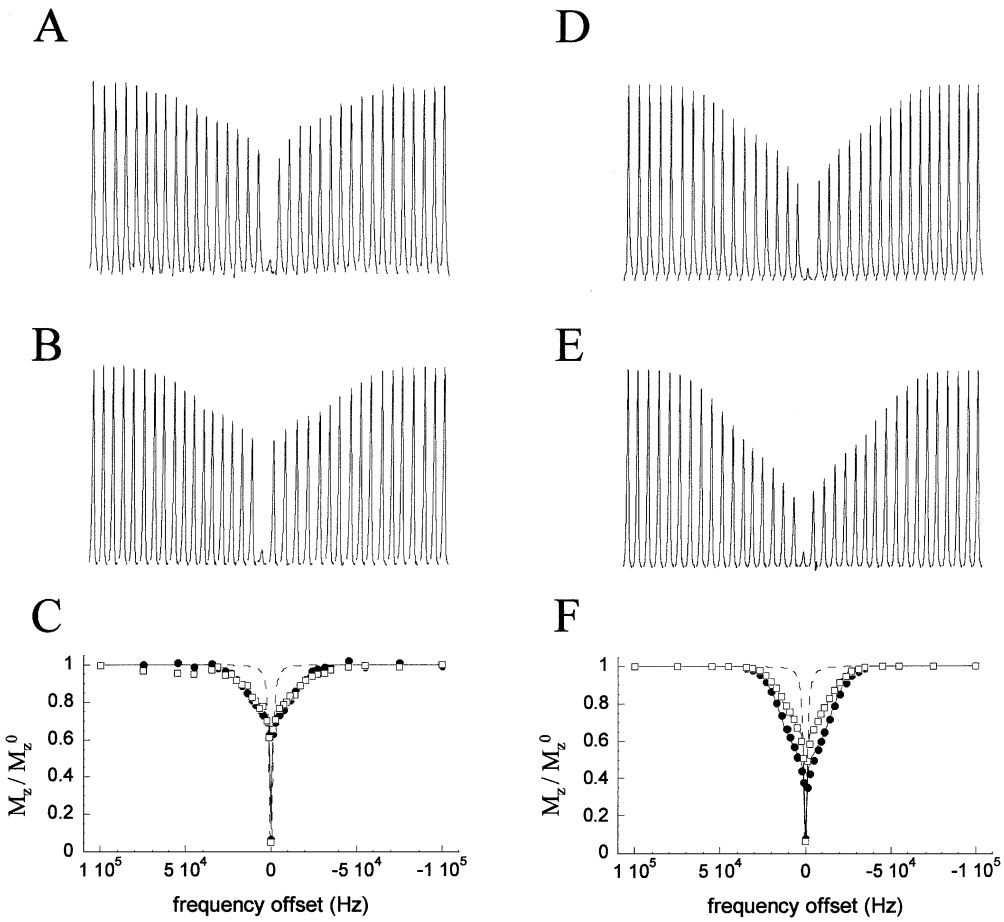


**FIG. 2.** Off-resonance magnetization transfer to the creatine methyl protons and the protons of mobile water in excised skeletal muscle. Panels A and B show the Cr methyl peak as a function of the frequency of saturation in $H_2O$- and $D_2O$-equilibrated muscle, respectively. The relative intensities of the Cr methyl signal in A (□) and B (●) are plotted in C together with the fit according to a two-pool exchange model (—) and the direct spill-over curve (- - -). Panels D–F show similar data for the protons of mobile water. The spectra in D and E were recorded in $H_2O$- and $D_2O$-equilibrated muscle, respectively. Panel F shows the relative $H_2O$ and HDO signal intensities of D (□) and E (●) with the fit according to a two-pool exchange model (—) and the direct spillover curve (- - -).

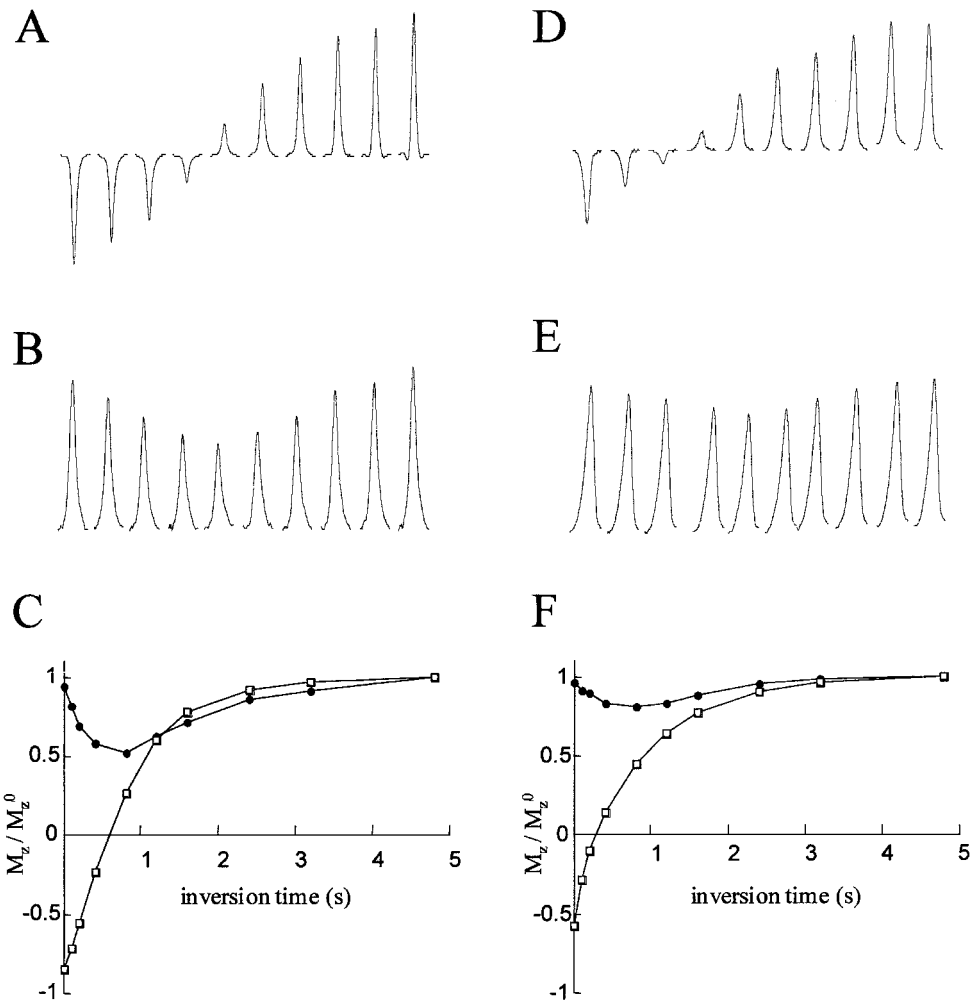


**FIG. 3.** Magnetic coupling between mobile water protons and Cr methyl protons in excised skeletal muscle, equilibrated in $H_2O$-containing (A–C) and $D_2O$-containing solution (D–F). Panels A and D depict the water signal as a function of the time after selective inversion of the water resonance. Panels B and E show the Cr methyl resonance for the same inversion recovery times. Panels C and F depict the relative intensities of the water signal (□) and the Cr methyl signal (●) in $H_2O$- and $D_2O$-equilibrated muscle, respectively.

by 80%, these data suggest that the immobile proton pool to which the mobile water protons are magnetically coupled had decreased by 70% upon storage of excised muscle in $D_2O$ solution.

The transfer of magnetization from mobile water protons to Cr methyl protons was also compared between $H_2O$- and $D_2O$-equilibrated muscle, using inversion transfer. Figures 3A and 3D show the inversion recovery of the water proton magnetization in $H_2O$- and $D_2O$-equilibrated muscle, respectively. Selective inversion of water resulted in an efficient magnetization transfer to Cr in $H_2O$-equilibrated muscle (Fig. 3B). The effect was strongly reduced in the $D_2O$-equilibrated tissue (Fig. 3E). The decreases in Cr signal intensity were approximately 50 and 20% at maximum in $H_2O$- and $D_2O$-soaked muscle, respectively (compare Figs. 3C and 3F). Selective inversion of the Cr proton signal did not result in a significant transfer to water (data not shown).

## DISCUSSION

The $^1$H NMR experiments on *ex vivo* rat hindleg muscle equilibrated in a creatine-containing $H_2O$ solution were indicative of a strong off-resonance MT effect on the creatine methyl protons. Similarly, the magnetic coupling between the mobile protons of water and the Cr protons that had been detected *in vivo* persisted in excised muscle. These observations prompted us to test the previously suggested involvement of exchangeable protons in the *in vivo* MT phenomenon (5). Substitution of approximately 80% of the NMR-detectable protons by deuterons only marginally affected the off-resonance MT characteristics of Cr. This suggests that exchangeable protons, both in the muscle tissue and within the creatine molecule itself as well as in water, play a minor role in the generation of the off-resonance MT effect on the Cr methyl signal in $^1$H NMR. Our findings on the modification of the water off-resonance MT dispersion curves

with solvent composition are in agreement with previous reports (e.g., see Refs. *15–17*).

The magnetic coupling of the mobile water protons to the Cr protons as probed by selective water inversion was less efficient in $D_2O$-equilibrated muscle. This is most probably due to the reduced size of the mobile water proton pool. It is plausible that this pool size argument also is the reason why inversion transfer from Cr to water protons is not observed.

Taken together, the present data suggest that exchangeable protons play an insignificant role in the magnetic coupling of creatine protons in skeletal muscle. Two major possibilities remain (see Fig. 1): (i) direct intermolecular transfer from immobilized protons to the tCr methyl protons, via cross-relaxation; and (ii) chemical exchange of tCr molecules between a mobile and immobile state. Since cross-relaxation has an $r^6$ dependence on the distance, the first mechanism presumably also requires transient immobilization of the tCr molecule.

While the mechanism that forms the basis of the off-resonance MT effect of creatine/phosphocreatine in skeletal muscle is unsolved, the biological implications of the phenomenon also remain to be established. Due to the prominent role of the creatine kinase system in the energy metabolism of excitable tissues, however, it is important to fully understand the biophysical characteristics of PCr and Cr *in vivo*. Further studies are therefore warranted to identify the mechanism(s) underlying the MT effect on PCr and Cr *in situ*.

## ACKNOWLEDGMENTS

These studies were supported by a grant from the Netherlands Foundation for the Earth and Life Sciences (ALW) with financial aid from the Netherlands Organization for Scientific Research (NWO). The *in vivo* NMR instrument used in this study is part of the European Large-Scale Facility for Biomolecular NMR (Bijvoet Center, University Utrecht) that is supported by the Access to Research Infrastructure program of the European Union.

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