Origin of octameric creatine kinases

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Abstract Mitochondrial creatine kinase (MiCK) occurs primarily as an octameric form localized in the mitochondrial intermembrane compartment in vertebrate tissues and echinoderm spermatozoa (both deuterostome groups). The octameric quaternary structure is thought to play important functional and enzyme targeting roles. We have found that the spermatozoa of the protostome polychaete *Chaetopterus variopedatus* contain three distinct isoenzymes of creatine kinase (CK) termed CK1, CK2 and CK3. CK3 appears to be present only in the sperm head/midpiece complex where mitochondria are restricted and has a subunit relative molecular mass (M_r) of 43.4 kDa. Gel permeation chromatography using Superdex 200HR showed that CK3 has a native M_r of 344.9 kDa indicating that this enzyme exists as an octamer. Electron micrographs of negatively stained CK3 preparations show structures which are virtually identical to those that have been seen for octameric vertebrate MiCK. The above observations show that CK3 from *C. variopedatus* displays great similarities to MiCKs from vertebrates and echinoderms. Octamerization of CK is not an advanced feature. The evolution of octameric subunit association is ancient and occurred prior to the divergence of protostomes and deuterostomes.

Key words: Mitochondrial creatine kinase; Octamer; Evolution; Spermatozoon

1. Introduction

Creatine kinase (CK) catalyzes the reversible transfer of phosphate from creatine phosphate (CP) to ADP yielding ATP. The CK/CP system is primarily found in cells with high and variable metabolic outputs such as muscles, neurons, spermatozoa, transport epithelia and photoreceptors [1]. In vertebrates CK is found as a number of isoenzymes – cytosolic MM, MB and BB and two mitochondrial forms, the so-called ubiquitous mitochondrial CK (Mia-CK) and the ubiquitous sarcomeric CK (Mib-CK) [1,2]. Both MiCKs possess important functional and structural features including localization in the intermembrane space, higher relative concentrations in the vicinity of contact sites, and electrostatic interactions with the outer portion of the inner mitochondrial membrane (for an extensive review see [1]). A number of workers have provided evidence for a direct coupling between MiCKs and the adenine nucleotide translocase (reviewed in [1]).

Cytosolic CKs are dimeric. In contrast, Wallimann and coworkers showed that MiCKs exist primarily as octamers [3–5]. Isolated MiCKs slowly dissociate into dimers under certain conditions, and this process is greatly accelerated by addition of creatine, ADP, Mg^{2+} and NO_3^-, forming the transition state analog complex (TSAC) [6]. It has been suggested that octamerization is physiologically relevant in that it influences binding to the inner membrane and may modulate mitochondrial energy flux [7]. The structural correlates of octamerization have been investigated. An N-terminal heptapeptide has been shown to be important in octamer formation [8]. Furthermore, the octamer is stabilized by both polar and hydrophobic components [9]. Publication of a crystal structure of apo- and ATP-MiCK has provided much insight into the interaction of subunits [10].

In addition to vertebrates, considerable attention has been focused on the role of the CK/CP system in the motility of primitive spermatozoa of echinoderms. These cells are highly polarized in that high energy phosphate must traverse a considerable diffusion distance between ATP source (mitochondria in midpiece) and ATP sink (dynein ATPase). In an elegant series of studies Tombes and coworkers [11,12] showed that sea urchin spermatozoa contain both mitochondrial and flagellar isoenzymes of CK. Sperm CK activity is obligatory for energy transport and normal flagellar motility. These workers found that sea urchin MiCK differed from vertebrate MiCKs in that detergents were required to extract the echinoderm form and that its M_r was considerably lower than the M_rs for vertebrate MiCKs. Recently, Wyss et al. [13] revisited the sea urchin system and found in fact that the MiCK is indeed an octamer and that this MiCK octamer is quite stable and does not dissociate when the TSAC is formed [13]. Because vertebrates and echinoderms are deuterostomes, it was suggested that the octameric enzyme evolved around the time of the radiation of this group [13].

In addition to echinoderms, CK is present in a number of other higher and lower invertebrate groups [14]. Of particular interest are the polychaetes (marine worms) which as a group have at least six different phosphagen (guanidino) kinases present in their somatic tissues [14]. However, regardless of which phosphagen kinase is found in the somatic tissues, spermatozoa always have CK only [14]. Recently, Kamp et al. [15] found two isoenzymes of CK in the sperm of the polychaete *Arenicola marina*, a head/midpiece form and a flagellar form. The subunit structure was not characterized. In the present communication, we show that the spermatozoa of the polychaete *Chaetopterus variopedatus* contain three distinct isoenzymes of CK – a dimer (CK1), a large CK (CK2) and an octamer (CK3). The octamer is restricted to the head/midpiece and is likely the mitochondrial form. CK3 has similar physicochemical properties to vertebrate and sea urchin MiCKs. Octamerization is clearly not an advanced feature of MiCKs but reflects a rather ancient origin more than 670 million years ago [16] prior to the protostome/deuterostome split.

2. Materials and methods

Specimens of *C. variopedatus* were collected on mud flats near Alli-

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gator Point, FL. Animals were maintained in recirculating aquaria prior to experiments. Sperm suspensions were obtained by piercing sperm-laden parapodia with a syringe. Biochemicals were from Sigma Chemical (St. Louis, MO) and Boehringer Mannheim (Indianapolis, IN).

Spermatozoa suspensions were centrifuged at 2000×*g* for 10 min and the supernatant discarded. The resulting pellet was resuspended in approximately 10 volumes of 25 mM Tris-HCl (pH 8)-−1 mM EDTA–1 mM DTT–2% glycerol–1% Triton X-100 and then homogenized at 70% full force using a Polytron (Brinkman, Westbury, NY) homogenizer. The resulting homogenate was centrifuged at 16 000×*g* for 30 min and the supernatant dialyzed exhaustively against 5 mM potassium phosphate buffer (pH 7) containing 2% glycerol and 1 mM DTT (buffer A). The sample was then applied to a hydroxyapatite (Bio-Gel HT Gel, Bio-Rad, Hercules, CA) column (2.5×22 cm; 86 ml) previously equilibrated in buffer A. After extensive washing, protein was eluted using a linear gradient of 5–400 mM potassium phosphate (400 ml). CK activity was assayed as described in Strong and Ellington [17] using CP as substrate.

Cellulose acetate electrophoresis and subsequent CK active staining were conducted using Beckman (Irvine, CA) membranes as previously described [17] with crude extracts of whole spermatozoa and body wall muscle. Sperm were also fractionated in order to assess the qualitative nature of CK activity in head/midpiece and flagellar fractions. Sperm were fractionated into head/midpiece and tail enriched fractions as previously described [17]. Samples were then suspended in a small volume of 2.5 mM Tris-HCl (pH 8) containing 0.1 mM EDTA, 1.4 mM 2-mercaptoethanol and 0.1% Triton X-100, sonicated at 40 W for 30 s and centrifuged at full force in a microfuge for 2 min. The supernatants were applied to Sepraphore III membranes (Gelman Sciences, Ann Arbor, MI).

Gel permeation chromatography (GPC) of the native isoenzymes was done using a Superdex 200HR 10/30 column (Pharmacia Biotech, Piscataway, NJ) equilibrated with 50 mM sodium phosphate buffer (pH 7) containing 150 mM NaCl, 0.5 mM EDTA and 1.4 mM 2-mercaptoethanol. GPC was conducted using a Beckman System Gold apparatus (Irvine, CA). To evaluate octamer stability, the high native Mr peak eluting from Superdex 200HR was collected and concentrated to 75 μl by spin filtration (Ultrafree, Millepore, Bedford, MA). A 30 μl aliquot of this highly pure sample was added to 30 ml of 50 mM sodium phosphate buffer (pH 7) containing 150 mM NaCl, 0.5 mM EDTA, 3 mM 2-mercaptoethanol and 5% glycerol (buffer B), mixed and the sample was injected into the Superdex 200HR column. A 30 μl aliquot of the above initial concentrate was added to 30 ml of buffer B containing 16 mM ADP, 20 mM $MgCl_2$, 80 mM creatine and 200 mM $NaNO_3$ (pH of solution had been adjusted to pH 7), mixed and incubated on ice for 60 min. Wyss et al. [13] have previously shown that these conditions promote formation of the TSAC and conversion of vertebrate MiCK to constituent dimers. After incubation, the sample was injected into the Superdex 200HR column.

Transmission electron microscopy was conducted on negative stained CK preparations. Purified CK was diluted to 9 μg/ml in buffered saline borate (BSB) composed of 133 mM NaCl, 162 mM boric acid and 35 mM NaOH. Electron microscopy was conducted using the negative stain 'sandwich' technique of Roux [18]. The carbon film was floated on droplets of the protein sample (10 s), washing solution (20% BSB, 15 s) and 2% uranyl formate (60 s). The film was picked up with a 600-mesh copper grid (Polysciences Inc., Worthington, PA), allowed to dry, and viewed on a JEOL 1200EX electron microscope. Electron micrographs were taken at ×100 000.

Protein assays and SDS-polyacrylamide gel electrophoresis were performed as previously described [17].

3. Results

Polychaete CK eluted as three peaks on hydroxyapatite – the first two overlapped somewhat (Fig. 1). This pattern of elution was highly reproducible. Tubes 47, 50 and 60 (corresponding to peak tubes for each CK fraction) were individually concentrated by centrifugal filtration. Cellulose acetate electrophoresis showed that the CK in tube 47 migrated slowly towards the anode while fraction 50 contained a minor

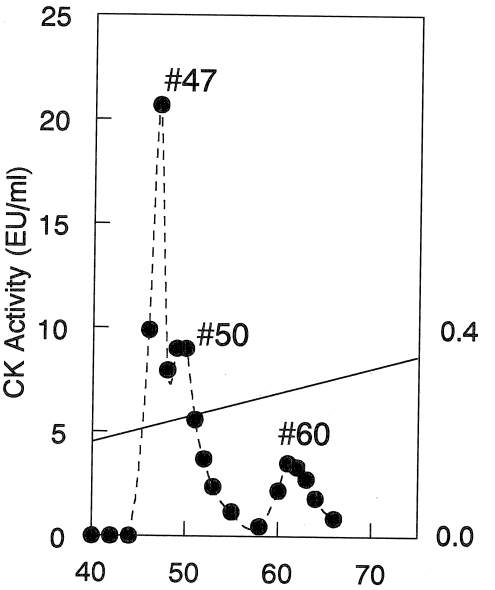

Fig. 1. CK elution profile (EU = μmol/min) versus fraction on a hydroxyapatite column. Scale on right ordinate corresponds to molarity of the potassium phosphate in the gradient.

band corresponding to the CK in #47 as well as a highly anodal form and a trailing minor band (Fig. 2A). These two major CKs were denoted CK1 (form in #47) and CK2 (major band in #50). CK1 had the same electrophoretic mobility as the CK from extracts of the body wall musculature (Fig. 2A). Fraction 60 showed an intense CK band which migrated slightly towards the cathode. This CK form was denoted CK3. Cellulose acetate electrophoresis of head/midpiece and tail enriched fractions showed that CK1 was present in both fractions, CK2 predominated in tail fractions and CK3 was found predominantly in the head/midpiece fraction (Fig. 2B). Thus, it seems likely that CK3 is the mitochondrial isoenzyme while CK2 is a flagellar specific form.

SDS-PAGE of the above fractions showed that the fraction containing CK1 (#47) (Fig. 2C) contained one major protein which migrated somewhat faster than ovalbumin. Fraction 50 contained a number of major protein bands one corresponding to CK1, the major band in fraction 47, plus a number of others one of which migrated somewhat slower than β-galactosidase. Fraction 60 had one intense, major band which corresponds to CK3 but there were minor contaminants (Fig. 2C). Subunit M_rs were determined by 10% SDS polyacrylamide gels. The subunit M_rs of CK1 and CK3 were 41.39 ± 0.49 kDa (S.D., $n = 3$) and 43.42 ± 0.65 kDa (S.D., $n = 3$), respectively. The approximate M_r of the slowly migrating protein in fraction 50, which we believe is CK2, was 130 kDa.

Native M_rs of the CK1 and CK3 were determined on a Superdex 200HR column using the following marker proteins: thyroglobulin (669 kDa), ferritin (440 kDa), aldolase (158 kDa), rabbit muscle creatine kinase (81 kDa) and ovalbumin (43 kDa). In the case of fraction 60, the major protein peak was collected, concentrated by spin filtration and rechromatographed to confirm that it represented CK3. The native M_rs of CK1 and CK3 were 87.09 ± 4.23 (S.D., $n = 4$) and 344.89 ± 4.75 (S.D., $n = 6$), respectively. Thus, CK1 exists as a dimer and CK3 appears to be octameric. LYS-C endopro-

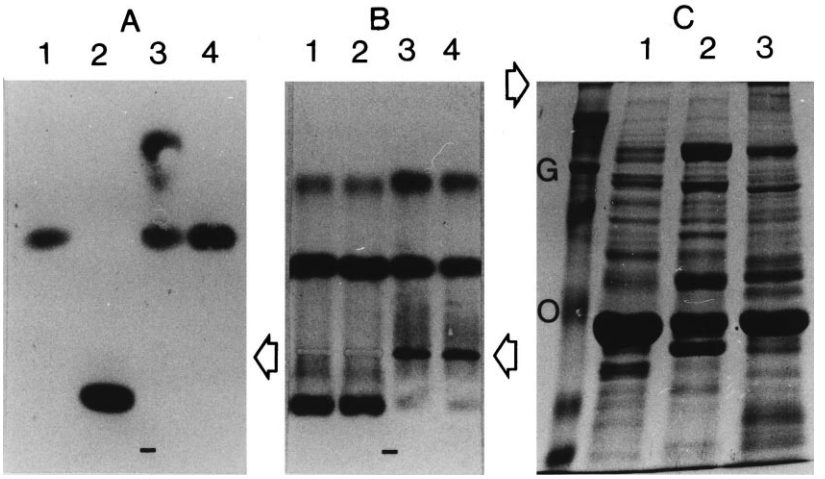

Fig. 2. Electrophoresis of CKs from *C. variopedatus* sperm. A: Cellulose acetate electrophoresis of a crude extract of *C. variopedatus* body wall muscle (lane 1), fraction 60 (lane 2), fraction 50 (lane 3), and fraction 47 (lane 4) from the column run depicted in Fig. 1. Arrow on bottom right indicates origin and minus sign indicates cathode end. B: Cellulose acetate electrophoresis of extracts of head/midpiece enriched (lanes 1 and 2) and tail enriched (lanes 3 and 4) samples of *C. variopedatus* sperm. Arrow on bottom right indicates origin and minus sign indicates cathode end. C: SDS-polyacrylamide gel electrophoresis of fractions 47 (lane 1), 50 (lane 2), and 60 (lane 3) from the chromatographic run depicted in Fig. 1. Lanes were deliberately overloaded to identify contaminants in each sample. 'G' corresponds to position of β-galactosidase (118 kDa) and 'O' corresponds to position of ovalbumin (45 kDa). Prestained standards were from Bio-Rad (Hercules, CA). Arrow on top left indicates origin.

teinase peptide maps of CK1 and CK3 showed that each had many unique peptides – these proteins are clearly products of different CK genes (data not shown). Contamination of the fraction containing CK2 precluded definitive analysis of this protein. However, preliminary GPC on Superdex 200HR showed that this form elutes after CK3 and before CK1 indicating an intermediate native M_r.

To further investigate the nature of the apparent CK3 octamer, Superdex 200HR fractions containing CK3 were concentrated and reinjected into the GPC column. The resulting protein absorbance profile displayed a large symmetrical peak in the octamer range but there was a small but detectable peak in the general region of where the dimers should elute (Fig. 3). Conversion of CK3 into the TSAC resulted in a slight reduction in the size of the octamer peak coincident with an increase in the size of the lower M_r peak consistent with conversion of octamers to dimers (Fig. 3).

Transmission electron micrographs of negative stained preparations of CK3 confirmed the octameric quaternary structure (Fig. 4a,b). Octamers were easily detectable. A central protein-free core (Fig. 4a,b) was present as has been previously observed in vertebrate MiCK [3].

4. Discussion

Sperm of the polychaete *C. variopedatus* have activities of CK in the order of 16.6 μmol/min/mg protein, which is highly comparable to activities in echinoderm sperm [19]. Sperm of this polychaete species contain three distinct isoenzymes of CK as judged by native and denatured electrophoretic differences and peptide mapping. Echinoderm spermatozoa also have multiple isoenzymes. A large single polypeptide consisting of three CK domains with an M_r of 126–145 kDa [20], which is myristoylated for binding with the axonemal mem-

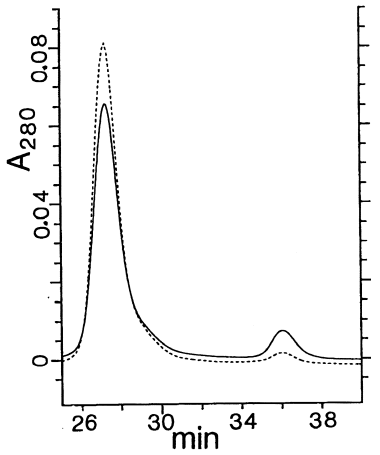

Fig. 3. Protein absorbance profile (280 nm) of the highly purified octomeric CK3 on Superdex HR200 before (dotted line) and after (solid line) conversion to the TSAC. Flow rate of column was 0.4 ml/min.

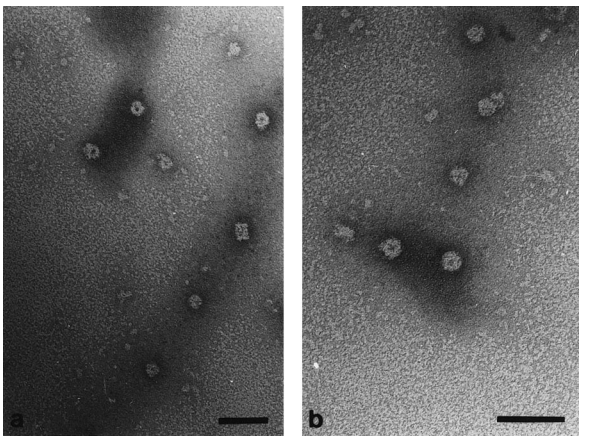

Fig. 4. a,b: Electron micrographs of octameric CK from the sperm of *C. variopedatus*. Bars correspond to 50 nm.

brane [21], is restricted to the flagella. An octameric, mitochondrial isoform is present [13]. Our results show that both flagellar (CK2) and mitochondrial (CK3) isoforms of CK, as well as a third ubiquitously distributed form (CK1), are present in *C. variopedatus* sperm. The CK2 and CK3 isoenzymes have strikingly similar M_rs to their echinoderm counterparts. Although CK2 remains poorly characterized, it is tempting to speculate that it exists as a contiguous trimer consisting of a three CK domain in a single polypeptide.

C. variopedatus octameric CK (CK3) shows great similarities to corresponding forms in vertebrates and in echinoderms. In the first place, native and subunit M_rs fall within the range of values for previously investigated MiCKs [1]. Secondly, polychaete CK3 migrates towards the cathode in cellulose acetate electrophoresis indicating that this protein has a p*I* greater than pH 8. Kamp et al. [15] also observed that the CK from the polychaete *Arenicola marina* sperm mitochondrial fractions had a p*I* of 7.9. Vertebrate MiCKs typically have similarly high p*I*s [1], giving the protein a net positive charge under physiological pH values which may facilitate binding to the outer portion of the inner mitochondrial membrane [1]. Interestingly, all previously investigated echinoderm MiCKs have p*I*s of 6.2 and below [12,13]. We further observed that when *C. variopedatus* octameric CK was equilibrated with the Superdex 200HR running buffer there was a slight conversion of octamers into what appear to be constituent dimers. This process was enhanced somewhat when the enzyme was converted into the TSAC (Fig. 3). Formation of the TSAC produces a rapid dissociation of vertebrate MiCKs into dimers [6] but is ineffective in echinoderm MiCK although high salt conditions could destabilize the octamer [13]. Finally, electron micrographs of the polychaete octameric CK display great similarities to morphology of MiCK from the vertebrates. On balance, octameric CK from the polychaete *C. variopedatus* has properties which closely resemble MiCKs from deuterostomes.

The present communication reports for the first time the presence of an octameric CK in a lower invertebrate. Previously described octameric MiCKs were from vertebrates and echinoderms, both deuterostome animals which diverged at least 670 million years ago from the other major animal line, the protostomes [16]. Polychaetes such as *C. variopedatus* have close affinities to other protostome groups including molluscs and minor marine groups such as echiuroids, sipunculids and brachiopods [22]. Given the similarities of *C. variopedatus* octameric CK to vertebrate/echinoderm MiCKs (as discussed above), it seems unlikely that octameric subunit organization evolved independently in these groups. More likely, octameric CKs evolved before the divergence of protostomes and deuterostomes indicating that octameric subunit structure is a primitive character of this enzyme family. To further clarify functional and evolutionary issues, we are currently in the process of cloning and sequencing the cDNAs for the major CK isoenzymes in *C. variopedatus* sperm.

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