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Separation of Mitochondrial Creatine Kinase (MiMi-CK) from Cytosolic Creatine Kinase Isoenzymes by Cibachrome-Blue Affinity Chromatography

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Abstract. The mitochondrial isoenzyme of creatine kinase (MiMi-CK) was separated by affinity chromatography on Cibachrome-Blue-Sepharose (Sepharose-Blue, Pharmacia). While the soluble CK isoforms (BB-CK and MM-CK) were specifically eluted by raising the pH of the column buffer from pH 6.0 to pH 8.0, MiMi-CK remained bound under these conditions but was specifically eluted by subsequent addition of ADP to the pH 8.0 buffer. This one-step method allows a fast and efficient separation of MiMi-CK from MM-and BB-CK isoenzymes and at the same time an enrichment of MiMi-CK by about 50-fold. Since MiMi-CK can be assayed separately after isolation by affinity chromatography on Sepharose-Blue, this method may be of clinical importance.

Introduction

High concentrations of creatine kinase (CK; EC 2.7.3.2) are found in heart, skeletal muscle and brain [1]. The muscle isoenzyme, MM-CK, is specific for striated muscle but is also found in electric organ of *Torpedo* [4, 5], whereas the brain isoform, BB-CK, present predominantly in neural tissues, smooth muscle, blood cells [23] has also been found recently in several tissues of high-energy metabolism, e.g., in spermatozoa [2, 3] and in the photoreceptor cells of the retina [6]. Interestingly, the mitochondrial isoenzyme, MiMi-CK [7], which is only found in mitochondria [8] and which differs in electrophoretic mobility, kinetic parameters and immunological properties from the three other CK isoforms (MM-CK, MB-CK and BB-CK) [21, 22], was, however, always found in parallel with the latter, mostly cytosolic, CK isoforms [6, 8]. Furthermore, small amounts of

CK have been localized at strategic intracellular locations where energy is extensively consumed. This has led to the proposal of a phosphoryl-creatine shuttle from the mitochondria to the intracellular locations of high energy demand [for review see ref. 9].

In order to obtain more information on the biochemistry, structure and localization of mitochondrial CK, we tried to find a simple way of purifying MiMi-CK in bulk quantities. However, during purification of MiMi-CK from chicken hearts the mitochondrial preparations were always contaminated by soluble BB-CK which is the most abundant CK isoform in this tissue [1, 10]. We developed a simple one-step affinity method by which MiMi-CK is separated from the other CK isoforms and which yields, after subsequent elution from the Sepharose-Blue matrix with a pulse of ADP, highly enriched MiMi-CK. This is demonstrated here by CK zymograms and immunoblotting.

Materials and Methods

Extraction of Mitochondrial Creatine Kinase

Fresh chicken hearts trimmed free of fat, cut into pieces and put into a meat grinder were suspended in 220 mmol/l mannitol, 70 mmol/l sucrose, 10 mmol/l HEPES, 0.2 mmol/l EDTA, 1 mmol/l 2-mercaptoethanol (BME), 1 mmol/l NaN_3 at pH 7.4 at a 1:7 ratio (w/v) followed by homogenization in a Polytron mixer (Kinematica, Kriens, Switzerland). After centrifugation of the homogenate for 10 min at 600 *g* and resuspension of the pellet in the same buffer followed by an additional centrifugation the two low-speed supernatants were combined and centrifuged for 20 min at 18,000 *g* to pellet down the mitochondria. After washing twice the crude mitochondrial pellet with the above buffer and swelling them by two consecutive washing cycles with distilled water, MiMi-CK was extracted from the pellet consisting mostly of swollen mitochondria and mitoplasts by an overnight incubation at 4 °C with 0.1 mmol/l sodium phosphate buffer at pH 9.0 containing 5 mmol/l BME.

Affinity Chromatography on Sepharose-Blue

The supernatant containing MiMi-CK as well as contaminating BB-CK, after a centrifugation for 30 min at 45,000 *g*, was dialysed against 50 mmol/l sodium phosphate, 5 mmol/l $MgCl_2$, 1 mmol/l BME and 1 mmol/l NaN_3 at pH 6.0 and loaded onto a Sepharose-Blue column (Pharmacia, Sweden; a 2.5 cm × 8 cm column was used for approximately 200 mg of extracted protein) that had been preequilibrated with the same buffer at pH 6.0. After loading of the extract, the column was rinsed with pH 6.0 buffer until the optical density at 280 nm was less than 0.05. Subsequently, the column buffer was changed to pH 8.0. Contaminated BB-CK was eluted as soon as the pH value approached pH 8.0. After rinsing the column with at least two more column volumes of pH 8.0 buffer, 10 mmol/l ADP was added to the pH 8.0 buffer and mitochondrial CK was specifically eluted by this cofactor.

Other Methods

Cellulose polyacetate electrophoresis of native CK-isoenzymes followed by enzymatic staining by overlay-gel technique via the enzyme-coupled staining for CK were performed as described [11]. To block adenylate kinase (myokinase) activity [12], 0.3 mmol/l diadenosine pentaphosphate (Boehringer, Mannheim, FRG) was added to the overlay-gel. PAGE in the presence of $NaDodSO_4$ was performed according to *Lämmli* [13] and electrophoretic transfers as well as immunoblots according to *Towbin and Staehelin* [14] as modified by *Wallimann* et al. [15]. Antibodies prepared against purified BB-CK and MiMi-CK were prepared and characterized in this laboratory [3, 16].

Results and Discussion

MiMi-CK can be extracted almost quantitatively from swollen heart mitochondria by phosphate buffer [8]. However, even highly enriched preparations of mitochondria obtained by differential centrifugation of homogenized chicken heart muscle tissue were still consistently contaminated by cytosolic BB-CK. In order to avoid extensive losses of mitochondria during repeated washing cycles

and thus to avoid a reduction in the yield of MiMi-CK, an affinity chromatography method was established by which MiMi-CK was separated efficiently from contaminating BB-CK. This method allowed preparation of MiMi-CK with bulk quantities of crude mitochondria even though they were significantly contaminated with cytosolic BB-CK. As shown in figure 1a, phosphate extract of crude chicken heart mitochondria was applied to a Sepharose-Blue column at pH 6.0 using the buffer described under Materials and Methods. Both CK isoenzymes present in the extract (fig. 1a, b, lanes 1) were quantitatively adsorbed to the affinity matrix under these conditions. Elevation of the pH of the column buffer from pH 6.0 to pH 8.0 yielded a peak of CK activity that was identified by cellulose polyacetate electrophoresis and subsequent staining for CK activity as being the brain-type, BB-CK, of creatine kinase (fig. 1a, lane 2). Omission of phosphoryl creatine (CP), the substrate for CK, abolished the staining seen in figure 1a and addition of diadenosine-pentaphosphate inhibited any staining due to myokinase [12] indicating that the enzyme band appearing in figure 1a was indeed CK and not adenylate kinase. The identification of isoenzyme species was further confirmed by co-electrophoresis of chicken brain and muscle extracts containing almost exclusively the BB- and MM-CK isoforms, respectively. In addition, immunoblotting experiments using subunit-specific rabbit anti-chicken B-CK and Mi-CK antibodies revealed subunit-type characteristics of the isoforms involved (fig. 1b). As shown earlier, in chicken the B-CK subunit runs on $NaDodSO_4$-PAGE with an apparent M_r of 43,000, and Mi-CK exhibiting a slightly faster electrophoretic mobility runs with an apparent M_r of 42,000 [2, 3].

If in a second step, 10 mmol/l ADP was included in the pH 8.0 column buffer, the MiMi-CK isoenzyme was specifically eluted at a specific activity of 80–110 EU min^{-1} mg^{-1} of protein (fig. 1, second peak, MiMi-CK) and identified by zymograms (fig. 1a, lane 3) and immunoblotting (fig. 1b, lane 3). With this purification method were not only the two isoforms of CK separated, but also the peak fractions were eluted at a specific activity that was higher by a factor of 20–40 as compared to the samples that were loaded onto the Sepharose-Blue column. Routinely, more than 90% of MiMi-CK were eluted by 10 mmol/l ADP. By a similar technique the muscle-type MM-CK from chicken could be separated from the mitochondrial-type MiMi-CK (not shown).

Sepharose-Blue affinity chromatography in combination with ammonium sulfate fractionation and gel permeation chromatography allowed to purify bulk quantities of highly enriched, native mitochondrial CK in a short period of time. By employing this methodology we have obtained evidence that native mitochondrial CK is an octamer consisting of 4 MiMi-CK dimers [17].

The method described for the separation of mitochondrial CK from the cytosolic forms of this enzyme may be of clinical importance since in solid-phase radioimmunoassays up to 100 U of MiMi-CK per liter of serum, i.e. about 20% of the total CK activity, were measured in sera of patients with myocardial infarction [18] confirming earlier reports [19, 20] on the presence of MiMi-CK in sera of patients with heart disease and a marked reduction in MiMi-CK activity in skeletal muscle of chickens with hereditary muscular dystrophy. Thus, as *Blum* et al. [18] pointed out, MiMi-CK could turn out to be a more specific and sensitive

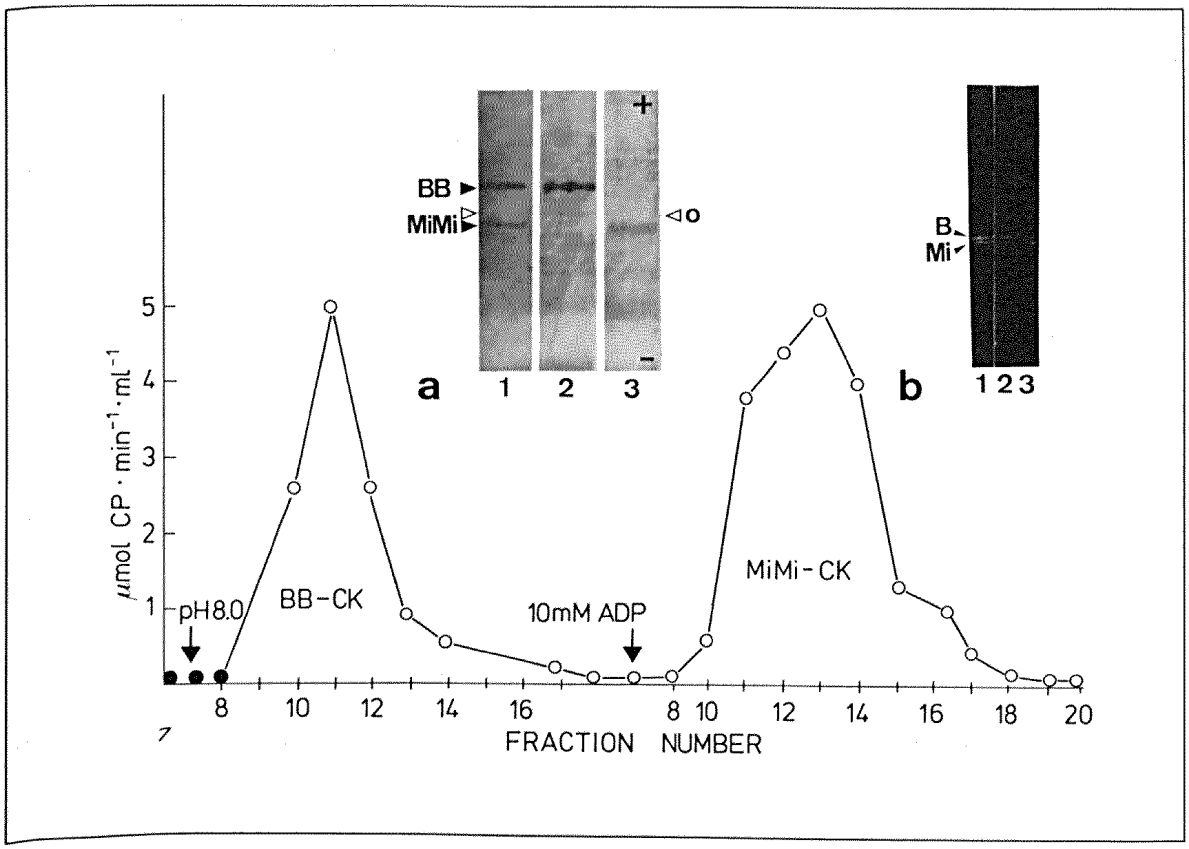

Fig. 1. Elution profile of CK activity from Sepharose-Blue column and identification of CK isoenzymes. Phosphate extract of crude mitochondria from chicken heart that contained MiMi-CK as well as BB-CK was loaded onto a Sepharose-Blue column at pH 6.0. Elution of BB-CK was facilitated by a change of column buffer to pH 8.0 (first peak, BB-CK) followed by elution of mitochondrial CK after addition of 10 mmol/l ADP to the pH 8.0 column buffer (second peak, MiMi-CK). CK activity of individual fractions is given in micromoles CP per minute and milliliter.

Inset **a** Cellulose polyacetate electrophoresis of a phosphate extract from crude chicken heart mitochondria which contained mitochondrial CK (MiMi-CK) as well as BB-CK before application onto a Sepharose-Blue column shown after staining in the presence of 0.3 mmol/l diadenosine-pentaphosphate for CK activity (**a**, lane 1); after changing the pH value of the column buffer from pH 6.0 to pH 8.0 (fraction 12 of the first peak in **a**, lane 2), and after addition of 10 mmol/l ADP to the pH 8.0 buffer (fraction 12 of the second peak in **a**, lane 3). O = origin of sample application. Note the different electrophoretical mobility of MiMi-CK and BB-CK. BB-CK (**a**, lane 2) and MiMi-CK (**a**, Lane 3) were separated by Sepharose-Blue affinity chromatography.

Inset **b** Immunoblots of a phosphate extract from crude mitochondria that contained MiMi-CK as well as BB-CK before application onto the Sepharose-Blue column (**b**, lane 1), immunoblot of fraction 13 of first peak (**b**, lane 2), and immunoblot of fraction 13 of the second peak (**b**, lane 3) all after $NaDodSO_4$-PAGE in 10% acrylamide, electrophoretic transfer to nitrocellulose paper and subsequent incubation with a mixture of both rabbit anti-chicken B-CK and rabbit anti-chicken MiMi-CK antibodies at dilutions of 1:100 each followed by 1:100 diluted FITC-conjugated goat anti-rabbit IgG (Cappel Labs, Dynatech, Kloten, Switzerland). The mixture of CK isoenzyme subunits present in **b**, lane 1 (B-CK and MiMi-CK subunits with apparent M_r of 43,000 and 42,000, respectively) was subsequently separated by Sepharose-Blue affinity chromatography in B-CK (**b**, lane 2) after a pH 8.0 step, and in MiMi-CK subunits (**b**, lane 3) after complementation of the pH 8.0 buffer with 10 mmol/l ADP.

biochemical marker for the severity of muscle diseases than the determination of total CK activity in sera and biopsy specimens. The one-step separation and enrichment of MiMi-CK from other CK isoforms reported here could greatly facilitate this analysis.

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