Expression of active octameric chicken cardiac mitochondrial creatine kinase in *Escherichia coli*

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Sarcomeric mitochondrial creatine kinase (Mi-c-CK) of chicken was expressed in *Escherichia coli* as a soluble enzyme by using an inducible phage-T7 promoter. Up to one third of the protein in *E. coli* extracts consisted of soluble recombinant Mi-c-CK in an enzymically active form. Approx. 20 mg of nearly-homogenous Mi-c-CK was isolated in a two-step isolation procedure starting with 1 litre of isopropyl β-D-thiogalactopyranoside-induced *E. coli* culture, whereas previous attempts to express other CK genes in *E. coli* have resulted in 20-fold lower yields and inclusion-body formation. Selection of the Mi-c-CK expression plasmid on media containing kanamycin rather than ampicillin extended the time period of maximal Mi-c-CK expression. Recombinant Mi-c-CK displayed an identical N-terminal amino acid sequence, identical Km for phosphocreatine and Vmax values, the same electrophoretic behaviour and the same immunological cross-reactivity as the native enzyme isolated from chicken heart mitochondria. The recombinant Mi-c-CK had the same molecular mass as native chicken Mi-c-CK in m.s. analysis, indicating that post-translational modification of the enzyme in chicken tissue does not occur. As judged by gel-permeation chromatography and electron microscopy, recombinant enzyme formed predominantly octameric oligomers with the same overall structure as the chicken heart enzyme. Furthermore, the enzymes isolated from both sources formed protein crystals of space group P422, when grown in the absence of ATP, with one Mi-c-CK octamer per asymmetric unit. The indistinguishable X-ray-diffraction patterns indicate identical structures for the native and recombinant proteins.

INTRODUCTION

Creatine kinase (CK; EC 2.7.3.2) reversibly transfers the phospho group of phosphocreatine to ADP, regenerating ATP. The enzyme exists in different isoforms, which are expressed tissue-specifically and localized subcellularly in an isoenzyme-specific manner [for a review, see Wallimann et al., 1992]. At least four genes located on different chromosomes encode the members of the CK family (Mariman et al., 1987; Klein et al., 1991). The 'cytosolic' M- and B-CK isoenzymes form homo- and hetero-dimers (Eppenberger et al., 1983). Additional isoforms are restricted to the mitochondria (Jacobs et al., 1964; Jacobus & Lehninger, 1973), where at least two tissue-specific isoforms exist. The basic 'sarcomeric' Mi-c-CK is found in heart and skeletal muscle, whereas the more acidic 'ubiquitous' Mi-c-CK is present in most other tissues (Hossle et al., 1988; Haas & Strauss, 1990; Klein et al., 1991; Payne et al., 1991).

Many functional and structural studies have been carried out with the mitochondrial creatine kinase isoenzymes isolated from heart and brain tissue of chicken and other species [for reviews, see Wallimann et al., 1989, 1992], and recently crystallites of Mi-c-CK have been obtained (Schnyder et al., 1990, 1991). Mi-c-CK is bound to the mitochondrial inner membrane (Scholte et al., 1973) and is enriched in mitochondrial contact sites (Adams et al., 1989). Despite the well-conserved amino-acid sequence between all four CK isoforms (Hossle et al., 1988; Klein et al., 1991), Mi-c-CK extracted from isolated mitochondria exists in an octameric form with a molecular mass of 340 kDa and a dimeric form, whereas the cytosolic isoenzymes form dimers only (Schlegel et al., 1988b; Wys et al., 1990). As shown recently, octamers bind distinctly better to model membranes (Rojo et al., 1991) and to mitoplasts (Schlegel et al., 1990) and the octameric form was the only one found in isolated contact sites (Adams et al., 1989). Thus it is likely that the functional form of Mi-CK, especially within the contact sites of mitochondria, is the octamer (Quemener et al., 1988; Brdiczka, 1991; Wallimann et al., 1992).

To probe further the structure and function of Mi-c-CK, we expressed the Mi-c-CK cDNA in *Escherichia coli* using the phage-T7 RNA polymerase system developed by Studier et al. (1990, and references cited therein). Large quantities of soluble enzyme were purified to near-homogeneity from *E. coli* extracts, and several biochemical properties of the recombinant protein were compared with native Mi-c-CK isolated from chicken heart mitochondria. The recombinant enzyme preparation was shown to be identical within experimental error with the native enzyme.

MATERIALS AND METHODS

*E. coli* strains, plasmids and DNA manipulations

*E. coli* strain BL21(DE3)pLysS and expression vector pET-3b have been described (Studier et al., 1990). *E. coli* strain TG1, media and standard DNA manipulations have already been described (Sambrook et al., 1989). In the Mi-c-CK cDNA (Hossle et al., 1988; a gift from Dr. J. C. Perriard of this Institute), an NdeI site was inserted at the 5'-end of the sequence encoding the mature Mi-c-CK protein, creating a new initiating methionine codon; the BamHI site within the coding sequence was destroyed, and a new BamHI site was created in the 3'-flanking sequence of the gene. Plasmid pRF23 was constructed by insertion of the NdeI–BamHI fragment, containing the complete sequence of the mature Mi-c-CK, between the NdeI and BamHI site of vector pET-3b; additionally, the EcoRV/EcoRI fragments of pET-3b were deleted and an XhoI linker inserted at the PvuII site of pET-
Expression and purification of Mi₄-CK

A 2 ml portion of 2XYT medium was inoculated with BL21(DE3)pLysS cells freshly transformed with pRF23 or pRF72. The two plasmids were selected on media containing ampicillin (100 µg/ml) or kanamycin (25 µg/ml) respectively. Cells were grown at 37 °C to an A₅₉₀ of about 0.5 and then induced by the addition of 0.4 mM isopropyl β-D-thiogalactopyranoside (IPTG). Growth was continued for the time period indicated in the individual experiments. Cells were then harvested by centrifugation, washed in 0.9% NaCl, resuspended in lysis buffer [50 mM-Tris(pH 8.0)/10 mM-EDTA/100 mM-NaCl/15% (w/v) sucrose] and sonicated twice for 1 min. Cell debris was removed by centrifugation. For preparative purpuses 1 litre of induced cells was used (about 2.5 g wet weight of cells). The cells were sonicated twice for 1 min in 20 ml of lysis buffer each time, then cell debris was removed by centrifugation at 17000 g for 15 min (crude extract). The supernatant was diluted with 1 vol. of Blue Sepharose loading buffer [50 mM-sodium phosphate (pH 6.5)/1 mM-MgCl₂/2 mM-2-mercaptoethanol/0.2 mM-EGTA] and the pH was adjusted to 6.5. The crude extract was absorbed at 45 ml/h to a 75 ml Blue Sepharose column equilibrated in loading buffer and extensively washed. Creatine kinase activity was eluted with elution buffer [50 mM-sodium phosphate (pH 8.0)/1 mM-MgCl₂/2 mM-2-mercaptoethanol/0.2 mM-EGTA] containing 10 mM-ADP. Pooled fractions were concentrated by ultrafiltration (Amicon Diaflo PM30; Amicon Corp., Lexington, MA, U.S.A.) and dialysed against Mono S buffer [25 mM-sodium phosphate (pH 7.0)/1 mM-2-mercaptoethanol/0.2 mM-EGTA] (Blue Sepharose fraction). For the final purification step, 10 mg of protein were absorbed on a Mono S HR5/5 column (Pharmacia), equilibrated in Mono S buffer, and CK activity was eluted with a NaCl gradient from 50 to 450 mM. Fractions containing the highest creatine kinase activity were pooled (Mono S fraction). Mi₄-CK isolation from chicken heart mitochondria has been described (Schlegel et al., 1988a). Protein concentrations were determined with the Bio-Rad protein assay (Bradford, 1976), with BSA as standard.

Characterization of Mi₄-CK

Mi₄-CK activities were determined by measurement of the H⁺ release in the reaction phosphocreatine + ADP → creatine + ATP, as described (Wallimann et al., 1984). Characterization of the Mi₄-CK oligomer pattern by FPLC Superose 12 HR 10/30 gel-permeation chromatography and cellulose polyacetate electrophoresis has been described (Schlegel et al., 1988b). Dimerization of octameric Mi₄-CK by transition-state analogues (4 mM-ADP/5 mM-MgCl₂/20 mM-creatine/50 mM-KNO₃; pH 7.2) has been described (Schlegel et al., 1988b). N-Terminal protein-sequence analysis was done with an ABI model 470A automated sequenator with on-line detection of phenylthiohydantoin derivatives (James et al., 1990). Electrospray mass spectra were generated using a Finnigan MAT model 710 triple quadrupole mass spectrometer (Finnigan, Mat; San José, CA, U.S.A.) with a 20 kV conversion dynode and 400 m/z mass range; data was routinely acquired with a mass accuracy of ±0.01 %, Mi₄-CK crystallization and sample preparation for electron microscopy were performed as described by Schnyder et al. (1988, 1990). Kinetic measurements using 3.0 µg of Mi₄-CK enzyme after fractionation on Mono S were done in a pH-stat by varying the phosphocreatine concentration at constant ADP and MgCl₂ concentration of 4 mM and 10 mM respectively. The values for the Kₘ for phosphocreatine and Vₘ₉ₐₓ were determined by linear regression in an Eadie–Hofstee plot of four series of independent measurements.

RESULTS AND DISCUSSION

Expression and purification of the chicken mitochondrial creatine kinase

The coding sequence of the mature Mi₄-CK (Hoselle et al., 1988) was fused to the T7-phage promoter in the expression vector pET-3b (Studier et al., 1990; see the Materials and methods section) to create Mi₄-CK expression plasmid pRF23. To express and purify mature Mi₄-CK, a 1 litre culture of BL21(DE3)pLysS cells containing plasmid pRF23 was grown under the inducing conditions for 4.5 h, 2.5 g cells (wet weight) were harvested and lysed. Mi₄-CK activity was found solely in the supernatant of the E. coli lysate at a specific activity of 27 units/mg protein (Table 1). Chromatography on Blue Sepharose, followed by FPLC Mono S chromatography (see the Materials and methods section) resulted in a 3-fold enrichment of Mi₄-CK, which was judged by PAGE to be a nearly homogeneous protein preparation (Fig. 1). The maximal specific activity of Mono S peak fraction was around 115 units/mg. Pooled Mono S fractions had a somewhat lower specific activity of 55–80 units/mg, depending on the isolation and the length of storage at 4 °C. Minor low-molecular-mass contaminants were detected on overloaded SDS/polyacrylamide gels (results not shown). A further purification by gel-permeation chromatography could be included to remove these impurities. E. coli itself does not contain any detectable CK activity. Cross-contamination with other CK isoenzymes, as it might occur when purifying Mi-CK from animal tissue, is therefore not a problem. Isolation of 20 mg of Mi₄-CK from chicken heart mitochondria requires at least 0.5 kg of heart tissue and longer purification times.

So far, two other CK proteins have been expressed in E. coli, namely rabbit M-CK (Chen et al., 1991) and CK from the electric organ of Torpedo californica (Babbitt et al., 1990). However, both proteins could only be expressed and isolated using special precautions, since cultures containing the expression plasmids grown at 37 °C yielded mainly insoluble protein aggregates (inclusion bodies). In addition, the yield of purified enzyme from these expression systems was in the range of only 1 mg/litre of E. coli culture, around 20-fold lower than the yield of Mi₄-CK. For these two CKs, protein-folding pathways may favour inclusion-body formation. We observed that deletion of as little as 30 amino acids (including six positively and five negatively charged residues) from the C-terminus of the Mi₄-CK

Table 1. Purification of Mi₄-CK from E. coli strain BL21(DE3)pLysS transformed with expression plasmid pRF23

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Protein (mg)</th>
<th>Total CK activity (units)</th>
<th>Specific CK activity (units/mg)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude E. coli lysate</td>
<td>149.6</td>
<td>4010</td>
<td>26.8</td>
<td>100</td>
</tr>
<tr>
<td>Blue Sepharose fraction</td>
<td>32.8</td>
<td>3190</td>
<td>60.4</td>
<td>80</td>
</tr>
<tr>
<td>Mono S fraction</td>
<td>28.0</td>
<td>2052</td>
<td>73.3</td>
<td>51</td>
</tr>
</tbody>
</table>

1992
resulted in a highly expressed, but totally insoluble, protein (results not shown). Identification of regions in the primary structure that differ between soluble rabbit M-CK (Chen et al., 1991), the truncated insoluble Mi₃-CK, soluble Mi₃-CK and soluble chicken B-CK (R. Furter, unpublished work) might point to important factors governing the formation of inclusion bodies for creatine kinases expressed in E. coli.

Stability of Mi₃-CK expression plasmid pRF72 in E. coli.

Selection for ampicillin resistance to maintain plasmids in E. coli cultures is effective only during initial stages of culture growth, since the β-lactamase, the enzyme conferring ampicillin resistance, is secreted into the medium and the ampicillin is degraded rather quickly. Plasmids can be lost at a particularly high rate if the expressed foreign gene product is toxic for E. coli (Studier et al., 1990). To test whether selection for kanamycin can improve plasmid stability and the amount of Mi₃-CK expressed from the Mi₃-CK expression plasmid, a kanamycin gene was added to plasmid pRF23 to form pRF72 (see the Materials and methods section). A small amount of Mi₃-CK is produced under non-inducing conditions (results not shown). Under non-inducing conditions all the plasmids tested (pBR322, pET-3b, pRF23 and pRF72) were stably maintained in strain BL21(DE3)pLysS during 18 h of growth under ampicillin or kanamycin selection (results not shown), indicating that Mi₃-CK per se is not highly toxic for the host cell. During the first 3 h after induction with IPTG, however, viable cell counts dropped sharply and all plasmids harbouring a phage-T7 promoter, i.e. pET-3b, pRF23 and pRF72, were rapidly lost. When selecting for ampicillin resistance under inducing conditions, more than 99% of the cells lost plasmid pRF72 after 5 h. Only 0.1% of the cells remained ampicillin-resistant after overnight growth of the culture. After growth for 5 h in medium containing kanamycin and IPTG, maximally 95–97% of cells lost plasmid pRF72. However, when growth was continued overnight, the percentage of plasmid-containing colonies increased with time up to 100%, indicating that cells that maintained their plasmids during the first 5 h were selectively propagated during longer growth periods. These kanamycin-resistant cells could be newly induced to express normally high amounts of Mi₃-CK (results not shown), showing that neither the plasmids nor the chromosomally encoded T7 RNA polymerase gene were inactivated by deletion or other conditions.

Fig. 1. SDS/PAGE and immunoreactivity of native and recombinant Mi₃-CK

Individual fractions of a Mi₃-CK purification were separated on a 10% polyacrylamide/SDS gel and stained with Coomassie Brilliant Blue R-250 (lanes 2–5). Lane 1, positions of molecular-size markers (Bio-Rad, low-Mᵢ standards); lane 2, purified Mi₃-CK from chicken heart mitochondria (Mono S fraction); lane 3, purified recombinant Mi₃-CK isolated from strain BL21(DE3)pLysS transformed with pRF23 (Mono S fraction); lane 4, Blue Sepharose fraction of the recombinant Mi₃-CK; lane 5, crude lysate of E. coli strain BL21(DE3)pLysS transformed with pRF23 used to isolate recombinant Mi₃-CK. (b) Immunological detection of Mi₃-CK in an E. coli lysate and a lysate from chicken heart mitochondria, separated on a 4–12.5% gradient polyacrylamide/SDS gel and stained after electroelcrophoretic transfer to nitrocellulose and immunoperoxidase staining with a monoclonal anti-chicken Mi-CK antibody (mAb 30; Schlegel et al., 1988b). Lane 6, Mi₃-CK isolated from E. coli strain BL21(DE3)pLysS transformed with pRF23 (Mono S fraction); lane 7, Mi₃-CK isolated from chicken heart mitochondria (Mono S fraction); lane 8, positions of molecular-size markers (Sigma 7B; prestained standards).

Fig. 2. Mi₃-CK expression under different selection conditions

The Mi₃-CK expression plasmid pRF72 was grown on media containing either ampicillin (100 µg/ml; ■, ●) or kanamycin (25 µg/ml; □, ○). At zero time IPTG was added to induce Mi₃-CK expression. CK activity in aliquots of the cultures was determined after the time intervals indicated. Shown in the figure is one representative experiment. ■ Specific activity (units/mg) of an extract from a culture grown on medium containing ampicillin. □ Specific activity (units/mg) of an extract from a culture grown on medium containing kanamycin. ● Total yield of CK activity (units/ml) of culture of the ampicillin-selected culture. ○ Total yield of CK activity (units/ml) of culture of the kanamycin-selected culture.

Overexpression of chicken mitochondrial creatine kinase in Escherichia coli
Table 2. Comparison of kinetic parameters of Mib-CK isolated from chicken heart mitochondria and E. coli

<table>
<thead>
<tr>
<th>CK source</th>
<th>$K_a$ for phosphocreatine (mM)</th>
<th>Specific activity (units/mg)</th>
<th>$V_{max}$ (units/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native Mib-CK</td>
<td>1.41 ± 0.04</td>
<td>52.3 ± 1.5</td>
<td>66.6 ± 0.7</td>
</tr>
<tr>
<td>Recombinant Mib-CK</td>
<td>1.41 ± 0.07</td>
<td>56.1 ± 0.9</td>
<td>63.0 ± 1.3</td>
</tr>
</tbody>
</table>

The dimeric form of all mitochondrial creatine kinases studied so far can associate to form octamers (Schlegel et al., 1988; Schnyder et al., 1988; Quemeneur et al., 1988; Wyss et al., 1990; Belousova et al., 1991). This feature distinguishes the mitochondrial isoenzymes clearly from the cytosolic isoforms which are only found as dimers. A detailed analysis of the
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REFERENCES


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