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PURIFICATION AND CELL-FREE TRANSLATION OF A UNIQUE HIGH MOLECULAR WEIGHT FORM OF THE BRAIN ISOZYME OF CREATINE PHOSPHOKINASE FROM MOUSE*

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Summary: Mouse brain creatine kinase was purified to homogeneity and shown to consist of two polypeptide chains of 50,000 daltons. This protein thus differs in size from all other creatine kinase molecules purified to date including the mouse muscle enzyme which shows a molecular weight between 39,000 and 42,000. The high molecular weight isozyme has been shown to represent the primary translation product of creatine phosphokinase mRNA from mouse brain. The unusual size of this creatine phosphokinase subunit provides unique tools for the study of the differential regulation of creatine kinase gene expression and for the study of subunit interactions in creatine kinase isozymes.

INTRODUCTION

Creatine phosphokinase (EC 2.7.3.2), an enzyme which plays a pivotal role in energy metabolism, has been isolated from a wide range of species (1,2). The enzyme exists as a dimer composed of 2 subunits. Two different types of monomers denoted M - (muscle type) and B - (brain type) have been identified in all species examined and usually possess similar or identical molecular weights of 40,000 daltons (3). The M and B monomers appear to be the products of at least two separate genes as evidenced by different amino acid compositions and differential regulation of the corresponding mRNAs in vivo (4,5). Considerable attention in recent years has focused on the possible physiological significance of multiple isozymic forms of CPK (3,6).

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The abbreviations used are: NaCl/Pi, phosphate buffered saline, 0.14 M NaCl, 10 mM sodium phosphate, pH 7.2, CPK, creatine phosphokinase.

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CPK expression has previously been shown to be developmentally regulated in myogenic cells (7). In the course of studying the regulation of CPK expression in a mouse derived muscle cell line, BC_3H1, we found that antibodies directed against human CPK precipitated from BC_3H1 extracts not only a protein of molecular weight 40,000 (M-CPK) but also smaller quantities of a protein of molecular weight 50,000 daltons (8). We now report that this higher molecular weight polypeptide represents mouse B-CPK. The mature enzyme and the primary translation product of B-CPK mRNA are indistinguishable in molecular weight. Mouse M-CPK, in contrast, exhibits a monomer molecular weight of 40,000 daltons in agreement with previous reports from other species (3,9). The large difference in molecular weight between the two isozymic forms of mouse CPK makes cells from this species a particularly interesting system for the study of CPK gene expression. Furthermore, the large difference in molecular weight between the mouse M- and B-CPK isozymes in addition to the large difference between mouse B-CPK and B-CPK in other species suggests that comparative structural information of these polypeptides will yield useful information regarding subunit interactions in the enzymatically active dimeric form.

MATERIALS AND METHODS

Purification of mouse brain creatine phosphokinase. Fraction 1 - Male Swiss-Webster mice were sacrificed by cervical dislocation and brains were removed and placed on ice. Approximately 15 gm wet weight of brain was obtained from 26 mice. Brains were well minced, followed by homogenization and filtration through cheesecloth as described by Armstrong et al. (10).

Fractions 2 and 3 - Ethanol precipitation followed by ammonium sulfate precipitation of the filtered homogenate was performed essentially as described (10) with the exception that a 50%-70% ethanol precipitation was used instead of 55%-60%. The ammonium sulfate pellet was resuspended to 5 mls with 50 mM Tris-HCl, pH 7.5, 5 mM β-mercaptoethanol, 0.1 M NaCl (column buffer).

Fraction 4 - The dissolved CPK (Fraction 3) was loaded onto a 2.5 x 100 cm Sephacryl S-100 column (Pharmacia Fine Chemicals, Toronto, Ontario) equilibrated with column buffer. The sample was eluted at 45 ml/hr and was collected in 5.3 ml fractions. Fractions were then assayed for CPK activity and absorbance at 280 nm was determined.

Fraction 5 - Fractions from the Sephacryl S-100 column containing CPK activity were pooled and loaded onto a 2.5 x 12 cm DEAE-cellulose column preequilibrated with column buffer. Protein was then eluted with a 500 ml linear gradient of 0.1-0.5 M NaCl in column buffer at a flow rate of 24 ml/hr. Five ml fractions were collected and fractions containing CPK activity

were pooled and concentrated by Amicon filtration. Protein was measured according to a modification of the Lowry procedure (11).

Creatine Phosphokinase Assay - CPK activity was assayed using the Sigma Creatine Phosphokinase Reaction Kit (Product number 45-1). All reactions were linear with respect to time and protein concentration.

Electrophoresis - For isozyme analysis of purified CPK, electrophoresis was performed on cellulose acetate strips as described (8). Mouse brain CPK was diluted to 0.04 U/ml in 50 mM Tris-HCl, 10 mM β-mercaptoethanol, pH 7.5, and 20 µl were applied to each strip. Following electrophoresis, specific enzyme staining was performed by overlaying the cellulose acetate strips with reaction strips which had been presoaked in CPK reaction mixture (Bio-Dynamics/Boehringer Mannheim, Inc.). CPK isozymes were visualized by ultraviolet fluorescence following incubation for 15 min at 37°C. Nonspecific background fluorescence was determined by omitting creatine phosphate from the reaction mixture. CPK standards, prepared as crude murine tissue extracts, were obtained from Helena Laboratories, Beaumont, Texas.

All polyacrylamide gel electrophoresis was performed according to Laemmli (12) and employed 10% polyacrylamide gels. Samples were prepared for electrophoresis as described previously (8). Following electrophoresis, gels were fixed and stained. For analysis of radiolabelled polypeptides, gels were treated with Enhance (New England Nuclear, Boston) according to manufacturer's instructions. Gels were then dried and exposed to Kodak X-Omat AR film for fluorography. Proteins used for M_r markers were: phosphorylase b, 92,500; bovine serum albumin, 68,000; ovalbumin, 43,000; carbonic anhydrase, 31,000; soybean trypsin inhibitor, 21,500.

Extraction of RNA - For extraction of total cellular RNA, mice were sacrificed by cervical dislocation and brains were removed and placed on ice. Brains were then minced and disrupted by polytron homogenization for 30 seconds in 4 volumes (wt/vol) of 8 M guanidine HCl, 20 mM sodium acetate, pH 5.0. The remaining steps were performed as described previously (8), according to a modification of the 8 M guanidine HCl technique of Cox and Deeley *et al.* (13,14).

Cell-Free Protein Synthesis and Immunoprecipitation of in vitro-Synthesized Creatine Phosphokinase - Total cellular RNA from mouse brain, at a final concentration of 200 µg/ml, was translated in a micrococcal nuclease-digested rabbit reticulocyte lysate system (New England Nuclear, Boston) as described previously (8). Following incubation for 60 min at 37°C, a 2 µl aliquot was removed from each translation reaction for analysis of total translation products by 10% polyacrylamide gel electrophoresis.

Immunoprecipitation of translation reactions with anti-human-BB-CPK antiserum (Organon Diagnostics, Inc.) was performed as described previously (8).

RESULTS

The purification of the brain form of mouse CPK is summarized in Table I. 2.7 mg of BB-CPK were obtained from approximately 15 gm of mouse brain (wet weight), or 1.7 gm total protein, representing a yield of 20% of the initial enzyme activity and a 120-fold purification. The final protein product possessed a specific activity of 190 µmol/min/mg, in agreement with the values

TABLE I

Purification of Mouse Brain Creatine Kinase

Fraction	Volume ml	Total Activity units (%)	Spec. Activity units/mg	Purification
1. Crude homogenate	50	2700 (100)	1.6	
2. 50-70% EtOH, 0.01 M $MgSO_4$, dialysis	19	1290 (48)	34	21
3. 0.70 saturation $(NH_4)_2SO_4$ precipitate	5	1370 (61)	39	25
4. Sephacryl S-100	69	1240 (46)	73	46
5. DEAE-cellulose	41	520 (19)	190	120

reported for the brain isozymes from calf and chicken (1,2). The relatively large amount of CPK activity which was lost in the initial centrifugation step can probably be attributed to the mitochondrial form of the enzyme since 20-25% of the CPK activity in mouse brain is localized in the mitochondrial fraction (15).

Chromatography on Sephacryl S-100 (Figure 1) resulted in an approximate 2-fold enrichment in specific activity over the preceding step and eliminated a significant amount of the low molecular weight protein contaminants (<100,000 daltons). DEAE-cellulose ion exchange chromatography eliminated a large amount of contaminating protein. In addition to separating BB-CPK from the

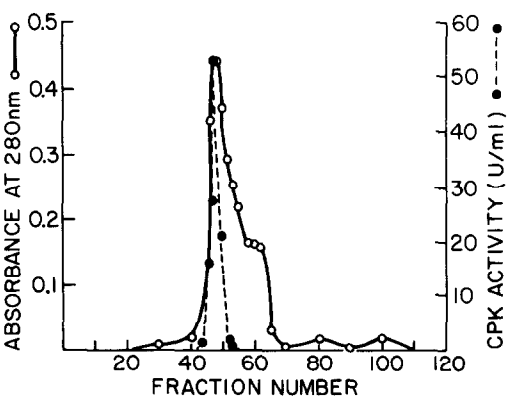

Figure 1. Chromatography of CPK on Sephacryl S-100. Fraction 3 was chromatographed on a Sephacryl S-100 column as described in Materials and Methods. Fractions 38-51 were pooled for further fractionation.

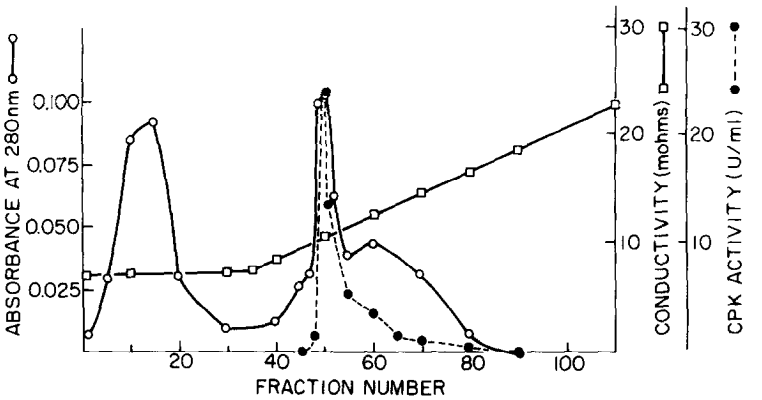

Figure 2. Chromatography of CPK on DEAE-cellulose. CPK fractions eluted from Sephacryl S-100 (Fig. 1) were chromatographed on DEAE-cellulose as described in Materials and Methods. Fractions 48-56 were concentrated by Amicon ultrafiltration to yield purified CPK.

majority of other contaminating proteins, this step also eliminates any possible traces of the muscle isozyme due to the large charge differences between the two isozymic forms (16). BB-CPK, which exhibits a low isoelectric point (17), was eluted at approximately 1.75 M NaCl (Figure 2). The enzyme obtained from the pooled peak fractions was concentrated by Amicon filtration and its purity was analyzed on 10% NaDod/SO_4 polyacrylamide gels (Figure 3, lane 7). The protein showed an apparent molecular weight of 50,000 daltons and contained only minor protein contaminants.

Cell-Free Translation and Immunoprecipitation

We considered the possibility that the apparently large molecular weight of mouse B-CPK was the result of post-translational modification. In order to test this possibility, total cellular RNA was extracted from mouse brain and translated in a rabbit reticulocyte lysate system. Immunoprecipitation of cell-free translations with rabbit anti-BB-CPK antiserum followed by NaDod/SO_4 polyacrylamide gel electrophoresis of the immunoprecipitated proteins, gave a single radioactive polypeptide which comigrated exactly with the mature form of mouse brain CPK (Figure 3, compare lanes 3 and 5). Unlabeled BB-CPK effectively competed for binding of the anti-BB-CPK antiserum to the 50,000 dalton translation product (Figure 3, lane 4). Immunoprecipitation with non-immune rabbit serum did not result in the precipitation of any radioactive

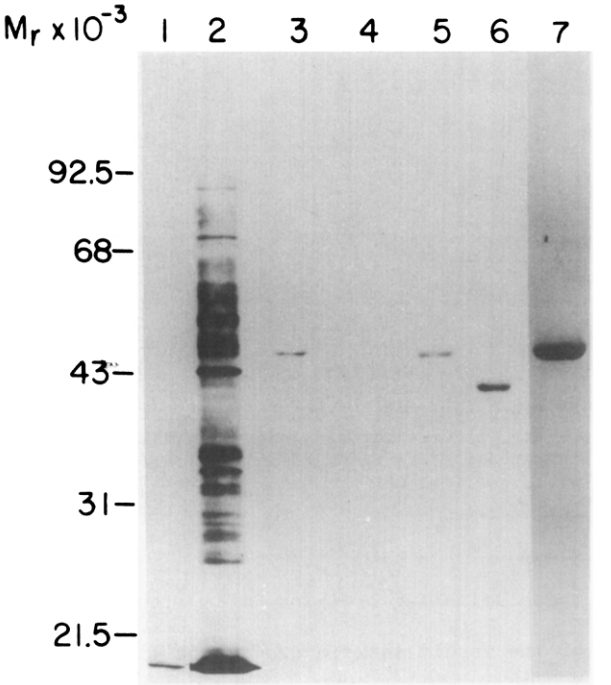

Figure 3. Polyacrylamide gel electrophoresis of CPK. Total cellular RNA was isolated from mouse brain and translated in a rabbit reticulocyte cell-free translation system as described in Materials and Methods. In vitro synthesized B-CPK was immunoprecipitated from 50 µl translations with anti-BB-CPK antiserum. Total cell-free translation products and immunoprecipitated proteins were analyzed on 10% polyacrylamide gels. Lanes: 1, endogenous translation, without added RNA; 2, total translation products of mouse brain RNA; 3, immunoprecipitation of cell-free translation with anti-BB-CPK antiserum; 4, immunoprecipitation of cell-free translation in the presence of excess unlabelled mouse brain CPK; 5, mouse B-CPK stained with Coomassie blue; 6, mouse M-CPK stained with Coomassie blue; 7, 10 µg of mouse brain CPK (Fraction 5).

polypeptides (data not shown). The large difference in apparent molecular weight between the M- and B-CPK isozymes from mouse can be clearly seen in lanes 5 and 6 in which the two purified CPK monomers were stained with Coomassie blue. Mouse M-CPK had a molecular weight of approximately 40,000 daltons and has been shown to comigrate with the primary translation product of the M-isozyme (see ref. 8).

Cellulose Acetate Electrophoresis

In order to demonstrate the lack of contamination of the purified mouse BB-CPK by other isozymic forms, the purified protein as analyzed by cellulose acetate electrophoresis. Using this technique it was found that purified mouse BB-CPK migrated as a single band of enzyme activity and co-

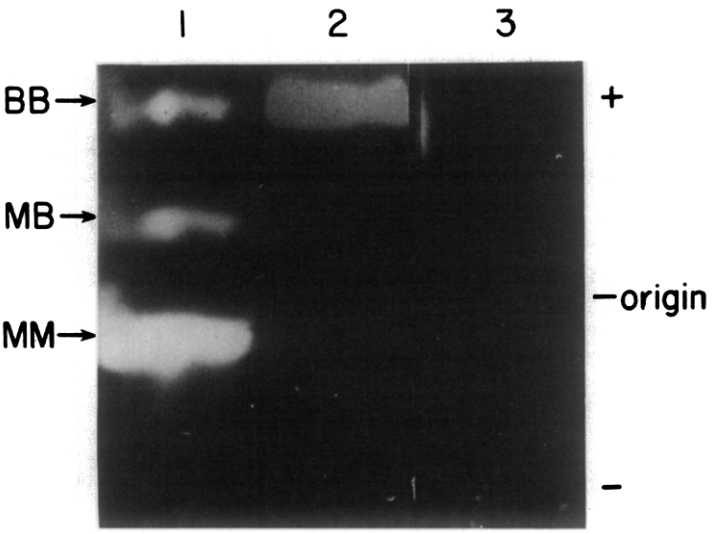

Figure 4. Cellulose acetate electrophoresis of CPK. 0.04 units of mouse brain CPK (Fraction 5) were applied to cellulose acetate strips and CPK activity was determined after electrophoresis as described in Materials and Methods. Strips: 1, mouse CPK standards; 2, purified mouse BB-CPK; 3, purified mouse BB-CPK stained with CPK reaction mixture without creatine phosphate for determination of nonspecific fluorescence.

migrated with the BB-isozyme from a mouse tissue extract (Figure 4, strips 1 and 2). No enzyme staining was observed in the absence of creatine phosphate (Figure 4, strip 3).

DISCUSSION

The purification and properties of the muscle and brain isozymes of CPK have been described for a variety of vertebrate species. The two isozymic forms demonstrate different catalytic, immunologic and physical properties in addition to differences in primary amino acid sequences (2,3,6). In most species which have been examined, however, the apparent molecular weights of the M and B monomers within the given species are similar or identical and demonstrate a range of approximately 78,500-85,100 daltons (3).

In the present study, the BB-isozyme of creatine phosphokinase was purified from mouse brain and was found to exhibit an apparent monomer molecular weight of approximately 50,000 daltons or 100,000 daltons for the enzymatically active dimeric form. This is 20% larger than the mouse muscle isozyme which demonstrates an apparent molecular weight of 80,000 daltons. Despite the similarity in molecular weights of the M and B isozymes within a

given species, other investigators have also reported the separation of the two forms under denaturing conditions. Caravatti et al., for example, reported the resolution of the muscle and brain monomers from chicken on 10% polyacrylamide gels (7). The degree of subunit separation, however, was very slight and only indicated minor differences in molecular weight between the two subunits. The high degree of subunit heterogeneity observed for the mouse isozymes in the present study is striking in comparison to previous reports from other species. Furthermore, the 50,000 dalton B-CPK monomer is the highest molecular weight form of CPK described to date.

A variety of potential mechanisms could explain the high degree of subunit heterogeneity between the mouse CPK isozymes. The large difference in molecular weight (10,000 daltons) between the M and B isozymes suggests that the brain isozyme possesses a longer polypeptide chain and therefore is encoded by a gene (and mRNA) significantly different from that encoding for the M-CPK isozyme. Post-translational protein processing and/or partial proteolytic degradation during the purification procedure of either isozyme would appear to be ruled out as possible causes for the size heterogeneity between the isozymes since the primary translation products of both M- and B-CPK comigrate with the catalytically active enzyme (Ref. 8 and Figure 3). The additional 10,000 dalton segment on the B-isozyme makes this particular enzyme a unique protein for the study of CPK subunit interactions since this larger polypeptide not only forms a BB dimer but also an active MB dimer. Whether or not the high molecular weight form of B-CPK has some significance to its role in energy metabolism within the cell remains to be determined.

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