Vol. 151, No. 1, 1988 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 29, 1988 Pages 408-416

DISTINCT TISSUE SPECIFIC MITOCHONDRIAL CREATINE KINASES FROM CHICKEN BRAIN AND STRIATED MUSCLE WITH A CONSERVED CK FRAMEWORK

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Received January 8, 1988

SUMMARY. cDNA clones for chicken mitochondrial creatine kinase (Mi-CK) were isolated from a λgt11 leg muscle cDNA library and sequenced. The deduced amino acid sequence showed 6 blocks of extensive homologies with the cytosolic creatine kinases and contained twenty N-terminal amino acids, with characteristic features of part of a mitochondrial presequence. The mature enzyme contained 380 amino acids with a calculated M_r of 43'195. RNA hybridization analysis showed corresponding Mi-CK transcripts in cardiac and skeletal muscle, but not in brain RNA. Within the 30 N-terminal amino acids purified brain Mi-CK contained 10 changes with respect to cardiac Mi-CK. Thus multiple isoproteins of mitochondrial creatine kinases of brain and striated muscle are encoded by multiple mRNA's. © 1988 Academic Press, Inc.

Three different types of subunits of creatine kinases (CK) have been described in higher vertebrates . The M-CK and the B-CK cytosolic subunits combine to form the enzymatically active MM-CK, MB-CK and BB-CK dimers which are expressed differentially during development and in the adult tissues (1,2). A third type of subunit, Mi-CK, is observed in mitochondria of tissues with high energy turnover (3) where a metabolic circuit has been postulated between the mitochondrial CK, producing phosphoryl creatine, and the cytosolic CK forms which are able to utilize this energy-rich compound to regenerate the ATP pools locally within the cells (4).

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Although mitochondrial CK catalyzes the same reaction as the cytosolic forms it shows different behaviour in many respects. Its isoelectric point is much more basic (5), specific antibodies against Mi-CK do not crossreact with the native cytosolic forms (5,6) and Mi-CK neither forms dimers with M-CK nor with B-CK (7). If the complex biology of the CKs is to be understood, detailed structural information like the primary sequence is of great importance. Except for some short N-terminal amino acid sequences (7, 8, 9) no data are available on Mi-CK. Here we report the molecular cloning and the amino acid sequence for the mature Mi-CK from chicken striated muscle. We also report the surprising evidence for distinct Mi-CK isoproteins in mitochondria from chicken striated muscle and brain.

MATERIALS AND METHODS

Chicken leg cDNA library (10) was screened with affinity purified rabbit anti-chicken cardiac Mi-CK antibody (11) and the DNA of the identified phages was purified and subcloned using methods already described (10).

DNA sequencing of inserts and restriction fragments subcloned into bacteriophage M13 was carried out by the dideoxy method (12). The resulting data were analyzed using the DNAstar software package.

Preparation of cellular RNA and RNA blot hybridization. RNA from various chicken tissues was isolated by the guanidine isothiocyanate method (13) denatured by glyoxylation and run on 1.2 % agarose gels. The blotted RNA was hybridized to a Mi-CK-specific probe radiolabelled by nick translation to a specific activity of about 10^8 cpm/ug as described (10).

N-terminal sequence analysis of FPLC-purified Mi-CK (5,14) from chicken brain and heart was performed on an Applied Biosystems 470A gas/liquid-phase microsequenator. Phenylthiohydantoin derivatives of amino acids were identified by reverse-phase HPLC on a Model 120A on-line PTH analyzer (Applied Biosystems).

RESULTS AND DISCUSSION

All the clones but one, obtained by immuno-screening contained only the sequences 3' to an endogenous EcoRl site. However, these carboxy terminal sequences already gave enough information to ascertain that these clones contained CK sequences but were neither B-CK nor M-CK sequences. One clone,

Mi-7a, contained sequences extending beyond the internal EcoRl site towards the 5'end of the corresponding mRNA. This segment was isolated and used as a radioactive probe to rescreen the same cDNA library. One of the resulting clones, Mi-33a, was found to contain all sequences necessary for encoding the N-terminal part of the mature Mi-CK protein. The segment downstream from the internal EcoRl site to the 3'end obtained in the first round of screening was ligated to the 5'upstream cDNA segment from clone Mi-33a at the common EcoRl site. In order to ensure that this cDNA construct was an accurate copy of the Mi-CK mRNA it was used in a Sl nuclease protection experiment. The end-labelled cDNA probe was fully protected up to its 5' end by RNA from heart and skeletal muscle (not shown; 15). It therefore could be concluded that the ligated cDNA construct represented a faithful copy of the Mi-CK mRNA. The resulting nucleotide sequence (figure 1) and the protein sequence deduced (figure 2) represent Mi-CK from striated muscle by the following criteria:

- the β-galactosidase fusion protein expressed from the Mi-7a λgtll clone reacts with a polyclonal as well as with several monoclonal antibodies against purified cardiac Mi-CK (not shown; 15)
- a polyclonal anti-cardiac Mi-CK antibody was affinity purified on a Mi-7a fusion protein matrix and was subsequently shown to react specifically with mitochondria in ultrathin frozen sections of striated chicken muscle (not shown; 15).
- there is extensive homology between the deduced Mi-CK sequence and the amino acid sequence of M-CK and B-CK. Especially well conserved is the peptide around cysteine 278 of Mi-CK, corresponding to cysteine 283 of the cytosolic CK which has been implicated with the active site of the enzyme (16).
- The sequence of the 29 N-terminal amino acids determined by direct protein sequencing of purified cardiac Mi-CK matches exactly the Mi-CK amino acid segment between Thr-1 and Leu-29, deduced from the cDNA sequence (figure 2).

The nucleotide sequence coding for Mi-CK from chicken striated muscle as shown in figure 1 contains 1307 nucleotides. However, it is not complete at the 5' end because the initiation codon for the synthesis of the putative Mi-CK precursor is absent from this clone. Hybridization experiments (figure 3) have allowed an estimation of mRNA size to be in the range of 1600 nucleotides. Since this estimate also includes the poly A tail of 100-200 residues it is likely that about 200 nucleotides are missing at the 5'end. The open reading frame at the 5' terminus codes for 20 amino acids with characteristics that correspond to the carboxy terminal hydrophobic part of a model

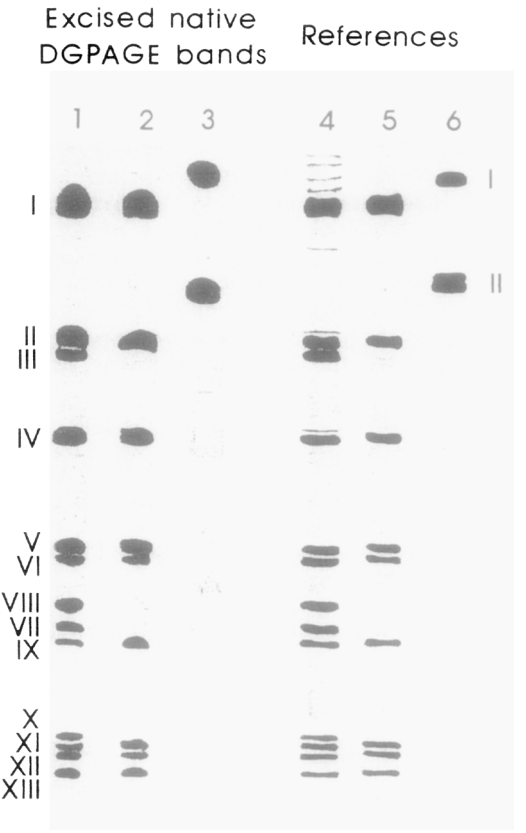

Figure 2. Two-dimensional polyacrylamide gel electrophoresis of cytochrome c oxidase. From the 1st dimension native DGPAGE gel supplemented by Triton X-100 (cf.fig.1, lanes 1-3) bands named below were excised and denatured; then polypeptides were resolved by 2nd dimension SDS-DPAGE: lane 1, band 1 of freshly prepared beef heart enzyme (BH-1), lane 2, major band of alkaline treated beef heart enzyme (BH-AT), lane 3, single band of Paracoccus dentrificans enzyme (PD) were applied. References: lane 4, freshly prepared, lane 5, alkaline treated bovine cytochrome oxidase, either containing 5 µg of protein, lane 6, 2 µg of freshly prepared bacterial enzyme were applied.

from liquid szintillation counting and bicinchoninic acid assay are (0.68 ± 0.05) g Triton X-100 per g oxidase in accordance to (14) for the subunit III depleted enzyme and (1.04 ± 0.08) g dodecyl maltoside per g oxidase respectively. Taking into account that the amount of bound detergent to the water soluble calibration proteins is negligibly low as shown e.g. for bovine serum albumin (20), the molecular masses of cytochrome c oxidase including heme-A, metal atoms and tightly bound phospholipids can be calculated from the apparent M_r-values and the binding of detergent. The corrected molecular masses are 286,000 in Triton X-100 and 152,000 in dodecyl maltoside representing a dimer and monomer respectively. For comparison the molecular mass of the alkaline treated monomer (147,144) is calculated from the protein chemical

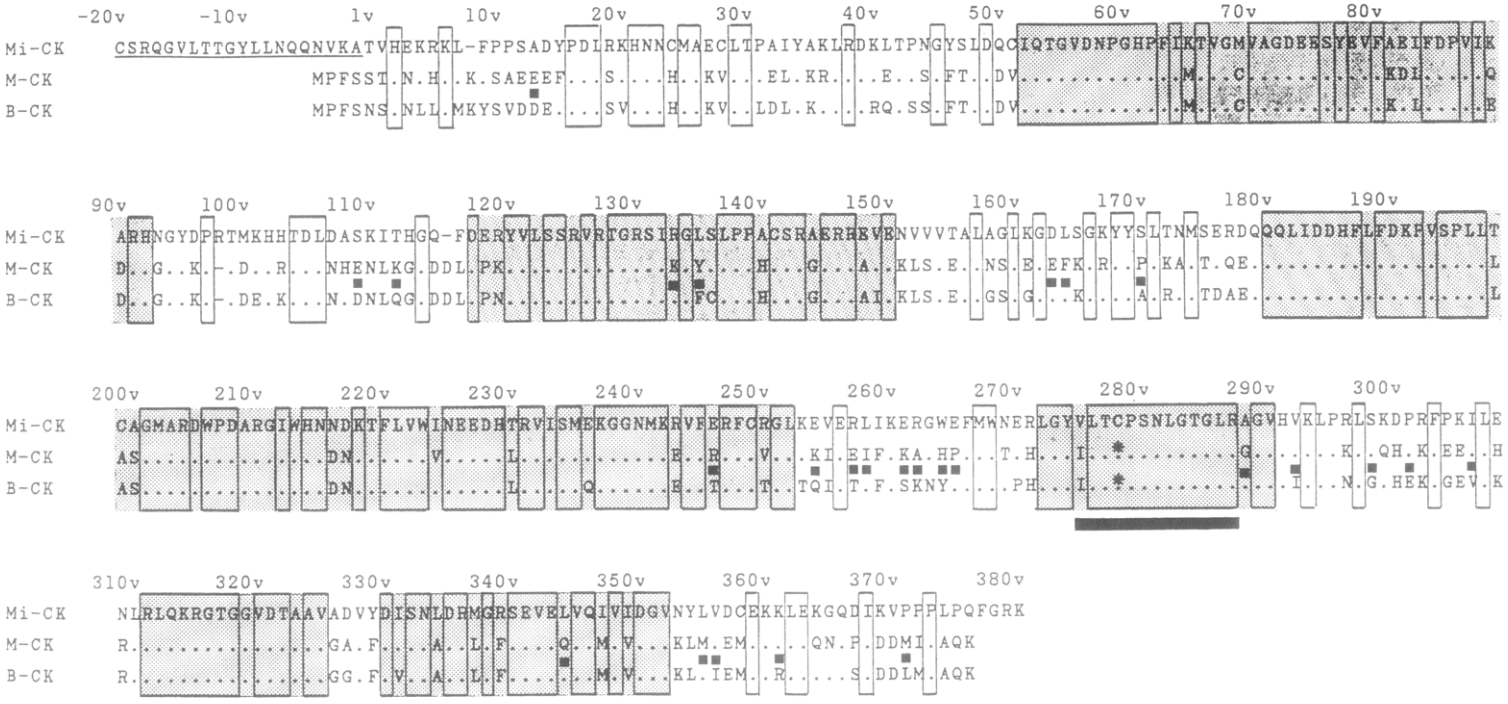

Figure 2. Comparison of the amino acid sequence of Mi-CK with M-CK and B-CK. The Mi-CK sequence from striated chicken muscle is listed. Dots indicate identical amino acids of M-CK and/or B-CK sequences with Mi-CK. For best fit alignment of homology, compensatory gaps of single amino acids are indicated by a hyphen (-). Amino acids homologous in all 14 CK sequences (19,20,21,22,23) are boxed. The shaded segments represent the putative CK-framework (see table II). Isotype specific residues for M-CK or B-CK are indicated by a black square (■). The part of the putative presequence of Mi-CK is underlined. The putative active site region of cysteine 278 (*), corresponding to cysteine 283 in cytosolic CKs (16), is underscored with a black bar.

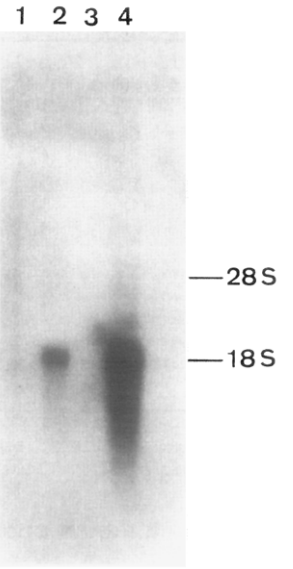

Figure 3. Size of mature Mi-CK mRNA and tissue specific expression. RNA (5 µg of polyA$^+$ RNA) from gizzard (1); heart (2), brain (3) and leg muscle (4) was resolved on a 1.2 % agarose gel, blotted onto Gene Screen membrane and hybridized with the radioactively labelled Mi-7a cDNA probe. The processed membrane was autoradiographed.

presequence for protein-import into the intermembrane space of mitochondria (17). It is therefore postulated that the chicken Mi-CK is synthesized as a precursor and that the 20 extra amino acids at the N-terminus are likely part of the presequence also suggested by the cell free translation of dog heart Mi-CK mRNA (18). Comparison with the N-terminal amino acid sequence obtained by direct sequencing of purified cardiac Mi-CK protein (table II) allowed the localization of the N-terminus at threonine 1. The open reading frame extends to position 1202 where the first stopcodon UGA occurs followed by two others in the same reading frame. A single polyadenylation signal was found at position 1285, 17 bp in front of the first A residue of the poly A tail. The nucleotides from number 62-1201 encode the mature Mi-CK from striated muscle. The resulting protein sequence comprises 380 amino acids with a calculated molecular weight of 43'195. Mi-CK is further characterized by its content of 8 cysteine residues compared to only 4 and 5 of M-CK and B-CK, respectively.

The nucleotide sequence corresponding to the coding region of the mature Mi-CK has been compared to the coding regions of M-CK and B-CK nucleotides and no extensive homologies were detected. However, at the protein level 66 % of the amino acid

Table I: Conserved and variable segments of CK isoproteins

Block:	Aminoacids	Numbers of residues	Homology to B-CK	Homology to M-CK
V	1- 51	52	43 %	53 %
C	52- 92	41	85 %	83 %
V	93-119	27	41 %	41 %
C	120-150	31	81 %	84 %
V	151-179	29	52 %	41 %
C	180-252	73	86 %	86 %
V	253-271	19	42 %	42 %
C	272-290	19	95 %	89 %
V	291-310	20	45 %	60 %
C	311-325	15	100 %	100 %
V	326-329	4	25 %	25 %
C	330-352	23	74 %	74 %
V	353-380	28	36 %	32 %

Conserved segments are designated by a C, the more variable sequences by a V. The amino acid positions are derived from the Mi-CK sequences shown in figure 2.

positions in the Mi-CK sequence correspond to B-CK, while 67 % are identical with the M-CK amino acids. This degree of homology is clearly much lower than that of 82% between chicken M-CK and B-CK (10). Nevertheless the homologies of the chicken enzymes respect a pattern that is characteristic for all CK sequences known to date (19,20,21,22,23). These homologous sequence segments are shown as boxes in figure 2. As shown in table I there are 6 regions of increased homology ranging from 100 % to 74 % of conserved residues (shaded areas) and interspersed are 7 variable segments, where the homology drops to as low as 25 %. Two of the latter comprise the N-terminus and C-terminus. It is likely that the more conserved segments guarantee the framework for the enzyme while the variable ones are characteristic for the isotypes or represent species-specific features.

Mitochondrial creatine kinase activity was found in mitochondrial preparations from chicken skeletal muscle, heart and brain, while no activity was detectable in smooth muscle from gizzard (5). A RNA blot was hybridized with a Mi-CK specific radioactive cDNA probe, the resulting autoradiograph is shown in figure 3. As expected no hybridization signal for gizzard RNA (lane 1) and significant hybridization for cardiac (lane 2) and leg muscle (lane 4) was observed. Surprisingly no signal was obtained in lane 3 where equal amounts of brain RNA had been applied. Even if the hybridization conditions were adapted to low stringency still no signal emerged. In addition to enzymatic activity immunoblot analysis of brain extracts had shown that there was material crossreacting with specific antibodies against cardiac Mi-CK confirming that Mi-CK is also expressed in brain. The failure to detect hybridization on RNA blots may have

Table II: Comparison of N-terminal sequences of mitochondrial CK

Mi-CK	chicken	brain	GERQRRR--YPPSAEYPDLRKHNNXMASXLT
Mi-CK	chicken	heart	TVHEKRKL-FPPSADYPDLRKHNNCMAECLT
Mi-CK	rat	heart (9)	DAREQCKL-FPPHADYPDLRKHNNCMAECLT
Mi-CK	human	heart (7)	EVREQPRL-FPPSADYPDLRKHNNCMA
M-CK	chicken	(24)	PFSSTHNKHKLKFSAEEEFPDLSKHNNHMAKVLT
B-CK	chicken	(10)	PFSNSHNLLKMKYSVDDEYPDLSVHNNHMAKVLT

The sequences of the chicken heart and brain Mi-CK were derived by direct protein sequencing (this paper). The sources of the other sequences are indicated by the references in the brackets. Conserved residues are boxed. X: No residue was identified at this position, which is compatible with presence of a Cys residue at this position.

been due to low levels of accumulation of Mi-CK mRNA. Even with the more sensitive S1 nuclease protection assays no signal was obtained for brain RNA (not shown). Therefore, one had to assume that Mi-CK mRNA from striated muscle and brain were quite different sequences and that such differences would very likely be reflected also in the protein sequence. Therefore, Mi-CK was also purified from brain (14) and N-terminal amino acids were determined by direct protein sequence analysis.

The comparison of the 30 N-terminal residues of cardiac with brain Mi-CK showed that the brain Mi-CK is a clearly distinct isoprotein that differs in 10 out of 30 N-terminal amino acids analyzed (table II). It thus can be concluded that Mi-CK from striated chicken muscle and from brain are distinct Mi-CK isoproteins that arise by a genetic mechanism. Further work is underway in order to understand how these Mi-CKs are generated and how well they are adapted to isotype-specific tasks within cells.

Acknowledgements: The assistence of T. Müller and P. Gautschi for the protein sequence analysis is gratefully acknowledged. This work was supported by the Swiss National Science Foundation grant No. 3.491-0.83 and No. 3.086-0.81 and a grant from the Muscular Dystrophy Association of America to JCP.

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