Structure of the Mitochondrial Creatine Kinase Octamer: High-Resolution Shadowing and Image Averaging of Single Molecules and Formation of Linear Filaments under Specific Staining Conditions

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Abstract. The combination of high-resolution tantalum/tungsten (Ta/W) shadowing at very low specimen temperature (~250°C) under ultrahigh vacuum (<2 × 10^-9 mbar) with circular harmonic image averaging revealed details on the surface structure of mitochondrial creatine kinase (Mi-CK) molecules with a resolution <2.5 nm. Mi-CK octamers exhibit a cross-like surface depression dividing the square shaped projection of 10 × 10 nm into four equally sized subdomains, which correspond to the four dimers forming the octameric Mi-CK molecule. By a combination of positive staining (with uranyl acetate) and heavy metal shadowing, internal structures as well as the surface relief of Mi-CK were visualized at the same time at high resolution. Computational image analysis revealed only a single projection class of molecules, but the ability of Mi-CK to form linear filaments, as well as geometrical considerations concerning the formation of octamers by four equal, asymmetric dimers, suggest the existence of at least two distinct faces on the molecule. By image processing of Mi-CK filaments a side view of the octamer differing from the top-bottom projections of single molecules became evident showing a funnel-like access each form the top and bottom of the octamer connected by a central channel. The general structure of the Mi-CK octamer described here is relevant to the localization of the molecule at the inner–outer mitochondrial contact sites and to the function of Mi-CK as an “energy channeling” molecule.

Mitochondrial creatine kinase (Mi-CK, EC 2.7.3.2) (Jacobs et al., 1964; Jacobus and Lehninger, 1973), a member of the creatine kinase isoprotein family, had been proposed to form octameric oligomers (Jacobs, 1974; Jacobs and Graham, 1978), which have recently been characterized by biochemical and biophysical means (Schlegel et al., 1988a,b; Schnyder et al., 1988). Mi-CK from chicken cardiac muscle, investigated by gel-permeation chromatography, analytical ultracentrifugation and direct mass measurement with the scanning transmission electron microscope (STEM), exhibited an M_r of 328,000–340,000. Thus, the molecules consist of eight identical subunits (Schlegel et al., 1988a,b; Schnyder et al., 1988) each with a M_r of 43,195, as had been derived from a full-length cDNA clone (Hossle et al., 1988). The octameric form of Mi-CK is not restricted to chicken cardiac mitochondria, but had also been shown in chicken brain and mammalian heart (Schlegel et al., 1988a,b; Quemeneur et al., 1988; Lipskaya et al., 1989; Wyss et al., 1990).

The Mi-CK isoenzyme is specifically localized in mitochondria, where it is bound to the outer side of the inner mitochondrial membrane (Scholte et al., 1973). Recent biochemical data suggest that the octamer of Mi-CK is specifically accumulated in mitochondrial microcompartments where inner and outer membrane are in close contact (Adams et al., 1989). These findings are supported by direct immunogold localization of Mi-CK along the internal cristae, as well as at the periphery of mitochondria (Schlegel et al., 1988a).

Functional coupling of Mi-CK with the ATP/ADP-translocase, which translocates ATP generated by oxidative phosphorylation from the matrix through the inner membrane in exchange for ADP, has been demonstrated by several laboratories (Saks et al., 1980, 1985; Erickson-Viitanen et al., 1982; Jacobus, 1985; Brooks and Suelter, 1987; see Bessman and Carpenter, 1985).

Mi-CK is an enzyme thought to act as an ATP converter and supplier of phosphocreatine (PCr) from within the mito-
chondria to the cytoplasm, with PCr representing an intermediate storage and transport form of energy. In excitable tissues with sudden high and fluctuating energy demand, e.g., cardiac and skeletal muscle, brain, photoreceptor cells of the retina, spermatozoa and other cells, Mi-CK is expressed at high levels concomitantly to “cytosolic” CK isoforms (muscle-type M-CK and/or brain-type B-CK) (for review see Wallimann et al., 1989). Some of the cytosolic isoforms are also partially compartmentalized and localized at intracellular sites of high energy demand where they locally transphosphorylate Pcr into ATP. The creatine formed in this way is then transported back into the intermembrane space of mitochondria. Mi-CK again convert creatine via matrix-generated ATP to Pcr, which diffuses from mitochondria to the cytoplasm. This is the basis for the PCR-shuttle model proposed by several authors (Wallimann, 1975; Saks et al., 1978; Bessman and Geiger, 1981; Wallimann and Eppenberger, 1985; Jacobus, 1985).

Recent ideas about muscle energetics concerning the subcellular localization of CK isoenzymes as well as the structure-function relationship of the Mi-CK octamer, provide a more general PCR-circuit model representing an intricate network for energy buffering, energy transport and regulation of local ATP levels in muscle cells (Wallimann et al., 1989).

From the point of physiological as well as pathophysiological significance of CK, the elucidation of the molecular structure of Mi-CK seems of utmost importance. In addition to the conventional EM repertoire, such as negative staining and conventional heavy metal shadowing used in an earlier study of Mi-CK (Schnyder et al., 1988), high resolution shadowing at very low specimen temperature under ultra-high vacuum conditions (UHV) (Gross et al., 1985) was applied here. Due to the considerable improvement provided by this method, new structural details on the surface of individual Mi-CK octamers became apparent.

High resolution shadowing of positively stained and subsequently freeze-dried Mi-CK molecules exhibited further details concerning the central cavity and the arrangement of the subunits in the octamers. Under defined staining conditions it was possible to generate one-dimensional aggregates of Mi-CK molecules, which are displaying projections of side view of Mi-CK octamers.

**Materials and Methods**

**Isolation of Mi-CK and Preparation of Molecules for EM**

Mi-CK was isolated from chicken hearts according to the procedure of Schlegel et al. (1988a) and stored in small aliquots at a high protein concentration (10 mg/ml) under liquid nitrogen. Immediately before use the sample was diluted in storage buffer (25 mM NaPi, 50 mM NaCl, 2 mM betamercaptoethanol (BME), 0.2 mM Na2EDTA, 1 mM Na2O, pH 7.2) to a concentration of 20 μg/ml, adsorbed for 30 s onto glow-discharged carbon-coated (6-10 nm thickness) 400 mesh copper grids and washed with distilled water as described elsewhere (Schnyder et al., 1988).

**High-Resolution Heavy Metal Shadowing**

After the washing steps, excess water was removed by blotting with damp filter paper. For rapid freezing, the grids were quickly plunged into liquid nitrogen and, while submerged in liquid nitrogen, inserted into the specimen table. The table was then transferred via a counterflow loading device into the precooled specimen stage of the airlock of the UHV machine (BAF 500 K; Balzers Union, Balzers, Lichtenstein). The samples were freeze-dried in the airlock at −80°C for 2 h (p < 10−6 mbar). After freeze-drying, the table was manipulated into the precooled specimen stage (−80°C) of the UHV chamber and after having reached p < 2 × 10−5 mbar the specimen temperature was lowered to −250°C. Shadowing was performed either unidirectionally at an elevation angle of 45° or rotationally at an elevation angle of 30° with 0.7 nm tantalum/tungsten (Ta/W). To stabilize the evaporated metal film, an additional layer of 5 nm C was deposited perpendicular ("C-backing"). Film thickness and deposition rate were measured with a quartz crystal film monitor (QSG 301; Balzers) and a rate meter (QRG 301; Balzers), respectively. Evaporation was monitored by a pneumatically driven shutter, allowing reproducible deposition. After shadowing, the specimens were warmed up to room temperature and brought via atmospheric conditions into the transmission electron microscope.

**High-resolution UHV Shadowing of Positively Stained Mi-CK Octamers**

To exploit the specific advantages of two techniques for contrasting of molecules, a combination of uranyl acetate staining and freeze-drying/shadowing was worked out. The grids with the adsorbed molecules were washed as described, transferred to a drop of acidic uranyl acetate and freeze-dried/shadowed were put on a drop of distilled water for an additional min to remove unbound stain ("positive staining"). Excess liquid was removed and the grids plunged in liquid nitrogen. The subsequent steps were identical to the procedure described above for high-resolution shadowing.

**Formation of Mi-CK Filaments**

Under certain conditions "positive staining" of Mi-CK resulted in the formation of long filamentous structures. Mi-CK (10 mg/ml) was diluted 100 times with neutral uranyl acetate (2% uranyl acetate in a solution of 0.15 M sodium oxalate, adjusted to pH 7.4 with 5% NH3) and incubated overnight. Excess stain was removed by dialysis against distilled water for at least 2 h. Filaments formed in this way were then adsorbed onto glow-discharged carbon films and, without further washing, the samples were either air-dried or were freeze-dried (−80°C) and subsequently C-backed. Freeze-drying and C-coating were performed in a freeze-etch unit (BAF 400T; Balzers) (p < 5 × 10−7 mbar).

**EM**

The specimens were examined in a Philips 420 transmission electron microscope equipped with a liquid nitrogen cooled anticontamination device. Micrographs were taken at an acceleration voltage of 100 kV on Agfa-Gevaert Scientia films at a primary magnification of 49,000. The magnification was calibrated with the known lattice dimensions of a periodic test specimen (hexagonally packed intermediate layer from Deinococcus radio-durans). Pictures were routinely taken at 500-700 nm underfocus and checked for corrected astigmatism by optical diffraction.

**Image Processing of Single Molecules**

For image processing, suitable micrographs were scanned on an Optronics drum-type digitizer (Photomation P1700, Chelmsford, MA) with 25 μm sampling distance, corresponding to 0.52 nm on the specimen scale. The molecules were selected and aligned translationally relative to a centrosymmetric reference, which was synthesized according to the shape and intensity distribution of the molecules. The aligned images were surrounded with a circular mask and submitted to the circular harmonic averaging (CHA) procedure, a recently developed method for the processing of single macromolecules (Kunath and Sack-Kongehl, 1989). The rotational alignment and averaging is performed with the circular harmonic components allowing data compression and noise reduction. Additionally, the symmetry can be deduced from the rotational power spectrum (for details, see Winkler et al., 1990).
Figure 1. High-resolution unidirectional shadowing of Mi-CK octamers. Mi-CK octamers (20 μg/ml) were freeze-dried and unidirectionally shadowed at −250°C with 0.7 nm Ta/W at 45° elevation. The molecules show a round to square-shaped appearance and their surface is divided by a cross-like indentation into four hill-like subdomains. The molecule’s height estimated from the shadow casts approximates the side length of the square (10 nm).

Figure 2. High-resolution rotary shadowing of Mi-CK octamers. Mi-CK octamers (20 μg/ml) were freeze-dried and rotary shadowed at −250°C with 0.7 nm Ta/W at 30° elevation. Roundish to square particles (top-bottom view), sharply divided into four subdomains are representing the projection of dimers. (Inset) Averaged motif of 112 octamers.

Results

Ta/W shadowing at −250°C under UHV conditions applied to isolated single Mi-CK molecules revealed submolecular features that had not been seen before by conventional methods. After unidirectional metal shadowing with 0.7 nm Ta/W at an elevation angle of 45°, Mi-CK octamers appeared as square particles subdivided by a cross-like indentation into four subdomains with a “hill-like” surface relief structure on each quadrant (Fig. 1). This appearance strongly supports a fourfold symmetry of the Mi-CK molecule (Schnyder et al., 1988). The shadow length was similar to both side lengths of the molecule, therefore, a quasicubic shape had to be assumed for the Mi-CK octamer. The relief appearance after unidirectional shadowing changed with the orientation of the individual Mi-CK octamers relative to the evaporation source and the intercept of the two crossbars at the center of the molecule often appeared brighter than the bars themselves (Fig. 1). This was due to the lack of metal deposition on the molecule’s surface, indicating the existence of a central depression.

If Mi-CK octamers were rotary-shadowed with Ta/W at −250°C and at 30° elevation, the three-dimensional impression was lost, but the surface and the circumference of the individual molecules were visualized with even greater detail (Fig. 2). The molecules generally showed a roundish to square-shaped projection and a clear subdivision by a central “white cross” into four domains. The crossbars were <2.5 nm thick and mostly perpendicular to each other as well as to the sides of the molecules. After image processing, in the averaged motif built up from 112 octamers (Fig. 2, inset) the fourfold symmetry with the axis centered in the middle of...
the projection of the molecule was evident. The cross of low density dividing the molecule into four sectors (subdomains), was slightly rotated clockwise with respect to the assumed square shape. Each sector showed a very dense border line of accumulated heavy metal as well as a moderately dense tip oriented towards the center of the molecule projection. The low density zone in between those contrast maxima indicates an additional depression in each subdomain.

The information in the averaged image was mainly represented by symmetry components with n = 0, 4, and 8. Therefore, other symmetry components were considered as noise contributions and were suppressed. The significant components extended up to spatial frequencies of 1/2.5 nm⁻¹, which was the resolution limit for these images. The averaged structure was verified by comparing independent data sets whereby no significant differences between individual sets were found.

Since shadowing or negative staining give rather distinct information about macromolecules, e.g., shadowing replicates exclusively surface structures, whereas negative staining visualizes a projected view of the volume, we tried here to combine the two methods. The adsorbed Mi-CK molecules were first soaked in uranyl acetate solution and unbound stain as well as stain that was not trapped in intramolecular cavities was removed by a single washing step ("positive staining"). Thereafter, the stained molecules were frozen, freeze-dried and rotary shadowed as described in Materials and Methods. Such positively stained molecules after rotary shadowing with 0.7 nm Ta/W at 30° elevation angle were depicted in Fig. 3. The particles were very high in contrast, especially at the circumferential area. The central dark spot seen in negatively stained molecules (Schnyder et al., 1988) was also obvious, but the intramolecular subdivision into four quadrants was less pronounced if compared with shadowed-only molecules. The averaged motif of 135 selected molecules after CHA (Fig. 3, inset) was round to square shaped, showed very dense borders and a fourfold axis protruding from the electron dense center of the molecule became evident. Four low density regions around the heavily stained center divide the molecule into its subdomains.

Direct incubation of Mi-CK octamers in stain solution followed by removal of unbound uranyl acetate by dialysis against pure water resulted in the formation of regular one-dimensional aggregates of Mi-CK molecules (Figs. 4 and 5). These individually long ribbonlike filaments were rigid, unbranched and showed no lateral additions. The filaments shown in Fig. 4 were air-dried after adsorption on carbon grids. Since excess stain had been removed by the dialysis step, the structures were no longer stabilized by surrounding stain and lateral shrinkage upon air-drying as well as increased electron beam sensitivity was observed. The structural integrity, however, of the Mi-CK ribbons was significantly improved if the filaments were freeze-dried and stabilized with a carbon film (C-backing). This is demonstrated in Fig. 5, where many more details concerning the molecular arrangement of these filaments are obvious. The insets in Figs. 4 and 5 represent stretches of Mi-CK ribbons after computational straightening and averaging over 20-unit cells. From the laser diffraction pattern the repetitive distance along the axis of air- and freeze-dried filaments was calculated to be 16.8 nm, representing the distance between every second stain spot along the axis of the filament (Figs. 4 and 5, inset, see bars). This value is roughly twice the dimension of an octamer obtained from single molecule studies and may show the side projection of the arrangement of two octamers stacked onto their fourfold faces with an alternating orientation along the filament axis. Thus, the 8.4 nm represents the height of the octamer when associated into filaments.

Among all the staining solutions tried, besides uranyl acetate, only sodium phosphotungstate at pH 7.0 was able to induce linear Mi-CK aggregates. The structural quality, however, of the filaments generated by phosphotungstate was inferior since they were bent like spaghetti (with a repeating distance of ~190 Å) and were wrapped in an envelope of stain often obscuring any molecular details (data not shown). The unexpected finding of "one-dimensional crystals" of Mi-CK molecules indicate the existence of at least two mo-
lecular faces on the octamer, which differ in their physicochemical properties. Thus, a preferential association of two compatible surfaces during each elongation step leads to the formation of linear filaments.

Surprisingly, Mi-CK incubated in neutral uranyl acetate or sodium phosphotungstate solution retained 20 and 100% of its enzymatic activity, respectively, but after dialysis when filaments were formed, the enzymatic activity was totally lost. Any attempts to disintegrate these filamentous structures to recover their enzymatic activity by redialysis against high or low salt buffers at a broad pH range have failed so far. The material either clumped together or remained as rigid filaments. In general, filaments formed by uranyl acetate, after dialysis against water, were stable for months at 4°C.

**Discussion**

High-resolution shadowing at very low specimen temperature under UHV conditions, using Ta/W as shadowing material, resulted in a significant improvement of the resolution of structural details on the surface of Mi-CK octamers. Unidirectional shadowing of Mi-CK molecules yielded dis-
tinct shadow casts and a pronounced surface topology, which lead to the suggestion that the octamer is of quasicubic nature. Each octamer showed a crosslike indentation dividing the surface into four hill-like subdomains which must correspond to the four dimers forming the octameric Mi-CK structure. The crosslike indentations appeared especially crisp when rotary shadowing was applied. Some structural heterogeneity of the individual octamers was presumably introduced during sample preparation before freeze-drying and high-resolution shadowing. It is likely that this variability reflects some structural disorder within the octamers, i.e., stages of partial disintegration, rather than indicating the existence of different views of the same molecule.

It was assumed that a combination of conventional staining techniques with high-resolution shadowing might reveal more structural details, thus Mi-CK molecules were positively stained and additionally shadowed by heavy metal. This technique allowed to get at the same time information about the surface as well as the interior structure of the molecule; however, it also complicated the interpretation of the images. The central stain spot also observed by negative staining was persistent, but the partition of the molecule into four subdomains by the crosslike indentation was different from that seen after rotary shadowing alone. It seems that residual uranyl acetate bound to the periphery of the molecules was responsible for the quite different overall appearance of these octamers as compared with shadowed-only octamers.

Whatever technique applied to visualize Mi-CK molecules by negative staining (Schnyder et al., 1988) or by the methods described herein, visual inspection and computer classification using CHA always showed one single projection of octamers only. Furthermore, a multivariate statistical analysis (Van Heel, 1989) of 1,250 molecules (as shown in Fig. 2) failed to demonstrate more than one class of molecule views (Winkler, H., unpublished results). This indicates either that the Mi-CK octamer is cubic with six structurally identical faces at a resolution of 2.5 nm or that the octamers adsorb always with the same side(s) onto the support. Several approaches altering the adsorption properties of the molecules to the support, e.g., by using different plastic films, glucose embedding and polylysine treatment of the carbon support prior to molecule adsorption were unsuccessful in showing different faces of the octamer.

Since octamers can disintegrate into dimers without any stable intermediates (hexamers or tetramers) it is reasonable to believe that dimers are the building blocks of the octamer (Schlegel et al., 1988). This has been corroborated by experiments concerning heterooctamer formation between two different Mi-CK isoenzymes (Wyss et al., 1990) and infers that the top and bottom faces of the octamer must be different from the four side faces, assuming a parallel side-to-side arrangement of the "banana-shaped" dimers. Experimental support for this idea is gained from the appearance of linear Mi-CK filaments shown here. It is obvious that the faces of the octamers at which propagation occurs must differ in their physicochemical properties relative to the other faces.

The most straightforward interpretation of the Mi-CK filaments (Figs. 4 and 5) is given by the assumption of a simple face to face aggregation of octamers by contacts of their fourfold faces (seen in Figs. 2 and 3). Thereby, two octamers per 16.8-nm repeat are stacked with an alternating polarity along the filaments, giving rise to two different types of intermolecular interactions (top-top and bottom-bottom). Alternatively, since some regions of the filaments in Figs. 4 and 5 suggest a slightly different orientation of the same arrangement as above with slightly differing side projections of adjacent octamers, the molecules could be stacked onto their fourfold faces with a difference of 45° in the orientation of the molecules along the filament axis, representing a view of individual octamers generally ± 22.5° off the filament axis.

In both cases, the “height” of the octamer in the filament would be 8.4 nm, which corresponds to the shadow length of unidirectionally shadowed single molecules (Fig. 1). According to the assumptions made above, the structure of the filaments represents projections of the parallel alignment of dimers within the octamer. Thus, the stain-filled funnel-like depressions at the top and bottom of adjacent molecules account for the dumbbell-shaped side view of the octamer. The fine traces of stain between the dimers represent a central channel connecting the funnel-like access from top and bottom of the octamer as postulated earlier (Wallimann et al., 1989).

The above interpretation is supported by the findings that Mi-CK retains its oligomeric nature in stain solution and pure water (data not shown) and even shows partial or full activity, depending on the stain used. The loss of enzymatic activity is a result of filament formation and not a consequence of dialysis against water per se. Since the enzyme is active in pure water, it is likely that it is the association of Mi-CK into "one-dimensional crystals" itself that leads to a blocking of the enzyme activity. This implies that the access of the substrates to the active center would be restricted to the top and bottom face of the octamer. Further evidence for the octamer as building unit was gained from preliminary x-ray studies of Mi-CK crystals (Schnyder et al., 1990a), where a tetragonal unit cell with the dimensions of $a = b = 17.1$ nm and $c = 15.0$ nm was found and a packing of eight octamers per unit cell was suggested (Schnyder et al., 1990b).

Despite the straightforward interpretation of filament formation by a linear association of individual octamers, one cannot rule out that this process may be more complex, e.g., involving a rearrangement of Mi-CK dimers as a consequence of incorporation of stain. Indications that dimers may also be the building blocks came from filaments generated by phosphotungstate, which were often only a few subunits long, were loosely arranged and coexisted with free Mi-CK dimers (data not shown).

Although the Mi-CK filaments shown in Figs. 4 and 5 were obtained under artificial conditions, they gave for the first time experimental evidence that the Mi-CK octamer possesses two distinct faces with different physicochemical properties and, according to the above interpretation, also with different structural appearance. It is conceivable that those faces involved in filament formation are also responsible for the preferential adsorption of single molecules onto the support films giving rise to one class of single molecules only. Thanks to the formation of linear Mi-CK filaments, however, one was able to show also a sideview of the Mi-CK octamer, which shows a funnel-like access at the top and bottom connected by a small channel. This interpretation fits with the projection images of single molecules ad-
sorbed on their top or bottom faces and is fully in line with the proposed function of the Mi-CK octamer as a mitochondrial energy channeling molecule (Wallimann et al., 1989).

The occurrence of Mi-CK "aggregates" has been described earlier when Mi-CK molecules were centrifuged for 16 h at 100,000 g and the pellet was sectioned and stained with uranyl acetate (Farrell et al., 1972). These authors reported a "tightly packed linear structure forming parallel aggregates" demonstrating a regular periodicity of 7 nm, and related these structures to the intracristal rods observed in sections of beef heart mitochondria as a result of different fixatives (Hall and Crane, 1971). A structural correlation of our in vitro filaments to these findings is obvious.

Structurally and functionally different faces on the Mi-CK octamers may also be inferred from their localization at the outer side of the mitochondrial inner membrane as well as from the fact that a significant portion of Mi-CK is found at contact sites, where outer and inner membrane are in close proximity (Adams et al., 1989). Recently, it was shown that Mi-CK molecules seem to be able to interact simultaneously with outer and inner membrane phospholipids in a model membrane system (Rojo et al., 1990). In addition, Mi-CK may form a multienzyme energy channeling complex with the ATP/ADP translocase (Wallimann et al., 1989) as well as with an outer membrane pore protein (Adams et al., 1990).

We hope to get further insight about the orientation of the molecules by cryoelectron microscopy of ice-embedded Mi-CK octamers ("bare-grid methods"). A detailed structural analysis by x-ray diffraction of Mi-CK crystals that have been obtained (Schnyder et al., 1990b) is underway in our laboratory.

We wish to dedicate this paper to the late Dr. Wolfgang Kunath who died early this year.

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