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THE FURTHER HETEROGENEITY OF CREATINE KINASE

PRESENCE OF ISOENZYMES OF CATHODIC MOBILITY IN RAT TISSUES

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Summary

Supernatant fractions from rat tissue homogenates were examined for creatine kinase (EC 2.7.3.2) isoenzymes by agarose gel electrophoresis at pH 8.6. The distribution of the various isoenzymes, and their relative contributions to the total tissue creatine kinase, is stated. A previously unreported isoenzyme associated with brain and kidney preparations is described. It is a minor contributor to brain creatine-kinase activity but is the major isoenzyme for kidney. The presence of an additional isoenzyme for cardiac tissue was confirmed; this isoenzyme exhibits similar levels of activity to that of the MB isoenzyme.

Introduction

The dimeric structure and the presence of three isomer forms, MM, MB and BB, of creatine kinase (EC 2.7.3.2) were established by Burger et al. [1] and Dawson et al. [2] Jacobs et al. [3] and later other investigators [4—8] found evidence for the even further heterogeneity of creatine kinase. They observed that the creatine-kinase activity of certain subcellular fractions exhibited electrophoretic mobilities different from that of MM, MB and BB. However, not all investigators were able to confirm these findings [9—12].

We have examined creatine-kinase isoenzymes by agarose electrophoresis and a nitroblue tetrazolium stain. The results confirm the previously reported finding of an additional isoenzyme of cathodic mobility associated with cardiac tissue. Furthermore, evidence is presented of a hitherto unreported creatine-kinase isoenzyme of cathodic mobility associated with kidney and brain tissues.

Methods

Sprague-Dawley rats were killed by exsanguination and the skeletal muscle (rectus abdominis), heart, brain and kidney were homogenized in 4 vols. of

their weight of ice-cold isotonic saline containing 1.0 mM β-mercaptoethanol. Supernatants obtained following centrifugation at 20 000 × *g* for 30 min were examined by electrophoresis using an agarose membrane, Pfizer Pol-E-film (1% agarose and 5% sucrose), in 0.05 M Veronal buffer pH 8.6 at 4° C. One μl material was applied and a distinct band separation was obtained in 10 min with an electric field of 10—15 V/cm.

The creatine-kinase substrate contained at pH 6.8 Tris · HCl buffer 50 mM, creatine phosphate 20 mM, ADP 0.5 mM, magnesium chloride 10 mM, D-glucose, 20 mM, NADP, 0.3 mM, β-mercaptoethanol, 5 mM, hexokinase, 2 I.U. per ml and glucose-6-phosphate dehydrogenase, 2 I.U. per ml. The staining solution consisted of 25 mg nitroblue tetrazolium and 1.5 mg phenazine methosulfate dissolved in 100 ml deionized water.

Following electrophoresis, the membranes were washed with 0.01 M Tris buffer, pH 6.8, containing 1.5 mM β-mercaptoethanol, incubated at 37° C for 10—15 min in the substrate solution and then rapidly washed three times with deionized water. Membranes were then transferred to the staining solution and incubated for 10 min. Excess stain and residual substrate were removed by careful washing with deionized water and the membranes were left to dry at 50° C. The conditions for electrophoresis and staining allowed for the detection of $5 \cdot 10^{-5}$ I.U. of creatine kinase activity as measured at 37° C.

Total creatine-kinase activity was determined on a Dupont Automatic Clinical Analyzer employing the method of Oliver [13] and included dithioerythritol as enzyme activator. The diluent was isotonic saline containing 1.5 mM β-mercaptoethanol and bovine albumin (1 mg per ml).

The contribution of each isoenzyme band to the total tissue creatine-kinase activity was determined by transmission densitometry, of the prepared membrane at 608 nm. The intensity of stain was correlated with enzyme activity by comparing with serial dilutions of known MM type creatine kinase activity as standards.

Results

The results of determinations for total creatine-kinase activity for the various tissue extracts are given in Table I. Electrophoresis of these tissue supernatants showed (Fig. 1) that in addition to MM, MB and BB, isoenzyme bands of cathodic mobility were present. The one, associated with heart extract, had a relative mobility of —0.24 with respect to BB at pH 8.6, and showed comparable staining to that of the MB fraction (Fig. 1, Table II). This isoenzyme of cathodic mobility was also found when human cardiac autopsy material was examined by a similar procedure (Fig. 2). The cathodic band associated with brain and kidney extracts has a mobility of —0.14 with respect to BB at pH 8.6 (Fig. 1). The degree of staining of the cathodic band associated with brain extract suggests that it is only a minor contributor to total creatine-kinase activity; in contrast, for the kidney, it was at least equivalent to that of BB (41—50% compared to 18—36%), considered to be the major isoenzyme of kidney creatine kinase. Heart extracts frequently exhibited BB although it was of low activity (< 1%, Table II). None of the above described bands could be detected in the absence of creatine phosphate from the staining procedure.

TABLE I

TOTAL CREATINE KINASE ACTIVITIES OF VARIOUS RAT TISSUES

The values are the range of duplicate experiments at 37°C

Tissue	Creatine kinase activity (I.U./g wet weight of tissue)
Skeletal muscle	2400—2800
Brain	210— 350
Heart	680—1250
Kidney	50— 140

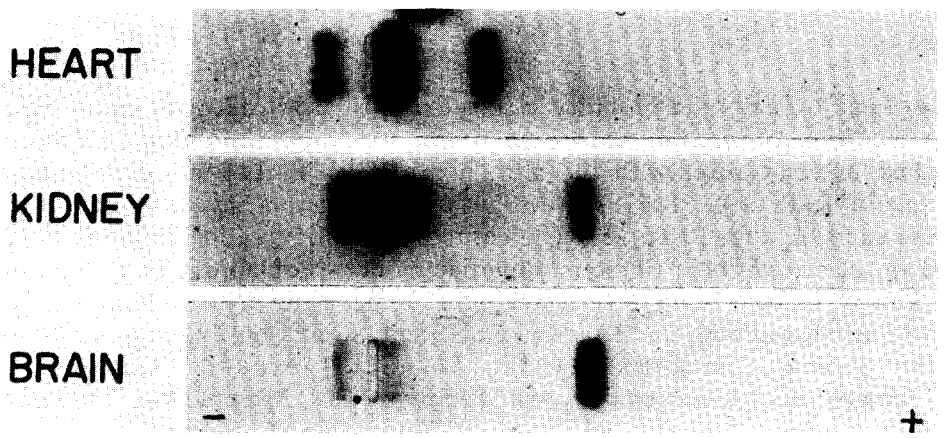

Fig. 1. Electrophoretograms of rat tissue supernatant fractions showing creatine kinase activities.

TABLE II

CREATINE KINASE ISOENZYMES OF RAT TISSUE EXTRACTS

The figures are the percentage contributions of each isoenzyme fraction to the total tissue creatine kinase. See text for methods.

Isoenzyme band	Skeletal muscle	Brain	Heart	Kidney
BB	n.d. [a]	97—99 [b]	0— 1	18—36
MB	n.d.	n.d.	11—12	n.d.
MM	100	1—2	60—81	23—32
Isoenzyme band of realtive mobility —0.14 to BB	n.d.	0.5—1	n.d.	41—50
Isoenzyme band of relative mobility —0.24 to BB	n.d.	n.d.	8—27	n.d.

[a] None detected.
[b] Range of duplicate experiments.

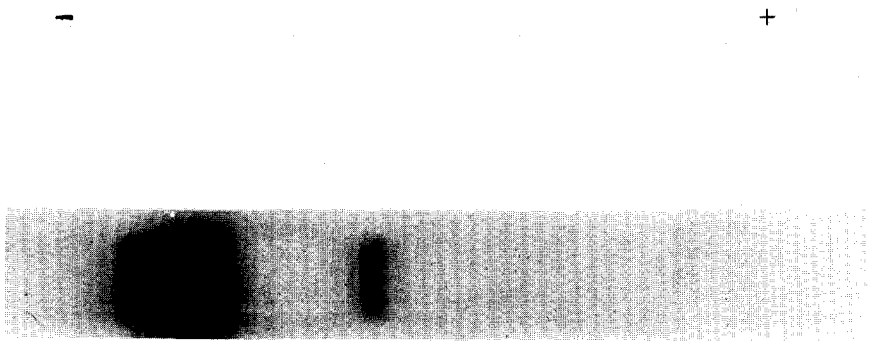

Fig. 2. Electrophoretogram of human, 22-h autopsy, cardiac-tissue supernatant fraction, showing creatine kinase activities.

Discussion

The band of cathodic mobility associated with heart extracts could have originated from organelles. Isoenzyme bands of cathodic mobility have been described in association with the mitochondrial [6—8, 14] and myofibrillar fractions of heart tissue [4,6]; however, other investigators have been unable to confirm such findings [9,15,16]. The cathodic band of creatine-kinase activity found on electrophoresis of brain supernatant does not appear to have been previously described. Some investigators find brain-tissue creatine-kinase activity to be associated with the BB isoenzyme fraction only, but other studies suggest the presence of additional fractions. Wood [17] observed multiple bands using cellulose acetate electrophoresis; Wolintz et al. [10] found two bands, of mobilities similar to those of MM and BB; Madsen [5] found two bands, the one being BB and the other of somewhat less anodic mobility; and Lapin et al. [18], using acrylamide gel electrophoresis, observed up to six bands of creatine-kinase activity associated with the high-speed supernatant and mitochondrial fractions of adult mouse brain.

Also, a band of cathodic mobility associated with kidney tissue does not appear to have been previously described. However, there have been only a few studies on kidney creatine-kinase isoenzymes. Dawson and Fine [9], Allard and Cabrol [19] and Smith [11] all found BB to be the major isoenzyme fraction, but Jockers-Wretou and Pfleiderer [20] found it to be MM.

Differences due to animal species seem an unlikely explanation of the present findings. All the above-quoted studies were carried out on tissues isolated from the rat and human, and despite all these numerous reports a species-specific creatine-kinase isoenzyme has never been observed. It is suggested that the ability to detect additional isoenzyme bands in the present experiments was due to the sensitive procedure employed.

Although the role of thiol groups in cathodic isoenzyme activity was not investigated, a sulfhydryl agent was found necessary to develop a sufficiently sensitive stain for visualizing these isoenzymes. Furthermore, β-mercaptoethanol protected against inactivation of all isoenzyme bands. These findings suggest that the isoenzymes of cathodic mobility are similar to MM, MB and BB in their requirement for thiol groups at the site of catalytic activity.

One other aspect of the present study requires comment. The results of scanning membranes were corrected against type MM creatine kinase of known activity that had also been subject to electrophoresis. Nevertheless, because of the inherent limitations of quantifying by densitometry, the values obtained for the various isoenzyme fractions should be considered as close approximation only.

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