CREATINE KINASE: EVIDENCE FOR A DIMERIC STRUCTURE[1]

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Electrophoretic studies have recently made it clear that the enzyme creatine kinase is one of an increasingly large group of enzymes known to occur in several distinct forms. Most authors (Burger et al., 1964; Deal and van Breeman, 1964; Eppenberger et al., 1964) have observed three electrophoretic varieties: one variety characteristic of skeletal muscle, and another of the brain. A third enzyme, intermediate in migration, is found, in addition to either or both of the others, in the heart of some adult species. Eppenberger et al. (1964) have described the ontogeny of these enzymes in the rat and chicken. The electrophoretic patterns of crude extracts of chicken muscle, heart, and brain are shown in Fig. 1. In common with other investigators, we have found it a general rule that all muscle from adult mammals or chickens contains only one creatine kinase called for convenience "muscle enzyme". Brain contains exclusively the "brain enzyme". Chicken heart extracts contain the "brain enzyme" and may contain the intermediate enzyme. In mammals heart extracts

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usually contain largely the "muscle enzyme" and occasionally the intermediate enzyme. In all extracts adenylic kinase is also usually visualized (Fig. 1). In birds other than the chicken the findings are more complex and will be reported elsewhere.

In addition to these creatine kinases from the soluble portion of the cell, Jacobs et al. (1964) have described a separate mitochondrial enzyme. The reports of Kar and Pearson (1965) and of Sjöval and Voigt (1964), indicating additional electrophoretic bands, are at variance with these findings.

To date there are little published data on these enzymes apart from those on electrophoretic migration. Wood (1963) has crystallized the brain enzyme from ox brain and has compared some of its properties with those reported by Kuby et al. (1954) in their original study of the rabbit muscle enzyme. We are aware of no other published studies of the enzyme from brain, although the rabbit muscle enzyme has been the subject

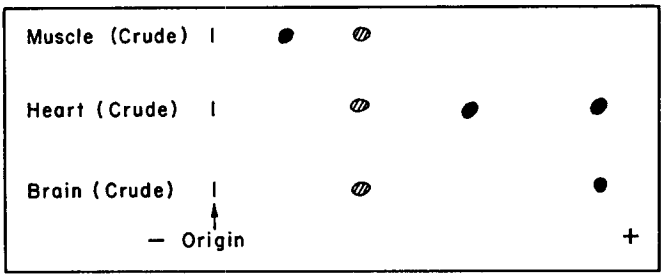

Fig. 1. Electrophoretic patterns (Fine et al., 1963) obtained with crude extracts (homogenization in 0.1 M Tris, pH 7.5, followed by centrifugation at 100,000 x g) of skeletal muscle (1), heart (2), and brain (3) from the adult chicken. The amount of the intermediate enzyme found in heart extracts depends in part on the age of the chicken. The staining medium consisted of Tris buffer, 0.1 M, pH 7.5, 15 ml.; $MgCl_2$, 0.1 M, 1.0 ml.; glucose, 0.1 M, 1.0 ml.; hexokinase and glucose 6-phosphate dehydrogenase, partially purified from yeast, 0.6 ml.; creatine phosphate, 0.1 M, 0.6 ml.; ADP, 0.05 M, 0.5 ml.; TPN, 0.035 M, 0.5 ml.; Nitro BT, 10 mg./ml., 1.0 ml.; and PMS, 1 mg./ml., 0.12 ml. In crude extracts of these tissues adenylic kinase is also visualized by this method (indicated by hatched areas); it can be identified as such after elution from the gel and can be partially inhibited by the addition of AMP, 0.1 M, 1.0 ml., to the staining medium.

of a number of investigations. Among these are several suggesting that creatine kinase is an enzyme with a dimeric structure. Dance and Watts (1962) found that after tryptic digestion only half the expected number of lysine plus arginine spots was found on fingerprint analysis. Mahowald et al. (1962), Thomson et al. (1964) and Mahowald (1965) have reported that there are two cysteine residues per mole of enzyme which appear to be concerned with the active site. Optical rotatory dispersion data indicate that 2 moles of ADP are bound per mole of enzyme (Kaegi and Li, 1965).

We have purified two creatine kinases from the chicken, one from muscle and a second from both brain and heart. Details of the purification procedure will be presented elsewhere. Substantial catalytic differences have been found between the two enzymes. By dissociating the enzyme molecules in concentrated guanidine or by freezing and thawing in the presence of salt and sodium phosphate as has been accomplished with a number of dehydrogenases (Chilson et al. 1965), the two enzymes can be induced to form an intermediate "hybrid" enzyme. On electrophoresis, the artificially formed hybrid migrates to a distance halfway between the two parental enzymes. Its catalytic properties are intermediate as well. It therefore resembles the naturally occurring intermediate enzyme found in chicken heart.

Our findings with the purified enzymes are shown in Fig. 2. After the enzymes were dissociated in 6.5 M guanidine, mixed, and reactivated by dilution or by dialysis, an intermediate enzyme was found. This enzyme was then separated from the others by chromatography on DEAE-cellulose, and after separation was free of the parental forms.

Kinetic studies were performed on the two parental enzymes, the artificially formed hybrid and a sample of the naturally occurring hybrid enzyme separated by chromatography. These findings are summarized in Tables I and Ia which show that the hybrid enzyme is intermediate in

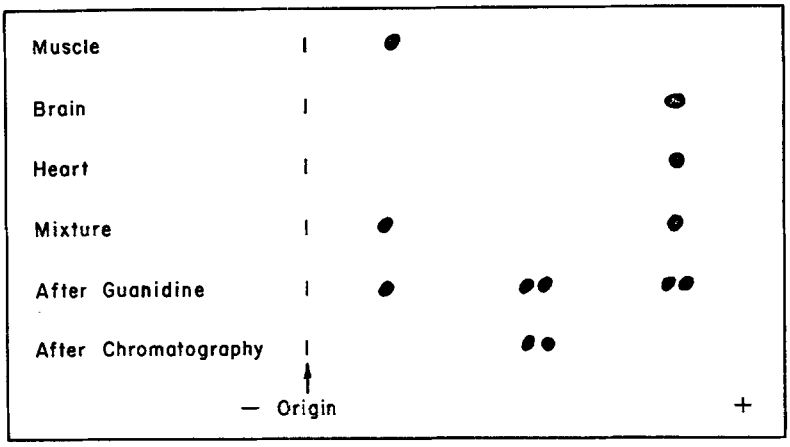

Fig. 2. Electrophoretic patterns at pH 7.0 obtained with purified chicken creatine kinases. Slot 1 shows the enzyme from muscle, Slot 2 the enzyme from brain, and Slot 3 the same enzyme purified from heart. Equal amounts of the muscle and brain enzymes were mixed (Slot 4), dissociated in buffered 6.5 M guanidine containing 0.1 M mercaptoethanol, and dialyzed. Slot 5 shows the product, with appearance of the intermediate or hybrid enzyme. This enzyme was then separated from the others by chromatography on DEAE cellulose, as shown in Slot 6.

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its rate of reaction in both the forward and reverse directions between the two parental forms and is similar to the naturally occurring intermediate enzymes.

It was of interest that, after dissociation by guanidine or after freezing and thawing, both the hybrid enzyme and the purified brain enzyme showed a double spot on electrophoresis. It seemed clear that the double spot was contributed by the brain enzyme for two reasons: 1) The double spot was usually seen when the brain enzyme had been reversibly denatured with guanidine or frozen and thawed in the presence of salt and sodium phosphate, although occasionally a double spot in the position of the brain enzyme was seen with crude extracts that had been frozen and stored in the absence of salt. 2) We have formed a hybrid molecule between the chicken brain enzyme and a purified sample of human muscle creatine kinase, indicating that the source of the muscle enzyme

Table I

Ratio of rates of reaction with high and low concentrations of creatine phosphate

The assay medium in a final volume of 3 ml. contained Tris buffer, pH 7.5, 250 mM; TPN, 0.15 mM; $MgCl_2$, 3.3 mM; glucose, 3.3 mM; ADP, 0.5 mM; glucose-6-phosphate dehydrogenase (Sigma) 5 µg; hexokinase (Sigma) 4 µg; and one of two concentrations of creatine phosphate. High concentration refers to 3.3 mM and low to 1.0 mM; the high/low ratio is reported. The assay is similar to that of Oliver (1955) and Nielsen and Ludvigsen (1963).

The *in vitro* hybrid was that formed by guanidine treatment of a mixture of brain and muscle enzymes, while the natural hybrid was isolated by chromatography from a crude extract of heart.

	High/low
Muscle enzyme	3.7
Brain enzyme	2.05
Heart enzyme	1.95
Hybrid (*in vitro*)	2.8
Hybrid (natural)	2.7

Table Ia

Ratio of rates of reaction with high and low concentrations of creatine

The assay medium (Rotthauwe, H. W. and Cerqueiro-Rodriguez, M. (1964)), in a final volume of 3 ml, contained triethanolamine buffer, pH 7.6, 50 mM; ATP 4 mM, $MgCl_2$ 4 mM, phosphoenolpyruvate 0.8 mM, $DPNH_2$ 0.3 mM, pyruvic kinase 1.5 Int. Units, lactic dehydrogenase 3.5 Int. Units, and one of two concentrations of creatine. High concentration refers to 25 mM and low to 6.25 mM; the high/low ratio is reported.

	High/low
Muscle enzyme	2.05
Brain enzyme (from heart)	1.15
Hybrid (*in vitro*)	1.55
Equal mixture of muscle and brain enzymes	1.50

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was not important in the hybridization process. This inter-species hybrid also showed a double spot.

It was of some interest that the rate of reactivation of the creatine

kinases studied here, as indicated by the percent of the original activity recovered after dilution, varied considerably. (See Table II). At all enzyme concentrations tested, the "brain enzyme" was more rapidly and completely reactivated than was the "muscle enzyme". The hybrid molecule

Table II

In each case the enzyme was inactivated in buffered 6.5 M guanidine containing 0.1 M mercaptoethanol, and reactivation was by 25fold dilution into buffered 0.1 M mercaptoethanol. The muscle enzyme had a specific activity of 250 units/mg, while the brain enzyme had a specific activity of 200 units/mg.

Enzyme	Concentration, units/ml., in inactivation mixture	% reactivated
Expt. A. Assayed 1 hr after dilution		
Muscle	19	13
Muscle	900	58
Brain	27	46
Brain	67	67
Brain (isolated from heart)	700	71
Hybrid (*in vitro*)	12.4	40
Expt. B. Assayed 2 hrs after dilution		
Muscle	52	13
	103	20
	206	49
	310	65
	515	74
Rabbit muscle[*]	52	4
	280	39
	460	44

[*] Purchased from Boehringer Mannheim Corp., New York, N. Y.

was slightly less reactivated than the brain enzyme. There was a clear-cut concentration dependence of the total enzyme reactivated on the concentration of enzyme in the original aliquot; with progressive dilution of the original enzyme there was less recovery of activity. At all concentrations tested the enzyme from rabbit muscle was less fully reactivated.

The findings presented here indicate that creatine kinase is an enzyme whose structure is that of a dimer, as suggested by Eppenberger et al. (1964) and Thomson et al. (1964). The muscle enzyme may be composed of two identical subunits (M-M), the brain enzyme of two identical but different subunits (B-B), and the hybrid molecule of two subunits, one of either kind (M-B). The hybrid enzyme, whether it occurs naturally or is formed in vitro, is intermediate in kinetic qualities and in electrophoretic mobility between the two parental or unmixed forms. Hence, the nature of the multiple forms of creatine kinase resembles that found with the lactic dehydrogenases in that a hybrid enzyme occurs in vivo (Cahn et al., 1962; Appella and Markert, 1961).

Further properties of the creatine kinases will be described elsewhere.

REFERENCES

Appella, E. and Markert, C. L., Biochem. Biophys. Research Communs., 6, 171 (1961).

Burger, A., Richterich, R., and Aebi, H., Biochem. Z., 339, 305 (1964).

Cahn, R. D., Kaplan, N. O., Levine, L., and Zwilling, E., Science, 136, 962 (1962).

Chilson, O., Kitto, G. B., and Kaplan, N. O., Proc. Natl. Acad. Sci. U.S., 53, 1006 (1965).

Dance, N., and Watts, D. C., Biochemical J., 84, 114 P (1962).

Deal, D. H., and van Breeman, J. F. L., Clin. Chim. Acta, 10, 276 (1964).

Eppenberger, H. M., Eppenberger, M., Richterich, R., and Aebi, H., Develop. Biol., 10, 1 (1964).

Fine, I. H., Kaplan, N. O., and Kuftinec, D., Biochemistry, 2, 116 (1963).

Jacobs, H., Heldt, H. W., and Klingenberg, M., Biochem. Biophys. Res. Communs., 16, 516 (1964).

Kaegi, J. H. R., and Li, T. K., Federation Proc., 24, 285 (1965).

Kar, N. O. and Pearson, C. M., Am. J. Clin. Path., 43, 207 (1965).

Kuby, S. A., Lardy, L., and Noda, H. A., J. Biol. Chem., 209, 191 (1954).

Mahowald, T. A., Biochemistry, 4, 732 (1965).

Mahowald, T. A., Noltmann, E. A., and Kuby, S. A., J. Biol. Chem., 237, 1535 (1962).

Nielsen, L. and Ludvigsen, B., J. Lab. Clin. Med., 62, 159 (1963).

Oliver, I. T., Biochem. J., 61, 116 (1955).

Rotthauwe, H. W. and Cerqueiro-Rodriguez, M., Clin. Chim. Acta, 10, 134 (1964).

Sjövall, K., and Voigt, A., Nature, 202, 701 (1964).

Thomson, A. R., Eveleigh, J. W., and Miles, B. J., Nature 203, 267 (1964).

Vassella, F., Richterich, R., and Rossi, E., Pediatrics, 65, 322 (1965).

Wood, T., Biochemical J., 87, 453 (1963) and 89, 210 (1963).