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Hearts of some Antarctic fishes lack mitochondrial creatine kinase

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ABSTRACT

Creatine kinase (CK; EC 2.7.3.2) functions as a spatial and temporal energy buffer, dampening fluctuations in ATP levels as ATP supply and demand change. There are four CK isoforms in mammals, two cytosolic isoforms (muscle [M-CK] and brain [B-CK]), and two mitochondrial isoforms (ubiquitous [uMtCK] and sarcomeric [sMtCK]). Mammalian oxidative muscle couples expression of sMtCK with M-CK, creating an energy shuttle between mitochondria and myofibrils. We hypothesized that the expression pattern and activity of CK would differ between hearts of red- and white-blooded Antarctic notothenioid fishes due to their striking differences in cardiac ultrastructure. Hearts of white-blooded icefishes (family Channichthyidae) have significantly higher mitochondrial densities compared to red-blooded species, decreasing the diffusion distance for ATP between mitochondria and myofibrils and potentially minimizing the need for CK. The distribution of CK isoforms was evaluated using western blotting and maximal activity of CK was measured in mitochondrial and cytosolic fractions and tissue homogenates of heart ventricles of red- and white-blooded notothenioids. Transcript abundance of sMtCK and M-CK was also quantified. Overall, CK activity is similar between hearts of red- and white-blooded notothenioids but hearts of icefishes lack MtCK and have higher activities of M-CK in the cytosol compared to red-blooded fishes. The absence of MtCK may compromise cardiac function under stressful conditions when ATP supply becomes limiting.

1. Introduction

Creatine kinase (CK) plays a central role in energy flux, catalyzing the reversible transfer of phosphate between creatine (Cr) and ADP in the following reaction:

$$MgATP^{2-} + creatine \leftrightarrow MgADP^{-} + PCr^{2-} + H^{+}.$$

CK functions as a temporal buffer, maintaining high levels of ATP as demand fluctuates, and as a spatial buffer, facilitating the diffusion of high-energy phosphates between sites of energy production and utilization. The smaller size of phosphocreatine (PCr) compared to ATP results in a diffusion coefficient that is 1.4-fold higher than that of ATP at 37 °C (de Graaf et al., 2000; Wallimann et al., 1992).

In birds and mammals, there are four isoforms of CK that are developmentally regulated and expressed in a tissue-specific fashion (Ventura-Clapier et al., 1998). Muscle (M) and brain (B) CK isoforms are localized to the cytosol and form homo- and hetero-dimers (MM-, MB- and BB-CK). The sarcomeric (sMtCK) and ubiquitous (uMtCK) isoforms are localized to the mitochondrial intermembrane space. Here they associate with anionic lipids of the inner mitochondrial membrane, particularly cardiolipin, and are localized within cristae junctions between the inner and outer membranes (Schlattner et al., 2004). The mitochondrial isoforms form octomers adjacent to the adenine nucleotide translocase (ANT) within the inner membrane and the voltage-dependent anion channel (VDAC) within the outer mitochondrial membrane (reviewed by Schlattner et al., 2006). ATP produced in the mitochondrial matrix by ATP synthase is exchanged for ADP through the ANT. Sarcomeric MtCK and uMtCK transfer the high-energy phosphate from ATP to creatine within the intermembrane space, producing PCr, which diffuses through VDAC into the cytosol, and ADP which maintains the activity of ATP synthase and mitochondrial respiration. ADP generated by ATP hydrolysis in the cytosol diffuses through VDAC into the matrix, also contributing to high ADP levels within the mitochondrial matrix. The addition of creatine and 1 mM ADP or ATP to permeabilized cardiac cells in mice stimulates respiration but not in mutants lacking sMtCK, suggesting that mitochondrial CK is essential for maximally stimulating oxidative phosphorylation (Kay et al., 2000). Mitochondrial CK also minimizes the production of reactive oxygen species (ROS) by maintaining high concentrations of ADP in the matrix. This maintains a low membrane potential and high flux through the electron transport chain (ETC), minimizing electron leakage (Meyer et al., 2006).

The localization and expression of CK differ between oxidative and glycolytic muscles in mammals. Glycolytic fast-twitch muscle, with a primary reliance on glycolysis, possesses an abundance of MM-CK but low levels of mitochondrial isoforms of CK (Yamashita and Yoshioka, 1991).

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MM-CK associates with the M-band of sarcomeres where it rapidly rephosphorylates ADP, providing a steady supply of ATP for the myofibrillar actin-activated Mg^{2+} ATPase (Wallimann et al., 1984). Higher levels of MM-CK are associated with the M-band in glycolytic muscle compared to oxidative skeletal muscle, suggesting that CK primarily serves as an energy buffer and energy regeneration system in glycolytic muscle (Yamashita and Yoshioka, 1991). Oxidative and cardiac muscles in contrast express high levels of sMtCK along with MM-CK, creating an energy shuttle between mitochondria and myofibrils (Yamashita and Yoshioka, 1991).

While the expression pattern and activity of CK are well described for mammals, less is known about CK expression in teleost fishes. Previous studies have shown that creatine increases mitochondrial affinity for ADP in cardiac myocytes of trout but not cod, suggesting the absence of mitochondrial CK in cod hearts, perhaps because of its lower energy demand compared to trout (Birkedal and Gesser, 2003). Hearts of hypoxia tolerant fishes have higher activities of CK compared to hypoxia intolerant species, suggesting that CK may be most critical in teleosts under stressful conditions when ATP supply may not meet demand (Christensen et al., 1994).

Antarctic icefishes (family Channichthyidae) of the suborder Notothenioidei are singular among vertebrates for their lack of the circulating oxygen-binding protein, hemoglobin (Hb) as adults (Ruud, 1954). Moreover, 6 of the 16 species of icefishes possess golden-colored hearts due to their lack of expression of the intracellular oxygen-binding protein myoglobin (Mb) (Moylan and Sidell, 2000; Sidell et al., 1997). Mb facilitates the diffusion of oxygen from blood to mitochondria and stores oxygen within most aerobically-poised muscles of vertebrates (Wittenberg, 1970). The loss of Hb and Mb in icefishes is correlated with striking alterations in cardiac and oxidative skeletal muscle architecture that enhance oxygen delivery to mitochondria, and which might also alter the abundance and distribution of CK (O'Brien and Sidell, 2000; O'Brien et al., 2003).

Muscles of icefishes devoid of Mb are paradoxically mitochondria-rich. Mitochondria occupy 36.5% of the volume of cardiac ventricular myocytes in the icefish *Chaenocephalus aceratus* compared to 15.9% in the red-blooded *Gobionotothen gibberifrons*, 22.8% in *Notothenia rossi* (+Hb) and 25.0% in *Notothenia coriiceps* (+Hb) (Feller et al., 1985; Johnston et al., 1983; Johnston and Harrison, 1985; O'Brien and Sidell, 2000). In oxidative pectoral adductor muscle of icefishes, mitochondrial volume density ranges between 39 and 53% compared to 25 and 31% in red-blooded fishes (O'Brien and Mueller, 2010). Typically, mitochondrial density scales with aerobic metabolic capacity, yet this relationship does not always hold true for Antarctic notothenioids. Aerobic metabolic capacity, estimated from maximal activities of citrate synthase (CS) and cytochrome c oxidase (CCO), is equivalent among hearts of red- and white-blooded fishes (Johnston and Harrison, 1985; O'Brien and Sidell, 2000). Alterations in mitochondrial ultrastructure provide insight to this apparent conundrum. Mitochondria from muscles of icefishes lacking both Hb and Mb are larger, and have a lower density of inner membranes (cristae) compared to red-blooded notothenioids (Johnston et al., 1998; O'Brien and Sidell, 2000; O'Brien et al., 2003). The surface-to-volume ratio of cardiac mitochondria from the icefish *C. aceratus* is 1.9-fold lower than the red-blooded species *N. coriiceps*, and the cristae surface density is 1.3 to 1.5-fold lower in icefishes compared to red-blooded species (O'Brien and Sidell, 2000; Urschel and O'Brien, 2008). The net result is that the density of ETC components is equivalent per g wet mass ventricular tissue between red- and white-blooded notothenioids but spreads out over a larger surface area in hearts of icefishes. Because oxygen solubility is approximately 4-fold higher in phospholipids compared to water, the extensive mitochondrial phospholipid bilayer network in icefish muscles serves as a low-resistance route for oxygen diffusion, likely compensating for the lack of Mb and enhancing oxygen diffusion rates (Gennis, 1989; Smotkin et al., 1991). The enlargement of the mitochondrial compartment in oxidative muscles of icefishes also decreases the diffusion distance for oxygen between blood plasma and CCO, and for ATP between mitochondria and myofibrils, the latter which might diminish the demand for CK.

To determine if alterations in cardiac ultrastructure associated with the loss of Hb impact CK distribution and activity, we evaluated the distribution of CK isoforms in hearts of the icefishes *C. aceratus*, *Chionodraco rastrospinosus* and *Champsocephalus gunnari*, and in the red-blooded notothenioids *N. coriiceps* and *G. gibberifrons* using western blotting. Maximal activity of CK was measured in ventricular homogenates and mitochondrial and cytosolic fractions. Transcript levels of sMtCK and M-CK were quantified in hearts of *C. aceratus* and *N. coriiceps* using quantitative real-time PCR (qRT-PCR).

2. Materials and methods

2.1. Animals

C. aceratus (Lönnberg), *C. gunnari* (Lönnberg), *C. rastrospinosus* (Dewitt and Hureau), *G. gibberifrons* (Lönnberg) and *N. coriiceps* (Richardson) were collected in Dallmann Bay, Antarctica (64°10′S, 62°35′W) by benthic trawl or with baited traps deployed from the ARSV *Laurence M. Gould* (*LMG*) during the austral fall of 2007. Animals were held in circulating seawater tanks at 0 ± 0.5 °C on board the *LMG* and then transferred to aquaria at the U.S. Antarctic research station, Palmer Station where they were maintained in circulating seawater tanks at 0 ± 0.5 °C. Animals were anesthetized in MS-222 (1:7500 in sea water) and sacrificed by brain pithing.

2.2. Mitochondrial isolation

Hearts were quickly excised and placed in ice-cold Ringer solution (260 mmol L^{-1} NaCl, 2.5 mmol L^{-1} $MgCl_2$, 5 mmol L^{-1} KCl, 2.5 mmol L^{-1} $NaHCO_3$, 5 mmol L^{-1} NaH_2PO_4, pH 8.0). Hearts were allowed to contract several times to remove blood from the ventricular lumen. Heart ventricles were diced into 1–2 mm-sized blocks on an ice-cold stage and homogenized in 9 volumes of ice-cold isolation buffer (140 mmol L^{-1} KCl, 10 mmol L^{-1} EDTA, 5 mmol L^{-1} $MgCl_2$, 20 mmol L^{-1} HEPES, pH 7.3) using a Tekmar Tissuemizer (Teledyne Tekmar, Cincinnati, OH, USA) (10 s, low setting). Homogenization was completed using an ice-cold Tenbroeck ground glass homogenizer (Wheaton, Millville, NJ, USA). Tissue homogenates were centrifuged for 5 min at 500 *g* at 4 °C. Supernatant was decanted and centrifuged for 10 min at 9000 *g* at 4 °C; 1 mL of supernatant (cytosolic fraction) was removed and immediately frozen in liquid nitrogen. The remaining supernatant was discarded and the mitochondrial pellet gently resuspended in 10 mL homogenization buffer. Resuspended pellets were centrifuged at 500 *g* (5 min, 4 °C). The supernatant was decanted and centrifuged at 9000 *g* for 10 min at 4 °C. Mitochondrial pellets were resuspended in 10 mmol L^{-1} $NaPO_4$ pH 7.0 and immediately frozen in liquid nitrogen. Mitochondrial and cytosolic fractions were stored at −80 °C until further use.

2.3. Western blots

Protein levels of uMtCK, sMtCK and MM-CK were detected in tissue homogenates and mitochondrial and cytosolic fractions from hearts of *C. aceratus*, *C. gunnari*, *C. rastrospinosus*, *G. gibberifrons* and *N. coriiceps*. Protein concentrations were determined as described by Lowry et al. (1951). Proteins were diluted in SDS-PAGE loading buffer (10 mmol L^{-1} $NaPO_4$, 1.25% SDS, 20 mmol L^{-1} DTT, 4% glycerol, 0.0125% bromophenol blue, pH 6.8) and incubated at 95 °C for 2 min. 100 μg (for sMtCK and uMtCK) or 3 μg of cytosolic proteins (for MM-CK) were separated on 10% polyacrylamide gels (uMtCK), 12% polyacrylamide gels (MM-CK) or 15% polyacrylamide gels (sMtCK). 10 μg (sMtCK and uMtCK) or 2 μg (MM-CK) of homogenates from rat hearts were also separated on gels as a positive control. Proteins were transferred to Hybond-P PVDF membranes (GE Healthcare,