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Communication

Amino-acid composition of pyruvate kinase M_2 isoenzyme variants from rat liver and Morris hepatoma 7777[o]

Jan Ignacak[1✉], Maria Gumińska[1*] and Janusz Steczko[2☆]

[1]*Department of General Biochemistry, Institute of Medical Biochemistry, Collegium Medicum, Jagiellonian University, Kraków, Poland,*
[2]*Institute of Catalysis and Surface Chemistry, Polish Academy of Sciences, Kraków, Poland*

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Cytosolic fractions B (salted out between 51–70% ammonium sulphate saturation) from rat liver and Morris hepatoma 7777, containing pyruvate kinase (EC 2.7.1.40) M_2 isoenzymes, were purified by affinity chromatography on Blue Sepharose CL-6B. When compared by polyacrylamide gel electrophoresis at pH 8.3, all three M_2 pyruvate kinase variants from Morris hepatoma 7777 had lower mobilities ($\alpha_2, \beta_2, \gamma_3$) than the three corresponding variants ($\alpha_1, \beta_1, \gamma_2$) from normal rat liver.

Using an automatic amino-acid analyser, significant differences in selected amino-acid content have been found in corresponding highly purified γ_3 and γ_2 variants from Morris hepatoma and normal rat liver, respectively. The γ_3-variant of the Morris hepatoma M_2 isoenzyme had twice the amount of L-tyrosine and L-cysteine, and a content of L-serine higher by 20% than the corresponding γ_2 variant of the normal rat liver M_2 isoenzyme. It contained, however, significantly less dicarboxylic amino acids which explains its lower electrophoretic mobility. It showed also a decrease (by about 10%) in several other amino-acid content, corresponding to a 10% decrease in the tumour enzyme molecular mass.

Among three pyruvate kinase isoenzymes — L, M_1, M_2 — the most interesting are the M_2 isoenzymes. Their variants can be separated by polyacrylamide gel electrophoresis [1, 2, 3].

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[✉]Corresponding author: J. Ignacak, Institute of Medical Biochemistry, Collegium Medicum, Jagiellonian University, Kopernika 7, 31-034 Cracow, Poland; tel. (48 12) 422 7400; fax: (48 12) 422 3272.

[☆]Present address: Hemo Cleanse, Inc., 2700 Kent Avenue, West Lafayette, Indiana 47906, U.S.A.

It was suggested that interconvertible forms may be products of binding electrically charged low molecular effectors such as FDP [4] or even ATP [5]. They can be also caused by serine or tyrosine phosphorylation by a protein kinase [6–10] especially since the tumour M_2 isoenzyme contains more phosphate [11]. Also the possibility of a different degree of protein sialization cannot be excluded [11]. However, in the case of the tumour γ_3 variant changes in amino-acid content were expected not only because of its sensitivity to L-cysteine [2, 3, 12] during the multi-stage process of neoplastic transformation [3], but also because of an increase in the number of chemically assayed thiol groups [11]. In the normal rat liver pyruvate kinase M_2 isoenzyme, insensitive to exogenous L-cysteine, this amino-acid occurred in much smaller amounts [11].

Because of a greater electrophoretic mobility of M_2 isoenzyme pyruvate kinase variants from normal rat liver (α_1, β_1, γ_2) than from Morris hepatoma 7777 (α_2, β_2, γ_3) [2] which are more cathodic, differences in amino-acid content in comparable variants were expected.

In the present study results of amino acid estimation in comparable variants of pyruvate kinase from normal rat liver (α_1, β_1, γ_2) and Morris hepatoma 7777 (α_2, β_2, γ_3) are presented.

MATERIALS AND METHODS

Materials. Morris hepatoma 7777 obtained thanks to the courtesy of Professor dr A. Koj (Institute of Molecular Biology, Jagiellonian University) was transplanted subcutaneously in Buffalo rats. Solid tumours about 2 cm in diameter were collected on the 14th day after transplantation. For comparison normal livers of Buffalo rats were used.

Enzyme separation, purification and molecular mass estimation. Liver and hepatoma pieces were homogenised at 4°C in a Potter-Elvehjem glass homogenizer with 20 mM Tris/HCl buffer, pH 7.4, containing 115 mM KCl, 10 mM $MgCl_2$ and 2 mM EDTA. The homogenates were centrifuged for 10 min at 20000 $\times g$ at 4°C. The supernatants were fractionated by ammonium sulphate precipitation as described previously [13, 14]. Fractions B of pyruvate kinase M_2 isoenzyme were precipitated between 51–70% of ammonium sulphate saturation. Both protein fractions B, from the supernatants of rat liver and Morris hepatoma, obtained by the salting-out procedure were centrifuged, dissolved in the homogenization buffer, and dialysed overnight at 4°C against the same buffer to remove ammonium sulphate. For enzyme purification by affinity chromatography on Blue Sepharose CL-6B (Pharmacia Fine Chemicals, Uppsala, Sweden) [15] 25% gel was used. The elution pattern was described before [14]. Combined samples showing pyruvate kinase activity were dialysed and concentrated using an Amicon PM 10 membrane.

Molecular masses of pyruvate kinase isoenzymes were determined as described previously [14] by sodium dodecyl sulfate gel electrophoresis [16].

Disc electrophoresis and pyruvate kinase activity measurement. Purified fractions were used for disc electrophoresis. Vertical electrophoresis was performed in 7.5% polyacrylamide gel (acrylamide and bisacrylamide from Koch-Light Laboratories Ltd., Colnbrook, England) according to Davis [17] in Tris/glycine buffer, pH 8.3, μ = 0.45. Electrophoresis was carried out for 2 h at 4°C with a current of 3 mA per gel tube 7 mm wide. Gels were treated in parallel. For the localisation of protein zones, one gel from each pair was stained with 0.5% Amido Black 10 B (Merck, Darmstadt, Germany) in 7% acetic acid and destained with 7% acetic acid [17].

For enzymatic studies the remaining gels were cut into slices approx. 1.5 mm thick. Each slice was transferred to a separate test tube and extracted for 24 h at 4°C with

Tris/HCl buffer 75 mM, pH 7.4, containing 100 mM KCl, 25 mM $MgCl_2$ and 2 mM EDTA for determination of pyruvate kinase activity.

Pyruvate kinase activity was determined spectrophotometrically at 340 nm according to Bücher & Pfleiderer [18] as described previously [19].

Amino acid estimation. Variants from normal rat liver and Morris hepatoma 7777, collected after polyacrylamide gel electrophoresis, were hydrolysed with 6 M HCl containing 0.05% (v/v) 2-mercaptoethanol (Merck, Darmstadt, Germany) in vacuum, for 24 h, 48 h and 72 h, 100°C [20-22]. Amino-acid content was estimated using DURRUM D-500 automatic amino-acid analyser. The results were expressed as the number of amino-acid residues per enzyme subunit molecule.

Protein determination. Protein was determined according to Lowry *et al.* [23].

RESULTS

The electrophoretic patterns of pyruvate kinase M_2 isoenzyme variants separated comparatively from normal rat liver and Morris hepatoma 7777 are presented in Fig. 1. All three variants ($\alpha_2, \beta_2, \gamma_3$) from Morris hepatoma had lower mobility than the corresponding variants ($\alpha_1, \beta_1, \gamma_2$) from normal rat liver [2].

The results of comparative amino acid estimations are presented in Table 1.

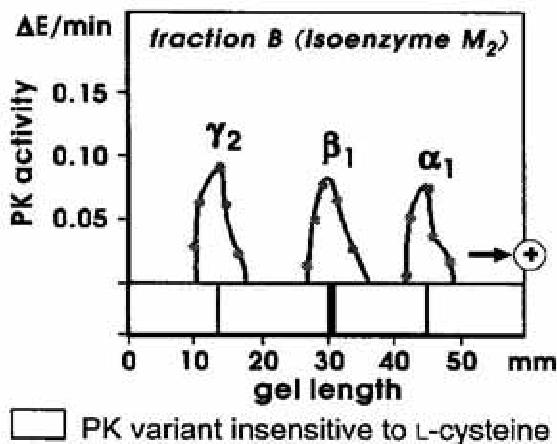
Only three amino acids were found in the same amounts in each of the three variants of normal liver and tumour fractions of M_2 isoenzymes, namely, L-methionine, L-phenylalanine and L-histidine. L-Isoleucine, L-threonine, L-proline and L-arginine occurred in slightly lower (by about 10%) amounts than in normal liver supernatants corresponding to 1-3 residues per one subunit of the enzyme from the neoplastic material. A considerable decrease in the amount of L-alanine, L-glycine, L-valine and L-leucine was observed, especially in variant α_2 of the neoplastic fraction B. It should be noted that L-glutamic and L-aspartic acids occurring in significantly decreased amounts may lower protein mobility. The sum of these acidic amino acids was much higher in the more mobile variants of M_2 pyruvate kinase from normal rat liver than in the corresponding variants of the tumour M_2 isoenzyme. It was reduced in normal α_1, β_1 and γ_2 variants from 97, through 93 to 90 residues per mol of subunit, while in the slower migrating α_2, β_2 and γ_3 variants of the Morris hepatoma M_2 isoenzyme, it was reduced from 92 through 79 to 74 residues per mol of subunit, respectively. Thus, the amount of acidic amino acids in the α_2 tumour variant was lower by five, in β_2 by twelve and in the γ_3 variant by sixteen residues, respectively, in comparison to variants of the normal liver isoenzyme. Therefore, the γ_3 variant was the most alkaline, and at pH 8.3 it remained on the starting position.

L-Tyrosine and L-cysteine content was twice as high in the γ_3 variant of the hepatoma M_2 isoenzyme as in γ_2 from normal liver. The L-serine content was increased by about 20%. Although all tumour variants differed from normal variants in amino-acid content, the most significant changes were noted in the γ_3 variant of M_2 pyruvate kinase isoenzyme, which may be regarded as the tumour specific variant.

DISCUSSION

The content of amino acid in pyruvate kinase has been determined by other investigators [24, 25], but owing to a genetically different material, different level of purity and different assay technique, these results are not comparable with ours.

The results of amino acid assay of particular variants of pyruvate kinase M_2 isoenzymes from closely comparable materials of rat liver and Morris hepatoma 7777 indicate that the decrease in electrophoretic mobility of purified variants of tumour pyruvate kinase may



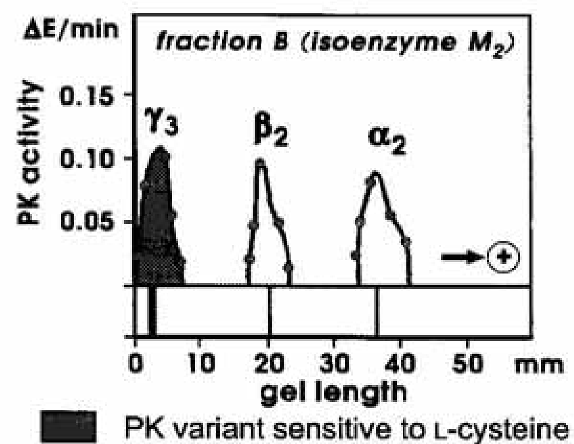


Figure 1. Electrophoretic pattern of purified pyruvate kinase from cytosolic fraction B of normal rat liver and Morris hepatoma 7777.

Polyacrylamide gel electrophoresis was performed in Tris/glycine buffer (pH 8.3). Plots of ΔE/min *vs.* gel length in mm. Hatched area – isoenzyme sensitive to L-cysteine.

be caused not only by a lower amount of sialic acid [11], but also by a lower content of dicarboxylic amino acids. The sum of L-glutamic and L-aspartic acids in the most mobile variants of the normal liver M_2 isoenzyme was 97 (in α_1), 93 (in β_1), and 90 (in γ_2), while in the slower migrating variants (α_2, β_2 and γ_3) from Morris hepatoma, these sums were significantly lower, and amounted to 92, 81 and 74 residues per protein subunit, respectively. An especially significant difference was found in the tumour γ_3 variant which remained in the gel at the starting position. Although the method used was not able to differentiate between free dicarboxylic amino acids and their amides, the regularity observed does not seem to be limited in a considerable way.

The slowest γ_3 variant from Morris hepatoma showed also a two-fold higher content of L-cysteine, which may account for its sensitivity to exogenous L-cysteine [1–3]. This result is in agreement with the results of a chemical assay of thiol groups [11]. It should be noted, however, that the increased L-cysteine content is only one factor increasing the tumour enzyme variant affinity to thiol groups, because the inhibitory effect of L-cysteine on its activity is stereospecifically connected with L-cysteine only and not with D-cysteine [12]. Thus it requires a stereospecific allosteric site in the protein molecule.

In contrast to the γ_2 variant, variant γ_3 also contains over two-fold higher amount of L-tyrosine, and an increased amount of L-serine, the amino acids which may undergo phosphorylation. Phosphorylation of pyruvate kinase isoenzyme M_2 by serine kinase [6–8,10] as well as by tyrosine kinase [9], independent on cAMP, was recently described. It was suggested that reversible phosphorylation may regulate pyruvate kinase activity [26].

It should be also noted that the sum of remaining amino-acid residues is lower by about 10% in variants of tumour pyruvate kinase, suggesting that their polypeptide chains are shorter by about 10%. This conclusion is supported by the fact that subunit molecular mass of the tumour enzyme (49 kDa) is lower by about 10% than that of the normal liver M_2 isoenzyme (54 kDa) [14].

In addition to shortening of the chain and minor (up to 10%) changes in amino-acid con-

Table 1. Number of amino-acid residues per protein subunit of electrophoretic variants of pyruvate kinase M_2 isoenzymes from normal rat liver and Morris hepatoma 7777.

Amino acid	Rat liver			Morris hepatoma 7777			Differences in M_2 isoenzyme of Morris hepatoma		
	α_1	β_1	γ_2	α_2	β_2	γ_3	α_2	β_2	γ_3
							no changes		
Met	2	2	2	2	2	2	0	0	0
Phe	16	16	16	16	16	16	0	0	0
His	8	8	8	8	8	8	0	0	0
							a small decrease		
Ile	17	14	17	15	13	14	-2	-1	-3
Arg	17	15	17	14	15	14	-3	0	†3
Thr	29	28	29	26	26	27	-3	-2	-2
Pro	22	25	21	22	19	20	0	-6	-1
							distinct decrease		
Gly	42	34	40	33	31	37	-9	-3	-3
Ala	60	62	59	45	50	50	-14	-12	-9
Val	33	31	32	27	27	28	-6	-4	-4
Leu	33	28	34	27	26	29	-6	-2	-5
Lys	22	21	23	23	19	18	+1	-2	-5
Asp+Asn	45	43	41	41	38	38	-4	-5	-3
Glu+Gln	52	50	49	51	41	36	-1	-7	-13
							an increase		
Ser	40	58	35	44	61	42	+4	+3	+7
Tyr	4	11	6	3	7	14	-1	-4	+8
Cys	11	12	7	11	13	14	0	+1	+7
Total	453	458	436	409	414	407	-44	-39	-29
% of changes							-10	-10	-10
Subunit molecular mass 54 kDa					49 kDa				

tent, the most important are changes exceeding 10%, proving the formation of M_2 isoenzyme mutants – most alkaline in γ_3 variant, with the lowest amount of dicarboxylic amino acids, and with the highest content of L-tyrosine, L-cysteine and L-serine, and in variant α_2, with the lowest content of L-glycine and L-alanine. These changes are probably a result of multistep DNA mutations and oncogen activation [27]. They change regulatory properties of the tumour enzyme which participates in the altered strategy of neoplastic cell metabolism.

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