Formation of an Unusual Hybrid in the Development of Human Adenosine 5'-Triphosphate-Creatine Phosphotransferase

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1. Starch-gel analysis of extracts from adult human muscle, heart and brain reveals a hybrid creatine kinase with an abnormally high electrophoretic mobility. 2. Hybridization *in vitro* confirms that the postulated hybrid is formed from a combination of brain- and muscle-type enzyme sub-units. 3. The relative electrophoretic mobility of the hybrid is not affected by changing the starch concentration in the gel or by the buffer system used, or by electrophoresis in thin layers of Sephadex. 4. It is concluded that hybrid formation results in a net increase on the dimeric enzyme of from 4 to 6 negative charges. 5. During development a sharp increase in the rate of production of muscle-type enzyme sub-units occurs in heart at 10–13 weeks' gestation and in muscle at 18–20 weeks' gestation. The latter change is accompanied by a relative decrease in the concentration of brain-type sub-units.

Vertebrate creatine kinase (ATP-creatine phosphotransferase, EC 2.7.3.2) is composed of two dissociable sub-units (Dawson, Eppenberger & Kaplan, 1965), each of molecular weight about 40000 (Noda, Kuby & Lardy, 1954; Moreland, Watts & Virden, 1967; Yue, Palmieri, Olson & Kuby, 1967; Watts, 1964). One type of sub-unit, B, forms the enzyme, BB, found in brain, whereas another type, M, forms the enzyme, MM, found in the white fibres of skeletal muscle. The two sub-unit types will combine to form a hybrid, BM (or MB), and this form has been found in heart muscle and the red fibres of skeletal muscle. The three enzyme forms can readily be distinguished by electrophoresis and the hybrid, BM, has a mobility that is generally found to be intermediate between those of the brain and muscle types, for example, in the chicken, rat, rabbit and mackerel (Dawson *et al.* 1965; Eppenberger, Eppenberger, Richterich & Aebi, 1964; Eppenberger, Eppenberger & Kaplan, 1967; Burger, Eppenberger, Weissman & Richterich, 1963).

Studies on the distribution of human creatine kinase in various tissues have been carried out with agar-gel (Burger, Richterich & Aebi, 1967; Sjöval & Voigt, 1964) and starch-gel (Sjöval & Voigt, 1964; Rosalki, 1965) electrophoresis. The purpose of the research reported below was to study the changes in creatine kinase forms that occurred during development. This led to a more detailed study of the hybrid, which was found to have an electrophoretic mobility much closer to that of the brain enzyme

rather than being a mean of the mobilities of the brain and muscle enzymes as expected.

MATERIALS AND METHODS

Tissue samples. Adult heart, muscle and brain were obtained from post mortems, usually 1–5 hr. after death. Foetal tissues were obtained from surgical abortions. The tissues were stored at -18° until required, and extracts prepared by grinding approx. 1g. of frozen tissue with a little acid-washed sand and 5 ml. of ice-cold tris-citrate buffer, pH 8.6 (see below), in a precooled mortar. The insoluble matter was removed by centrifugation at 4° (approx. 20000g for 30 min.), and the supernatant concentrated overnight to about 0.5 ml. by dialysis under reduced pressure (Virden & Watts, 1964) against 50 ml. of the same buffer at 2° . Where other buffers were subsequently used for electrophoresis the sample was dialysed against the appropriate buffer.

Buffers and electrophoretic techniques. Horizontal starch-gel electrophoresis was carried out at 2° by the method of Smithies (1955). For most experiments a slightly modified version of the Poulik (1957) discontinuous buffer system was used. Connaught starch (Connaught Medical Research Laboratories, Toronto, Ont., Canada) was made up in a buffer, pH 8.6, containing 5.25g. of recrystallized tris base and 1.05g. of citric acid in 1 l. in the quantity recommended for the particular starch batch. This same buffer was also used for tissue extraction, as mentioned above. The buffer vessels contained borate buffer, pH 8.5 (23.35g. of boric acid dissolved in approx. 500 ml. of distilled water, adjusted to the required pH with saturated NaOH solution and made up to 1 l.). The continuous buffer systems used were borate, pH 8.4 (Smithies, 1959), phosphate, pH 6.8 (Gammack, Huehns, Shooter & Gerald, 1960), and a tris-citrate buffer

system, pH 7.8, in which the buffer vessels contained 20 mM-citric acid and 82.4 mM-tris base and the gel was made up in the same buffer diluted fourfold.

Creatine kinase activity in the gel was determined by the negative-staining technique for creatine (Virden & Watts, 1964).

Horizontal electrophoresis on Sephadex G-100 [Pharmacia (G.B.) Ltd., London, W.13] was carried out with the discontinuous tris-citrate buffer system, pH 8.6. Washed Sephadex was equilibrated by stirring with several changes of tris-citrate buffer and the clear supernatant poured off before the Sephadex slurry was transferred to a Desaga thin-layer spreader. Standard plates (20 cm. $\times$ 20 cm.) were spread 0.5 mm. thick and the enzyme sample (20 μ l.) was applied from a micro-pipette as a streak about 5 cm. long. Electrophoresis was carried out on a Mini 65 Phaeograph with the thin-layer plate raised from the refrigerated bed of the machine by an aluminium block 1.25 in. thick but insulated from the plate by a polythene sheet. In this way the thin-layer plate could be maintained close to 0° and electrical connexions with the buffer vessels could be made by filter-paper wicks in the usual way. Electrophoresis was carried out for 2 hr. at 12.6 v/cm. (constant voltage). After electrophoresis Sephadex was transferred from the appropriate regions of the plate (determined in preliminary experiments) to centrifuge tubes and assayed for creatine kinase activity as described by Virden & Watts (1964).

Hybridization of creatine kinases. These experiments were carried out by using slight modifications of the procedure described by Dawson *et al.* (1965). A mixture of the concentrated tissue extracts was made 8 M with respect to urea with a 10 M-urea solution that had been stored over Bio-Deminolit (British Drug Houses Ltd., Poole, Dorset) mixed-bed resin, and 0.3 M with respect to sodium mercaptoacetate, pH 8.5. The mixture was then dialysed under reduced pressure at 2° against two changes of 50 ml. of tris-citrate buffer, pH 8.6, for 24 hr. before analysis by starch-gel electrophoresis.

RESULTS

The relative electrophoretic mobilities of creatine kinase from adult human brain, heart and skeletal muscle, with the discontinuous tris-citrate buffer system, are shown in Fig. 1. Brain contains only a single, very electropositive component, whereas skeletal muscle most usually had a broad, slowly migrating band, which in some preparations could be resolved into three components, and a weak band of activity that migrated just behind the brain enzyme. In one very fresh muscle sample a trace of the brain enzyme form could also be detected. Adult heart muscle contains only the slowest of the three slowly migrating bands, but very much more of the form that runs just behind the brain enzyme together with a small amount of the brain enzyme.

Raising or lowering the starch concentration in the gel by 25% (w/w) altered the absolute mobilities of the enzyme components, but did not alter their mobilities relative to each other. The electrophoretic mobilities in various other buffer systems

Fig. 1. Starch-gel-electrophoresis patterns of creatine kinase from adult human muscle, heart and brain. Electrophoresis was carried out with the discontinuous borate/tris-citrate buffer system, pH 8.6, for 5 hr. at 2.5 mA/cm. (constant current) at 2°.

Fig. 2. Effect of altering the buffer system on the electrophoretic mobilities of creatine kinase from: M, muscle; H, heart; B, brain. The buffer systems were (a) phosphate, pH 6.8; (b) tris-citrate, pH 7.8; (c) borate, pH 8.4. Details of the buffers are given in the Materials and Methods section. Electrophoresis was carried out for 5 hr. at 2.5 mA/cm. (constant current) at 2°.

are shown in Fig. 2. Essentially the same distribution of the creatine kinases was obtained by electrophoresis of crude extracts on thin-layer Sephadex. When the appropriate enzyme forms were eluted from the Sephadex, concentrated by vacuum dialysis and reanalysed on starch gel, each migrated to the expected position in the gel. There was no evidence of interconversion of one form into another or that the degree of purity affected the electrophoretic mobility.

Developmental changes were followed from 9 to 20 weeks' gestation and the findings are outlined in Table 1. When thin-layer-Sephadex analysis was used instead of the relatively insensitive gel stain all three of the enzyme forms could be detected in skeletal muscle. In general, however, foetal muscle contained only the slowest of the three slowly migrating sub-components. With the exception of brain, which contains only one enzyme form, Table 1 should be interpreted not in terms of the

Table 1. *Distribution of the various forms of creatine kinase in human foetal tissues at various stages of development*

Age of foetus (weeks)	Sample no.	Muscle			Heart			Brain		
		MM	MB	BB	MM	MB	BB	MM	MB	BB
9		+	+	Trace	Trace	+	-	-	-	+
10	1	+	+	-	-	+	-	-	-	+
10	2	+	+	-	-	+	-	-	-	+
10	3	+	+	-	Trace	+	-	-	-	+
13	1	+	+	-	+	+	-	-	-	+
13	2	+	+	-	+	+	-	-	-	+
13	3	+	+	-	+	+	-	-	-	+
16		+	+	-	+	+	-	-	-	+
18-20		+	-	-	+	+	-	-	-	+
20	1	+	-	-	+	+	-	-	-	+
20	2	+	-	-	+	+	-	-	-	+

Fig. 3. Starch-gel analysis of the hybridization of muscle and brain creatine kinase. 1, Muscle enzyme, hybridized; 2, brain enzyme, hybridized; 3, muscle enzyme plus brain enzyme, but urea omitted from the hybridization mixture; 4, muscle enzyme plus brain enzyme, hybridized; 5, heart, enzyme, unhybridized control. For the experimental details see the Materials and Methods section and the Results section. Electrophoresis was carried out as described in Fig. 1.

presence or absence of particular components but as indicating marked changes in the contents of these enzymes in the tissues.

Hybridization experiments were initially carried out with a mixture of brain and 20-week foetal muscle supernatants, as only single enzyme components could be detected in these extracts. The results (Fig. 3) unambiguously showed that a hybrid was formed that had the electrophoretic mobility characteristic of heart muscle. Simultaneously run controls in which the brain and muscle extracts alone were put through the hybridization procedure gave only the initial enzymes and no new

enzyme bands were formed. No hybridization occurred if urea was omitted from the hybridization mixture. Hybridization of the fast enzyme component of heart muscle did not result in the formation of detectable amounts of the brain and muscle types. In a second series of experiments attempts were made to produce three hybrids from the three slowly migrating creatine kinase components of adult muscle. In all cases only one, identical, hybrid was formed. The two faster components of the slowly migrating creatine kinase tended to be selectively inactivated by the hybridization procedure, particularly when the concentration of sodium mercaptoacetate was raised to 6mM. The brain enzyme was also inactivated to a small extent by the hybridization procedure, but not enough to prevent the detection of enzyme activity unless the initial enzyme concentration was very low.

DISCUSSION

The very fast and very slowly migrating forms of creatine kinase (Fig. 1) can readily be equated with the BB and MM types respectively, though the cause of three sub-components in the latter is unknown. Slight evidence that they are storage artifacts does not seem to be the entire explanation, as similar bands were also found in biopsy samples, but did not seem to occur in stored foetal samples. The fast form found in adult heart and skeletal muscle seemed to migrate much too fast to be readily accepted as the BM hybrid, which would be expected to have properties intermediate between the component types (Dawson *et al.* 1965; Eppenberger *et al.* 1964). Similarly, studies on other enzymes such as lactate dehydrogenase (Wilkinson, 1965), alkaline phosphatase (Robson & Harris, 1965; Beckman, Björling & Christodoulou, 1966) and catalase (Beckman, 1966) have revealed hybrids

with the expected intermediate electrophoretic mobilities. However, hybridization experiments showed that an artificial hybrid creatine kinase could be formed that was more like the brain type than the muscle type in its electrophoretic properties and was indistinguishable from the major creatine kinase component of heart muscle (Fig. 3). The equilibrium for the reaction appears to greatly favour hybrid formation, which is largely why the brain enzyme, which had the lower concentration, could not be detected after hybridization with the muscle enzyme. The equilibrium position is also shown in the 9–10-week foetal heart extracts, which contain the hybrid form almost exclusively (Table 1).

It seemed possible that the abnormal electrophoretic behaviour of the hybrid might be due to the buffer system used, as R. K. Scopes (personal communication) has informed us that use of the discontinuous tris-citrate buffer system rapidly results in the loss of any buffering power in the gel as electrophoresis proceeds. Analysis in other buffer systems and at other pH values (Fig. 2) excludes this possibility. An alternative possibility was that, if the dimeric creatine kinase had an elongated shape (the frictional ratio for rabbit creatine kinase is 1.28; Noda *et al.* 1954), then fusion of a relatively highly charged sub-unit to one with a relatively small charge might result in linear orientation of the hybrid in an electric field and facilitate passage through the gel. If this were so, altering the gel composition would have a significant effect on the relative electrophoretic mobilities of the three enzyme types. Altering the gel composition by $\pm 25\%$ was without this effect and, further, the same general electrophoretic pattern was observed by electrophoresis on thin layers of Sephadex G-100. It can only be concluded that the hybrid form of human creatine kinase results from the combination of the two parent sub-units in such a way as to expose relatively more negative charges on the molecule. From comparison with the effect of iodoacetamide in blocking single negative charges in rabbit muscle creatine kinase (Watts, 1964) and arginine kinase (Virden & Watts, 1964), as a rough estimate, it is concluded that a net increase of between 4 and 6 negative charges occurs.

Developmental changes in the rate of production of B and M creatine kinase sub-units are indicated by Table 1. There is no production of M sub-units in brain, at least from 9 weeks' gestation onwards. Where the hybrid occurs it seems certain that careful investigation will reveal evidence of the parental enzyme types, BB and MM, though they may not have a physiological significance at low concentrations. In heart muscle there is a sharp increase in the production of M sub-units between 10 and 13 weeks'

gestation as assessed by the staining intensity of the enzyme in the gel, whereas in skeletal muscle (pooled leg muscles) this increase does not occur until after 18–20 weeks and is accompanied by an apparent decrease in the production of B sub-units. This latter change appears to correlate with the onset of muscle-fibre production (Ochoa & Mair, 1968), whereas a contractile heart is present from an early stage of development. It seems that M sub-units are particularly associated with the contractile elements, which suggests a functional involvement (Watts, 1968). The specific function of B sub-units is still obscure.

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REFERENCES

- Beckman, L. (1966). *Monographs in Human Genetics: Isozyme Variations in Man*, vol. 1, p. 62. Basle: S. Karger.
- Beckman, L., Björling, G. & Christodoulou, C. (1966). *Acta genet., Basel*, **16**, 59.
- Burger, A., Eppenberger, M. E., Weissman, U. & Richterich, R. (1963). *Helv. physiol. Acta*, **21**, C6.
- Burger, A., Richterich, R. & Aebi, H. (1967). *Biochem. Z.* **339**, 305.
- Dawson, D. M., Eppenberger, H. M. & Kaplan, N. O. (1965). *Biochem. biophys. Res. Commun.* **21**, 346.
- Eppenberger, H. M., Eppenberger, M. E., Richterich, R. & Aebi, H. (1964). *Developmental Biol.* **10**, 1.
- Eppenberger, M. E., Eppenberger, H. M. & Kaplan, N. O. (1967). *Nature, Lond.*, **214**, 239.
- Gammack, D. B., Huehns, E. R., Shooter, E. M. & Gerald, P. S. (1960). *J. molec. Biol.* **2**, 372.
- Moreland, B., Watts, D. C. & Virden, R. (1967). *Nature, Lond.*, **214**, 458.
- Noda, L., Kuby, S. A. & Lardy, H. A. (1954). *J. biol. Chem.* **209**, 203.
- Ochoa, J. & Mair, W. G. P. (1968). *Proc. 4th Symp. Current Research in Muscular Dystrophy*. London: Pitman Medical Publishing Co. (in the Press).
- Poulik, M. D. (1957). *Nature, Lond.*, **180**, 1477.
- Robson, E. B. & Harris, H. (1965). *Nature, Lond.*, **207**, 1257.
- Rosalki, S. (1965). *Nature, Lond.*, **207**, 414.
- Sjöval, K. & Voigt, A. (1964). *Nature, Lond.*, **202**, 701.
- Smithies, O. (1955). *Biochem. J.* **61**, 629.
- Smithies, O. (1959). *Biochem. J.* **71**, 585.
- Virden, R. & Watts, D. C. (1964). *Comp. Biochem. Physiol.* **13**, 161.
- Watts, D. C. (1964). *Abstr. 1st Meet. Fed. Europ. biochem. Soc., London*, A13.
- Watts, D. C. (1968). In *Advances in Comparative Biochemistry and Physiology*, p. 1. Ed. by Lowenstein, O. New York: Pergamon Press Inc.
- Wilkinson, J. H. (1965). *Isoenzymes*, p. 43. London: E. and F. N. Spon Ltd.
- Yue, R. H., Palmieri, R. H., Olson, O. E. & Kuby, S. A. (1967). *Biochemistry*, **6**, 3204.