Brain Isoenzyme of Creatine Kinase

II. SPECIES SPECIFICITY OF ENZYME AND PRESENCE OF INACTIVE FORM IN STRIATED MUSCLE OF RABBIT AND MAN*

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Antibodies specific for the brain isoenzymes (BB) of creatine kinase were prepared by the injection of the highly purified rabbit enzyme into roosters. The antibodies were found to cross-react only partially with BB of rat, guinea pig, sheep, monkey, and man, demonstrating that these proteins are not entirely identical with respect to their antigenic sites. A greater degree of species specificity seems to exist for BB than for the muscle isoenzyme (MM) suggesting that BB may have undergone considerable evolutionary modification. Developmentally mature rabbit cardiac and skeletal muscles contained large quantities of an enzymatically inactive BB which can be detected immunochemically. It is antigenically identical with active rabbit BB but the two proteins can be separated by DEAE-cellulose chromatography. Enzymatically inactive BB is also present in human skeletal muscle. The presence of MM in muscle provides sufficient catalytic function and may have permitted the evolution of inactive BB in an additional (noncatalytic) metabolic role.

Owing to its dimeric structure, soluble creatine kinase (EC 2.7.3.2) exists as three isoenzymes: MM, MB, and BB (1). It is generally believed, although unproved, that the M and B subunits are coded on different genes. Amino acid composition, finger print analysis, and immunochemical reactions reveal a high degree of structural homology between the MM isoenzymes of different species (2-8). Indeed, the MM isoenzyme has been found to be an organ-specific, rather than a species-specific, antigen (5). The BB isoenzyme is less well studied in this respect.

During ontogeny the changing isoenzyme pattern in skeletal and cardiac muscle is similar among various mammalian species (9-12). In the very young fetus only BB exists. As ontogeny proceeds, MB activity gradually appears followed by MM activity. BB activity diminishes and, shortly after birth, the adult pattern appears in which MM activity predominates together with lesser amounts of MB and only a trace of BB. The transition is less complete in heart than in skeletal muscle. Falling BB activity is not associated with a declining rate of isoenzyme synthesis because, despite almost no BB enzymatic activity in skeletal muscle of the 1-2-day-old rabbit, the incorporation of [14C]leucine into BB was as high or higher than into MM or MB (13).

We have used an immunochemical approach to examine the antigenic similarity of the BB isoenzymes of various mammals. The same antibodies have enabled us to demonstrate an enzymatically inactive BB isoenzyme in adult striated muscle of rabbit and man.

EXPERIMENTAL PROCEDURES

A number of experimental techniques have been used as described (14). These include the automated fluorescent creatine kinase assay, protein assay, production of antibodies to rabbit BB, agar gel double immunodiffusion, and antibody inhibition of enzyme activity. Unless specifically noted, all reagents were of analytical grade.

Biological Material and Tissue Extracts

Rabbits - Male California white rabbits (2 to 3 kg) were killed by an overdose of sodium pentobarbital administered intravenously. Tissues were removed immediately, rinsed free of blood with cold running water, and transported to the laboratory on ice. In the cold, gastrocnemius was trimmed free of fat, connective tissue, nerves, and blood vessels and the atria and great vessels were removed from the heart. Brain was freed of dura. The tissues were homogenized at 2°C in 5 volumes of doubly distilled deionized water containing 0.014 M 2-mercaptoethanol. A Sorvall Omni-Mixer (Ivan Sorvall Inc., Newtown, Conn.) was used to homogenize muscle and heart after these tissues were minced with scissors. A glass tube and motor-driven Teflon pestle were used to homogenize brain. The homogenates were frozen and stored at −20°C until thawed for study. All investigations were conducted on the tissue supernatants (10,000 × g for 30 min at 2°C).

Human - Normal brain was obtained at autopsy. It was frozen and stored at −20°C and pieces were chopped off and thawed for use as required. Gastrocnemii were obtained from a 5-month fetus following hysterotomy for therapeutic abortion. At autopsy, normal gastrocnemius was taken from a 5-year-old female child. Samples of other developmentally mature muscles were obtained by biopsy. All muscle samples were trimmed of extraneous tissue as described for rabbit and were studied immediately or frozen and stored at −20°C. Mincing with scissors and aqueous homogenization were ultimately

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† During course of this research, Fellow of the Medical Research Council of Canada, and subsequently, of the Muscular Dystrophy Association of Canada.

‡ Creatine kinase in a dimer and there are two different subunits. Hence, the brain, muscle, and hybrid isoenzymes are designated BB, MM, and MB, respectively.
performed as described for rabbit. Again, investigations were conducted on the tissue supernatants (10,000 \times g_{max} for 30 min at 2°C).

Miscellaneous Species—Sheep brain was kindly provided by Dr. Cecil Pace-Acquaia (Hospital for Sick Children). Rhesus monkey brain was a gift from Dr. Donald Hill (Hospital for Sick Children). These specimens, together with the brain of rat and guinea pig, were frozen and stored at -20°C. Aqueous homogenization was performed as described for rabbit. Tissue supernatants (10,000 \times g_{max} for 30 min at 2°C) were studied.

Cellulose Acetate Electrophoresis for Assay of Creatine Kinase Isoenzyme Activities

Creatine kinase isoenzymes of tissue extracts were separated by cellulose acetate electrophoresis using the Gelman Sepratek system (Gelman Instrument Co., Ann Arbor, Mich.). Electrophoresis was conducted in Tris/barbital buffer (ionic strength 0.05, pH 8.8) or 30% cellulose acetate electrophoresis using the Gelman Sepratek system. The brain was a gift from Dr. Donald Hill (Hospital for Sick Children). Those specimens, together with the brain of rat and guinea pig, were frozen and stored at -20°C. Aqueous homogenization was performed as described for rabbit. Tissue supernatants (10,000 \times g_{max} for 30 min at 2°C) were studied.

Immunoochemical Quantitation of BB

Serial dilutions of tissue extracts were made to 1:1024. The highest dilution which gave an unaltered immunoprecipitation reaction with full-strength immune plasma or serum was noted with the aid of a hand lens. Immunoreactivity is expressed as the reciprocal of this dilution in antigen units (AU). For example, a dilution of 1:64 represents 64 antigen units.

Separation of Creatine Kinase Isoenzymes by DEAE-cellulose Column Chromatography

This procedure was performed throughout at 2°C. Extract from two rabbit hearts was fractionated at pH 7.0 with solid (NH4)2SO4 (enzyme grade, Schwarz/Mann, Orangeburg, N. Y.). The 31 to 70% fraction, collected by centrifugation at 45,000 \times g_{max}, was dissolved in 2 ml of 0.02 M Tris/HCl (pH 8.0) containing 0.014 M 2-mercaptoethanol and was dialyzed exhaustively against this buffer. The retentate was applied to a column (1 x 15 cm) of DEAE-cellulose (14) which had previously been equilibrated with the same buffer. At a flow rate of 60 ml/h, the chromatogram (Fig. 5) was developed with a linear concentration gradient of NaCl produced by continuously adding 400 ml of 0.4 M NaCl in starting buffer to an equal volume of starting buffer. Fractions (2 ml) were pooled as indicated in Fig. 5. The protein in each pool was precipitated at 70% (NH4)2SO4 and recovered by centrifugation for 45 min at 45,000 \times g_{max}. Each sample was then dissolved in 1 ml of starting buffer and dialyzed exhaustively against this buffer.

RESULTS

Cross-reactivity of Brain Creatine Kinase from Other Species with Anti-rabbit BB Immune Plasma—In agar gel double immunodiffusion studies there was an immunoprecipitation reaction between anti-rabbit BB immune rooster plasma and brain extracts of guinea pig, rat, sheep, monkey, and man. However, these reactions were weaker than, and only partially identical with, the reaction between the immune plasma and rabbit brain extract (Fig. 1). The precipitin line for rabbit spurred over the lines for all other species, whereas these latter did not break through the precipitin line for rabbit.

Fig. 2 illustrates the effects of anti-rabbit BB immune rooster plasma on creatine kinase (CK) activity in brain extracts of rabbit (O), monkey (•), man (△), guinea pig (▲), rat (□), and sheep (○). Varying dilutions of extracts were added to a fixed amount of immune plasma (constant final volume). Incubation was for 1 h at 25°C and 18 h at 2°C. Residual creatine kinase activity was assayed in 2000 x g supernatant. Note that immune plasma inhibited all creatine kinases but was most effective against rabbit enzyme. Effects were not due to precipitation as none was observed for species other than rabbit. Greater inhibition of rabbit brain creatine kinase was also not related to precipitation since inhibitory effect was fully manifest after incubation for 1 h at 25°C when precipitation was minimal.

Compared to rabbit, the concentration of creatine kinase activity at which 50% inhibition occurred was lower for the other species. These effects were not due to precipitation as none was observed except with rabbit brain extract. However, the inhibitory effect of the immune plasma on rabbit brain extract was also fully manifest in the absence of precipitation (14).

Enzymatically Inactive Brain Isoenzyme in Heart and Skeletal Muscle—Despite the low enzymatic activity of BB in rabbit heart and gastrocnemius compared to rabbit brain (Fig. 3), its protein was detectable in the muscular tissues (Fig. 4).
FIG. 3 (left). Cellulose acetate electrophoresis of creatine kinase isoenzymes in extracts of developmentally mature rabbit tissues: 1, heart; 2, brain; 3, gastrocnemius (dilution = 1/2); and 4, gastrocnemius (full strength). Procedure described in text. Three applications of extracts made at cathodal end of strip. Note trace of BB activity in extracts of gastrocnemius (absent in diluted extract) and heart compared to brain extract. MB activity was greater in heart than gastrocnemius. MM activity was greater in gastrocnemius than heart. Control strip (phosphorylcreatine omitted from enzyme stain) did not show any bands.

FIG. 4 (right). Double immunodiffusion analysis conducted under same conditions as in Fig. 1. Unstained precipitin lines photographed after 48 h. Center well contained anti-rabbit BB immune rooster plasma. Peripheral wells contained (A) rabbit heart extract (dilution = 1/2), (B) rabbit gastrocnemius extract, (C) rabbit brain extract, and (D) purified rabbit BB. Precipitin lines stained for protein after 48 h (14). Note single immunoprecipitin reaction of identity between center well and all peripheral wells. Similar distances between center well and precipitin lines for heart, gastrocnemius, and brain indicate that all tissue extracts contained at least similar amounts of antigenic BB protein. Compare with enzymatic activity of BB in these tissues (Fig. 5).

TABLE I

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Specific activity</th>
<th>Ratio B:C</th>
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<tbody>
<tr>
<td></td>
<td>A. Total creatine kinase</td>
<td>B. Enzymatic BB</td>
</tr>
<tr>
<td>Brain</td>
<td>11,500</td>
<td>11,500</td>
</tr>
<tr>
<td>Heart</td>
<td>52,200</td>
<td>1,300</td>
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<tr>
<td>Gastrocnemius</td>
<td>237,000</td>
<td>230</td>
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* Values expressed are means for five rabbits. Standard errors in brackets. Activities in the tissue extracts are expressed per g of protein.

* AU, antigen units.

TABLE II

<table>
<thead>
<tr>
<th>Pool</th>
<th>BB antigenic specific activity*</th>
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<tr>
<td>A</td>
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<td>B</td>
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<tr>
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<td>F</td>
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<tr>
<td>G</td>
<td>6</td>
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* Antigen units (AU) per g of protein.
FIG. 5. Separation of creatine kinase (CK) isoenzymes of rabbit heart at 2° by chromatography on column (1 x 15 cm) of DEAE-cellulose. Starting buffer was 0.02 M Tris/HCl (pH 8.0) containing 0.014 M 2-mercaptoethanol. Linear salt gradient elution established by continuously adding 400 ml of 0.4 M NaCl in starting buffer to equal volume of starting buffer. Flow rate was 60 ml/h (down) and 2-ml fractions were collected. Solid line denotes absorbance at 280 nm and broken line indicates creatine kinase activity. Gradient (---) was measured using conductivity meter. Fraction Pools A to G were made. Protein in each pool was concentrated by precipitation at 70% (NH₄)₂SO₄. After recovery and dialysis of each protein pool, they were examined by cellulose acetate electrophoresis for creatine kinase isoenzymes. See inset and note absence of BB and trace of MB and MM in Pool E. (S denotes sample prior to chromatography.) Pool G contained only traces of three isoenzymes. Control strip (phosphorylcreatine omitted from enzyme stain) showed traces of activity in cathodal region where MM was observed in all pools.

DISCUSSION

There are substantial quantities of immunoreactive BB in mature rabbit and human striated muscle despite the relatively low enzymatic activity of this isoenzyme. The reported high rate of [¹⁴C]leucine incorporation into BB of newborn rabbit gastrocnemius (13) may, therefore, represent rapid synthesis of the inactive protein. The decrease of BB activity in mammalian striated muscle (9-12) during development may be related to an inactivation of the isoenzyme rather than to a decrease in its synthesis or an increase in its degradation.

There are clearly two forms of the BB isoenzyme since an active and an inactive fraction could be separated by DEAE-cellulose column chromatography. Their different binding affinities for the ion exchanger do not necessarily indicate major differences in their primary structure. A conformational change could be sufficient to account for their apparent difference in effective negative charges. Both forms behave identically in agar gel double immunodiffusion studies suggesting that they have similar diffusional and antigenic properties.

The enzymatically inactive BB we observed did not appear to be an in vitro artifact; nor is it likely to have resulted from aging (15-17) since we worked with young animals. Moreover, Perry (18) has reported a resurgence of BB enzymatic activity in the skeletal muscle of very old rabbits. Conversion of inactive BB to the active form does not seem to occur in dystrophic rabbit skeletal muscle even though BB activity increases in this tissue (19). Instead, both the active and inactive fractions increase proportionately in the diseased muscle (19). The mechanism of inactivation and the relationship between the active and inactive fractions remains unknown.

The apparent species specificity of BB contrasts with the organ specificity of MM (5). In striated muscle MM has catalytic advantages over BB in that it is more stable (20) and less subject to phosphorylcreatine inhibition (21). The pressure of natural selection on BB has no doubt been relaxed in striated muscle since MM provides a viable phenotype.

Consequently, one might expect BB to display properties of enzymatic instability and conformational variability as well as species differences in amino acid composition and antigenicity. Indeed, some of these features have been documented for BB in this paper and in other reports (4, 14, 20, 22). The gene for the B subunit may be perpetuated in the genome because BB has selective neutrality (23).

On the other hand, perhaps in striated muscle BB performs an unknown metabolic role that is related to its inactivation.

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