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Purification and stability of octameric mitochondrial creatine kinase isoform from herring (*Clupea harengus*) organ of vision

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ABSTRACT

Creatine kinases (CKs) constitute a large family of isoenzymes that are involved in intracellular energy homeostasis. In cells with high and fluctuating energy requirements ATP level is maintained via phosphocreatine hydrolysis catalyzed by creatine kinase. In contrast to invertebrates and higher vertebrates, in poikilothermic vertebrates the adaptations for the regulation of energy metabolism by changes in the oligomeric state of CK isoforms are not well known. The present study aimed at identification of herring eye CK isoforms and focuses on factors affecting the CK-octamer stability. In addition to the CK octamer, three different dimeric isoforms of CK were detected by cellulose acetate native electrophoresis. Destabilization of octamer was studied in the presence of TSAC substrates and about 50% of octamers dissociated into dimers within 24 h. Moreover, we found that the increase of temperature from 4 °C to 30 °C caused rapid inactivation of dimers in TSAC-treated samples but did not affect octameric structures. In a thermostability assay we demonstrated that octamers retain their activity even at 50 °C. Our results indicate that destabilization of the octameric structure can lead to loss of enzyme activity at higher temperatures (above 30 °C). Furthermore, our results based on N-terminal sequence analysis suggest that probably the mitochondrial s-type CK, rather than u-type, is predominantly expressed in herring eye. In conclusion the existence of four various CK isoforms in one organ may reflect complex regulation of energy metabolism in the phototransduction process in teleost fishes.

1. Introduction

In cells with high and fluctuating energy requirements (skeletal muscle, kidney, brain, neurons, electric organs, retina photoreceptor cells and spermatozoa) ATP level is maintained via the phosphocreatine/creatine (PCr/Cr) shuttle catalyzed by creatine kinase (CK). CK belongs to a family of phosphoryl transfer enzymes called phosphagen kinases, which play a central role in cellular energy homeostasis. It is widely distributed in both invertebrate and vertebrate groups (reviewed in Wallimann et al., 2011; Ellington, 2001). In higher vertebrates there are two CK isoform gene groups, each coding for proteins targeted to different intracellular compartments: cytosolic (CytCK: muscle M-CK and brain type B-CK) and mitochondrial (MtCK: sarcomeric sMtCK and ubiquitous uMtCK). Generally, cytosolic isoenzymes form dimeric structures, while mitochondrial ones exist as octamers (Mühlebach et al., 1994).

In fishes, tissue distribution and level of expression of the CK isoenzymes may vary from their counterparts in higher vertebrates. The ectotherms are highly susceptible to multiple environmental changes, like those in temperature, salinity, pressure, pH, water availability, ionic strength or radiation that affect all metabolic reactions and biochemical pathways in cells. In some cases the expression of various enzyme isoforms is likely related to environmental acclimation (Hochachka and Somero, 2002). The CK isoforms may differ in amino acid sequence, which reveals some potential differences in protein structure and immunological, biochemical and physico-chemical properties. For instance, three CK isoenzymes (CK-A, CK-C, CK-D) were identified in muscle, stomach and testis, while CK-B was confined to neural tissue of green sunfish (*Lepomis cyanellus*) (Fisher and Whitt, 1978, 1979). Three different muscle-type CKs – M1, M2 and M3 – were found in carp muscle and revealed to have various thermal stabilities depending on temperature conditions (Sun et al., 1998, 2002). Furthermore, the CK isoforms detected in gills of tilapia (*Oreochromis mossambicus*) were confirmed to be functionally coupled to Na^+–K^+-ATPase activity, providing ATP for the ATPase reaction that maintains physiological ion balance. This function is especially important in the event of transition from fresh to seawater (Weng et al., 2002; Lin et al., 2003). Fish CK isoenzymes have also been characterized

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in trout skeletal muscle (Eppenberger et al., 1971), stomach (Perriard et al., 1972), and germ cells (Saudrais et al., 1996), zebrafish muscle (Harder and McGowan, 2001), Antarctic icefish white muscle (Winnard et al., 2003), and white skeletal muscle and stomach wall muscle in some members of the family of *Acipenseridae* (Kuz'min, 2008). Multiple isoforms, mostly of cytosolic origin, were also found in channel catfish tissues (Liu et al., 2001).

Baltic herring (*Clupea harengus*) is an excellent model for studying the fish CK biochemical properties because of high activity and variability of CK isoenzymes existing in its tissues. Moreover, because of strong environmental gradients (salinity and temperature) in the Baltic Sea it has the potential for local adaptation via environmentally induced selection (Teacher et al., 2012). In our previous studies we confirmed the existence of cytosolic and mitochondrial CK isoforms in herring muscle and spermatozoa and suggested the presence of the energy PCr/Cr shuttle in fishes. We identified four cytoplasmic isoforms of CK in herring tissues: three homodimers and one heterodimer specific for the brain and eye. Mitochondrial isoforms were also present in the muscle, stomach, brain, eye and sperm. The sperm-type CK isoform was distinctive for male gonads only (Grzyb and Skorkowski, 2005, 2006).

Previous studies showing localization of creatine kinase isoforms support the existence of a PCr circuit in the highly polar photoreceptor cells of higher vertebrates (Haverkamp et al., 2000; Acosta et al., 2005). In higher vertebrates BB-CK is usually co-expressed with uMtCK (Wallimann et al., 2011). In chicken retina photoreceptor cells BB-CK is distributed within the inner and outer segments of photoreceptor cells, whereas uMtCK is strictly located within the mitochondria of the inner segment (Wallimann et al., 1986). Only a few reports regarding creatine kinase isoenzymes that are expressed in fish eye are available and these concern: green sunfish (Fisher and Whitt, 1979), zebrafish (Xu et al., 2000) and mandarin fish (Zhang et al., 2010). In contrast to higher vertebrates and invertebrates, in poikilothermic vertebrates adaptations in regulation of energy metabolism by the changes of the oligomeric state of CK isoforms are not well known. Especially the octameric mitochondrial CK isoforms from fishes have not been studied extensively. The latest studies indicate a lack of mitochondrial CKs in hearts of some Antarctic fishes (O'Brien et al., 2014).

This report describes results from studies on identification of mitochondrial CK isoform purified from the eye of Baltic herring with particular focus on characterization of the octamer/dimer transitions including its thermal stability.

2. Materials and methods

2.1. Fish and eye extract preparation

Baltic Sea herrings (*C. harengus*) were caught by local fishermen during the spring season. 20 g of herring eyes was homogenized with 15 mL of buffer containing: 50 mM Na-phosphate, 150 mM NaCl, 0.2 mM EDTA, 2 mM BME, 1 mM NaN_3 and 1% Triton X-100 at pH 7.2. The homogenate was centrifuged for 20 min at 14,000 ×*g*. The resulting supernatant was used for subsequent purification procedures.

2.2. Measurement of CK activity

CK activity was measured according to an Alpha Diagnostics, Warsaw spectrophotometric procedure kit No. C6512.

2.3. Measurement of protein levels

Protein concentration was determined by the Coomassie Blue method (Spector, 1978).

2.4. Gel filtration chromatography

The CK octamer was purified according to the procedures described in Grzyb and Skorkowski (2005, 2006). Gel filtration experiments were performed on Sephacryl S-300 in buffer A containing: 50 mM Na-phosphate, 150 mM NaCl, 0.2 mM EDTA, 2 mM BME, and 1 mM NaN_3 at pH 7.2. Thyroglobulin (669,000), ferritin (440,000), catalase (232,000), aldolase (158,000), albumin (67,000), chymotrypsinogen A (25,000) and ribonuclease A (13,700) (SIGMA) were used as M_r standards and the void volume was determined by Blue Dextran 2000. Approximately 23 mL of herring eye extract was applied onto a Sephacryl S-300 column at 4 °C. The peaks at 518 and 605 mL of elution volume (76 and 88 fractions) corresponded to CK1 (octamer) and CK2 (dimer), respectively. After gel filtration the fraction with CK octamer was collected and dialyzed against buffer B containing: 50 mM Na-phosphate, 1 mM $MgCl_2$, 1 mM NaN_3, 1 mM BME, and 0.2 mM EDTA, pH 6.5.

2.5. Affinity chromatography on Blue-Sepharose CL-6B

Dialyzed material of CK octamer was loaded onto a Blue Sepharose column previously equilibrated with buffer B. The column was rinsed with 100 mL of buffer B and subsequently with 50 mL of buffer B with pH 8.0 (buffer C). The CK octamer was eluted with a linear salt gradient by mixing 50 mL of buffer C with 50 mL of 1 M KCl in buffer C. The fraction with CK octamer was concentrated to 2.5 mL with a collodion bag apparatus and then desalted on the PD-10 desalting column (Amersham Pharmacia Biotech).

2.6. Anion exchange chromatography

After Blue Sepharose chromatography the enzyme was loaded onto a DEAE-Sephacel column equilibrated with buffer C. Although the CK octamer did not bind to the column, the remaining contaminants did. The fraction with CK octamer was concentrated with a collodion bag apparatus and used for further experiments or kept in 40% glycerol in 0.1 M Na-phosphate buffer, pH 7.1 at −20 °C.

2.7. Cellulose acetate electrophoresis

CK-samples were applied in the middle of the cellulose acetate membranes by a sample applicator (Helena Super Z-12 Applicator kit, Helena Laboratories). Electrophoresis was performed at 200 V for 20 min at 4 °C in electrode buffer (0.2 M glycine, 0.025 M Tris, pH 8.5). After electrophoresis membranes were stained for CK activity. The staining solution contained: 1 mg NBT (p-Nitro blue tetrazolium chloride), 0.1 mg PMS (phenazine methosulfate), 5 mL of medium for CK activity assays (Alpha Diagnostics, No. C6512), 3 mL of 2% agarose and 2 mL of redistilled water. The reaction was stopped by 7% acetic acid.

2.8. SDS polyacrylamide gel electrophoresis

Sodium dodecyl sulfate (SDS) polyacrylamide electrophoresis was run according to the method of Laemmli (1970). To determine a molecular weight marker SDS-7B from SIGMA and cat. no. 26617 from Thermo Scientific were used.

2.9. Western blot analysis

The immunostaining was performed using the polyclonal goat anti-sMtCK and anti-uMtCK antibodies (sc15169, sc15165 from Santa Cruz Biotechnology) and AP-conjugated anti-goat antibody. The samples were resolved on SDS-polyacrylamide gels and transferred to a PVDF membrane in buffer containing 25 mM Tris–Base, 150 mM glycine, and 10% methanol, pH 8.3. The membrane was incubated for 60 min in a blocking buffer (5% milk in TBS buffer; TBS buffer: 50 mM Tris–