PURIFICATION OF MITOCHONDRIAL CREATINE KINASE: TWO INTERCONVERTIBLE FORMS OF THE ACTIVE ENZYME

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SUMMARY

The purification of creatine kinase from beef heart mitochondria is described. The purified enzyme appears as a single band after electrophoresis on SDS gels. Electrophoresis on cellulose acetate followed by staining for creatine kinase activity shows two forms of the enzyme. The slower migrating (m-1) form upon concentration is converted to the more rapidly migrating form (m-2). The reverse conversion occurs if the m-2 is incubated with β-mercaptoethanol. These results are consistent with a reversible oxidation of protein sulfhydryl group(s).

Creatine kinase in mammalian heart cells has been shown to exist in mitochondria (1), myofibrils (2), and in the cytosol (3). The first observation of a mitochondrial isozyme of creatine kinase was that of Jacobs et al. (1). Bessman and Fonyo studied the enzyme in pigeon breast muscle mitochondria and concluded the enzyme did not utilize intramitochondrial ATP as a substrate but required extramitochondrial adenine nucleotides (4).

Since these early studies there have been many reports documenting the existence of mitochondrial creatine kinase. A recent study by Jacobus and Lehninger (5) using rat heart mitochondria demonstrated that the enzyme will use ATP generated in the mitochondria and that the phosphorylation of creatine by mitochondrial creatine kinase occurs outside the atractyloside sensitive ADP-ATP carrier system. They concluded that in the presence of physiological concentrations of creatine and ADP that creatine phosphate is the end product of oxidative phosphorylation.

Similar results have been reported by Saks et al. (6). These authors also studied the effect of Mg^{++} concentration on the activity of mitochondrial creatine kinase and found that at low Mg^{++} the synthesis of creatine phosphate

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is favored, while at higher Mg^{++} the synthesis of ATP is stimulated.

It seems very likely that the mitochondrial creatine kinase isozyme may play a major role in controlling the flow of high energy phosphates between the mitochondria and the myofibrils in heart cells.

We report here the purification of the creatine kinase from beef heart mitochondria. The apparently purified enzyme exists in two electrophoretically separable forms, a fast moving cathodal form (m-2) and a slower cathodal form (m-1). The distribution of these two forms depends upon the extent of reduction of the enzyme sulfhydryl groups.

MATERIALS AND METHODS

Isolation of mitochondria: Beef heart mitochondria were isolated by differential centrifugation as described previously (7). Tissue was homogenized in 3 volumes of 0.25 M sucrose with 0.05 M Tris-HCl pH 7.4, 1×10^{-3} M EGTA, 1×10^{-3} M β-mercaptoethanol.

Electrophoresis of creatine kinase isozymes: The method employs Gelman Sepraphore III cellulose acetate strips, prepared in 0.06 M Tris-bartital buffer, pH 8.8, supplemented with 1 mM dithiothreitol. Separation was achieved using 300 V for 3 hrs at 4°C. Strips were then stained as previously described (9) and the NADH produced was measured by fluorometric scanning. In addition to the complete staining mix, some strips were stained with a mix lacking creatine phosphate, as a control for adenylate kinase. No reaction was detected in these controls.

Enzyme assays: Creatine kinase was assayed by the coupled reactions described by Rosalki (10) using Calbiochem CPK-Max Pack reagent.

Protein assays: Protein was assayed by the method of Bradford (11) using Coomasie Brilliant Blue G-250.

Affinity chromatography: A column containing 2.5 ml Agarose-hexane-adenosine-5'-triphosphate (P-L Biochemicals) was employed, using 0.03 M Tris-HCl, pH 8.3, 3×10^{-4} M EGTA, 1.3×10^{-3} M β-mercaptoethanol, at a flow rate of 20 ml/hr. Elution was with a gradient of ATP, 0-10 mM, in a total volume of 100 ml; 2.9 ml fractions were collected.

Polyacrylamide gel electrophoresis: Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis was performed according to Weber and Osborn (8) with the following modifications. The proteins were prepared by incubating at 60°C for 30 minutes in gel buffer plus 1% dithiothreitol and 6 M urea, adding an equal volume of tracking dye in glycerol and applying 100 µl of this resulting solution to a gel. Usually 10 µg of protein was applied per gel. The gels were 7-1/2% acrylamide, made by mixing 5 ml 30% acrylamide, 0.8 ml 2% methylenebisacrylamide, 10 ml gel buffer, 3 ml water, 1 ml freshly made ammonium persulfate (12 mg per ml) and 10 µl of N,N,N',N'-tetramethylethylenediamine. Gel tubes were 5 mm internal diameter, 12.7 cm in length. Staining was done at 60°C for 30 minutes. Destaining was done by incubating the gels in a 10% glacial acetic acid - 50% methanol solution.

TABLE 1

	Total Protein (Mgs)	Total Creatine Kinase (Units)	Specific Activity (U/mg)	(%) Isozyme Distribution MM	m-1	m-2
1. Mitochondrial Extract	783	6,742	8.61	17	12	71
2. First Ethanol Precipitate	161	2,310	14.4	0	33	67
3. Second Ethanol Precipitate with $MgSO_4$	17.3	1,296	74.9	0	33	67
4. DEAE Fraction 1	2.34	260	111	0	37	63
5. DEAE Fraction 2	1.56	65	41.7	0	80	20

RESULTS AND DISCUSSION

Table I shows the purification of mitochondrial creatine kinase from 75 grams of beef heart mitochondria. The mitochondria, in a total volume of 100 ml in sucrose, were diluted with 175 ml of 0.1 M Na-phosphate buffer, pH 7.5, containing 10^{-3} M dithiothreitol, and stirred in the cold overnight. The mitochondria were removed by centrifugation and washed three times with 100 ml volumes of Na-phosphate buffer, pH 7.5. The pooled extract and washes were diluted with the same buffer to give 10 units/ml total creatine kinase activity (700 ml total volume; fraction 1). Electrophoresis of the extract from even well-washed mitochondria always reveals some activity, apparently MM, near the origin (Fig. 2a).

Most of the purification occurs during the alcohol fractionations. Ethanol (95%) was added dropwise at about 5 ml/min with stirring on ice. Each successive addition increased the ethanol concentration by 5% up to a final concentration of 45% ethanol. The precipitates were dissolved in 0.1 M Tris, pH 7.5, containing 1×10^{-3} M β-mercaptoethanol. The fractions between 30-40%

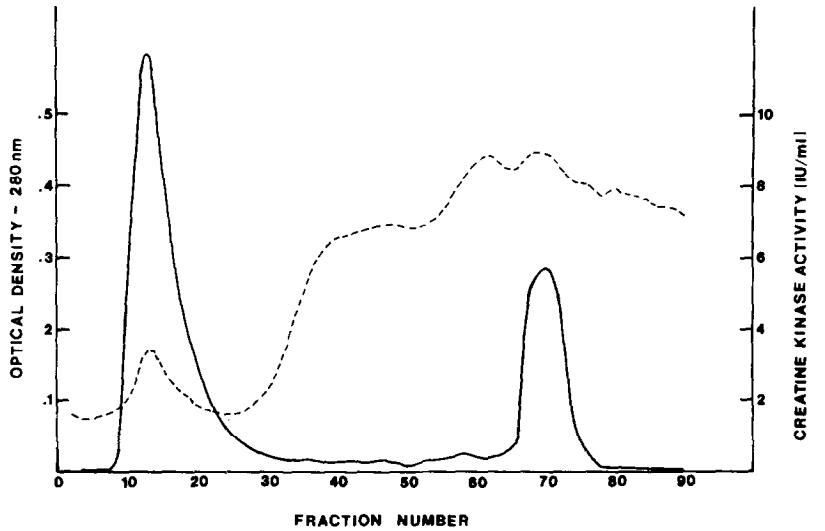

Fig. 1: Elution pattern of mitochondrial creatine kinase from a DEAE-Sephadex column. (——) enzymatic activity; (---) OD 280. Details are in the text.

ethanol had the highest specific activity and these were combined as fraction 2 $MgSO_4$ was added to give a final concentration of 0.1 M. Ethanol was added as before at approximately 7-8% increments in ethanol concentration. The two fractions at 21% and 28% contained most of the activity, and these were pooled and dialyzed against 0.1 M Tris-HCl, pH 9.0, containing 1×10^{-3} M EGTA, 1×10^{-3} M β-mercaptoethanol (fraction 3).

This sample was put on a 35 x 2 cm column of DEAE Sephadex A-50 which had been equilibrated with 0.1 M Tris-HCl, pH 9.0, 1×10^{-3} M EGTA and 1×10^{-3} M β-mercaptoethanol. The column was washed with 100 ml of the buffer and then eluted with a 600 ml gradient of NaCl, 0-0.5 M at a flow rate of 20 ml per hour; 5 ml fractions were collected. As shown in Fig. 1, two peaks of creatine kinase activity were recovered. The first (DEAE-1) appearing in the void volume, contains 24% of the activity and 16% of the protein applied. The second peak, at fraction 70 (DEAE-2) contains 6% of the activity and 10% of the protein applied. The electrophoretic distribution in the first fraction, shown in Fig. 2b, is the same as that applied to the column. This distribution, with

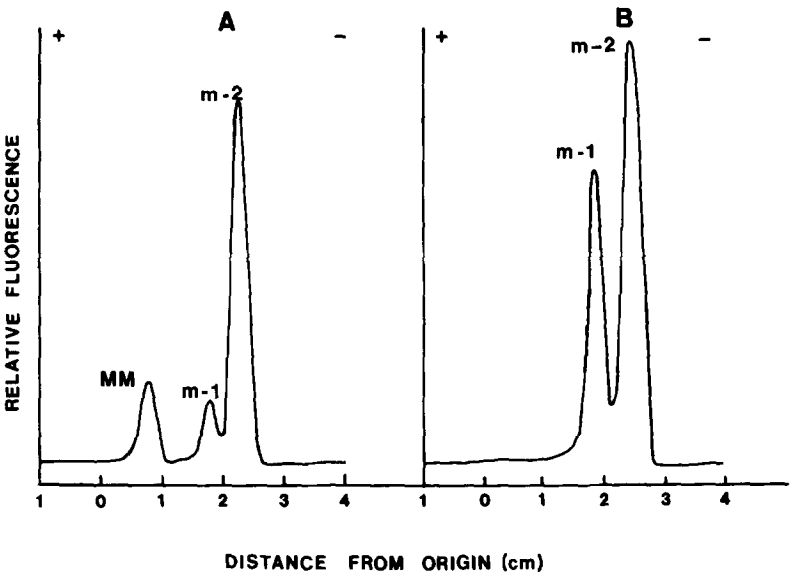

Fig. 2: a: Electrophoretic pattern of the initial extract from mitochondria showing a small amount of MM creatine kinase and both mitochondrial forms.

b: Electrophoretic pattern of the first fraction of creatine kinase eluted from the DEAE-Sephadex column (Table I, 4).

about twice as much of the faster migrating form (m-2) as of the slower (m-1), remained relatively constant throughout the isolation (Table 1). An exception to this is the second fraction off the DEAE column which in addition to its lower specific activity, also displays an altered ratio of the two forms, with 80% of the m-1 form and 20% of the m-2 form. Only a single band was seen on SDS gel electrophoresis of the material from DEAE-1.

When a sample of the apparently pure mitochondrial creatine kinase from fraction DEAE-1 was passed through an agarose-ATP affinity column, the enzyme which was eluted at about 5 mM ATP was found to consist entirely of the slower m-1 form (Fig. 3a). When this enzyme was stored in its diluted form, it remained entirely as m-1. However, upon 3-fold concentration of a portion of the enzyme with an Amicon concentrator to 0.075 mg/ml protein, it was seen to convert to a mixture of 63%, m-1, 37%, m-2 (Fig. 3b). Another similar sample which was concentrated to a higher degree (about 5X) showed a higher proportion

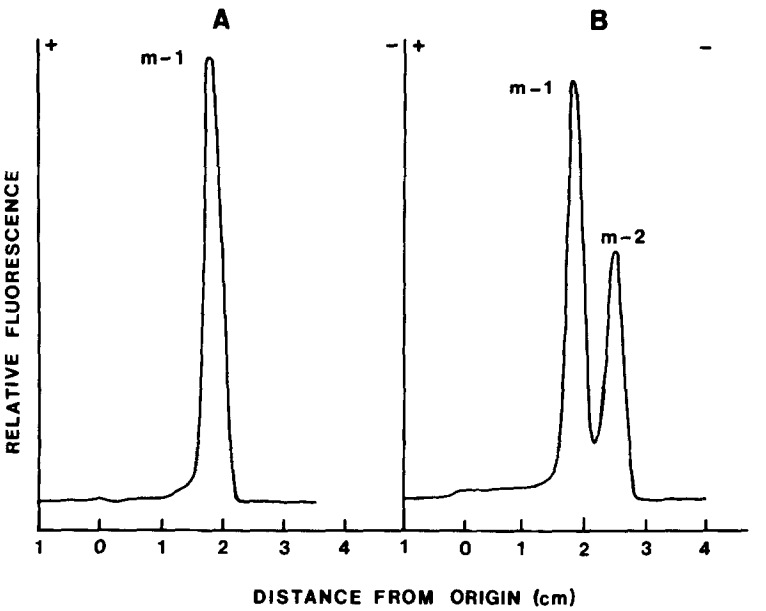

Fig. 3: a: Electrophoresis of creatine kinase after passage through the ATP-agarose affinity column. Only the m-1 form is present.

b: Electrophoresis of the same enzyme after concentration.

of the m-2 (59%). Even higher proportions (70%) have been seen in concentrates which started as an approximately equal mixture of the two forms.

When the concentrated preparation shown in Fig. 3b was incubated for 3 hrs with 100 mM β-mercaptoethanol, the total activity was not changed significantly, but the proportion of the fast-migrating form was reduced from 37% to 19%.

These results are consistent with a reversible oxidation of sulfhydryl groups in m-1 which results in the conversion to m-2. Since the electrophoresis is carried out at pH 8.8, the formation of a disulfide would effectively remove 2 negative charges and this modified enzyme would migrate more rapidly toward the cathode. We do not know whether this disulfide is intramolecular or intermolecular, however, the fact that concentration of m-1 results in conversion to m-2 suggests it may be intermolecular.

Perhaps the most interesting question is whether or not the two forms of the mitochondrial enzyme are both present in vivo and whether the two forms

might have different physiological roles. Both forms have been present in extracts of mitochondria isolated from the hart tissues of all species we have examined: beef, rabbit, and guinea pig. As we have previously reported, no mitochondrial form of creatine kinase is seen in fetal moust hearts (12).

Studies are underway to further characterize these enzymes and elucidate their role in vivo.

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