Acta Cryst. (1998). D**54**, 989–990

Purification, crystallization and preliminary crystallographic analysis of bovine cytosolic brain-type creatine kinase

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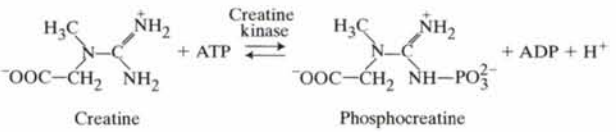
(*Received 14 October 1997; accepted 14 January 1998*)

Abstract

Creatine kinase (E.C. 2.3.7.2) is an important enzyme in energy metabolism which catalyzes the reversible transfer of a phosphoryl group between phosphocreatine and ADP to give ATP. Large quantities of a brain-type creatine kinase have been isolated from bovine photoreceptor cells and crystals suitable for X-ray diffraction analysis have been obtained by hanging-drop vapor diffusion. Crystals grow as tetragonal bipyramids in space group $P4_32_12$ with cell dimensions $a = b = 96.49$, $c = 108.42$ Å and diffract to at least 2.7 Å resolution.

1. Introduction

The primary source of energy for many processes in living cells, including retinal photoreceptor cells, is ATP. However, even though the cellular pools of ATP are rather small, no significant decrease of ATP concentration is observed during cell activation (muscle contraction, brain stimulation or phototransduction in retina). This is due to a continuous ATP regeneration from phosphocreatine (PCr) by the action of creatine kinase (CK) (Watts, 1973; Kenyon & Reed, 1983; Wallimann *et al.*, 1992; Hemmer *et al.*, 1993). This enzyme catalyses the reversible phosphoryl transfer reaction between PCr and ADP.



Two types of cytosolic creatine kinase subunits are known, a brain-type (B) and a muscle-type (M), which have an amino-acid identity of about 80% with each other. In solution the 43 kDa subunits form homodimers [(BB) or (MM)] or heterodimers (MB). A mitochondrial membrane-associated form of creatine kinase, with about 65% sequence identity to the cytosolic isoforms, has also been characterized.

Recently the structure of the octameric mitochondrial chicken creatine kinase has been determined at 3.0 Å (Fritz-Wolf *et al.*, 1996). Herein we report the isolation, crystallization and initial X-ray studies of a bovine brain-type creatine kinase at 2.7 Å. The determination of the structure of bovine retinal creatine kinase should help to give more insight into the catalytic mechanism of these important enzymes, and may allow a definitive identification of the residues involved in catalysis.

Fig. 1. Crystals of bovine retinal creatine kinase. The size of the large single crystal is approximately 500 × 200 × 200 µm.

2. Methods, results and discussion

2.1. *Purification of bovine retinal creatine kinase*

2.1.1. *Preparation of crude retinal extract.* Bovine retinal creatine kinase was isolated as follows. All procedures were performed in the cold room or in an ice bath. Typically 400 frozen retinas were thawed at room temperature until the consistency of ice cream was reached. Then 200 ml of ice-cold buffer *A* (10 m*M* Tris–HCl, pH 7.6, 1 m*M* β-mercaptoethanol, 1 m*M* PMSF, and 1 m*M* sodium benzamidine) with 60 m*M* NaCl was added and the mixture stirred with a magnetic stirrer for a further 15 min at 277 K. The mixture was passed through a 50 ml syringe equipped with a 18-gauge needle and the homogenate centrifuged at 25 000*g* for 30 min. The supernatant was collected and the pellet subjected to two more extractions with 200 ml buffer *A* following centrifugation at 25 000*g*. The combined supernatant from those three extraction was then used as the starting material for creatine kinase purification.

2.1.2. *DEAE–cellulose batch chromatography.* The combined supernatant was applied to 200 ml DEAE–cellulose pre-equilibrated with buffer *A*. This was washed with 500 ml buffer *A* + 60 m*M* NaCl and the creatine kinase containing fractions were then eluted with 500 ml buffer *A* + 200 m*M*

Table 1. *X-ray data-collection statistics*

No. of crystals	1
Resolution (Å)	23–2.7
Wavelength (Å)	1.54
Space group	$P4_32_12$
Unit cell (Å)	a = 96.49
	b = 96.49
	c = 108.42
Temperature of data collection (K)	293
No. of observed reflections	44300
No. of unique reflections	14143
Completeness of all data (%)	97.5
R_{sym} for all data (%)	4.7
Completeness of outer shell (2.80–2.70 Å) (%)	95.8
R_{sym} in outer shell (%)	20.1

NaCl. The protein solution was dialyzed over night against 2 × 5 l of buffer *B* (1 m*M* K-phosphate, pH 6.8, 1 m*M* PMSF, 1 m*M* benzamidine, 0.1% sodium azide and 1 m*M* β-mercaptoethanol).

2.1.3. *Hydroxyapatite chromatography*. The protein solution was then applied to a Macroprep Ceramic Hydroxyapatite column (Biorad, Typel, 40 µm, 2.6 × 10 cm) equilibrated in buffer *B* at a flow rate of 1 ml min^{-1}. The column was washed with 100 ml buffer *B* before eluting the protein with a linear gradient from 1 to 100 m*M* K-phosphate buffer (2 × 300 ml). Creatine kinase was identified by SDS–PAGE as the major band (~45 kDa) eluting at approximately 30 m*M* phosphate. Fractions containing creatine kinase were pooled and dialyzed over night against 2 × 5 l of buffer *A*.

2.1.4. *Fractogel Q-Sepharose Fast Flow chromatography*. The pooled creatine kinase was applied to a Q-Sepharose Fast Flow column (Pharmacia, 2.6 × 15 cm,) preequilibrated in buffer *A* at a flow rate of 1.5 ml min^{-1}. After washing with 100 ml buffer *A* a linear gradient (600 ml) from 0 to 350 m*M* NaCl in buffer *A* was applied and 8 ml fractions were collected. Creatine kinase elutes as the major peak around 150 m*M* NaCl. Fractions containing creatine kinase were pooled and dialyzed over night against 5 l water containing 10 m*M* β-mercaptoethanol, 1 m*M* PMSF and 0.02% sodium azide. The protein was then concentrated using an Amicon concentrator (YM-10 membrane) up to 10–15 mg ml^{-1} and used in subsequent crystallization trials. Starting from 400 bovine retinas approximately 20 mg of bovine creatine kinase could be obtained. The purity of the protein assessed by SDS–PAGE was greater than 98%.

2.1.5. *N-terminal Edman degradation*. The purified protein was identified as bovine brain-type creatine kinase by N-terminal microsequencing. Using ~20 µg of the concentrated protein solution ten cycles of automated Edman degradation were performed yielding a protein sequence identical to the N-terminal sequence of dog brain creatine kinase. Creatine kinase is a highly conserved enzyme, the percentage sequence identity between human brain-type creatine kinase and dog brain-type creatine kinase is 96.3% (bovine brain-type creatine kinase has not been fully sequenced).

2.2. *Crystallization and data collection*

Bovine retinal creatine kinase was crystallized using the hanging-drop vapor-diffusion method at 277 K. Initial conditions were obtained using an incomplete factorial screen (Jancarik & Kim, 1991).

A typical experiment was as follows: 3 µl of the concentrated protein solution (10 mg ml^{-1}) was mixed with an equal volume of crystallization buffer (100 m*M* Hepes–HCl pH 7.5, 1.86 *M* ammonium sulfate and 1.8% PEG 1000) and disposed over 600 µl reservoir containing crystallization buffer. Tetragonal bipyramids grew within a week to a size of approximately 500 × 200 × 200 µm (Fig. 1).

A native data set extending to 2.7 Å was collected at room temperature on a Mar imaging-plate system mounted on a GX-21 rotating anode from a single crystal. Data were processed with *MOSFLM* (Leslie *et al.*, 1986) and merged with programs from the *CCP*4 package (Collaborative Computational Project, Number 4, 1994) to give an overall R_{sym} of 4.7% on data to 2.7 Å (Table 1). Calculation of the crystal volume per unit mass of protein, V_m (Matthews, 1968), showed that there was one molecule in the asymmetric unit with a solvent content of 58%.

The brain-type creatine kinases have about a 65% amino-acid sequence identity with the mitochondrial creatine kinase whose structure has been determined (Fritz-Wolf *et al.*, 1996). Using the coordinates of the mitochondrial creatine kinase (PDB code 1crk) a molecular-replacement solution was obtained (full details will be reported elsewhere) which confirmed the space group as $P4_32_12$ (as had been suspected from analysis of heavy-atom derivatives). The crystal structure of the cytosolic bovine brain creatine kinase, which we have isolated and crystallized, should help to provide further understanding of the catalytic mechanism of this important class of enzymes.

Part of this work was supported by a fellowship of the American Heart Association to AL.

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