Re-evaluation of the structure and physiological function of guanidino kinases in fruitfly (*Drosophila*), sea urchin (*Psammechinus miliaris*) and man

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Purification and biophysical characterization of mitochondrial creatine kinase (Mi-CK) from sperm of the sea urchin *Psammechinus miliaris*, as well as gel-permeation chromatography of human heart Mi-CK demonstrate that these two Mi-CK isoenzymes form highly symmetrical octameric molecules with an M_r of approx. 350000, a value similar to that found for all other Mi-CK isoenzymes investigated so far. The absolute evolutionary conservation of this oligomeric form from sea urchins to mammals points both to its essentiality for Mi-CK function and to an important role of octameric Mi-CK in the energy metabolism of tissues and cells with high and fluctuating energy demands. To investigate whether a similar physiological principle also operates in an even more distantly related animal phylum, the arginine kinase (ArgK) isoenzyme system of *Drosophila* flight muscle was investigated with two independent subcellular fractionation procedures and subsequent analysis of the fractions by SDS/PAGE, immunoblotting and native isoenzyme electrophoresis. In contrast with a previous report [Munneke and Collier (1988) Biochem. Genet. **26**, 131–141], strong evidence against the occurrence of a Mi-ArgK isoenzyme in *Drosophila* was obtained. The findings of the present study are discussed in the context of CK and ArgK function in general and of structural and bioenergetic differences between vertebrate striated muscles and arthropod flight muscles.

INTRODUCTION

Creatine kinase (CK) isoenzymes participate in the energy metabolism of tissues with high and fluctuating energy demands like heart, skeletal muscle, brain, retina or spermatozoa, by catalysing the reversible transfer of the γ-phosphate group of ATP to creatine (Cr) to yield ADP and phosphocreatine (PCr). Two principal functions have been assigned to the CK/PCr/Cr system (for reviews, see [1,2]): (i) in fast-twitch skeletal muscles, high levels of PCr and of CK activity in the cytosol efficiently buffer [ATP] and [ADP] during short periods of intense work; (ii) in heart, slow-twitch skeletal muscles or spermatozoa, all of which depend on a more continuous delivery of 'high-energy phosphates' to sites of ATP utilization, mitochondrial (Mi-) CK located in the mitochondrial intermembrane space and cytosolic CK coupled to ATP-requiring processes operate in opposite directions and thus form a 'shuttle' (transport) device for high-energy phosphates [3]. According to this model, PCr and Cr increase considerably the capacity of high-energy phosphate transport, since they are present in higher intracellular concentrations and also diffuse more quickly than ATP and ADP.

While the 'buffer function' of the CK/PCr/Cr system relies only on a cytosolic CK isoenzyme, the 'transport function' may depend on both cytosolic and Mi-CK isoenzymes. This notion is supported by the observation that, in cardiac muscle, the proportion of Mi-CK is far higher (up to 35% of total CK activity) than in fast-twitch skeletal muscles (0.5–2%). Thus the buffer and transport functions should be regarded as extreme situations of a highly flexible and adaptable system, with the physiological requirements of a tissue determining the relative importance of either function and, consequently, the proportion of Mi-CK.

Most Mi-CK isoenzymes investigated so far (bovine, chicken, pig, pigeon, rabbit and rat Mi-CK) form octameric molecules that are composed of eight identical subunits with an M_r of $\approx$ 43000 each (for a review, see [2]). Mi-CK octamers are highly symmetrical (space group P422) cube-like molecules displaying a side length of 10 nm and a central channel through the molecule [4]. The highly symmetrical Mi-CK octamers are able to mediate contacts between two opposing membranes [5] and are in fact accumulated in mitochondrial contact sites [6,7]. This specific localization may be an essential prerequisite for the physiological function(s) of Mi-CK (see [2],[8] and [9]).

The finding of distinct mitochondrial and cytosolic guanidino kinase isoenzymes and the conservation of the octameric structure of Mi-CK over a wide evolutionary time span suggests that Mi-CK and its highly symmetrical octameric structure are required for the transport function of the CK/PCr/Cr system. To examine this possibility, we conducted two sets of experiments: (i) for native sea-urchin (*Psammechinus miliaris*) sperm and human heart Mi-CK, M_r values of 180000–250000 have been reported, suggesting tetrameric or hexameric molecules [10–13]. Since sea-urchin sperm and human heart Mi-CK at present represent the 'evolutionary cornerstones' of Mi-CK research, we re-investigated the molecular size and structure of these two isoenzymes; (ii) in arthropods, arginine kinase (ArgK), phosphoarginine (PArg) and Arg substitute for CK, PCr, and Cr and appear to fulfil physiological functions similar to those of the CK/PCr/Cr system in vertebrates. An important difference, however, is that the oxidative capacity is considerably higher in

Abbreviations used: CK, creatine kinase; Mi-CK, mitochondrial CK; Mi_b-CK, Mi-CK isoenzyme from chicken heart; Cr, creatine; PCr, phosphocreatine; ArgK, arginine kinase; PArg, phosphoarginine; PMSF, phenylmethanesulphonyl fluoride; 2-ME, 2-mercaptoethanol; IFM, indirect flight muscle; GdmCl, guanidinium chloride; GPC, gel-permeation chromatography.

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insect flight muscle than in vertebrate striated muscle ([14,15]; but see [6–18]). Thus, if the transport function requires that the Mi-CK or Mi-ArgK content correlates with the oxidative capacity of a tissue, as one would expect, then insect flight muscle should contain a relatively large amount of Mi-ArgK. Munneke and Collier [19] have reported that *Drosophila* flight muscle does contain a 'Mi-ArgK' isoenzyme, but the fraction amounted to less than 3% of total ArgK activity. Unfortunately, no attempt was made by these workers to determine the molecular size and structure of this isoenzyme in order to rule out the possibility that the putative mitochondrial fraction was a cytosolic ArgK contaminant. We therefore re-investigated the ArgK isoenzyme system in *Drosophila* flight muscle.

MATERIALS AND METHODS

Purification of sea-urchin sperm Mi-CK

Purification of Mi-CK from sperm of the sea urchin *P. miliaris* was based on the procedure described by Tombes and Shapiro [13]. All purification steps were performed at 4 °C. Release of sperm into solution A (450 mM NaCl/10 mM KCl/50 mM $MgCl_2$/1 mM EGTA/15 mM Tris/15 mM Hepes/1 mM NaN_3, pH 8.0; 15–90 ml/animal) was induced by intracoelomic injection of 0.5 M KCl in solution A. Sperm were pelleted by centrifugation at 5000 *g* for 10 min, resuspended in 12–60 ml of solution A/animal and again centrifuged at 5000 *g* for 10 min. After resuspension in 4–30 ml of solution A/animal and homogenization of the sperm with ten strokes of a glass/Teflon homogenizer, sperm heads were separated from the tails by two consecutive centrifugations at 1500 *g* for 10 min. The pellets of the first and second centrifugation step were resuspended in 4–30 ml of solution A and 6–25 ml of solution B per animal respectively [solution B is 5% (v/v) Nonidet P-40/10% (v/v) glycerol/5 mM $MgCl_2$/125 mM KCl/1 mM EGTA/1 mM 2-mercaptoethanol (2-ME)/0.2 mM ADP·Na_2/0.2 mM phenylmethanesulphonyl fluoride (PMSF)/20 mM Hepes/10 mM Tris/1 mM NaN_3/soybean trypsin inhibitor (10 mg/l), pH 7.0].

Sperm heads in solution B were homogenized with ten strokes of a glass/Teflon homogenizer, stirred for 1–2 h on ice, and homogenized again. After centrifugation for 1 h at 104500 *g*, the supernatant, containing most of the Mi-CK, was dialysed extensively against solution C (15 mM Hepes/15 mM Tris/0.5 mM EGTA/0.2 mM ADP·Na_2/1 mM 2-ME/10 mM KCl, pH 8.0). In this solution of low ionic strength and containing neither detergent nor glycerol, sea-urchin sperm Mi-CK precipitates rather selectively (Figure 1 below). Mi-CK was pelleted at 30600 *g* for 30 min, resuspended in a small volume of solution D (10% glycerol/25 mM NaH_2PO_4/50 mM NaCl/2 mM 2-ME/0.2 mM EDTA, pH 7.0) and loaded directly on to a Mono S HR 5/5 cation-exchange chromatography column (FPLC, Pharmacia). Mi-CK was eluted with a linear salt gradient from 0.05 to 1 M NaCl.

Enrichment of human heart Mi-CK

All steps were performed at 4 °C. A 2.5 g portion of human heart tissue was homogenized with a glass/Teflon homogenizer in 23 ml of solution E (220 mM D-mannitol/70 mM sucrose/10 mM Hepes/0.2 mM EDTA/1 mM 2-ME/1 mM NaN_3, pH 7.4). After a low-speed centrifugation at 1000 *g* for 10 min to remove cellular debris and nuclei, mitochondria were pelleted at 15000 *g* for 15 min, resuspended in 5 ml of solution E, and pelleted again. The pellet was resuspended in 1 ml of solution F (83 mM NaH_2PO_4/0.05% Triton X-100, pH 7.5). The suspension was stirred for 30–45 min and sonicated repeatedly (Branson 1200 sonifier). Then the membranes were pelleted at 14000 *g* for 60 min. The supernatant containing soluble Mi-CK was sterile-filtered and used directly for gel-permeation chromatography (GPC). Dimeric human heart Mi-CK was obtained by mixing three parts of the sterile-filtered Mi-CK extract with 1 part of solution G (16 mM ADP/20 mM $MgCl_2$/80 mM Cr/200 mM KNO_3/50 mM NaH_2PO_4/150 mM NaCl/0.2 mM EDTA/2 mM 2-ME/1 mM NaN_3, pH 7.2) 4 h before GPC.

Investigation of the ArgK system of *Drosophila*

For the experiment shown in Figure 4 (below), the cytosolic and mitochondrial fractions of *Drosophila* were prepared exactly as described by Munneke and Collier [19]. In short, whole flies were homogenized, and the cytosolic ('S6 fraction') and mitochondrial fractions were prepared by a differential centrifugation procedure. Mitochondrial proteins were solubilized by addition of 1% Triton X-100.

For the experiments shown in Figure 5 (below), the fractionation of *Drosophila* flight-muscle fibres was adapted from a previously published procedure [20]. For each fractionation, fibre bundles of dorsal longitudinal indirect flight muscle (IFM) were dissected from three thoraces of 3–5-day-old (adult) *Drosophila* immersed in cold mineral oil (pre-cooled in an ice bucket). The corresponding 36 individual IFM fibres were isolated from the bundles, mechanically skinned, and combined again. Separation into a cytosolic, an organelle (mitochondria, T-tubule system and sarcoplasmic reticulum) and a cytomatrix fraction was attained by consecutively transferring the combined skinned fibres through a series of six 15 μl drops of relaxing solution, all under oil. The relaxing solution ($[Ca^{2+}] = 10$ nM) contained 18 mM MgATP, 1 mM free Mg^{2+}, 5 mM EGTA and 20 mM Bes, pH 7.0, and was adjusted to *I*0.15 mol/litre with potassium methanesulphonate.

Incubation for 20 min in drop 1, which additionally contained 50 mM sucrose and 100 μM leupeptin, allowed all readily diffusible protein (mostly ArgK and glycolytic enzymes) to diffuse out of the fibres. Subsequently, the fibres were rinsed by incubation in drops 2 and 3 for 20 min and 5 min respectively. Immersion of the fibres for 60 min in drop 4, which additionally contained 0.5% (w/v) Triton X-100, allowed mitochondria, T-tubule system and sarcoplasmic reticulum to be solubilized and the proteins comprising these organelles to diffuse out of the fibres. After rinsing in drops 5 and 6 for 10 min and 5 min respectively the remaining fibre mass was transferred to 10 μl of SDS/PAGE sample buffer and sonicated for 15 s. Drops 1–6 were also transferred to 10 μl of SDS/PAGE sample buffer. All samples were stored at −20 °C until analysis.

Other methods

GPC was performed on a FPLC Superose 12 column (Pharmacia). Solution H (50 mM NaH_2PO_4/150 mM NaCl/0.2 mM EDTA/2 mM 2-ME/1 mM NaN_3, pH 7.2) served as running buffer for the experiments with human heart Mi-CK; 885 parts of solution H were mixed with 115 parts of glycerol (87%) for the experiments with sea-urchin sperm Mi-CK in order to avoid precipitation of the protein.

In immunoblotting experiments, proteins were separated by SDS/PAGE (4% stacking gel; 10% running gel), semi-dry-blotted on to nitrocellulose membranes at a constant current of 1 mA/cm² for 105 min, incubated consecutively with a specific primary antibody and with a horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (Nordic, Biogenzia Lemania AG, Lausanne, Switzerland), and specifically stained

with 4-chloronaphthol as peroxidase substrate. Polyclonal rabbit antibodies against chicken Mi_b-CK, chicken B-CK, 'tail' CK from the sea urchin *Strongylocentrotus* (kindly given by Dr. A. Quest), *Drosophila* ArgK [21], *Drosophila* myosin light chain, and the β-subunit of *Drosophila melanogaster* ATP synthase ([22]; kindly provided by Professor R. Garesse) were used as primary antibodies.

CK activity was measured by the pH-stat method in the direction of ATP synthesis, as described previously [23]. Protein concentrations were determined as described by Bradford [24], with BSA (Pierce) as standard. Two-dimensional gel electrophoresis, cellulose polyacetate electrophoresis and N-terminal amino acid sequencing were performed as described in [25], [26] and [23] respectively. Results are given as means $\pm$ S.D.

RESULTS

Purification of sea-urchin sperm Mi-CK

The purification of *P. miliaris* sperm Mi-CK was based on the method developed by Tombes and Shapiro [13] for *Strongylocentrotus* sperm Mi-CK. Briefly, the procedure involves four steps: rudimentary differential centrifugation, extraction of Mi-CK from the sperm membranes by high concentrations of detergent, precipitation of Mi-CK in a low-ionic-strength buffer and cation-exchange chromatography (for details, see [27]).

Owing to the large difference in M_r, sea-urchin sperm Mi-CK ($M_r \approx 45000$) and cytosolic tail CK ($M_r \approx 145000$) can easily be discriminated on SDS/polyacrylamide gels (Figure 1; [13,28–32]). In addition, Coomassie Blue staining (Figure 1a) as well as immunoblotting experiments with a variety of antibodies against CK isoenzymes (Figures 1b–1d) demonstrated that *Psammechinus* sperm Mi-CK is resolved into two bands. This compares favourably well with the situation in *Strongylocentrotus* sperm, where 2,4-dinitrofluorobenzene labelling revealed three Mi-CK bands with M_r values of 44000, 47000 and 50000 [13,28].

When the upper and lower bands of *Psammechinus* Mi-CK were excised from SDS/polyacrylamide gels and re-electrophoresed separately, no interconversion became apparent (Figure 1e). Partial CNBr digestion of the upper and lower band of Mi-CK, on the other hand, gave rise to very similar peptide patterns, with just a few distinctive bands (results not shown). These data suggest that the upper and lower band of *Psammechinus* Mi-CK are derived from the same gene, but differ in biochemical and biophysical properties due, e.g., to differential splicing, post-translational modification, removal of a signal peptide at the N-terminus or partial proteolytic fragmentation.

Incubation of *Psammechinus* sperm Mi-CK in a low-ionic-strength buffer resulted in selective precipitation of Mi-CK (Figure 1a, lanes 2 and 3). Mono S cation-exchange chromatography (Figure 1a, lane 4) not only caused further enrichment of Mi-CK, but largely reduced the proportion of the upper Mi-CK band as well. Purified *Psammechinus* sperm Mi-CK displayed a specific activity of 177 ± 18 units/mg of protein ($n = 3$), which agrees with the value of 180 units/mg of protein (25 °C, pH 6.5) reported for *Strongylocentrotus* sperm Mi-CK [13]. Purified *Psammechinus* sperm Mi-CK was enzymically stable at 4 °C for several weeks, provided that 10% glycerol and sufficient amounts of 2-ME were present in the storage solution. Omission of glycerol in all instances caused immediate precipitation of Mi-CK (see also [13]). Probably on account of this pronounced tendency to precipitate, all attempts to separate sea-urchin sperm CK isoenzymes by cellulose acetate or agar gel electrophoresis failed, even when 10% glycerol was included in the electrophoresis buffer.

Surprisingly, antibodies against chicken cytosolic (B-) and Mi_b-CK stained an additional band in between sea-urchin tail and Mi-CK (Figure 1; marked by a star). Whether this band represents a further (possibly duplicated) CK or ArgK isoenzyme or whether staining is due to accidental cross-reactivity remains to be established. Nevertheless, the high specificity of most of the antibodies raised against chicken CK isoenzymes also for sea-

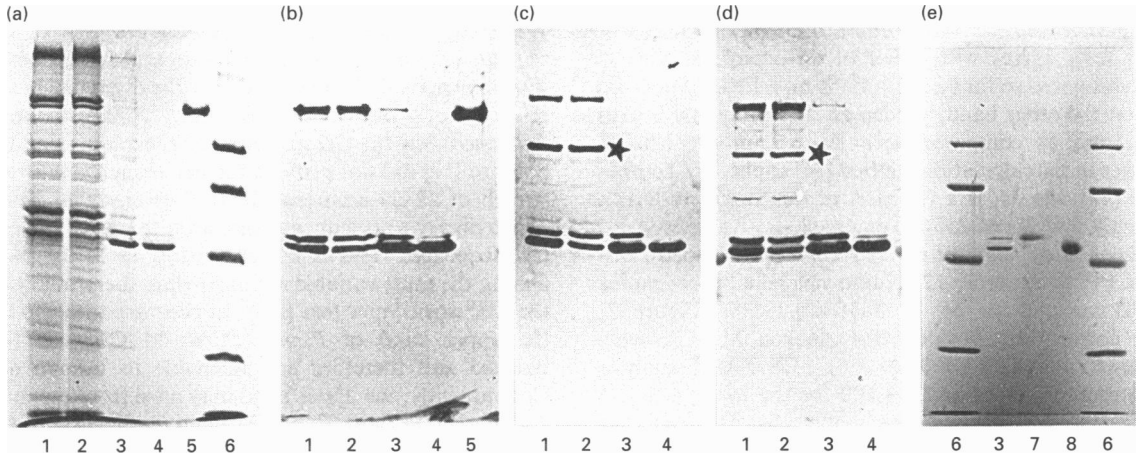

Figure 1 Purification of sea-urchin sperm Mi-CK: documentation by SDS/PAGE and immunoblotting

Mi-CK was purified from sea-urchin sperm as described in the Materials and methods section. Lanes 1, Mi-CK extracted from the sperm membranes; lanes 2 and 3, supernatant (lane 2) and pellet fraction (lane 3) of the centrifugation step after selective precipitation of Mi-CK in a low-ionic-strength solution; lanes 4, Mi-CK after Mono S cation-exchange chromatography; lanes 5, purified sperm tail CK of the sea urchin *S. purpuratus*; lanes 6, low-M_r standard from Bio-Rad comprising phosphorylase *b* (M_r 97400), BSA (66200), ovalbumin (42700), carbonic anhydrase (31000) and soybean trypsin inhibitor (21500); lanes 7 and 8, re-electrophoresis of the upper and lower band of *P. miliaris* Mi-CK after excision of the respective band from a (first) SDS/polyacrylamide gel. (**a** and **e**) Coomassie blue staining; (**b–d**) gels like the one shown in (**a**) were semi-dry-blotted on to nitrocellulose membranes and 'stained' with the following antibodies: (**b**) antibody directed against *Strongylocentrotus* tail CK; (**c**) anti-chicken B-CK antibody; (**d**) anti-chicken Mi_b-CK antibody. A band which is stained by antibodies against both chicken B- and Mi_b-CK and which may actually represent an additional (possibly duplicated) CK isoenzyme is marked by a star.

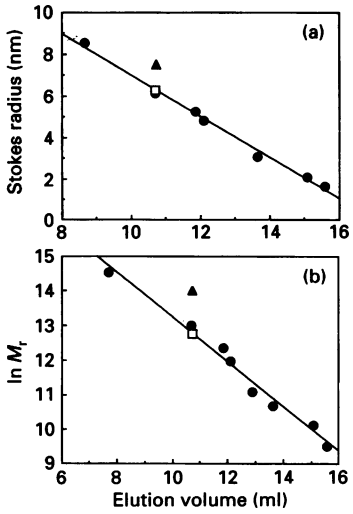

Figure 2 Octameric structure of sea-urchin sperm Mi-CK

The Stokes radius (**a**) as well as the M_r (**b**) of *Psammechinus* sperm Mi-CK (□) were determined by GPC on an FPLC Superose 12 column (Pharmacia). The marker proteins (●) used were (gel filtration calibration kit of Pharmacia): (**a**) thyroglobulin (8.5 nm), ferritin (6.1 nm), catalase (5.22 nm), aldolase (4.81 nm), ovalbumin (3.05 nm), chymotrypsinogen A (2.09 nm) and RNase A (1.64 nm); (**b**) Blue Dextran 2000 (M_r 2000000), ferritin (440000), catalase (232000), aldolase (158000), BSA (66200), ovalbumin (43000), chymotrypsinogen A (25000) and RNase A (13700). For comparison, the elution position of chicken Mi_b-CK octamers is indicated by ▲.

urchin sperm tail and Mi-CK hints at a high degree of primary and secondary structure conservation of CK from sea urchins to mammals (see also [33]).

Biophysical characterization of sea-urchin sperm Mi-CK

Tombes and Shapiro [13] used two, seemingly independent, methods for the estimation of the molecular size of *Strongylocentrotus* sperm Mi-CK: sedimentation velocity ultracentrifugation revealed a sedimentation coefficient of 12.4 S, a value that fully agrees with those of all octameric Mi-CK molecules investigated so far (see [2]). GPC on a FPLC Superose 12 column, on the other hand, yielded an unusually low Stokes radius of 4.5 nm as compared with 5.6–6.5 nm for Mi-CK octamers. Since in the calculation method [34] applied by Tombes and Shapiro [13], the M_r is a function of the Stokes radius, a correspondingly low M_r of 235000 emerged.

In the present study, GPC of native *Psammechinus* sperm Mi-CK also on a FPLC Superose 12 column yielded a Stokes radius of 6.28 ± 0.09 nm and an M_r of 353000 ± 19000 (Figure 2; $n = 7$). The corresponding values for chicken Mi_b-CK were 6.25 ± 0.02 nm and 346000 ± 4000 ($n = 3$). SDS/PAGE analysis revealed a protomer M_r of 44400 ± 600 for the lower ($n = 11$) and one of 47600 ± 700 for the upper band ($n = 8$) of *Psammechinus* Mi-CK. Electron micrographs of *Psammechinus* Mi-CK, relative to those of chicken Mi_b-CK [35–37], were characterized by inferior resolution, a fact that is most likely due to the high concentrations of glycerol present in the sea-urchin Mi-CK samples. Nevertheless, negatively stained sea-urchin sperm Mi-CK molecules appeared cube-like in shape, displaying a 4-fold symmetry and a central cavity, or even a channel, through the molecule (not shown; see [27]). These structural features are highly reminiscent of negatively stained Mi-CK octamers from other species. Surprisingly, in one instance, *Psammechinus* Mi-CK spontaneously formed small stretches of linear filaments (see [27]) similar to the ones previously observed for chicken Mi_b-CK [2,4]. To conclude, these results strongly suggest that sea-urchin sperm Mi-CK also forms octameric molecules, and that the underestimation of the M_r by Tombes and Shapiro [13] is due to an artefact in the determination of the Stokes radius.

As far as the dimer/octamer ratio of Mi-CK is concerned, formation of a transition-state analogue complex of *Psammechinus* Mi-CK (Mi-CK + MgADP + Cr + nitrate) did not result in dissociation of octamers into dimers, in contrast with most other Mi-CK isoenzymes investigated so far. While incubation with 0.5 M KI or 1 M guanidinium chloride (GdmCl) caused complete dissociation of chicken Mi_b-CK octamers into dimers, again no effect on *Psammechinus* sperm Mi-CK octamers was observed. However, slight dissociation of *Psammechinus* sperm Mi-CK occurred with 1 M KI or 2 M GdmCl, and almost complete dissociation with 1.5 M KI or 3 M GdmCl. These findings may be due either to a higher stability of sea-urchin sperm Mi-CK octamers or to the presence of 10% glycerol, which was shown to influence the stability of other oligomeric proteins (see [38]). The latter hypothesis, however, seems unlikely, since 10% glycerol had no obvious effect on the stability of chicken Mi_b-CK octamers (not shown).

Two-dimensional gel electrophoresis revealed pI values of 5.95 ($n = 4$), 6.05 ($n = 4$) and 5.8-5.9 ($n = 4$) for the lower band of *Psammechinus* Mi-CK, the upper band of *Psammechinus* Mi-CK, and *Strongylocentrotus* tail CK respectively (not shown; see [27]). Whereas Mi-CK gave clearly defined spots, *Strongylocentrotus* tail CK was resolved into several subspecies. For unknown reasons, our values are somewhat lower than the previously reported pI values of 6.2 for the mitochondrial and of 6.45–6.9 for the flagellar tail CK isoenzymes of *Strongylocentrotus purpuratus* [13,30].

Tombes and Shapiro [13] determined the N-terminus of *Strongylocentrotus* Mi-CK to be Ala-Ala-Asn-Gly. Edman degradation of the lower band of *Psammechinus* Mi-CK, after covalent modification of the Cys residues, yielded the N-terminal sequence: Ala-Ala-Asn-Cys-Thr-Leu-Cys-Glu-Asp-Gly-Ser-Tyr-Thr-Pro-Val-Tyr-Gly-Gln-Lys-Lys-Asn-Phe-(Leu)-(Leu). When modification of the Cys residues was omitted before sequencing, only the first four amino acids could be determined. This may actually be the reason for the discrepancy at the fourth residue of the sequences for *Strongylocentrotus* and *Psammechinus* Mi-CK and may also explain why Tombes and Shapiro [13] did not provide further sequence information. The stretch of 22–24 amino acids of *Psammechinus* Mi-CK shown above displays no sequence similarity to any of the other known CK sequences. This is not surprising, since the N-terminus is among the most variable regions within the primary structure of the CK isoenzymes (see [33]). In contrast with the lower band, the upper band of *Psammechinus* Mi-CK was N-terminally blocked and therefore not amenable to Edman degradation. Consequently, the upper band may arise from the lower band of *Psammechinus* Mi-CK by covalent modification at the N-terminus. As a matter of fact, N-terminal myristoylation has recently been demonstrated for *Strongylocentrotus* tail CK, with this modification mediating the binding of this CK isoenzyme to the sperm tail membrane [30,31].

Molecular size of human heart Mi-CK

The low amounts of tissue available precluded purification of human heart Mi-CK to homogeneity. Instead, Mi-CK was simply extracted from enriched mitochondria and its molecular

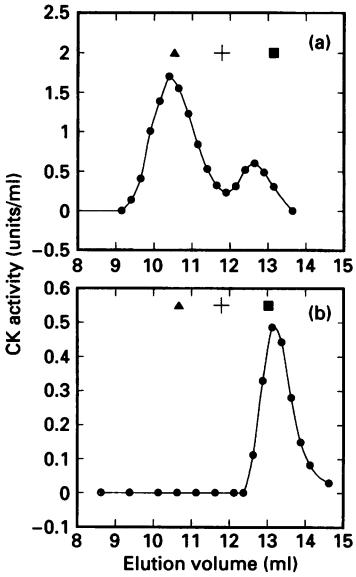

Figure 3 GPC of human heart Mi-CK

The molecular size of human heart Mi-CK, extracted from isolated mitochondria by phosphate, was analysed by GPC on an FPLC Superose 12 column. Fractions (250 μl each) were collected. The CK activity in each fraction was measured by the pH-stat method. ▲, ■ and + mark the elution positions of octameric chicken Mi_b-CK, dimeric chicken Mi_b-CK, and catalase (M_r 232000) respectively. (**a**) Freshly extracted human heart Mi-CK. (**b**) Human heart Mi-CK after a 4 h incubation with MgADP, Cr and nitrate, substances known to induce dissociation of Mi-CK octamers into dimers (for a review, see [2]).

size determined by GPC on a FPLC Superose 12 column. The eluate was collected in 250 μl fractions, and the CK activity in each fraction was determined by the pH-stat method.

Freshly extracted human heart Mi-CK (Figure 3a) gave an elution pattern virtually identical with that of chicken heart Mi_b-CK, with a large peak that was almost exactly co-eluted with octameric chicken Mi_b-CK, and with a smaller second peak at about the elution position of dimeric chicken Mi_b-CK (see Figure 5 in [35] and Figure 2 in [39]). On incubation with MgADP, Cr and nitrate {these conditions being known to induce dissociation of Mi-CK octamers into dimers (for a review, see [2])}, human heart Mi-CK was eluted as a single peak from the gel filtration column at a position coinciding with that of dimeric chicken Mi_b-CK (Figure 3b). Therefore it is quite obvious that human heart Mi-CK also forms both octameric and dimeric molecules which, depending on the experimental conditions, are readily interconvertible. As already discussed for rat heart Mi-CK [35], the underestimation of the M_r of human heart Mi-CK oligomers in previous studies [10–12] may be explained by partial dissociation of the (labile) Mi-CK octamers during conventional gel-filtration runs. This problem has now been overcome by the use of a (fast) FPLC Superose column.

The arginine kinase system of *D. melanogaster*

Munneke and Collier [19] reported that both a cytosolic and a mitochondrial ArgK isoenzyme exist in *D. melanogaster*. Their experimental evidence for the Mi-ArgK isoenzyme, however, is problematical. Munneke and Collier [19] separated the cytosolic and mitochondrial components of whole flies by differential centrifugation. When each component was electrophoresed separately on cellulose acetate membranes, 'Mi-ArgK' migrated somewhat more slowly than its cytosolic counterpart.

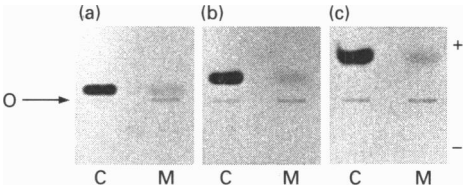

Figure 4 Cellulose polyacetate electrophoresis of *Drosophila* ArgK

Cytosolic (C) and mitochondrial (M) fractions, prepared from whole flies by the procedure of Munneke and Collier [19], were electrophoresed on cellulose acetate membranes at a constant voltage of 100 V for 15 (**a**), 30 (**b**) or 45 min (**c**). ArgK activity was subsequently revealed by the overlay-gel technique. Note the indistinguishable electrophoretic mobility of ArgK from both fractions. O, origin (point of sample application).

Quantification of the immunological cross-reactivity by the technique of microcomplement fixation suggested "that the isozymes differ by about one residue" (per 39000-M_r monomer) and "are the product of a single gene". Given the reliability of the microcomplementation technique, a sequence difference of only 0.3% must be within the range of error, rendering the conclusions of the study questionable.

To re-examine the conclusions of Munneke and Collier [19], we prepared cytosolic and mitochondrial fractions of *Drosophila* exactly as described by those workers. In our hands, electrophoresis of the fractions under a variety of conditions (varying the voltage applied, duration of electrophoresis, buffer composition or pH) produced bands of identical electrophoretic mobility (Figure 4). However, adding to the samples large concentrations of salts caused 'banana-shaped' bands and an apparent shift in electrophoretic mobility similar to those reported by Munneke and Collier [19] (results not shown). It is highly likely, therefore, that disturbances of the electric field caused by components present in the mitochondrial sample created the difference in mobility observed. Furthermore, the relative proportion of the mitochondrial fraction loaded per lane in Figure 4 was far higher than that of the cytosolic fraction, implying that the ArgK activity associated with mitochondria represents such a small percentage ($\ll 3\%$) of the total ArgK activity that contamination of the mitochondrial fraction by ArgK from the cytosol or from other organelles cannot be ruled out.

Nevertheless, homogenization of whole flies instead of isolated flight muscle may have obscured the presence of a Mi-ArgK isoenzyme. To circumvent this problem, cytosolic, organelle (mitochondria, T-tubule system and sarcoplasmic reticulum) and cytomatrix fractions were prepared from isolated single fibres of *Drosophila* flight muscle using a microdiffusion method that has been applied successfully to single muscle fibres of *Drosophila*, frog and rabbit [20,40,41]. Combined with SDS/PAGE and silver staining of the gel, this method allows a clean separation of subcellular compartments and direct revelation of their protein components from isolated, split muscle fibres (Figure 5a). Contamination of each fraction by components from the other subcellular compartments was virtually absent.

Experiments with control antibodies (against the β-subunit of the mitochondrial ATP synthase of *Drosophila* for the organelle fraction and against *Drosophila* myosin light chain for the cytomatrix fraction) confirmed the identity of each cellular fraction (Figures 5d and 5e). As can be deduced from Figure 5(c), $\leqslant 3\%$ of the total ArgK activity was associated with the organelle fraction of *Drosophila* flight-muscle fibres. The cytomatrix fraction completely lacked ArgK protein. Furthermore, the

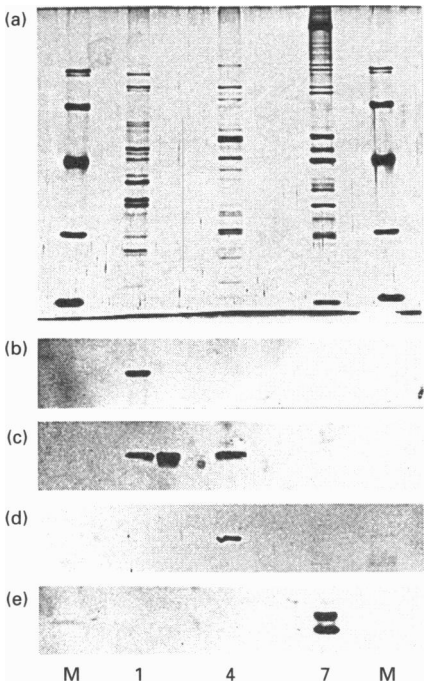

Figure 5 Fractionation of *Drosophila* flight-muscle fibres: analysis by SDS/PAGE and immunoblotting

Fibres of *Drosophila* flight muscle were skinned and separated into a cytosolic, organelle (mitochondria, T-tubule system and sarcoplasmic reticulum) and cytomatrix fraction by consecutive incubation in different solutions, as described in the Materials and methods section. Lane 1, cytosolic fraction; lanes 2 and 3, wash steps after removal of the cytosolic components; lane 4, organelle fraction; lanes 5 and 6, wash steps after solubilization and removal of the organelle components; lane 7, cytomatrix fraction; lane M, low-M_r standard (see Figure 1). (**a**) Silver staining; (**b–e**) immunoblots stained with antibodies against *Drosophila* ArgK (**b** and **c**), the β-subunit of *D. melanogaster* ATP synthase (**d**) and against *Drosophila* myosin light chain (**e**). In all lanes, the contents of 1.8–3 *Drosophila* flight muscle fibres were applied, except for (**c**), lanes 2–7, where 20 times the amount of protein was applied (contents of 36 muscle fibres).

electrophoretic mobilities of cytosolic and 'mitochondrial' ArgK on SDS/polyacrylamide gels (Figure 5c) were indistinguishable, confirming the results from cellulose acetate electrophoresis. It is therefore likely that the ArgK associated with the organelle fraction of *Drosophila* represents either cytosolic ArgK bound to the mitochondrial outer membrane or, more likely, cytosolic ArgK bound to the sarcoplasmic reticulum or to other membranes. In fact, significant amounts of MM-CK in vertebrate striated muscle are bound to the sarcoplasmic reticulum and to the plasma membrane, where they are functionally coupled to the Ca^{2+}-ATPase and the Na^+/K^+-ATPase respectively (for a review, see [1]).

DISCUSSION

In the present investigation, sea-urchin sperm and human heart Mi-CK were found by GPC (Figures 2 and 3) and electron microscopy (of *Psammechinus* Mi-CK; not shown here; see [27]) to form octameric molecules with an M_r of approx. 350000 like all other Mi-CK isoenzymes studied so far. The evolutionary conservation of the highly symmetrical octameric structure of Mi-CK from sea urchins to man strongly suggests that this oligomeric form of Mi-CK is essential to its function. This evolutionary conservation, together with the fact that sea urchins also have distinct mitochondrial and cytosolic CK isoenzymes, corroborates the conclusion drawn from sequence comparisons [33] that duplication of an ancestral CK gene, giving rise to a primordial Mi-CK and a primordial cytosolic CK gene, must have occurred before sea urchins and vertebrates started to diverge.

The present experiments also demonstrate that energy metabolism is organized differently in arthropods and vertebrates. In oxidative striated muscle of vertebrates, approx. 5–10% and up to 35% of total CK activity is bound to the myofibrillar M-line and to the mitochondrial inner membrane respectively [2,42]. It has been suggested that these two spatially segregated CK compartments constitute the producing and receiving end of a shuttle (transport) system for high-energy phosphates (for a review, see [1]). In contrast, *Drosophila* flight muscle, although containing large amounts of cytosolic ArgK, seems to lack both M-line-bound ArgK [21] and Mi-ArgK (the present study). It is therefore tempting to speculate that facilitation of high-energy phosphate transport from mitochondria to myofibrils is not a major function of ArgK in arthropods, or that a Mi-ArgK isoenzyme is not a prerequisite for the transport function of the ArgK system in insect flight muscle as it is in oxidative vertebrate striated muscle.

We hypothesize that Mi-CK becomes important for the transport function of the CK/PCr/Cr system only (or primarily) in cells displaying large diffusion distances between sites of ATP production and ATP utilization. In favour of this interpretation, experimental depletion of muscle Cr by feeding rats the Cr analogue 3-guanidinopropionic acid resulted in an adaptive 40–50% reduction in mean fibre size of type II muscle fibres to a value near that of smaller type I muscle fibres [43]. In contrast, the mean cross-sectional area of type I muscle fibres was not affected by Cr depletion. Furthermore, the importance of the CK/PCr/Cr system for efficient high-energy phosphate transport has only been proven for the highly polar sea-urchin sperm cell, which displays diffusion distances of up to 100 μm from the mitochondrion in the midpiece to the very end of the sperm tail. *Drosophila* flight muscle, on the other hand, is characterized by rows of mitochondria (making up 30–40% of the muscle volume) that line and envelope individual myofibrils, as well as by an intimate association between terminal tracheoles and mitochondria [17,44,45]. Thus *Drosophila* flight muscle possesses very short diffusion distances for oxygen and high-energy phosphates, negating the need for a Mi-ArgK isoenzyme in these muscles.

The results presented here invite further examination of the physiological functions of the CK/PCr/Cr and ArgK/PArg/Arg systems, with particular attention to Mi-CK. One can only speculate at this point that Mi-CK serves, in addition to its involvement in high-energy phosphate transport, some other physiological function yet to be defined.

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