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# EVIDENCE FOR SIGNIFICANT QUANTITIES OF CREATINE KINASE MM ISOENZYME IN HUMAN BRAIN

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## Summary

The isoenzymes of creatine kinase (ATP : creatine *N*-phosphotransferase, EC 2.7.3.2) in human brain were studied. Approx. 30% of total creatine kinase activity is due to the MM isoenzyme. The identity of this isoenzyme was confirmed by several techniques and shown not to be of mitochondrial origin.

## Introduction

Creatine kinase (ATP : creatine *N*-phosphotransferase, EC 2.7.3.2) exists as three electrophoretically distinct isoenzymes designated BB, MB and MM. Each isoenzyme consists of two subunits of either the M (muscle) or B (brain) type. The BB isoenzyme occurs predominantly in brain and the MM in skeletal muscle. Cardiac muscle contains both the MM and the hybrid MB isoenzymes [1]. There have been many reports on the typing of creatine kinase isoenzymes from the brain [2—5]. Using electrophoretic methods to separate the three isoenzymes, Sjövall and Voigt [3] analysed aqueous extracts of human tissue and reported the presence of the BB isoenzyme and another band which they identified as adenylate kinase (ATP : AMP phosphotransferase, 2.7.4.3), as this band disappeared if creatine phosphate was absent from the assay medium.

Using similar techniques, Van der Veen and Willebrands [6] reported trace amounts of the MM isoenzyme in rat brain extracts and Klein et al. [4] found small amounts of the MM isoenzyme in canine brain.

Other authors [7,8] whilst purifying creatine kinase from brain failed to report the presence of any isoenzyme other than BB. Bulcke and Sherwin [9] did not detect any MM isoenzyme in rabbit brain extracts using immunological techniques.

In this report we demonstrate the presence of significant quantities of an active MM isoenzyme in human brain by means of both electrophoretic and immunological techniques.

## Materials and Methods

Creatine kinase was isolated from human brain, taken at autopsy as soon as possible after death (less than 12 h) from myocardial infarction. The meninges and vessels were removed, the brain was cut into 2-cm cubes, washed in 0.1 M ammonium acetate (pH 9.5) and homogenised in the same buffer. Creatine kinase was partially purified by ethanol fractionation, $(NH_4)_2SO_4$ fractionation, DE-52 (DEAE-cellulose) ion-exchange chromatography, essentially as described by Keutel et al. [5]. Sephadex G200 gel filtration was performed on a column previously calibrated with standards of known molecular weight, namely catalase, bovine albumin, ovalbumin and myoglobin.

Mitochondria from human brain were prepared by the method of Basford, omitting Nagarse from the isolation medium [10]. Oxygen uptake was measured polarographically with a Clark type electrode using succinate as the substrate. Creatine kinase was extracted from mitochondria by suspension of the mitochondria in 0.1 M ammonium acetate (pH 9.5), sonication and then centrifugation at 15 000 $\times g$. The supernatant fraction contained the solubilised mitochondrial creatine kinase.

Animals were killed by cervical dislocation. Their brains were immediately removed, washed in 0.1 M ammonium acetate (pH 9.5)/1 mM $\beta$-mercaptoethanol, homogenised in the same buffer (2 ml/g tissue) and centrifuged for 20 min at 15 000 $\times g$. Isoenzyme electrophoresis was performed on the supernatant fractions.

Creatine kinase MM isoenzyme was isolated from human psoas muscle by the method of Keutel et al. [5].

Creatine kinase activity was measured on a Centrifichem System 400 centrifugal analyser (Roche) using an optimised method (Boehringer, Mannheim GmbH Diagnostica). Normal serum values by this method are 0—50 I.U./l. Adenylate kinase activity was measured by the method of Adam [11].

Electrophoretic separation of creatine kinase isoenzymes was carried out on cellulose acetate strips using a barbitone buffer (pH 8.6, ionic strength 0.75). Isoenzyme bands were visualised by the sandwich technique [4] and examined under ultraviolet light.

Hybridisation of creatine kinase subunits was carried out essentially by the method of Keutel et al. [5]. Samples containing equal activities of MM and BB isoenzymes (1 I.U./ml) after Sephadex G200 gel filtration, were incubated in 8 M urea for 5 min at 37°C and then dialysed at 4°C for 16 h against 0.6 M $NH_4HCO_3$/1 mM $\beta$-mercaptoethanol. The mixture was then analysed for creatine kinase isoenzymes.

Anti-creatine kinase-M subunit antibodies were obtained from E. Merck, Darmstadt and dialysed against 0.6 M $NH_4HCO_3$/1 mM $\beta$-mercaptoethanol before use. Equal volumes of sample and antibody were incubated for 10 min at 20°C before measurement of the creatine kinase activity.

Thermal inactivation studies were performed by incubating samples (DE-52 eluates) containing creatine kinase activity at 37°C. Aliquots were withdrawn at the indicated time intervals, cooled immediately to 0°C and the creatine kinase activity measured.

## Results

Electrophoresis of a supernatant of a whole human brain homogenate showed the presence of rapidly and slowly migrating bands corresponding to MM and BB (Table I). A similar picture was found with whole brain homogenates of rat, rabbit and guinea pigs (Table I).

Preparative ion exchange chromatography of the partially purified brain extract (Fig. 1) showed the presence of 2 discrete fractions with creatine kinase activity and a further fraction with adenylate kinase activity. It can be seen that the peak of adenylate kinase activity does not correspond with that of the earlier eluting creatine kinase fraction. Adenylate kinase migrates more anodally than the creatine kinase MM isoenzyme on electrophoresis [2] and would be more tightly bound to the ion exchange column. Fractions were pooled as shown, concentrated by dialysis against solid polyethylene glycol and subjected to gel filtration on a previously calibrated Sephadex G200 column. The creatine kinase activity of the two pools 1 and 3 (Fig. 1) both eluted in a fraction corresponding to a molecular weight of approximately 83 000 which is in close agreement with the documented molecular weight of 80 000 for creatine kinase [12]. Adenylate kinase has a molecular weight of 21 000 [13].

Pools 1 and 3 containing creatine kinase activity were chromatographed separately on Sephadex G200. The eluate was assayed for creatine kinase activity. Selected fractions containing creatine kinase activity were then assayed in the presence and absence of antibodies to the M subunit. These antibodies reduced the creatine kinase activity of the fractions derived from pool 1 to less than 5% of the original activity but had no effect on creatine kinase activity of fractions derived from pool 3 (Table II). Pool 2, which contained both creatine kinase and adenylate kinase activities (Fig. 1) was assayed for these activities both with and without anti-M antibodies present. Creatine kinase activity was reduced from 202 I.U./l to 4 I.U./l by the addition of these antibodies, whilst adenylate kinase activity was 92 I.U./l without antibodies and 120 I.U./l in the presence of antibodies.

TABLE I

MOBILITIES FROM POINT OF ORIGIN OF CREATINE KINASE ISOENZYMES RELATIVE TO HUMAN ALBUMIN ON CELLULOSE ACETATE ELECTROPHORESIS

| | | | | | |
|---|---|---|---|---|---|
| BB (human brain) | +1.14 | | | | |
| MB (human myocardium) | | +0.58 | | | |
| MM (human muscle) | | | | −0.10 | |
| Mitochondrial (human brain) | | | | | −0.16 |
| Whole brain (human) | +1.14 | | | −0.10 | |
| Cerebrum | +1.14 | | | −0.10 | |
| Cerebellum | +1.14 | | | −0.10 | |
| Basal ganglia | +1.14 | | 0.0 | −0.10 | |
| Whole brain (guinea pig) | +1.14 | | | −0.10 | |
| Whole brain (rabbit) | +1.14 | | | −0.10 | |
| Whole brain (rat) | +1.14 | | 0.0 | −0.10 | |
| Serum from myocardial infarct patient | | +0.58 | | −0.10 | |
| Hybridisation experiment | +1.14 | +0.58 | | −0.10 | |

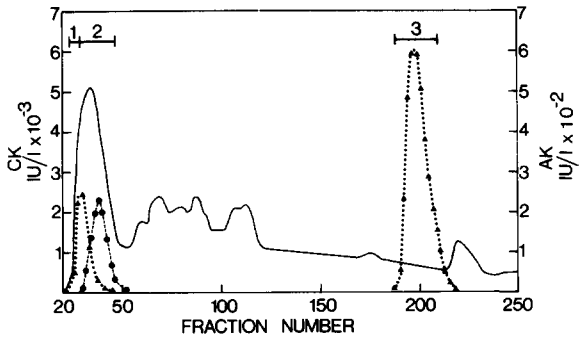


Fig. 1. DE-52 ion exchange chromatography of partially purified brain homogenate. $A_{280}$, ———; creatine kinase (CK), ▲· · · · · ▲; Adenylate kinase (AK), ● - - - - - ●. Samples were pooled as indicated by the solid bars (top).

Hybridisation experiments between partially purified (DE-52 eluates) MM and BB fractions showed the production of an MB isoenzyme that migrated between the original MM and BB bands on cellulose acetate and had the same mobility as an MB band from a myocardial infarct patient (Table I).

Further confirmation of the identity of the MM fraction isolated from human brain was provided by thermal inactivation studies of the MM and BB fractions relative to a MM isoenzyme isolated from human muscle. These studies showed that, whereas the BB isoenzyme was rapidly inactivated by incubation at 37°C, these conditions had little effect on the activity of MM isoenzymes from either muscle or brain (Fig. 2).

In order to exclude the possibility that the MM isoenzyme was of mitochondrial origin, mitochondria were prepared from human brain. Succinate dehydrogenase was used as a mitochondrial marker. (The isolated

TABLE II

EFFECT OF ANTI-M-ANTIBODIES ON CREATINE KINASE ACTIVITIES AFTER ELUTION FROM SEPHADEX G-200

| Fraction | Creatine kinase activity (I.U./l) | |
|---|---|---|
| | Without anti-M | With anti-M |
| Pool 1 | | |
| Fraction 64 | 257 | 16 |
| Fraction 66 | 350 | 14 |
| Fraction 70 | 448 | 27 |
| Fraction 72 | 406 | 21 |
| Fraction 74 | 303 | 11 |
| Pool 3 | | |
| Fraction 62 | 501 | 504 |
| Fraction 64 | 703 | 757 |
| Fraction 66 | 678 | 735 |
| Fraction 68 | 647 | 656 |
| Fraction 70 | 459 | 493 |

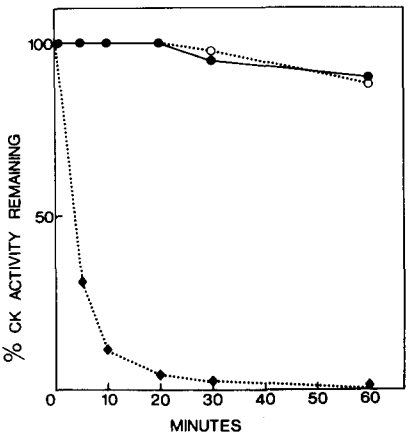


Fig. 2. Thermal inactivation of creatine kinase by incubation at 37°C. ●———●, MM (muscle); ○ · · · · · ·○, MM (brain); ◆ · · · · · ·◆, BB.

mitochondria consumed 1.5 nmol oxygen/mg protein per min, whilst both the post mitochondrial supernatant and the original brain homogenate consumed less than 0.1 nmol oxygen/mg protein per min). Table I shows that on electrophoresis the mitochondrial isoenzyme migrates more cathodally than the MM isoenzyme. Anti M antibodies had little effect on its activity reducing it from 537 I.U./l to 494 I.U./l. This contrasts with the effect of these antibodies on the MM isoenzyme.

Isoenzyme electrophoresis was performed on extracts of selected regions of the human brain (Table I). Activity of the BB isoenzyme is significantly greater than that of the MM isoenzyme in both cerebellum and cerebrum but this relationship is reversed in basal ganglia.

## Discussion

From our data (Fig. 1) we calculate that 31% of the total creatine kinase activity extracted from human brain using 0.1 M ammonium acetate is due to the MM isoenzyme. This result may seem surprising in view of the fact that other authors [2—8] have either failed to detect or demonstrated only small amounts of the MM isoenzyme in brain tissue. Possible explanations for this are (a) different extraction techniques since it is known that the proportion of creatine kinase activity extracted is dependent on the conditions used (salt concentration, type of buffer, pH [5]); (b) selection of different regions of the brain since we have shown that the MM isoenzyme is predominantly found in basal ganglia; (c) a failure by some authors to use quantitative methods. Bulcke and Sherwin [9], using a phosphate-buffered saline extraction buffer, did not detect any MM isoenzyme in rabbit brain using immunological detection procedures and concluded that MM was an organ-specific antigen. In our studies, however, creatine kinase M subunit antibodies abolished creatine kinase activity of the MM isoenzyme isolated from human brain (Table II).

Since it has been shown that approximately 30% of the myocardial creatine

kinase is situated on the outer surface of the inner mitochondrial membrane [14], the possibility existed that the MM isoenzyme, isolated by us, was of mitochondrial origin, even though this creatine kinase isoenzyme is known to migrate more cathodally than MM [15]. We have demonstrated that brain mitochondrial creatine kinase also migrates more cathodally than MM. Moreover, anti-M-antibodies, which abolished MM activity, have little effect on mitochondrial creatine kinase activity. Furthermore mitochondrial creatine kinase could not be extracted from mitochondria under the conditions used to isolate creatine kinase from whole brain, namely extraction of homogenised tissue with 0.1 M ammonium acetate (pH 9.5) unless the mitochondrial suspension was sonicated.

Leroux et al. [16] have reported the presence of a labile form of creatine kinase, creatine kinase Z, in extracts of human heart and brain. Creatine kinase Z electrophoresed on agarose between the MM and MB isoenzymes and on storage at 4°C and after chromatography on DEAE-cellulose changed into a form with identical electrophoretic properties to the MM isoenzyme. Creatine kinase Z could only be isolated in the presence of β-mercaptoethanol; in addition freezing destroyed the creatine kinase Z activity [16]. Since our isolation procedures were performed in the presence of β-mercaptoethanol and the isolated MM isoenzyme was stable to freezing and thawing, it would seem unlikely that this MM isoenzyme originated from the labile creatine kinase Z.

Armstrong et al. [17,18] have recently reported the presence of catalytically active and inactive BB isoenzymes in human and rabbit muscle thus demonstrating that at least the BB isoenzyme is not organ specific. Thus muscle cells synthesise both the M and the B subunits of creatine kinase and not surprisingly the hybrid MB isoenzyme is also present. We found extremely low creatine kinase activity eluting from DE-52 in fractions 70—74 (unpublished data). Total activity of this fraction was less than 1% of total MM activity. This was presumably due to an active MB isoenzyme.

The hybridisation experiment is additional evidence for the occurrence of the MM isoenzyme in brain. We have shown that a functionally active MB isoenzyme could be produced from M and B subunits isolated from human brain. Since only a small amount of the MB isoenzyme could be detected in the original extract, it is possible that the synthesis of M and B subunits is compartmentalised.

In the field of diagnostic enzymology it has been assumed that the isoenzymes of creatine kinase are organ-specific [1]. Our work and that of Armstrong et al. [17,18] indicates that this specificity may not be as clear cut as has been previously thought. Thus brain cells synthesise not only the BB isoenzyme, but also MM and mitochondrial isoenzymes. After cerebral vascular accidents it has been shown that the elevated serum creatine kinase is of the muscular type [19]. It has been assumed that this creatine kinase is not of cerebral origin and the failure to detect the BB isoenzyme is due to the impermeability of the blood-brain barrier [1]. We would like to suggest that this muscular isoenzyme does indeed originate from the brain and the failure to detect the brain isoenzyme is due to its rapid thermal inactivation at body temperature (Fig. 2).

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