

³¹P NMR Saturation Transfer Study of the Creatine Kinase Reaction in Human Skeletal Muscle at Rest and during Exercise

Jean-François Goudemant, Marc Francaux, Isabelle Mottet, Roger Demeure, Merence Sibomana, Xavier Sturbois

The creatine kinase reaction has been studied by ³¹P NMR in exercising human calf muscle. Quantitative analysis of high energy phosphates and saturation transfer study of the creatine kinase flux in the direction of ATP synthesis (V_{iso}) were performed at rest and during exercise. As expected, exercise induced a [PCr] decrease (from 28.5 ± 0.9 to 21.9 ± 1.5 mM, $P < 0.01$) matched by a P_i increase (from 4.5 ± 0.2 to 8.9 ± 1.8 mM, $P = 0.06$). pH, and [ATP] remained unchanged. V_{iso} did not change from rest (12.4 ± 0.9 mM s⁻¹) to moderate exercise and decreased at the highest exercise level (8.4 ± 1.4 mM s⁻¹, $P = 0.006$). This observation differs from the prediction of the creatine kinase rate equation, showing an increase in the flux with exercise intensity. Computations suggest that this discrepancy arises from metabolite compartmentalization and/or from the reaction kinetics of a dead end complex stabilized by planar anions.

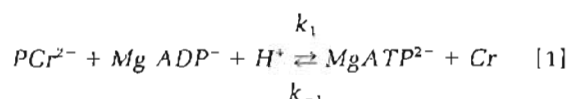
Key words: energy metabolism; creatine kinase; human skeletal muscle.

INTRODUCTION

The ³¹P NMR has proven to be a helpful tool in the study of energy metabolism in skeletal and cardiac muscle, by allowing a noninvasive and continuous monitoring of phosphorus compounds. In addition to this classical approach, the NMR technique can be used to study enzyme kinetics *in vivo*, by the magnetization transfer method (1). The creatine kinase (CK) reaction, which is intimately implicated in energy metabolism, has been widely studied by this approach in cardiac cells (2–12). In contrast, the kinetics of this enzyme has not been investigated to the same extent in the skeletal muscle (13–17).

Creatine kinase catalyses the reversible transfer of a high energy phosphate group from phosphocreatine (PCr) to adenosine diphosphate (ADP) (forward direc-

tion), or from adenosine triphosphate (ATP) to creatine (Cr) (reverse direction) (Eq. [1]):



where k_1 and k_{-1} are the rate constants of the reaction in the forward and reverse directions, respectively. Because of the high flux of CK with respect to those of ATPases, the first role attributed to this reaction is energy buffering: it ensures a stable ATP concentration by replenishment from PCr pool, and allows energy storage by conversion of ATP into PCr. Another possible role of CK is related to its isozymes localized in muscle cells. According to the hypothesis of the PCr-Cr shuttle (2–4, 18–20), the energy transfer from ATP production sites (mitochondria) to ATP consumption sites (myofibrils and ion transport sites) could be facilitated by the presence of CK isozymes at these sites. This concept has been referred to as a spatial buffering of energy (19). Several authors (5–10) demonstrated the validity of this hypothesis in cardiac muscle cells, by showing a coupling between the reaction velocity and the energy production-degradation processes; however, the shuttle hypothesis is still controversial since other investigators (11, 12) were unable to see this coupling. In skeletal muscle, previous works (13–15) have established that the creatine kinase reaction flux does not change or even decrease with work. The origin of this observation is unclear. The aim of this study is to understand the lack of relationship between ATP synthesis and creatine kinase kinetics in skeletal muscle. The comparison of enzymatic flux predicted *in vitro* (13) with experimental flux recorded in the complex intracellular conditions found *in vivo* can improve our understanding of energy metabolism during exercise.

We applied the NMR kinetics study to human muscle. Although specific problems are brought by human studies, including the limitation of maximum voluntary force development and muscle heterogeneity, human investigations are highly attractive due to the important implications in functional evaluation in pathology and exercise physiology. In contrast to previous studies (13, 14), the present work is run at rest and at different levels of exercise to delineate the pattern of the flux change with the increase in work. Five series of CK fluxes and metabolite concentrations were recorded from rest to the highest exercise level sustainable for the period required by the saturation transfer measurement. A complete time dependent saturation transfer (TDST) sequence was used to ensure the quality of the kinetics data. Experimental

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From the Department of Radiology and Medical Imagery, RMN Experimental Laboratory (J-F., I.M., R.D.), Unité d'Education Physique (M.F., X.S.), and Unité de Tomographie par Positrons (M.S.), Catholic University of Louvain, Louvain-La-Neuve, Belgium.

Address correspondence to: Professor X. Sturbois, Catholic University of Louvain, Belgium. Unité d'Education Physique, Place Pierre de Coubertin, 1, B-1348, Louvain-La-Neuve, Belgium.

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results were then compared with the prediction of the CK rate equation determined *in vitro* (21–24). The discrepancy between the experimental observations and the calculations increased our understanding of metabolic changes occurring during exercise.

MATERIAL AND METHODS

General

^{31}P NMR spectra were recorded on a Bruker Biospec spectrometer (horizontal bore) working at 4.7 Tesla and 81 MHz for ^{31}P , equipped with a 50-mm diameter surface coil appropriate for both ^1H and ^{31}P . Six 22- to 28-year-old healthy male volunteers were placed in a supine position with their legs in the magnet. The left calf muscle was firmly immobilized on the coil by means of a specially designed setup that allowed us to study mainly the gastrocnemius muscle, with probable contribution from neighboring muscles. The subjects were studied at rest during dynamic exercises consisting of plantar flexions at 0.5 Hz frequency. The foot was placed in a pedal connected to a weight by means of a pulley system (Fig. 1). The linear displacement of the pedal (~ 8 cm) was continuously monitored to be certain that the work performed was proportional to the weight applied. Exercise intensity was varied by using weights of 2.5, 5, 7.5, and 10 kg. Experiments were run in several sessions. No changes in $[P_i]/[\text{PCr}]$ and pH_i were observed before exercise, confirming that the recovery period between examinations was sufficient to ensure the restoration of parameters before starting a new exercise. These experiments were approved by the committee on the ethical use of human subjects of our institution.

Quantification and Intracellular pH Measurement

After the fine tuning of the probe with the subject properly installed, magnetic field homogeneity was optimized on the free induction decay (FID) of water protons. The linewidth at half height of the water signal was typically ~ 50 Hz. ^{31}P signals were acquired after a pseudo-90° pulse on 1024 points with a 6000-Hz spectral width. The pseudo-90 used (130- μs pulse duration) is equal to 2 times the pulse duration required to achieve a 90° pulse in the center of the coil. This pulse is the

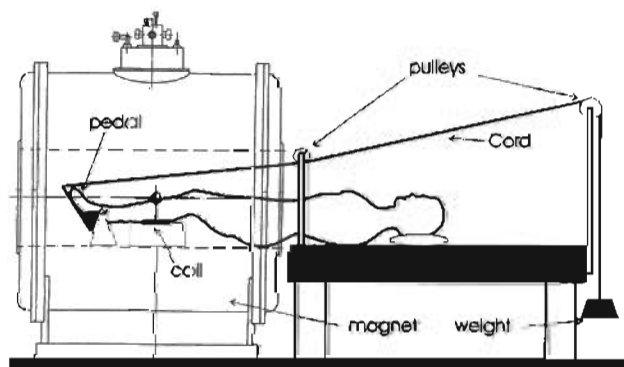


FIG. 1. Experimental setup used for the exercise protocol on the human calf muscle.

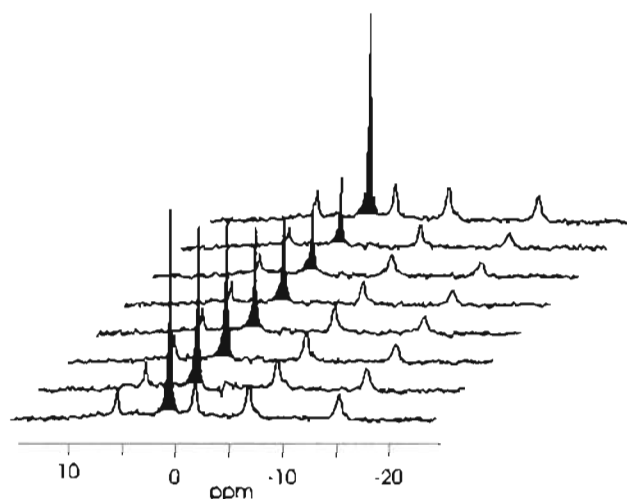


FIG. 2. Sample of the NMR spectra acquired during the saturation transfer experiment (repetition time = 8 s, pseudo-90°, number of scans = 12, saturation of the $[\gamma\text{-P}]\text{ATP}$). The lines are from left to right: P_i , PCr, $[\gamma\text{-P}]\text{ATP}$, $[\alpha\text{-P}]\text{ATP}$, and $[\beta\text{-P}]\text{ATP}$. The saturation durations applied on the $[\gamma\text{-P}]\text{ATP}$ are 0, 0.5, 1, 2, 3, 5, and 7.5 s. The first spectrum, recorded without saturation of the $[\gamma\text{-P}]\text{ATP}$, is repeated after the last spectrum to highlight the decrease of the PCr peak.

optimum irradiation necessary to yield to the maximum signal intensity and allows the recording of a signal mainly originating ~ 3 cm deep in the muscle. Quantification of peak areas was performed by fitting in the time domain (25). The Fourier transform of the data presented in Fig. 2 was calculated on 8192 points after zero filling and exponential multiplication (line broadening = 5 Hz).

A relative quantification of PCr, ATP, and P_i was achieved at rest and during exercise from the first spectrum recorded for each kinetics measurement. This spectrum corresponds to the data recorded without irradiation of the $[\gamma\text{-P}]\text{ATP}$ line, and it was acquired after a pseudo-90° pulse with a repetition time of 8 s. A factor was calculated to relate the area of the $[\beta\text{-P}]\text{ATP}$ peak in the rest spectrum of each subject to the concentration of 8.2 mM classically reported for this compound in the skeletal muscle at rest (26, 27). The quantification during exercise was achieved by calculating the ATP concentration from this factor and from the area of the $[\beta\text{-P}]\text{ATP}$ line. Similarly, the P_i and PCr concentrations at rest and during exercise were estimated by comparing the area under their peaks with that of the $[\beta\text{-P}]\text{ATP}$ resonance. To reduce errors due to changes in the quality of the spectra caused by leg movements, the spectra were scaled by comparison to the signal of a reference (methylene phosphonic acid, Merck, B-1900 Overijse, Belgium) contained in a small tube located at the center of the coil. Thus, this reference was not used for absolute quantification but to ensure the correspondence between spectra recorded on different subjects and at various exercise levels.

Since the quantification was performed on slightly saturated spectra (repetition time = 8 s), a correction of peak areas was needed. Saturation factors were calculated from the apparent longitudinal relaxation times ($T_{1\text{app}}$)

of the phosphorus compounds. $T_{1\text{ app}}$ were measured in separate experiments by the progressive saturation method. According to this technique, several spectra with different repetition times (RT) between successive pseudo-90° pulses were recorded, and the peak areas of the resonances were plotted against the RT. A monoexponential fit (Eq. [2]) of these data to the three parameters a , $T_{1\text{ app}}$ and M_0 (the maximum magnetization observed, corresponding to the fully relaxed condition) allowed the $T_{1\text{ app}}$ to be calculated:

$$M(t) = M_0[1 - a \exp^{-RT/T_{1\text{ app}}}] \quad [2]$$

For this measurement, 9 spectra of 16 or 32 scans were recorded on each subject, with RT ranging from 0.1 to 20 s.

The intracellular pH (pH_i) was calculated from the chemical shift of the P_i peak (δ_{P_i}) with respect to the PCr peak (28) according to the classical equation (Eq. [3]) (13):

$$\text{pH} = 6.75 + \log\left(\frac{\delta_{P_i} - 3.27}{5.69 - \delta_{P_i}}\right) \quad [3]$$

ADP concentration was determined from the apparent equilibrium constant ($K_{\text{eq app}} = K_{\text{eq}}[\text{H}^+]$) of the creatine kinase reaction (Eq. [4]):

$$[\text{ADP}] = \frac{[\text{Cr}][\text{ATP}]}{[\text{PCr}]K_{\text{eq}}[\text{H}^+]} \quad [4]$$

A value of 166 at 38°C and pH 7 has been reported by Lawson and Veech (29) for the $K_{\text{eq app}}$ (ionic strength 0.25 mole kg^{-1} and $[\text{Mg}^{2+}] = 1 \text{ mM}$). $[\text{H}^+]$ is calculated from the pH_i measured on NMR spectra. The Cr concentration is calculated from the concentration of the total Cr pool, generally reported to be 42.5 mM in the human skeletal muscle (26, 27).

Kinetics Measurements

The kinetics measurements aim to estimate the pseudo-first order rate constant k_{for} (forward direction) of the phosphorus exchange between PCr and $[\gamma\text{-P}]\text{ATP}$ (Eq. [5], derived from Eq. [1]):

$$k_{\text{for}} = k_1[\text{MgADP}^-][\text{H}^+] \quad [5]$$

The flux of the reaction (V_{for}) is calculated from this pseudo-first order rate constant (k_{for}) and the PCr concentration by means of Eq. [6]:

$$V_{\text{for}} = k_{\text{for}}[\text{PCr}] \quad [6]$$

The pseudo-first order rate constant k_{for} was measured by the TDST method (28). Saturation pulses were applied on the $[\gamma\text{-P}]\text{ATP}$ peak, and different spectra were recorded after the saturation duration (t) (Fig. 2). The pseudo-first order rate constant was calculated by fitting the area of the nonirradiated exchanging resonance (PCr peak) against this duration according to Eq. [7]:

$$M(t) = M_{\infty}[1 + k_{\text{for}}T_1 \exp^{-t/T_{1\text{ app}}}] \quad [7]$$

where M_{∞} is the magnetization remaining after a long saturation period, T_1 is the intrinsic relaxation time of the exchanging unsaturated nucleus, and $[T_{1\text{ app}}]^{-1} = [T_1]^{-1} + k_{\text{for}}$. This $T_{1\text{ app}}$ is the apparent T_1 value involving the effect of the exchange on the relaxation mechanism generally recorded by inversion recovery or saturation recovery (see above). The parameters fit to Eq. [7] are M_{∞} , $(k_{\text{for}}T_1)$ and $T_{1\text{ app}}$.

In these experiments, the saturation is achieved by a DANTE pulse sequence (30) consisting of a train of short pulses (10 μs) separated by a 490- μs delay. Mirrored irradiations (Fig. 3) in double irradiation experiments proved that the selectivity of this sequence was adequate: the nonirradiated spectrum (noted A in Fig. 3) is not different from the mirrored irradiation spectrum (C) as assessed by the difference spectrum $F = A - C$. Figure 3 also shows that a small shift (20 Hz) of the irradiation frequency (spectrum D), likely to occur with leg movement for example, has no effect on the magnetization transfer of the P nucleus from PCr to $[\gamma\text{-P}]\text{ATP}$ (difference spectrum $E = B - D$). These measurements prove the accuracy of the saturation transfer in the exercising human calf muscle. Finally, the saturation transfer technique used is appropriated for kinetics measurements with a surface coil, as used here, since full resonance saturation was obtained in spite of the inhomogeneous B_1 field of the coil. Moreover, similar methods have been previously used by others (24, 31).

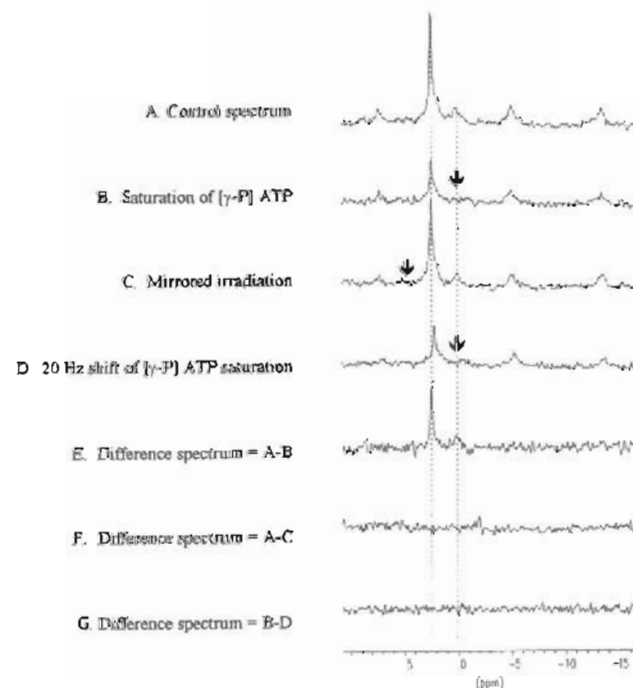


FIG. 3. Validation of NMR measurement of the CK kinetics on human calf muscle. Spectrum A is the control spectrum recorded without irradiation (repetition time = 8 s, pseudo-90°, number of scans = 12). Spectra B-D are acquired during saturation transfer experiment (saturation duration = 7.5 s) with change of irradiation frequency as indicated by the arrow position. Difference spectra (E-G) are presented to highlight the changes between spectra A-D.

A set of seven saturation durations was used for the kinetics study: 0-s (spectrum without saturation, also used for the relative quantification), 0.5-, 1-, 2-, 3-, 5-, and 7.5-s saturations were applied in random order, to overcome time-dependent change in signal intensity. In this protocol, the exercise was started 3 min before measurements to ensure a stable metabolic condition during the experiment (~12 min). Dummy scans were not used to avoid excessive lengthening of the experiment duration, unless they would be preferred due to the partial saturation of our NMR data. However, the error resulting from this omission is very small, since only slight (PCr and P_i) or no (ATP) saturation occurs in our experimental conditions (see below). Possible fluctuations of the metabolic condition and of metabolite concentrations were overcome by partitioning the 12 acquisitions required for each saturation duration into 3 blocks of 4 scans accumulated discontinuously. This acquisition in several interleaved blocks allowed the recording of the mean value of concentration and kinetic parameters over the entire duration of the experiment and reduced the influence of an imperfect steady state of the exercise on the NMR measurement.

Calculation of the Predicted CK Flux from the Rate Equation of the Creatine Kinase

The predicted velocity of the creatine kinase reaction was calculated from the rate equation determined by Morrison and James (21) and Morrison and Cleland (22), depicted in Eq. [8]:

$$V_{\text{for}} = \frac{V_{\text{max}}^{\text{for}} [\text{MgADP}] [\text{PCr}]}{K_m^{\text{(ADP)}} K_i^{\text{(PCr)}} D} \quad [8]$$

where

$$D = 1 + \frac{[\text{MgATP}]}{K_i^{\text{(ATP)}}} + \frac{[\text{Cr}]}{K_i^{\text{(Cr)}}} + \frac{[\text{MgADP}]}{K_i^{\text{(ADP)}}} + \frac{[\text{PCr}]}{K_i^{\text{(PCr)}}} + \frac{[\text{Cr}][\text{MgATP}]}{K_m^{\text{(ATP)}} K_i^{\text{(Cr)}}} + \frac{[\text{PCr}][\text{MgADP}]}{K_m^{\text{(ADP)}} K_i^{\text{(PCr)}}}$$

This equation requires on the reactant concentrations ([MgATP], [MgADP], [PCr], and [Cr]), the maximum velocity of the creatine kinase reaction in the skeletal muscle ($V_{\text{max}}^{\text{for}}$) and the dissociation constant of the binary and ternary complexes of enzyme-substrate, K_i and K_m . To consider the ternary dead end complex formation enzyme-MgADP-Cr and enzyme-MgATP-PCr in the control of enzyme kinetics, the following terms (Eq. [9]) have to be added to the parameter D . The inhibitory constant implicated in dead end complexes formation are K_i .

$$\frac{[\text{Cr}][\text{MgADP}]}{K_i^{\text{(Cr)}} K_i^{\text{(ADP)}}} \text{ and } \frac{[\text{PCr}][\text{MgATP}]}{K_i^{\text{(PCr)}} K_i^{\text{(ATP)}}} \quad [9]$$

Finally, the stabilization of the enzyme-MgADP-Cr complex by planar anions (32–34) requires the replacement of the corresponding term in Eq. [9] by (Eq. [10]):

$$\frac{[\text{anion}][\text{Cr}][\text{MgADP}]}{K_i^{\text{(anion)}} K_m^{\text{(ADP)}} K_i^{\text{(Cr)}}} \quad [10]$$

where $K_i^{\text{(anion)}}$ is the anion inhibitory constant.

The K_m , K_i , and K_i values used are those of McFarland *et al.* (32) for the skeletal muscle, and are listed in Table 1. The maximal velocity of the reaction in the forward direction ($V_{\text{max}}^{\text{for}}$) is derived from the concentration of creatine kinase in human muscle (35), known to be 2588 U g⁻¹. Since 1 U is defined as the formation of 1 μmole ATP per minute (pH 7.4 and 30°C) and the Q_{10} of creatine kinase is 2 (5, 32, 36), it can be concluded that $V_{\text{max}}^{\text{for}}$ in human muscle is 60 $\mu\text{mole s}^{-1} \text{g}^{-1}$. Furthermore, since the cytosolic water content is classically reported to be 40–60% of muscle wet weight, $V_{\text{max}}^{\text{for}}$ must be close to 100–150 mM s^{-1} . Using the cytosolic water content of 67% of wet weight used by Rees *et al.* (13) leads to a $V_{\text{max}}^{\text{for}}$ value of 90 mM s^{-1} . A $V_{\text{max}}^{\text{for}}$ value close to 100 mM s^{-1} thus seems appropriate for human skeletal muscle.

Statistical Analysis of the Results

Results are expressed as means $\pm$ standard error of the mean (SEM). Repeated measures ANOVA was used to test the effect of exercise level. When a significant effect was found, a contrast analysis was performed to determine the differences among work levels. The limit of significance was taken to be $P = 0.05$. All statistical analyses were performed by using the SYSTAT software (Systat Inc, 60201 Evanston, IL).

RESULTS

The apparent longitudinal relaxation time measured on the six subjects gave the following results: 2.5 ± 0.4 s for P_i , 2.5 ± 0.2 s for PCr and 1.0 ± 0.1 s for the $[\beta\text{-P}]\text{ATP}$. Since the corresponding saturation factors calculated from acquisition conditions are 0.96 for PCr and P_i and 1 for ATP, so the large repetition time relative to the $T_{1\text{app}}$ leads only to a slight saturation of the PCr and P_i resonances.

Results of the relative quantification are listed in Table 2. As expected (37), a [PCr] decrease ($-23 \pm 6\%$, $P < 0.01$) is observed during exercise, and is matched by a possible increase in the P_i content ($104 \pm 44\%$, $P = 0.06$). The ATP concentration is not significantly modified by exercise, whereas the calculated ADP level at maximum workload shows a significant enhancement with respect to the rest value. The sum of phosphorylated compounds calculated from the NMR spectra is not significantly altered by exercise, indicating that no loss of phosphate occurs. The exercise protocol did not significantly modify the intracellular pH.

Kinetics measurements clearly demonstrate the phosphate transfer between PCr and $[\gamma\text{-P}]\text{ATP}$ (Figs. 2 and 3). The pseudo-first order rate constant k_{for} and the intrinsic T_1 of PCr are presented in Table 2. T_1 and k_{for} are not

Table 1
 K_m , K_i , and K_i values (mM) Used in the Predicted V_{for} Calculation (32)

	MgADP	PCr	MgATP	Cr	Anion
K_m	0.04	1.11	0.057	3.8	
K_i	0.135	3.9	3.5	554	
K_i	0.015	3.9	3.2	58	0.1–0.4

Table 2

Results of the Relative Quantification (Concentrations in mM), pH_i, Kinetics (k_{for} in s⁻¹, T_1 in s), Flux (V_{for} in mM s⁻¹) Calculations during Rest and during Four Different Levels of Exercise

	rest	2.5 kg	5 kg	7.5 kg	10 kg	Anova
[P]	4.5 ± 0.2	5.2 ± 0.4	6.3 ± 0.4	8.1 ± 2.2	8.9 ± 1.8	NS ^b
[PCr]	28.5 ± 0.9	28.7 ± 1.5	26.9 ± 1.2	26.0 ± 2.0	21.9 ± 1.5 ^a	P=0.008
[ATP]	8.2	9.1 ± 0.5	8.7 ± 0.6	8.2 ± 0.8	7.9 ± 0.7	NS
[ADP]	0.027 ± 0.003	0.029 ± 0.004	0.035 ± 0.006	0.033 ± 0.004	0.048 ± 0.006 ^a	P=0.02
[P]/[PCr]	0.159 ± 0.008	0.180 ± 0.009	0.24 ± 0.02 ^a	0.32 ± 0.09	0.39 ± 0.06 ^a	P=0.015
Sum [P]	57.6 ± 0.9	61.3 ± 3.2	59.4 ± 2.9	58.7 ± 5.2	54.6 ± 5.3	NS
pH _i	7.04 ± 0.01	7.04 ± 0.01	7.05 ± 0.01	7.02 ± 0.02	7.02 ± 0.04	NS
k_{for}	0.44 ± 0.03	0.41 ± 0.02	0.49 ± 0.01	0.47 ± 0.05	0.38 ± 0.04	NS
T_1 , PCr	3.9 ± 0.3	4.2 ± 0.2	3.4 ± 0.2	4.0 ± 0.4	4.8 ± 0.4	NS
measured V_{for}	12.4 ± 0.9	11.8 ± 1.0	13.1 ± 0.6	12.1 ± 1.5	8.4 ± 1.4 ^a	P=0.006
predicted V_{for} (A)	20.4 ± 0.8	20.8 ± 1.0	22.5 ± 1.2	21.1 ± 1.1	24.1 ± 1.9	NS
predicted V_{for} (B)	12.0 ± 0.7	12.1 ± 1.2	13.8 ± 1.1	13.3 ± 1.4	16.7 ± 1.9	NS

Quantification was performed by assuming a value of 8.2 mM for ATP concentration at rest. The predicted V_{for} was calculated from the rate equation of the CK by using the experimental metabolite concentrations, the constants presented in Table 1 and a $V_{\text{max}}^{\text{CK}}$ of 100 mM s⁻¹. Predicted V_{for} (A) refers to the calculation of the rate equation with no dead end complex formation, while predicted V_{for} (B) refers to the calculation involving ternary dead end complex formation.

^a Denotes statistical difference between the measurement conducted during exercise and rest condition ($P < 0.05$, ANOVA analysis and contrast analysis)

^b NS, nonsignificant.

significantly modified with exercise in comparison to rest. V_{for} calculated from PCr concentration and k_{for} remained identical at the three lowest exercise levels and significantly decreased at the highest exercise level.

DISCUSSION

The relative quantification shows the well-known pattern of concentration changes with exercise. This pattern supports the transfer function proposed by Chance *et al.* (38), since a plot (not presented) of the workload versus the $[P_i]/[PCr]$ ratio shows the classical hyperbolic behavior. Since exercise induced no changes in either ATP concentration, the sum of phosphorylated compounds, or the pH_i, the energy production still relies on aerobic metabolism, and the protocol used here is consistent with a mild exercise that never led to exhaustion. Clearly, the work range used here is limited, since only the 10-kg exercise values are statistically different from the rest value. However, the heaviest weight (10 kg) is the maximum load possible considering the duration of the experiment required for saturation transfer measurement (12 min); voluntary contractions with a heavier load would be achievable for shorter experiment durations, but only with a subsequent temporal reduction of the NMR measurement and thus the quality of the spectroscopic data.

The validity of kinetics measurements as performed here is widely recognized (39), including when surface coils are used (24, 31), and the accuracy in the forward direction has proven to be adequate. Moreover, the *in vivo* flux of CK is high enough to ensure the equilibrium condition of the reaction (13), which is a prerequisite for the NMR measurement. However, it must be noted that Wallimann (40) reported that the accuracy of the NMR measurement of CK flux has to be reconsidered, since experiments on mice expressing reduced levels of myofibrillar creatine kinase (M-CK) showed that the NMR detectable flux through the CK reaction is dependent on the M-CK level expression. The flux was only detectable

if the M-CK level was above half that of normal muscle. This suggests that the bounding of the enzyme to subcellular structures may impair the NMR detection of the flux. However, this suggestion remains hypothetical and remains to be confirmed. In cardiac muscle, creatine kinase is substantially bounded, according to the shuttle hypothesis, and fluxes of these functionally coupled enzymes have been observed (2–4, 18–20). In skeletal muscle, half of the CK pool is bound to myofibrils and to mitochondria, and the rest of the enzyme pool is likely bound to other structures (41). In both cases, a significant flux is observed by NMR. Therefore, in accordance with our present knowledge, the *in vivo* flux recorded by the NMR approach can be considered to adequately reflect the unidirectional flux of muscle cell CK.

The exercise levels used here show little effect on CK kinetics, since k_{for} is not significantly modified in comparison to rest values, and since V_{for} is only significantly decreased at the highest work level. This observation is in agreement with the findings of Rees *et al.* (13) and of Le Rumeur *et al.* (14), who demonstrated a decrease in CK flux with exercise. In addition to these works, where only one exercise intensity was investigated, our protocol demonstrates that the flux decrease is only detectable at the highest workload, and rules out a simple dependence on global substrate concentrations of the muscle cells (which showed progressive changes with exercise intensity, Table 2).

The decrease of V_{for} also argues against a major role for energy channeling (the shuttle hypothesis) for the creatine kinase reaction. A functional coupling between energy production and the CK reaction for energy transfer between production and consumption sites would lead to an increase in V_{for} with increasing aerobic energy synthesis. In cardiac muscle, 67% (5) and 42% (7) increases of the CK flux have been reported for workload enhancements of 2.3 and 2.9 times, respectively. This coupling between creatine kinase flux and ATP synthesis has been interpreted as the shuttle hypothesis (7, 8). In muscle, since V_{for} does not increase with exercise, enzy-

matic control of the creatine kinase reaction appears to be unrelated to a shuttle function. This conclusion was also reached by Shoubridge *et al.* (15), Rees *et al.* (13), and Le Rumeur *et al.* (14), but differs from the view of Meyer *et al.* (19). Alternatively, if the shuttle function of CK is not the major role of this reaction in skeletal muscle, its function must be energy buffering. In this view, the decrease in flux recorded with exercise is surprising, but it should be emphasized that its amplitude (-32%) is not sufficient to reduce the buffering function of the enzyme, since its velocity remains well above the ATPases fluxes (known to be in the range of $0.5\text{--}1.6\text{ mM s}^{-1}$, (15)).

The absence of a direct functional coupling between creatine kinase flux and ATP synthesis rate implies that the enzymatic control of the reaction simply relies on the concentration of reactants in the vicinity of the enzyme and does not involve a local control by a multi-enzymatic system. This assumption can be tested by comparing the experimental flux of the CK reaction to a calculated flux estimated from *in vitro* experiments. Accordingly, this calculated forward flux of the CK reaction is the flux predicted by the rate equation delineated by Morrison and James (21) and Morrison and Cleland (22) in aqueous solutions. As McFarland *et al.* (32) did in cat muscle experiments, we used this approach and considered three cases of enzymatic control of the reaction: (a) no dead end complexes formed; (b) dead end ternary complexes formed; or (c) a particular dead end quaternary complex formed, implying anions fixation on the enzyme (32–34).

It appears from the rate equation of Morrison and James (21) and Morrison and Cleland (22) that ADP is the most significant reactant implicated in the control of the reaction, since its intracellular concentration is close to or smaller than its K_m value. In contrast, the PCr, Cr, and ATP are present at high concentration with respect to their K_m values. To emphasize the role of these reactants on enzymatic control, Fig. 4 (top) presents a simple model of changes in metabolite concentration with a $[P_i]$ increase (42, 43) as observed during exercise. When work and therefore $[P_i]$ increase, the energy stores of [PCr] are depleted to maintain normal ATP content. Additionally, these changes in the energy balance lead to an increase in [ADP]. Figure 4 (bottom) uses these calculations to present the relationship between the predicted flux and the [ADP]. In the simplest case of enzymatic control considered (a), an increase in the flux is first predicted with the increase in [ADP], but high [ADP] (above 0.2–0.3 mM) leads to a further decrease in predicted V_{for} . This decrease has been observed by Shoubridge *et al.* (15) in *in vitro* experiments above an [ADP] of 0.18 mM. When considering the dead end complexes formation (b), the pattern of predicted flux change is similar, with a small reduction in flux intensity. These pictures of the predicted flux contrast with the stability of the measured flux observed for the three moderate exercise levels and with the significant decrease of V_{for} recorded at the highest exercise level. With involvement of anions in the enzyme-Cr-MgADP dead end complex (c), the prediction of flux changes with exercise is completely different, since the predicted V_{for} decreases with [ADP] increases.

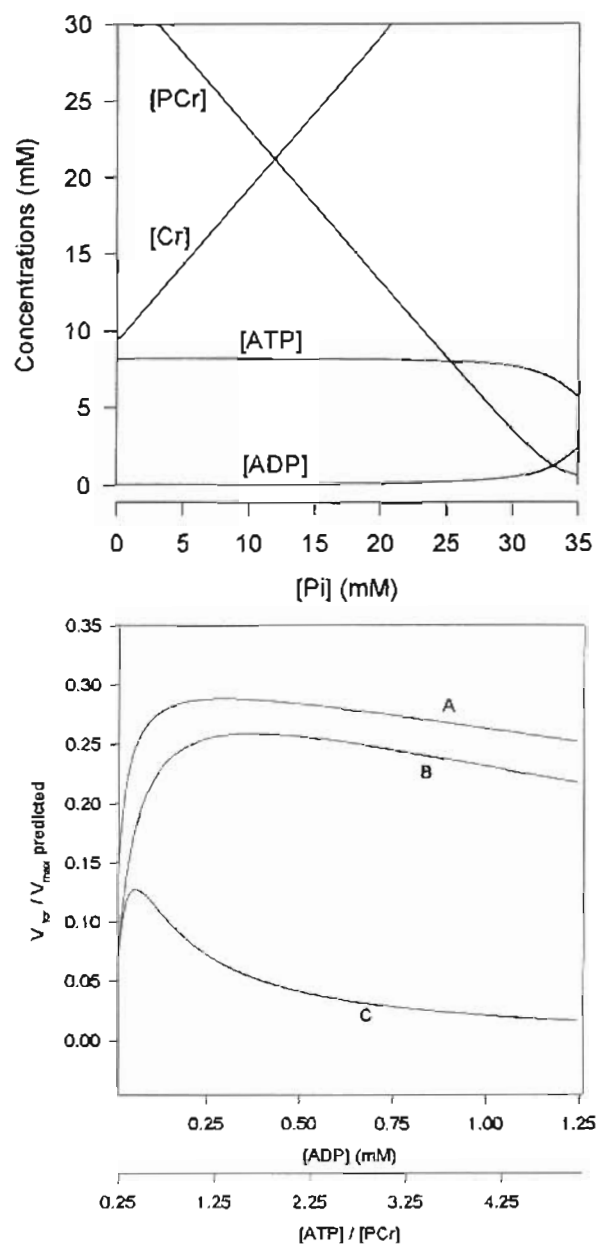


FIG. 4. (Top) Theoretical estimation of metabolite concentration changes with exercise. (Bottom) Effect on the predicted V_{for} (Eq. [8], according to Morrison *et al.* (21, 22), McFarland *et al.* (32)). Predicted V_{for} is calculated (A) with the rate equation without dead end complexes, (B) with dead end complexes, and (C) with dead end complexes stabilized by planar anions. The model corresponds to the resolution of Eq. [11]–[14], as a function of $[P_i]$ ranging from 0 to 30 mM (the value used in Eq. [11] is determined from our quantification, whereas the value used in Eqs. [12]–[14] are derived from the literature (26, 27) and (29) respectively):

$$[\text{PCr}] + [P_i] + 3[\text{ATP}] + 2[\text{ADP}] = 57.7 \text{ mM}, \quad [11]$$

$$[\text{Cr}] + [\text{PCr}] = 42.5 \text{ mM}, \quad [12]$$

$$[\text{ADP}] + [\text{ATP}] = 8.2 \text{ mM}, \quad [13]$$

$$\frac{[\text{ATP}][\text{Cr}]}{[\text{ADP}][\text{PCr}]} = 166 \quad [14]$$

This pattern is thus closer to the experimental observation.

To clarify the discrepancy between predicted kinetics and experimental observations, the following three sections will examine: (i) the effect of substrate concentration, (ii) the effect of changes in CK kinetics, and (iii) the involvement of dead end quaternary complex as proposed by McFarland *et al.* (32).

Change in Substrates Concentrations

Because of the significant role of [ADP] in the enzymatic control of the CK reaction, a decrease or a very large increase in the concentration of this compound could account for the decrease of CK flux with exercise.

A first hypothesis explaining V_{for} changes is that the [ADP] seen by the enzyme is lower than the global [ADP] estimated from spectroscopic data. The rate equation involving ternary dead end complexes formation shows that the [ADP] must be decreased from 0.027 mM at rest (calculated from experimental data) to 0.021 mM at the highest exercise level to account for the 32% decrease of CK flux. A decrease in [MgADP], which is the effective substrate of the enzyme (13), could be caused by a loss in Mg^{2+} during heavy exercise. However, this hypothesis has been rejected because even a large loss in Mg^{2+} will not result in a decrease in [MgADP] since the dissociation constant of MgADP is so small (13).

A local decrease in [ADP] could also be related to intracellular heterogeneity. Since the [ADP] presented here is calculated, errors in this parameter could originate from inaccuracy in the $K_{eq,app}$ or in the creatine ([Cr]_{tot}) and adenosine ([A]_{tot}) pools. Changes in these parameters, and their subsequent effects on [ATP], [ADP], [PCr], [Cr] and predicted V_{for} , are considered in Table 3. It can be seen from these calculations that the hypothesis of a decrease of ADP implies local changes of -32%, +122%, and -87% for the [Cr]_{tot}, $K_{eq,app}$, and [A]_{tot}, respectively. In our opinion, the changes in [Cr]_{tot} and $K_{eq,app}$ are conceivable, whereas the -87% variation of [A]_{tot} seems to be excessive. For example, the $K_{eq,app}$ change could be easily explained by a local pH change, since a pH variation of only 0.35 units leads to a $K_{eq,app}$

variation from 166 to 304 (Table 3). These changes could result from changes in substrate distribution inside the cell, rather than from global content changes, since no adenosine or phosphate losses are recorded in our mild exercise protocol.

On the other hand, the second hypothesis of a large increase in [ADP] above ~0.2 mM (Fig. 4) would imply very large changes in [Cr]_{tot}, [A]_{tot}, and $K_{eq,app}$. An increase in the [Cr]_{tot} leading to a very important increase in [ADP] is not a viable hypothesis, because of the extent of the [Cr]_{tot} required (ca 250 x) to fulfill the CK flux reduction expected (Table 3). In addition, the hypothetical increases of [A]_{tot} or $K_{eq,app}$ adequately lead to high [ADP], but fail to reduce the predicted V_{for} because of the involvement of other reactants in the control of the reaction. Thus, the hypothesis of a large increase in [ADP] during exercise to explain the V_{for} decrease is irrelevant. Only the first hypothesis of a local [ADP] decrease is a valuable suggestion, since it could be achieved by the intracellular heterogeneity.

The compartmentalization of metabolites presented above could also be as a result of different muscular fiber types, in which pH, or substrate pools differ (44, 45), or display different evolution patterns with changes in energy turnover. However, studies on humans show similar activities of CK in different fiber types (46). Similar [ATP] and [PCr] have been reported at rest and during cycling exercise (47) for the fast and slow twitch human fibers. It should be noted that a different situation was found in nonhuman mammalian muscle by Meyer *et al.* (44), Pette *et al.* (48), and Kushmerick *et al.* (49). Even if different concentrations of CK substrate exist in human muscle, resulting in differences in CK flux with fiber type, the global CK flux recorded should be related to the global phosphate concentrations measured. This global CK flux should thus be the weighted mean of the CK fluxes found in the different fiber types. On the other hand, recruitment of different fiber types occurring with modification of exercise intensity (the oxidative slow-twitch fast fiber are first recruited at low workload, and the fast-twitch glycolytic fibers are then mobilized at higher workload (50)) could affect the quantitative CK flux with exercise. However, the qualitative evolution of

Table 3

The Effect of the Total Concentration in Creatine ([Cr]_{tot}), in Adenosine Compounds ([A]_{tot}), $K_{eq,app}$, and $V_{for,max}$ on [ATP], [ADP], [PCr], [Cr], and predicted V_{for}

	[Cr] _{tot} (mM)	[A] _{tot} (mM)	$K_{eq,app}$	$V_{for,max}^{or}$ (mM)	[ATP] (mM)	[ADP] (mM)	[PCr] (mM)	[Cr] (mM)	Predicted V_{for} (mM s ⁻¹)
Experimental data (classical values of [Cr] _{tot} , [A] _{tot} , or $K_{eq,app}$ are used)									
Rest	42.5	8.2	166	100	8.2	0.027	28.5	14	12.0
Exercise	42.5	8.2	166	100	8.15	0.048	21.9	20.6	16.7
Hypothesis: calculations with changes in [Cr] _{tot} , [A] _{tot} , $K_{eq,app}$, or $V_{for,max}$ during exercise									
[Cr] _{tot} decrease	29.0	8.2	166	100	8.18	0.016	21.9	6.8	8.1
[Cr] _{tot} increase	10798	8.2	166	100	2.05	6.15	21.9	10776	8.1
[A] _{tot} decrease	42.5	1.12	166	100	1.11	0.006	21.9	20.6	8.1
$K_{eq,app}$ increase	42.5	8.2	369	100	8.18	0.021	21.9	20.6	8.1
$V_{for,max}$ change	42.5	8.2	166	50	8.15	0.047	21.9	20.6	8.1

This table shows the changes in [Cr]_{tot}, [A]_{tot}, $K_{eq,app}$, and $V_{for,max}$ necessary to achieve the same 32% reduction in V_{for} as observed during the highest exercise load as compared with rest (right hand column). Computations are run for rest and exercise conditions (top), and changes in parameters (in bold characters) are applied to the exercise situation (bottom). The equations used for the calculations are: [Cr] + [PCr] = [Cr]_{tot}, [ADP] + [ATP] = [A]_{tot}, and [ADP] = [Cr]/[ATP]/[PCr] $K_{eq,app}$.

the metabolic parameters should be similar in both fiber types: to explain a decrease in flux in the whole muscle, a decrease in [ADP] in some fibers during exercise should be proposed but is highly improbable. Thus, although further work is required to prove this assumption, fiber heterogeneity does not seem to be a valuable suggestion for explaining the kinetics of CK during exercise. Moreover, McFarland *et al.* (32) working on the homogeneous cat soleus muscle, consisting of slow oxidative fibers, recorded CK kinetics close to ours.

Change in Creatine Kinase Kinetics or Activity

In addition to substrate compartmentalization changes, possible modification of the reaction kinetics should be considered. Changing the kinetics parameters employed, i.e., K_m , K_i , and K_p by using constants proposed by others (21, 24, 51), does not modify the overall pattern of flux changes with substrate concentration, but only modifies the amplitude of the predicted fluxes. For example, changes in the constants used for the rate equation are therefore not responsible for the discrepancy between experimental and calculated fluxes. Change in creatine kinase activity ($\sim 50\%$) as proposed in Table 3 is unlikely, since it would imply an enzyme loss. This might be caused by membrane damage; however, this is not the case in our mild exercise protocol where total phosphate content remains unchanged. Alternatively, a decrease in CK activity might be due to P_i accumulation; however, this has been ruled out by Williams *et al.* (52).

Involvement of Anion in the CK Kinetics

Finally, enzyme kinetics can be influenced by increased enzyme-Cr-ADP dead end complex formation due to stabilization by planar anions (33, 34). This idea was recently tested by McFarland *et al.* (32) on *in vivo* data recorded on cat skeletal muscle. Figure 5 shows that with the K_i of 0.25 mM and the anion concentration of 26 mM proposed by these authors, the rest and low level exercises observed experimentally agree well with the predicted V_{for} . However, at the maximum exercise level tested here, the predicted value is slightly increased whereas the observed value undergoes a significant decrease in flux (Fig. 5, top). Using the K_i values of 0.1–0.4 mM proposed by McFarland *et al.* (32) gives the same result. The correspondence between predicted and measured flux (Fig. 5, bottom) is reached when a K_i value of 0.025 mM is used in the computation. Since this correspondence occurs for a K_i value not largely different than that of the 0.1–0.4 mM range previously reported from *in vitro* analysis (32), we conclude that the anion influence on the *in vivo* CK kinetics is a possible explanation for the CK flux pattern observed here with exercise. However, the precise value of K_i or anion concentration used are unknown, since K_i is derived from *in vitro* experiments and since the precise nature of these anions remains unclear. McFarland *et al.* (32) presumed that these anions are chloride and bicarbonate, and that their total concentration is about 26 mM.

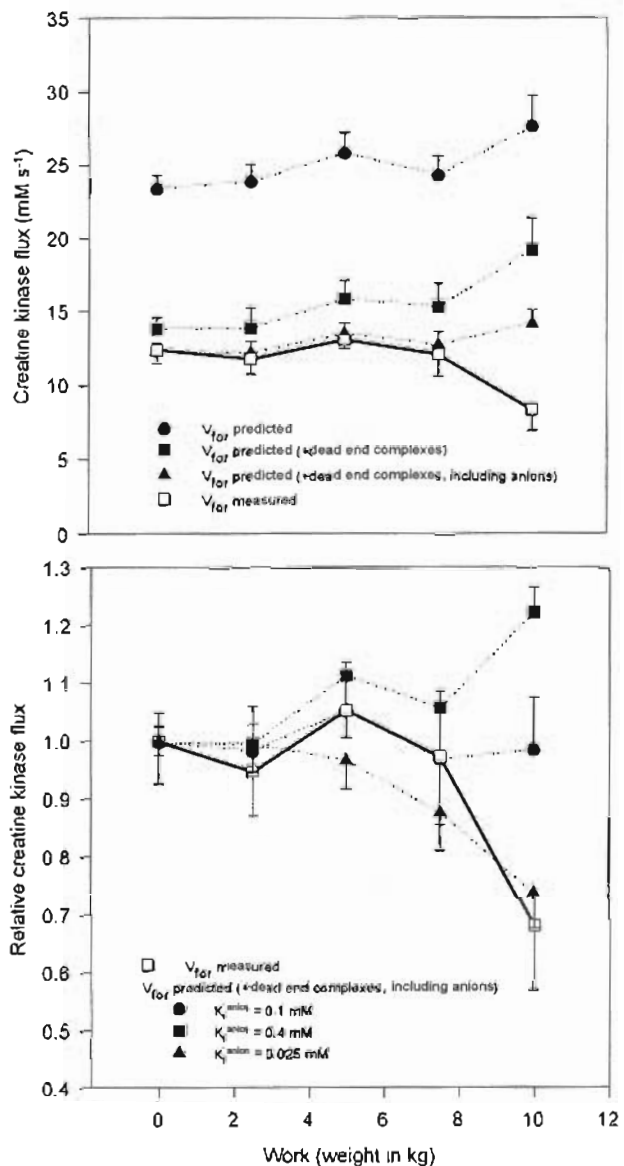


FIG. 5. Comparison between the experimentally measured fluxes and the predicted fluxes predicted by the rate equation. (Top) Absolute value of the creatine kinase flux (the calculated flux including dead end complexes formation with stabilization by planar anions is calculated for a $K_i^{\text{anion}} = 0.25$ mM, and an [anion] = 26 mM). (Bottom) Relative creatine kinase fluxes measured or calculated for the dead end complex formation with stabilization by planar anions ($K_i^{\text{anion}} = 0.1, 0.4$, or 0.025 mM, [anion] = 26 mM).

CONCLUSIONS

The kinetics measurements performed on human skeletal muscle at various exercise levels suggest that no direct functional coupling exists between CK flux and ATP synthesis rate. Therefore, during exercise, the shuttle function is not the major role of this reaction in skeletal muscle, its function being instead energy buffering. The decrease in flux recorded with exercise is not sufficient to reduce the buffering function of this enzyme, since its velocity remains well above the ATPase fluxes, known to be in the range of 0.5–1.6 mM s⁻¹.

We investigated the control of the CK reaction by its reactants by comparing of rate equation calculation with experimental data and with an equilibrium model of intracellular concentrations. This analysis shows that local changes in metabolite pools or in pH_i are adequate to explain the flux decrease through a decrease of [ADP]. We further show that an increase of [ADP] is not likely. Additionally, our data recorded on humans agree with that of McFarland *et al.* (32) in isolated cat muscle, suggesting an involvement of a quaternary dead end complex formation in the reaction kinetics.

We conclude that our utilization of rate constants determined *in vitro* is justified because of the close agreement between the calculated and the observed CK flux. According to the complex creatine kinase isozyme distribution (3, 53), our work emphasizes the discrepancy between the organization of the CK system and its functional role in skeletal muscle.

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