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Two different B-type creatine kinase subunits dimerize in a tissue-specific manner

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Brain-type creatine kinase B-CK (EC 2.7.3.2) was purified from several chicken tissues, e.g. cardiac muscle, brain, gizzard and retina. Two major monomeric chicken B-CK subunits, designated Bb (basic) and Ba (acidic), which differ in isoelectric point, were separated by chromatofocussing in the presence of 8 M urea on a MonoP column. The two subunits were shown by peptide mapping, amino acid analysis and partial sequencing, as well as by immunological criteria, to be distinct B-CK polypeptides. The N-terminal sequence of 30 amino acid residues of Bb correspond entirely to data derived from a B-CK c-DNA clone termed H4 [(1986) Nucleic Acids Res. 14, 1449–1463], whereas the N-terminus of the acidic Ba species was blocked. Native dimeric B-CK isoenzymes obtained from these tissues were separated by ion exchange chromatography on a MonoQ column yielding two B-CK dimer populations, type-I and type-II B-CK, varying in relative proportions. Quantitation of the CK activity peak ratios of these two populations revealed the existence of a tissue-specific, post-translational mechanism regulating B-CK dimerization in neural tissues. Tissue-specific dimerization of the two distinct B-CK monomer species may represent a means of specifying the intracellular distribution of the dimeric B-CK subspecies.

Creatine kinase isoenzyme; Heterogeneity of brain-type creatine kinase; Tissue-specific dimerization of B-CK; Subcellular compartmentation

1. INTRODUCTION

Proteins often exist in tissue- or even cell compartment-specific isoforms. While essentially fulfilling the same structural and/or catalytic role, these isoenzymes differ in order to respond more efficiently to the specialized metabolic requirements of their local environments. Isoenzymes, therefore, endow an organism with greater metabolic flexibility and precision. In synthesizing isoenzymes, organisms have exploited several different mechanisms [1]. The simplest employ different genes to encode proteins which can differ in a number of aspects, but display the same basic catalytic activity. Creatine kinase (CK) is an example of such a multilocus enzyme [2].

Creatine kinase plays a critical role in the energy metabolism of tissues or of cellular systems with sudden changes in energy requirements, such as skeletal and cardiac muscle, brain, photoreceptor cells and spermatozoa (for review, see [3]). Most higher vertebrates express two 'cytosolic' CK isoforms, brain-type B-CK and muscle-type M-CK. Active, dimeric cytosolic isoforms are expressed in a tissue-specific manner [4]. In chicken, the more ubiquitous B-CK is present in adult brain, smooth muscle, heart and several other tissues as well as in embryonic cells [5]. M-CK is accumulated in adult chicken skeletal muscle [6], but contrary to mammalian heart where both M- and B-CK are expressed to yield significant amounts of MB-heterodimers, only B-CK is found in chicken cardiac muscle [7]. A third type of CK isoenzymes, mitochondrial creatine kinases (Mi-CK), strictly confined to the mitochondria, are co-expressed with B- or M-CK [8].

Compartmentalization of CK isoenzymes, however, is not restricted to the mitochondrial isoform. Cytosolic isoenzymes are associated with distinct structural and functional subcellular components where ATP is generally consumed [3]. For instance, M-CK is associated with the M-line of skeletal and cardiac muscle [9], where it is functionally coupled to the myofibrillar actin-activated Mg-ATPase [10]. This association is isoenzyme-specific, since only MM-CK, but neither MB- or B-CK, bind to the M-band of myofibrils [11,12]. Although targets with which B-CK might be functionally coupled have not been identified, B-CK has been found in association with synaptic vesicles [13,14] and with post-synaptic membranes of *Torpedo* electrolytes [13,15,16].

Apart from the tissue-specific B- and M-CK isoforms, additional B-CK subspecies with slightly different isoelectric points have been detected within the B-CK isoform itself from birds [17]. It was shown that purified chicken B-CK gives rise to two main protein spots on two-dimensional gels [5]. These two major B-

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CK subunit species, which now have been termed Bb and Ba are present in approximately equal amounts in several chicken tissues (brain, heart, gizzard) [5]. A similar 1:1 B-CK monomer ratio was obtained by in vitro translation of poly A^+ mRNA, both by a cell-free system and in frog oocytes [5,12,18]. So far these B-CK subspecies have evaded further characterization.

Here we report on the isolation and characterization of the two monomer species by chromatofocussing in the presence of urea and present data showing that Ba and Bb are similar, but distinct protein species which dimerize in a tissue-specific fashion. A possible role of this mechanism in determining the subcellular distribution of B-CK isoenzymes is discussed.

2. MATERIALS AND METHODS

2.1. *Preparation of native brain-type creatine kinase*

All reagents used were of analytical grade quality. Enzyme purification steps were performed at 4°C, unless stated otherwise. Native B-CK dimer populations were prepared as described [19]. In short, chicken tissues were homogenized and extracted in MSH buffer (220 mM mannitol, 70 mM sucrose, 10 mM Hepes, 1 mM EGTA, 5 mM $MgCl_2$, 2 mM β-mercaptoethanol (bME), at pH 7.4). The 13 000 × g supernatant was subsequently fractionated by a 50% (at pH 7.0) to 65% (at pH 6.2) ammonium sulfate cut, dialyzed against BS 7.0 buffer (20 mM sodium phosphate, 1 mM EGTA, 5 mM $MgCl_2$, 2 mM bME at pH 7.0), adjusted to pH 6.3 and loaded onto a Sepharose blue column (Pharmacia, Sweden) equilibrated in BS buffer at pH 6.3. B-CK was preferentially eluted by BS 6.3 buffer containing 5–10 mM ADP. Fractions containing B-CK activity at 70% purity were pooled (BS pool) and precipitated by 80% ammonium sulfate at pH 6.2. After dialysis against BT 6.8 buffer (18 mM-Tris, 0.2 mM EGTA, 1 mM $MgCl_2$, 2 mM bME at pH 6.8), samples were separated on an FPLC MonoQ HR 5/5 anion exchange column (Pharmacia).

2.2. *Preparation of Ba and Bb monomers*

Chicken brain-type B-CK monomers Ba and Bb were separated by chromatofocussing of BS pool material on an FPLC MonoP HR 5/20 column in the presence of 8 M urea at room temperature. Per run on the MonoP column, equilibrated in BT buffer containing 8 M urea at a flow rate of 0.5 ml/min, 2 mg of protein were separated. B-CK was eluted in a pH gradient generated on the MonoP column by changing to PB-5.0 buffer (10% v/v of Polybuffer 74 (Pharmacia), 4 mM $MgCl_2$, 10 mM bME and 8 M urea at pH 5.0).

2.3. *Creatine kinase assay*

Creatine kinase activity measured by pH-stat assay [10] was expressed in IU (one international unit corresponding to 1 μmole of CP per minute at pH 7.0 and 25°C).

2.4. *SDS polyacrylamide gel electrophoresis and immunoblotting*

2D-gel analysis of B-CK samples was performed as previously described [19]. Proteins separated by SDS-PAGE [20] were first stained with Coomassie blue (CB) and, if necessary, by subsequent silver-staining [21]. 2D-gels were either CB-stained or electroblotted onto nitrocellulose [22]. After reversible staining for protein with Ponceau red-S dye, the blots were immuno-stained with goat anti-chicken B-CK antibodies at 1:50 to 1:250 dilution followed by horseradish peroxidase-conjugated second antibody (Cappel, Dynatech Prod. Zürich) at 1:2000 dilution and vizualized by peroxidase staining using 4-chloro-1-naphthol as a substrate.

2.5. *Antibody preparation and affinity purification*

Polyclonal antibodies against purified B-CK (type-II) were raised in goats. The animals received 3 initial immunizations: day 1, 1.5 mg in complete Freund's adjuvant; day 20, 1.5 mg in a mixture of complete and incomplete adjuvant; and day 35, 1.5 mg in incomplete adjuvant. Antisera, isolated from blood obtained 20–30 days after the third immunization, reacted specifically on immunoblots with B-CK, present in different tissue extracts separated by SDS-PAGE.

Anti-B-CK antibodies were cross-adsorbed by passing 0.2–0.5 ml aliquots of anti-B-CK serum through a type-I B-CK affinity column, prepared by covalently coupling 3 mg of the B-CK subspecies to CNBr-activated Sepharose (Pharmacia). Bound IgG enriched in antibodies specific for the basic Bb B-CK subunit species was eluted by 1 M propionic acid at pH 2.3 followed by immediate neutralization with Tris base.

2.6. *Protein characterization by partial digestion*

B-CK subunits were characterized by partial proteolytic or chemical fragmentation followed by peptide mapping on 5–15% polyacrylamide gradient gels. Purified Ba and Bb monomers were individually digested by thermolysin (Sigma) in 0.1 M ammonium carbonate, 6 mM $CaCl_2$, 0.2% SDS at 56°C; by trypsin (Sigma) in 0.15 M sodium phosphate, 0.1% SDS, pH 7.2 at room temperature; by V8 protease (Boehringer Mannheim) in 0.125 M sodium phosphate; 0.1% SDS, pH 7.0 at 37°C; or by cyanogen bromide in 50% formic acid at room temperature.

2.7. *Quantitative amino acid analysis*

Quantitative amino acid analysis, kindly performed by Mrs M. Wirth, ETH Zurich, was done on a Biotronic LC 6000 E analyser using a Mitsubishi CK 10S ion exchange column [23]. For the quantitation of serine and threonine a time course of acid hydrolysis from 12 to 120 h was chosen and the respective amino acids quantitated at each time point.

2.8. *N-terminal amino acid analysis*

Amino acid sequencing was done on an Applied Biosystems 470A gas/liquid-phase microsequenator by Dr P. Böhlen and later by direct sequencing of the individual B-CK spots after separation by electrophoresis on two-dimensional gels and electroblotting of the spots onto glass-fibre filters by Dr J. Vanderkerckove [24].

3. RESULTS

3.1. *Monomer separation*

In order to characterize the subunits present in B-CK dimers, the monomers were separated by chromatofocussing on an FPLC MonoP column. The purity of B-CK peak fractions obtained by separation on the MonoP column (not shown) was comparable to that of B-CK isolated on the MonoQ column [19]. A typical MonoP profile of B-CK is shown in fig.1A and the B-CK monomer composition of the corresponding MonoP fractions is shown after 2D-gel analysis in fig.1B. The peak ratios of the two major B-CK subunit species is 1:1 (compare peak size of fractions (a) and (c) of fig. 1A). An additional minor B-CK subunit species, Bb*, was apparent in intermediate fractions ((b) of fig.1A).

3.2. *Characterization of Ba and Bb monomers of B-CK*

Isolated Ba and Bb monomers were characterized in several different ways as summarized in table 1. The monomers had identical M_r values, but differed in their isoelectric points and, as suspected, eluted from a

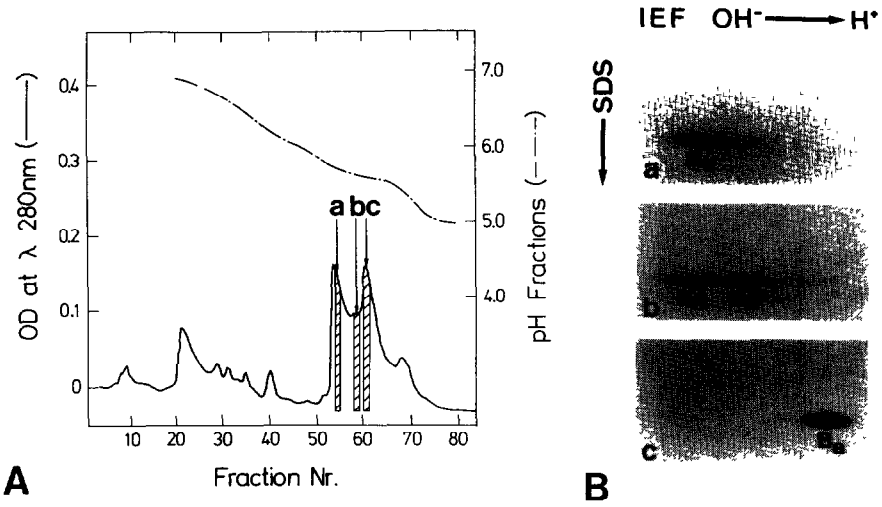

Fig.1. Separation of B-CK subunits Ba and Bb by chromatofocussing and characterization on 2D-gels. (A) Material eluted from Blue Sepharose affinity chromatography was further purified by chromatofocussing on a MonoP HR 5/20 column (Pharmacia) in the presence of 8 M urea. The pH of the eluted fractions is indicated by a dashed line and the recorder tracing of absorbance at 270 nm by a solid line. Two peaks of equal size were resolved corresponding to the individual B-CK monomers Bb and Ba which were eluted at pH 5.7 and pH 5.6, respectively. Fractions (0.5 ml each) indicated by the shaded areas (a, b and c) were analyzed on 2D-gels. (B) Approximately 3 μg of protein from MonoP fractions (a, b and c) were separated on 2D-gels and stained with Coomassie blue. In the initial peak only basic Bb monomers (a) were seen, while the second peak contained exclusively acidic Ba monomers. (c) Intermediate fractions contained a yet unidentified species, called Bb*, in addition to Bb monomers (b).

chromatofocussing column at different pH values. The overall amino acid composition was nearly identical except for serine, where a difference of 3 residues was found. Deviations in values determined for other amino acid residues were within the 5% error range. For example, the threonine content, analyzed by the same time course procedure as used for serine, was found to be identical (18 residues) for both monomers.

Table 1

Summary of comparison between Ba and Bb monomers showing the two monomers to be similar proteins, but with distinct amino terminal regions

Characterization	Monomer Ba	Monomer Bb
M_r (SDS-PAGE)	identical	(43 kDa)
IEP (2D-gels)	6.1	6.5*
Eluent pH (MonoP, fig.1A)	5.6	5.7
Serine residues per 380 amino acids	18	21
Partial cleavage of polypeptides (fig.2B)	some peptide fragments (trypsin, BrCN) of Bb migrate with lower M_r than those of Ba	
N-terminal amino acid analysis	blocked N-terminus	identical to H4 B-CK cDNA sequence**
N-terminal sequence of two internal peptides at position 90 and 120	identical	
Immunoreactivity with polyclonal anti-B-CK, cross-absorbed on type-I B-CK (fig.2A)	Yes	No

* See [5]
** See [25]

While partial cleavage of Ba and Bb with thermolysin and V8 protease gave identical peptide patterns (fig.2B; V8 digest not shown), a few of the peptides obtained by digestion of Bb-CK with trypsin and BrCN had slightly lower apparent M_r values (fig.2B, lanes 2 and 6, arrowheads) compared to those of the corresponding cleavage products of Ba-CK (fig.2B, lanes 1 and 5). N-terminal amino acid sequence analysis of Bb versus Ba revealed a difference at this end (table 1). Sequence data obtained for the first 30 amino acids of Bb-CK agreed precisely with cDNA-derived sequence data of the B-CK cDNA clone H4 [25], except that the N-terminal methionine encoded by this mRNA was no longer present in the mature protein. However, Ba-CK isolated and analyzed under the same conditions was blocked at the N-terminus showing that the N-termini of the two B-CK subunits, Ba and Bb, were indeed different.

If the N-terminally blocked Ba species was subjected to chemical cleavage by 70% formic acid (at 40°C for 24–28 h) at specific sites where an aspartic acid residue is followed by a proline, two fragments with readable N-termini were obtained. With the exception of two uncertainties indicated by X, the two internal amino acid sequences obtained, DPVIEDXHGG and DPNYVLSSRVXT, were in complete agreement with the amino acid sequence following the Asp(D)/Pro(P) sites at positions 90 and 120 predicted from the H4 B-CK cDNA. Thus, despite differences at the N-terminus the two monomer species were found to have identical sequences at two internal sites accessible to analysis, which is consistent with the similarity of the peptide patterns found between Ba and Bb B-CK monomer

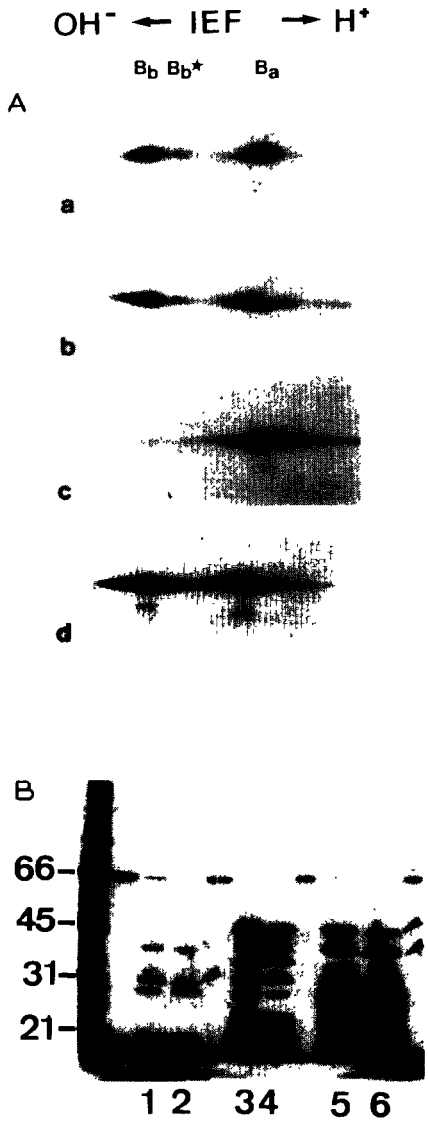

Fig.2. Ba and Bb monomers are similar, but distinct proteins. (A) Differential immunoreactivity of Ba and Bb B-CK subunit species with cross-adsorbed anti-B-CK antibodies. Areas of interest from parallel 2D-gels with purified chicken B-CK after electrophoretic transfer onto nitrocellulose membranes are shown. The two B-CK subunit species, Ba and Bb, as well as the modified Bb species termed Bb*, are present, as revealed after staining for protein by Ponceau red-S (panel a); and after immunostaining with polyclonal goat anti-chicken B-CK antiserum at 1:250 dilution (panel b). The anti-B-CK serum recognizes both B-CK spots with equal intensity. Immunostaining with pooled breakthrough fractions (at 1:50 dilution) of the same serum, but after adsorption on a type-I B-CK affinity column shows that the cross-adsorbed antibody specifically labels the acidic Ba spot (panel c), demonstrating partial immunological divergence of the two B-CK monomers. Antibodies eluted from the Bb-CK affinity column by propionic acid at pH 2.3 and subsequently diluted 1:250 recognize both spots, indicating the presence of common epitopes on Bb and Ba (d). (B) Comparison of peptide fragments of Ba and Bb B-CK subunit types on silver-stained 5–15% gradient polyacrylamide SDS gels. Parallel samples of separated Ba (lanes 1, 3, 5) and Bb (lanes 2, 4, 6) B-CK subunits digested under identical conditions are shown in pairs after cleavage by CNBr for 10 min (lanes 1, 2); by thermolysin for 10 min (lanes 3,4); and by trypsin for 10 min (lanes 5, 6). Arrowheads indicate slight differences between Ba and Bb B-CK subunit-types in electrophoretic mobility of the corresponding peptides visible after digestion with CNBr (lanes 1, 2) and trypsin (lanes 5, 6).

subspecies. Polyclonal goat anti-chicken B-CK antiserum was cross-adsorbed on a type-I B-CK affinity matrix (fig.2A), on which only Bb monomers or derivatives thereof were present (fig.3, panel C(b)). Antiserum flowthrough fractions from this column specifically recognized Ba monomers (fig.2A(c)). On the other hand, antibodies recovered by elution from the matrix with propionic acid recognized both Ba and Bb monomers (fig.2A(d)). This clearly illustrates that the two monomers have both similar as well as divergent immunological epitopes.

3.3. *Tissue-specific dimerization*

A comparison of MonoQ elution profiles between cardiac and brain B-CK (fig.3A,B) or gizzard and retina B-CK (table 2), revealed striking tissue-specific differences in the type-I to type-II peak ratios. While B-CK purified from heart and gizzard both showed a peak ratio of approximately 1:3, this ratio was about 1:10 for B-CK derived from brain and retina. The differences were entirely tissue-specific (table 2) and did not depend on the extraction conditions (salt, detergent, etc., not shown). The specific activity was constant throughout both peaks. For all tissues a B-CK monomer ratio of 1:1 was seen on 2D-gels at all steps during purification before separation on MonoQ. A representative example from brain tissue is shown in fig.3, panel C(a). While type-I peaks contained B-CK dimers exclusively composed of Bb and Bb*, the latter most likely representing derivatives of Bb [26] (fig.3, panel C(b)), both Ba- and Bb-containing B-CK dimers were present in type-II peak fractions (fig.3, panel C(c)). The same 1:1 monomer ratio as described above was obtained by chromatofocussing of partially purified enzyme on MonoP (fig.1A).

4. DISCUSSION

While having identical apparent M_r values, B-CK monomers Ba and Bb are easily distinguished on 2D-gels. The two monomers have p*I* values of 6.1 and 6.5, respectively (table 1). This p*I* difference is too large to be explained on the basis of a simple post-translational modification; e.g. the addition of one phosphate group would result in a p*I* change of 0.05–0.1 units in the acidic direction. On the other hand, the observed p*I* difference between Bb and Bb* could easily be explained by such a modification, i.e. Bb* representing modified Bb. Evidence supporting this interpretation is provided elsewhere [26].

In vitro translation of crude poly A^+ mRNA from different B-CK-containing chicken tissues always yielded both major species in a 1:1 ratio [5,12], supporting the concept that Ba and Bb are different proteins. Since CK dimers are extremely stable and can only be dissociated into monomers under denaturing conditions [4], Ba and Bb were separated from one another by

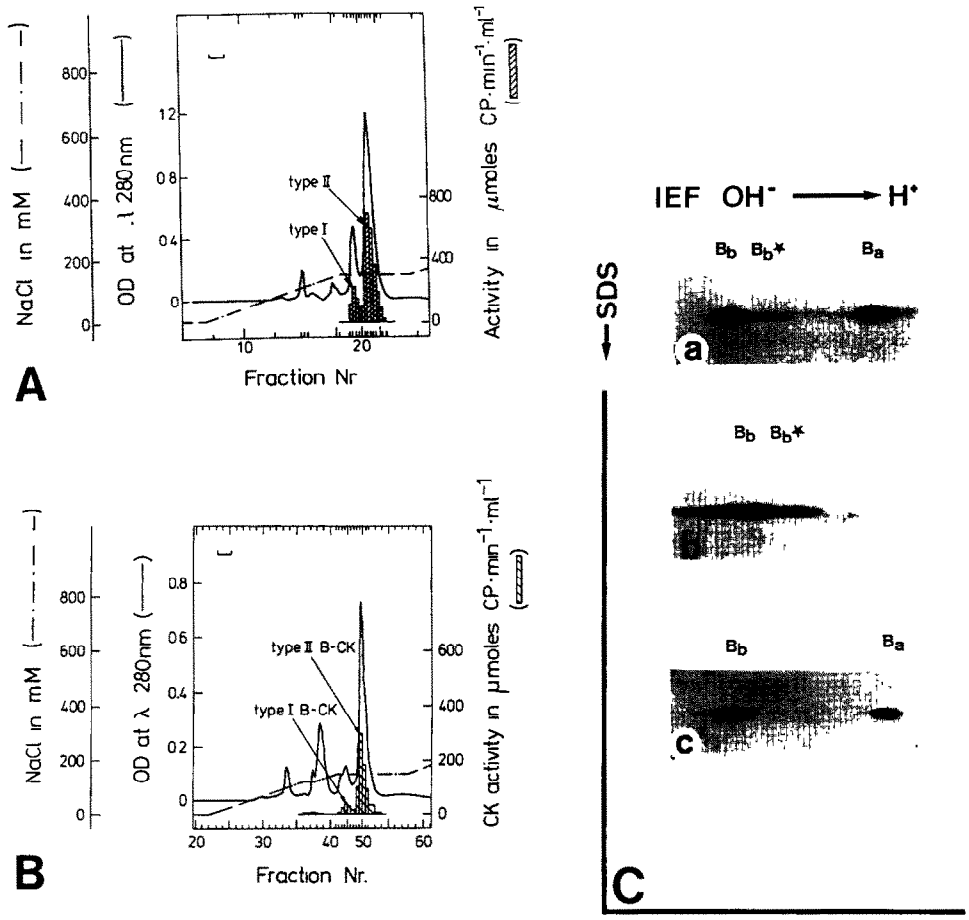

Fig.3. Two B-CK peaks (type-I and -II) obtained by MonoQ chromatography show distinct monomer composition. (A) Elution profile of chicken cardiac muscle B-CK activity (IU/mg, shaded area) and protein (solid line) eluted by a salt gradient (dashed line) from a Pharmacia FPLC MonoQ HR 5/5 column. Two B-CK peaks were eluted by an isocratic step at 150 mM NaCl. The length of the bar in the upper left hand corner represents 1 ml elution volume. For optimal resolution the fraction size was varied. (B) This panel shows a representative elution profile of brain B-CK on the MonoQ column. Figure specifications are the same as in panel A. (C) Characterization of monomer species present in individual brain B-CK peaks by 2D-gel electrophoresis. Panel (a) shows 3–5 μg of CB-stained B-CK protein, while in panel (b) and (c) 1 μg of silver-stained protein are shown. Separation in the first direction by isoelectric focussing between pH 5 and 8, indicated along the horizontal axis, was followed by separation according to M_r on SDS-PAGE. Material from the Blue Sepharose column, highly enriched in B-CK is shown in panel (a). The ratio of Ba:Bb species was still close to 1:1. Additionally, a species termed Bb* was often resolved. Type-I B-CK peak fractions (b) contained only B-CK dimers composed of Bb or Bb*. In contrast, type-II B-CK peak fractions (c) contained dimers composed of both major monomers Ba and Bb.

Table 2

Tissue-specific dimerization of Ba and Bb monomers

Chicken-tissue extract	MonoQ type-I to type-II peak ratios	
Heart	1:3–4	(fig.3A)
Gizzard	1:3–4	(see [26])
Brain	1:9–10	(fig.3B)
Retina	1:9–10	(see [19])

B-CK activity ratios clearly differ in a tissue-specific manner. While in muscle-like tissues (heart, gizzard) the type-I to type-II B-CK peak ratio reflects random association of Ba and Bb monomers, neuronal tissues differ in this respect (see section 4). In all cases studied, both monomers were present in a 1:1 ratio in the B-CK pool fractionated on the MonoQ column. The specific B-CK activity in both peaks was identical

chromatofocussing in the presence of 8 M urea. Subsequent protein characterization revealed that the individual B-CK monomers are indeed similar (two identical internal peptide sequences, identical overall amino acid composition (except for serine) and digest patterns with thermolysin and V8 protease), but distinct proteins (different serine content, different N-termini, differential immuno-reactivity with polyclonal antibodies) which are encoded by different mRNAs [28].

Together with the information obtained by comparison of the total amino acid composition, the proteolytic cleavage pattern and the immunological cross-reactivity of the two B-CK subunit species, Ba and Bb (summarized in table 1), the complete correspondence of more than 20 internal amino acid residues sequenced at two different sites is direct proof that Ba is indeed a

B-CK polypeptide highly homologous to Bb, but with a different N-terminus. Due to N-terminal blockage of the Ba monomer, no direct comparison of the N-terminal amino acid sequences could be made. It is important to note that as far as the total amino acid composition is concerned, the serine content was the only significant difference between Bb and Ba, with Ba containing at least 3 serine residues less than Bb (table 1). Such a difference in serine content, although totalling a number of 4, corresponds to the cDNA-derived N-terminal amino acid sequence difference between the cDNA clone H4, coding for Bb, and a recent cDNA clone 18c, most likely coding for the Ba subunit [27].

Ba and Bb were shown to dimerize in a tissue-specific fashion. In all cases the monomer ratio was 1 : 1 prior to separation of the dimer species, ruling out the possibility that selective loss of distinct enzyme sub-populations may account for the difference in type-I to -II B-CK peak ratios obtained on the MonoQ column. No deviations were obtained even when extraction conditions varied considerably. Since CK dimers do not dissociate under native conditions [4], the probability that an equilibrium between B-CK dimer species might complicate the interpretation of these experiments can be excluded. The type-I to type-II cardiac and gizzard B-CK peak ratio corresponds to what would be expected by random association of Ba and Bb monomers present at a 1 : 1 stoichiometry. The peak ratio observed for neural tissues, like brain and retina, cannot be explained on this basis, but rather indicates that in these tissues a mechanism ensures the almost exclusive pairing of Ba with Bb monomers to form preferentially heterodimeric B-CK molecules.

This is the first time to our knowledge that such a mechanism has been described for creatine kinase. In general, it is assumed that oligomerization of multimeric isoenzymes is a random association process [2]. Post-translational modulation of oligomeric isozyme composition, however, could possibly lead to more precise subcellular targeting of the enzyme. Since different CK isoenzymes generally appear to be associated with distinct subcellular compartments [3], it is suggested that tissue-specific dimerization of B-CK monomers might represent a mechanism for specifying the intracellular distribution of this cytosolic isoform.

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