# Příloha 2 - Výsledky

2.1 Levels of energy-related metabolites in intact and isolated perfused-superfused rat skeletal muscles. (Štefl et al. 1994)

2.2 Creatine kinase reaction in skinned rat psoas muscle fibers and their myofibrils. (Gregor et al. 1999)

2.3 Substrate channelling in a creatine kinase system of rat skeletal muscle under various pH conditions. (Gregor et al. 2003)

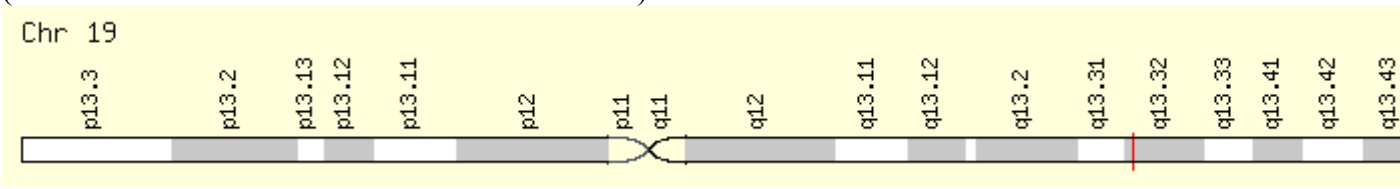
2.4 Creatine kinase binds more firmly to the A-band of rabbit skeletal muscle myofibrils in the presence of its substrates. (Žurmanová et al. 2007 - accepted)

# GeneCard for protein-coding CKM GC19M050501

| Aliases | Descriptions | External Ids |
|---|---|---|
| CKMM [2, 3, 5, 6] | Creatine kinase M chain [3] | HGNC: 1994[1] |
| EC 2.7.3.2 [3] | Creatine kinase M-type [3] | Entrez Gene: 1158[2] |
| M-CK [2, 3] | creatine kinase, muscle [1, 2, 5] | UniProt: P06732[3] |
| | | Ensembl: ENSG00000104879[8] |

Chromosome: **19**
Entrez Gene cytogenetic band: **19q13.2-q13.3** Ensembl cytogenetic band: **19q13.32**

Gene in genomic location: bands according to Ensembl, locations according to GeneLoc
(and/or **Entrez Gene** and/or **Ensembl** if different)




*GeneLoc location for GC19M050501:*

Start: **50,501,511** bp from pter
End: **50,517,974** bp from pter
Size: **16,463** bases
Orientation: **minus** strand

**UniProt/Swiss-Prot:** KCRM_HUMAN, P06732 (See protein sequence)

- **Size**: 381 amino acids; 43101 Da
- **Subunit**: Dimer of identical or non-identical chains. With MM being the major form in skeletal muscle and myocardium, MB existing in myocardium, and BB existing in many tissues, especially brain
- **Subcellular location**: Cytoplasm

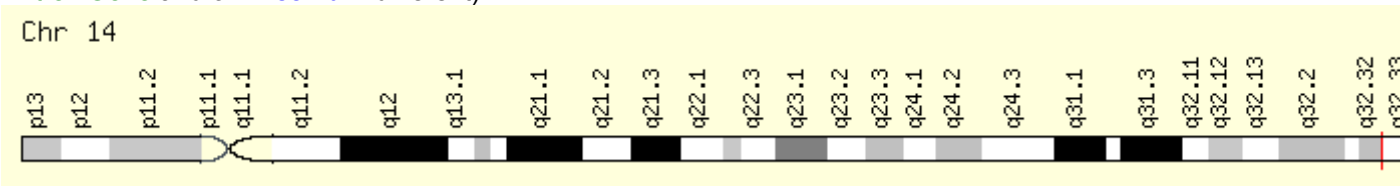
**REFSEQ proteins:** NP_001815.2

# GeneCard for protein-coding CKB GC14M103055

| | Descriptions | External Ids |
|---|---|---|
| B-CK [2, 3] | Creatine kinase B chain [3] | HGNC: 1991[1] |
| CKBB [2, 3, 5, 6] | Creatine kinase B-type [3] | Entrez Gene: 1152[2] |
| EC 2.7.3.2 [3] | creatine kinase, brain [1, 2, 5] | UniProt: P12277[3] |
| | | Ensembl: ENSG00000166165[8] |

Chromosome: 14
Entrez Gene cytogenetic band: 14q32 Ensembl cytogenetic band: 14q32.32

Gene in genomic location: bands according to Ensembl, locations according to GeneLoc (and/or Entrez Gene and/or Ensembl if different)




GeneLoc location for GC14M103055:

Start: 103,055,749 bp from pter
End: 103,058,923 bp from pter
Size: 3,174 bases
Orientation: minus strand
RefSeq genomic assemblies:
NC_000014.7 NT_026437.11

UniProt/Swiss-Prot: KCRB_HUMAN, P12277 (See protein sequence)

- Size: 381 amino acids; 42644 Da
- Subunit: Dimer of identical or non-identical chains. With MM being the major form in skeletal
  muscle and myocardium, MB existing in myocardium, and BB existing in many tissues, especially
  brain
- Subcellular location: Cytoplasm

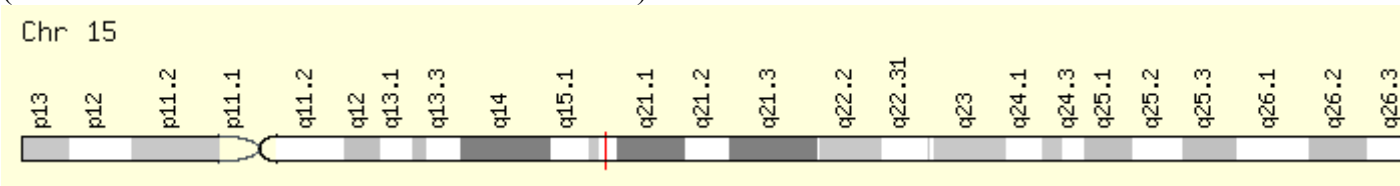
REFSEQ proteins: NP_001814.2

GeneCard for protein-coding CKMT1A GC15P041773

| Aliases | Descriptions | External Ids |
|---|---|---|
| CKMT [3] | Acidic-type mitochondrial creatine kinase [3] | HGNC: 31736[1] |
| **CKMT1** [2] | Creatine kinase, ubiquitous mitochondrial precursor [3] | Entrez Gene: 548596[2] |
| EC 2.7.3.2 [3] | U- MtCK [3] | UniProt: P12532[3] |
| Mia-CK [3] | creatine kinase, mitochondrial 1A [1, 2] | Ensembl: ENSG00000166998[8] |
| UMTCK [2] | | |

Chromosome: **15**
Entrez Gene cytogenetic band: **15q15** Ensembl cytogenetic band: **15q15.3**

Gene in genomic location: bands according to Ensembl, locations according to GeneLoc
(and/or **Entrez Gene** and/or **Ensembl** if different)




*GeneLoc location for GC15P041773:* (*about GC identifiers*)

Start: **41,772,376** bp from pter
End: **41,778,712** bp from pter
Size: **6,336** bases
Orientation: **plus** strand

RefSeq genomic assemblies:
NC_000015.8 NT_010194.16

# GeneCard for protein-coding CKMT1B GC15P041674

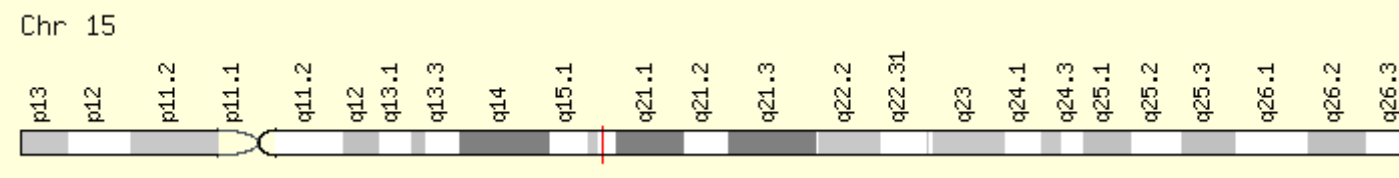
**Aliases**
CKMT [2, 3]
**CKMT1** [2]
EC 2.7.3.2 [3]
Mia-CK [3]
UMTCK [1, 2]

**Descriptions**
Acidic-type mitochondrial creatine kinase [3]
Creatine kinase, ubiquitous mitochondrial precursor [3]
U- MtCK [3]
creatine kinase, mitochondrial 1B [1, 2]

**External Ids**
HGNC: 1995[1]
Entrez Gene: 1159[2]
UniProt: P12532[3]
Ensembl: ENSG00000168775[8]

Chromosome: **15**
Entrez Gene cytogenetic band: **15q15** Ensembl cytogenetic band: **15q15.3**

Gene in genomic location: bands according to Ensembl, locations according to GeneLoc
(and/or **Entrez Gene** and/or **Ensembl** if different)




GeneLoc location for GC15P041674:

Start: **41,672,544** bp from pter
End: **41,678,896** bp from pter
Size: **6,352** bases
Orientation: **plus** strand

**UniProt/Swiss-Prot:** KCRU_HUMAN, P12532

- **Size**: 417 amino acids; 47037 Da
- **Subunit**: Exists as an octamer composed of four MTCK homodimers
- **Subcellular location**: Mitochondrion; mitochondrial inner membrane; intermembrane side
- **Miscellaneous**: Mitochondrial creatine kinase binds cardiolipin

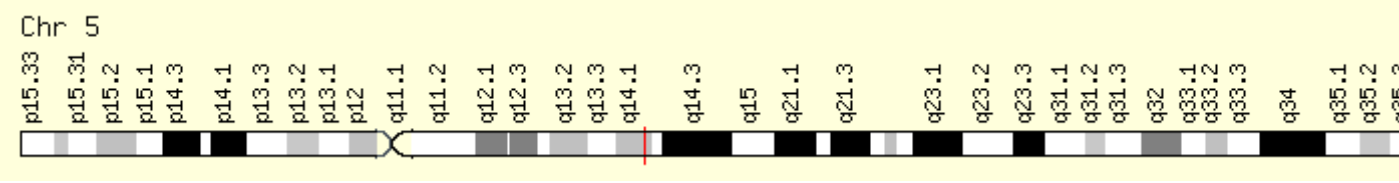
**REFSEQ proteins:** NP_066270.1

# GeneCard for protein-coding CKMT2 GC05P080564

| Aliases | Descriptions | External Ids |
|---|---|---|
| EC 2.7.3.2 [3] | Basic-type mitochondrial creatine kinase [3] | HGNC: 1996[1] |
| Mib-CK [3] | Creatine kinase, sarcomeric mitochondrial precursor [3] | Entrez Gene: 1160[2] |
| SMTCK [1, 2, 5] | S- MtCK [3] | UniProt: P17540[3] |
| | creatine kinase, mitochondrial 2 (sarcomeric) [1, 2, 5] | Ensembl: ENSG00000131730[8] |

Chromosome: **5**
Entrez Gene cytogenetic band: **5q13.3** Ensembl cytogenetic band: **5q14.1**

Gene in genomic location: bands according to Ensembl, locations according to GeneLoc (and/or **Entrez Gene** and/or **Ensembl** if different)




GeneLoc gene densities for chromosome 5

GeneLoc location for GC05P080564: (about GC identifiers)

Start: **80,564,907** bp from pter
End: **80,597,973** bp from pter
Size: **33,066** bases
Orientation: **plus** strand
RefSeq genomic assemblies:
NC_000005.8 NT_006713.14

**UniProt/Swiss-Prot:** KCRS_HUMAN, P17540 (See protein sequence)

- **Size**: 419 amino acids; 47520 Da
- **Subunit**: Exists as an octamer composed of four **CKM**T2 homodimers
- **Subcellular location**: Mitochondrion; mitochondrial inner membrane; intermembrane side
- **Miscellaneous**: Mitochondrial creatine kinase binds cardiolipin

**REFSEQ proteins:** NP_001816.1

**1:** Physiol Res. 1994;43(3):175-80. Links

Erratum in:
Physiol Res 1994;43(4):257.

# Levels of energy-related metabolites in intact and isolated perfused-superfused rat skeletal muscles.

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Adenosine 5'-triphosphate (ATP), phosphocreatine (PCr), creatine (Cr), inorganic phosphate (Pi), lactate (LAC), pyruvate (PYR) and glycogen as glucose (GLU) were determined and free adenosine 5'-diphosphate (ADP) was calculated from ATP:creatine phosphokinase (CPK) reaction in the gracilis muscle of cold-acclimated rats in vivo, and in completely isolated muscles under medium perfusion and superfusion in vitro, using the freeze-clamping method. The mean in vivo levels (mumol/g w.w.) were: ATP 4.8, PCr 12.0, Cr 7.8, Pi 16.1, LAC 1.6, PYR 0.09, GLU 22.9, ADP 0.62 x 10(-3). Isolation of the muscle (about 11 min of anoxia followed by perfusion in the air with a high pO2 medium) decreased macroergic phosphate levels (ATP 3.0, PCr 8.3). In isolated muscles perfused with a high pO2 medium (99 kPa O2, perfusion rate 70 microliters/min) and simultaneously superfused with a low pO2 medium (6.2 kPa O2, 2.3 ml/min) at 28 degrees C in vitro the levels of metabolites were (mumol/g w.w.): ATP 3.1, PCr 8.5, Cr 5.6, Pi 9.2, LAC 2.1, PYR 0.19, GLU 6.6, ADP 0.44 x 10(-3). The mean steady oxygen uptake of the isolated muscle was 97 nmol O2 x min-1 x g-1 w.w. Thus, the levels of macroergic phosphates and their derivatives are lower after isolation and perfusion of the muscle, but the creatine charge [PCr]/([PCr]+[Cr]) remains stable (0.61 in vivo versus 0.60 in the isolated muscle). This indicates that the steady-state and high energy status of the isolated perfused-superfused gracilis muscle is maintained [corrected].

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# Creatine Kinase Reaction in Skinned Rat Psoas Muscle Fibers and Their Myofibrils

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## Summary

The aim of this study was to evaluate myofibrillar creatine kinase (EC 2.7.3.2) activity on the background of the effect of substrate channeling by myosin ATPase and to compare it with creatine kinase (CK) activity of whole skinned fibers. In order to assess CK activity, skinned fibers were prepared from the rat psoas major muscles defined by light microscopy. The activity in permeabilized fibers after treatment with saponin, Triton X-100 and $Ca^{2+}$-free medium reached 2.80, 6.97 and 3.32 μmol ATP $min^{-1}$ $mg^{-1}$ protein, respectively, when a coupled enzyme assay system with external hexokinase and glucose-6-phosphate dehydrogenase was used. Transmission electron microscopy (TEM) revealed a possible interference among activities of sarcolemmal, sarcoplasmic, myofibrillar and mitochondrial CK from persisting structures. For evaluation of the myofibrillar CK itself, a pure myofibrillar fraction was prepared. Fraction purity was confirmed by TEM and by enzymatic assays for marker enzymes. Two procedures, i.e. the coupled enzyme assay and the evaluation of phosphocreatine (PCr) concentration before and after the CK reaction, were used for measurement of CK activity in this fraction. The procedures resulted in 3.2 nmol ATP $min^{-1}$ $mg^{-1}$ protein and 7.6 nmol PCr $min^{-1}$ $mg^{-1}$ protein, respectively. These alternative approaches revealed a discrepancy between the reacting portions of PCr by more than 50 %, which provides information about the size of the effect, generally described as substrate channeling.

**Key words**

Muscle • Skinned fibers • Myofibrils • Creatine kinase • Substrate channeling

## Introduction

The molecular aspects of muscle energetics are considered currently in relation to three groups of problems: mechanisms of force generation, chemo-mechanical conversion during contraction and operation of enzyme molecules in energetic reactions. A particular role among the energetic reactions is played by the creatine kinase reaction (Lohmann 1934), which maintains the ATP concentration stable during muscular activity. The reaction is catalyzed by different isoenzymes of creatine kinase (CK) within the different



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compartments in a muscle fiber. Ubiquitous mitochondrial CK isoenzymes ($Mi_a$ and $Mi_b$ CK) (Jacobs *et al.* 1964, Hossle *et al.* 1988, Payne *et al.* 1991) are coupled to oxidative phosphorylation (Wyss *et al.* 1992). The cytosolic creatine-kinase isoenzymes (MM and BB CK) (Eppenberger *et al.* 1967, Pickering *et al.* 1985) in the cytosol are free. The last form is bound to subcellular structures such as the sarcolemma, sarcoplasmic reticulum or myofibrils (for review see Wallimann *et al.* 1992). It is functionally coupled to the ATPases.

The myofibrillar CK yields 5-10 % MM CK in skeletal muscles (Wallimann *et al.* 1977, 1984), depending on the muscle fiber type. It is localized mostly at the myofibrillar M-band of sarcomeres. This localization was described as being isoenzyme specific (Wallimann *et al.* 1983a). Only MM CK, and not BB CK or heterodimer MB CK, is located in this region. The MM CK molecules are functionally coupled to myosin ATPase and probably to glycolytic enzymes associated with the filaments (Wegmann *et al.* 1992).

Since the myofibrils make up functionally in a straightforward manner the macroscopic integrative behavior of a skeletal muscle fiber (Friedman and Goldman 1996), they can be considered as the simplest structure which still preserves a cellular function, and simultaneously enables access to the molecular level of energetically significant enzymes.

The present work evaluates the creatine-kinase activity in skinned muscle fibers and in isolated purified myofibrils on the background of the effect of substrate channeling between MM CK and myosin ATPase. Our activity data together with data on the structural role of CK in the myofibrillar architecture (Wallimann *et al.* 1983b, Strehler *et al.* 1983) represent a starting point for a further structural operation study of myofibrillar CK molecules integrated in the macroscopic chemical reaction.

## Methods

*Animals and muscle preparation*

Muscle fibers and myofibrils were obtained from the psoas major muscle of Wistar male rats (350- 480 g). Under general anesthesia (50 mg $kg^{-1}$ of sodium pentobarbital, i.p.), both psoas muscles were prepared and thereafter the rat was sacrificed. Freshly dissected intact muscle fibers were morphologically inspected by light microscopy. Transversally cut fiber bundles of about 2 mm in diameter and 2-3 cm long were kept on ice and used for the following procedures.

*Skinning procedures*

Single fiber segments or small bundles of several (up to ten) fiber segments were separated from fresh fiber bundles and chemically skinned using three methods: saponin, Triton X-100 and $Ca^{2+}$-free medium according to the modified procedure described by Thirlwell *et al.* (1994). The composition of the skinning $Ca^{2+}$-free medium was calculated by means of a computer program, using the equilibrium constants of Fabiato (1981). The $Ca^{2+}$-free medium contained (in mM): 6.34 $MgCl_2$, 76.55 KCl, 100 N,N-bis (2-hydroxyethyl)-2-aminoethanesulfonic acid (BES), 5 sodium azide, 5 ethylene glycol-bis(β-amino-ethyl ether)-N′, N′, N′, N′-tetraacetic acid (EGTA), 5 $Na_2$ ATP and 10 $Na_2$ creatine phosphate. Muscle fibers were incubated with the medium on ice under gentle stirring for 24 h. Two other skinning solutions contained in addition Triton X-100 (1 % vol/vol) or saponin (50 μg $ml^{-1}$) and with these fibers were skinned on ice under gentle stirring for 3 and 1.5 h, respectively.

*Enzymes activity measurements*

The CK activity was directly measured using a coupled assay system consisting of hexokinase and glucose-6-phosphate dehydrogenase which resulted in the formation of NADPH (Rosalki 1967). One enzyme unit (IU) is defined as the amount of an enzyme necessary to catalyze the formation of 1 μmol ATP per min. Samples were incubated in 37.42 mM 2,2',2''-nitrilotriethanol (triethanolamine, TEA) buffer, pH 7.5 containing (in mM): 7.72 $MgCl_2$, 38.57 glucose, 0.26 $Na_2$ ADP, 0.57 NADP, 5.86 IU $ml^{-1}$ hexokinase and 1.37 IU $ml^{-1}$ glucose-6-phosphate dehydrogenase. After a 2-min incubation (in order to determine a possible contamination by myokinase activity), the reaction was started by addition of 0.1 mM creatine phosphate.

The method does not allowed an evaluation of maximum enzyme activities, nevertheless it provided sufficient data for the relative comparison of CK activities under different conditions used in this study.

Myosin ATPase activity was determined according to Arrio-Dupont (1988) in a 50 mM TEA buffer, pH 7.2, containing (in mM): 75 KCl, 10 $MgCl_2$, 0.1 EGTA, 50 μM phosphoenolpyruvate, 4 $Na_2$ ATP, 10 μM NADH, 1.25 IU $ml^{-1}$ pyruvate kinase and 1.25 IU $ml^{-1}$ lactate dehydrogenase. The reaction was started by adding a sample aliquot.

Contaminant enzyme activities of pyruvate kinase and lactate dehydrogenase were determined using standard spectrophotometric methods (Ventura-Clapier *et al.* 1995). Succinate dehydrogenase activity was

measured by the spectrophotometric method with $K_3Fe(CN)_6$ (Veeger *et al.* 1969). Lysosomal marker activity of acid phosphatase was measured by the fixed time method using *in vitro* diagnostic assay (Lachema Diagnostica).

*Protein determination*

Proteins were determined by the method of Lowry *et al.* (1951), using bovine serum albumin as a standard.

*Preparation of myofibrillar fraction*

The rat psoas major muscle, cut into small pieces immediately after killing of the animal, was transferred into a homogenization medium (in mM): 100 KCl, 1 EGTA, 5 [ethylenedinitrilo]tetraacetic acid (EDTA), 0.1 β-mercaptoethanol, 0.1 phenylmethylsulfonyl fluoride (PMSF) and 3 sodium azide (at pH 7.0) with 50 % (vol/vol) glycerol. After penetration of glycerol (about one hour), the samples were replaced into a glycerol-free homogenization medium and then homogenized according to Wallimann *et al.* (1984). The homogenates were allowed to stand on ice for 20 min and then centrifuged at 1500 *g* for 10 min. This washing procedure was repeated seven times. After the washing procedure, the myofibrillar fraction was incubated with 1% Triton X-100 (vol/vol) to eliminate the proteins loosely associated with myofibrils and other possible membrane contaminants (Solaro *et al.* 1971). After this, washing was again repeated for seven times.

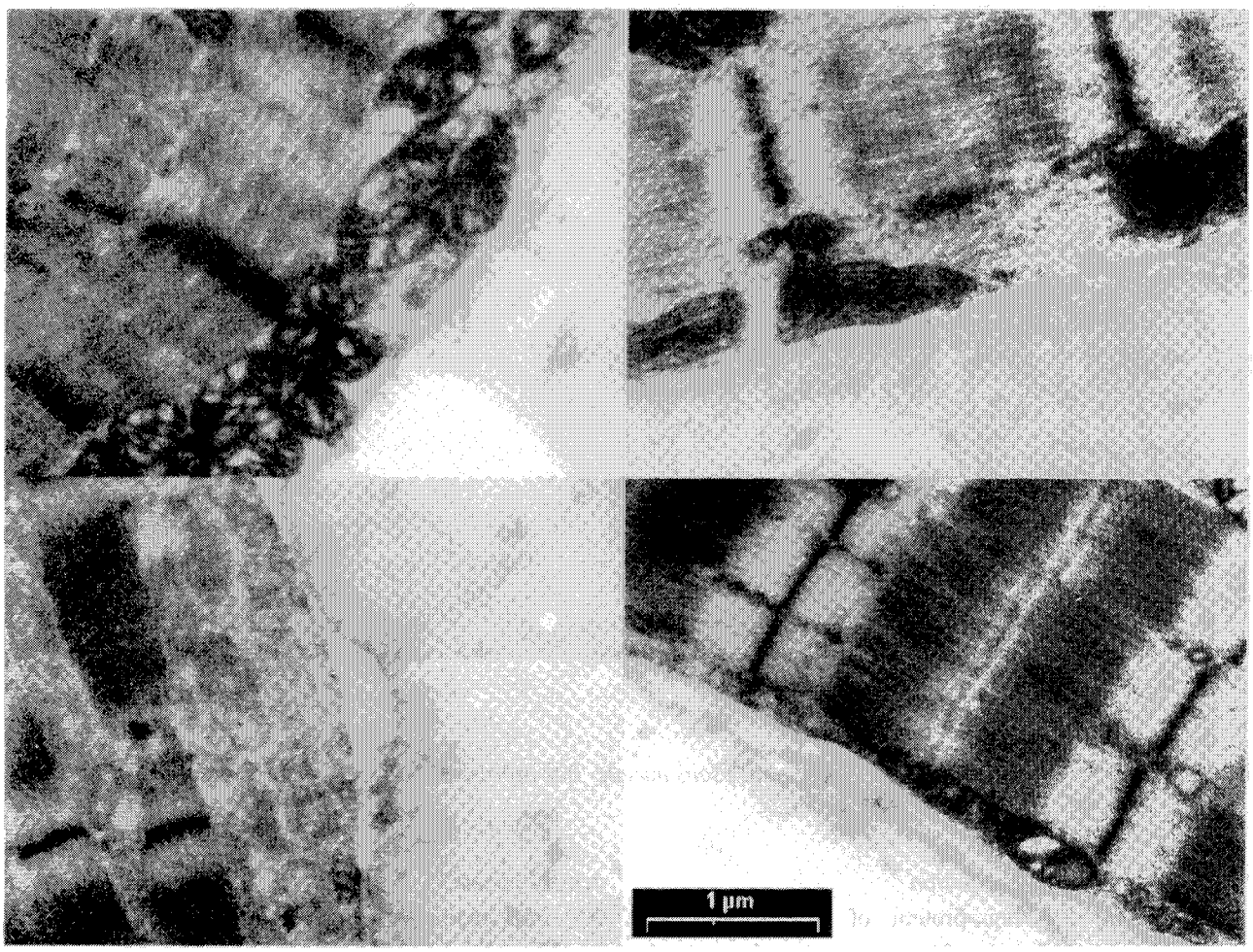


**Fig. 1.** *Photomicrographs of the skinned fibers prepared from rat psoas muscle. In the upper-left field is a fiber incubated in a $Ca^{2+}$-free medium, the upper-right field represents a fiber treated with saponin, lower-left field shows a fiber incubated in Triton and a non-skinned fiber is presented in the lower-right field. Bar represents 1 µm.*

*Electron microscopy (TEM)*

Extensively washed skinned fibers or the myofibrillar fraction were fixed (12 h at 4 °C) in glutaraldehyde (3 % in a cacodylate buffer, pH 7.2). After fixation, the suspension was washed twice with the cacodylate buffer and gently centrifuged. The samples were postfixed with 1 % $OsO_4$ at 4 °C for 3 h. The fixed material was dehydrated in ethanol and acetone series. The samples were embedded into Vestopal W resin (Fluka). Thin sections were cut on a LKB Ultratome 1 and contrasted with uranyl acetate and lead citrate according to Reynolds (1963). Electron microscopic examinations were carried out in a Philips CM100 equipped with Gatan 673 wide angle CCD camera.

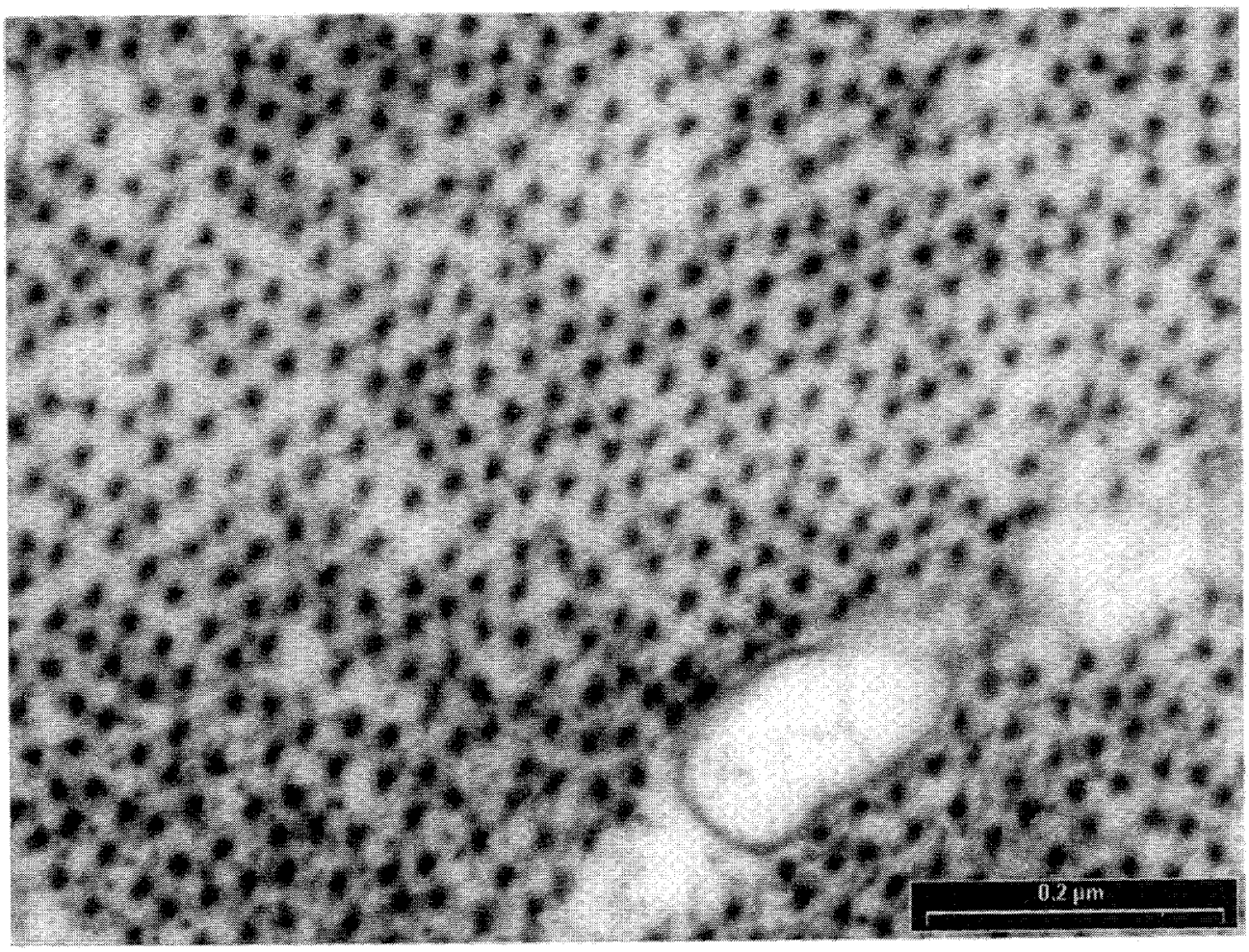


**Fig. 2.** *Transverse section of a fiber from the rat psoas muscle. Bar represents 0.2 µm.*

*Substrate channeling evaluation*

Using 2.5 mg protein of the myofibrillar fraction as a sample, the CK activity was determined during 30 min incubation in the presence of coupled enzymes. The incubation was performed in 37.42 mM TEA buffer, pH 7.5 containing (in mM): 7.72 $MgCl_2$, 38.57 glucose, 1.4 $Na_2$ ADP, 0.57 NADP, 5.86 IU $ml^{-1}$ hexokinase and 1.37 IU $ml^{-1}$ glucose-6-phosphate dehydrogenase. The reaction was started by addition of 0.53 mM creatine phosphate. Absorbance was measured continuously, using Shimadzu recording spectrophotometer UV-1601.

The amount of phosphocreatine before and after incubation was compared in parallel samples in the same solution. After incubation, sample proteins were precipitated with perchloric acid. The pH of the sample

was adjusted to 7.0 and the amount of phosphocreatine was then spectrophotometricaly determined using the coupled enzyme system.

All enzymes were obtained from Boehringer (Germany), substrates and chemicals were from Sigma (USA).

## Results

*Skinned fibers*

Single muscle fibers observed in the light microscope take their origin from tendons on the transverse processes of lumbar vertebrae and run without interruption for a suprisingly long distance of up to 11 mm, towards their tendon insertion on the lesser trochanter of the femur. Their diameter remains the same, namely 13 μm along the whole length of the cylindrical fibers.

The skinned fibers prepared by three skinning procedures manifested very good permeability of their sarcolemma. Exogenous substrates as well as enzymes penetrated into the fibers. Direct measurements of creatine kinase activity comprising particular activities of all isoforms gave approximately the same values. The total CK activity reached 2.80, 6.97 and 3.32 μmol ATP $min^{-1}$ $mg^{-1}$ protein in fibers treated with saponin, Triton X-100 and the $Ca^{2+}$-free medium, respectively. Relatively small discrepancies among the activities confirmed that sarcolemmal permeabilization by three skinning procedures was similar.

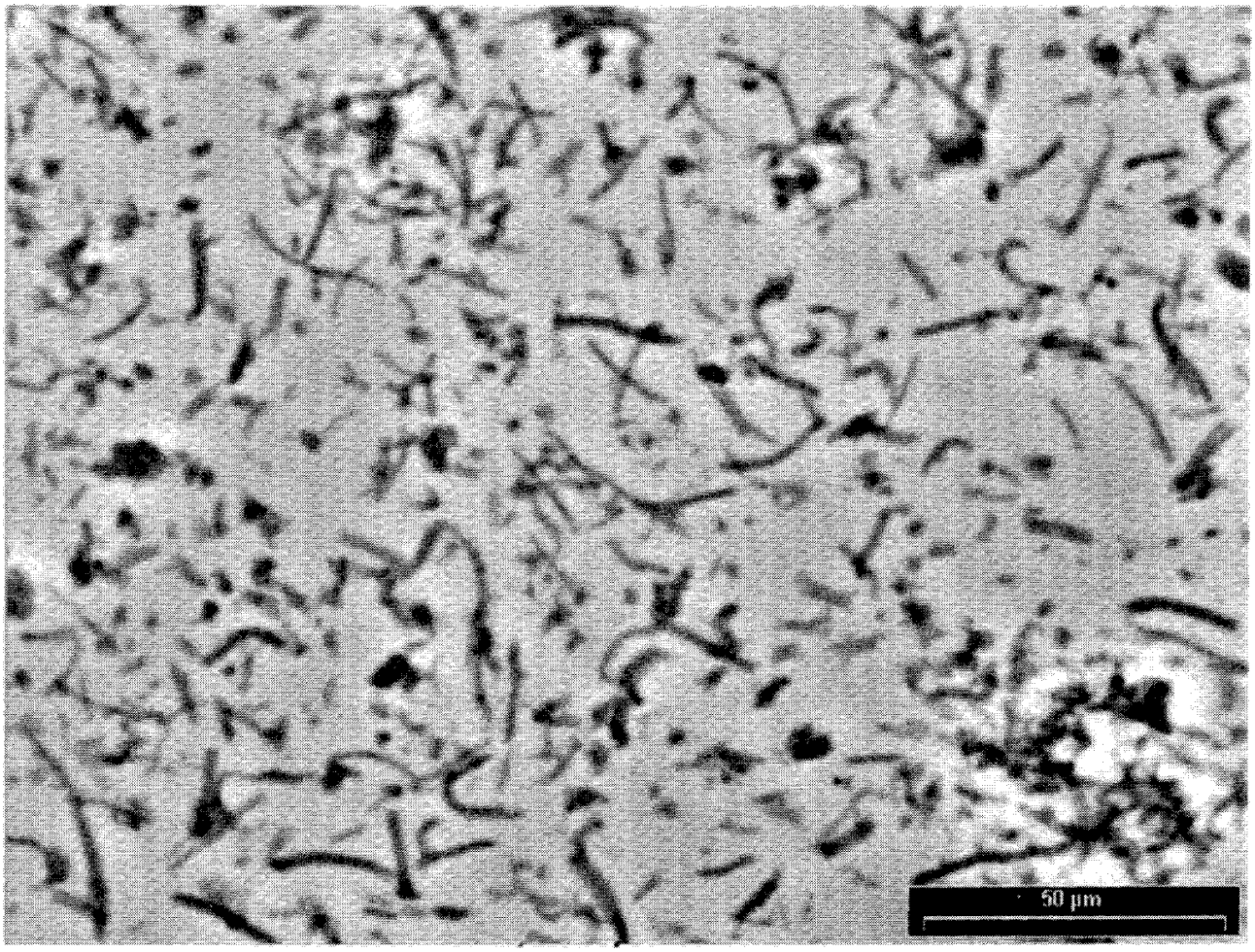


**Fig. 3.** *A photomicrograph (phase contrast) of the pure myofibrillar fraction prepared from the rat psoas muscle. Bar represents 50 μm.*

A TEM comparison of skinned fibers prepared by the different procedures, is shown in Figure 1. In all preparations, the skinned fibers retained their proper contractile apparatus and the samples had a typical pattern of sarcomeres which is characteristic for skeletal muscles under physiological conditions. The

mitochondrial matrix was removed in all samples, however, the Triton treatment was the most efficient. The M-line with myofibrillar CK can be recognized in treated fibers as well as in control samples. None of the skinning procedures removes the basal membrane around the muscle fibers and TEM shows remaining structures of the sarcoplasmic reticulum, mitochondria and the sarcolemma. Statistical evaluation of six electron micrographs (Fig. 2) of cross-sectioned myofibrils gives the distance between two thick myosin filaments: centre-to-centre as 31.5 nm, surface-to-surface 14.4 nm.

*Myofibrillar fraction*

The myofibrillar fraction represented by light microscopy contains uniform myofibrillar fragments (Fig. 3) of considerable purity, without any contaminant. The enzymes activity profile (Table 1) of the prepared myofibrillar fraction shows low or no activity of all contaminant enzyme markers. The specific activity of myofibrillar CK reached 0.003 IU $mg^{-1}$ protein, which corresponds to 0.75% of CK activity measured in a homogenate. The CK activities during each step of preparation of the myofibrillar fraction are shown in Figure 4. In accordance with previous observations (Wallimann *et al.* 1984), direct measurement gives a stable activity value of the myofibrillar bound enzyme after five washing cycles. However, the washed CK activity was significantly lower in each further washing, the portion of the removed CK stays suprisingly constant in each washing step. This also applies to the value of the remaining bound CK after up to 10 washing cycles.

**Table 1.** Specific activities of CK and four contaminant enzymes (as indicators of the fraction purity) in the pure myofibrillar fraction. The percentage of enzyme activity is calculated from the total activity measured in the myofibrillar fraction and the total activity in the whole muscle homogenate. Creatine kinase activities were measured using an enzyme-coupled assay (1) and evaluation of PCr concentration (2).

| | μmol $min^{-1}$ $mg^{-1}$ protein | % |
|---|---|---|
| pyruvate kinase | 0.0015 | 0.05 |
| lactate dehydrogenase | 0.0030 | 0.08 |
| acid phosphatase | – | – |
| succinate dehydrogenase | 0.2760 | 9.58 |
| creatine kinase[1] | 0.0032 | 0.31 |
| creatine kinase[2] | 0.0076 | 0.75 |

When the myofibrillar fraction was incubated in a low ionic strength medium, most of the myofibrillar portion of CK was eluted. The removal of the bound enzyme was monitored by direct measurement of CK activity and by SDS denaturing electrophoresis. After protein collection with centricon concentrator (Amicon, cut-off 30 000), the recovery of the CK specific activity gives 0.0048 IU $mg^{-1}$ protein corresponding to 1.2 % of the CK activity measured in a homogenate.

Further checking of the myofibrillar fraction purity using TEM (picture not shown) revealed neither contamination by other subcellular particles, nor stacked myofibrillar bundles.

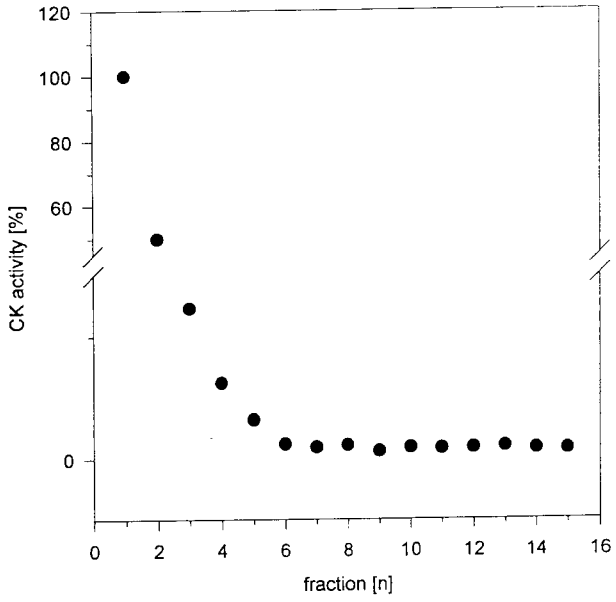


**Fig. 4.** *Portion of MM CK bound to the myofibrils. Creatine kinase activity is expressed as percentage of the total activity (100%, measured in the whole muscle homogenate, which represents fraction No. 1). n is the fraction number of each washing step of the preparation of pure myofibrils.*

*Substrate channeling evaluation*

Repeated activity measurements of myofibrillar CK were performed using 2.5 mg protein of the myofibrillar fraction as a sample after 30 min incubation in the presence of coupled enzymes (Table 1). Two procedures, i.e. the enzyme-coupled assay and the evaluation of phosphocreatine (PCr) concentration before and after CK reaction, were used to quantify substrate channeling. Changes in ATP concentration (evaluated by

the enzyme-coupled assay) were the same as those obtained in PCr concentrations for expression of CK activity. The enzyme-coupled assay allowed measurement of free accessible concentrations of ATP entering the CK reaction, i.e. reduced by ATPase activity of myosin. Measurements of PCr concentration before and after CK reaction avoid the competitive effect of myosin ATPase. Thus, a comparison of the two procedures could be used for quantification of substrate channeling at the level of myofibrillar CK. The former procedure gave results of 3.2 nmol $min^{-1}$ $mg^{-1}$ protein, while the latter gave values of 7.6 nmol $min^{-1}$ $mg^{-1}$ protein of reacted ATP and PCr, respectively. These alternative approaches revealed a discrepancy between reacting portions of PCr of more than 50 % (4.4 nmol $min^{-1}$ $mg^{-1}$ protein), indicating the extent of substrate channeling of the CK reaction by myofibrillar ATPase *via* ATP.

## Discussion

Skinned fibers are considered to be an ideal model for studies dealing with muscle physiology at the cellular level under persisting native conditions. This experimental model has been successfully used for studies in the field of muscle contraction (Hilber and Galler 1997, Coonan and Lamb 1998, Veigel *et al.* 1998), signal transduction (Kuznetsov *et al.* 1996, Galler *et al.* 1997) and energy metabolism (Potma and Stienen 1996, O'Gorman *et al.* 1997, Sahlin *et al.* 1998). Nevertheless, skinned fibers have some structural and biochemical disadvantages for further studies of the behaviour of myofibrillar CK molecules in a macroscopic energy reaction. These disadvantages make it necessary to simplify muscle fibers to a purely myofibrillar fraction.

The structural disadvantage of whole muscle fibers consists in the restricted accessibility of enzyme molecules in fiber bundles to external substrates or testing enzymes and by their restricted diffusion within a fiber through the actomyosin molecular sieve (Arrio-Dupont *et al.* 1997, Tanner *et al.* 1992). In spite of this fact, CK activity measured in the skinned fibers is found to be higher as it is measured for myofibrillar CK in the pure myofibrillar fraction from the whole muscle homogenate. The unexpected difference is due to the high content of non-myofibrillar CK isoenzymes.

Possible interference of Mi-CK activity (5-20 % of the cellular CK, Wegmann *et al.* 1992), due to persisting mitochondria observed by TEM microscopy, is one of the biochemical obstacles for evaluation of pure myofibrillar CK activity in the energetically active skinned fibers.

However, contrary to the skinned fibers, pure myofibrillar fraction exhibits ideal biochemical homogeneity and well defined relations between structure and function. Further washing procedures together with Triton treatment significantly reduced the content of contaminating enzymes. Fraction purity, documented by marker enzyme activities (Table 1) was achieved at the expense of a relatively low yield of myofibrillar CK. The previously described relative portion of the myofibrillar CK activity, with respect to the total CK activity, represented 5 to 10 % (Wallimann *et al.* 1977, 1984). In our preparations, 5 % activity values (see Fig. 4), considered as the total CK localized in myofibrils, was repeatedly obtained during 4-5 wash cycles. The portions of myofibrillar CK removed between washing cycles 5 and 10 correspond to the activity of loosely bound CK localized mostly to the I-band of the sarcomere (Wegmann *et al.* 1991, 1992). The remaining CK activity measured after 10 washing cycles represented unremovable, tightly bound myofibrillar CK localized to the M-band (Wallimann *et al.* 1983b, Stolz and Wallimann 1998).

Substrate channeling represents another biochemical obstacle which restricts the determination of absolute activity of the myofibrillar CK isoenzyme. Even in the pure myofibrillar fraction, this effect has been observed. Arrio-Dupont (1988) first described substrate channeling of myofibrillar CK in frog ventricular cells. This problem was noted in several papers (e.g. Saks *et al.* 1994, 1996), without being quantitatively evaluated. Direct measurement of total PCr consumed in the CK reaction, gives a reasonable estimation of the channeling effect in our experiments. The extent of 60 % ATP channeling is in good correlation with the results obtained by comparison of CK activity determined in the pure myofibrillar fraction and after elution in a low ionic strength medium. Due to elution of all myofibrillar CK (92-96 %, Wallimann *et al.* 1984), the portion of myofibrillar CK activity rose to twofold values. The biochemical role of substrate channeling has been extensively discussed elsewhere (Cornish-Bowden and Cárdenas 1992, Ovádi 1995, Kholodenko *et al.* 1996).

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## References

ARRIO-DUPONT M: An example of substrate channeling between co-immobilized enzymes. Coupled activity of myosin ATPase and creatine kinase bound to frog heart myofilaments. *FEBS Lett* **240:** 181-185, 1988.

ARRIO-DUPONT M, FOUCAULT G, VACHER M, DOUHOU A, CRIBIER S: Mobility of creatine phosphokinase and β-enolase in cultured muscle cells. *Biophys J* **73:** 2667-2673, 1997.

COONAN JR, LAMB GD: Effect of transverse-tubular chloride conductance on excitability in skinned muscle fibers of rat and toad. *J Physiol London* **509:** 551-564, 1998.

CORNISH-BOWDEN A, CÁRDENAS ML: Channeling can affect concentrations of metabolic intermediates at constant net flux: artifact or reality? *Eur J Biochem* **213:** 87-92, 1992.

EPPENBERGER HM, DAWSON DM, KAPLAN NO: The comparative enzymology of creatine kinases. *J Biol Chem* **242:** 204-209, 1967.

FABIATO A: Myoplasmic free calcium concentration reached during the twitch of an intact isolated cardiac cell and during calcium-induced release of calcium from the sarcoplasmic reticulum of a skinned cardiac cell from the adult rat or rabbit ventricle. *J Gen Physiol* **78:** 457-498, 1981.

FRIEDMAN AL, GOLDMAN YE: Mechanical characterization of skeletal muscle myofibrils. *Biophys J* **71:** 2774-2785, 1996.

GALLER S, HILBER K, GÖBESBERGER A: Effects of nitric oxide on force-generation proteins of skeletal muscle. *Pflügers Arch* **434:** 242-245, 1997.

HILBER K, GALLER S: Mechanical properties and myosin heavy chain isoform composition of skinned skeletal fibers from a human biopsy sample. *Pflügers Arch* **434:** 551-558, 1997.

HOSSLE JP, SCHLEGEL J, WEGMANN G, WYSS M, BÖHLEN P, EPPENBERGER HM, WALLIMANN T, PERRIARD J-C: Distinct tissue specific mitochondrial creatine kinases from chicken brain and striated muscle with a conversed CK framework. *Biochem Biophys Res Commun* **151:** 408-416, 1988.

JACOBS H, HELDT HW, KLINGENBERG M: High activity of creatine kinase in mitochondria from muscle and brain. Evidence for a separate mitochondrial isoenzyme of creatine kinase. *Biochem Biophys Res Commun* **16:** 516-521, 1964.

KHOLODENKO BN, SAKAMOTO N, PUIGJANER J, WESTERHOFF HV, CASCANTE M: Strong control on the transit time in metabolic channeling. *FEBS Lett* **389:** 123-125, 1996.

KUZNETSOV AV, TIIVEL T, SIKK P, KAAMBRE T, KAY L, DANESHRAD Z, ROSSI A, KADAJA L, PEET N, SEPPET E, SAKS VA: Striking differences between the kinetics of regulation of respiration by ADP in slow-twitch muscles in vivo. *Eur J Biochem* **241:** 909-915, 1996.

LOHMANN K: Über die enzymatische Aufspaltung der Kreatinephosphorsäure; zugleich ein Beitrag zum Chemismus der Muskelkontraktion. *Biochem Z* **271:** 264-277, 1934.

LOWRY OH, ROSEBROUGH NJ, FARR AL, RANDALL RL: Protein measurement with Folin phenol reagent. *J Biol Chem* **193:** 265-275, 1951.

O'GORMAN E, PIENDL T, MÜLLER M, BRDICZKA D, WALLIMANN T: Mitochondrial intermembrane inclusion bodies: the common denominator between human mitochondrial myopathies and creatine depletion, due to impairment of cellular energetics. *Mol Cell Biochem* **174:** 283-289, 1997.

OVÁDI J: *Cell architecture and metabolic channeling.* Springer Verlag, New York, 1995, pp 1-250.

POTMA EJ, STIENEN GJ: Increase in ATP consumption during shortening in skinned fibers from rabbit psoas muscle: effects of inorganic phosphate. *J Physiol London* **496:** 1-12, 1996.

PAYNE RM, HAAS RC, STRAUSS AW: Structural characterization and tissue-specific expression of the mRNAs encoding isoenzymes from two rat mitochondrial creatine kinase genes. *Biochim Biophys Acta* **1089:** 352-361, 1991.

PICKERING L, PANG H, BIEMANN K, MUNRO H, SCHIMMEL P: Two tissue-specific isozymes of creatine kinase have closely matched amino acid sequences. *Proc Natl Acad Sci USA* **82:** 2310-2314, 1985.

ROSALKI SB: An improved procedure for serum creatine phosphokinase determination. *J Lab Clin Med* **69:** 696-705, 1967.

REYNOLDS ES: The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *Cell Biol* **17:** 208-212, 1963.

SAHLIN K, TONKONOGI M, SÖDERLUND K: Energy supply and muscle fatigue in humans. *Acta Physiol Scand* **162:** 261-266, 1998.

SAKS VA, KHUCHUA ZA, VASILYEVA EV, BELIKOVA OY, KUZNETSOV AV: Metabolic compartmentation and substrate channeling in muscle cells. Role of coupled creatine kinases in in vivo regulation of cellular respiration – a synthesis. *Mol Cell Biochem* **133-134:** 155-192, 1994.

SAKS VA, VENTURA-CLAPIER R, ALIEV MK: Metabolic control and metabolic capacity: two aspects of creatine kinase functioning in the cells. *Biochim Biophys Acta* **1274:** 81-88, 1996.

SOLARO RJ, PANG DC, BRIGGS FN: The purification of cardiac myofibrils with Triton X-100. *Biochim Biophys Acta* **245:** 259-262, 1971.

STOLZ M, WALLIMANN T: Myofibrillar interaction of cytosolic creatine kinase (CK) isoenzymes: allocation of N-terminal binding epitope in MM-CK and BB-CK. *J Cell Sci* **111:** 1207-1216, 1998.

STREHLER EE, CARLSSON E, EPPENBERGER H., THORNELL LE: Ultrastructural localization of M-band proteins in chicken breast muscle as revealed by combined immunocytochemistry and ultramicrotomy. *J Mol Biol* **166:** 141-158, 1983.

TANNER JW, THOMAS DD, GOLDMAN YE: Transients in orientation of fluorescent cross-bridge probe following photolysis of caged nucleotides in skeletal muscle fibers. *J Mol Biol* **223:** 185-203, 1992.

THIRLWELL H, CORRIE JET, REID GP, TRENTHAM DR, FERENCZI MA: Kinetics of relaxation from rigor of permeabilized fast-twitch skeletal fibers from the rabbit using a novel caged ATP and apyrase. *Biophys J* **67:** 2436-2447, 1994.

VEEGER C, DER VARTANIAN DV, ZEYLEMAKER WP: Succinate dehydrogenase. *Meth Enzymol* **13:** 81-90, 1969

VEIGEL C, VON MAYDELL RD, KRESS KR, MOLLOY JE, FINK RH: The effect of ionic strength on the kinetics of rigor development in skinned fast-twitch skeletal muscle fibers. *Pflügers Arch* **435:** 753-761, 1998.

VENTURA-CLAPIER R, KUZNETSOV AV, D'ALBIS A, VAN DEURSEN J, WIERINGA B, VEKSLER VI: Muscle creatine kinase-deficient mice. I. Alterations in myofibrillar function. *J Biol Chem* **270:** 19914-19920, 1995.

WALLIMANN T, TURNER DC, EPPENBERGER HM: Localization of creatine kinase isoenzymes in myofibrils. I. Chicken skeletal muscle. *J Cell Biol* **75:** 297-317, 1977.

WALLIMANN T, MOSER H, EPPENBERGER HM: Isoenzyme-specific localization of M-line bound creatine in myogenic cells. *J Muscl Res Cell Motil* **4:** 429-441, 1983a.

WALLIMANN T, DOETSCHMAN TC, EPPENBERGER HM: Novel staining pattern of skeletal muscle M-lines upon incubation with antibodies against MM-creatine kinase. *J Cell Biol* **96:** 1772-1779, 1983b.

WALLIMANN T, SCHLÖSSER T, EPPENBERGER, HM: Function of M-line-bound creatine kinase as intramyofibrillar ATP regenerator at the receiving end of the phosphorylcreatine shuttle in muscle. *J Biol Chem* **259:** 5238-5246, 1984.

WALLIMANN T, WYSS M, BRDICZKA D, NICOLAY K: Intracellular compartmentation, structure and function of creatine kinase isoenzymes in tissues with high and fluctuating energy demands: the 'phosphocreatine circuit' for cellular energy homeostasis. *Biochem J* **281:** 21-40, 1992.

WEGMANN G, HUBER R, ZANOLLA E, EPPENBERGER HM, WALLIMANN T: Differential expression and localization of brain-type and mitochondrial creatine kinase isoenzymes during development of the chicken retina: Mi-CK as a marker for differentiation of photoreceptor cells. *Differentiation* **46:** 77-87, 1991.

WEGMANN G, ZANOLLA E, EPPENBERGER HM, WALLIMANN T: In situ compartmentation of creatine kinase in intact sarcomeric muscle: the actomyosin overlap zone as a molecular sieve. *J Muscl Res Cell Motil* **13:** 420-435, 1992.

WYSS M, SMEITINK J, WEVERS R, WALLIMANN T: Mitochondrial creatine kinase: a key enzyme of aerobic energy metabolism. *Biochim Biophys Acta* **1102:** 119-166, 1992.

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# Substrate channelling in a creatine kinase system of rat skeletal muscle under various pH conditions

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Experimental Physiology

# Substrate channelling in a creatine kinase system of rat skeletal muscle under various pH conditions

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**The aim of this study was to evaluate myofibrillar creatine kinase (CK) activity and to quantify the substrate channelling of ATP between CK and myosin ATPase under different pH conditions within the integrity of myofibrils. A pure myofibrillar fraction was prepared using differential centrifugation. The homogeneity of the preparation and the purity of the fraction were confirmed microscopically and by enzymatic assays for contaminant enzyme activities. The specific activity of myofibrillar CK reached 584 ± 33 nmol PCr $min^{-1}$ $mg^{-1}$ at pH 6.75. Two methods were used to detect CK activity: (1) measurement of direct ATP production, and (2) measurement of PCr consumption. This method of evaluation has been tested in experiments with isolated creatine kinase. No discrepancy in CK activity between the methods was observed in the pH range tested (6.0–7.5). However, the same procedures resulted in a significant discrepancy between the amounts of reacted PCr and produced ATP within the pure myofibrillar fraction. This discrepancy represents the portion of ATP produced by the CK reaction, which is preferentially channelled to the myosin ATPase before diffusing into the bulk solution. The maximum evaluated difference reached 42.3% at pH 6.95. The substrate channelling between myofibrillar-bound CK and myosin ATPase was evaluated under various pH levels within the physiological range and it reached a maximum value in a slightly acidic environment. These results suggest that ATP/ADP flux control by the CK system is more important at lower pH, corresponding to the physiological state of muscle fatigue.** *Experimental Physiology* (2003) **88.1**, 1–6.

Cells with high and fluctuating energy demands (e.g. muscle tissue) require an effective system for metabolic control and energy transfer. The most effective system, integrating energy metabolism into one efficiently regulated metabolic network, is the creatine kinase (CK) shuttle (for review see Walliman *et al.* 1992; Saks *et al.* 1996). Creatine kinase (EC 2.7.3.2) controls the near-equilibrium (Kushmerick, 1983) CK reaction:

$$ATP + Cr \rightarrow PCr + ADP + \alpha H^{+}, \quad (1)$$

in heart, skeletal muscle, brain and smooth muscle.

The spatial organization of creatine kinase isoenzymes has been long recognized, and striated muscle cells are the best example of energy metabolism compartmentation. The CK isoenzymes are localized into energy-producing and energy-utilizing sites, where they are functionally coupled with ATP synthesis (mitochondria, cytosol) or ATP-consuming processes (myofibrils, sarcoplasmic reticulum). This organization of the CK system ensures the regulation of local concentrations of ADP and ATP, maintenance of the optimal ATP/ADP ratio, regulation of adenylate nucleotide fluxes and protection of the adenine nucleotides cellular pool from degradation.

It can be seen from eqn (1) that first, the position of the CK reaction equilibrium should be affected by cytoplasmic pH, and second, the CK reaction evidently deviates from equilibrium, and its regulation should be described in terms of non-equilibrium thermodynamics (Mejsnar *et al.* 1992; Maršík & Mejsnar, 1994). The regulation can be realized by conformational changes of the CK molecule, when its reactive 'closed' conformation is not achieved merely by the substrate-induced energy-minimizing principle (Mejsnar *et al.* 2002).

Stated in another way, any ATPase system that evokes a unidirectional net reverse CK flux towards ATP, by the splitting of ATP will shift the CK reaction out of equilibrium. The functional coupling of myosin ATPase and myofibrillar CK by substrate channelling (Arrio-Dupont, 1988; Gregor *et al.* 1999), which is defined as direct transfer of ATP between active sites of these enzymes, emphasizes the key role of the phosphocreatine/creatine



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kinase system in muscle energy metabolism. Substrate channelling has been proposed to decrease the transit time of substrates, prevent loss of substrates by diffusion, protect labile substrates from solvent and forestall the entry of intermediates into competing metabolic pathways (Ovádi, 1995). Substrate channelling also plays a significant role in the control of metabolic fluxes (Kholodenko & Westerhoff, 1993; Kholodenko *et al.* 1994). A pure myofibrillar fraction that possesses CK activity as well as ATPase activity by myosin (Gregor *et al.* 1999) makes it possible to study the behaviour of the two energy systems in combination and evaluate substrate channelling under different conditions.

In this paper, using a pure myofibrillar fraction, we define at this subcellular level of organization first, the discrepancy between PCr consumption and apparent ATP production in the CK reaction, the difference between these representing substrate channelling between myofibrillar-bound CK and myosin ATPase, and second, the dependence of the degree of substrate channelling upon varying pH.

## METHODS

Myofibrils were obtained from the psoas major muscle of male Wistar rats weighing 350–480 g. The rats were anaesthetized by an intraperitoneal injection of 50 mg $kg^{-1}$ thiopental. Left and right psoas muscles were dissected, and then the rats were killed by an overdose of anaesthetic. The experiments were performed in accordance with local ethical committee guidelines.

The psoas major muscle was cut into small pieces and incubated in a homogenization medium for 1 h. After penetration of glycerol, the pieces were placed into a glycerol-free homogenization medium and then homogenized according to Wallimann *et al.* (1984). The homogenate was allowed to stand on ice for 20 min and then centrifuged at 1500 *g* for 7 min. The washing procedure was repeated six times. After this, the myofibrillar fraction was incubated with 1% Triton X-100 for 5 min (to eliminate the proteins loosely associated with the myofibrils and other possible membrane contaminants; Solaro *et al.* 1971) and washing was again repeated seven times.

The activity of free CK (Roche Diagnostics GmbH) and the activity of myofibrillar-bound CK were determined using an enzyme-coupled assay system, consisting of hexokinase and glucose-6-phosphate dehydrogenase (Gerhardt, 1983). The incubation was performed in imidazol acetate buffer (115 mM imidazol, 11.5 mM magnesium acetate, 2.3 mM EDTA) containing: 2.3 mM ADP, 5.8 mM AMP, 11.5 $\mu$M Ap5A, 23 mM D-glucose, 2.3 mM NADP, 23 mM NAC, 3.5 i.u. $ml^{-1}$ hexokinase (HK) and 2.3 i.u. $ml^{-1}$ glucose-6-P dehydrogenase (Glu-6-PDH).

Parallel samples were incubated in the same solution with a known concentration of PCr (no NADP, glucose, HK or Glu-6-PDH were added). After this incubation, sample proteins were precipitated with 6% perchloric acid. The sample was neutralized by 5.21 M $K_2CO_3$ up to 7.3 pH. The concentrations of PCr and ATP after CK reaction were then spectrophotometrically measured using the coupled enzymatic method (Trautschold *et al.* 1985; Wahlefeld & Siedel, 1985) and the decrease in total PCr concentration was calculated. To assess the pH dependence, measurements were performed in imidazole acetate buffers within the pH range 6.0–8.0.

Protein concentrations were determined by the method of Lowry *et al.* (1951), using bovine serum albumin as a standard.

The results are expressed as means ± standard errors of the mean (S.E.M.). Differences between two groups (apparent ATP production and PCr consumption measurements) were compared by a one-way analysis of variance (ANOVA) after confirmation of the normal distribution of the variable ($\chi^2$ test). Significance was accepted for $P < 0.05$. The dependence of the substrate channelling on pH was evaluated by the polynomial regression function (using the Simfit program of W. G. Berdsley, University of Manchester, UK).

## RESULTS

Activity measurements of free CK incubated without myofibrillar contractile proteins were performed to give control values of activity with respect to pH. Two procedures, i.e. an enzyme-coupled assay and an evaluation of the phosphocreatine concentration before and after CK reaction, were used to evaluate substrate channelling. The enzyme-coupled assay allowed direct measurement of the freely accessible concentration of ATP originating in the CK reaction (apparent ATP production). Analyses of changes in the total PCr concentration (PCr entering the CK reaction) avoid the competitive effect of myosin ATPase (Fig. 1) and represent the true extent of the reaction. A comparison of these alternative methods enabled us to determine the discrepancy between the reacting portion of PCr and apparent ATP production and thus quantify the substrate channelling at the level of myofibrillar CK.

In order to preclude the effect of different pH levels on the enzyme-coupled assay and measurement of the decrease in total PCr concentration, free CK was put through the substrate channelling evaluation. Using external CK as a control, no divergence was found between the two alternative CK reaction measurements (ANOVA, $P > 0.90$), and both curves had the same pH dependency. No substrate channelling was indicated within the pH range used (6.0–7.5; Fig. 2). In parallel with the decrease of total PCr concentration, the ATP increase was evaluated. This measurement revealed partial non-specific hydrolysis of PCr due to pH alteration during protein precipitation. Although the hydrolysed portion was small (about 8%), calibration with an internal standard was used in all subsequent experiments. These measurements evaluated methods for substrate channelling quantification under various pH conditions and confirmed their suitability for experiments with myofibrillar fraction.

The purity and uniformity of the myofibrillar fragments in the prepared myofibrillar fraction were checked by light microscopy. Myofibrillar fragments and other subcellular particles were counted in the microscope field under ×100 magnification. The preparation was considered as uniform when ballast compounds did not exceed 5% of the total counted particles. The myofibrillar fraction was also screened for any activity of contaminant marker enzymes as indicators of fraction purity. No acid phosphatase activity

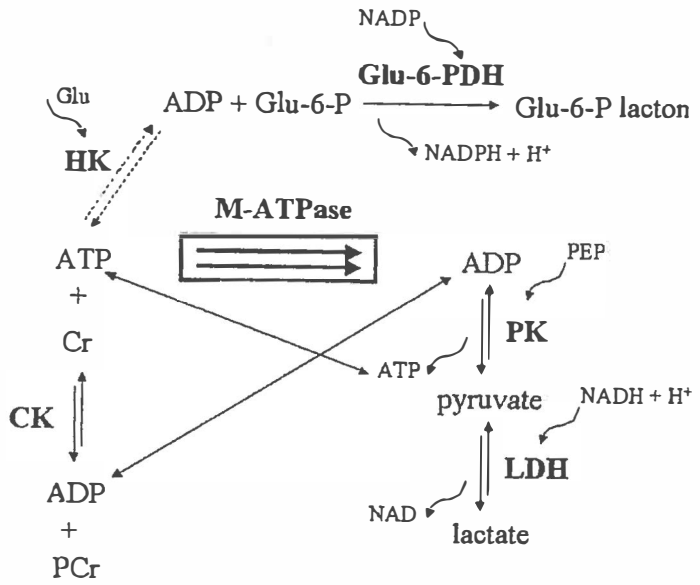


**Figure 1**

Scheme of the coupled activity of myosin ATPase (M-ATPase) and myofibrillar creatine kinase (CK). Dashed arrows represent the competitive measurement of CK activity via an enzyme-coupled assay (PCr, phosphocreatine; Cr, creatine; HK, hexokinase; Glu, glucose; Glu-6-P, glucose-6-P; Glu-6-PDH, glucose-6-P dehydrogenase). Double-headed arrows represent constraints for CK activity evaluation via ADP measurement (PEP, phosphoenolpyruvate; PK, pyruvate kinase; LDH, lactate dehydrogenase).

and negligible background activities of pyruvate kinase, lactate dehydrogenase and succinate dehydrogenase were detected. The specific activity of myofibrillar CK reached 584 ± 33 nmol $min^{-1}$ $mg^{-1}$ (pH 6.75), which corresponded to 0.42 % of CK activity measured in the homogenate.

The substrate channelling evaluation, using myofibrillar bound CK as a sample, showed a significant difference between the decrease of total PCr concentration and apparent ATP production, as measured by the enzyme-coupled assay (Fig. 3). At the optimum pH of 6.75, apparent CK activity reached 377 ± 28 nmol (mg protein)$^{-1}$ $min^{-1}$, which corresponds to a real CK activity value of 584 ± 33 nmol (mg protein)$^{-1}$ $min^{-1}$. The substrate channelling was thus evaluated, and 34.7 %, corresponding to 207 ± 10 nmol (mg protein)$^{-1}$ $min^{-1}$, represents ATP consumption by myosin ATPase. This difference was significant within the tested pH range of 6.0–8.0 (ANOVA, $P < 0.01$).

As shown in Fig. 4, the level of substrate channelling increased within the pH interval of 6.00–7.25, and then continuously declined with increasingly alkali pH. The best fit was attained with the weighted least squares polynomial which results in a quadratic function ($n = 36$, weight = $1/s^2$) (Simfit program). The maximum of the function was reached at pH 6.95. This analysis was confirmed by a cubic spline fitting which resulted in function maximum at pH 6.97. The maximum value of substrate channelling was measured at pH 7.25, where 205 nmol (mg protein)$^{-1}$ $min^{-1}$ of ATP is not freely accessible for enzyme-coupled evaluation. This substrate channelling value corresponds to 42.3 % of the total PCr entering the CK reaction.

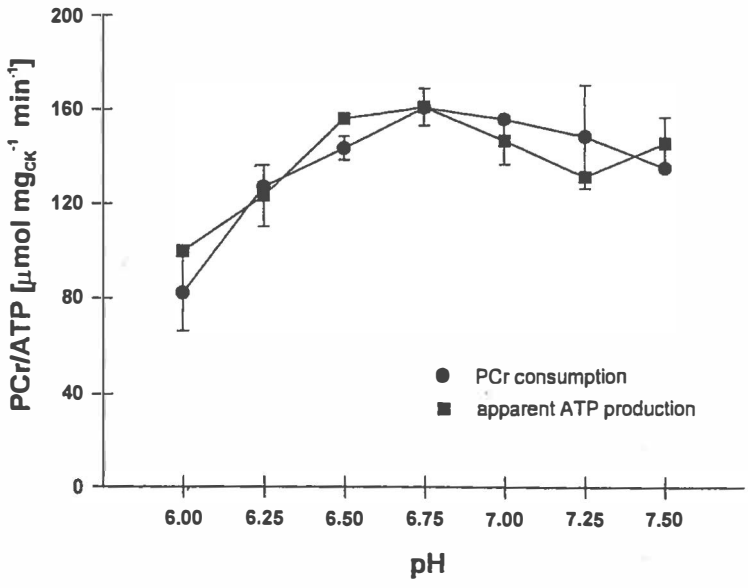


**Figure 2**

The pH dependency of direct ATP production (apparent ATP production; ■) and PCr consumption (●) measurements in creatine kinase reaction with free CK (the two curves do not differ; ANOVA $P > 0.90$; $n = 3$ for each point). Symbols and bars are means ± S.E.M.

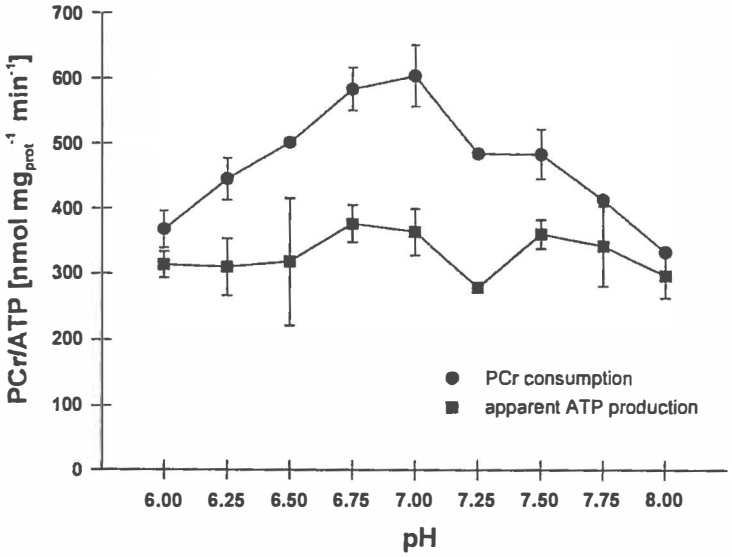


**Figure 3**
The pH dependency of direct ATP production (apparent ATP production; ■) and PCr consumption (●) measurements in creatine kinase reaction in a myofibrillar fraction (apparent ATP production is significantly lower; ANOVA $P < 0.01$; $n = 4$ for each point). Symbols and bars are means ± S.E.M.

## DISCUSSION

The energy source necessary for muscle contraction comes from two main metabolic processes: glycolysis and the tricarboxylic acid (TCA) cycle. Excess protons, formed as a by-product of glycolysis, have been implicated in intracellular pH change and the development of one form of muscle fatigue (Chase & Kushmerick, 1988; Cooke *et al.* 1988; Pate & Cooke, 1989; Allen *et al.* 1995). If the rate of pyruvate production (from glycolysis) exceeds the rate of its oxidation through the TCA cycle, the excess pyruvate is converted into lactic acid, which dissociates into lactate and $H^+$ at physiological pH. The transient alkalization due to proton consumption associated with net PCr hydrolysis after twitch contraction has also been detected in muscle fibres (Adams *et al.* 1990). Despite an effective muscle $pH_i$ regulation mechanism and buffer capacity (Aickin, 1986), the metabolic build-up of $H^+$ within the muscle lowers the pH and may reduce muscle force as well as result in dramatic reductions in the free energy of ATP hydrolysis.

A decrease in pH within the muscle cell due to anaerobic metabolism and the accumulation of lactic acid has been documented many times. Non-invasive NMR studies revealed different values of acidification; however, all results are within a pH range of 6.0–7.0 (Meynial-Denis *et al.* 1993; Mizuno *et al.* 1994; Damon *et al.* 2002). Iwanaga *et al.* (1991) described an even more significant pH decrease after the contraction of muscle using $^{31}$P-MRS spectra measurement. The activity of hydrogen ions is a parameter of fundamental importance in the regulation of cell metabolism. In cells that undergo significant changes of intracellular pH (e.g. muscle fibres), the behaviour of enzymes (and pathways) with expressive pH dependences within the physiological range of $pH_i$ should conform to their pH activity profiles. This is the case for such

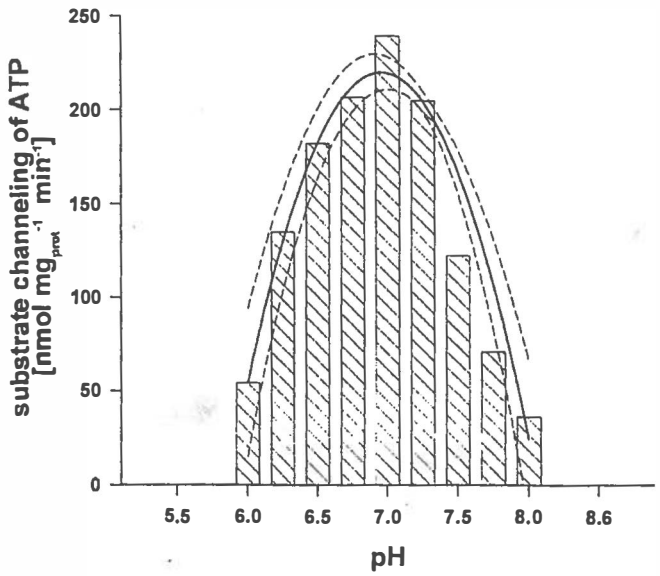


**Figure 4**
The effect of different pH levels on substrate channelling between myofibrillar CK and myosin ATPase. Substrate channelling was expressed as a difference in PCr consumption and apparent ATP production from Fig. 3. Columns represent means ($n = 4$), continuous line represents weighted least squares polynomial fit ($n = 36$) and dashed lines represent the 95 % confidence interval of the corresponding function (quadratic function reaching maximum at pH 6.95).

important pathways as glycolysis or protein synthesis (Busa, 1986). Although some recent findings cast doubt upon the role of acidification in the mechanism of fatigue (Wiseman *et al.* 1996; Saugen *et al.* 1997; Chin & Allen, 1998), changes in pH strongly influence all physiological processes in the muscle. Examples of processes influenced by changes in pH are the steady contractile properties of muscle, $Ca^{2+}$ release from the sarcoplasmic reticulum, $Ca^{2+}$ binding to troponin C and cross-bridge cycling (Allen *et al.* 1995; McComas, 1996).

The method of measuring direct ATP production and PCr consumption was successfully used for substrate channelling quantification (Gregor *et al.* 1999). Our former experiments estimate that more then 50 % of ATP produced in the CK reaction is channelled towards myosin ATPase at pH 7.5. The present results, achieved by a modified method for maximum CK activity evaluation, yielded 42.3 % as a maximal value of substrate channelling at pH 7.25. The latter estimated amount of channelled ATP corresponds better to physiological reality in the muscle cell.

The compartmentalization of cellular energy metabolism is ensured by the distribution of adenine nucleotides, as well as other substrates, between subcellular compartments surrounded by membranes. During the last decade a high degree of compartmentalization within the cytoplasm has been recognized, which is not defined by internal membranes. Extraorganellar substrate pool heterogeneity is due to the existence of different microcompartments without clear physical boundaries. One of the main effects of cytoplasmic microcompartmentalization is the possibility of a direct transfer of a substrate between the active sites of two enzymes that catalyse sequential reactions, without complete mixing with the rest of cytosol (Anderson, 1999). The phenomenon of substrate channelling has been widely reported for individual enzymes, within multi-enzyme complexes and whole metabolic pathways (Ovádi, 1995). The creatine kinase system participates in direct crosstalk between organelles through the compartmentalization of adenine nucleotides. The direct substrate channelling of ATP and ADP between mitochondria and the sarcoplasmic reticulum and between mitochondria and myofibrils has been described (Kaasik *et al.* 2001). Similarly, the direct transfer of ATP between CK and myosin ATPase takes place at the myofibrillar end of the creatine kinase system (Arrio-Dupont, 1988).

Because CK isoenzymes ensure the functional and dynamic compartmentalization of adenine nucleotides in tissues with high energy demands, substrate channelling at the myofibrillar end of the CK shuttle would undoubtedly play a key role in regulation and feedback control in energy metabolism in the muscle. The mechanism of coupling of two main enzymes – CK and myosin ATPase – by proton transfer along F-actin has already been proposed (Khan *et al.* 1989). The pH dependence of substrate channelling, presented in this work, emphasizes the tight cooperation of these enzymes. Similarly Harkema & Meyer (1997) described a role for pH decrease in the control of respiration and intramitochondrial potential for ATP synthesis in skeletal muscle, and CK regulation via the AMP-activated protein kinase pathway is also pH sensitive (Ponticos *et al.* 1998).

In the present work, the coupling between CK and myosin ATPase varied in dependence on pH. The pH dependence of ATP channelling was best approximated with a quadratic function, which reached a maximum value at pH 6.95. This pH value corresponds to the usual acidification range due to muscle contraction when intracellular pH in muscle falls from pH 7.2 at rest to pH 6.7 after exercise (Sullivan *et al.* 1994). One of the most physiologically important consequences of lactic acid accumulation and the consequent intracellular acidification in contracting muscle is a decrease in the free energy of ATP hydrolysis (GATP) (Combs & Ellington, 1995). This energetic state makes increased demands on the tight regulation of effective energy supply to the sites of its consumption. A decrease in intracellular pH in muscle participates in the activation of the AMP-activated protein kinase pathway (Ponticos *et al.* 1998). Not surprisingly, the pH optima for CK (pH 6.0 in the direction of ADP phosphorylation) and myosin ATPase (pH 5.9) also belong to the acidic range of pH (Kameyama *et al.* 1985; Wyss *et al.* 1992). It seems that, similar to interorganellar channelling (Kaasik *et al.* 2001), the ATP channelling between myofibrillar CK and myosin ATPase is a dynamic function, which could reach different values in dependence on temporal physiological conditions such as changes in the intracellular pH. The phenomenon of substrate channelling thus plays two physiological roles: it ensures a more efficient ATP supply at lower pH, which prevents optimal energy yield from ATP hydrolysis, and it ensures a unidirectional flux when the CK reaction is shifted out of equilibrium.

Adams GR, Foley JM & Meyer RA (1990). Muscle buffer capacity estimated from pH changes during rest-to-work transitions. *J Appl Physiol* 69, 968–972.

Aickin CC (1986). Intracellular pH regulation by vertebrae muscle. *Annu Rev Physiol* 48, 349–361.

Allen DG, Westerblad H & Lännergren J (1995). The role of intracellular acidosis in muscle fatigue. *Adv Exp Med Biol* 384, 57–68.

Anderson KS (1999). Fundamental mechanisms of substrate channeling. *Methods Enzymol* 308, 111–145.

Arrio-Dupont M (1988). An example of substrate channeling between co-imobilized enzymes. *FEBS Lett* 240, 181–185.

Busa WB (1986). Mechanisms and consequences of pH-mediated cell regulation. *Annu Rev Physiol* 48, 389–402.

Chase PB & Kushmerick MJ (1988). Effects of pH on contraction of rabbit fast and slow skeletal muscle fibers. *Biophys J* 53, 935–946.

Chin ER & Allen DG (1998). The contribution of pH-dependent mechanisms to fatigue at different intensities in mammalian single muscle fibers. *J Physiol* 512, 831–840.

Combs CA & Ellington WR (1995). Graded intracellular acidosis produces extensive and reversible reductions in the effective free energy change of ATP hydrolysis in a molluscan muscle. *J Comp Physiol* B 165, 203–212.

Cooke R, Franks K, Luciani GB & Pate E (1988). The inhibition of rabbit skeletal muscle contraction by hydrogen ions and phosphate. *J Physiol* 395, 77–97.

Damon BM, Gregory CD, Hall KL, Stark HJ, Gulani V & Dawson MJ (2002). Intracellular acidification and volume increases explain R(2) decreases in exercising muscle. *Magn Reson Med* 47, 14–23.

Gerhardt W (1983). Phosphotransferases: Creatine kinase. In *Methods of Enzymatic Analysis*, vol. III, 3rd edn, ed. Bergmeyer HU, pp. 508–518. Verlag Chemie, Weinheim.

Gregor M, Mejsnar J, Janovská A, Žurmanová J, Benada O & Mejsnarová B (1999). Creatine kinase reaction in skinned rat psoas muscle fibers and their myofibrils. *Physiol Res* 48, 27–35.

Harkema SJ & Meyer RA (1997). Effect of acidosis on control of respiration in skeletal muscle. *Am J Physiol* 272, C491–500.

Iwanaga K, Yoshimitsu H, Kamata T & Sairyo K (1991). 31P-MRS study of change in intracellular pH during sustained static contractions in human. *Ann Physiol Anthropol* 10, 83–90.

Kaasik A, Veksler V, Boehm E, Novotova M, Minajeva A & Ventura-Clapier R (2001). Energetic crosstalk between organelles. Architectual integration of energy production and utilization. *Circ Res* 89, 153–159.

Kameyama S, Ichikawa H, Sunaga Y, Nakata S, Saito Y, Eiki T & Watanabe S (1985). Biochemical characteristics of cardiac myosin: the pH dependence of Ca-ATPase activity, and that of the absorption spectrum of 2,4,6-trinitrophenyl groups attached to myosin. *J Biochem* 97, 625–632.

Khan LA, Raj M & Amin M (1989). Coupling of the enzyme activities of myosin ATPase and creatine kinase and its role in muscular contraction. *Indian J Biochem Biophys* 26, 148–152.

Kholodenko BN, Cascante M & Westerhoff HV (1994). Control theory of metabolic channelling. *Mol Cell Biochem* 133–134, 313–331.

Kholodenko BN & Westerhoff HV (1993). Metabolic channelling and control of the flux. *FEBS Lett* 320, 71–74.

Kushmerick MJ (1983). Skeletal muscle: Energetics of muscle contraction. In *Handbook of Physiology*, section 10, *Skeletal Muscle*, pp. 189–236. *Am Physiol Soc*, Bethesda.

Lowry OH, Rosebrough NJ, Farr AL & Randall RL (1951). Protein measurement with Folin phenol reagent. *J Biol Chem* 193, 265–275.

McComas AJ (1996). *Skeletal Muscle: Form and Function*. Human Kinetics Publishers, Champaign.

Maršík F & Mejsnar J (1994). The balance of entropy underlying muscle perfomance. *J Non-Equilib Thermodyn* 19, 197–216.

Mejsnar J, Kushmerick MJ & Williams DA (1992). Phosphocreatine and ATP concentration increase during flow stimulated metabolism in non-contracting muscle. *Experientia* 48, 1125–1127.

Mejsnar J, Sopko B & Gregor M (2002). Myofibrillar creatine kinase activity implied from the 3D model. *Physiol Res* 51, 35–41.

Meynial-Denis D, Mignon M, Foucat L, Bonnet Y, Bielicki G, Renou JP, Lacourt P, Lacourt A & Arnal M (1993). Use of superfused rat skeletal muscle for metabolic studies: assessment of pH by 31P n.m.r. *Biochem J* 293, 399–405.

Mizuno T, Takanashi Y, Yoshizaki K & Kondo M (1994). Fatigue and recovery of phosphorus metabolites and pH during stimulation of rat skeletal muscle: an evoked electromyography and *in vivo* 31P-nuclear magnetic resonance spectroscopy study. *Eur J Appl Physiol Occup Physiol* 69, 102–109.

Ovádi J (1995). *Cell Architecture and Metabolic Channeling*. Springer-Verlag, Heidelberg.

Pate E & Cooke R (1989). Addition of phosphate to active muscle fibers probes actomyosin states within the powerstroke. *Pflugers Arch* 414, 73–81.

Ponticos M, Lu QL, Morgan JE, Hardie DG, Partridge TA & Carling D (1998). Dual regulation of the AMP-activated protein kinase provides a novel mechanism for the control of creatine kinase in skeletal muscle. *EMBO J* 17, 1688–1699.

Saks VA, Ventura-Clapier R & Aliev MK (1996). Metabolic control and metabolic capacity: two aspects of creatine kinase functioning in the cell. *Biochim Biophys Acta* 1274, 81–88.

Saugen E, Vøllestad NK, Gibson H, Martin PA & Edwards RH (1997). Dissociation between metabolic and contractile responses during intermittent isometric exercise in man. *Exp Physiol* 82, 213–226.

Solaro RJ, Pang DC & Briggs FN (1971). The purification of cardiac myofibrils with Triton X-100. *Biochim Biophys Acta* 245, 259–262.

Sullivan MJ, Saltin B, Negro-Villar R, Duscha N, & Charles HC (1994). Skeletal muscle pH assessed by biochemical and 31P-MRS methods during exercise and recovery in men. *J Appl Physiol* 77, 2194–2200.

Trautschold I, Lamprecht W & Schweitzer G (1985). Nucleotides, coenzymes and related compounds: UV-method with hexokinase and glucose-6-phosphatase. In *Methods of Enzymatic Analysis*, vol. VII, 3rd edn, ed. Bergmeyer HU, pp. 346–357. Verlag Chemie, Weinheim.

Wahlefeld AW & Siedel J (1985). Metabolites 3: Lipids, amino acid-related compounds: creatinine and creatine. In *Methods of Enzymatic Analysis*, vol. VIII, 3rd edn, ed. Bergmeyer HU, pp. 488–507. Verlag Chemie, Weinheim.

Walliman T, Schlösser T & Eppenberger HM (1984). Function of M-line-bound creatine kinase as intramyofibrillar ATP regenerator at the receiving end of the phosphorylcreatine shuttle in muscle. *J Biol Chem* 259, 5238–5246.

Walliman T, Wyss M, Brdiczka D & Nicolay K (1992). Intracellular compartmentation, structure and function of creatine kinase isoenzymes in tissues with high and fluctuating energy demands: the 'phosphocreatine circuit' for cellular energy homeostasis. *Biophys J* 281, 21–40.

Wiseman RW, Beck TW & Chase PB (1996). Effect of intracellular pH on force development depends on temperature in intact skeletal muscle from mouse. *Am J Physiol* 271, C878–886.

Wyss M, Smeitink J, Wevers RA & Wallimann T (1992). Mitochondrial creatine kinase: a key enzyme of aerobic energy metabolism. *Biochim Biophys Acta* 1102, 119–166.

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**Substrate channelling in a creatine kinase system of rat skeletal muscle under various pH conditions**

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# Creatine kinase binds more firmly to the A-band of rabbit skeletal muscle myofibrils in the presence of its substrates

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*Running title*: Myofibrillar creatine kinase exchange

**Abstract**

Creatine kinase (CK) (E.C. 2.7.3.2) buffers cellular ATP concentration during fluctuating ATP turnover. Muscle cytosolic CK isoform interacts with various subcellular structures where it is functionally coupled with relevant ATPases. However, how this interaction, affects its activity is not known. We have therefore studied the interaction of CK with myofibrils and the role of different conformational states of CK molecule induced by ATP, phosphocreatine, ADP and the ATP-creatine pair. Purified rabbit psoas myofibrils with CK specific activity of 0.4±0.02 IU/mg were used. The exchange rates between the myofibrillar A-band and its surroundings were measured with fluorofore conjugated CK (IAF) by the Fluorescence Lost in Photobleaching (FLIP) method within a very narrow pH range 7.1-7.15. For CK-IAF without docked substrates, the time derivative of the initial loss of the fluorescent signal within the A-band equalled -3.26 at the fifth second and the decrease reached 82% by the 67$^{th}$ second. For CK-IAF with added substrates, the derivatives fell into the range of -0.95 to -1.30, with respective decreases from 16 to 46% at the 67$^{th}$ second. The results show that the substrates slowed down the exchange rate. This indicates that the strength of the bond between CK and the A-band of myofibrils increased.

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## Introduction

The creatine kinase/phosphocreatine system plays an important role in muscle energetic metabolism. It is well compartmentalized and is a very efficient system of maintaining optimal ATP/ADP ratio during muscle contraction; for review see [ 1, 2]. The control of creatine kinase (CK) activity is realized by conformational changes of the CK molecule that are closely related to changes in the Gibbs free energy due to the concentrations of the substrates. However, the force field computation for three energetic states (the substrate-free CK molecule, the molecule conjugated with the MgATP complex, and the pair MgATP-creatine) revealed an inactive "open", reactive "closed", and a non-reactive "intermediary" conformation respectively [3]. The data have been confirmed experimentally by measuring anisotropies, lifetimes of the fluorescence by intrinsic tryptophans, and the radii of CK molecule gyration determined by the respective rotational correlation times [4]. These results propose the existence of an essential cellular component of the CK activity control, consisting of CK interaction with a given subcellular structures, (reviewed by Wallimann *et al.* [5]) and resulting in the conformational shift to the reactive "closed" form of the CK molecule.

The unique interaction with myofibrils involves two parts, namely the binding of the CK molecule to the myofibrillar M-band and the exchange of CK between the M-band and its cytosol surroundings, the both of which are still poorly understood. According to Stolz and Wallimann [6] the N-terminal region of MM-CK mediates its specific isoform interaction with sarcomeric M-bands. Two pairs of key amino acid residues at this region were determined on the dimeric MM-CK molecule. The first is represented by highly conserved lysine residues (K104, K115) which creates a strong binding site; the second (K8, K24) is a weak interacting site [7]. The specific bonding partners of MM-CK in the M-band were identified as myomesin and M-protein [8].

The labelled MM-CK binding with rather high affinity to the M-band during equilibration can be replaced rather rapidly by unlabelled enzyme from its surroundings [9]. In other words, from a thermodynamic point of view, the M-band – CK bond is at its physiological role in the stationary state, which is maintained by the exchange of CK between myofibrils and cytosol. The finding has two consequences. The first, under definite conditions, the exchange rate between myofibrils and their surroundings should indicate – in reciprocal proportion – the force that holds CK and M-band proteins together. The second, CK (with its docked substrates) has to be kinetically different from experimentally prepared CK (substrates free), in order to maintain the stationary state [10]. Furthermore, for the physiological role, the strength of the CK (with substrates) – M-band bond has to be stronger than the bond with the

CK (substrates free) – M-band, indicated by their exchange rates. Thus the experimental question arises how the interaction of the CK with myofibril is influenced by a conformational state of CK molecule, evoked by its binding with substrates.

Results of the present study, were obtained by confocal microscopy FLIP measurement, and the resultes show the stronger bond of the CK (with substrates) during its specific interaction with myofibrils in the A-band. In the stationary state, its exchange rate with the surroundings in real time is slower than in comparison with the exchange rate of the CK (substrates free) and with the rates for non-specifically bound CK.

## Materials and Methods

*Chemicals*

Creatine kinase from rabbit muscle (CK) was obtained from Roche Diagnostic GmbH, as well as all enzymes and substrates for enzyme coupled assays. Fluorescent probes and conjugates were from Molecular Probes (U.S.A), DC Protein assay and SDS-PAGE molecular weight standard from Bio-Rad Laboratories Inc.; all other chemicals were from Sigma-Aldrich.

Rabbit creatine kinase from skeletal muscle was conjugated with 5iodoacetamidofluorescein (IAF) as described previously by Gregor *et al.* [11].

*Solutions*

A) *no calcium isotonic solution* (mM): NaCl 121, fresh protease inhibitors – Phenylmethylsulphonylfluoride (PMSF) 0.1, $NaN_3$ 3 and Ethylene glycol-bis(beta-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) 1; EDTA 5, 1,4 Dithio-DL-threitol (DTT) 0.2; pH 7.0. B) *low ionic strength medium* (mM): 3-morpholinopropanesulfonic acid (MOPS) 20, Manitol 11, EDTA 4, DTT 5; pH 7.1. C) *relaxing buffer* (mM): HEPES 20, $MgCl_2$ 1.2, EGTA 5, EDTA 1, DTT 2, PMSF 0.1, $NaN_3$ 3, potassium propionate 100 and Di(Adenosin)Pentaphosphate ($Ap_5A$) - adenylate kinase inhibitor - 0.2 at pH 7.1.

*Isolation of myofibrils*

The experiments were performed in accordance with local ethical committee guidelines (Czech Ministry of Agriculture, No. 1020/437/A/99). Eight rabbits was used for a whole study.

Muscle tissue was obtained from a half year old male rabbit Chinchila weighing 2.8-3.2kg. The rabbit was killed by rapid cervical dislocation, and bleed. Left and right psoas muscles were isolated and immediately immersed into low $Ca^{2+}$ isotonic solution A and placed at room temperature. The muscles were cut into small pieces and transferred to solution A free of NaCl containing 100 mM KCl and 50% glycerol [12]. After 60 min glycerol penetration into the pieces of muscle the samples could be stored at –20°C for up to 2 months. Isolation of myofibril was performed by homogenisation at 4°C using solution A free of NaCl containing 100 mM KCl. Sample was twice homogenised by rotation homogenizer and centrifuged at 1000 g. After 20 min incubation of sediment on ice this washing cycle was repeated five times using glass homogenizer with teflon plunger [12,13]. The myofibrillar fraction was then incubated with 1% Triton X-100 (vol/vol) for 5 min and then seven washing cycles were repeated. We made following modification: $MgCl_2$ (2.5 mM) was added into medium from the third washing cycle in order to prevent the degradation of actin filaments.

Two sorts of myofibrils were used for the FLIP experiment: "*intact*" and "*treated*". In the case of "*intact*" myofibrils, the exchange of naturally bound CK in the A-band with external fluorescently labelled CK by 5-iodoacetamidfluorescein (CK-IAF) was measured directly after isolation. In the second case, *treated* myofibrils were incubated in a low ionic strength medium B for 15 minutes which removed the native CK molecules from the M-band. Subsequent reconstitution of the native CK by the external CK-IAF was performed according to Ventura-Clapier *et al.* [14]. This is stated in the Exchange experiments chapter.

A protein profile, obtained by elution using low ionic strength medium, was detected by SDS-PAGE electrophoresis (acryl amid gel 5% for stacking and 12% running gel, 210V, 90 min., Mini Protean 3-Bio Rad, Stained by silver according to Blum *et al.* [15].

Lengths of sarcomers were measured using the quantification mode of Leica Confocal Software (LCS Lite)

The protein concentration of myofibrils was determined by DC Protein Assay with bovine serum albumin as a standard.

*Exchange experiments*

The replacement of naturally bound CK in the A-band of sarcomere by external fluorescently labelled CK-IAF was performed according to Kraft *et al* [9] as follows. Purified myofibrils, in a concentration of 8-12 mg protein/ml, were incubated in a total volume of 100 µl of relaxing solution C with 2.5 µl of CK-IAF (5 mg protein / ml) added on a cover glass at 20°C for 10 minutes. This experiment was carried out either with or without CK substrates, adding each

one individually, and for ATP-creatine pair at resting physiological concentrations (5 mM ATP, 12 mM phosphocreatine, 8 mM creatine and 0.62 μM ADP) [16].

Substrates' presence in freshly purified myofibrils was tested, using enzymatic-coupled assays [17 , 18].

Myofibrils stained by conjugate Phalloidin-Alexa 633 were used for determining the location of CK-IAF.

*FLIP measurements and data evaluation*

The exchange interactions have been studied mainly by confocal microscopy, using the Fluorescence Lost in Photobleaching (FLIP) method according to scheme (Fig.1). The myofibrils equilibrated with CK-IAF were surrounded by a bleached region. The loss in the fluorescent signal in the central region of interest is due to the fact that, before the CK-IAF molecules reach the A-band sites of interaction, they have to pass the bleaching region. Therefore we evaluate the exchange between the fluorescent and the bleached CK-IAF molecules in the central region as the loss in the fluorescent signal in time (Fig. 2).

FLIP measurements were performed on a confocal microscope (Leica SP2 AOBS) with 63×/ 1.2 NA water-immersion objective operating with an Argon laser tuned to 488 nm (10 mW) to excite the IAF dye. Bleaching was performed in a closed band around the myofibril with a 488 nm line operating at 25% laser power. Fluorescence loss was monitored at a low laser intensity (at 5% laser power) in 1.686 sec intervals for 40 frames with a total acquisition time of 67 sec. The exchange of CK-IAF between the myofibrils and its surroundings was measured in a stationary state by evaluating the loss of the fluorescent signal in myofibrils. Myofibrils labelled under the same condition on actin filaments by Phalloidin-FITC conjugate were used for the bleaching control.

FLIP measurements were carried out in different substrate conditions, and repeated 5 to 20 times to aid the statistical evaluation. Data of fluorescent intensity decay obtained from A-bands for each group were averaged and normalized to 100% at the start of FLIP measurement. The experimental data of fluorescence decay were fitted to two-exponential curves. All the exchange rates presented in Fig. 5 and Fig. 6 are characterized for mutual comparison by two values: the derivative at the fifth second, and the percentage decrease of the fluorescence at the end of the experiment (67 seconds). The absolute value of the derivative, as well as the decrease, is proportional to the respective exchange rate.

The intensities of the signals are expressed as means ± S.E.M. "n" means number of myofibrillar preparations. The statistical evaluation was carried out by One Way Analysis of

Variance (ANOVA), nonparametric tests of Kruskal-Wallis, Mann-Whitney-U, Kolmogorov-Smirnov-D, and unpaired-t tests in five time intervals: 5, 10, 20, 40 and 60 sec after the start of the FLIP measurement. The differences are accepted for $P < 0.05$ (using the Bonferroni multiplex comparison method).

## Results

*Characterization of myofibrils*

Myofibrils were standardized for exchange rate measurements in the following three steps. First, in order to study the effects of substrates for muscle creatine kinase (CK) interaction in the A-band, their absence was verified after the isolation procedure. The enzyme assays confirmed the absence of ATP, ADP, phosphocreatine and creatine in both preparations of myofibrils (see Methods). Second, the ability of myofibrils to contract was proved by the length of sarcomers. Relaxed myofibrils had a resting length of 2.1±0.20 μm and after the contraction in presence of 5 mM ATP and 7 mM $CaCl_2$ they shortened to the length of 1.6±0.12 μm. Third, creatine kinase specific activity was determined for "*intact*" and "*treated*" myofibrils as 0.4±0.02 IU/mg protein and 0.01±0.008 IU/mg protein, respectively.

The SDS-PAGE protein profile (Fig. 3) shows that the treatment of myofibrils with a low ionic strength medium lead to elution of not only the CK (43 kD), but also eluted proteins with a higher molecular weight of approximately 120, 80 and 70 kD and at a lower molecular weight of 43 kD (Fig. 3). The elution of proteins significantly changed the behaviour of myofibrils in the exchange experiments. The FLIP measurements revealed irreversible binding of CK-IAF (substrates free) in contrast to the "*intact*" myofibrils. "*Treated*" myofibrils were excluded from further exchange measurements.

*Myofibrillar CK-IAF binding*

Location of CK-IAF binding in the A-band was confirmed by confocal microscopy. Staining actin filaments within the I-bands, in red, and labelling CK-IAF, in green, within myofibrils (Fig. 4a) showed alternating green and red stripes, confirming the place of bound CK-IAF between two I-bands.

Unspecific surface adhesion of CK-IAF molecules and their axial distribution within the lumen of myofibrils (which could affect the measurements of the exchange rate) were excluded by xyz projection with subsequent 3D reconstruction from the data. The resulting

visualization of the A-band by confocal microscopy showed "discs" of CK-IAF molecules, associated in the A-bands and without penetration in the axial direction (Fig. 4b). This result allowed us to study the real time exchange of CK-IAF molecules in the A-band fluorescence by the FLIP method just in the two-coordinate planar system x-y with respect to time.

*FLIP measurements evaluation*

Results presented in Fig. 5 show weak and specific interaction of substrate free CK-IAF within the A-band which differs from the control. Conjugate Phalloidin FITC tightly bound in the I-band was used as a control for CK-IAF bleaching in the A-band at the $67^{th}$ sec of the FLIP and the derivative (see Methods) equalled -0.96 and percentage a decrease of 40%. These two characterizing values are compared with fluorescence decays of CK-IAF (substrates free) molecules in the A-band, the I-band and close surroundings. The CK-IAF (substrates free) fluorescence decay in the A-band is characterized by the derivative -3.08, and the 80% decrease is significantly different ($P<0.05$) from Phalloidin-FITC bleaching control values. The fluorescence intensities in the I-band and close surroundings are very low and identical.

CK-IAF (with substrates) molecules demonstrate very slow fluorescence decay during the experiment, presented in Fig. 6. The decay is not significantly different from the bleaching control, nevertheless it is different ($P<0.05$) from the signal of CK-IAF (substrates free) molecules. The distinctive two values derivative/decrease for CK-IAF associated with the respective substrates are: ATP: -1.30 / 16%, phosphocreatine: -1.10 / 46%, ATP-creatine pair: -0.95/ 33%. The respective data for ADP (values are not shown in Fig. 6) equal 0.98 / 25%. There are not any significant differences among the substrates variants. The derivatives in this way fall within the range of -0.95 to -1.30 with the respective decreases ranging from 16% to 46%. The CK-IAF (substrates free) –3.26 / 82% were repeatedly measured for comparison, and both values which characterize the decay in Fig. 6 were close to each other.

*pH dependence of CK-IAF interaction in the A-band*

The value of pH has a strong influence on the CK-IAF interaction with the myofibrils. We did not observe a clear fluorescence signal in the A-band during the exchange experiment in acid pH (pH 6.8 to 6.9). In neutral pH, the exchange reaction occurs and reaches its maximum in a basic environment of pH 7.10-7.15. Within this pH range all the following FLIP experiments were performed. The fluorescent signal in the A-band was weak or absent at pH ≥7.2.

## Discussion

It is a problem of general interest whether the interaction of enzymes with structural proteins related to their intracellular compartmentalization plays, in general, a role in the cellular control of their activity. The essential physiological component (proposed in the Introduction of this paper) which controls muscle CK activity turns the attention toward the nature of the interaction of CK with myofibrils.

Myofibrils can be considered as the simplest structure that still preserves a cellular function (contraction). It simultaneously enables access to the molecular level of CK-myofibrillar interaction which could affect the enzyme activity. For such an interaction the following are decisive: an exchange of CK between the respective cellular compartments, and the bond that holds CK and A-band-proteins together. Both of which are within the scope of this paper.

### *Myofibrils as an experimental object*

Purified myofibrils reserve its physiological function, which is proved by its ability to contract, and CK activity (0.4±0.02 IU/mg) correspond to Ventura-Clapier *et al.* [14] and agrees with our previous findings [13].

Elution of CK from the myofibril (treated myofibrils) by low ionic strength medium was effective and decreased the specific CK activity by up to 0.01 IU/mg protein. The aim of elution was to obtain a simpler kinetic model. However, the FLIP experiment showed that reconstituted CK-IAF bound in A-band is fixed. And on the base of analyses of eluted proteins, the treated myofibrils were excluded from FLIP measurements. These SDS-PAGE results showed a loss of other proteins from myofibrils besides that of CK (43 kD ) (see Fig. 3). Namely actin (upper 43 kD band) together with CK (lower 43 kD band) was proved by mass spectroscopy (data not shown). Some other higher molecular weight (Mw) proteins about 70, 80 and 120 kD were detected by SDS-PAGE. At lower Mw two main bands about 30 and 20 kD appeared. 30 kD protein could be DRAL/FHL-2, which mediates targeting of CK with titin [19, 20]. Fig. 3 shows that a low ionic strength medium could harm the structure of sarcomers in the case of smaller proteins. This way could uncover some unspecific interaction for CK in the A-band. These results could also show that the smaller proteins are indispensable for

natural interaction of CK in the A-band as well as its main binding partners like myomesin (185 kD) and M-protein (165 kD) [8].

*pH dependence of CK-IAF interaction in A bands of myofibrils*

Results show a strong pH dependence of the CK interaction with myofibrils in the A-band. In a slightly acidic environment the bond of the native CK is so strong that it excludes the CK exchange between myofibrils and their surroundings. These findings correspond to a recent observation of a strong pH effect on the binding strength between CK and myomesin [8]. This fact can be hypothetically explained by the physiological function of CK, as a pH buffer [21] and ATP buffer, within the range of intracellular pH changes that occur upon muscle contraction [22]. Also energy channelling between creatine kinase and myosin ATPase, reaching its maximum at pH 6.95 [13], could help to explain the importance of a strong CK bond in the A-band during acidification. The exchange process reaches its maximum rate in a slightly basic environment (7.10 – 7.17), which is in accordance with previous data [14] that show the reconstitution of CK bound in the M-band at pH 7.1. A weaker interaction of CK could be also explained by its isoelectric points at a slightly basic pH [23, 24]. On the other hand Kraft *et al.* [9] used neutral pH for the exchange of CK molecules at skinned fibres. At this pH we observed only partial and slow exchange of CK-IAF molecules in the A-band.

*The role of substrates*

Results presented in Fig. 4a, b justify the FLIP method employed in the two coordinate planar system. Resulting data (Fig. 5 and Fig. 6) allow us to evaluate the exchange of CK, or reciprocally, to estimate the force of the bond. Under a slightly basic (pH 7.10 – 7.17) condition the exchange and equilibration between CK-IAF (substrates free) and the natively bound CK in the A-band is very fast (in seconds) (Fig. 5). The exchange rate in the presence of substrates is more than sixty times slower (in minutes) during equilibration. The FLIP experiments show, how the conformation change of the CK molecule, due to substrates binding, affects the process of exchange between the A-band and surroundings. This result indicates a stronger bond due to the presence of substrates. The ATP induced autophosphorylation of muscle CK [25] and nucleotidylation [26] can influence the interaction

with myofibrils, as well as the conformational change due to other substrates' binding [27, 3, 4].

In summary, the results show that within a narrow pH range the substrates slowed down the exchange rate of the CK molecule. This indicates that the strength of bond between the CK and A-band increased.

## Acknowledgements

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## References

1. Saks VA, Ventura-Clapier R, Aliev MK (1996) Metabolic control and metabolic capacity: two aspects of creatine kinase functioning in the cell. Biochim Biophys Acta 1274:81–88

2. Ventura-Clapier R, Veksler J, Hoerter JA (1994) Myofibrillar creatine kinase and cardiac contraction. Mol Cell Biochem 133/134:125-44.

3. Mejsnar JA, Sopko B, Gregor M (2002) Myofibrillar creatine kinase activity inferred from a 3D model. Physiol Res 51(1):35-41

4. Mejsnar JA, Herman P, Malacova D et al (2005) The substrate-dependent three conformations of muscle creatine kinase. Physiol Res 54:33P

5. Wallimann T, Wyss M, Brdiczka D et al (1992) Intracellular compartmentation, structure and function of creatine kinase isoenzymes in tissues with high and fluctuating energy demands: the 'phosphocreatine circuit' for cellular energy homeostasis. Biochem J 281(1):21-40

6. Stolz MT, Wallimann T (1998) Myofibrillar interaction of cytosolic creatine kinase (CK) isoenzymes: allocation of N-terminal binding epitope in MM-CK and BB-CK. J Cell Sci 111(9):1207-1216

7. Hornemann T, Stolz M, Wallimann T (2000) Isoenzyme-specific interaction of muscle-type creatine kinase with the sarcomeric M-line is mediated by NH(2)-terminal lysine charge-clamps. J Cell Biol 149(6):1225-1234

8. Hornemann T, Kempa S, Himmel M et al (2003) Muscle-type creatine kinase interacts with central domains of the M-band proteins myomesin and M-protein. J Mol Biol 332(4):877-887

9. Kraft T, Messerli M, Rothen-Rutishauser B et al (1995) Equilibration and exchange of fluorescently labeled molecules in skinned skeletal muscle fibers visualized by confocal microscopy. Biophys J 69(4):1246-1258

10. Prigogine I (1967) Introduction to Thermodynamics of Irreversible Processes. Interscience Publishers, New York, London, Sydney, pp 83-85

11. Gregor M, Kubala M, Amler E et al (2003) Frequency-Domain Lifetime Fluorometry of Double-Labeled Creatine Kinase. Physiol Res 52(5):579-585

12. Wallimann T, Schlosser T, Eppenberger HM (1984) Function of M-line-bound creatine kinase as intramyofibrillar ATP regenerator at the receiving end of the phosphorylcreatine shuttle in muscle. J Biol Chem 259(8):5238-5246

13. Gregor M, Janovska A, Stefl B et al (2003) Substrate channelling in a creatine kinase system of rat skeletal muscle under various pH conditions. Exp Physiol 88(1):1-6

14. Ventura-Clapier R, Saks VA, Vassort G et al (1987) Reversible MM-creatine kinase binding to cardiac myofibrils. Am J Physiol Cell Physiol 253:C444-C455

15. Blum M, Beier H, Gross MJ (1987) Improved silver staining of plan proteins, RNA and DNA in polyacrylamide gels. Electrophoresis 8(2):93-99

16. Stefl B, Mejsnar JA, Janovska A (1999) Energy metabolism of rat skeletal muscle modulated by the rate of perfusion flow. Exp Physiol 84:651-663

17. Gerhardt W (1983) Phosphotransferases: Creatine kinase. In: Bergmeyer HU (ed) Methods of Enzymatic Analysis, vol. III 3rd edn, Verlag Chemie, Weinheim, pp508-518

18. Wahlefeld AW, Siedel J (1985) Metabolites 3: Lipids, amino acid related compounds: creatinine and creatine. In: Bergmeyer HU (ed) Methods of Enzymatic Analysis, vol. VIII. 3rd edn, Verlag Chemie, Weinheim, pp488-507

19. Lange S, Auerbach D, McLoughlin P et al (2002) Subcellular targeting of metabolic enzymes to titin in heart muscle may be mediated by DRAL/FHL-2. J Cell Sci 115(24):4925-4936

20. Lange S, Agarkova D, Perriard JC et al (2005) The sarcomeric M-band during development and disease. J Muscle Res Cell Motil 26(6-8):375-379

21. Adams GR, Foley JM, Meyer RA (1990) Muscle buffer capacity estimated from pH changes during rest-to-work transitions. J Appl Physiol 69:968–972

22. Chase PB, Kushmerick MJ (1988) Effect of pH on contraction of rabbit fast and slow skeletal muscle fibers. Biophys J 53:935-946

23. Malacova D, Zurmanova J, Mejsnar J (2004) Three isoelectric points of the creatine kinase M-subunit, purified from myofibrils. Physiol Res 53:22P

24. Wright-Weber B, Held BC, Brown A et al (2006) Immunological and physical comparison of monomeric and dimeric phosphagen kinases: Some evolutionary implications. Biochim Biophys Acta 1760(3):364-71

25. Stolz M, Hornemann T, Schlattner et al (2002) Mutation of conserved active-site threonine residues in creatine kinase affects autophosphorylation and enzyme kinetics. Biochem J 363: 785-792

26. Satyajit SM, Boyd D, Haley E (1999). ATP Nucleotidylation of creatine kinase. Biochemistry 38:8492-8500

27. Lahiri SD, Wang PF, Babbitt PC et al (2002) The 2.1 Å structure of Torpedo californica creatine kinase complexed with the ADP-$Mg^{2+}$-$NO_3$-creatine transition-state analogue complex. Biochemistry 41(47):13861–13867

**Figure Legends**

*Fig. 1.* FLIP measurement. CK-IAF molecules bound into A-bands of myofibril after equilibration were surrounded by a bleached region (B). The loss in the fluorescent signal in the central region of interest (ROI) is due to the fact that, before the CK-IAF molecules from (O) outer surroundings of myofibril reach the A-band sites of interaction, they have to pass the bleaching region (B). The loss in the fluorescent signal shown in time sequence *Fig. 2* corresponds to the exchange rate between the fluorescent and the bleached CK-IAF molecules in the central region in A-bands during the FLIP measurement.

*Fig. 3.* Protein profiles of intact myofibrils (MF) and supernatant eluted from myofibrils using low ionic strength medium (E) are placed in the center. Molecular weight markers are placed on the left (high range Mw) and right (low Mw) side.

*Fig. 4.* Location of CK-IAF in the A-band of myofibriles visualized by confocal microscopy. a) Staining of actin filaments within the I-bands by Phalloidin Alexa 633 and CK-IAF marked in green. b) 3D reconstruction of CK-IAF associated in the A-band of myofibrils, without its penetration in the axial direction.

*Fig. 5.* Fluorescent intensities decrease from two compartments, which represent the respective rates of labelled molecules movement, evaluated in time by FLIP. The values expressed as means ±S.E.M. (n=6, for each point, number of myofibrillar preparations) represent: Conjugate Phalloidin-FITC tightly bound in the I-band as bleaching control value (▲), CK-IAF (substrates free) associated in the A-band (○). The movment of CK-IAF (substrates free) molecules is significantly different from the control ($P<0.05$).

*Fig. 6.* Fluorescent intensities decrease from the A- bands of myofibrils incubated with CK substrates, which represent the rate of labelled CK-IAF molecules movement, evaluated in time by FLIP. The values expressed as means ±S.E.M. (n=6, for each point, number of myofibrillar preparations) represent: CK-IAF - (ATP-Creatine pair) (▲), CK-IAF-ATP (◇), CK-IAF-Phosphocreatine (▽) and CK-IAF (substrates free) (○). The movement of CK-IAF molecules with substrates do not differ from the control ($P<0.05$) and CK-IAF (substrates free) differ from all the cases of substrate presence ($P<0.05$).

## Figures

Figure1

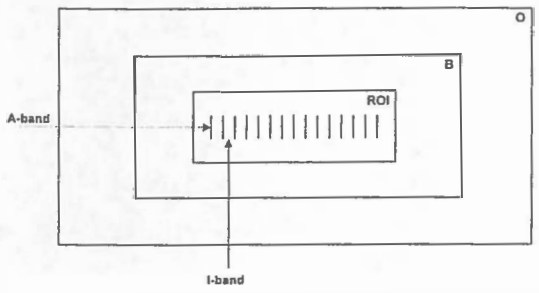



Figure 2

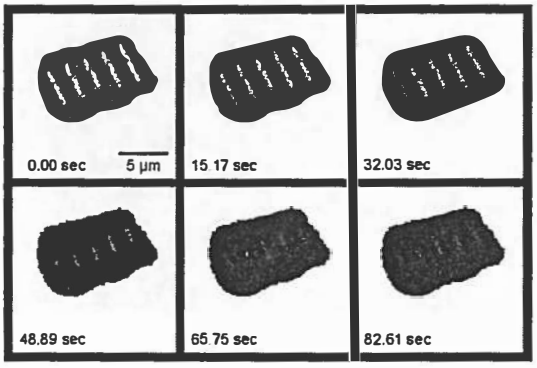



Figure 3

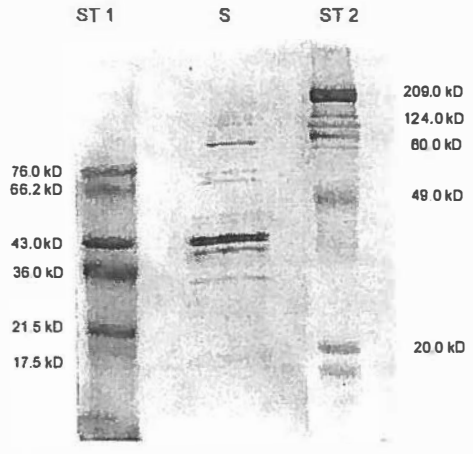


Figure 4a

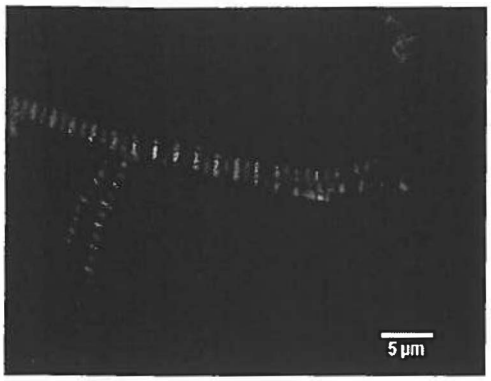



Figure 4b

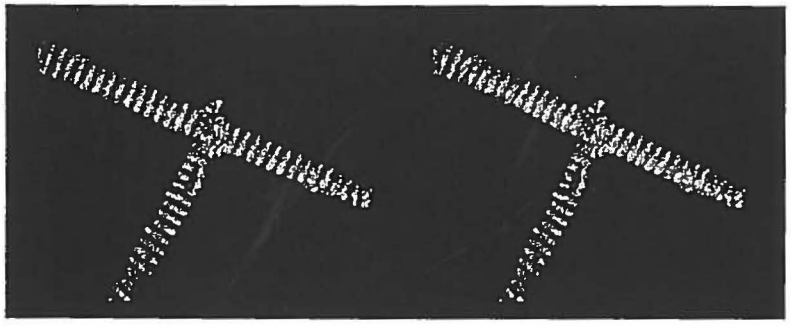


Figure 5

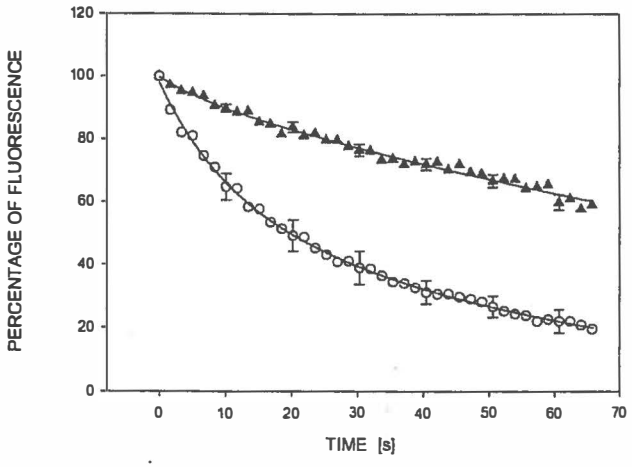
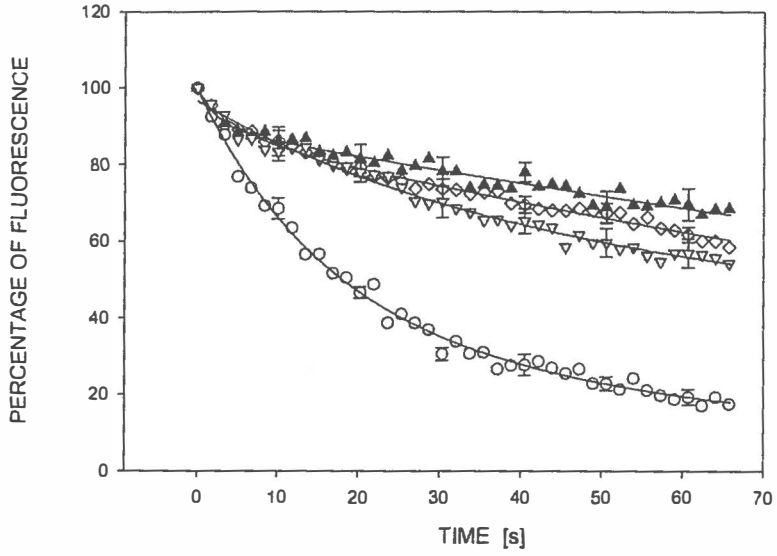


Figure 6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