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PURIFICATION OF FOUR PYRUVATE KINASE ISOZYMES OF RATS BY AFFINITY ELUTION CHROMATOGRAPHY

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Summary

1. Purification of four isozymes of pyruvate kinase (ATP:pyruvate 2-*O*-phosphotransferase, EC 2.7.1.40) L, M_1, M_2 and R was much improved to give good yields by affinity elution chromatography. The enzyme was eluted from a phosphocellulose column with 0.5 mM phosphoenolpyruvate. Types L, M_2 and R were stabilized with fructose 1,6-diphosphate throughout the purification procedures.

2. The isozymes were crystallized under various conditions: types L and R were readily crystallized from medium of low ionic strength, types L, M_1, and M_2 were crystallized from ammonium sulfate solution in different forms in the presence and absence of phosphoenolpyruvate. Type M_1 was also crystallized in different forms in the presence and absence of fructose 1,6-diphosphate.

3. Amino acid analyses showed that the compositions of types L and R, and of types M_1 and M_2, respectively, were very similar.

Introduction

There are at least three major isozymes of pyruvate kinase (ATP:pyruvate 2-*O*-phosphotransferase, EC 2.7.1.40) in mammals. They were purified from rat tissues for the first time in this laboratory and designated types M_1, M_2 and L [1,2]. There are also other multiple forms in various tissues. The L-M_2 hybrid set exists in the intestine and kidney [3], and the M_1-M_2 hybrid set in fetal muscle [4] and some adult tissues [5]. Another specific type (designated as type R [6]) exists in erythrocytes.

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The homogeneities of the purified samples were examined by disc gel electro- rather easily from the muscles of many species. Types L and M_2, however, are present in only small amounts in the tissues and are labile under ordinary conditions. Several workers have used two or more ion-exchanger column chromatographies and gel filtration into their purification procedures [7,8]. Affinity adsorption chromatography has also been attempted [9], and employed recently in the purifications of type L [10] and type M_2 [11] from human tissues. Moreover, affinity elution chromatography has been developed by Schultz et al. [12] for mouse type M_2 and by Scopes [13] for various glycolytic enzymes from rabbit skeletal muscles.

The present paper describes a new method for purification of pyruvate kinase isozymes from rat tissues; application of affinity elution chromatography greatly simplified the purification procedure, and types L, M_1, M_2 and R were purified completely by almost the same procedure. Various methods were also developed for crystallization of the isozymes.

Experimental Procedures

Materials. Disodium ADP and NADH were obtained from Oriental Yeast Co. Japan. Lactate dehydrogenase from rabbit muscle (5500 units/ml, 25°C) and calibration proteins for sodium dodecyl sulfate (SDS) electrophoresis (Combithek) were obtained from Boehringer Co. Tetrasodium fructose 1,6-diphosphate, grade I (for enzyme assay and for the electrophoretic running buffer) and grade II (for enzyme purification), and tricyclohexylamine phosphoenolpyruvate were obtained from Sigma Chemical Co. Phosphocellulose (capacity: 1.06 mequiv./g) was from Brown Co. All other reagents were standard commercial products. Distilled, deionized water was used in preparation of all solutions.

Enzyme assay. Pyruvate kinase activity was measured at 25°C with a coupled assay system similar to that described by Bücher and Pfleiderer [14]. The assay mixture contained final concentrations of 50 mM Tris · HCl buffer, pH 7.5, 100 mM KCl, 5 mM $MgSO_4$, 2 mM phosphoenolpyruvate, 2 mM ADP, 0.5 mM Fru-1,6-P_2, 0.18 mM NADH and 8 units/ml of lactate dehydrogenase. The samples were suitably diluted with the stabilizing buffer (homogenization buffer containing 0.5 mM Fru-1,6-P_2 and 1% bovine serum albumin) before assays. The homogenization buffer contained 20 mM Tris · HCl buffer, pH 7.5, 100 mM KCl, 5 mM $MgSO_4$, and 1 mM EDTA. Protein concentration was measured by method of Lowry et al. [15] using bovine serum albumin as a standard.

Electrophoresis. The method of thin-layer polyacrylamide gel electrophoresis described previously [3] was modified as follows. A thin layer (1 mm thick) of polyacrylamide gel (3.4% (w/v) acrylamide with 0.13% (w/v) bisacrylamide) was prepared on a glass (210 × 128 mm) and used after equilibration in running buffer (10 mM Tris · HCl, pH 7.5, 20 mM KCl, 2 mM $MgSO_4$, 0.4 mM Fru-1,6-P_2, 20 mM β-mercaptoethanol). Electrical connections were made between the gel plate and running buffer with filter paper made from glass fiber, which greatly reduced the electro-osmotic effect of the bridges. A constant current of 5—6 mA per cm width of the gel layer was applied for 7—8 h. Pyruvate kinase activity was detected by a modification of the method of Susor and Rutter [4].

The homogeneities of the purified samples were examined by disc gel electrophoresis in the presence of sodium dodecyl sulfate by the method of Fairbanks et al. [16] with minor modifications.

Amino acid analyses. The samples were hydrolyzed in distilled 5.7 M HCl in vacuo for 24 or 72 h at 110°C. All analyses were performed by the method of Spackman et al. [17] using a Beckman Amino Acid Analyzer, Model 120 B, equipped with an accelerator.

Enzyme sources. Sprague-Dawley albino rats of both sexes were used; type M_1 enzyme was prepared from their skeletal muscle and type L from their liver. A high level of type L was induced in the liver by feeding the animals on high carbohydrate diet [1]. Type R was prepared from erythrocytes, collected by decapitation of animals and washed with saline. Type M_2 was prepared from AH-130 Yoshida ascites hepatoma cells maintained in female Sprague-Dawley albino rats. The cells were well washed with saline. All tissues were used after storage at −20°C.

Results

Preliminary experiments on phosphocellulose chromatography of the isozymes

Conditions for chromatography of pyruvate kinase isozymes on phosphocellulose were investigated. During purification, phosphate buffer, pH 6, containing 2 mM $MgSO_4$ and 10 mM β-mercaptoethanol was used in preparation of all solutions, and 0.2 mM Fru-1,6-P_2 was also used for isozymes other than type M_1 throughout this study as stabilizing agents. Under these conditions column chromatography can be carried out at room temperature without loss of activity.

Phosphocellulose was washed successively with five or six volumes of 0.2 M KOH, then 0.2 M HCl and finally with deionized water, therafter it was resuspended in 10 mM phosphate buffer and adjusted to pH 6.0 by 2 M KOH. After enough equilibration with 10 mM phosphate buffer, pH 6, 2 mM $MgSO_4$, 10 mM β-mercaptoethanol, with or without 0.2 mM Fru-1,6-P_2, the cellulose was packed into a column. All four types, R, L, M_1 and M_2, were adsorbed similarly to a phosphocellulose column at low ionic strength (10 mM phosphate buffer, pH 6) in the presence of the stabilizing agents. Fig. 1 depicts the elution patterns of types L and M_2. The sample of type L used was the 25—45% $(NH_4)_2SO_4$ fraction of a crude extract of liver and the sample of type M_2 was the 45—75% fraction of a crude extract of ascites tumor cells. These samples were dissolved in, and dialyzed against 10 mM phosphate buffer, pH 6, and applied to phosphocellulose columns. Then the columns were washed with 10 mM phosphate buffer and eluted with a linear concentration gradient of 10—140 mM phosphate buffer, pH 6. In the absence of phosphoenolpyruvate in the elution buffer, type L and type M_2 began to be eluted at 2.5 and 5.5 mS electric conductance, respectively (Figs. 1a and 1c). In the presence of 0.5 mM phosphoenolpyruvate, type L and type M_2 began to be eluted at 1.8 and 2.5 mS electric conductance, respectively, and their elution profiles were steep (Figs. 1b and 1d). Type R had a similar elution profile to that of type L.

Type M_1 began to be eluted at 4.9 mS electric conductance without phosphoenolpyruvate, but its elution peak was very broad (data not shown). This

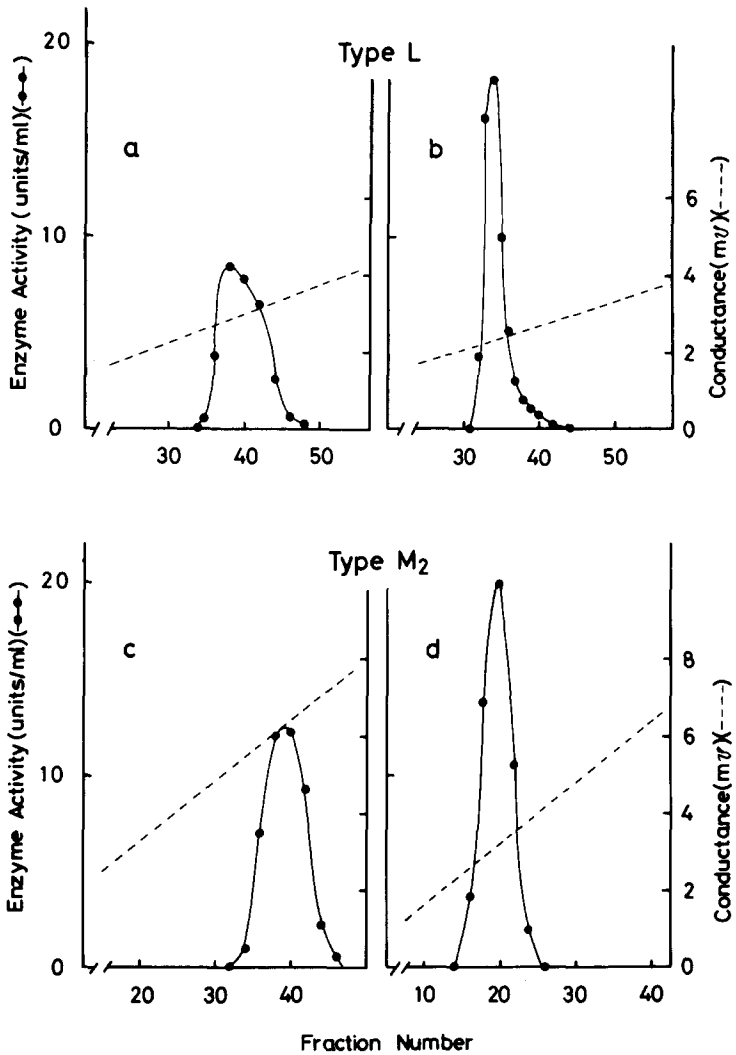

Fig. 1. Phosphocellulose chromatography of types L and M_2 pyruvate kinase. ●———●, pyruvate kinase activity; - - - - - -, electric conductance. Ammonium sulfate fractions of crude tissue extracts were applied to a phosphocellulose column. The enzyme was eluted with a linear concentration gradient of 10—140 mM phosphate buffer, pH 6, containing stabilizing agents, in the presence (b, d) or absence (a, c) of phosphoenolpyruvate. For details see text. (mʊ = mS)

disadvantage was avoided by using a linear concentration gradient of 0—0.2 M KCl in 10 mM phosphate buffer for elution. Under these conditions type M_1 was eluted at 4.2 and 12 mS electric conductance of elution buffer with and without 0.5 mM phosphoenolpyruvate, respectively. These results show that addition of phosphoenolpyruvate to the elution buffer greatly accelerated the elutions of all four types of pyruvate kinase from the phosphocellulose column.

Purification of the pyruvate kinase isozymes from various rat tissues

(a) Purification of type R pyruvate kinase from rat erythrocytes. About 4 l

of frozen erythrocytes were thawed and lyzed by adding three volumes of 5 mM phosphate buffer, pH 6, containing 2 mM $MgSO_4$, 10 mM β-mercaptoethanol and 0.2 mM Fru-1,6-P_2. The hemolyzate was adjusted to pH 5 with 2 M acetic acid and centrifuged at $2 \cdot 10^4 \times g$ for 30 min at 0°C. The resulting supernatant was rapidly adjusted to pH 6 with 2 M NaOH and brought to 50% saturation of ammonium sulfate. The precipitate obtained by centrifugation was dissolved in 3 l of 40 mM phosphate buffer, pH 6. The preparation was again fractionated with ammonium sulfate and the 25—45% $(NH_4)_2SO_4$ fraction was dissolved in, and dialyzed against 10 mM phosphate buffer, pH 6, at 4°C.

Phosphocellulose (600 ml), previously equilibrated with the same buffer, was added to the enzyme solution with stirring for 10 min, then it was packed in a column and was washed with the same buffer, increasing the concentration of phosphate buffer stepwise to 33 mM (2 mS). The enzyme was completely eluted with a high concentration of phosphate buffer (100 mM). The material precipitated from the solution with 50% saturation of ammonium sulfate was dissolved in, and dialyzed again and applied to a phosphocellulose column. The column was washed with a linear concentration gradient of 10—33 mM phosphate buffer, and then with 33 mM phosphate buffer until the absorbance of the eluate at 280 nm became almost zero. The enzyme was specifically eluted with 33 mM phosphate buffer containing 0.5 mM phosphoenolpyruvate

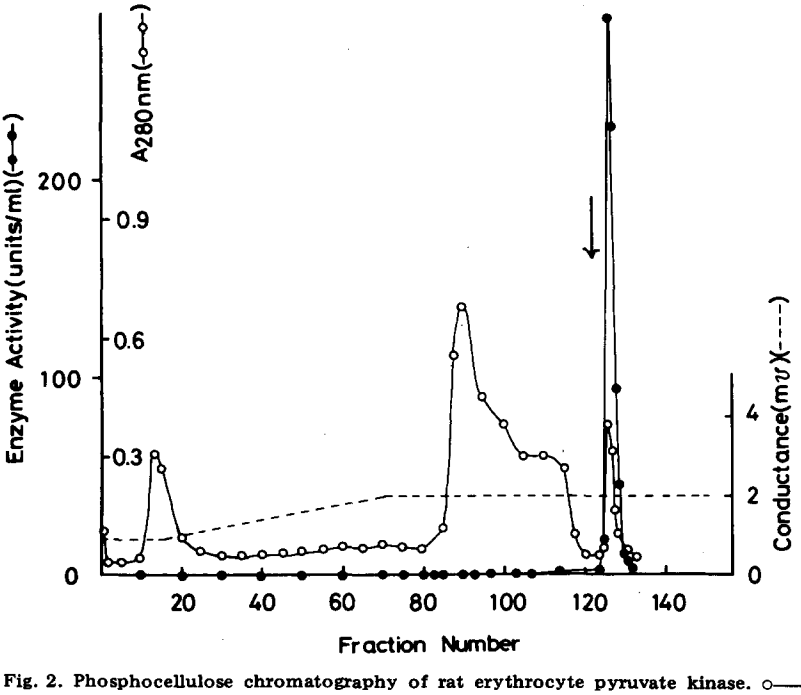

Fig. 2. Phosphocellulose chromatography of rat erythrocyte pyruvate kinase. ○———○, adsorbance at 280 nm; ●———●, pyruvate kinase activity; ------, electric conductance. Pyruvate kinase obtained by preliminary batchwise chromatography was applied to a phosphocellulose column. The column was washed with a linear concentration gradient of 10—33 mM (2 mS) phosphate buffer, pH 6, containing stabilizing agents and then with 33 mM phosphate buffer. Then, the enzyme was eluted specifically with 0.5 mM phosphoenolpyruvate. The arrow indicates the point of addition of 0.5 mM phosphoenolpyruvate to 33 mM phosphate buffer.

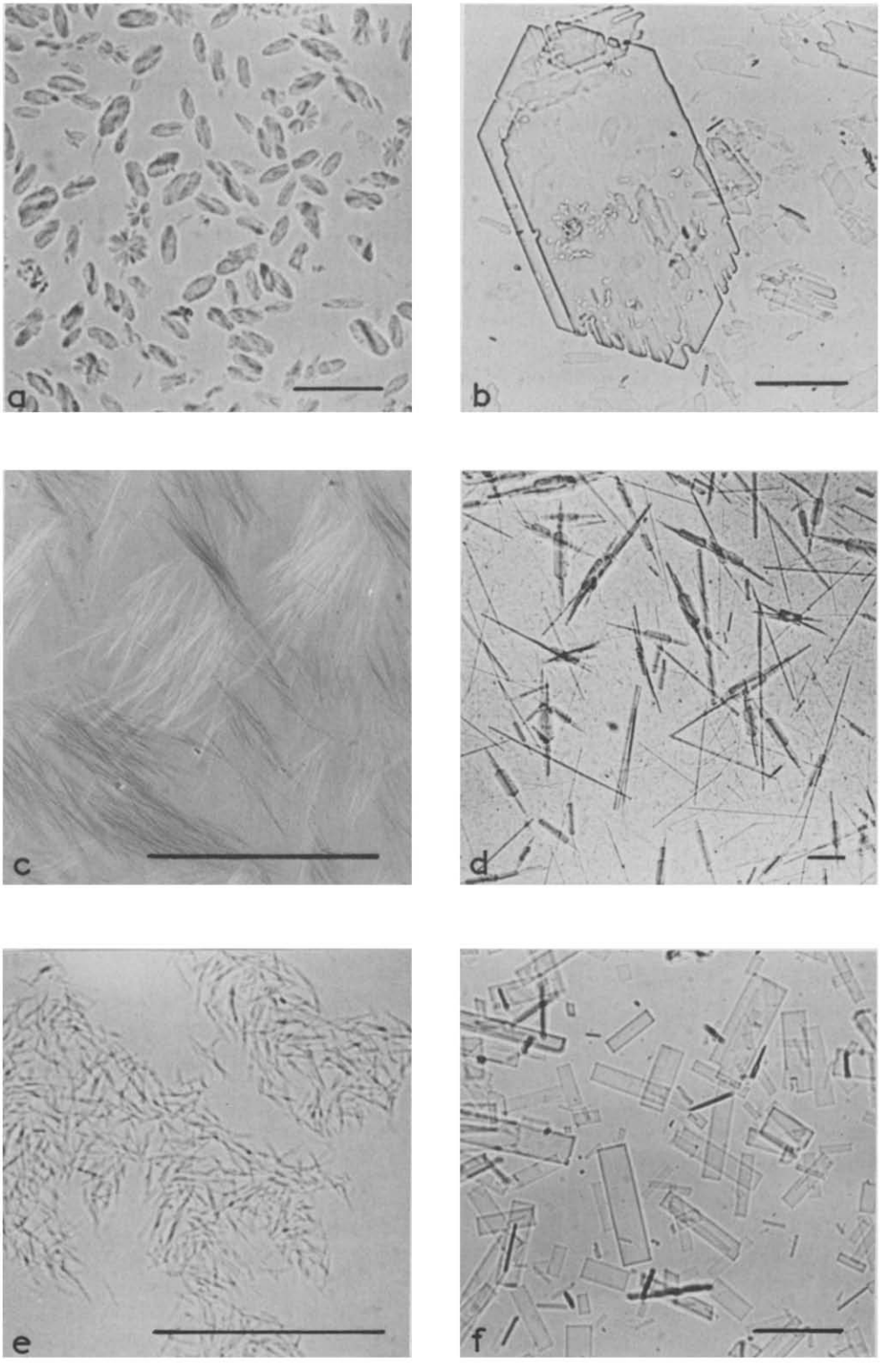
a
b
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TABLE I

PURIFICATION OF TYPE R PYRUVATE KINASE FROM RAT ERYTHROCYTES

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Recovery (%)
Hemolyzate	$382 \cdot 10^4$	34 380	0.009	100
50% $(NH_4)_2SO_4$ fraction	$79 \cdot 10^3$	34 020	0.43	99
25—45% $(NH_4)_2SO_4$ fraction	$34 \cdot 10^3$	31 680	0.93	92
Phosphocellulose chromatography	$2 \cdot 10^3$	26 880	13.4	78
Phosphocellulose chromatography (phosphoenolpyruvate elution)	62	19 870	320	58
Crystallization	55	16 850	307	49

(Fig. 2). Fractions with pyruvate kinase activity were combined and brought to 50% saturation of ammonium sulfate. The resulting precipitate was dissolved in a small volume of 50 mM phosphate buffer, pH 6, and the solution was dialyzed against the same buffer. Clusters of short needle-shaped crystals appeared within a few hours (Fig. 3a). The purification of rat erythrocyte pyruvate kinase is summarized in Table I.

(b) Purification of type L pyruvate kinase from rat liver. About 3 kg of rat liver was homogenized with three volumes of homogenization buffer containing 0.2 mM Fru-1,6-P_2 and 10 mM β-mercaptoethanol, and the homogenate was centrifuged ($2 \cdot 10^4 \times g$). The 33—45% $(NH_4)_2SO_4$ fraction of the crude extract was dissolved in, and dialyzed against 40 mM phosphate buffer and fractionated with 20% (v/v) to 40% (v/v) saturation of acetone (—15°C) containing 1 mM dithiothreitol. Subsequent purification was carried out by a batchwise chromatography on phosphocellulose (1000 ml). The enzyme was eluted with 33 mM phosphate buffer containing 0.5 mM phosphoenolpyruvate. It was proved that only one step of the chromatography sufficed for purification, since the following crystallization under a similar condition to that of type R could efficiently increase the specific activity of the enzyme (Table II).

(c) Purification of type M_2 pyruvate kinase from AH-130 Yoshida ascites hepatoma cells. About 500 g of frozen AH-130 Yoshida ascites hepatoma cells were thawed and lyzed by adding two volumes of homogenization buffer containing 10 mM β-mercaptoethanol and 0.2 mM Fru-1,6-P_2. The 45—75% $(NH_4)_2SO_4$ fraction of the crude extract was purified by a batchwise chromatography on phosphocellulose (400 ml) by elution with 70 mM phosphate buffer containing 0.5 mM phospoenolpyruvate (Table III). Type M_2 from intestine was separated from L-M_2 hybrids by phosphocellulose column chromatography

Fig. 3. Crystals of the four types of pyruvate kinase. (a) Crystals of type R formed by dialysis against 50 mM phosphate buffer, pH 6, containing stabilizing agents (0.4 mM Fru-1,6-P_2, 2 mM $MgSO_4$ and 10 mM β-mercaptoethanol). (b—f) Crystals formed by adding ammonium sulfate. Crystals of type L obtained from 50 mM phosphate buffer, pH 6, containing stabilizing agents and 1 mM phosphoenolpyruvate (b). Crystals of type M_2 from 50 mM phosphate buffer, pH 6, containing stabilizing agents in the absence (c) and presence (d) of 1 mM phosphoenolpyruvate. Crystals of type M_1 from 50 mM phosphate buffer, pH 6, containing 2 mM $MgSO_4$ and 10 mM β-mercaptoethanol in the absence (e) and presence (f) of 1 mM phosphoenolpyruvate. The bars represent 50 μm.

TABLE II

PURIFICATION OF TYPE L PYRUVATE KINASE FROM RAT LIVER

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Recovery (%)
Crude extract	$227 \cdot 10^3$	$340 \cdot 10^3$	1.5	100
33—45% $(NH_4)_2SO_4$ fraction	$126 \cdot 10^3$	$272 \cdot 10^3$	2.0	80
20—40% Acetone fraction	$163 \cdot 10^2$	$163 \cdot 10^3$	10	48
Phosphocellulose chromatography (phosphoenolpyruvate elution)	504	$126 \cdot 10^3$	250	37
Crystallization	157	$816 \cdot 10^2$	520	24

in the absence of phosphoenolpyruvate (unpublished data) and then purified as described above.

(d) Purification of type M_1 pyruvate kinase from rat muscle. About 3.5 kg of rat skeletal muscle was homogenized with three volumes of homogenization buffer containing 10 mM β-mercaptoethanol. The 55—70% $(NH_4)_2SO_4$ fraction of the crude extract was dissolved in, and dialyzed against 40 mM phosphate buffer, pH 6, containing 2 mM $MgSO_4$ and 10 mM β-mercaptoethanol. The preparation was fractionated with 30% (v/v) to 38% (v/v) saturation of acetone (—15°C) containing 10 mM β-mercaptoethanol. The enzyme solution was again fractionated with ammonium sulfate (55—70%) and then applied to a phosphocellulose column (280 ml). The column was washed with buffer of stepwise increasing electric conductance to 9 mS by addition of KCl to 10 mM phosphate buffer, maintaining the pH at pH 6. Then, the enzyme was eluted with 10 mM phosphate buffer containing 170 mM KCl (12 mS). It was again applied to a phosphocellulose column. The column was washed with a linear gradient of ionic strength of 0—80 mM (6 mS) KCl in the same buffer and eluted with the latter containing 0.5 mM phosphoenolpyruvate (Table IV).

Purified types L, M_1 and M_2 were stable for a year on storage at —70°C in 140 mM phosphate buffer, pH 6, containing stabilizing agents, at protein concentrations of 3—10 mg per ml.

Crystallization

Crystallization of purified type R was achieved as described above (Fig. 3a). Purified type L was also crystallized similarly and its crystalline form was the same as that of type R.

TABLE III

PURIFICATION OF TYPE M_2 PYRUVATE KINASE FROM AH-130 CELLS

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Recovery (%)
Crude extract	$120 \cdot 10^2$	$677 \cdot 10^2$	5.6	100
45—75% $(NH_4)_2SO_4$ fraction	$338 \cdot 10$	$643 \cdot 10^2$	19	95
Phosphocellulose chromatography (phosphoenolpyruvate elution)	78	$406 \cdot 10^2$	520	60

TABLE IV

PURIFICATION OF TYPE M_1 PYRUVATE KINASE FROM RAT MUSCLE

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Recovery (%)
Crude extract	$138 \cdot 10^3$	$690 \cdot 10^3$	5	100
55—70% $(NH_4)_2SO_4$ fraction	$607 \cdot 10^2$	$621 \cdot 10^3$	10	90
30—38% Acetone fraction	$907 \cdot 10$	$483 \cdot 10^3$	53	70
55—70% $(NH_4)_2SO_4$ fraction	$650 \cdot 10$	$455 \cdot 10^3$	70	66
Phosphocellulose chromatography (KCl elution)	$146 \cdot 10$	$366 \cdot 10^3$	251	53
Phosphocellulose chromatography (phosphoenolpyruvate elution)	763	$290 \cdot 10^3$	380	42

Types R and L were also crystallized under another condition: when purified enzyme samples dissolved in 50 mM phosphate buffer, pH 6, containing stabilizing agents and 1 mM phosphoenolpyruvate at a protein concentration of about 10 mg per ml, were slowly mixed with solid ammonium sulfate until the solution became slightly turbid, and then stored at 4°C. For a few weeks, types R and L crystallized as hexagonal plates (Fig. 3b). In the absence of phosphoenolpyruvate, type L crystallized as short fine needles.

Type M_2 was crystallized from 50 mM phosphate buffer containing stabilizing agents and 1 mM phosphoenolpyruvate by addition of solid ammonium sulfate, as shown in Fig. 3d. In the absence of phosphoenolpyruvate, type M_2 was also crystallized as very thin needles (Fig. 3c).

Type M_1 was crystallized as small needles from 50 mM phosphate buffer, pH 6, containing 2 mM $MgSO_4$ and 10 mM β-mercaptoethanol by adding solid ammonium sulfate (Fig. 3e). In the presence of phosphoenolpyruvate, type M_1 easily crystallized in large rectangular plates (Fig. 3f). In the presence of Fru-1,6-P_2, it crystallized in similar but longer rectangular plates.

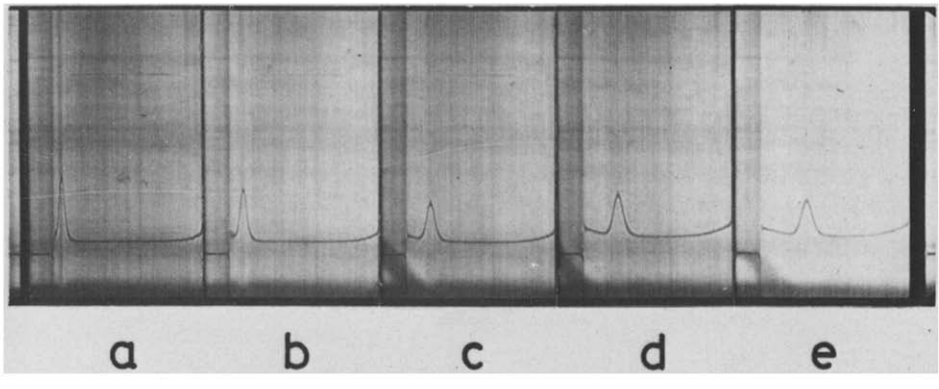

Fig. 4. Sedimentation velocity profiles of type R pyruvate kinase. The ultracentrifugal measurement was carried out with a Beckman-Spinco Ultracentrifuge, Model E. The protein (5 mg/ml) was in 100 mM phosphate buffer, pH 6, containing stabilizing agents and 5% saturation of ammonium sulfate. The speed was 52000 rev./min and the temperature was 25°C. Centrifugation is from left to right: a, 5 min; b, 10 min; c, 20 min; d, 30 min and e, 40 min. The $s_{20,w}$ was 7.8 S.

Ultracentrifugal analyses

As seen in Fig. 4, on sedimentation analysis of purified type R showed a single symmetrical peak with an $s_{20,w}$ value of 7.8 S, indicating that it was in a homogeneous state. The other purified types also showed single symmetrical peaks (data not shown). The $s_{20,w}$ values of types L, M_1 and M_2 were 9.4 S, 9.6 S and 10.8 S, respectively.

Electrophoresis

Purified type L and type R each gave a single band on thin-layer polyacrylamide gel electrophoresis and the mobilities of these bands differed from those of the native enzyme (Fig. 5). Purified type L migrated further to the cathode than the crude enzyme and when a mixture of purified type L and crude type L was subjected to electrophoresis, the band of purified type L separated clearly from that of crude type L. Purified type R gave a single band with nearly the same mobility as that of purified type L, although crude type R gives three bands on electrophoresis. When a mixture of purified type R and crude type R from adult rat was subjected to electrophoresis, purified type R migrated to nearly the same position as R_3. Purified type M_1 and type M_2 migrated to the same positions as the respective crude samples.

On disc gel electrophoresis in the presence of sodium dodecyl sulfate (data not shown), the purified types L, M_1, and M_2 gave single bands. Type R, gave two adjacent bands, but these may reflect heterogeneity in the subunit molecular weight of purified type R rather than the presence of impurity [18].

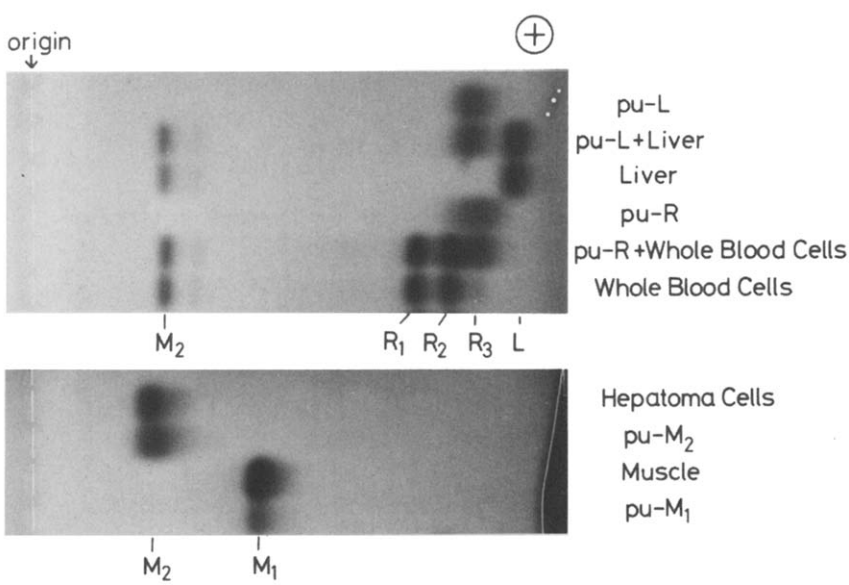

Fig. 5. Electrophoretic patterns of purified samples of the four types of pyruvate kinase. Purified samples were compared electrophoretically with the original crude extracts. Each sample was diluted with stabilizing buffer to an enzyme concentration of about 3 units per ml and subjected to electrophoresis. A mixture of equal volumes of purified and crude enzyme solution was also subjected to electrophoresis. pu-, purified.

TABLE V

AMINO ACID ANALYSES OF PURIFIED ISOZYMES OF PYRUVATE KINASE

The amino acid compositions of four types of pyruvate kinase were analyzed as described in Experimental Procedures. The values for valine and isoleucine are the averages of duplicate determinations after 72 h hydrolysis and those for all other amino acids, except threonine and serine, are the averages of duplicate analyses after 24 and 72 h. Those for threonine and serine were obtained by extrapolation to zero time of the mean of duplicate analyses. Values represent numbers of residues (to the nearest integer) per 60 000 mol. wt. of pyruvate kinase isozymes. The molecular weight was calculated from the subunit sizes of these isozymes determined by sodium dodecyl sulfate gel electrophoresis using Combithek (Boehringer) as marker proteins. Half-cystine and tryptophan were not included in the calculation. Int. 5 shows type M_2 from the intestine.

Amino acid	Type M_1	Type M_2 (Int. 5)	Type L	Type R
Lysine	38	39	23	21
Histidine	13	13	12	10
Arginine	35	35	40	34
Aspartic acid	51	51	43	46
Threonine	25	22	26	29
Serine	29	20	33	35
Glutamic acid	57	56	63	69
Proline	23	25	26	31
Glycine	43	45	46	46
Alanine	62	63	62	59
Valine	47	51	55	53
Methionine	18	16	11	11
Isoleucine	38	43	43	42
Leucine	45	45	46	46
Tyrosine	11	11	9	9
Phenylalanine	16	16	17	17

Amino acid analyses

Table V shows the amino acid contents of the purified isozymes of pyruvate kinase. On the basis of their compositions the isozymes could be divided into two groups, one consisting of types L and R, the other of types M_1 and M_2. The differences between the two groups were quite definite, but the two isozymes in each group were very similar *.

Discussion

Purification of pyruvate kinase by the affinity procedure has been described by several workers. The affinity adsorption method using special immobilized ligands, such as Blue dextran [9,10], Cibacron Blue F3G-A [19], and derivatives of adenine nucleotides [20], has given good results, but in practice this method has various disadvantages: it is difficult to find suitable ligands, preparation of ligand-bound matrix is time consuming and the adsorptive capacity is generally low. Alternatively, the affinity elution method [21], appears much simpler and easier, because it does not require any special adsorbent and already been applied to the purification of pyruvate kinase. Carminatti et al. [22] and Chern et al. [23] adsorbed the enzyme to a CM-cellulose column and

* While manuscript of this paper was in preparation, we learned that Berglund et al. [29] reported the comparison of amino acid compositions between pig kidney and pig muscle pyruvate kinase.

eluted it with Fru-1,6-P_2. Marie et al. [24] eluted human type L with ATP and Fru-1,6-P_2 from a CM-Sephadex column. Schulz et al. [12] reported the selective elution of mouse type M_2 from a phosphocellulose column with various phosphate compounds. However, although these studies demonstrated the value of affinity elution, they mostly did not take full advantage of the method. Indeed, several purification procedures, including ion-exchange and gel chromatography, were introduced before and after the affinity procedure and consequently the enzyme recoveries were poor.

In developing an effective affinity method, the choice of a suitable effector and the optimal pH and ionic strength of the elution medium are the most critical factors. In this work these parameters were determined for each isozyme in preliminary experiments, as shown in Fig. 1. Among the compounds tested as effectors, phosphoenolpyruvate was found to be the most effective [12]. It is also necessary to take conditions for enzyme stabilization into account. Thinking that types L, R, and M_2 were unstable, several workers have added glycerol, sucrose and a low concentration of Fru-1,6-P_2 as stabilizers. Types L, R, M_1 and M_2 are indeed very unstable in the dilute maleate buffer, pH 6, hitherto employed in the purification [1], but in dilute phosphate buffer, pH 6, was found that they were rather stable in the presence of 2 mM Mg^{2+}, 0.2 mM Fru-1,6-P_2 and 10 mM β-mercaptoethanol; Fru-1,6-P_2 being especially necessary for stabilizing types L, R, and M_2.

Large crystals of type M_1 from human muscle or rabbit muscle were easily obtained [25], but the only report on the crystallization of other mammalian isozymes, is our previous paper [1]. Phosphoenolpyruvate was found to promote the crystallization of purified pyruvate kinase isozymes from ammonium sulfate solutions and also to affect the size and form of the crystals. Interestingly, Fru-1,6-P_2 also affected the size and form of crystals of type M_1. This finding supports the existence of an interaction between type M_1 and Fru-1,6-P_2 [12,26], although no definite allosteric change of kinetics of type M_1 by Fru-1,6-P_2 has been demonstrated. Types L and R readily crystallized from dilute medium in the presence of Fru-1,6-P_2 and type L sometimes even crystallized in the fractionation tubes immediately after affinity elution chromatography. This characteristic provided a useful method for the last step in enzyme purification, especially in large scale purification.

Thin-layer polyacrylamide gel electrophoresis clearly showed that purified type L moved more slowly than native enzyme and purified type R moved to nearly the same position as type R_3 of erythrocytes. These results imply that the enzymes may be modified during the purification. The purified enzyme, however, showed the same kinetic behavior as the native one.

The homogeneities of the purified enzymes were shown by ultracentrifugal analysis and disc gel electrophoresis in the presence of sodium dodecyl sulfate. Type R, however, appeared heterogeneous (with an additional slower band) on disc gel electrophoresis [18,27]. Marie et al. [18] concluded that human type R is a heterotetramer, designated as L_2L_2', but further investigations are required on a molecular level.

The amino acid compositions of pyruvate kinase isozymes have been investigated by several workers, but the reported compositions of the isozymes cannot be compared, because the samples were purified from different animal

species. Very recently, however, Harkins et al. [11] purified type M_2 from human kidney and compared its amino acid composition with those of human type M_1 [28] and human type R [23]. They indicated a difference between the amino acid compositions of types M_1 and M_2, and suggested that the two isozymes were the products of separate genes. In this work we analyzed the amino acid compositions of all four types (i.e. types L, R, M_1 and M_2) from rats simultaneously. The data clearly show that these isozymes may be divided into two groups; one consisting of types L and R and the other of types M_1 and M_2. Further investigations such as peptide mapping and amino acid sequence analysis are required to elucidate the structural relationship between the isozymes.

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