Physiol. Res. 52: 579-585, 2003

Frequency-Domain Lifetime Fluorometry of Double-Labeled Creatine Kinase

M. GREGOR[1], M. KUBALA[2,3], E. AMLER[3], J. MEJSNAR[1]

[1]Department of Physiology and Developmental Biology, Faculty of Science, [2]Institute of Physics, Charles University, [3]Institute of Physiology, Czech Academy of Sciences, Prague, Czech Republic

Received July 31, 2002
Accepted November 1, 2002

Summary

Myofibril-bound creatine kinase EC 2.7.3.2 (CK), a key enzyme of muscle energy metabolism, has been selected for studies of conformational changes that underlie the cellular control of enzyme activity. For fluorescence spectroscopy measurements, the CK molecule was double-labeled with IAF (5-iodoacetamidofluorescein) and ErITC (erythrosin 5'-isothiocyanate). Measurement of fluorescence resonance energy transfer (FRET) from fluorescein to erythrosin was used to obtain information about the donor-acceptor pair distance. Frequency-domain lifetime measurements evaluate the donor-acceptor distance in the native CK molecule as 7.8 nm. The Förster radius equals 5.3 nm with the resolution range from 0.2 to 1.0 nm. Erythrosin-fluorescein labeling (EFL) was tested for artificial conformational changes of the CK molecule with high-salt concentration treatment. The transition distance, defined by His-97 and Cys-283 and derived from a 3D model equals 0.766 nm for the open (inactive) form and 0.277 nm for the closed (reactive) form of the CK molecule. In this way, the resolution range of the used spectroscopy method is significant, concerning the difference of 0.489 nm. Nevertheless, the CK enzyme activity, assessed by the hexokinase-coupled assay, was diminished down to 1 % of the activity of the native enzyme. EFL is suitable for description of conformational behavior implied from the regulation of creatine kinase. However, the observed inhibition restricts EFL to studies of conformational changes during natural catalytic activity.

Key words

Creatine kinase molecule • Conformational change • FRET • Frequency-domain lifetime fluorometry

Introduction

Creatine kinase is a key enzyme of muscle metabolism and the creatine kinase/phosphocreatine system plays a complex role in cell energy metabolism (Walliman *et al.* 1992, Saks *et al.* 1996). Different isoenzymes of CK ensure effective energy transport from the sites of production (mitochondria, glycolysis) to the sites of utilization (kinases, ATPases). The myofibril-bound CK izoenzyme is localized at the sarcomeric M-band, where it is functionally coupled to the myosin ATPase. Through Lohmann's reaction (Lohmann 1934):

$$Mg\ ADP + PCr \rightarrow Mg\ ATP + Cr$$

the creatine kinase continuously and efficiently replenishes ATP utilized during muscle contraction, as well as recuperates phosphocreatine.

Muscle creatine kinase is a homodimeric enzyme, consisting of two identical and freely dissociable

Fax +420 241 062 164
E-mail: physres@biomed.cas.cz http://www.biomed.cas.cz/physiolres

M subunits. Each subunit (with molar mass of 43 kDa) contains one active-site domain. Although much is known about the kinetics and enzymology, the three-dimensional structure of the active site of CK has only recently been determined (Fritz-Wolf *et al.* 1996). Extensive investigation has been carried out to understand the mechanism of action of CK and several amino acid residues involved in the catalytic mechanism or in the binding site have been identified. Among them, the highly reactive Cys-283 was found to play a significant role during catalysis, although the mechanism of its action remains unclear.

The control of creatine kinase activity is affected by conformational changes of the CK molecule, in addition to slight changes in the concentration of the substrates in the reaction, which is thought to be at or near equilibrium in the muscle cell (Kushmerick 1983). Former studies revealed at least two main conformational states of CK, a switch between them was induced by substrate binding. Without its substrates, the enzyme molecule assumes an inactive "open" form. According to Forstner *et al.* (1998) and Raimbault *et al.* (1996) the CK molecule upon binding of the MgATP or MgADP complex takes up an exclusively reactive "closed" conformation and no other substrate can induce conformational change. Recent results indicated that the protein secondary structure remained relatively stable during nucleotide binding (Granjon *et al.* 2001).

The 3D model of myofibrillar CK (Mejsnar *et al.* 2002) was constructed from the coordinates of mitochondrial CK for further quantitative measurements of the conformational changes with the derived consequences for its activity. The existence of a third, intermediate, conformation indicates that the substrate-induced energy minimizing conformational changes does not represent a sufficient condition for CK activity and that some other controlling component is involved at the cellular level. In order to describe the physicochemical behavior of the creatine kinase molecule, we prepared fluorescently labeled CK. *In vitro* fluorescence spectroscopy evaluates the methods and provides good prospects for future conformational measurements in intact muscle fibers.

Methods

Rabbit creatine kinase (Roche Diagnostics GmbH, 50 μM) was conjugated with 100 μM IAF (5-iodoacetamidofluorescein, Molecular Probes) in imidazole buffer (50 mM imidazole, 20 mM KCl, pH 7.5). The conjugation was terminated after 2 h. by addition of a 10-fold molar excess of DTT. Labeled CK was separated from the free dye and DTT in a homemade Eppendorf column (9x250 mm) packed with Sephadex G-25 equilibrated in 20 mM Tris buffer (pH 9.0). The CK-IAF was then concentrated by ultrafiltration (Centricon, molar mass cut-off 30 kDa) at 25 μM and stock solution of ErITC (Molecular Probes) was added to the final concentration of 35 μM. The reaction was terminated after 2 h by freshly prepared 1.5 M hydroxylamine (pH 8.5). Non-bound ErITC was removed by gel filtration (homemade Eppendorf column, 9x250 mm, Sephadex G-25) and the sample was concentrated using Centricon ultrafiltration in 20 mM Tris buffer (pH 7.5).

The degree of labeling was determined from absorption measurements at 280, 500 and 540 nm. Corresponding extinction coefficients were calculated by comparison with normalized spectra provided by the manufacturer (Molecular Probes). The protein extinction coefficient was estimated spectrophotometrically and the ratio of labeling was calculated according to the Lambert-Bear law. Protein integrity and purity of double-labeled CK was confirmed by SDS electrophoresis.

Using cyanogen bromide and trypsin cleavage (Webb *et al.* 1997) and HPLC separation of protein fragments, the labeling stoichiometry calculated from absorbance was corroborated. Cleavage mixture was applied on the reverse phase HPLC column (4.6x25 mm, Supercosil LC-304) and separated using gradient elution (30 min from 1 % to 30 % acetonitrile at flow rate 1 ml/min). Protein fractions with IAF and ErITC were detected using the fluorescence detector at 485/515 nm and 540/555 nm, respectively.

The specific activity of labeled CK was directly measured using a coupled assay system for ATP determination consisting of hexokinase and glucose-6-phosphate dehydrogenase which resulted in the formation of NADPH (Rosalki 1967, Gregor *et al.* 1999).

For fluorescence experiments, labeled CK (final concentration 3 μM) was incubated with various salt concentrations for one night at 4 °C in 10 mM Tris-acetate buffer (pH 8.0), in order to test this labeling for artificial conformational changes.

Lifetime measurements were performed in the phase-domain setup using spectrofluorometer K2 (ISS). Excitation wavelength was 490 nm, emission was sampled through a 538-559 nm filter. Excitation bandpass was 16 nm. Samples were dissolved in 10 mM Tris buffer (pH 8) at room temperature. Data were analyzed by the least-squares method.

Creatine kinase was labeled with IAF only and the sample was compared with another, labeled with both IAF and ErITC. Förster resonance energy transfer (FRET, Förster 1948) was used to calculate the distances between binding sites for IAF and ErITC. The energy transfer rate is given by

$$k_T = \frac{1}{\tau_D}\left(\frac{R_0}{r}\right)^6 = \frac{1}{\tau_{DA}} - \frac{1}{\tau_D} \quad (1)$$

where R_0 is the Förster radius (a characteristic distance for D-A pair, at which FRET is 50 % efficient) and r is the distance between donor and acceptor, τ_{DA} and τ_D are the lifetimes of donor in the presence and absence of the acceptor, respectively. One can easily rearrange the equation to

$$r^6 = R_0^6 \frac{\tau_{DA}}{\tau_D - \tau_{DA}} \quad (2)$$

However, in a case when the lifetimes show a Lorentzian distribution

$$L(\tau|\tau_0, w) = \frac{1}{\pi w} \frac{1}{1 + \left(\frac{\tau - \tau_0}{w}\right)^2} \quad (3)$$

where τ_0 is a peak center and w is the full width at half maximum (FWHM), it is useful to perform the calculation with mean values of reciprocal lifetimes τ_{ave}. These are given as

$$\frac{1}{\tau_{ave}} = \int_{-\infty}^{\infty} L(\tau|\tau_0, w)\frac{1}{\tau} d\tau \quad (4)$$

The integrand is singular at $\tau = 0$, but the integral exists in the Cauchy sense and can be evaluated as

$$\frac{1}{\tau_{ave}} = \frac{\tau_0}{\tau_0^2 + w^2} \quad (5)$$

that can be rearranged to

$$\tau_{ave} = \tau_0 + \frac{w^2}{\tau_0} \quad (6)$$

Values of mean lifetimes obtained in this way are then inserted into eq. (2) and the donor-acceptor distance is calculated.

Results

The degree of creatine kinase labeling, characterized as the ratio of the number of fluorophores per enzyme molecule, was calculated from absorption spectra. The absorbance maxima at 280, 500, and 540 nm give a ratio of CK:ErITC:IAF equal to 1:0.61:1.75. Trypsin and cyanogen bromide cleavage of labeled CK confirmed the calculated donor-to-acceptor ratio. HPLC separation of protein fragments assigned fluorescence peaks to each fluorophore. From the elution profile ErITC to IAF was equal to ratio 1:3 (data not shown). SDS electrophoresis revealed high protein purity and no protein degradation had occurred during the labeling procedure (Fig. 1). Specific activity measurement of labeled CK revealed a considerable inhibition effect of IAF conjugation. In spite of that, ErITC binding to CK has a minor effect on specific activity, which corresponds to 76.7 % of the non-labeled enzyme (Table 1).

Table 1. Specific activities of labeled and non-labeled CK. The percentage was expressed from the activity of the non-modified enzyme.

	[μmol/min/mg]	%
IAF-CK	0.62	0.4
ErITC-CK	132.57	76.7
IAF-ErITC	2.75	1.6
Non-labeled CK	172.82	100

The Förster distance for IAF and ErITC as a donor-acceptor pair was calculated from the emission spectrum of IAF and absorption spectrum of ErITC. Using values of quantum yield of donor QY = 0.4 and index of refraction of proteins n = 1.54, we obtained R_0 = 5.345 nm. When bound to creatine kinase, IAF shows a single exponential decay with a Lorentzian distribution. A better fit was obtained when we added the second component. This component displays a lifetime of 0 ns and a preexponential factor $\alpha = 0.026$ which indicates the presence of a small amount of scattered light. The calculated lifetime was then $\tau = 4.549$ ns and FWHM of

the Lorentzian distribution was 0.344 ns. IAF was quenched in the presence of ErITC. From the absorption spectrum we estimated that the ratio of bound probes IAF:ErITC = 2.89:1. We must therefore consider the fact that there is one population of the unquenched donor and another of the quenched donor. The ratio of these populations is 1.89:1. We assumed that the lifetime and distribution of unquenched population is the same as in the absence of ErITC. Hence we held these values fixed in the calculation. Because of the presence of scattered light a three-component fit was used. The surface of χ_R^2 showed many local minima, so we chose a solution where the ratio of preexponential factors for unquenched: quenched population of the donor was close to 1.89:1. The distance between the binding sites for IAF and ErITC was calculated using eqs. (2) and (6). The phase-domain lifetime measurements evaluated the donor-acceptor distance at the native CK molecule as 7.8 nm.

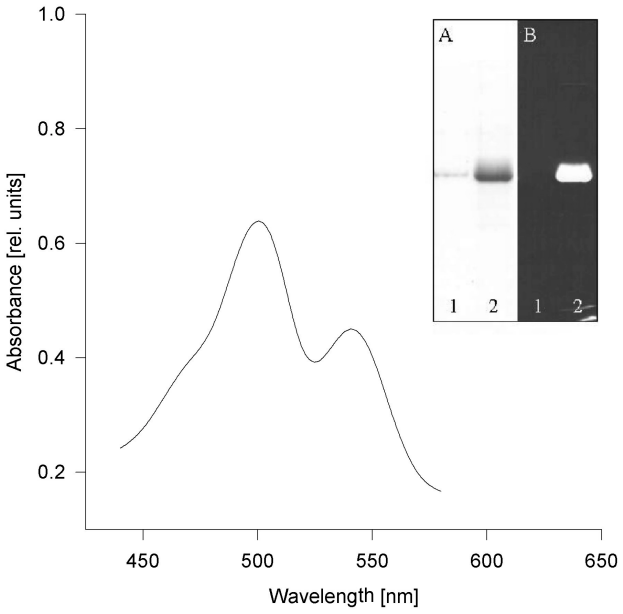

Fig. 1. *Labeling of native creatine kinase. Fluorescence emission spectra of creatine kinase conjugates of IAF (maximum 500 nm) and ErITC (maximum 540 nm). Protein purity (panel A) and free dye portion (panel B) were determined by SDS electrophoresis. Lanes 1 represent unlabeled CK, lanes 2 fluorescently modified enzyme under UV light.*

The suitability of erythrosin-fluorescein CK labeling for fluorescence energy transfer experiments was tested in the presence of different concentrations of KCl. The specific activity of native CK was measured as a function of KCl concentration ranging from 0 to 5 M. No effect was observed up to 1 M KCl but the activity was considerably diminished above 3 M KCl.

Double-labeled CK was incubated with KCl at the required concentration and the fluorescence lifetime of IAF was recorded by phase-domain fluorometer (Table 2). We observed three distributions of lifetimes, one of the unquenched donor (τ_1), one of the donor quenched by ErITC due to FRET (τ_2) and a small amount of scattered light with a lifetime 0 ns (τ_3). Distributions of τ_1 and τ_2 had a Lorentzian shape. The mean distance between donor and acceptor, calculated as described in the Methods, continually decreased from 7.8 nm in the absence of KCl to 5.5 nm in 5 M KCl. We checked that an increasing concentration of KCl had no influence on the fluorescence of IAF in the absence of ErITC. Steady-state intrinsic tryptophan fluorescence was recorded upon excitation at 300 nm and wavelength of maximum of emission spectra (λ_{max}) was monitored. The observed red shift in λ_{max} from 331 nm for the native CK to 337 nm in the presence of 5 M KCl corresponds to increasing polarity of the tryptophan environment.

The results obtained clearly indicate the suitability of erythrosin-fluorescein labeling for description of conformational changes of the creatine kinase molecule. Measurements of Förster resonance energy transfer on CK have been successfully used to describe the distance between tryptophans and IAEDANS (5-[2-(iodoacetyl)-aminoethylamino]naphthalene-1-sulphonic acid, Clottes *et al.* 1994, Couthon *et al.* 1997), tryptophans and dansyl (Grossman 1991), tryptophans and IAANS (2-[4-(iodoacetamido)anilino]naphthalene-6-sulphonic acid, Grossman *et al.* 1992) and during measurements of the active site distance by combined labeling with IAF (5-iodoacetamidofluorescein), IAEDANS, CPM (7-diethylamino-3-(4-maleimidyl-phenyl)-4-methylcoumarin, DABIA (4-[4-(dimethyl-amino)phenylazophenyl]-iodoacetamide) and IAANS (Grossman 1989, 1990). However, evaluation of the efficacy of FRET requires a comparison of fluorescence from two differently labeled samples. While it is difficult to believe that the concentration of the donor in both experiments will be exactly the same, monitoring of steady-state intensity (which depends on the dye concentration) can lead to values of energy transfer efficiency deflected by some artifacts. We circumvent these problems by measuring the donor lifetimes, a characteristic independent of the concentration.

Table 2. Effect of salt concentration on the lifetimes and mean IAF-ErITC distance (R). τ_1 represents the lifetime of unquenched donor, τ_2 the lifetime of donor quenched by ErITC due to FRET and τ_3 the lifetime of a small amount of scattered light, α is the relative abundance, FW is the full width of Lorentzian distribution at half maximum.

KCl [M]	τ_1 [ns]	FW	α_1	τ_2 [ns]	FW	α_2	τ_3 [ns]	α_3	χ^2	R [nm]
0.0	4.549	0.344	0.630	4.134	0.050	0.330	0	0.040	6.062	7.8
1.0	4.549	0.344	0.625	4.032	0.254	0.339	0	0.036	5.854	7.5
2.0	4.549	0.344	0.635	3.758	0.387	0.318	0	0.047	7.072	7.0
3.0	4.549	0.344	0.627	3.540	0.883	0.328	0	0.045	6.524	6.9
3.5	4.549	0.344	0.614	2.988	0.458	0.322	0	0.064	6.538	6.0
4.5	4.549	0.344	0.605	2.731	0.050	0.308	0	0.087	5.948	5.7
5.0	4.549	0.344	0.579	2.462	0.050	0.295	0	0.126	5.696	5.5

The estimated dependence of donor-acceptor distance on the concentration of KCl can be interpreted as ionic-strength-induced conformational change. We observed a large step in the calculated distances between 3 M and 3.5 M concentrations of KCl. This suggests that the deformation at this ionic strength becomes critical in areas of contact between the subunits and results in efficient monomer-monomer dissociation. This is supported by the fact that the distance of 6.0 nm is the first in a row which can be explained by the existence of both labels on the same subunit (which is 6.4 nm in diameter). After dissociation of the subunits, the energy transfer to the acceptor on the other subunit becomes impossible and the mean distance is rapidly shortened. This conclusion is further supported by steady-state fluorescence data obtained from tryptophan fluorescence measurement and the observed red shift in λ_{max}. Again, a large shift of the emission maximum was observed between 3 M and 3.5 M concentrations of KCl, indicating that some Trp residues become suddenly exposed to an aqueous phase. Although our measurements do not allow estimation of specific affected residue(s), these results strongly support our conclusions from measurements of FRET.

The 3D creatine kinase model analysis implies the existence of at least three distinct conformational forms. Without its substrates, the enzyme molecule takes an inactive "open" form. Upon binding of the MgATP complex, the CK molecule takes a reactive "closed" conformation. The difference between these two basic forms could be characterized by the distance of His-97 (triggers phosphate group transfer) and Cys-283 (essential amino acid for catalysis) from primary sequence. The transition distance, defined by His-97 and Cys-283 equals 0.766 nm for the open form and 0.277 nm for the closed form of the CK molecule. Subsequent binding of creatine yields a non-reactive "intermediate" conformation. The results indicate that the substrate-induced energy minimizing conformational changes do not represent a sufficient condition for CK activity and that some other controlling component is involved at a cellular level in the transition from the intermediate to the closed structure of the molecule.

Discussion

The observed loss of enzyme activity after binding of IAF corresponds to the formerly described modification of a highly reactive sulfhydryl group on the creatine kinase molecule (Brown and Cunningham 1970, O'Sullivan 1971, Henkin 1977). This group can be modified by a number of sulfhydryl-specific reagents (Kenyon and Reed 1983) and covalent binding can lead to a complete loss of activity in dependence on the modification moiety. Labeling stoichiometry of 1.75 IAF per CK molecule is in agreement with the presence of another accessible sulfhydryl group on the CK surface (Schnyder *et al*. 1995, Wang *et al*. 1996, Tanaka *et al*. 1997). The sulfhydryl group responsible for the inactivation was identified as Cys-282 near the creatine kinase active site (James *et al*. 1990, Sheikh *et al*. 1993, Olcot *et al*. 1994), but the role it plays in catalysis is still unclear (Furter *et al*. 1993, Lin *et al*. 1994). The effect of gross modifications can be rationalized by the structure in terms of a steric conflict with the substrate. Cys-282 may not be catalytically important but it may be involved in synergism between binding of the two substrates or in a hinge movement for the enzyme to become active.

These hypotheses comply with our recent findings that iodoacetamide modification does not interfere with the binding of the fluorescent ATP analogue. The inhibition effect of IAF thus makes it possible to describe conformational changes induced by substrate binding or related to regulation (e.g. phosphorylation) although it precludes description of the conformational behavior of CK during catalysis.

Acknowledgements

This work has been supported by the Ministry of Education, Czech Republic Grant No 113100003.
Preliminary results were presented at the Congress of Czech and Slovak Physiological Societies, České Budějovice, 2001 (Gregor *et al*. 2001).

References

BROWN CS, CUNNINGHAM LW: Reaction of reactive sulfhydryl groups of creatine kinase with dansyl chloride. *Biochemistry* **9:** 3878-3885, 1970.

CLOTTES E, COUTHON F, DENOROY L, VIAL C: Creatine kinase compactness and thiol accessibility during sodium dodecyl sulfate denaturation estimated by resonance energy transfer and 2-nitro-5-thiocyanobenzoic acid cleavage. *Biochim Biophys Acta* **1209:** 171-176, 1994.

COUTHON F, CLOTTES E, VIAL CH: High salt concentrations induce dissociation of dimeric rabbit muscle creatine kinase. Physico-chemical characterization of the monomeric species. *Biochim Biophys Acta* **1339:** 277-288, 1997.

FORSTNER M, KRIECHBAUM M, LAGGNER P, WALLIMANN T: Structural changes of creatine kinase upon substrate binding. *Biophys J* **75:** 1016-1023, 1998.

FÖRSTER T: Zwischenmolekuläre Energiewanderung und Fluoreszenz. *Ann Phys* **6:** 55-75, 1948.

FRITZ-WOLF K, SCHNYDER T, WALLIMANN T, KABSCH W: Structure of mitochondrial creatine kinase. *Nature* **381:** 341-345, 1996.

FURTER R, FURTER-GRAVES EM, WALLIMANN T: Creatine kinase: the reactive cysteine is required for synergism but is nonessential for catalysis. *Biochemistry* **32:** 7022-7029, 1993.

GRANJON T, VACHERON MJ, VIAL C, BUCHET R: Mitochondrial creatine kinase binding to phospholipids decreases fluidity of membranes and promotes new lipid-induced beta structures as monitored by red edge excitation shift, laurdan fluorescence, and FTIR. *Biochemistry* **40:** 6016-6026, 2001.

GREGOR M, MEJSNAR J, JANOVSKÁ A, ŽURMANOVÁ J, BENADA O, MEJSNAROVÁ B: Creatine kinase reaction in skinned rat psoas muscle fibers and their myofibrils. *Physiol Res* **48:** 27-35, 1999.

GREGOR M, MIKŠÍK I, MEJSNAR J: Fluorescence spectroscopy of creatine kinase molecule. *Physiol Res* **50:** P6, 2001.

GROSSMAN SH: Resonance energy transfer between the active sites of rabbit muscle creatine kinase: analysis by steady-state and time-resolved fluorescence. *Biochemistry* **28:** 4894-4902, 1989.

GROSSMAN SH: Resonance energy transfer between the active sites of creatine kinase from rabbit brain. *Biochim Biophys Acta* **1040:** 276-280, 1990.

GROSSMAN SH: Conformational heterogeneity of creatine kinase determined from phase resolved fluorometry. *Biophys J* **59:** 590-597, 1991.

GROSSMAN SH, FRANCE RM, MATTHEIS JR: Heterogeneous flexibilities of the active site domains of homodimeric creatine kinase: effect of substrate. *Biochim Biophys Acta* **1159:** 29-36, 1992.

HENKIN J: Photolabeling reagent for thiol enzymes. Studies on rabbit muscle creatine kinase. *J Biol Chem* **252:** 4293-4297, 1977.

JAMES P, WYSS M, LUTSENKO S, WALLIMANN T, CARAFOLI E: ATP binding site of mitochondrial creatine kinase. Affinity labelling of Asp-335 with C1RATP. *FEBS Lett* **273:** 139-143, 1990.

KENYON GL, REED GH: Creatine kinase: structure-activity relationships. *Adv Enzymol Relat Areas Mol Biol* **54:** 367-426, 1983.

KUSHMERICK MJ: Energetics of muscle contraction. In: *Handbook of Physiology. Skeletal Muscle.* American Physiological Society, Bethesda, USA, 1983, pp 189-236.

LIN L, PERRYMAN MB, FRIEDMAN D, ROBERTS R, MA TS: Determination of the catalytic site of creatine kinase by site-directed mutagenesis. *Biochim Biophys Acta* **1206:** 97-104, 1994.

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

MEJSNAR J, SOPKO B, GREGOR M: Myofibrillar creatine kinase activity inferred from a 3D model. *Physiol Res* **51**: 35-41, 2002.

OLCOTT MC, BRADLEY ML, HALEY BE: Photoaffinity labeling of creatine kinase with 2-azido- and 8-azidoadenosine triphosphate: identification of two peptides from the ATP-binding domain. *Biochemistry* **33:** 11935-11941, 1994.

O'SULLIVAN WJ: The reaction of creatine kinase with dithiobisnitrobenzoic acid. Formation of derivatives of the enzyme. *Int J Protein Res* **3:** 139-147, 1971.

RAIMBAULT C, BUCHET R, VIAL C: Changes of creatine kinase secondary structure induced by the release of nucleotides from caged compounds. An infrared difference-spectroscopy study. *Eur J Biochem* **240:** 134-142, 1996.

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

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.

SCHNYDER T, TITTMANN P, WINKLER H, GROSS H, WALLIMANN T: Localization of reactive cysteine residues by maleidoyl undecagold in the mitochondrial creatine kinase octamer. *J Struct Biol* **114:** 209-217, 1995.

SHEIKH S, MUKUNDA K, KATIYAR SS: Evidence for proximal cysteine and lysine residues present at the nucleotide domain of rabbit muscle creatine kinase. *Biochim Biophys Acta* **1203:** 276-281, 1993.

TANAKA N, TONAI T, KUNUGI S: Site-specific modification of rabbit muscle creatine kinase with sulfhydryl-specific fluorescence probe by use of hydrostatic pressure. *Biochim Biophys Acta* **1339:** 226-232, 1997.

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.

WANG HR, BAI JH, ZHENG SY, WANG ZX, ZHOU HM: Ascertaining the number of essential thiol groups for the folding of creatine kinase. *Biochem Biophys Res Commun* **221:** 174-180, 1996.

WEBB T, JACKSON PJ, MORRIS GE: Protease digestion studies of an equilibrium intermediate in the unfolding of creatine kinase. *Biochem J* **321:** 83-88, 1997.

Reprint requests

Martin Gregor, Department of Physiology and Developmental Biology, Faculty of Sciences, Charles University, Viničná 7, CZ-128 44 Praha 2, Czech Republic, e-mail gregor@natur.cuni.cz