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Crystallization of Mitochondrial Creatine Kinase

GROWING OF LARGE PROTEIN CRYSTALS AND ELECTRON MICROSCOPIC INVESTIGATION OF MICROCRYSTALS CONSISTING OF OCTAMERS*

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Mitochondrial creatine kinase isolated from chicken cardiac muscle was crystallized by vapor diffusion techniques. Depending on the growth conditions, fine needles and platelets as well as large single crystals appeared after a few days. Large crystals were shown to diffract to at least 3.2 Å resolution (Schnyder, T., Winkler, H., Gross, H., Sargent, D., Eppenberger, H. M., and Wallimann, T. (1990) *Biophys J.* 57, 420 and thus are suited for a detailed X-ray analysis in the future. The relatively high density of single crystals measured by a linear organic solvent density gradient indicates a tight packing of mitochondrial creatine kinase molecules within the crystals. Microcrystals, however, were subjected to electron optical examination either after prefixation with glutaraldehyde followed by conventional negative staining or by freeze-fracturing crystals in mother liquor and heavy metal replication with platinum/carbon. In both cases the crystals exhibited a square lattice with parameters of a = b = 139 Å and a = b = 132 Å in negatively stained and replicated crystals, respectively. No other lattice parameters were found, suggesting that these microcrystals represent a quasi-cubic three-dimensional lattice, which is in accordance with the finding that the building blocks of the crystals are the cube-like octamers described (Schnyder, T., Engel, A., Lustig, A., and Wallimann, T. (1988) *J. Biol. Chem.* 263, 16954–16962). Digital image processing applied to electron micrographs of crystals clearly revealed the arrangement of mitochondrial creatine kinase octamers in the crystal lattice as well as the subdivision of the octamer into its subdomains at a resolution of 23 Å.

Creatine kinase (CK[1]; EC 2.7.3.2) transphosphorylates reversibly the phosphoryl group of phosphocreatine to ADP to regenerate ATP. The enzyme exists in different isoforms and is responsible for various functions concerning the energy metabolism in cells of high fluctuating energy demand, *e.g.* muscle, brain, photoreceptors, and spermatozoa (for review, see Wallimann and Eppenberger, 1985; Wallimann *et al.*, 1989). Three of the CK forms, the so-called "cytosolic" isoenzymes (BB-, MB- and MM-CK), are dimeric and are expressed in a tissue-specific manner. Whereas brain-type BB-CK seems to be more or less ubiquitous, muscle-type MM-CK has been considered as a specific marker enzyme for differentiated muscle cells although small amounts of this isoenzyme may also be found in nonmuscle tissue, as demonstrated recently by Hamburg *et al.* (1990). The heterodimer, MB-CK, represents a transition form during muscle development in which the early BB-CK is replaced by the MM-CK. However, MB-CK is permanently expressed in adult cardiac muscle of mammals (for review, see Eppenberger *et al.*, 1983) and thus is used widely as a clinical post-heart infarction marker.

A fourth form, the mitochondrial creatine kinase (Mi-CK) (Jacobs *et al.*, 1964) is restricted to the mitochondria and also exists in tissue-specific isoforms (Hossle *et al.*, 1988; Haas *et al.*, 1989; Haas and Strauss, 1990; Wyss *et al.*, 1990).

Mi-CK is bound to the mitochondrial inner membrane (Scholte *et al.*, 1973) and was found to be accumulated also in mitochondrial contact sites (Adams *et al.*, 1989). The mitochondrial isoenzymes of CK are clearly different from the cytosolic forms in immunological properties, amino acid composition, cDNA sequence, and oligomeric state. Nevertheless, on the amino acid sequence level an extensive overall homology of 66 and 67% was found between Mi-CK and B- and M-CK, respectively, with six regions within the Mi-CK molecule showing 75–100% sequence homology with the cytosolic isoforms (Hossle *et al.*, 1988). A detailed characterization of the Mi-CK isolated from chicken heart muscle and brain by biochemical and biophysical means has been published (Schlegel *et al.*, 1988a, 1988b; Schnyder *et al.*, 1988; Wyss *et al.*, 1990). Mi-CK extracted from isolated mitochondria was shown to exist in an octameric form with a molecular mass of 340 kDa. At low protein concentrations and alkaline pH, the octamer dissociates slowly into stable dimers of 84 kDa. The dimer/octamer equilibrium is reversible and can be influenced by substrate combinations (Schlegel *et al.*, 1990; Wyss *et al.*, 1990). Even though the reason for the occurrence of these two oligomeric states, which differ only slightly in their kinetic behavior (Lipskaya *et al.*, 1989), is not clear, a marked difference in the pH dependence of their rebinding properties to the mitochondrial inner membrane has been demonstrated (Marcillat *et al.*, 1987; Schlegel *et al.*, 1990), and exclusively the octameric form was found in isolated contact sites (Adams *et al.*, 1989; Kottke *et al.*, 1990). Thus, we believe that the functional form of Mi-CK within mitochondria is the octamer (Schlegel *et al.*, 1990; Wallimann *et al.*, 1989; Wallimann and Eppenberger, 1990).

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[1] The abbreviations used are: CK, creatine kinase; B- and M-CK refer to the subunits of brain- and muscle-type CK, respectively, giving rise to three functional cytosolic CK isoforms, BB-, MB-, and MM-CK, whereas Mi-CK stands for the mitochondrial CK isoenzyme which is specifically located within mitochondria; PEG, polyethylene glycol; Pt/C, platinum/carbon; SDS, sodium dodecyl sulfate.

The structure of the Mi-CK octamer was studied extensively on the electron microscopic level. Negatively stained molecules exhibit a square projection with a central heavily stained portion (about 25 Å in diameter). The side length of the particles is about 100 Å, and the height, estimated by unidirectional shadowing with heavy metal, approximates the side length. These data, taken, together with hydrodynamic measurements by analytical ultracentrifugation, demonstrate that a globular or cube-like shape can be assumed for the Mi-CK octamer (Schnyder *et al.*, 1988). Improved metal shadowing techniques for specimen preparation revealed significantly more information on the surface relief of freeze-dried octamers, and a clear subdivision of the square projection into four subdomains by a cross-like surface depression was demonstrated (Schnyder *et al.*, 1989). Thus, the Mi-CK octamer is composed of four elongated dimers arranged around a central cavity. Independent of the technique of sample preparation, computational image processing revealed only one projection class of particles. However, geometrical considerations as well as the occurrence of linear Mi-CK filaments, obtained under specific conditions, are indicative for the existence of at least two distinct faces on the cube-like octamer which differ in their physicochemical properties and their structural appearance. Indeed, a second view of the octamer, representing the side projection, has been found recently by image processing of Mi-CK filaments composed of Mi-CK octamers stacked on top of each other along their 4-fold axis (Schnyder *et al.*, 1991a).

A detailed analysis of the structure of Mi-CK on a molecular level should be possible by x-ray diffraction analysis of protein crystals. To date, preliminary x-ray diffraction data including some space group information have been published for various crystals of the muscle isoform, MM-CK (McPherson, 1973; Burgess *et al.*, 1978; Takasawa *et al.*, 1981; Gilliland *et al.*, 1983; Hershenson *et al.*, 1986). Crystallization of BB-CK purified from calf brain (Keutel *et al.*, 1968) and of Mi-CK from human heart muscle (Blum *et al.*, 1983) were also reported, but none of these crystals seemed to be suitable for further analysis by x-rays so that no space groups could be determined for the latter small protein crystals.

Here, we report on the crystallization of Mi-CK from chicken heart purified according to a published procedure (Schlegel *et al.*, 1988a). In addition, the growth of large single crystals suitable for x-ray diffraction analysis and density measurements of these crystals in an organic solvent gradient suited for dense protein crystals are described. The high density of Mi-CK crystals indicates a tight packing of Mi-CK octamers in these crystals. An electron optical investigation of Mi-CK microcrystals was performed by contrasting them with stain solution as well as by freeze-fracture/replication of crystals in mother liquor followed by computer-based image processing. The lattice constants were calculated, and it could be shown that the building blocks of the crystals are indeed Mi-CK octamers. These data support the fact that under a variety of conditions the Mi-CK enzyme exists as an octamer, representing the same oligomeric form of the enzyme which is compatible with the proposed function of Mi-CK as an "energy-channeling molecule" for energy translocation in mitochondria (Wallimann *et al.*, 1989).

EXPERIMENTAL PROCEDURES

Isolation of Mi-CK—The enzyme was isolated and purified from chicken myocardium essentially according to the published procedure (Schlegel *et al.*, 1988a), concentrated to 13–14 mg/ml in a Centricon microconcentrator (Amicon), and stored in small aliquots in liquid nitrogen. Immediately before use the protein solution (30–100 μl) was dialyzed for 1 h at 4 °C against 100 ml of 25 mM phosphate buffer containing 50 mM NaCl, 2 mM β-mercaptoethanol; 0.2 mM Na_2EDTA, 1 mM NaN_3 at a pH ranging from 6.0 to 7.6 in a Bethesda Research Laboratories microdialysis system (model 1200MA) connected to a Vario-Perpex peristaltic pump at maximum flow rate.

Crystallization of Mi-CK—Microcrystals were grown by vapor diffusion using the hanging drop technique at ammonium sulfate reservoir concentrations ranging from 30 to 38% at pH 7.4–7.6 after a few days. Tiny crystal platelets as well as large single crystals were obtained using PEG 1000 (Merck) at reservoir concentrations ranging from 12 to 26% at pH 6.0–7.6 after 7–10 days. All crystals were grown by equilibration of a reservoir solution with precipitants against a drop with an initial protein concentration of 6.5–7 mg/ml containing half of the precipitant concentration of the reservoir.

Other Methods—The proteinaceous nature of the crystals was verified either by staining of washed crystals with 0.1% Amido Black (Sigma) dissolved in 35% acetic acid or by solubilization of the crystals in sample buffer (60 mM Tris/HCl, pH 6.8, 10% glycerol, 5% SDS, 10% β-mercaptoethanol) and analysis on SDS-polyacrylamide gel electrophoresis (Schlegel *et al.*, 1988a). In addition, the enzyme activity of dissolved crystals was also measured by the titrimetric pH-stat method at room temperature (Wallimann *et al.*, 1984) in the absence of bovine serum albumin but in the presence of 1 mM β-mercaptoethanol in the assay mixture. Protein concentrations were determined according to the standard procedures of the BCA reagent method (Pierce Chemical Co.) and the Bio-Rad method (Bradford, 1976) using bovine serum albumin as a standard.

Estimation of the density of large single crystals was performed by a density gradient obtained by overlaying 9 × 1 ml of water-saturated bromobenzene/toluene mixtures (covering a range of approximately 1.18–1.43 g/cm^3 as calculated from the published density values (*Merck Index*) for the anhydrous organic solvents). The resulting density gradient was calibrated with droplets of CsCl solution of known densities (measured by a digital precision density measurement apparatus, DMA 10, Anton Paar K. G., A-8054 Graz, Austria) and was shown to be linear. Crystals were blotted off from excess mother liquid and immediately transferred by a glass fiber on top of the density gradient. After 10–30 min settling time, depending on the crystal form, the crystals reached their final position. The density of the crystals was calculated from the linear regression of the CsCl droplet calibration. The positions of the crystals and the CsCl droplets remained stable over several hours, and the linearity of the gradient was reproducible.

Preparation of Mi-CK Crystals for Electron Microscopy—Drops containing large amounts of tiny crystals as obtained by PEG 1000 were transferred into reservoir solution wherein crystals were washed and centrifuged gently several times.

Negative Staining—About 5 μl of the washed and concentrated crystal suspension was placed onto a glow-discharged carbon-coated copper grid. After 30 s, the liquid was blotted off from underneath, and a drop of 2% aqueous glutaraldehyde was added for 60 s. After washing by three drops of distilled water, the remaining crystal fragments were stained with acidic uranyl acetate for 15 s and air dried subsequently.

Cryofixation and Heavy Metal Replication—Drops of about 2 μl of the crystal suspension were placed on gold discs, frozen by plunging them in liquid nitrogen and, while submerged in liquid nitrogen, mounted on a specimen table. The table was introduced into the precooled specimen stage (−170°C) of a Balzers BAF 400 T freeze-etching unit using the counterflow-loading device. Fracturing and deep etching (3 min) were performed at −100°C at a vacuum of 5×10^{-7} mbar. Prior to replication the sample temperature was lowered to −130°C. A layer of approximately 10 Å of Pt/C was deposited onto the fracture plane at an elevation angle of 45° either by unidirectional or by rotary shadowing. The heavy metal layer was stabilized by a 150 Å-thick carbon coat evaporated under normal incidence. The thickness of the metal layers was measured by a quartz crystal film monitor (QSG 301). The gold discs were withdrawn from the stage at evaporation temperature, the replicas floated off on water, and mounted without further washing onto copper grids.

Electron Microscopy and Image Processing—Micrographs were taken on a Philips EM 420 or CM12 transmission electron microscope at a primary magnification of 47,000 and 45,000 ×, respectively. The magnification was calibrated with the HPI layer as a test specimen (Gross *et al.*, 1985). Images were recorded on Agfa-Gevaert Scientia films at 500 nm underfocus. Suitable areas were digitized on an Optronics scanner (Photoscan P-1000 HS) with 25-μm sampling raster, corresponding to 0.52–0.55 nm/pixel at the specimen level. The digitized data were processed with the Semper image processing

system. Areas of 512 × 512 pixels were averaged by using correlation or crystallographic averaging (Fourier filtration).

RESULTS AND DISCUSSION

Mi-CK molecules isolated and purified from chicken heart muscle appeared as a single band on SDS-polyacrylamide gel electrophoresis after silver staining. The specific activity of the enzyme was 91–131 enzyme units·min·mg of protein, similar to the value published (Schlegel *et al.*, 1988a), and almost exclusively octamers were found when the enzyme was examined by electron optical methods (Schnyder *et al.*, 1988).

By vapor diffusion in the presence of ammonium sulfate in a broad pH range (pH 6.6–8.8) Mi-CK generally crystallized as needles; at lower precipitant concentration and lower pH, crystallization tended to result in a few crystalline clusters whereas at higher concentrations of precipitant many fine needles appeared. The activity of Mi-CK dialyzed against phosphate buffer was constant over the pH range of 6.6–8.8 used for crystallization. The influence of several additives (glycerol, cystein, substrates) on crystal growth was found to be marginal.

Mi-CK crystallization was significantly improved with PEG as precipitating agent. Many small squared platelets or staples of sheets were formed at pH 6.0–6.4 and 12–20% PEG 1000 in the reservoir solution (Fig. 1*A*). At pH 6.6–7.2 and reservoir concentrations of 16–18 PEG 1000, large single crystals occurred either as needles (about 0.5 × 0.5 × 1 mm in dimension) or as rectangular plates (about 0.5 × 1 × 1 mm) (Fig. 1, *B*, and *C*). Rectangular rods or square plates were formed in the presence of 5 mM ATP (Fig. 1*D*). The use of higher molecular weight PEG (2,000, 4,000, 6,000, and 20,000) as precipitation agents resulted in fewer, tiny square platelets.

Crystallization seemed to be rather insensitive to temperature, both when ammonium sulfate or PEG 1000 was used and was found to occur in a temperature range between 4 and 37°C. When using PEG 1000 at temperature around 4°C many tiny platelets (microcrystals) appeared which were suitable for electron microscopy.

Mi-CK crystals were weakly birefringent, transparent, and rather soft in handling. Washed crystals were readily stained with Amido Black and showed, after SDS-polyacrylamide gel electrophoresis and Coomassie Blue staining, a single band with an apparent M_r of 43,000 corresponding to the Mi-CK monomer (data not shown). Therefore, one can assume that these crystals consist of Mi-CK protein.

Microcrystals obtained from ammonium sulfate precipitation were dissolved in phosphate buffer and rechromatographed on a Mono S fast protein liquid chromatography column (Schlegel *et al.*, 1988a). After salt elution, a single peak appeared at the position at which Mi-CK octamers were to be expected, and the octameric nature of the Mi-CK molecules was confirmed additionally by electron microscopy (data not shown). Rechromatographed Mi-CK exhibited a specific activity of 80 enzyme units·ml·mg of protein and could be reutilized successfully for further crystallization setups, indicating that the enzymatic activity of Mi-CK was preserved in the crystal for several months.

Preliminary density estimations revealed that the large Mi-CK crystals were too dense to be resolved by the Ficoll density gradient method published by Bode and Schirmer (1985). Therefore, a linear organic density gradient was generated by water-saturated bromobenzene/toluene mixtures, and it was found that the sinking velocity of the crystals was highly dependent on their gross morphology. However, after individual settling time, all crystals remained at a constant position even for several days (initially colorless crystals became brown upon oxidation) corresponding to a density of 1.27 ± 0.01 g/cm^3 as calculated from a linear regression of the positions of the CsCl calibration drops (Fig. 2). This value is indicative for a very densely packed arrangement of molecules in the crystals.

In order to investigate the arrangement of the crystal and to estimate the lattice constant, microcrystals were subjected to electron optical examination. The crystals were quite stable in the mother liquor over months but were readily dissolved in buffers lacking ammonium sulfate or PEG. For electron microscopic studies, especially when negative staining was applied, microcrystals had to be freed from precipitating agents. To limit solubilization of crystals during washing with pure water, microcrystals were fixed with glutaraldehyde prior to the washing and staining steps. Fig. 3 shows a fragment of a Mi-CK crystal, presumably one molecule layer thick, after negative staining with uranyl acetate. Individual Mi-CK octamers, resulting from partial solubilization of the crystals, were clearly visible in the background, strengthening the argument that Mi-CK octamers are the building blocks of the protein crystals. Fig. 4*a* shows a large crystal fragment, which represents several crystal planes and therefore was highly electron dense. The optical diffraction pattern shows a square lattice (a = b = 139 Å) with spots until 1/23 Å^{-1}. In order to avoid any projectional information contributed by planes from

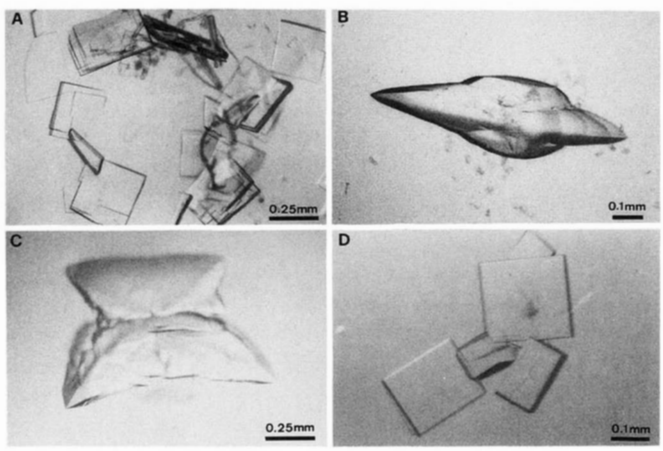

FIG. 1. **Protein crystals of Mi-CK.** Mi-CK was crystallized by the vapor diffusion technique in hanging drops using PEG as precipitation agent. Small platelets were formed at pH 6.0–6.4 with PEG 1000 at reservoir concentrations between 12 and 20% (*A*). Large single needles (*B*) and butterfly-shaped crystals (*C*) appeared at pH 6.6–7.2 at PEG concentrations between 16 and 18%. With 5 mM ATP present in the hanging drop, rectangular crystal blocks were generated (*D*).

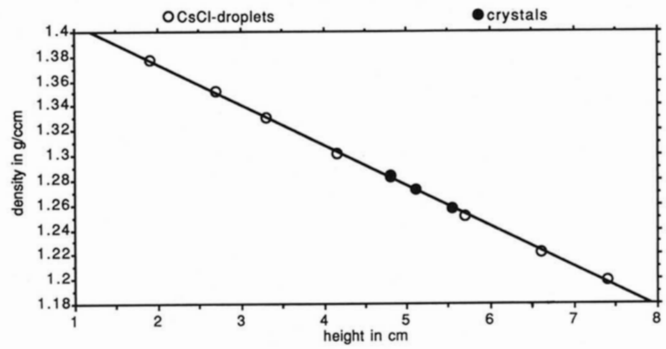

FIG. 2. **Estimation of the density of single Mi-CK crystals.** Linear regression of CsCl droplets of known density (*open circles*) was used for calibration of the organic solvent gradient in order to evaluate the density of the protein crystals (*filled circles*). The mean value for several Mi-CK crystals of different morphology was 1.27 ± 0.01 g/cm^3.

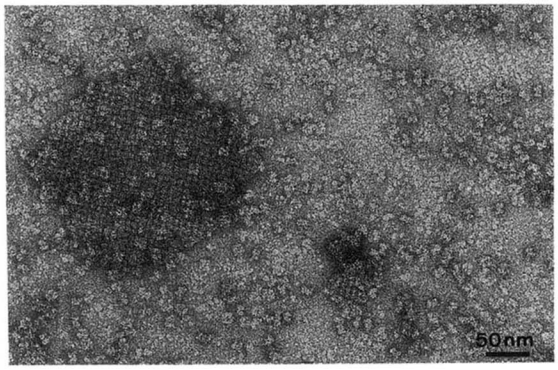

FIG. 3. **Electron optical investigation of microcrystals.** Microcrystals of Mi-CK (tiny platelets or needles) were negatively stained with uranyl acetate and examined by electron microscopy. A crystal fragment was surrounded by single, mostly square shaped particles of single Mi-CK octamers. The octameric nature of the enzyme is preserved upon dissolution of the crystal, indicating that the higher oligomeric species of Mi-CK is the building block of the protein crystals.

the interior of the crystal, as could be the case after negative staining, heavy metal replication of freeze-fractured crystals in mother liquor was applied. It was, however, difficult to find well fractured crystal planes, for often parts of the crystal surface were buried in nonvolatile PEG 1000 or were either distorted with respect to the image plane or to the evaporation source. Fig. 4*b* shows a replica of the surface of a deep etched Mi-CK crystal fracture after conventional rotary metal shadowing with Pt/C at an elevation angle of 45°, displaying in a square lattice molecules arranged with calculated unit cell parameters of a = b = 132 Å. Crystal surfaces of microcrystals replicated by unidirectional Pt/C shadowing at 45° elevation angle gave rise to a three-dimensional impression of the octamers arranged in the crystal plane (Fig. 4*c*). The same lattice dimensions of 132 Å were calculated from the optical diffraction pattern. Generally, the lattice constants of the freeze-fractured/replicated crystals were about 5% lower than those from negatively stained crystals.

The images of the freeze-etched and metal-replicated specimen were averaged with correlation methods, ensuring that slight lattice distortions were compensated. For negatively stained samples, standard correlation averaging failed to reveal the alternating arrangement of the molecular units, which was, however, achieved readily by Fourier filtration. The reconstructed images are presented in Fig. 4 (*a–c, lower right*). The interpretation of the averaged images refers to the study of single molecules since Mi-CK octamers appear square shaped (100 × 100 Å), showing a central stain-filled cavity, when negatively stained (Schnyder *et al.*, 1988) and a cross-like depression on the surface when heavy metal shadowed under high resolution conditions (Schnyder *et al.*, 1989). These features were also obvious in the averaged pattern. An octamer occupies a unit cell of 139 × 139 Å as seen in negatively stained crystals. The electron-transparent domains represent the subunits whereas the central cavity of the molecule and the intermolecular space are filled with stain and are therefore completely electron opaque (Fig. 4*a, lower right*). In negatively stained crystals the surface was dominated by low and high protein densities, and the fact that surface replication yielded a similar crystal arrangement led us to conclude that the crystal planes in the c-dimension are either stacked in register or staggered by a half of unit cell.

In the freeze-fractured/replicated samples (Fig. 4*b, lower right*) the cross-like surface indentation observed in single molecules, giving rise to the windmill-wheel appearance

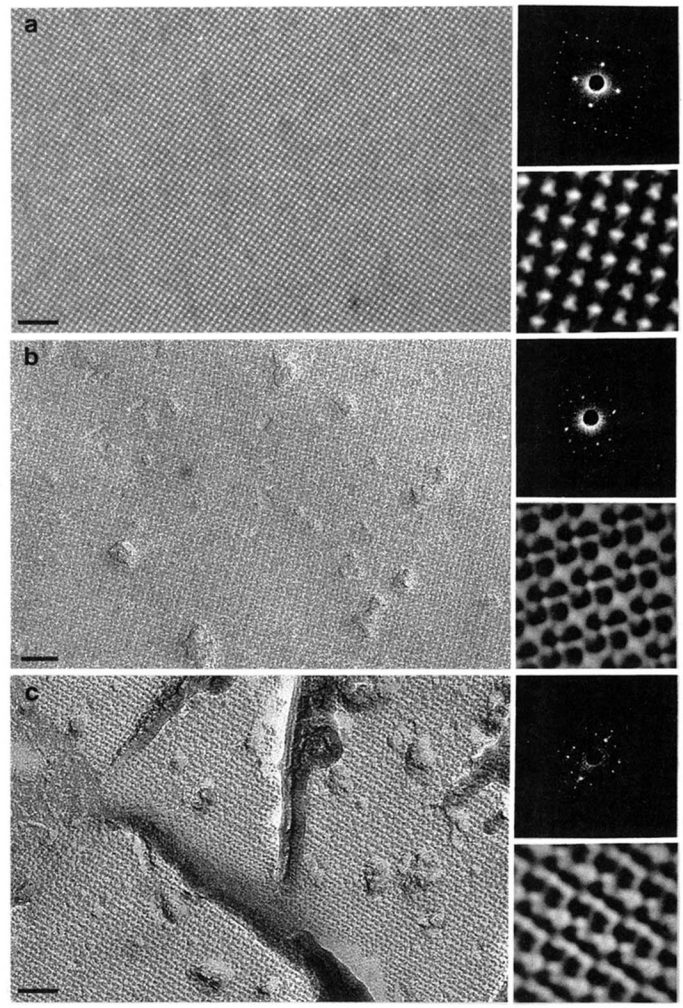

FIG. 4. **Microcrystals visualized by different sample preparation techniques.** Mi-CK crystals are shown with their optical transforms (*upper right*) and averaged images (*lower right*). *Bars* are 50 nm. *a*, glutaraldehyde-prefixed negatively stained sample showing a square lattice with unit cell parameters a = b = 139 Å. Note: in the processed image (*lower right*) units consisting of four main protein densities corresponding to the four dimers (*light*) are arranged around a central negative stain-filled cavity (*dark*), each unit resembling isolated negatively stained Mi-CK octamers (compare Fig. 3, above, and Fig. 4*A* of Schnyder *et al.*, 1988) which are the building blocks of the crystal. *b*, crystals in mother liquor freeze-etched and rotary shadowed with Pt/C at a 45° elevation angle exhibiting a square lattice with lattice constants a = b = 132 Å. Note: in the processed image (*lower right*) the unit cell is occupied by one octamer with a windmill-wheel-like appearance where the four dimers (*dark*) are arranged around a central indentation (*light*). *c*, crystals in mother liquor freeze-fractured and unidirectionally shadowed (Pt/C, 45° angle) displaying identical lattice constants as shown in *b*. Note the regular packing of octamers (as above) in the crystal.

(Schnyder *et al.*, 1991a), is clearly visible. The centers of the molecules showed little metal deposition, indicating a central depression on the surface of the octamers. In the reconstructed image of unidirectionally shadowed fracture planes (the evaporation direction was approximately perpendicular to a crystal axis) the subdivision of octamers into subunits as well as the demarcation between adjacent octamers were prominent (Fig. 4*c, lower right*). The low amount of evaporated metal in the center of an octamer was also obvious by unidirectional shadowing, and the windwill-wheel appearance of the individual Mi-CK molecules was even more pronounced by the shadow-casting effect as compared with rotary shadowed samples.

CONCLUSIONS

Mi-CK obviously crystallizes as octamers in a square lattice as visualized by electron microscopy using different sample preparation techniques. The averaged images in all cases exhibited a quasi-4-fold symmetric arrangement of the four subdomains within the octamers arranged in the lattice (Fig. 4, *a–c*) differing slightly from those obtained from the study of single molecules, where the octamer is divided into four equally structured subdomains by a 4-fold axis protruding the center of the molecule (Schnyder *et al.*, 1991a). This is most probably due to conformational constraints between octamers arranged within the crystals, which is most obvious in metal-replicated samples. In Fig. 4*b* the molecules are twisted with respect to the lattice plane, giving rise to a staggered packing of Mi-CK octamers. A staggering of the octamers in the third dimension is indicated by unidirectionally shadowed metal replicas of microcrystals shown in Fig. 4*c*. The calculated lattice parameters represent center-to-center distances between octamers, having a side length of 100 Å in accordance with the single molecule studies (Schnyder *et al.*, 1991a). The parameters found for negatively stained and heavy metal-replicated fragments are comparable, and the slight variance in unit cell dimensions is most likely caused by different sample preparation procedures rather than being indicative of the existence of two distinct crystal forms of Mi-CK grown under the very same conditions.

The structural details of the Mi-CK octamer within the crystal justify the conclusion that the octamer is made up of dimers arranged around a central region of low protein density. Since no other type of layer packing was found by electron microscopy using different sample preparation techniques, a quasicubic three-dimensional lattice, built up from cube-like octamers (Schnyder *et al.*, 1988; 1991a), has to be assumed for Mi-CK microcrystals. Our recent findings by preliminary x-ray studies of large single crystals (Schnyder *et al.*, 1990a), in which a tetragonal packing of eight octamers/unit cell with the parameters a = b = 171 Å and c = 150 Å was observed (Schnyder *et al.*, 1990c), show that microcrystals and large single crystals are formed by a different packing mode of octamers.

The Mi-CK crystals analyzed so far by x-ray are all tetragonal and belong to space groups $P42_12$ and P422, depending on the presence of ATP (Schnyder *et al.*, 1991b), which are different from those reported for MM- or BB-CK (no space group has yet been reported for Mi-CK, for the only Mi-CK crystals from human cardiac muscle produced so far (Blum *et al.*, 1983) were too small for x-ray analysis). Since both of these crystal types diffract up to a resolution of 0.32 nm a detailed structural analysis of Mi-CK by x-ray crystallography seems possible now (Schnyder *et al.*, 1991b). Structural details of the Mi-CK octamer will hopefully shed some light on the structure of this energy-channeling enzyme important for cellular energetics (Wallimann *et al.*, 1989; Wallimann and Eppenberger, 1990).

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