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QUARTERLY

N-Acetylneuraminic acid, phosphate and thiol groups of pyruvate kinase isoenzymes from Morris hepatoma 7777 and normal rat liver

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The highest amount of *N*-acetylneuraminic acid (AcNeu) was found in pyruvate kinase isoenzyme L from normal rat liver (24 moles/mole of enzyme tetramer), with the highest electrophoretic mobility. On the other hand, isoenzyme M_2 from Morris hepatoma 7777, with the lowest electrophoretic mobility, had the lowest AcNeu content (5 moles/mole of enzyme tetramer). This tumour isoenzyme M_2 of pyruvate kinase was, however, characterised by the highest phosphate content (12 moles/mole protein), in comparison to isoenzyme L (3 moles/mole protein) or normal liver isoenzyme M_2 (6 moles/mole protein). This could indicate a regulatory change caused by reversible enzyme phosphorylation and dephosphorylation or sialization and desialization. Despite these differences, the sum of the two negatively charged residues was lower in tumour pyruvate kinase isoenzyme M_2, with the slowest migration rate, than in normal rat liver isoenzyme M_2.

Moreover, isoenzyme M_2 from tumour material, in comparison with isoenzyme M_2 from normal rat liver, had a twice as high content of thiol groups (20 moles/mole protein), especially of free and superficially located ones, than the isoenzyme M_2 from normal liver (10 moles/mole protein). This may explain abnormal susceptibility of tumour isoenzyme M_2 to stereospecific inhibition by exogenous L-cysteine, and indicate genetically dependent changes in amino-acid content of tumour enzyme which take place during cell tumourigenic transformation.

Pyruvate kinase (EC 2.7.1.40) is a key glycolytic enzyme with regulatory properties, directly involved in ATP formation [1]. Two isoenzymes of pyruvate kinase — type L (fraction A) and M_2 (fraction B) [2] found in normal rat liver and Morris hepatoma 7777 [3, 4] differ significantly from each other. In the previous study [3] it was found that over-expressed tumour pyruvate kinase M_2 isoenzyme (fraction B) from cytosol of Morris hepatoma 7777 is more alkaline than the liver M_2 isoenzyme. It shows at pH 8.3 three isoenzyme variants: α_2, β_2, γ_3 with slower electrophoretic mobility than that of the corresponding pyruvate kinase fraction B from normal rat liver (variants: α_1, β_1, γ_2). This

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Abbreviation: AcNeu, *N*-acetylneuraminic acid

observation suggests a significant diminution in acidic group content, influencing the hepatoma pyruvate kinase negative charge. Tumour isoenzyme M_2 of pyruvate kinase loses its sensitivity to ATP as a negative effector [5] and acquires a stereospecific sensitivity to inhibition by L-cysteine [3–7]. This sensitivity, seen in tumour pyruvate kinase only, may indicate increased thiol group content in the protein regulatory centre, capable of exogenous L-cysteine binding.

Pyruvate kinase fractions A (isoenzyme L) and B (isoenzyme M_2) from cytosol of both normal rat liver and Morris hepatoma 7777, obtained by salting out procedure and purified by affinity chromatography [4], represent a closely comparable and highly purified experimental material. Prior to comparative studies of dicarboxylic amino-acid content in pyruvate kinase isoenzymes, both from normal rat liver and Morris hepatoma 7777, the amounts of *N*-acetylneuraminic acid (AcNeu) and phosphate were determined, as they may influence the electric charge of isoenzyme molecules; the number of thiol groups, variations of which may explain differences in enzyme sensitivity to L-cysteine, was also determined.

MATERIAL AND METHODS

Material

Morris hepatoma 7777, obtained by courtesy of Professor 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 and purification

The liver and hepatoma pieces were homogenised at 4°C in a Potter-Elvehjem glass homogenizer in Tris/HCl buffer (20 mM) pH 7.4, containing KCl (115 mM), $MgCl_2$ (10 mM) and EDTA (2 mM) (buffer for homogenisation). The homogenates were centrifuged for 10 min at 20000 × *g* at 4°C. The supernatants were fractionated by ammonium sulfate precipitation. Pyruvate kinase fractions A (isoenzyme L) were collected between 21–30%, and B (isoenzyme M_2) between 51–70% of ammonium sulfate saturation as described previously [4, 8].

For enzyme purification affinity column chromatography was used [4]. The proteins bound to Blue Sepharose CL-6B (Pharmacia Fine Chemicals, Uppsala, Sweden) were subsequently washed with 250 ml of starting buffer: Tris/HCl, pH 7.4 (75 mM) containing 100 mM KCl, 25 mM $MgCl_2$ and 2 mM EDTA. For pyruvate kinase elution the starting buffer was supplemented with 10 mM ADP (Boehringer, Mannheim, Germany). Combined samples showing pyruvate kinase activity [9, 10] were dialysed and concentrated using Amicon PM 10 membrane. Highly purified isoenzymes were used for enzyme estimation and chemical analysis.

Methods

N-Acetylneuraminic acid (AcNeu). For hydrolysis of pyruvate kinase proteins (fractions A and B, from both materials) the samples were heated for 10 min with 5% trichloroacetic acid in a boiling water bath. AcNeu content was determined by the 2-thiobarbiturate Warren's method [11]. The coloured product obtained was extracted with cyclohexanone. Absorbancy was measured spectrophotometrically at 549 nm. As a standard the AcNeu (Koch-Light Laboratories Ltd. Colnbrook, England) solution in 5% trichloroacetic acid without prior hydrolysis was used.

Inorganic phosphate. For mineralization, pyruvate kinase protein samples were heated on a sand bath with 5 M sulfuric acid (p.a.). A few droplets of 30% hydrogen peroxide were used for sample decolorisation. After dilution and adjustment of samples to pH 4.0, inorganic phosphate was determined according to Lowry-Lopez's method, using ascorbate as a reducing compound [12].

Thiol groups. The number of thiol groups was determined at 495 nm from diminishing mercuric acetate fluorescence (FMA) following the reaction with protein [13]. The total numebr of thiol groups was measured after

prior hydrolysis of disulfide bridges with 1 M NaOH. Superficially arranged thiol groups were determined with 5,5-dithio-bis-(2-nitrobenzoic) acid (DTNB) according to Ellman's method [14], modified by Robyt *et al.* [15]. As a standard reduced glutathione was used [16, 17].

L-Cysteine content was compared in fractions A and B of the two studied enzymes, highly purified by affinity chromatography on Blue Sepharose CL-6B.

All results were expressed in moles per mole of pyruvate kinase protein. Molecular masses of pyruvate kinase isoenzymes were determined previously [4]. Protein content was determined according to Lowry *et al.* [18].

RESULTS AND DISCUSSION

Characterisation of the fractions obtained, presented in the previous works [3, 4], is collected in Table 1. Different electrophoretic mobility of pyruvate kinase isoenzyme molecules may be a result of different amino-acid content with lower or higher pI values, or may be a result of phenotypic modification of proteins by their phosphorylation or dephosphorylation and sialization or desialization. Such changes, which can also modify the enzyme activity, may occur under the effect of various protein kinases or protein phosphatases. Fractions A with higher electrophoretic mobility corresponded to isoenzyme L of pyruvate kinase, and fractions B with lower mobility represented isoenzyme M_2. Isoenzymes M_2 from normal liver and rat hepatoma differ in their properties, among them in electrophoretic mobility. Tumour M_2 isoenzyme containing three "interconvertible" variants $\alpha_2, \beta_2, \gamma_3$, is more alkaline than the corresponding liver M_2 isoenzyme [3]. Using highly purified, comparable material it was possible to answer the question whether the changes observed were connected with phenotypic modification of protein molecules or could be a result of genetical alterations.

N-Acetylneuraminic acid and phosphate in pyruvate kinase isoenzymes

The greatest numebr of *N*-acetylneuraminic acid (AcNeu) molecules (approximately 24 moles per mole of pyruvate kinase protein) was found in fraction A from normal rat liver, with the greatest electrophoretic mobility (Table 2). This AcNeu content was almost by 50% higher than in pyruvate kinase fraction A from Morris hepatoma and fraction B from normal liver (about 14–15 moles). The lowest amount of AcNeu (about 5 moles) was found in pyruvate kinase fraction B of hepatoma with the lowest electric charge and lowest electrophoretic mobility. These differences in protein electric charge

Table 1. Characterisation of pyruvate kinase isoenzymes from rat liver and Morris hepatoma 7777, used in chemical studies [3, 4]

Pyruvate kinase (isoenzyme)	Activity* IU/mg protein	Purification grade	Electrophoretic variants [1]	Subunit molecular mass** (kDa)
Rat liver				
fraction A (L)	201	251	$\alpha_1, \beta_1, \gamma_1$	50
fraction B (M_2)	46	58	$\alpha_1, \beta_1, \gamma_2$	54
Morris hepatoma 7777				
fraction A (L)	295	118	$\alpha_1, \beta_1, \gamma_2$	52
fraction B (M_2)	762	305	$\alpha_2, \beta_2, \gamma_3$	49

*Measured spectrophotometricaly according to Bücher & Pfleiderer [9] in kinetic modification [10].

**Measured by SDS/polyacrylamide gel electrophoresis of Laemmli [19] under reducing conditions [4].

could explain the smallest electrophoretic mobility of tumour isoenzyme variants at pH 8.3 [3].

Unlike the AcNeu content, the highest amount of phosphate (12 moles) was found in pyruvate kinase fractions B from hepatoma (Table 2). It was twice as high as in fraction B from normal rat liver (6 moles) and four times as high as in fractions A, both from normal rat liver and Morris hepatoma (3 moles).

Despite these differences, the sum of AcNeu and phosphate residues with negative electric charge was the highest in the pyruvate kinase fraction A from normal rat liver (27 moles), intermediate in fraction A of hepatoma (18 moles) and fraction B of normal rat liver (20 moles), and the lowest in the pyruvate kinase fraction B from rat hepatoma (17 moles). This could explain their different electrophoretic mobility [3].

phatase [27]. In addition to L isoenzyme phosphorylation, the chicken liver pyruvate kinase isoenzyme M_2 is also phosphorylated at serine residue but in a cAMP-independent reaction [22, 25]. Moreover, phosphorylation at tyrosine residues was observed in chicken liver cells transformed by the Rous sarcoma virus, containing *src*-oncogene, coding for phosphoprotein $pp60^{v\text{-}src}$ associated with protein kinase activity [22, 25]. Cell transformation causes a tenfold increase in total cell phosphotyrosine content, connected with phosphorylation of several glycolytic enzymes and an elevated rate of aerobic glycolysis. Although tyrosine phosphorylation can be considered as a post-translational regulatory change, it requires a genotypic change connected with an increase in tyrosine content.

Reversible phosphorylation of isoenzyme L of pyruvate kinase co-operates with glycogen

Table 2. *N*-Acetylneuraminic acid (AcNeu) and phosphate residues bound with pyruvate kinase fractions A and B from normal rat liver and Morris hepatoma 7777

Estimated compound	Pyruvate kinase fractions (ammonium sulfate saturation)		Rat liver (moles/mole protein)		Morris hepatoma 7777 (moles/mole protein)	
			$\bar{X}$	± SD	$\bar{X}$	± SD
AcNeu° (n = 18)	A (L)	(21–30%)	24	2.7	15*	3.8
	B (M_2)	(51–70%)	14	1.3	5**	1.1
Phosphate°° (n = 9)	A (L)	(21–30%)	3	0.14	3	0.34
	B (M_2)	(51–70%)	6	1.58	12***	1.03

°Estimated by Warren's method [11].

°°Determined by Lowry-Lopez method [12]; after mineralization of proteins.

Significance of differences between corresponding fractions of rat liver and Morris hepatoma 7777: * $0.05 < P < 0.1$; ** $P < 0.001$; *** $0.001 < P < 0.01$

However, no considerable differences were noticed in the activities of the purified variants of the same isoenzymes [3].

In other papers [20–26], it was suggested that phosphorylation of pyruvate kinase under the influence of an appropriate protein kinase may have a regulatory effect and may be associated with the transformation of the active form of pyruvate kinase into the inactive one [20]. Liver pyruvate kinase isoenzyme L (rat, pig) is phosphorylated by cAMP-dependent protein kinase at the serine residue [27] with inhibition of enzyme activity [28], and can be reactivated by protein phos-

breakdown only in liver. However, the situation is different in hepatomas. They do not accumulate glycogen, and have no glucose-6-phosphatase. Therefore only in normal liver, the phosphorylation of isoenzyme L of pyruvate kinase can be used in the mechanism of metabolic control. Isoenzyme M_2 seems not to be associated with the inhibition of aerobic glycolysis. Pyruvate kinase fraction B from hepatoma, containing the highest number of phosphate residues, had the greatest activity and affinity to 2-phosphoenolpyruvate [3]. Nevertheless, the problem requires further investigation. Recently differences in amino-

-acid content have been also found (Ignacak, Gumińska & Steczko, unpublished observations). The comparison of corresponding highly purified variants of normal liver and hepatoma has shown that tumour γ_3 variant with the slowest mobility contains 10% less aspartic acid and 26% less glutamic acid than the normal γ_2 variant.

Sulfhydryl and disulfide groups of pyruvate kinase

The effect of exclusive inhibition of tumour pyruvate kinase fraction B by L-cysteine suggests that this fraction contains the highest quantity of thiol groups capable of exogenous L-cysteine binding.

Pyruvate kinase fraction A (isoenzyme L) from normal rat liver showed higher concentration of the sum of sulfhydryl and disulfide groups measured by the fluorescence method in the presence of 1 M NaOH than did pyruvate kinase fraction B (isoenzyme M_2) from the same material (Table 3). Pyruvate kinase fraction A of hepatoma contained approximately equal amounts of the sum of sulfhydryl and disulfide groups, as fraction B of tumour enzyme in which a double amount of thiols was found (20 moles/mole protein) as compared with fraction B from normal rat liver (10 moles/mole protein).

Using the same fluorescence method, but in Tris/HCl buffer, pH 8.0, pyruvate kinase fraction A from normal rat liver showed twice as many free thiol groups (14 moles/mole protein) than pyruvate kinase fraction B from the same material (7 moles/mole protein) (Table 3). The results corresponded to the total sulfur content. It was found that in pyruvate kinase fraction A isolated from Morris hepatoma 7777 the content of thiol groups (15 moles/mole protein) was similar as in pyruvate kinase fraction A from normal liver, and slightly higher than in fraction B. The number of thiol groups in tumour pyruvate kinase fraction B was, however, almost twice as high (12 moles/mole protein) as in the corresponding pyruvate kinase fraction B from normal liver (7 moles/mole protein), which may explain greater affinity of this tumour enzyme to exogenous L-cysteine.

Determination of the superficially accessible thiol groups according to Ellman [14] (Table 3) revealed that pyruvate kinase fraction A from normal liver contained over twice as many of these groups (10 moles/mole pro-

Table 3. The sum of sulfhydryl and disulfide groups estimated by fluorescence method [13]. The number of thiol groups superficially located determined by Ellman's method [14] in pyruvate kinase fractions A and B from normal rat liver and Morris hepatoma 7777, expressed in moles per mole protein (tetramer)

Fractions (isoenzyme)	Pyruvate kinase (ammonium sulfate saturation)	n	Rat liver		Morris hepatoma 7777	
			$\bar{X}$	± SD	$\bar{X}$	± SD
			Sum of –SH and –S–S– groups (moles / mole protein)			
A (L)	(21–30%)	4	17	0.3	19	0.6
B (M_2)	(51–70%)	4	10	0.1	20*	0.9
			Thiol groups (moles / mole protein)			
A (L)	(21–30%)	4	14	0.2	15	0.3
B (M_2)	(51–70%)	4	7	0.1	12*	0.5
			Superficially located –SH groups (moles /mole protein)			
A (L)	(21–30%)	6	10	2.3	4**	1.7
B (M_2)	(51–70%)	6	4	1.8	9***	4.1

Significance of differences between corresponding fractions of rat liver and Morris hepatoma 7777: * $P < 0.001$; ** $0.001 < P < 0.01$; *** $0.01 < P < 0.02$

tein) as in pyruvate kinase fraction B (4 moles/mole protein). On the contrary, in the case of hepatoma enzyme, a greater number of externally accessible thiol groups was found in pyruvate kinase fraction B (9 moles/mole protein) than in pyruvate kinase fraction A (4 moles/mole protein). The number of thiol groups in pyruvate kinase fraction A from Morris hepatoma 7777 was half that in pyruvate kinase fraction A from normal rat liver and fraction B from hepatoma but the same as in pyruvate kinase fraction B from normal liver. Sensitivity of fraction B from Morris hepatoma to exogenous L-cysteine may be a consequence of twice as great content of superficial sulfhydryl groups. Amino-acid analysis of pyruvate kinase fraction B from hepatoma, demonstrated also twice as high L-cysteine content as in pyruvate kinase fraction B from normal liver (Ignacak, Gumińska & Steczko, unpublished observations).

When considering the capability of interaction of a greater number of superficial thiol groups with an exogenous sulfhydryl-containing compound, it should be stressed, however, that the tumour isoenzyme inhibition by L-cysteine is stereospecific [29]. D-Cysteine, as well as other thiol group reactive compounds (homocysteine, glutathione) which all are theoretically able to react with free thiol groups [29], are inactive in enzyme inhibition. Therefore, it may be concluded that, for inhibition of tumour pyruvate kinase fraction B, not only the thiol groups are necessary but the whole steric structure of L-cysteine or any other compound from which cysteine can be restored (3-mercaptopyruvate, penicillamine, *N*-acetylcysteine) [29]. Undoubtedly, the crucial role in this abnormal inhibitory effect is fulfilled by specific folding of the protein chain with L-cysteine residues enabling stereospecific binding of exogenous L-cysteine.

The amount of AcNeu as well as phosphate may differ in various proteins depending on isolation conditions and phenotypic changes during post-translational modification of enzyme protein. On the other hand, changes in the number of thiol groups indicate genetically dependent changes in primary protein structure. This was especially evident on comparing pyruvate kinase fractions B from normal liver and Morris hepatoma, indicating that, despite similar behaviour during the salting out procedure, these fractions cannot be identical not only with respect to their activity and electrical charge [3] but also with respect to L-cysteine content. These fractions differed also in exogenous L-cysteine, which acted as an inhibitor of the tumour pyruvate kinase fraction B only [8]. Thus, in addition to phenotypic modification of proteins by glycosylation, and phosphorylation, tumour enzymes which play an essential role in tumour metabolism show also genotypic changes in amino-acid content.

Much attention is focused nowadays on proteins appearing during neoplastic transformation of cells. Oncogens, such as e.g. from the *ras* family [30], lead to formation of altered oncoproteins. Therefore pyruvate kinase fraction B from hepatoma, especially its slowest tumour specific γ_3 variant sensitive to L-cysteine, should be regarded as one of the oncoprotein products. According to Weber [31, 32], some of newly formed enzymatic proteins modify the metabolic strategy of a transformed cell in such a way as to ensure supply of substrates and energy in tumours for uncontrolled cell divisions.

Pyruvate kinase variant γ_3, from fraction B of hepatoma seems to play such a role. It loses sensitivity to ATP [3, 5, 8]; in consequence glycolysis can be active in aerobic conditions, and Pasteur effect disappears or is significantly limited. The investigations of Gosalvez *et al.* [33] showed that in such cases not ATP but L-cysteine can restore the Pasteur effect; however, the role of this signal has not been fully elucidated. It should be stressed that tumour pyruvate kinase variant γ_3 differing in its properties from variant γ_1 from normal liver, resembles more closely the enzyme variant from other types of neoplastic cells, such as Ehrlich ascites tumour [5] or human bladder tumourigenic cell lines at the third stage of transformation [34], than the enzyme variant from comparable material of normal origin.

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