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Crystallization and Preliminary X-ray Analysis of Two Different Forms of Mitochondrial Creatine Kinase from Chicken Cardiac Muscle

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Crystals of mitochondrial creatine kinase isolated from chicken heart were grown by precipitation with polyethylene glycol 1000. The enzyme has been crystallized in the absence and presence of ATP in two different space groups. Crystals are tetragonal, with space group $P42_12$, $a = b = 171$ Å, $c = 150$ Å in the absence of ATP; and $P422$, $a = b = 101$ Å, $c = 114{\cdot}4$ Å in the presence of ATP. We suggest that there is one octamer (346 kDa) per asymmetric unit without ATP and one dimer (86 kDa) per asymmetric unit with ATP. Using synchrotron radiation, the octameric form diffracts to at least 3 Å resolution.

Creatine kinase (CK‡; EC 2.7.3.2) transphosphorylates ADP using the phosphoryl group of phosphocreatine to regenerate ATP (for a review, see Kenyon & Reed, 1983). The enzyme plays a fundamental role in the energy homeostasis of excitable cells with sudden high energy turnover: (1) as an energy buffering system, (2) as an energy transport and distributing system, and (3) as a regulatory system of subcellular, local ATP/ADP ratios (for a review, see Wallimann *et al.*, 1989). As for "cytosolic" CK, with isoforms BB, MB and MM-CK (Eppenberger *et al.*, 1964), mitochondrial creatine kinase (Mi-CK; Jacobs *et al.*, 1964) exists in isoforms, encoded by specific nuclear genes, which are expressed in a tissue-specific manner (Hossle *et al.*, 1988; Schlegel *et al.*, 1988*b*; Haas & Strauss, 1990). Mi-CK is localized at the inner mitochondrial membrane (Scholte *et al.*, 1973) and seems to be specifically accumulated in mitochondrial contact sites (Adams *et al.*, 1989). A functional coupling of Mi-CK to the ATP/ADP-translocase (Klingenberg, 1980) has been demonstrated (Jacobus & Lehninger, 1973; Saks *et al.*, 1985; Jacobus, 1985; Kuznetsov *et al.*, 1989).

Two Mi-CK isoenzymes from chicken cardiac muscle and brain have been extensively characterized by biochemical and biophysical means (Schlegel *et al.*, 1988*a*,*b*; Schnyder *et al.*, 1988; Wyss *et al.*, 1990). These results demonstrated that Mi-CK exists in two oligomeric forms: a banana-shaped dimer of 86 kDa and a cube-like octamer of approximately 340 kDa molecular mass, composed of four Mi-CK dimers. As seen by electron microscopy of negatively stained samples, the octameric particles have an edge-length of about 10 nm, and a central cavity approximately 2·5 nm in diameter (Schnyder *et al.*, 1988). New structural details of the surface relief of cardiac Mi-CK octamers, i.e. a 4-fold cross-like indentation, became evident by high-resolution metal shadowing of freeze-dried molecules (Schnyder *et al.*, 1990).

When extracted from isolated mitochondria, Mi-CK is almost entirely octameric, but tends to slowly dissociate into highly stable dimers, promoted by low protein concentrations especially at alkaline pH (Schlegel *et al.*, 1988*b*; Schnyder *et al.*, 1988). It is significant that the observed octamer/dimer equilibrium is reversible and is shifted towards dimer formation by different nucleotide (ATP and ADP) and substrate combinations (Marcillat *et al.*, 1987; Schlegel *et al.*, 1988*a*,*b*, 1990). In addition, it was shown recently that these two molecular forms of Mi-CK differ in their interactions with the inner mitochondrial membrane. The octamers reassociate readily with extracted mitoplasts over a broad pH range, whereas rebinding of dimers was strongly pH-dependent (Schlegel *et al.*, 1990).

Preliminary X-ray diffraction data has been reported for various crystals of the muscle isoform,

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‡ Abbreviations used: CK, creatine kinase; Mi-CK, mitochondrial creatine kinase.

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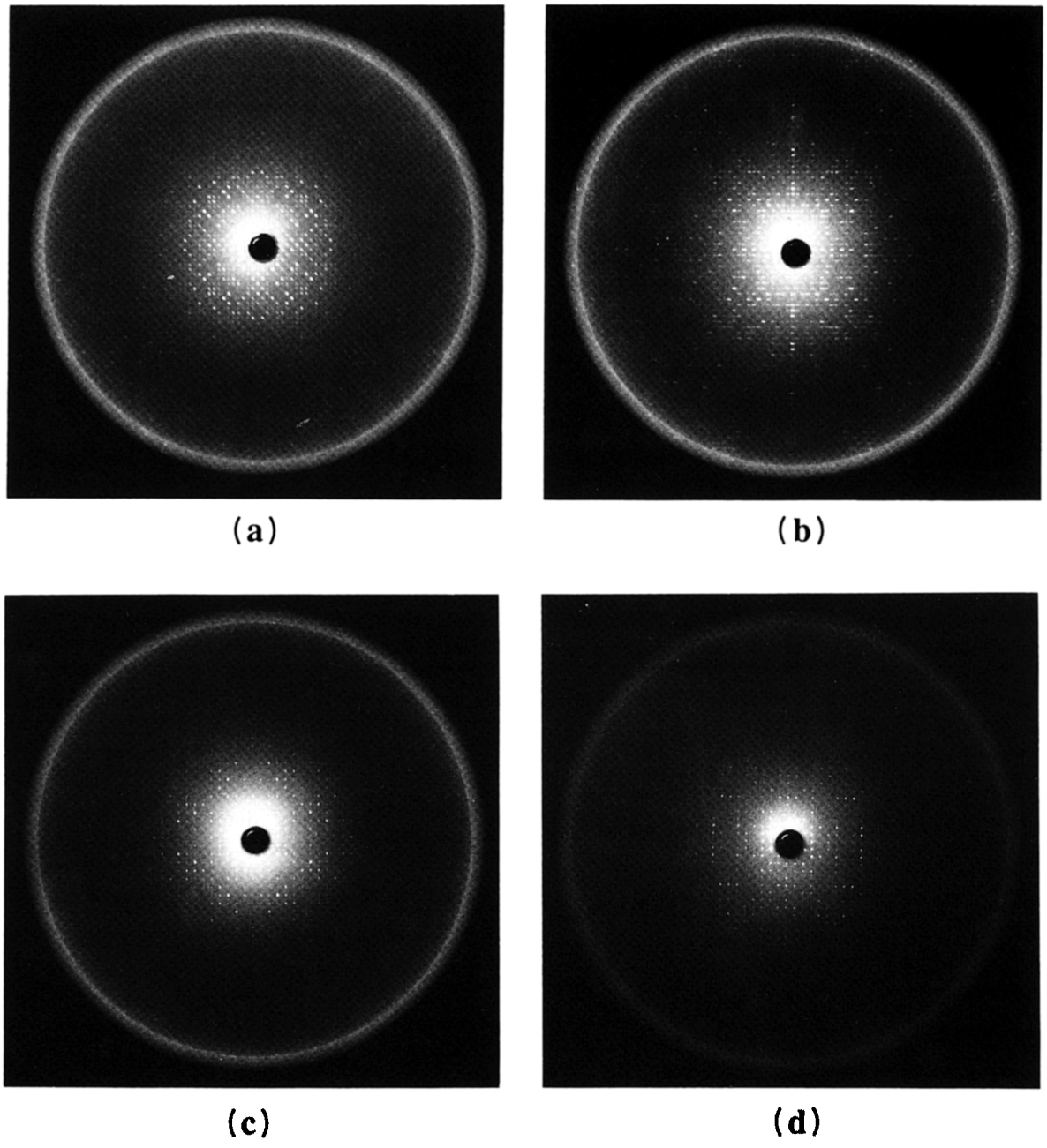

Figure 1. Precession photographs of 2 crystal forms of Mi-CK taken on a rotating X-ray source at 4 °C. The diffraction limit at the edge of the photograph is 3·7 Å (Ni-filtered CuKα radiation). The crystal volume exposed to X-rays is approximately 8-fold greater for (a) and (b) as compared to (c) and (d). (a) The *hk*0 projection of a crystal grown in the absence of ATP (octameric form). The space group is $P42_12$, $a = b = 171$ Å, $c = 150$ Å. (b) The *h*0*l* projection of the crystal in (a). (c) The *hk*0 projection of a crystal grown in the presence of ATP (dimeric form). The space group is $P422$, $a = b = 101$ Å, $c = 114$ Å. (d) The *h*0*l* projection of the crystal in (c).

MM-CK (McPherson, 1973; Burgess *et al.*, 1978; Gilliland *et al.*, 1983; Hershenson *et al.*, 1986). The appearance of crystals from purified calf brain BB-CK (Keutel *et al.*, 1968) and small crystalline needles of Mi-CK from human heart muscle (Blum *et al.*, 1983) were also published. In addition, Keto & Doherty (1968) reported on the appearance of thin square plates in preparations of a "particulate but not mitochondrial" fraction of CK from pig heart with a sedimentation coefficient of 11·7 S, which in hindsight was probably Mi-CK. However, none of these crystals seemed to be suitable for X-ray analysis nor have they been characterized any further. The problem of obtaining good quality crystals of the different CK-isoforms is most probably related to the considerable microheterogeneity found in purified MM-CK (Hershenson *et al.*, 1986) as well as in BB-CK (Quest *et al.*, 1990).

Here, we report the crystallization of chicken cardiac Mi-CK purified according to a recently developed procedure (Schlegel *et al.*, 1988*a*), allowing the isolation of Mi-CK that, in contrast to MM and BB-CK, shows a single polypeptide species if analyzed by two-dimensional gel electrophoresis

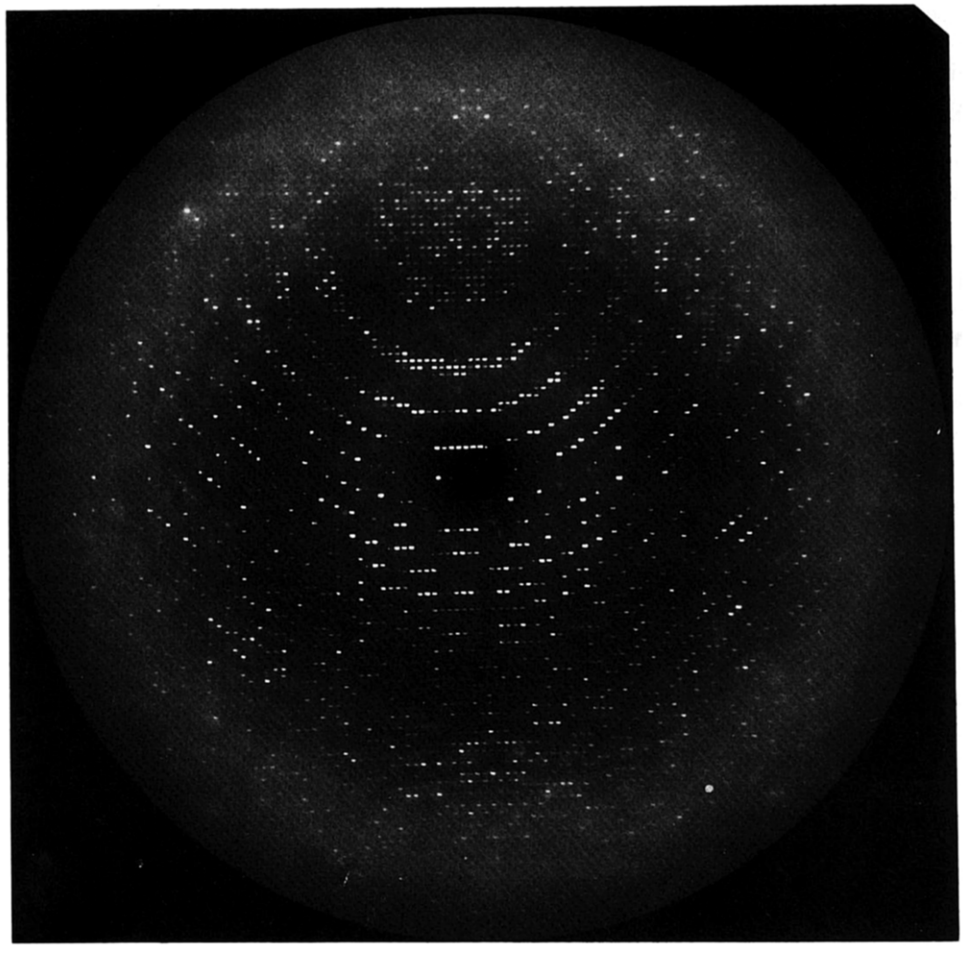

Figure 2. Oscillation photograph of a Mi-CK crystal grown in the absence of ATP taken using synchrotron radiation ($\lambda = 1{\cdot}55$ Å) at 4 °C. The film to crystal distance was 183 mm. The diffraction limit at the edge of the photograph is 3·0 Å.

(Schlegel *et al.*, 1988*b*). The enzyme was concentrated to 13 mg/ml in a Centricon microconcentrator (Amicon) and dialyzed against 25 mM-phosphate buffer containing 50 mM-NaCl at pH 6·7 to 7·2. Crystallization trials were performed using the hanging-drop vapor diffusion technique, where suitable crystals were grown under the following conditions: drops (10 μl) of 6·5 mg enzyme/ml in 8 to 9% (w/v) polyethylene glycol 1000 were equilibrated at room temperature against reservoirs (0·5 ml) of 16 to 18% polyethylene glycol 1000. Large single crystals appeared within one week either as needles (0·5 mm × 0·5 mm × 1 mm) or as rectangular plates (0·5 mm × 1 mm × 1 mm). Square plates or rectangular rods of lengths ranging from 0·2 mm to 1 mm were formed in the presence of 5 mM-ATP.

Both crystal forms diffract to at least 4 Å resolution as determined by precession photography at 4 °C (Fig. 1) using a Rigaku-FR X-ray generator. Although the highest resolution spots do not show up too well in Figure 1, it can be seen that the ATP-absent form is of space group $P42_12$, with $a = b = 171$ Å, $c = 150$ Å, while the ATP-present crystal form is of space group $P422$, with $a = b = 101$ Å, $c = 114{\cdot}4$ Å (1 Å = 0·1 nm). Neither of these two crystal forms of mitochondrial creatine kinase bears a close relationship to previously reported crystals of cytosolic creatine kinases.

The octameric form of the crystals diffracts to spacings of at least 3 Å resolution (Fig. 2) and are suitable for detailed X-ray structural analysis. This was confirmed by the excellent quality of oscillation photographs (Fig. 2) obtained at 4 °C using synchrotron radiation (Station X11, EMBL Outstation/DESY, Hamburg) from a crystal grown in the absence of ATP. Apparently, secondary radiation damage to the crystals limits the resolution observable at 4 °C with the rotating anode X-ray generator. Crystals of the dimeric form have not yet been examined by synchrotron radiation.

Two stable oligomeric forms of Mi-CK exist in solution: an octamer and a dimer. The enzyme is extracted from mitoplasts predominantly (greater than 90%) in the octameric form, but, depending on the conditions used, dissociates into stable dimers with no observable intermediates such as hexamers or tetramers (Schlegel *et al.*, 1988*a*). At protein concentrations below 0·5 mg/ml the equilibrium is shifted toward the dimeric state. The dissociation is markedly enhanced by the presence of ATP or ADP, and complete with the addition of creatine and nitrate together with ADP (Schlegel *et al.*, 1990). The later "dead-end complex" mixture is

known to induce a transition-state analog complex in CK (Milner-White & Watts, 1971). However, at the high protein concentrations used for crystallization, Mi-CK remains in the octameric state even in the presence of dimerization agents (Wyss & Schnyder, unpublished results).

The contents of the asymmetric unit for both crystal forms, in the presence and in the absence of ATP, appear to correspond to the relevant oligomeric states in solution. The densities of several crystals of both types were measured in water-saturated bromobenzene/toluene step gradients that were calibrated with droplets of cesium chloride solution of known density. The mean values obtained for both crystal forms were 1·27(±0·01) g/cm^3. The molecular weight of a subunit is 43,195 as determined from a cDNA clone of the protein (Hossle *et al.*, 1988). From the volume of the asymmetric unit, the crystal density, and the subunit molecular weight, we determined that two subunits per asymmetric unit is the only number that gives a value for the solvent fraction between 0 and 100%, as well as a reasonable value for the corresponding "effective" protein density, that is 23% and 1·38 g/cm^3, respectively, in the ATP-present crystal. For the ATP-absent crystals, eight subunits per asymmetric unit gives the most reasonable value for the solvent fraction and protein density at 17% and 1·34 g/cm^3, respectively (7 yields 27% solvent and 1·41 g/cm^3; 9 yields 6% solvent and 1·29 g/cm^3). It is possible that the packing of oligomers in the two crystal forms is quite similar, and that a small change in the quaternary structure of the molecule induced by ATP, which represents the octamer–dimer equilibrium, causes a molecular 4-fold axis of symmetry of the octamer to become a crystallographic axis.

These structural findings pointing to a nucleotide-induced conformational change in Mi-CK may be relevant to the proposed function of this mitochondrial enzyme as an octameric energy channeling molecule (see Walliman *et al.*, 1989).

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