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QUARTERLY

Comparison of pyruvate kinase variants from rat liver and Morris hepatoma 7777, obtained by an affinity chromatography on Blue Sepharose CL-6B*

Jan Ignacak and Maria Gumińska

Institute of Medical Biochemistry, N. Copernicus Medical Academy, Jagiellonian University School of Medicine, M. Kopernika 7, 31–034 Kraków, Poland

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Fractions A (salted out by ammonium sulphate between 21 - 30% saturation), and fractions B (salted out between 51 - 70% saturation) of pyruvate kinase (EC 2.7.1.40.) corresponding respectively to pyruvate kinase types L and M2 from rat liver and Morris hepatoma 7777 were purified by an affinity chromatography on Blue Sepharose CL-6B. Peaks of inactive proteins were eliminated and the enzyme fractions bound biospecifically to the gels were eluted by free ADP.

The molecular mass of purified hepatoma pyruvate kinase fraction B was smaller than that of liver pyruvate kinase fraction B. Morris hepatoma pyruvate kinase fraction B represented a variant of type M2, characterised by greatest affinity to 2-phosphoenolpyruvate as a main substrate and different sensitivity to low-molecular effectors in comparison with types L from both liver and hepatoma and in comparison with type M2 from normal rat liver. Only this hepatoma fraction B showed a tumour specific sensitivity to L-cysteine and was insensitive to normal signal molecules i.e. to ATP and fructose-1,6-diphosphate which influence liver pyruvate kinase activity. L-Cysteine inhibited the tumour fraction B of pyruvate kinase by decreasing its V_{max} and increasing the K_m values in relation to 2-phosphoenolpyruvate.

Previous studies showed that L-cysteine stereospecifically inhibits aerobic glycolysis in cytosol of tumour cells, but not of normal tissues [1]. In different tumours this effect was related to the presence of a pyruvate kinase (PK, EC 2.7.1.40.)[1] variant, sensitive to L-cysteine [2, 3].

From the two PK fractions A and B, of Ehrlich ascites tumour and normal mouse liver obtained by ammonium sulphate precipitation, only fraction B corresponding to type M2 and predominating in various tumours, was sensitive to L-cysteine inhibition, but not the fraction M2 from the normal liver [2 - 4].

For further comparative studies of these two PK fractions, both from the neoplastic and non-neoplastic materials, a sufficient amounts of the purified enzyme proteins are required. Therefore the aim of the present study was to obtain a purified PK fractions, sensitive and insensitive to L-cysteine, by the affinity chromatography on Blue Sepharose CL-6B, from the comparable materials: the normal rat liver and Morris hepatoma 7777, transplanted in

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[1]Abbreviations: PK, pyruvate kinase (EC 2.7.1.40.); PEP, 2-phosphoenolpyruvate

Buffalo rats. Blue Sepharose CL-6B, containing an immobilised dye Cibacrone-Blue F3G-A, and capable of binding biospecifically a number of enzymes [5], binds also PK [6] which requires an adenyl-containing substrate, due to the structural similarity of this polymer and adenyl nucleotide [5]. The bound PK separated from other proteins was eluted by free ADP solution [6].

Purified fractions A and B of PK from both materials, used for studies of enzymatic activity in the presence and absence of L-cysteine and other low molecular effectors, were also a subject of polyacrylamide gel electrophoretic studies in the presence of sodium dodecyl sulphate to determine the molecular mass of their subunits [7].

MATERIALS AND METHODS

Materials. Morris hepatoma 7777 was transplanted subcutaneously into Buffalo rats. Solid tumours, about 2 cm in diameter, were collected on the 14th day after transplantation. For comparison the normal livers of healthy Buffalo rats were used.

Chemicals. Blue Sepharose CL-6B was purchased from Pharmacia Fine Chemicals (Sweden), PEP ($\times$ 3Na $\times$ $7H_2O$) from Calbiochem (La Jola, Ca, U.S.A.), lactate dehydrogenase, ATP, ADP and fructose-1,6-diphosphate were obtained from Boehringer (Manheim, Germany), acrylamide and bisacrylamide from Koch-Light Laboratories Ltd (Colnbrook, England), L-cysteine from Serva Feinbiochemica (Heidelberg, Germany).The high molecular mass standard proteins, purchased from Sigma Chemical Company, were: bovine serum albumin, 68 kDa; γ-globulin, heavy chain, 50 kDa; γ-globulin, light chain, 23.5 kDa.

Enzyme assay. Liver and hepatoma pieces were homogenized in a Potter-Elvehjem glass homogenizer with Tris/HCl buffer (20 mM), pH 7.4, containing 115 mM KCl, 10 mM $MgCl_2$ and 2 mM EDTA (homogenization buffer). The homogenates obtained were centrifuged for 10 min at 20000 $\times g$ at 4°C.

Salting-out procedure. Supernatants obtained from rat liver and Morris hepatoma 7777 were fractionated by ammonium sulphate precipitation as described previously [3, 4]. Fractions A were collected between 21 - 30% and fractions B between 51 - 70% of ammonium sulphate saturation. All procedures were carried on at 4°C. Both protein fractions A and B, obtained by the salting-out procedure, were centrifuged and dissolved in the homogenization buffer, dialysed at 4°C overnight against the same buffer to remove ammonium sulphate, and used for the affinity chromatography.

Affinity chromatography. Four columns (2 cm $\times$ 1 cm) filled up with 25% gel of Blue Sepharose CL-6B were equilibrated with the starting buffer (75 mM Tris/HCl, pH 7.4, with 100 mM KCl, 25 mM $MgCl_2$ and 2 mM EDTA). Fractions A from the rat liver and Morris hepatoma 7777 (60 mg and 46 mg of protein, respectively, dissolved in 9.5 ml of the homogenization buffer) and fractions B (21 mg and 43 mg of protein, respectively, dissolved in 4 and 4.4 ml of the buffer) were passed throught the columns. Inactive proteins were eluted already by the first 10 ml of starting buffer, but the columns were subsequently washed with 250 ml of this buffer.

For elution of PK, the starting buffer was supplemented with 10 mM of ADP. Samples of a one-ml volume were collected. In each sample the protein content and the PK activity were determined. For further studies the combined samples showing the PK activity were dialysed and concentrated using an Amicon PM 10 membrane.

PK activity measurements. The PK activity was determined spectrophotometrically at 340 nm, according to Bücher & Pfleiderer [8], as described previously [9], under conditions of a pseudo-zero-order kinetics. In individual samples containing ADP after the affinity chromatography, 2-phosphoenolpyruvate (PEP) was added to the reaction mixture to start enzymatic measurements. The results were expressed in IU/mg protein. The K_m or $S_{0.5}$ values in relation to PEP were obtained from Lineweaver-Burk's or Hill's plots. The effects of L-cysteine, ATP and fructose-1,6-diphosphate were given as a percentage of control activity without effectors.

Protein assay. The protein content was determined according to Lowry *et al.* [10].

Molecular mass determination by sodium dodecyl sulphate gel electrophoresis. The gels were obtained by polymerization of 10% acry-

lamide with 1% bisacrylamide and 0.1% sodium dodecyl sulphate in 0.375 M Tris/HCl buffer (pH 8.8), under reducing conditions according to Laemmli [7]. Electrophoresis was carried out in tubes with a current of 3 mA per gel for 3 h. Protein was stained with Coomassie Blue [7].

RESULTS AND DISCUSSION

Table 1 presents the results of determination of PK activity and the protein content during successive steps of enzyme fractionation. The rat liver and Morris hepatoma 7777 homogenates contained comparable amounts of starting proteins. However, the specific activity of PK in tumour cytosol was four times higher than that in the normal rat liver, which is in agreement with the results of previous studies [2]. After the salting-out procedure with ammonium sulphate, in both materials studied as described previously [4], two PK fractions were obtained: fraction A (21 - 30% saturation) prevailed in normal rat liver and fraction B (51 - 70% saturation) prevailed in Morris hepatoma 7777.

Fraction A from the normal rat liver, corresponding to the PK type L [11, 12], represented approx. 70% of the cytosol activity. During purification on Cibacron-Blue, 99.6% of ballast proteins were eliminated and PK specific activity increased 250 times. Sodium dodecyl sulphate electrophoresis has shown that the molecular mass of fraction A subunit was 50 kDa (Fig. 1). It was slightly smaller than that determined by other authors for rabbit PK monomere of type L (52 kDa) [11, 13, 14].

Fraction A from rat hepatoma, represented 37% of total cytosol activity. During purifica-

Table 1

Purification of pyruvate kinase from the rat liver and Morris hepatoma 7777

Fresh tissue weights: rat liver - 5.5 g; Morris hepatoma 7777 - 5.3 g

Purification steps	Ammonium sulphate saturation (%)	Protein (total) (mg)	PK activity (total) (IU)	PK activity (spec.) (IU/mg of protein)	Purification grade	Yield (%)
Rat liver						
Homogenate	–	438	354	0.8	1.0	100.0
Cytosol	–	250	212	0.9	1.0	60.0
Crude fractions						
A	21 - 30	60	168	2.8	3.5	47.5
B	51 - 70	22	36	1.8	2.3	10.3
Purified fractions						
A	21 - 30	0.23	46	201	251.0	13.1
B	51 - 70	0.31	14	46	58.0	4.0
Morris hepatoma 7777						
Homogenate	–	479	1216	2.5	1.0	100.0
Cytosol	–	268	856	3.2	1.3	70.4
Crude fractions						
A	21 - 30	46	302	6.6	2.6	24.9
B	51 - 70	43	451	10.5	4.2	37.1
Purified fractions						
A	21 - 30	0.41	121	295	118.0	10.0
B	51 - 70	0.39	297	762	305.0	24.5

tion on Cibacron-Blue, 99.1% of protein was eliminated, so PK specific activity increased 118 times. Fraction A (type L) of hepatoma did not differ significantly from liver fraction A. Molecular mass of its subunit (52 kDa) (Fig. 1) was higher than in rat liver and was the same (52 kDa), as it is known for PK type L from other studies [13, 15].

Fraction B from the normal rat liver, corresponding to the type M2 [15, 16], represented approx. 38% of the cytosol activity. During purification on Cibacron-Blue, 98.5% of ballast protein was eliminated and PK specific activity increased 58 times. As it was shown by sodium dodecyl sulphate electrophoresis the molecular mass of fraction B subunit was 54 kDa (Fig. 1). It corresponded to the molecular mass of liver type M2 of the monomere (54 kDa) [11, 13].

Fraction B from rat hepatoma, represented 61.4% of the total cytosol activity. During purification on Cibacron-Blue, approx. 99.1% of ballast protein was eliminated and PK specific activity increased 305 times. Its molecular mass was much lower (49 kDa for subunit) (Fig. 1) in comparison to the molecular mass of normal liver PK fraction B (54 kDa) [11, 13].

After the affinity chromatography, the ratios of the absorbed to the eluted proteins were: 1:260 and 1:110 for fractions A of the liver and hepatoma, respectively, and 1:70 and 1:110, for fractions B of the same materials. The affinity chromatography is a fast method which yields purified enzymes with rather a moderate loss of their activity. Although PK belongs to a group of enzymes which lose their activity during purification [17], it was possible to obtain purified fractions in sufficient amounts for further studies.

In purified normal rat liver PK fraction, specific activity of type L (A) predominated (201 IU/mg of protein) over type M2 (B) (46 IU/mg of protein) (Table I). In purified rat hepatoma PK fractions specific activity of type L (A) was almost similar as in normal rat liver (295 IU/mg of protein), but a significant increase in a specific activity of the type M2 (B) was noted (762 IU/mg of protein) which pointed to its much greater turnover number.

The increase in type M2 during development of hepatoma was observed by many authors in cytosol [12, 16 - 18]. However, in addition to the quantitative increase in the tumour PK fraction B, a qualitative changes also appear.

The significant sigmoidal kinetics in relation to PEP were found only for the liver PK both fractions A and B, and hepatoma PK fraction A (Fig. 2). Only fraction B of PK from hepatoma showed a hyperbolic, i.e. linear kinetics in a double-reciprocal plot (Fig. 2).

The $S_{0.5}$ values for PEP in fractions A of liver and hepatoma PK were respectively 2.10 mM and 0.75 mM. They were in the range between 0.4 - 2.5 mM [15,18,19] found by various authors for type L of PK, but higher than for PK fraction A from the Ehrlich ascites tumour (0.45 mM) [4]. The $S_{0.5}$ or K_m values for fractions B (type M2) of liver and hepatoma PK were lower (1.40 and 0.55 mM) than for type L (fractions A), and were higher than the range of 0.2 - 0.8 mM found by other authors [13, 15, 20, 21] for type M2 PK. The lowest K_m value 0.55 mM for type M2 PK of hepatoma pointed to greater enzyme affinity to PEP as the main substrate in comparison with fraction A of hepatoma and both fractions A and B of liver PK. However, this K_m was still much higher than K_m value to PEP for fraction B from Ehrlich ascites tumour (0.19 mM) [4]. Probably elution of enzyme from Cibacron-Blue F3GA columns by ADP, which already saturates enzyme with this substrate,

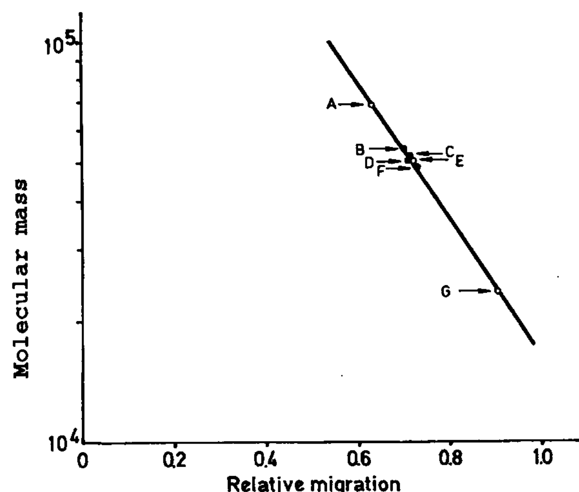

Fig. 1. Molecular mass determination by polyacrylamide gel electrophoresis.
Molecular mass markers (open circles): A, bovine serum albumin (68 kDa); E, γ-globulin heavy chain (50 kDa); G, γ-globulin light chain (23.5 kDa). Pyruvate kinases (closed circles): B, fraction B from rat liver (54 kDa); C, fraction A from Morris hepatoma (52 kDa); D, fraction A from rat liver (50 kDa); F, fraction B from Morris hepatoma (49 kDa)

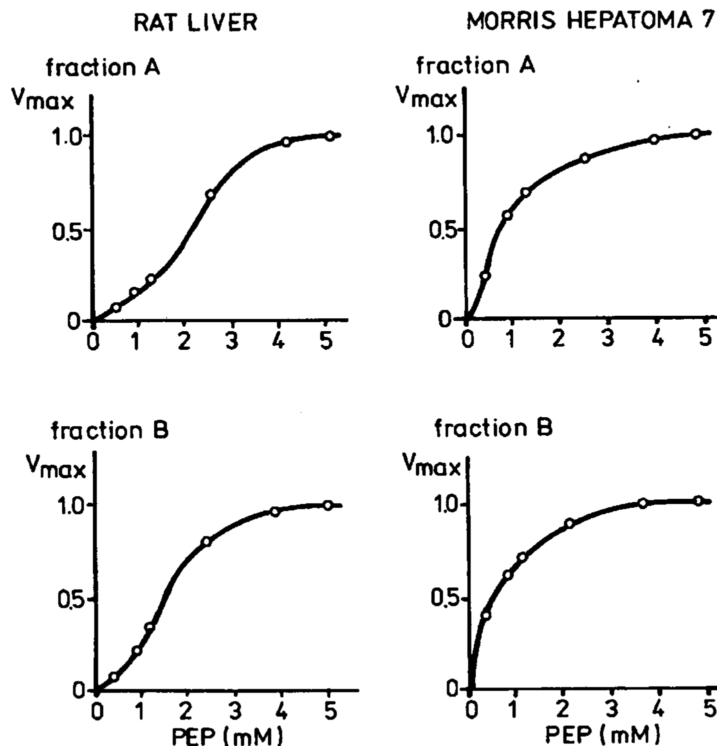

Fig. 2. Michaelis-Menten plots for rat liver and Morris hepatoma 7777 PK in relation to PEP.
Fraction A from rat liver (ammonium sulphate 21 - 30%) V_{max} = 201 IU/mg of protein, $S_{0.5}$ = 2.10 mM; fraction B from rat liver (ammonium sulphate 51 - 70%) V_{max} = 46 IU/mg of protein, $S_{0.5}$ = 1.40 mM; fraction A from Morris hepatoma 7777 (ammonium sulphate 21 - 30%) V_{max} = 295 IU/mg of protein, $S_{0.5}$ = 0.75 mM; fraction B from Morris hepatoma 7777 (ammonium sulphate 51 - 70%) V_{max} = 762 IU/mg of protein, K_m = 0.55 mM

and using PEP instead of ADP as a starting molecule make the results not completely comparable with those obtained in normal procedure.

The fraction B may contribute to elevated overall glycolytic rate in tumours. Thus the high rate of glycolysis in tumour might result not only from the increased activity concentration of PK as one of the key enzymes [22], but also from its enhanced affinity to PEP.

The sensitivity of all fractions obtained, were compared in relation to the commonly used signal molecules. M2 is usually regulated by the same effectors as L-PK [11, 23]. ATP as a negative effector of PK in feedback mechanism inhibits glycolytic energy formation [24], and fructose-1,6-diphosphate [25] in positive forward control, synchronizes PK activity with phosphofructokinase. The lack of sensitivity to fructose-1,6-diphosphate or ATP of tumour PK confirms the suggestion that tumour PK variant differs from M2-PK. The lack of sensitivity to ATP is in agreement with the previous observations of Gosalvez *et al.* [26], since tumour PK, remains very active even at low fructose-1,6-diphosphate and high ATP concentrations.

This points to a marked metabolic role of PK variant in altered tumour metabolism, i.e., in diminution in the Pasteur effect and in appearance of the Crabtree effect [21, 26, 27].

Previous studies pointed to a special role of L-cysteine [2]. In the present study, similarly as in crude PK fractions from mouse Ehrlich ascites tumour [4], only the fraction B from the Morris hepatoma 7777 was sensitive to L-cysteine (Fig. 3), while the fraction B from rat liver PK was insensitive to this amino acid. In the presence of 0.1 mM L-cysteine specific activity of PK fraction B decreased to 53 ± 11% of the control PK specific activity. The K_i calculated according to the Dixon plot at two different PEP concentrations was determined as 1.30 mM of L-cysteine (Fig. 4). L-Cysteine decreased the V_{max} and increased the K_m value (PEP) from 0.55 to 1.30 mM in hepatoma fraction B of PK, pointing to a mixed type of inhibition.

It has been found that the PK pattern of hepatoma is different from that of control normal

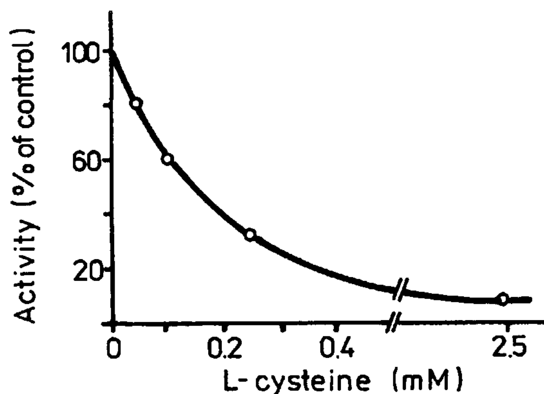

Fig. 3. Sensitivity of PK fraction B from Morris hepatoma 7777 to various concentrations of L-cysteine.
The results are expressed as a percentage of control activity without L-cysteine

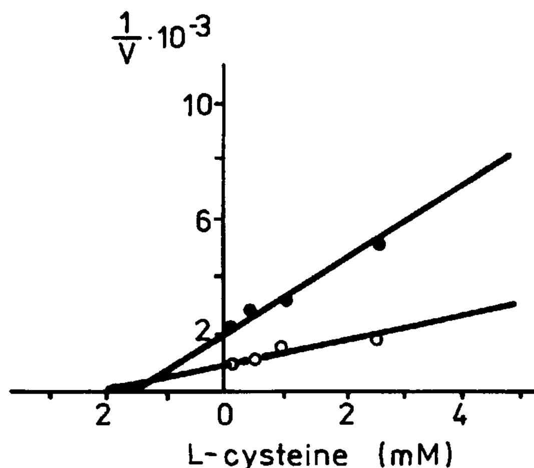

Fig. 4. The Dixon plot at two different PEP concentrations (○, 5 mM PEP; ●, 1.25 mM PEP) for fraction B of PK from Morris hepatoma 7777 (v = IU/mg of protein)

liver [28, 29] and becomes much more similar to PK pattern of Ehrlich ascites tumour. However, the metabolic role of this new L-cysteine-sensitive variant in tumours is not known.

In previous studies it has been found [30] that the sensitivity to L-cysteine may be used as a marker of multistage neoplastic transformation, since L-cysteine sensitive variant appeared in grade III cell lines with tumourigenic properties in nude mice [30].

The L, M and M2 types of PK are considered as products of three different genes [31]. They can be separated in polyacrylamide gel (the L-type as the fastest and M2-type as the slowest towards anode). The appearance of new sensitivity to L-cysteine points to a change in the primary structure of M2-type of PK and thus in cell genotype during the process of carcinogenesis [2]. Therefore the hepatoma fraction B in spite of some similarities with type M2 cannot be identified with the type M2, as had been reported in some earlier works [13, 15], neither it can be identified with the type M found in normal skeletal muscles [12]. The appearance of this fraction in the tumour materials supports the concept of altered enzymatic strategy in rapidly growing cells [32, 33], which require an increased ATP formation.

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