BBA 66390

PYRUVATE KINASE ISOZYMES IN ADULT TISSUE AND EGGS OF *RANA PIPIENS*

II. PHYSICAL AND KINETIC STUDIES OF PURIFIED SKELETAL AND HEART MUSCLE PYRUVATE KINASES

L. E. FLANDERS, J. R. BAMBURG AND H. J. SALLACH

Department of Physiological Chemistry, University of Wisconsin Medical School, Madison, Wisc. 53706 (U.S.A.)

(Received April 5th, 1971)

SUMMARY

1. Pyruvate kinase (ATP:phosphotransferase, EC 2.7.1.40) from skeletal muscle of *Rana pipiens* has been purified to homogeneity as judged by sedimentation velocity studies, immunoelectrophoresis, double diffusion experiments and acrylamide-gel electrophoresis. The enzyme has a molecular weight of approx. 220 000 as estimated by gel-filtration studies and a subunit molecular weight of approx. 55 000 as measured by acrylamide-gel electrophoresis in the presence of sodium dodecyl sulfate.

2. The properties of the frog skeletal and partially purified cardiac pyruvate kinases have been compared. Both enzymes have the same molecular weight as estimated by gel-filtration experiments and the cardiac enzyme cross-reacts with the antibody to the skeletal muscle pyruvate kinase. However, the cardiac enzyme exhibits different chromatographic and kinetic properties. Unlike the muscle enzyme, heart pyruvate kinase is retained on DEAE-Sephadex, displays temperature sensitivity, is inhibited by L-alanine and DL-phenylalanine and is activated by fructose 1,6-diphosphate. With respect to the extent of inhibition or activation by the latter compounds, present data suggest the possibility that there are two forms of the cardiac enzyme, one of which is highly sensitive, and a second, which is markedly less sensitive, to the effects of these modifiers.

INTRODUCTION

The occurrence of multiple forms of pyruvate kinase (ATP:pyruvate phosphotransferase, EC 2.7.1.40) has been demonstrated in different tissues of the rat[1-3] and frog[4]. The exact relationship between the different isozymes has not been fully elucidated to date. On the basis of their immunological properties, TANAKA *et al.*[1]

Abbreviation: Fru-1,6-P_2, fructose 1,6-diphosphate.

have established that there are at least two basic types of pyruvate kinase in rat tissues referred to as Types M and L, for muscle and liver, respectively. However, more recent work by several investigators has revealed the existence of electrophoretically distinct forms of Type M pyruvate kinase[2] and the occurrence of interconvertible forms of Type L from rat liver[5] and of pyruvate kinase from rat adipose tissue[6].

The form of pyruvate kinase found in rat skeletal muscle, heart and brain (Type M_1) displays Michaelis–Menten kinetics whereas another form (Type M_2), which is immunologically indistinguishable from Type M_1, is widely distributed in different tissues and exhibits sigmoidal kinetics as well as activation by fructose 1,6-diphosphate (Fru-1,6-P_2) (ref. 2). Phenylalanine has been reported to inhibit the activity of Type M pyruvate kinase in rat skeletal muscle[7] and rat uterus, seminal vesicles and testis[8]. Type L pyruvate kinase, found in liver and kidney, is under dietary and hormonal control; although it is immunologically distinct from Types M_1 and M_2, its kinetic properties resemble the latter in that it is activated by Fru-1,6-P_2, exhibits sigmoidal kinetics and in addition is inhibited by L-alanine. However, BAILEY *et al.*[5] have demonstrated the occurrence of two interconvertible forms of Type L pyruvate kinase from rat liver, one of which has less cooperativity with respect to substrate, phosphoenolpyruvate, and modifier, Fru-1,6-P_2. POGSON[6] has also demonstrated in rat adipose tissue the interconversion between two active forms of pyruvate kinase which involves a change in cooperativity with respect to phosphoenolpyruvate and altered sensitivity to fructose-1,6-P_2 activation. These results support the conclusion of TANAKA *et al.*[1] that Type L pyruvate kinase is an important control point in the glycolytic pathway.

The developing amphibian is being used in this laboratory as a model system for a study of biochemical differentiation. Hence, comparative studies on the properties of pyruvate kinases from adult tissues of *Rana pipiens* with those from the unfertilized egg and developing embryo are under investigation. In an earlier report[4], evidence was presented for the presence of five different forms of pyruvate kinase in the unfertilized egg which are also present in the adult. Furthermore, it was established that heart pyruvate kinase is electrophoretically different from the isozyme found in skeletal muscle. In view of this observation, further studies on the properties of frog heart and skeletal muscle pyruvate kinases have been carried out. The results reported here establish that unlike the adult rat, where to date no differences in the properties of cardiac and skeletal muscle pyruvate kinases have been reported, differences do exist in the properties of the corresponding enzymes from the frog. For example, frog heart pyruvate kinase has kinetic properties similar to those reported for Type L from rat liver but exhibits immunological cross-reactivity with Type M pyruvate kinase from frog skeletal muscle.

EXPERIMENTAL PROCEDURE

Materials

Grassfrogs, *R. pipiens*, were purchased from The Lemberger Co., Oshkosh, Wisc. All reagents and substrates were commercial products of the highest purity available. Pyruvate kinase (EC 2.7.1.40), aldolase (EC 4.1.2.7), and lactate dehydrogenase (EC 1.1.1.28) from rabbit muscle and beef liver glutamic dehydrogenase

(EC 1.4.1.2) were obtained from Sigma Chemical Co., Ferritin was purchased from Pentex and myoglobin from Calbiochem. DEAE-, CM-, and G-200 Sephadexes were obtained from Pharmacia.

Procedures

Protein determinations were performed according to the method of LOWRY *et al.*[9] using bovine serum albumin as the standard. During purification, pyruvate kinase was assayed as described previously[4]. In the kinetic studies reported here, the assay conditions used for a given experiment are detailed in the legends to the figures and tables. In control experiments, it was established that the concentration of K^+ (0.08 M) in the assay system was sufficient to preclude any additional effects on the kinetic measurements by other cations; thus, the sodium salt of phosphoenolpyruvate, the tetracyclohexylammonium salt of Fru-1,6-P_2 and a 1:250 dilution of lactate dehydrogenase (stored as an $(NH_4)_2SO_4$ suspension) were used routinely. Reaction rates were followed by measuring the change in NADH absorbance at 340 nm with a Beckman DU spectrophotometer in conjunction with a Gilford 2000 recorder equipped with an automatic cuvette changer and a circulating water bath for temperature control. All kinetic studies were performed with 26–90 units of pyruvate kinase with 1 unit of activity defined as that amount of enzyme which catalyzes a change of 0.001 in absorbance per min.

Prior to use, the Sephadex ion-exchange resins were treated according to the directions supplied by Pharmacia with the final equilibration carried out in the buffer to be used for column chromatography. Molecular weight determinations by gel filtration were performed on columns of Sephadex G-200 according to the method of DEVENCENZI AND HEDRICK[10].

Antibody production *via* injection of purified skeletal muscle pyruvate kinase (Type M) into rabbits and the partial purification of the anti-M pyruvate kinase antibody were accomplished as described by LEVY AND SOBER[11]. Antisera, collected after the injection of a total of 10.5 mg of purified enzyme over a 4-weeks period, gave a positive interfacial ring test[12] with a muscle pyruvate kinase preparation at a concentration of 0.1 μg/ml.

Immunoelectrophoresis was performed on 0.9% agar supported on glass slides. The buffer employed was 0.03 M L-asparagine adjusted to pH 8.0 with 2 M Tris. The electrophoresis was carried out at 4° for 5.5 h at 300 V. After the run was completed, the protein was allowed to diffuse 24 h at room temperature before applying the antibody to the trough. Double diffusion experiments were carried out in 0.9% agar according to the method of OUCHTERLONY[13]. The time required for a detectable precipitin band was 48 h. Acrylamide-gel electrophoresis in the presence of sodium dodecyl sulfate and the pretreatment of the proteins used for the runs was achieved by the method of WEBER AND OSBORN[14]. Myoglobin was used as the reference marker for the molecular weight calibration. Zone electrophoresis on cellulose acetate and the staining for enzyme and protein were carried out as described previously[4].

RESULTS

Purification of pyruvate kinase from frog skeletal muscle

The following general conditions were used unless otherwise stated. All steps

TABLE I

PURIFICATION OF PYRUVATE KINASE FROM *R. pipiens* SKELETAL MUSCLE

Fraction	*Activity (total units) ($\times 10^6$)*	*Total protein (mg)*	*Yield (%)*	*Specific activity ($\times 10^4$)*	*-Fold purified*
I. Extract	70	5000	—	1.4	—
II. $(NH_4)_2SO_4$ precipitate (45–60% satn.)	60	800	86	7.5	5.4
III. CM-Sephadex chromatography*	30	45	43	67	50
IV. $(NH_4)_2SO_4$ precipitate	28	32	40	87	62
V. DEAE-Sephadex chromatography*	22	17	31	133	95

* This step is quite variable with respect to yield. Recovery of 100% of the activity can occasionally be obtained for either Fraction III or V.

were carried out at 4–7°. Fractionations with $(NH_4)_2SO_4$ were made by the slow addition, with stirring, of the calculated amount of solid $(NH_4)_2SO_4$. The resulting suspensions were equilibrated with stirring for 15 min prior to centrifugation at 15 000 $\times$ *g* for 30 min. All $(NH_4)_2SO_4$ residues were dissolved in the appropriate buffer for the given purification step and were dialyzed against the same buffer until free of NH_4^+ as measured by Nessler's reagent. In chromatographic procedures, gels were equilibrated with the appropriate buffer prior to the pouring of the columns which were then washed with buffer before use. All gradients were made by dissolving KCl in the column buffer. Protein in column eluates was detected by absorbance at 280 nm. A summary of the purification procedure is given in Table I.

A typical enzyme preparation is made according to the following procedure: 1 g of muscle from the hind legs is homogenized in a Waring blendor for 1 min in 5 ml of homogenizing buffer consisting of 0.25 M sucrose, 0.025 M Tris–HCl, 2.5 mM EDTA and 1 mM β-mercaptoethanol (pH 7.5). Cellular debris is removed by centrifugation and the supernatant solution retained (Fraction I). This solution is brought to 45% saturation with $(NH_4)_2SO_4$, the precipitated protein removed by centrifugation and the resulting supernatant solution is then carried to 60% of saturation. The precipitated protein is recovered after centrifugation and the pellet (Fraction II) can be stored for 6 months at −20° with minimal loss in activity.

The 45–60% $(NH_4)_2SO_4$ pellet (Fraction II) is dissolved in 0.04 M imidazole–acetate buffer (pH 7.4) containing 1 mM EDTA and 10 mM β-mercaptoethanol. The volume of buffer used for the dissolution is that which results in a protein concentration of 10 mg/ml. The dialyzed fraction is then applied to a CM-Sephadex column which is equilibrated with the dialysis buffer; a bed volume of 1 l of resin is used for each 0.8 g of protein. After the protein solution has run into the column bed and the latter washed with 50 ml of buffer, a linear gradient of 0–0.15 M KCl is applied; a total volume of 2 l is used for a resin bed volume of 1 l. The enzyme elution profile is shown in Fig. 1A. The fractions comprising the main peak are pooled and concentrated by ultrafiltration to give a protein concentration of 2.5 mg/ml (Fraction III).

1 vol. of Fraction III is dialyzed for 48 h against 50 vol. of 52% saturated $(NH_4)_2SO_4$ solution to precipitate pyruvate kinase. (The resulting suspension is a convenient storage form for the enzyme; no loss in activity is observed for over a 1-month period). The precipitated protein (Fraction IV) is recovered by centrifugation and the supernatant solution discarded. The $(NH_4)_2SO_4$ precipitate is dissolved in 0.04 M imidazole–acetate buffer (pH 7.4) containing 1 mM EDTA, 10 mM β-mercaptoethanol and 0.5 mM Fru-1,6-P_2 to give a protein concentration of 5–7 mg/ml (approximate dissolution volume is about two-thirds that of the volume of the suspension centrifuged). After dialysis against the same buffer to remove NH_4^+, the solution is applied to a DEAE-Sephadex column (150 ml bed volume per 30 mg of protein) equilibrated with the same buffer. After washing the column with 10 ml of buffer, the enzyme is eluted with a linear gradient of 0–0.3 M KCl (300 ml total volume for a bed volume of 150 ml). The elution profile is shown in Fig. 1B. The fractions containing pyruvate kinase are pooled and concentrated *via* ultrafiltration to give a protein concentration of 4–8 mg/ml (Fraction V). This fraction can be stored at 4° for several weeks with no loss in activity.

Partial purification of frog heart pyruvate kinase

Pyruvate kinase in cardiac tissue is extracted by homogenization in the same buffer as that employed for skeletal muscle except that a tissue to buffer ratio of 1:2 (w/v) is used. An $(NH_4)_2SO_4$ fraction (30–60%) of the extract is made under conditions similar to those used for the skeletal muscle enzyme. Further purification of cardiac pyruvate kinase is achieved by chromatography on either DEAE- or CM-Sephadex. The buffer used in all subsequent steps is 0.04 M imidazole–acetate buffer

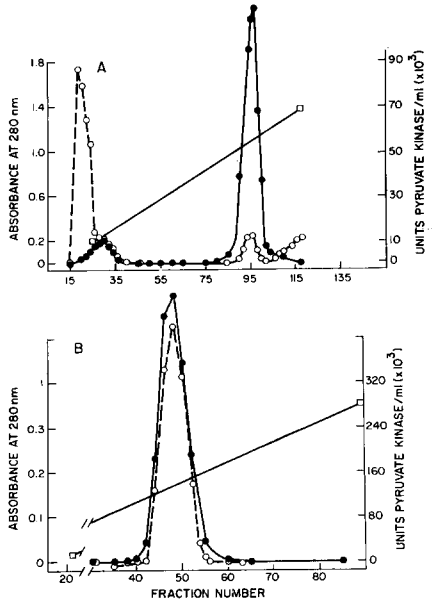
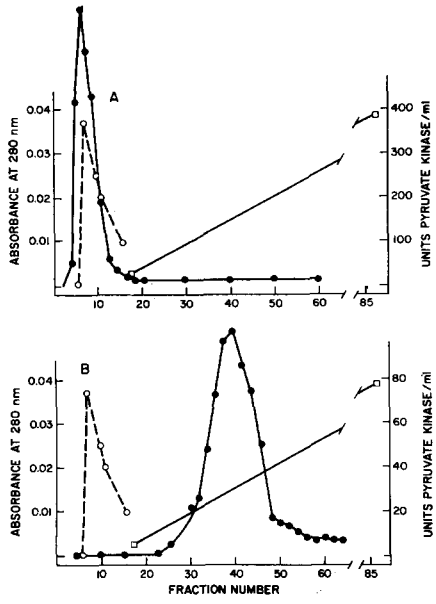


Fig. 1. Elution pattern for frog skeletal muscle pyruvate kinase (A) Fraction III, on CM-Sephadex and (B) Fraction V, on DEAE-Sephadex chromatography. ○ - - - ○, absorbance at 280 nm; ●—●, pyruvate kinase activity; □—□, KCl gradient. The column buffer in (B) contains Fru-1,6-P_2 (see text for description of buffer and column size).



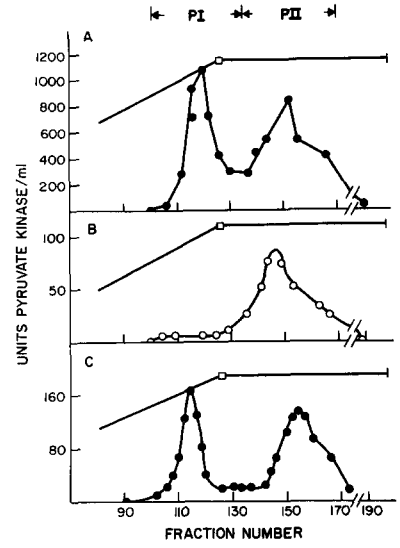


Fig. 2. Elution pattern for (A) frog skeletal muscle pyruvate kinase and (B) frog heart pyruvate kinase on DEAE-Sephadex chromatography in the absence of Fru-1,6-P_2. Both columns were of identical size (2.6 cm diameter, 100 ml bed volume). Both the skeletal muscle (Fraction I, specific activity = $2.1 \cdot 10^4$) and heart (30–60% $(NH_4)_2SO_4$ fraction, specific activity = $1.7 \cdot 10^4$) pyruvate kinase preparations applied consisted of 11–12 mg of protein. Recovery of activity (204 000 units for heart and 250 000 units for muscle) was 100%. Symbols are the same as in Fig. 1.

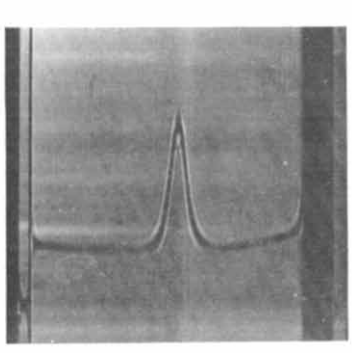
Fig. 3. Elution patterns for heart pyruvate kinase from CM-Sephadex. (A) The 30–60% $(NH_4)_2SO_4$ fraction (specific activity of preparation applied = $1.0 \cdot 10^4$). The active fractions were pooled separately and designated as PI and PII. Fractions PI (specific activity = $4.0 \cdot 10^4$) and PII (specific activity = $4.6 \cdot 10^4$) contained 22 000 and 25 000 units of activity respectively and each had comparable levels of protein (0.06 mg/ml). (B) Rechromatography of PII after concentrating by ultrafiltration and dialysis to remove the KCl. (C) Rechromatography of PI after concentrating by ultrafiltration and dialysis to remove the KCl. The early eluting peak in (C) contained 3100 units and the back peak 5000 units of activity. All three columns were of the same size, 1.8 cm × 42 cm. Flow rate (25 ml/h) and fraction volume (2.5 ml) were constant for the three runs. After termination of the gradients, column buffer with 0.15 M KCl was used to wash the column until all activity was eluted. Symbols as in Fig. 1.

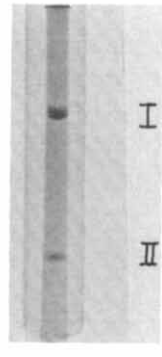
(pH 7.4) containing 1 mM EDTA and 10 mM β-mercaptoethanol. The 30–60% $(NH_4)_2SO_4$ fraction is dissolved in this buffer and dialyzed against the same buffer until free of NH_4^+. For DEAE-Sephadex chromatography, the enzyme solution (100 mg of protein per 100 ml bed volume of resin) is applied to the column and then eluted with a 1 l linear gradient of 0–0.15 M KCl. The enzyme is eluted at approx. 0.04 M KCl as a single peak (Fig. 2B). Chromatography on CM-Sephadex, under identical conditions, gives two peaks (I and II) of pyruvate kinase activity (Fig. 3A). Pyruvate kinase in the two fractions has identical electrophoretic mobilities on either cellulose acetate (0.04 M imidazole–acetate buffer (pH 7.4) containing 10 mM β-mercaptoethanol, 1 mM EDTA and 0.15 M KCl) or on acrylamide gel (0.034 Tris–glycine buffer (pH 9.3)). However, as discussed below, the pyruvate kinase in the two different fractions has different kinetic properties. In view of this observation, these two individual fractions (Peaks I and II) were rechromatographed separately on

CM-Sephadex columns under conditions identical to those employed for the first column. Pyruvate kinase in Peak II upon rechromatography is eluted from the second column in its original position (Fig. 3B) whereas 62% of that originally in Peak I is eluted in the position of Peak II when rechromatographed (Fig. 3C) with the remainder eluting in its original position. It can be estimated from the data presented in Fig. 3A that the separated fractions used for the second chromatographic experiments contained no more than 10% of the other fraction. Furthermore, the kinetic properties of the pyruvate kinase in Peak II (Fig. 3C) generated upon rechromatography of Peak I (Fig. 3A), are identical to those of the enzyme in the original Peak II (Fig. 3A). These results suggest, but obviously do not prove, that the two active fractions of pyruvate kinase found upon CM-Sephadex chromatography represent interconvertible forms of a single cardiac pyruvate kinase species. The theoretical basis for interconversions of this type[15] and their occurrence upon ion-exchange chromatography of proteins[16] have been discussed by other investigators.

Purity of skeletal muscle pyruvate kinase

The purified pyruvate kinase (Fraction V) was investigated by several different procedures to establish the purity of the preparation. In sedimentation velocity studies, the protein sedimented as a single symmetrical peak (Fig. 4). Treatment of the enzyme with 1% sodium dodecyl sulfate followed by acrylamide gel electrophoresis in sodium dodecyl sulfate (ref. 14) revealed only one protein component with a R_m similar to that of rabbit skeletal muscle pyruvate kinase which was used as an





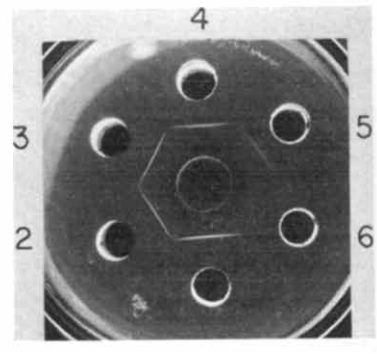


Fig. 4. Sedimentation velocity pattern for purified frog skeletal muscle pyruvate kinase. The run was carried out at 10° with a rotor speed 60 000 rev./min. The protein (5 mg) was dissolved in 1 ml of 0.04 M imidazole–acetate buffer (pH 7.4) containing 1mM EDTA and 10mM β-mercaptoethanol. Photograph was taken 64 min after reaching speed. Direction of sedimentation is from left to right.

Fig. 5. Polyacrylamide-gel electrophoresis of purified frog muscle pyruvate kinase in the presence of sodium dodecyl sulfate. The band labelled I is muscle pyruvate kinase while Band II is the myoglobin marker. A total of 10 μg of muscle pyruvate kinase was used for electrophoresis.

Fig. 6. Double diffusion antigen–antibody experiments with crude (Fraction I) and purified (Fraction V) skeletal muscle pyruvate kinase against anti-M pyruvate kinase in agar gel. The wells contained: (1) Fraction V, 5 mg protein/ml; (2) Fraction I, 10 mg protein/ml; (3) Fraction V, 1 mg protein/ml; (4) Fraction V, 0.5 mg protein/ml; (5) Fraction V, 0.1 mg protein/ml; (6) buffer (0.04 imidazole–acetate buffer (pH 7.4) containing 1 mM EDTA, 10 mM β-mercaptoethanol). Antibody to the enzyme was placed in the center well. Diffusion time was 24 h at room temperature.

TABLE II

A COMPARISON OF THE PHYSICAL PROPERTIES OF PYRUVATE KINASES FROM RABBIT SKELETAL MUSCLE AND *R. pipiens* SKELETAL AND HEART MUSCLE

	Rabbit muscle	*Frog muscle*	*Frog heart*
Elution volume from Sephadex G-200	70 ml	70 ml	70 ml
$s_{20,w}$	9.3*	9.2*	—
Subunit mol.wt.	57 200**	55 000***	—
Mol.wt.	237 000**	220 000†	220 000†

* Rabbit muscle and frog skeletal muscle (Fraction V) were sedimented in 0.01 M sodium acetate buffer (pH 7) at 20°. The protein concentrations for the rabbit and frog enzymes were 2.2 and 2.4 mg/ml, respectively.

** Data taken from STEINMETZ AND DEAL[17].

*** The subunit molecular weight was determined from sodium dodecyl sulfate gel electrophoresis using glutamic dehydrogenase, aldolase, and pyruvate kinase as standards. The molecular weight values used for these proteins were those listed by WEBER AND OSBORN[14].

† Calculated assuming the enzyme is composed of four subunits. The heart enzyme is judged to be the same molecular weight as the frog skeletal muscle enzyme on the basis of identical elution volumes upon Sephadex G-200 chromatography.

internal standard (Fig. 5). Double diffusion antigen–antibody reactions gave only one precipitin band which fused completely with that from a crude skeletal muscle extract (Fig. 6). Furthermore, immunoelectrophoresis with the purified enzyme at different concentrations revealed only one precipitin band in each case. On the basis of these results, the purified pyruvate kinase is considered to be homogeneous.

Comparative studies on the physical and kinetic properties of frog cardiac and skeletal muscle pyruvate kinases

As reported earlier[4], the electrophoretic mobilities of the cardiac and skeletal muscle pyruvate kinases are different. Their chromatographic properties on DEAE-Sephadex (Figs. 2A and 2B) further substantiate the different apparent charges on the enzymes from the two sources. Gel filtration studies (Table II) demonstrate that both enzymes appear to have the same molecular weight and are very similar in size to rabbit muscle pyruvate kinase which was used as an internal standard for these physical studies. The similar $s_{20,w}$ values obtained for both frog and rabbit skeletal muscle pyruvate kinases support the conclusion that these two enzymes have similar molecular weights. The molecular weight of the dissociated frog muscle pyruvate kinase was determined to be approx. 55 000 by sodium dodecyl sulfate electrophoresis thereby establishing that the frog skeletal muscle enzyme, like that of rabbit[17,18] is a tetramer. Comparable data for the cardiac enzyme must await its isolation in a homogeneous form; however, in view of the similarity in the molecular weights of the three enzymes, it seems probable that the cardiac enzyme also has a tetrameric structure.

Immunological cross-reactivity of the frog cardiac pyruvate kinase with the antibody to the skeletal muscle enzyme was observed. Double diffusion antigen–antibody experiments demonstrated that cardiac and skeletal muscle pyruvate kinases gave a single precipitin band which fused completely with no spurring. In addition, the activity of the two enzymes was inhibited to the same extent by the

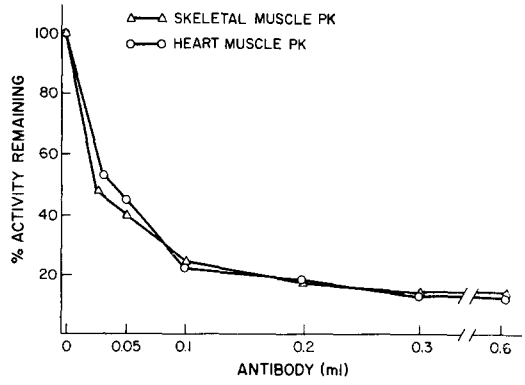


Fig. 7. Inhibition of frog skeletal muscle and heart pyruvate kinase activities with anti-M pyruvate kinase antibody. Antibody preparation was added to the assay system complete except for phosphoenolpyruvate and was allowed to stand for 1 min at room temperature before addition of the phosphoenolpyruvate (0.66 mM). The number of units of pyruvate kinase used was identical (25 units) for heart (pooled DEAE-Sephadex fractions) and the muscle (Fraction V). △—△, skeletal muscle pyruvate kinase; ○—○, heart muscle pyruvate kinase.

antibody to the skeletal muscle enzyme (Fig. 7). The decrease in activity of the cardiac enzyme in the presence of the antibody cannot be attributed to the presence of skeletal muscle pyruvate kinase in the heart preparation. As shown in Figs. 2A and 2B, the different elution profiles for the two enzymes from identical DEAE-Sephadex columns would result in the removal of any skeletal muscle pyruvate kinase from the heart preparation. In fact, the skeletal muscle form of pyruvate kinase has never been observed chromatographically or electrophoretically in any preparation of cardiac pyruvate kinase.

TABLE III

COMPARATIVE STUDIES ON THE ACTIVATION AND INHIBITION OF FROG SKELETAL MUSCLE AND HEART PYRUVATE KINASES

All studies except the 25° incubation experiment were carried out at 15°. Activation with Fru-1,6-P_2 and inhibition with L-alanine were performed at 35 μM phosphoenolpyruvate while the other experiments were done at 660 μM phosphoenolpyruvate. The levels of Fru-1,6-P_2, L-alanine and DL phenylalanine used were 0.5, 0.12 and 0.1 mM, respectively. For the inhibition studies, the effectors were added to a complete pyruvate kinase assay mixture except for phosphoenolpyruvate which was added 5 min later. Incubation at 25° was for 5 min. All assays contained 90 units of pyruvate kinase.

Preparation	*Activation with Fru-1,6-P_2**	*Percent of control activity*		
		L-*Alanine*	DL-*Phenylalanine*	*25° incubation*
Skeletal muscle	1.0	100	100	110
Heart; CM-Sephadex Fraction PI**	1.8	106	34	—
Heart; CM-Sephadex Fraction PII**	8.3	50	6	70

* The ratio of activity with Fru-1,6-P_2 to that without Fru-1,6-P_2.
** See Fig. 3A.

The pyruvate kinase from cardiac tissue, while possessing physical properties similar to those of the skeletal muscle enzyme, displays different kinetic properties in that, unlike the muscle enzyme, it is subject to activation by Fru-1,6-P_2 and phosphoenolpyruvate and inhibition by alanine and phenylalanine. However, the extent to which the activity of the cardiac enzyme is altered by these modifiers is dependent upon the isolation procedure. As outlined above, two different fractions of pyruvate kinase are obtained by chromatography on CM-Sephadex (see Fig. 3A). A comparison of the properties of these two fractions of cardiac pyruvate kinase (referred to as P-I and P-II) with those of purified skeletal muscle pyruvate kinase is given in Table III. Although both cardiac pyruvate kinase preparations are activated by Fru-1,6-P_2 at low phosphoenolpyruvate concentrations, that observed with the P-II fraction was 8-fold whereas that with the P-I fraction was around 1.8-fold. Different degrees of inhibition by alanine and phenylalanine were observed with the P-I and P-II fractions as well. On the other hand, the activity of the skeletal muscle enzyme was not altered by any of these compounds and this enzyme is less sensitive to inactivation by increased temperatures.

TABLE IV

THE LEVEL OF Fru-1,6-P_2 ACTIVATION FOR DIFFERENT PREPARATIONS OF FROG HEART PYRUVATE KINASE

All determinations were performed at 15° with 90 units of pyruvate kinase, 35 μM phosphoenolpyruvate and 0.5 mM Fru-1,6-P_2.

Treatment	*Activation with Fru-1,6-P_2*
Fresh heart tissue extract	6.6
Aged heart tissue extract*	1.7
45–60% $(NH_4)_2SO_4$ fraction ($(NH_4)_2SO_4$ removed by dialysis)	1.3
DEAE-Sephadex preparation**	1.8
CM-Sephadex preparation***	
Fraction PI	1.3
Fraction PII	8.3
PII, 24 h after elution*	2.8
Fraction PII dialyzed†	1.7
Fraction PI re-chromatographed on CM-Sephadex††	7.4

* Standing for 24 h at 4° in homogenization or column buffer (see text).
** See Fig. 2B.
*** See Fig. 3A.
† Dialyzed *vs.* column buffer (see text).
†† See Fig. 3C.

The differences in the properties of the two different fractions of cardiac pyruvate kinase obtained by CM-Sephadex chromatography suggested that there are two different forms of the enzyme, one of which is highly sensitive to the action of modifiers and the other which has undergone apparent desensitization to these compounds. Hence, the properties of the cardiac enzyme were examined at different stages of purification and after subjecting the enzyme preparation to different experimental conditions. The results are summarized in Table IV and, of the different pyruvate kinase preparations examined, a high level of Fru-1,6-P_2 activation is found only

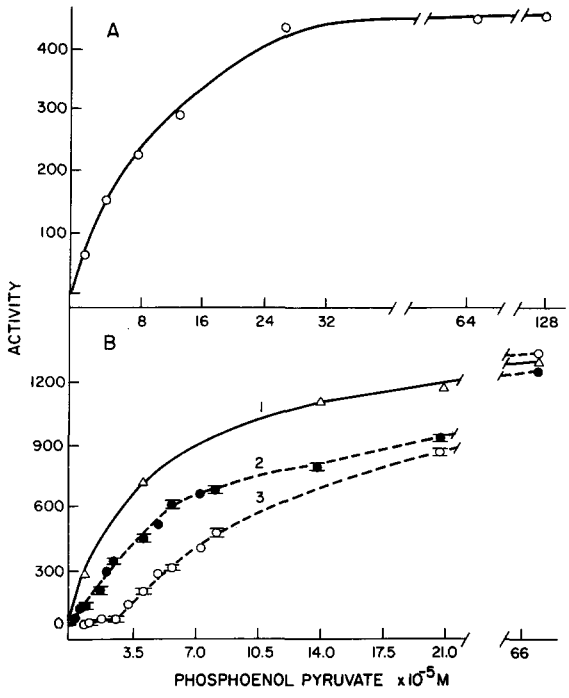


Fig. 8. The effect of increasing concentrations of phosphoenolpyruvate on the activity of (A) purified frog skeletal muscle pyruvate kinase and (B) frog heart pyruvate kinase in the presence and absence of Fru-1,6-P_2. The saturation kinetics for the muscle enzyme were performed with Fraction IV. Determinations have also been carried out on the most purified fraction (Fraction V and found to be identical to those shown in A. The curves in (B) depict the results for: heart pyruvate kinase from CM-Sephadex Fraction PII (Fru-1,6-P_2-sensitive) (○ - - - ○); heart pyruvate kinase from DEAE-Sephadex chromatography (Fru-1,6-P_2-insensitive) (●—●); heart pyruvate kinase activity from both sensitive and insensitive preparations measured in the presence of Fru-1,6-P_2 (0.5 mM) (△—△). All determinations (muscle and heart) were performed at 15° with 90 units of pyruvate kinase added to the cuvette. Standard deviations were calculated from five determinations for activity measurements made at phosphoenolpyruvate concentrations of 0.1 mM or lower and from three determinations at the higher phosphoenolpyruvate levels.

with fresh extracts of heart tissue and with the P-II fraction following chromatography on CM-Sephadex. Furthermore, when these two preparations are aged at 4° for 24 h and then assayed, they exhibit only a low level of Fru-1,6-P_2 activation. It should be emphasized that this apparent desensitization occurs without a loss in the total activity of the enzyme as measured at saturating levels of phosphoenolpyruvate. A kinetic interpretation for this change becomes apparent upon a comparison of the phosphoenolpyruvate saturation curves for pyruvate kinase preparations exhibiting either high or low Fru-1,6-P_2 activation (Fig. 8B). It can be seen that the preparation with a high Fru-1,6-P_2 activation displays sigmoidal kinetics whereas the one with low Fru-1,6-P_2 activation yields a hyperbolic curve. Both preparations exhibit the same level of activity in the presence of Fru-1,6-P_1 (Fig. 8B, Curve 1). Removal of the KCl by dialysis from the CM-Sephadex fraction (P-II) showing high Fru-1,6-P_2 activation converts the enzyme to a form showing a low level of Fru-1,6-P_2 activation (Table IV). The phosphoenolpyruvate saturation

curve for this desensitized preparation is hyperbolic (not shown) but the level of total activity in the presence of Fru-1,6-P_2 is unchanged. Hence, the extent of activation by Fru-1,6-P_2, measured at low phosphoenolpyruvate concentrations, is indicative of the form in which the enzyme exists. The sensitive form exhibits higher levels of Fru-1,6-P_2 activation and greater inhibition by alanine or phenylalanine whereas the effect of these modifiers on the activity of the desensitized form is markedly less. The decrease in Fru-1,6-P_2 sensitivity is accompanied by a conversion of the phosphoenolpyruvate saturation curve from a sigmoidal to a hyperbolic form.

To date, the only procedure established for effecting a conversion of the desensitized form of the enzyme to a sensitive one is by rechromatography of the former on CM-Sephadex (see Fig. 3C and Table IV). Attempts to effect the conversion by dialysis, or incubation, of the enzyme in buffers containing high concentrations of KCl (0.15 M), comparable to the concentration needed to elute the sensitive form of the enzyme from the CM-Sephadex, or in sucrose (0.25 M), have been unsuccessful with aged crude extracts and with fractions purified by DEAE- and CM-Sephadex (Fraction P-I) chromatography.

The above effects observed with the heart pyruvate kinase cannot be demonstrated with the skeletal muscle enzyme despite treatment under identical conditions. Thus, hyperbolic kinetics (Fig. 8A) are always observed with the skeletal muscle pyruvate kinase at all stages of purification. Furthermore, no activation or inhibition of the skeletal muscle enzyme is observed with the compounds that modify the activity of the heart pyruvate kinase (Table III).

DISCUSSION

The results presented here establish that in the adult grass frog, *R. pipiens*, heart pyruvate kinase is physically similar to but kinetically distinct from the skeletal muscle enzyme and therefore that these two forms of the enzyme are isozymes. In this respect the frog differs from the rat in that studies on the properties of cardiac pyruvate kinase of rat heart have shown it to be identical to the muscle type enzyme[1]. It has been reported that beef heart pyruvate kinase is activated by Fru-1,6-P_2 (ref. 19) but no data or experimental conditions were presented. The possible activation of beef heart pyruvate kinase by Fru-1,6-P_2 was investigated in this laboratory. Under our conditions, no activation of the beef heart enzyme could be demonstrated either with crude extracts or partially purified (50-fold) preparations but activation of the beef kidney pyruvate kinase was observed.

On the basis of the studies reported here, frog heart pyruvate kinase has many properties in common with the regulatory Type L pyruvate kinase of the rat in that both are activated by Fru-1,6-P_2 and are inhibited by L-alanine. Further similarities exist with respect to their phosphoenolpyruvate saturation profiles as several workers[1,5,6] have observed sigmoidal phosphoenolpyruvate saturation curves with rat Type L pyruvate kinases. Comparative studies of frog heart and frog liver pyruvate kinases cannot be made as yet since the latter tissue has been shown to contain several forms of pyruvate kinase[4]. However, in preliminary experiments crude liver preparations of pyruvate kinase from *R. pipiens* were found to be activated by Fru-1,6-P_2 and show sigmoidal phosphoenolpyruvate saturation curves. More definitive studies must await the separation of the individual isozymes found in frog liver

and a comparison of the properties of each with those of the pyruvate kinase found in heart. The phenomenon of a regulatory-type pyruvate kinase in heart tissue is not peculiar to *R. pipiens* since heart pyruvate kinase from *Rana catesbeiana* was examined in the present studies and it is also activated by Fru-1,6-P_2. The significance of the inhibition of frog heart pyruvate kinase by phenylalanine is not apparent at this time. To our knowledge, only rat Type M pyruvate kinases have been demonstrated to show phenylalanine inhibition[7,8] and this property has not been reported for rat Type L pyruvate kinases. In view of the properties described above for frog heart pyruvate kinase, it appears to resemble the Type M_2 described by TANAKA *et al.*[2]. A more quantitative treatment of the kinetics of the frog heart enzyme, in order to establish its relationship to either Type L or Type M_2, cannot be undertaken until the factors contributing to the desensitization of the enzyme are defined.

Several workers have reported apparent interconversions of Fru-1,6-P_2-sensitive and Fru-1,6-P_2-insensitive forms of pyruvate kinase from rat liver and adipose tissue. SUSOR AND RUTTER[3] found that pyruvate kinase activity in rat liver extracts was only slightly activated (1.5-fold) by Fru-1,6-P_2 but required Fru-1,6-P_2 for catalytic activity at low phosphoenolpyruvate concentrations after a second DEAE-cellulose chromatographic step. BAILEY *et al.*[5] observed an increase in the cooperativity of rat Type L pyruvate kinase towards phosphoenolpyruvate and Fru-1,6-P_2 upon incubation of the enyzme at 25°. This procedure was unsuccessful with the desensitized form of frog heart pyruvate kinase resulting in a 20% loss in activity without any change in its kinetic properties. POGSON[6] also observed alterations in rat adipose tissue pyruvate kinase activity to Fru-1,6-P_2 when the tissue was extracted in the presence (Fru-1,6-P_2-sensitive) or absence (Fru-1,6-P_2-insensitive) of EDTA and increased cooperativity toward phosphoenolpyruvate with the Fru-1,6-P_2-sensitive preparations. Addition or omission of EDTA to frog heart pyruvate kinase preparations was without effect on the level of Fru-1,6-P_2 activation. The parallelism between the observations of BAILEY *et al.*[5] and POGSON[6] and the different kinetic properties defined for the Fru-1,6-P_2-sensitive and -desensitized forms of frog heart pyruvate kinase would seem to support the conclusion that the results reported here do involve interconvertible forms of pyruvate kinase and are not artifacts of the experimental procedures used. Nevertheless, more direct experimentation is required to establish this point unequivocally.

The frog skeletal muscle pyruvate kinase appears to resemble Type M_1 of rat. Hyperbolic kinetics are always observed and its activity is not altered by the addition of modifiers or by temperature variations. In spite of the fact that no Fru-1,6-P_2 activation can be demonstrated, it appears that Fru-1,6-P_2 does interact with the frog skeletal muscle enzyme as shown by the differences in chromatographic properties in the presence or absence of Fru-1,6-P_2 (Figs. 1B and 2A). JOHNSON AND DEAL[20] have published indirect evidence for the binding of Fru-1,6-P_2 to rabbit skeletal muscle pyruvate kinase. The apparent change in the charge on the frog skeletal muscle enzyme could either be due to the added negative charge of Fru-1,6-P_2 or a conformational change resulting from bound Fru-1,6-P_2. Direct measurements are obviously needed to clarify this behavior. The only inconsistency between the properties of frog skeletal muscle pyruvate kinase and those of rat Type M (ref. 7,8) is the absence of phenylalanine inhibition in the frog system. No inhibition of the frog muscle was observed with phenylalanine levels up to 20 mM.

In our initial studies[4] on eggs of *R. pipiens*, we found five isozymes of pyruvate kinase none of which corresponded electrophoretically to the skeletal muscle type. The adult heart and liver forms did, however, correspond to certain of the egg isozymes. With the work presented here, it appears that in the early embryonic development of *R. pipiens*, the forms of pyruvate kinase are of the regulatory type and the potential exists then, for glycolytic control by this enzyme. The importance of this is based on the observations that carbohydrate utilization is at least the predominant source of energy at the early stages of amphibian embryonic development[21].

ACKNOWLEDGEMENTS

This study was supported in part by Research Contract No. AT(11-1)1631 from the U.S. Atomic Energy Commission and by Grant No. AM-00922 from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, U.S. Public Health Service. The technical assistance of Mr. E. Kmiotek in certain phases of this work is gratefully acknowledged.

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