J. Microbiol. Biotechnol. (2008), **18**(2), 295–298

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Overexpression, Purification, and Preliminary X-ray Crystallographic Analysis of Human Brain-Type Creatine Kinase

Bong, Seung Min, Jin Ho Moon, Eun Hyuk Jang, Ki Seog Lee, and Young Min Chi*

Division of Biotechnology, College of Life Sciences, Korea University, Seoul 136-713, Korea

Received: June 11, 2007 / Accepted: August 26, 2007

Creatine kinase (CK; E.C. 2.7.3.2) is an important enzyme that catalyzes the reversible transfer of a phosphoryl group from ATP to creatine in energy homeostasis. The brain-type cytosolic isoform of creatine kinase (BB-CK), which is found mainly in the brain and retina, is a key enzyme in brain energy metabolism, because high-energy phosphates are transfered through the creatine kinase/phosphocreatine shuttle system. The recombinant human BB-CK protein was overexpressed as a soluble form in *Escherichia coli* and crystallized at 22°C using PEG 4000 as a precipitant. Native X-ray diffraction data were collected to 2.2 Å resolution using synchrotron radiation. The crystals belonged to the tetragonal space group $P4_32_12$, with cell parameters of a=b=97.963, c=164.312 Å, and α=β=γ=90°. The asymmetric unit contained two molecules of CK, giving a crystal volume per protein mass (V_m) of 1.80 $Å^3$ Da^{-1} and a solvent content of 31.6%.

Keywords: Brain-type creatine kinase, shuttle system, energy homeostasis

Creatine kinase (CK; E.C. 2.7.3.2) is a member of the phosphagen (guanidino) kinase family and catalyzes the reversible phosphoryl transfer reaction between ATP and creatine, producing ADP and phosphocreatine [11]. For high-energy requiring biological processes in skeletal muscle, heart, and brain, phosphocreatine is used as a reservoir of high-energy phosphate to efficiently supply ATP for energy source. There are four major CK isoenzymes, and they have been characterized on the basis of differences in gene, amino acid sequence, tissue localization, and immunogenicity [20]. Two cytosolic isoenzymes, the brain type (B chain) and muscle type (M chain), can form homodimers (MM-CK and BB-CK) or heterodimers (MB-CK). In addition, there are two distinct mitochondrial isoforms that exist as octamers; ubiquitous Mi_a-CK in the brain and sarcomeric Mi_b-CK in muscle [5]. Both CKs located in the mitochondrial intermembrane space can transphosphorylate intramitochondrially produced ATP into phosphocreatine [5], which serves as an energy carrier between intracellular sites of ATP generation and sites of ATP consumption. The creatine kinase/phosphocreatine shuttle system functions as an energy transport through subcellularly compartmentalized CK isoenzymes as well as a temporal energy buffer. The brain-type cytosolic isoform of creatine kinase, BB-CK, exists mainly in the brain and retina [11], and is associated with ion transport pumps in the brain [10]. The major energy consuming process in neural cells is the transport of ions by the Na^+-K^+ ATPase [8, 9] and the ATP-gated K^+-channel [1, 3], which use about 50% of total brain energy. Therefore, BB-CK is a key enzyme in brain energy metabolism. In addition, improper regulation of CK is associated with neurodegenerative diseases such as Alzheimer's disease [2, 4], since the function of the brain seems to be linked with the creatine kinase/phosphocreatine system in a number of different ways. Crystal structures of the CK family that have so far been determined include the chicken Mi_b-CK [7], brain-type CK [6], rabbit muscle-type CK [17] and its transition-state analog complex [15], human ubiquitous Mi_a-CK [5], muscle-type CK [18], bovine brain-type CK [19], and TSAC structure of *Torpedo californica* [11]. The amino acid sequence homology between human BB-CK and chicken is almost 91%, whereas that of between human and bovine BB-CKs is 81%. Therefore, the overall structure of human BB-CK appears to be similar to the structure of chicken or bovine BB-CKs. Even though a number of crystal structures for the CK family have been solved and analyzed extensively, a definitive catalysis mechanism has not yet been elucidated: For example, the exact role of the negatively charged NEED (Asn^{230}, Glu^{227}, Glu^{232}, and Asp^{228}) motif that is important for substrate binding and catalysis; Glu232 forms a bidentate salt bridge, and is positioned to act as a base to remove a

*Corresponding author
Phone: 82-2-3290-3025; Fax: 82-2-921-3702;
E-mail: ezeg@korea.ac.kr

proton from the nucleophilic nitrogen [16]; and Cys283, which is related to substrate recognition and catalysis, interacts with the non-nucleophilic η-nitrogen [14]. In order to understand isoform specific substrate binding and catalysis in the human BB-CK, we overexpressed and crystallized the human BB-CK protein, and collected X-ray crystallographic data for determination of its three-dimensional structure.

The gene encoding human brain-type creatine kinase was cloned from a human cDNA library using polymerase chain reaction techniques. The oligonucleotide primers for forward and reverse directions were 5'-ATTGCCCATATG-CCCTTCTCCAACAGC-3' and 5'-ATACCGCTCGAG-TCATTTCTGGGCAGG-3', which have the NdeI or XhoI enzyme sites, respectively. The resulting DNA construct encodes human BB-CK, the full length protein (1–381 amino acids). The amplified DNA was inserted into the NdeI/XhoI-digested expression vector pET-28a for expression of N-terminal six histidine-tagged proteins. The plasmid was transformed into *E. coli* strain BL21 (DE3) (Novagen) for protein expression. The cells were grown in Luria-Bertani medium, containing 50 μl/ml of kanamycin, at 37°C. When the optimal density of the culture reached 0.45 at 600 nm, the recombinant BB-CK proteins were induced by 0.5 mM isopropyl-β-D-thiogalactopyranoside. After five hours at 37°C, the cells were harvested by centrifugation at 6,000 ×*g* for 30 min at 4°C. The cell pellet was resuspended in ice-cold lysis buffer [20 mM HEPES, pH 7.5, 300 mM NaCl, 5% (v/v) glycerol, 1 mM phenylmethylsulfonyl fluoride], and the cells were disrupted by sonication. The cell lysate was centrifuged at 13,000 ×*g* for 30 min at 4°C, and the supernatant fraction was passed through a 0.45-μm cellulose acetate syringe filter to remove the remaining cell debris. The supernatant was loaded to a Ni-NTA column (GE, U.S.A.) that had previously been equilibrated with buffer A [20 mM HEPES, pH 7.5, 300 mM NaCl, 5% (v/v) glycerol] and subsequently washed with buffer A containing 50 mM imidazole. Protein was eluted with a gradient of 50–500 mM imidazole in buffer A. Fractions containing BB-CK protein were pooled and concentrated. The concentrated protein suspension was exchanged with buffer B (20 mM MES, pH 6.5, 2 mM DTT) by repeated dilution and concentration on an Amicon Ultra concentrator. The protein suspension was then loaded onto a Q-sepharose ion-exchange column that had been pre-equilibrated with buffer B. The column was washed with buffer B and then eluted with a linear gradient of 150–400 mM NaCl in buffer B at a flow rate of 1 ml/min. The product homogeneity of the purified protein was judged by gel electrophoresis (Fig. 1): SDS-PAGE under denaturing conditions was carried out in 12% (v/v) polyacrylamide gels using Protein Marker (Bio-Rad) as a reference protein for molecular mass estimation. The protein suspension was concentrated

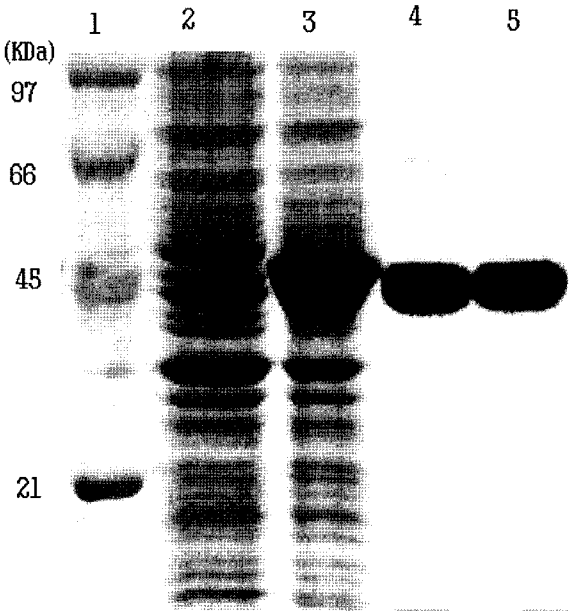

Fig. 1. SDS-PAGE of samples obtained during the purification of recombinant human BB-CK.
Lane 1, molecular mass protein marker (kDa); lane 2, crude extract of *E.coli* BL21 (DE3) before induction; lane 3, crude extract after induction with 0.5 mM IPTG; lane 4, pooled fractions after Ni-affinity chromatography; lane 5, A fraction eluted from Q-sepharose anion-exchange chromatography.

to 40 mg/ml for crystallization. The protein concentration was determined by using Bradford's method with bovine serum albumin as the standard. The initial crystallization screening was performed using Crystal Screen I and II kits (Hampton Research, U.S.A.) by the hanging-drop vapor-diffusion method at 22°C, by mixing 1 μl of protein

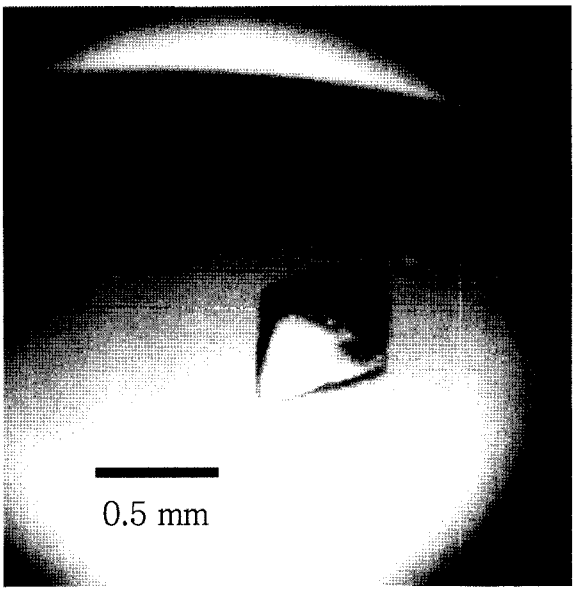

Fig. 2. A crystal from human brain-type creatine kinase for X-ray data collection.
Its dimensions are approximately 0.5×0.5×0.2 mm.

Table 1. Data collection statistics.

Wavelength (Å)	1.000
Space group	$P4_32_12$
Resolution range (Å)	50.0–2.2 (2.28–2.20)
Observed reflections	463,098
Unique reflections	41,184
Redundancy	11.3(9.4)
Completeness (%)	99.5(99.5)
R_{sym} (%)	14.4 (26.7)
I/sigma (I)	33.9 (9.15)

Values in parentheses are for the highest resolution shell.
$R_{sym}=\Sigma_{hkli}|I_{hkli}-\langle I_{hkl}\rangle|/\Sigma_{hkli}I_{hkli}$, where I is the observed intensity, <I> is the average intensity, and i counts through all symmetry-related reflections.

suspension and 1 μl of reservoir solution. Each hanging drop was placed over 0.5 ml of reservoir solution. The best crystallization conditions for human BB-CK protein consisted of 0.1 M Na-citrate, pH 6.5, 0.2 M ammonium acetate, and 30% PEG 4000. The crystals suitable for X-ray diffraction analysis grew to maximum dimensions of approximately 0.5×0.5×0.2 mm within 4 days (Fig. 2). The crystal was transferred into cryoprotection solution, containing 25% ethylene glycol, 0.1 M Na-citrate, pH 6.5, 0.2 M ammonium acetate, and 30% PEG 4000, before being flash-frozen in liquid nitrogen. X-ray diffraction data were collected from the cooled crystals using an ADSC Quantum CCD 210 detector at the beamline 4A at Pohang Accelerator Laboratory (Pohang, South Korea). Diffraction data were collected to 2.2 Å resolution. The raw data for native crystal were processed and scaled using the program HKL2000. The crystals belong to the tetragonal space group $P4_32_12$ with unit cell dimensions of a=b=97.963, c= 164.312 Å, and $\alpha=\beta=\gamma=90^{\circ}$. Assuming the presence of two molecules in an asymmetric unit, because BB-CK is a functional homodimer, the calculated Matthews coefficient (V_M) value was 1.80 $Å^3$ Da^{-1} with solvent content of 31.6% by volume [11]. Data collection statistics are given in Table 1. The crystal structure of human BB-CK protein has been solved by molecular replacement method using the program PHASER [13] with the coordinates of the chicken brain-type creatine kinase (PDB ID; 1QH4, a dimer model) as a search model. After rigid-body refinement, crystallographic R_{work} and R_{free} values from the initial model of MR solution were found to be 25.4% and 29.6%, respectively, for the resolution range of 50–3.0 Å. Crystallization experiments to obtain MgATP/creatine complex crystals are currently in progress. The structural details will be described in a separate paper.

REFERENCES

1. Abraham, M. R., V. A. Selivanov, D. M. Hodgson, D. Pucar, L. V. Zingman, B. Wieringa, P. P. Dzeja, A. E. Alekseev, and A. Terzic. 2002. Coupling of cell energetics with membrane metabolic sensing. Integrative signaling through creatine kinase phosphotransfer disrupted by M-CK gene knock-out. *J. Biol. Chem.* **277:** 24427–24434.
2. Aksenov, M. Y., M. V. Aksenova, R. M. Payne, C. D. Smith, W. R. Markesbery, and J. M. Carney. 1997. The expression of creatine kinase isoenzymes in neocortex of patients with neurodegenerative disorders: Alzheimer's and Pick's disease. *Exp. Neurol.* **146:** 458–465.
3. Crawford, R. M., H. J. Ranki, C. H. Botting, G. R. Budas, and A. Jovanovic. 2002. Creatine kinase is physically associated with the cardiac ATP-sensitive K+ channel *in vivo*. *FASEB J.* **16:** 102–104.
4. David, S., M. Shoemaker, and B. E. Haley. 1998. Abnormal properties of creatine kinase in Alzheimer's disease brain: Correlation of reduced enzyme activity and active site photolabeling with aberrant cytosol-membrane partitioning. *Mol. Brain Res.* **54:** 276–287.
5. Eder, M., K. Fritz-Wolf, W. Kabsch, T. Wallimann, and U. Schlattner. 2000. Crystal structure of human ubiquitous mitochondrial creatine kinase. *Proteins* **39:** 216–225.
6. Eder, M., U. Schlatter, A. Becker, T. Wallimann, W. Kabsch, and K. Fritz-Wolf. 1999. Crystal structure of brain-type creatine kinase at 1.41 Å resolution. *Protein Sci.* **8:** 2258–2269.
7. Fritz-Wolf, K., T. Schnyder, T. Wallimann, and W. Kabsch. 1996. Structure of mitochondrial creatine kinase. *Nature* **381:** 341–345.
8. Grosse, R., E. Spitzer, V. V. Kupriyanov, V. A. Saks, and K. R. Repke. 1980. Coordinate interplay between (Na+ + K+)-ATPase and creatine phosphokinase optimizes (Na+/K+)-antiport across the membrane of vesicles formed from the plasma membrane of cardiac muscle cell. *Biochim. Biophys. Acta* **603:** 142–156.
9. Guerrero, M. L., J. Beron, B. Spindler, P. Groscurth, T. Wallimann, and F. Verrey. 1997. Metabolic support of Na+ pump in apically permeabilized A6 kidney cell epithelia: Role of creatine kinase. *Am. J. Physiol.* **272:** C697–C706.
10. Kaldis, P., W. Hemmer, E. Zanolla, D. Holtzman, and T. Wallimann. 1996. Hot spots of creatine kinase localization in brain: Cerebellum, hippocampus and choroid plexus. *Dev. Neurosci.* **18:** 542–554.
11. Lahiri, S. D., P. F. Wang, P. C. Babbitt, M. J. McLeish, G. L. Kenyon, and K. N. Allen. 2002. The 2.1 Å structure of *Torpedo californica* creatine kinase complexed with the ADP-Mg^{2+}-NO^{3-}-creatine transition-state analogue complex. *Biochemistry* **41:** 13861–13867.
12. Matthews, B. W. 1968. Solvent content of protein crystals. *J. Mol. Biol.* **33:** 491–497.
13. McCoy, A. J., R. W. Grosse-Kunstleve, L. C. Storoni, and R. J. Read. 2005. Likelihood-enhanced fast translation functions. *Acta Crystallogr. D* **61:** 458–464.
14. Mcleish, M. J. and G. L. Kenyon. 2005. Relating structure to mechanism in creatine kinase. *Biochem. Mol. Biol.* **40:** 1–20.
15. Ohren, J. F., M .L. Kundracik, C. L. Borders, P. Edmistonb, and E. R. Viola. 2007. Structural asymmetry and intersubunit communication in muscle creatine kinase. *Acta Crystallogr. D* **63:** 381–389.
16. Pruett, P. S., A. Azzi, S. A. Clark, M. S. Yousef, J. L. Gattis, T. Somasundaram, W. R. Ellington, and M. S. Chapman. 2003.

The putative catalytic bases have, at most, an accessory role in the mechanism of arginine kinase. *J. Biol. Chem.* **278:** 26952–26957.

17. Rao, J. K., G. Bujacz, and A. Wlodawer. 1998. Crystal structure of rabbit muscle creatine kinase. *FEBS Lett.* **439:** 133–137.
18. Shen, Y. Q., L. Tang, H. M. Zhou, and Z. J. Lin. 2001. Structure of human muscle creatine kinase. *Acta Crystallogr. D* **57:** 1196–1200.
19. Tisi, D., B. Bax, and A. Loew. 2000. The three-dimensional structure of cytosolic bovine retinal creatine kinase. *Acta Crystallogr. D* **57:** 187–193.
20. Walliman, T. and W. Hemmer. 1994. Creatine kinase in non-muscle tissues and cells. *Mol. Cell. Biochem.* **133–134:** 193–220.