ISOZYMES OF PYRUVATE KINASE IN TISSUES AND EGGS OF RANA PIPIENS*

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SUMMARY

At least five isozymes of pyruvate kinase have been detected electrophoretically in certain tissue extracts from the grassfrog, Rana pipiens. The five individual isozymes occurring in egg have been separated by DEAE-Sephadex chromatography and compared to the isozymes in other tissues by electrophoresis. Evidence is presented that shows that there is probably no skeletal muscle type pyruvate kinase in egg. Extracts of pyruvate kinase from egg and liver are activated by FDP**

Pyruvate kinase isolated from adult rat tissues has recently been shown to exist in multiple molecular forms. One of these, the classical skeletal muscle enzyme, type M, displays Michaelis-Menton kinetics with PEP (Boyer, 1962; Tanaka et al., 1967a). Another form, electrophoretically distinct from type M is liver pyruvate kinase, type L, found so far only in liver and erythrocytes (Tanaka et al., 1967a & b, Taylor and Bailey, 1967) which displays sigmoid kinetics with PEP, allosteric activation by FDP, and inhibition by ATP. Under certain conditions, type L has been reported to undergo a transformation into a species which is no longer sensitive to FDP activation although retaining immunologic identity with type L (Tanaka et al., 1967b; Bailey et al., 1968; Susor and Rutter, 1968). In addition, there is a third electrophoretic type found in kidney and certain other tissues which is

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** Abbreviations used are: PEP, phosphoenolpyruvic acid; FDP, fructose-1,6-diphosphate; LDH, lactic dehydrogenase.

immunologically and kinetically similar to type M (Susor and Rutter, 1968; Pogson, 1968).

The importance of pyruvate kinase in glycolysis, the physiological regulation of type L for gluconeogenic purposes (Tanaka et al., 1967a) and its apparent heterogeneity in different adult tissues of the rat make this enzyme an interesting subject for physico-chemical studies from the developmental point of view in the system, Rana pipiens. In this paper evidence is presented for the existence of at least five isozymes of pyruvate kinase in the unfertilized egg and certain adult tissues of this species.

MATERIALS AND METHODS

Male and gravid female grassfrogs (Lemberger Co., Oshkosh, Wis.) were decapitated and liver, muscle and other tissues were removed. Tissue extracts were prepared by homogenizing in two volumes of 0.01 M imidazole acetate buffer (pH 7.4) containing 0.001 M EDTA, 0.01 M β-mercaptoethanol and 0.25 M sucrose. The homogenates were centrifuged in a Spinco Model L ultracentrifuge at 105,000 x g for 30 min to obtain supernatant solutions. Induction of ovulation and dejellying of eggs were achieved as in Adachi et al. (1968), except that the above buffer was used. The two types of egg preparations used were eggs plus ovary from non-induced females and ovulated eggs. Both preparations gave identical electrophoretic patterns.

Electrophoresis was performed on cellulose acetate strips (Gelman Sephraphore III, 2.5 x 17 cm) using three buffer systems (pH 7.4): Buffer A, 0.05 M imidazole acetate containing 0.25 M sucrose plus 0.5 mM FDP; buffer B, the same system without FDP; buffer C, 0.025 M imidazole acetate, 0.01 M KCl, 0.25 M sucrose, 0.001 M β-mercaptoethanol. Prior to electrophoresis all tissue extracts were passed through a G-25 Sephadex column previously equilibrated in the appropriate buffer. Electrophoretograms were run at 30 volts per cm for 3-5 hours at 4°C. Pyruvate kinase activity was detected by a modification of the method of Susor and Rutter (1968). After electrophoresis the strip was put on a glass plate and upon it was placed another

strip which had been soaked in a solution containing 0.05 M Tris-HCl (pH 7.4), 0.1 M KCl, 5 mM $MgCl_2$, 4 mM ADP, 4 mM PEP, 2 mM NADH, 0.01 mM FDP and about 200 units of LDH (Sigma). The opposing strips were then placed in a humidified plastic box and incubated at 37°C for 15-30 min. Pyruvate kinase activity was observed under UV light and recorded on photographic paper by contact printing using light through a 340 mμ filter.

Pyruvate kinase activity was assayed routinely according to the method of Bücher and Pfleider (1955) except that 0.46 mM ADP was employed in the assay system. A Gilford spectrophotometer was used to follow the decrease in absorbance at 340 mμ. One unit of activity is defined as that amount of enzyme which catalyzes a change of 0.001 in absorbance per minute.

Column chromatography on DEAE-Sephadex was used to separate the five isozymes of pyruvate kinase from eggs plus ovary. The crude extract of the egg-ovary preparation was subjected to a 42% (w/v) ammonium sulfate precipitation and the precipitate dissolved in and dialyzed against a buffer containing 0.09 M imidazole acetate (pH 7.4), 0.01 M β-mercaptoethanol, 0.001 M EDTA and 0.0005 M FDP. The dialyzed preparation (having ∿ 80,000 u/ml of activity) was applied to a column (22 x 1.4 cm) of DEAE-Sephadex A-50 equilibrated in the same buffer used for dialysis. Elution of the different isozymes was accomplished by first washing the column with 60 ml of buffer and then using a 0-0.15 M KCl linear gradient (300 ml) in the same buffer.

RESULTS AND DISCUSSION

Electrophoretic patterns obtained from egg-ovary, ovulated egg and liver extracts using buffer A (Fig. 1) show five distinct anodal bands of activity. The occurrence of these five isozymes was consistent in all individuals studied, the only variations being in the intensity of the individual bands. Electrophoresis of spleen, lung and kidney extracts also demonstrated five bands of activity which have the same mobilities as those of liver and egg, while testes contained only the first three of the five isozymes (Fig. 1). In this buffer, extracts from skeletal muscle, brain and

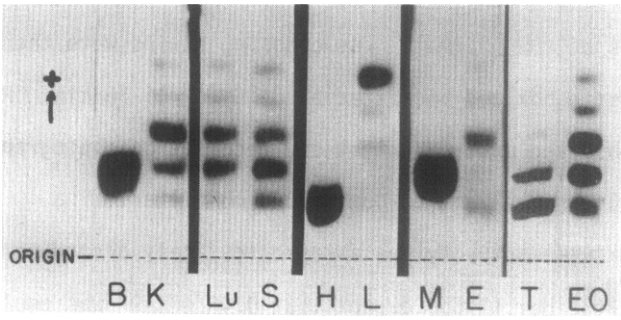

Figure 1. Zone electrophoretic resolution of pyruvate kinase activities in extracts of various tissues of the frog, Rana pipiens. Tissues are abbreviated as follows: B, brain; K, kidney; Lu, lung; S. spleen; H, heart; L, liver; M, skeletal muscle; E, unfertilized eggs; EO, eggs plus ovary. Electrophoresis was carried out in buffer A for 5 hrs. and pyruvate kinase activity was detected and photographed as described in the text. About 2,000 units per band of activity were applied.

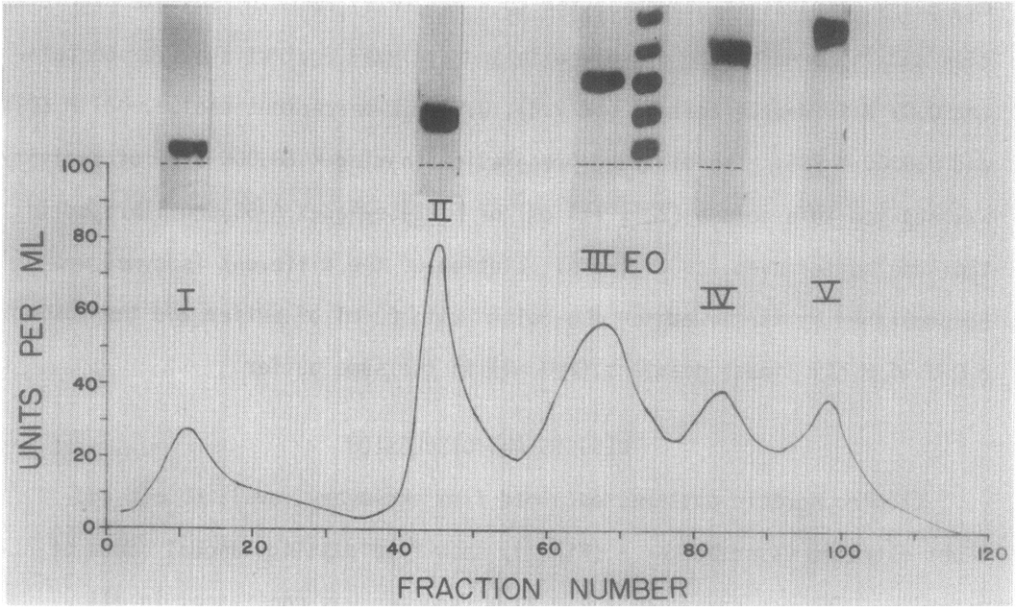

Figure 2. Separation of egg-ovary isozymes of pyruvate kinase by chromatography on DEAE-Sephadex. Experimental conditions are given in the text. Fractions (3 ml) containing each isozyme were combined, concentrated by ultrafiltration, and electrophoresed in buffer A as in Fig. 1.

heart (Fig. 1) show only one broad anodal band of activity on electrophoresis.

In order that the five isozymes of pyruvate kinases found in egg might

be further characterized, they were separated by chromatography on DEAE-Sephadex A-50 (Fig. 2). Each peak of activity was found to be homogeneous with respect to activity by electrophoresis in buffer A and each isozyme corresponded to its respective band in the crude extract.

If buffer B is used for electrophoresis, four of the five bands of egg, liver, spleen, lung and kidney still migrate toward the anode while one of the bands is cathodic. The single band of activity from heart has become slightly cathodic and that from skeletal muscle now migrates strongly toward the cathode.

An electrophoretic comparison of crude muscle pyruvate kinase with isolated isozymes I and II from egg-ovary in buffer C (Fig. 3) provides further evidence that the skeletal muscle enzyme is dissimilar to either of these two isozymes. Furthermore, under conditions where all five isozymes of pyruvate kinase in egg-ovary extracts are adsorbed on a column of DEAE-Sephadex A-50, the skeletal muscle enzyme is not retained indicating that none of the latter type occurs in egg.

Clarification of the electrophoretic relationships between pyruvate kinase activities in extracts of various tissues was obtained with buffer C which gives a better resolution than buffers A or B. In this system, heart and brain pyruvate kinases and isozyme I from egg-ovary remained at the origin whereas that from muscle was cathodic and isozyme II from egg-ovary was anodic (Fig. 3).

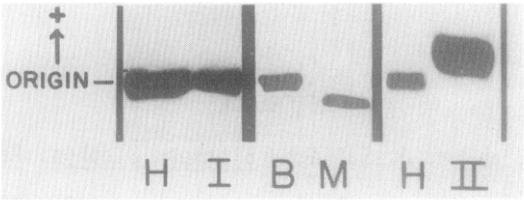

Figure 3. Electrophoretic comparison of separated isozymes I and II from egg-ovary and crude extracts of brain, heart and skeletal muscle. Electrophoresis was carried out for three hours in buffer C and pyruvate kinase activity was detected and photographed as described in the text.

Preliminary kinetic studies indicate that crude liver and egg pyruvate kinases are sensitive to FDP activation and ATP inhibition. The crude liver enzyme has a Km app. (PEP) of 1.0×10^{-4} M and displays sigmoid characteristics; with the addition of 0.5 mM FDP, it decreases to 1.6×10^{-5} M. Similarly, the crude egg enzyme displays a Km app. (PEP) of 1.0×10^{-4} M in the absence of FDP and 3.1×10^{-5} M in its presence. Purification and further kinetic studies on these isozymes are presently under way in this laboratory.

Multiple forms of pyruvate kinase were first suggested by Tanaka et al. (1967a) whose starch zone electrophoretograms of crude rat liver pyruvate kinase showed in certain cases aside from the major L_1 and M types, additional bands of activity which they labeled L_2 and L_3. To our knowledge there have been no previous reports of five isozymes of this enzyme in a given tissue as are shown here in certain tissues of Rana pipiens.

Steinmetz and Deal (1966) and Cottam et al. (1969) have shown by ultracentrifugation studies that rabbit muscle pyruvate kinase is composed of four nearly identical subunits. The existence of five isozymes of pyruvate kinase in certain tissues of Rana pipiens might be explained on the basis of a hybridization between two parental types, each with four subunits. It seems clear that heart, brain and isozyme I from egg-ovary are of a different basic type from muscle and all may represent one parental type while isozyme V, predominant for example in liver, may represent the other. The apparent absence of the muscle type in egg and other tissues where the five isozymes occur would suggest that muscle type is not taking part in these hybridizations.

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