

A comparison of *in vivo* catalysis by creatine kinase in avian skeletal muscles with different fibre composition

M.B. Smith^{*}, R.W. Briggs^{**}, E.A. Shoubridge^{***}, D.J. Hayes and G.K. Radda

Department of Biochemistry, Oxford University, South Parks Road, Oxford OX1 3QU (V.K.)

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The maximum activity of creatine kinase *in vitro* is similar in the pectoralis major muscle of the chicken and the duck. However, the flux (phosphocreatine to ATP) as measured by ³¹P saturation transfer NMR *in vivo* is almost 2-fold higher in the duck. This apparent discrepancy can be accounted for by the differences in the cytosolic free ADP concentrations in resting muscle.

The maximum activities *in vitro* of enzymes involved in cellular energy metabolism are often assumed to reflect the catalytic potential of the reaction *in vivo*. The actual activities expressed *in vivo*, however, may be modified by the intracellular environment and thus the validity of the assumption is difficult to determine. One can examine this problem using the technique of ³¹P saturation transfer NMR [1], with which one can measure the flux through moderately fast enzymes *in vivo*. In this study we have compared the flux *in vivo* through the creatine kinase reaction with maximum activities *in vitro* in the pectoralis major muscle in a system which relies predominantly on anaerobic-glycolytic metabolism (chicken) and in a predominantly aerobic system (duck).

Adult female Rhode Island Red chickens and

male or female green mallard ducks were anaesthetized through a face mask with 2–3% halothane in a 1:1 O₂:N₂O mixture. Anaesthesia was maintained with 0.5–1.0% halothane for *in vivo* NMR measurements. Chemical analyses were performed on separate groups of animals. In this case the animals were anaesthetized with 1 and 5 mg/kg gallamine triethiodide and 5 and 10 mg/kg alphaxolone-alphadolone for the chicken and duck, respectively, delivered by intravenous injection in the brachial vein.

The animals were placed in a specially constructed horizontal plexiglass cradle for NMR investigation. A nested surface coil pair [2] with a transmitter of 85 mm diameter and a receiver of 25 mm diameter was placed near the ventral portion of the sternum over the belly of the pectoral muscle. The nested coil configuration produces a homogeneous radiofrequency field throughout the sensitive volume of the receiver coil.

The ³¹P-NMR spectra were recorded at 32.5 MHz using an Oxford Research Systems TMR-32 spectrometer and 1.9 tesla, 20 cm diameter bore superconducting magnet. Magnetic field homogeneity was adjusted by observation of the proton signal from tissue water, either in the dual-coil

^{*} Present address: Department of Neurology, Henry Ford Hospital, Detroit, MI, U.S.A.

^{**} Present address: Departments of Radiology and Biological Chemistry, The Milton S. Hershey Medical Center, Pennsylvania State University, Hershey, PA, U.S.A.

^{***} To whom correspondence should be addressed.

mode or by using the smaller coil as both transmitter and receiver.

Longitudinal relaxation times (T_1 values) were measured in the presence of saturating irradiation at the resonance of the exchanging nuclear species. The progressive saturation technique [3] was used, with 15 delays, the longest of which was 10 s. The saturating frequency was noise-modulated to a bandwidth of 15–25 Hz.

Spectra were accumulated using the following parameters: 2048. data points, 4000 Hz sweep width, 256 ms acquisition time, 110 μ s 90° pulse length, 60–80 transients, and total experiment time of 1.2–1.6 h. A convolution difference routine was used to remove a small broad signal component. The time-domain data were then multiplied by a 10 Hz exponential filter to increase signal to noise, zero-filled to 4K data points, and Fourier transformed to the frequency domain. A three-parameter fitting function was used to analyse the relaxation data.

Saturation transfer measurements, from which the pseudo-first-order rate constants were calculated, were based on the original description by Forsen and Hoffman [4,5].

Muscles were freeze-clamped and stored at 77 K until analysed (1–2 weeks). ATP was measured in perchlorate-extracted homogenates as detailed by Bergmeyer [6]. Total creatine was measured according to the method of Bonas et al. [7]. Mitochondria were isolated essentially by the method described by Morgan-Hughes et al. [8] except that the protease step was omitted.

Creatine kinase activity was measured in the creatine to creatine phosphate direction [9] in 100 mM Tris-HCl (pH 9.0), and in the creatine phosphate to creatine direction [10] in 250 mM sucrose, 20 mM Tris-HCl (pH 7.4). Citrate synthase activity was measured in 100 mM Tris-HCl (pH 7.4) [8] and lactate dehydrogenase activity [11] was measured in 100 mM imidazole buffer (pH 7.4). Triton-X-100 was added (0.1–0.5%, v/v) to release maximal enzyme activities. Protein content was determined by the Lowry method [12]; crystalline bovine serum albumin was used to construct standard curves.

The maximum activities of creatine kinase in vitro in the pectoralis major muscle are similar in chickens and ducks (see Table I; however, this

TABLE I

ACTIVITIES OF CREATINE KINASE (CK), CITRATE SYNTHASE (CS) AND LACTATE DEHYDROGENASE (LDH) IN CHICKEN AND DUCK PECTORALIS MAJOR MUSCLE

Figures are means \pm S.D., with the number of animals in parentheses. Results are expressed in μ mol/min per g wet wt. Activities were measured at 25°C.

Enzyme	Chicken	Duck
CK (Cr \rightarrow PCr)	584 \pm 99 (7)	644 \pm 51 (6)
CK (PCr \rightarrow Cr)	1247 \pm 111 (8)	908 \pm 63 (3)
CS	3.40 \pm 0.77 (7)	63.9 \pm 8.7 (3)
LDH	2002 \pm 169 (4)	1144 \pm 166 (3)

assumes that the creatine kinases present in the chicken and duck are equally affected by Cl^- present in the assay buffer). Newsholme et al. [13] have reported that the maximal extractable activity of creatine kinase is similar in the breast muscle of several species of gallinaceous birds. Histochemical analysis [14] showed that each muscle was composed of a homogeneous population (> 95%) of type II (fast) fibre types (data not shown). These fibers are fast-glycolytic (type IIb) in the chicken and fast-oxidative glycolytic (type IIa) in the duck, as indicated by the relative activities of citrate synthase and lactate dehydrogenase in the whole muscle (Table I).

The intracellular pH and the relative concentrations of phosphorus-containing metabolites and creatine in the resting muscle are shown in Table II. The ATP and phosphocreatine concentrations are similar in both animals. The free ADP concentration, calculated from the creatine kinase equilibrium, is 2-fold higher in the duck than in the chicken. Because the free ADP concentration is calculated from several independent parameters, some of which have been measured in different animals, it is difficult to assign precise error terms; however, we estimate that the error is not greater than \pm 10%.

Fig. 1 shows the spectra obtained in a typical saturation transfer experiment on chicken pectoralis major muscle. A summary of the results of all experiments appears in Table III. Although there were marked differences in the equilibrium magnetizations of phosphocreatine in the presence

TABLE II

METABOLITE CONCENTRATIONS IN THE PECTORALIS MAJOR MUSCLE OF THE CHICKEN AND DUCK

The values are means \pm S.D. ($n = 3$, except where noted) and are expressed as $\mu\text{mol/g}$ wet wt. ATP and the total creatine were measured chemically. Creatine phosphate and inorganic phosphate concentrations were determined relative to the mean ATP value from fully relaxed NMR spectra. Free ADP values were determined using the creatine kinase equilibrium ($K_{\text{eq}} = 1.66 \cdot 10^9 \text{ M}^{-1}$ [22]). The free ADP concentration calculated represents the total unbound ADP content of muscle (i.e. ADP^{3-} , HADP^{2-} , MgADP^- and MgHADP). A free Mg^{2+} concentration of 1 mM was assumed as this is representative of values in the literature [23].

	Chicken	Duck
Total creatine (creatine + creatine phosphate)	39.4 k5.7	46.5 \pm 4.4
Creatine phosphate	31.9 k2.5	32.6 \pm 2.5
ATP	7.2 k0.6	8.2 k0.6
ADP (free)	0.011	0.023
Inorganic phosphate	< 0.75	1.6 \pm 0.1
Intracellular pH	7.03 \pm 0.04 (5)	7.03 \pm 0.04 (8)

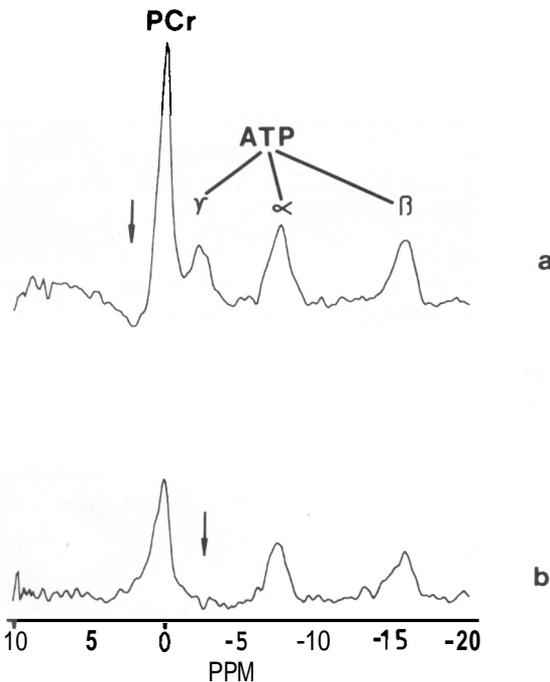


Fig. 1. Results of ^{31}P -NMR saturation transfer experiment on pectoralis muscle in vivo. (a) Control spectrum, irradiation at arrow; (b) saturating irradiation at ATP γ -phosphate. Resonance assignments as indicated.

TABLE III

SUMMARY OF CREATINE KINASE SATURATION TRANSFER MEASUREMENTS IN CHICKEN AND DUCK PECTORALIS MAJOR MUSCLE

M^+/M^0 = equilibrium magnetization of phosphocreatine in the presence of saturating irradiation at the ATP γ -phosphate resonance. T_{1M} = spin lattice relaxation time of phosphocreatine measured in the presence of saturating irradiation at the ATP γ -phosphate resonance. T_1 = calculated intrinsic T_1 for PCr. k = pseudo-first-order rate constant for the exchange phosphocreatine to ATP. F = exchange flux for same reaction ($F = k[\text{PCr}]$). All values are means \pm S.D. ($n = 6$).

	Chicken	Duck
M^+/M^0	0.52 \pm 0.06	0.35 \pm 0.05
T_{1M}	1.81 \pm 0.27	1.32 k0.17
T_1	3.44 \pm 0.27	3.86 \pm 0.65
k (s^{-1})	0.27 \pm 0.06	0.50 \pm 0.08
F ($\mu\text{mol} \cdot \text{g}^{-1} \cdot \text{s}^{-1}$)	8.65	16.16

of saturating irradiation at the ATP γ -phosphate resonance and in the measured T_1 values in both species, there were no significant differences in the calculated intrinsic T_1 . The creatine kinase flux in resting chicken muscle was $8.6 \mu\text{mol} \cdot \text{g}^{-1} \cdot \text{s}^{-1}$ and $16.2 \mu\text{mol} \cdot \text{g}^{-1} \cdot \text{s}^{-1}$ in the duck. Individually these flux measurements represent a composite of three parameters and we estimate that the error associated with the the flux measurement is $\pm 20\%$. Thus, despite the fact that the creatine kinase activities in vitro are the same in both species (Table I), the activity in vivo in resting muscle is 1.9-times greater in the duck.

The validity of the saturation transfer measurement rests on several assumptions. We assume that the reaction can be treated as a two-site exchange. It has been demonstrated that this model adequately describes the flux we have measured [15]. We also assume that phosphocreatine exists in a single compartment and that phosphocreatine is not involved in any other enzymatic interconversions, both of which appear to be valid assumptions.

There are a number of possible explanations for the fact that the creatine kinase flux in vivo in the duck is twice that of the chicken despite the fact that the total enzyme activity is the same in both species. Creatine kinase exists in several isozymic forms, some of which are intimately associated

with mitochondria [16]. If the substrates for the reaction at these local environments are different from those in the bulk cytoplasm, as has been suggested by Bessman and Gerger [17], then the kinetics of the enzyme may be altered. We have calculated that 12% of the total creatine kinase activity is associated with mitochondria in duck, while 0.3% of the total is associated with chicken mitochondria (Table IV, legend). Assuming that the fluxes of the different creatine kinase isozymes are additive in the overall flux measurement, then the mitochondrial creatine kinase flux would have to be about 10-times greater than the cytoplasmic creatine kinase flux to account for the difference we have observed.

The cytoplasmic phosphocreatine concentration which we have used to calculate the creatine kinase flux is based on the assumption that all the chemically determined ATP is NMR-visible. Whilst there is some evidence for this in skeletal muscle [18], there is also good evidence that a significant fraction of the total ATP pool is intramitochondrial [19] and hence NMR-invisible [20]. Since duck muscle contains many more mitochondria than chicken muscle, we investigated the possible influence of this invisible pool on our flux calculations. If we take 25 nmol/mg mitochondrial protein as a maximum estimate of the intra-mitochondrial pool [19] the creatine kinase fluxes then become 8.5 vs. **13.4** $\mu\text{mol} \cdot \text{g}^{-1} \cdot \text{s}^{-1}$ for the chicken and duck respectively, a ratio of 1.6.

TABLE IV

ACTIVITIES OF CREATINE KINASE (CK) AND CITRATE SYNTHASE IN ISOLATED MITOCHONDRIA FROM CHICKEN AND DUCK PECTORALIS MAJOR MUSCLE

Figures are means \pm S.D. ($n=3$). Results are expressed in nmol/min per mg mitochondrial protein at 25°C. The percentage of mitochondrial creatine kinase was determined by calculating the ratio of mitochondrial creatine kinase activity to citrate synthase activity, then multiplying this ratio by the whole muscle citrate synthase activity (Table I) and dividing by the whole muscle creatine kinase activity.

Enzyme	Chicken	Duck
CK (PCr \rightarrow Cr)	690 \pm 157	1940 \pm 149
CK (% total activity)	0.26 \pm 0.009	12.3 \pm 3.4
CS	824 \pm 39	1104 \pm 232

Applying the same correction to the calculation of the free ADP concentration we obtain 11.6 and 31.8 nmol/g wet wt. for the chicken and duck, respectively. It has been reported that a 3-fold change in free ADP concentrations from 9–28 μM results in a 1.6-fold increase in the equilibrium exchange flux in isolated creatine kinase *in vitro* [21]. It therefore appears that the differences in *in vivo* fluxes in chicken and duck muscle can be entirely explained by the differences in ADP concentration in resting muscle and the properties of the isolated enzyme.

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