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DIFFERENCE IN PHYSICAL AND CHEMICAL PROPERTIES BETWEEN MUSCLE-TYPE AND SPLEEN-TYPE ISOENZYMES OF PYRUVATE KINASE IN RAT[1)]

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SUMMARY: Muscle(M)-type and spleen(S)-type isoenzymes of pyruvate kinase were prepared to a homogeneous purity from skeletal muscles and the tumor (Rhodamine sarcoma) of rats, respectively. The respective types were composed of four of the identical subunit of approximately 60,000 daltons. Their amino acid compositions and peptide maps resembled each other, although there were some definite differences. The total numbers of dicarboxylic and basic amino acids were higher and lower with M-type than with S-type, respectively, in accordance with the difference in isoelectric point. Of the ninhydrin-positive spots on the peptide maps of their tryptic digests, 61 spots were common for both types, whereas 4 spots were specific to M-type and 5 spots to S-type.

The isoenzymes of pyruvate kinase in various mammalians have been extensively studied by many workers (1-23). We previously reported that those present in various tissues of rats, if freed of fructose 1,6-diphosphate (FDP), can be classified into the three types, liver(L)-type, muscle(M)-type and spleen(S)-type, that the spleens and Rhodamine sarcoma are abundant in the S-type isoenzyme, and that the S-type isoenzyme is increased in the livers when the animals have borne the tumor or when they have been injected with the chromatin prepared from either the spleens or the tumor (23). Imamura et al. (10) and Cardenas et al. (22) suggested on the basis of immunological studies and studies on amino acid composition, respectively, that the syntheses of the L-type and M-type isoenzymes are governed by different genes.

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The present report deals with studies on the molecular weights, isoelectric points (pI) and amino acid compositions of the M-type and S-type isoenzymes of pyruvate kinase in rats, and on the peptide maps of their tryptic digests.

MATERIALS AND METHODS

Adult, male albino rats of the Donryu strain (150-200 g) were used. Rhodamine sarcoma was transplanted on the back of the animals as described previously (24).

The S-type isoenzyme of pyruvate kinase was purified from Rhodamine sarcoma as reported previously (1). Its M-type isoenzyme was purified from skeletal muscles of rats as follows: Frozen muscles (1 kg) were homogenized with 2 liters of 10 mM Tris-HCl (pH 7.5) containing 10 mM 2-mercaptoethanol, 5 mM $MgCl_2$ and 1 mM ethylenediamine tetraacetate (Tris-MME buffer) by a Waring blender (model CB-2-10, Eberbach Corp., Michigan) at the medium speed for 2 min, followed by centrifugation at 14,000 x g for 20 min. The resultant supernatant (2 liters) ("extract") was subjected to fractionation by ammonium sulfate. The precipitate between 50% and 70% in saturation was collected, dissolved in Tris-MME buffer and dialyzed against the same buffer ("50-70% ammonium sulfate"). The dialyzed solution was heated at 60°C for 30 min, followed by centrifugation. The resultant supernatant ("heat-treatment") was passed through a DEAE-cellulose column (2.6 x 28 cm) equilibrated with Tris-MME buffer. The fraction that had not been adsorbed ("DEAE-cellulose non-adsorbed") was dialyzed against 5 mM potassium citrate buffer (pH 5.5) and applied on a CM-cellulose column (6.8 x 33 cm) equilibrated with the same buffer. The charged column was developed with a linear gradient of KCl from 0.1 M to 0.4 M in 4 liters of 5 mM the buffer. The fractions of the resultant eluate showing specific activities higher than 240 U/A_{280} were mixed. The mixture (1.4 liter) was concentrated to approximately 200 ml by a Diaflow filtration apparatus (model 402 with PM30 filter membrane, Amicon Corp., Lexington), followed by dialysis against 70 mM Tris-HCl (pH 7.5) containing 25% (v/v) glycerol and 10 mM 2-mercaptoethanol (Tris-GM buffer) ("CM-cellulose eluate"). The dialyzed solution was applied on a P-cellulose column (6.8 x 30 cm) equilibrated with Tris-GM buffer. The charged column was developed with the same buffer containing 10 mM ATP by an affinity chromatography described previously (25). The fractions of the resultant eluate showing the enzyme activity were mixed and concentrated to approximately 50 ml as described above ("P-cellulose eluate"). The concentrated solution was equally divided into aliquots, which contained approximately 10,000 units of pyruvate kinase in each. The resultant aliquots were separately subjected to isoelectric electrophoresis with 2% Ampholine carrier-ampholytes (pH 3.5-10) in 110-ml electrofocusing columns. Of the fractions thus obtained, those showing specific activities higher than 480 U/A_{280} were mixed ("isoelectric electrophoresis"), followed by dialysis against an excess of distilled water for 3 days in a cold room. The dialyzed solution was lyophilized. The activity of pyruvate kinase was measured by the method as described previously (1).

The lyophilized samples of the M-type and S-type isoenzymes were respectively hydrolyzed at 110°C for 24 h, 48 h and 72 h in 6 N HCl in glass tubes sealed under vacuum. The hydrolyzed samples were analyzed by an amino acid analyzer (model JLC-5AH, Japan Electron Optics Lab., Tokyo) according to the method of Moore and Stein (26). The lyophilized samples of the M-type and S-type isoenzymes (10 mg each) were respectively reduced and then S-carboxymethylated by the method of Crestfield et al. (27). With the resultant samples, their small parts were subjected to estimation of the completion of hydrolysis and determination of the content of half-cystine. To most of their remaining parts (8 mg each), 1.6 ml of 0.2 M ammonium bicarbonate buffer (pH 8.5) and 80 μg of trypsin were added, followed by incubating at 37°C. After 6

h, 40 μg of the proteinase was newly added, followed by further incubation for 6 h. The resultant solutions were lyophilized, dissolved in 10 μl of 20% (v/v) pyridine, and centrifuged. Appropriately small volumes of the resultant supernatants, which contained approximately 800 μg peptides/μl, were then subjected to a descending chromatography on cellulose thin layer plates (Merck 5716 plate, 20 x 20 cm) in n-butanol/pyridine/acetic acid/water (15:10:3:12, v/v) at 24°C for 6 h. The plates were dried at 50-60°C for 15 min, allowed to stand at room temperature for 1 h and then moistened with pyridine/acetic acid/water (1:10:89, v/v; pH 3.5). With the resultant plates, the second dimensional electrophoresis was carried out at 700 V and at 4°C for 85 min. The plates thus obtained were dried at 60°C for 15 min, sprayed with 0.2% ninhydrin in acetone, heated at 100°C for 10 min, and then photographed.

Sodium dodecylsulfate(SDS)-polyacrylamide gel electrophoresis was carried out in 10% gel disc (5 x 90 mm) by the method of Weber et al.(28).

Molecular-sieve chromatography was carried on a Sephadex G-200 column (1.5 x 90 mm) by the method of Andrews (29).

[^{14}C]FDP was obtained from the Radiochemical Centre, Amersham, trypsin that had been treated with L-(1-tosylamide-2-phenyl)ethyl chloromethyl ketone from Worthington Biochemical Corp., Freehold, New Jersey, and Ampholine carrier-ampholyte (pI 3-10) from LKB Produkter AB., Stockholm-Bromma.

RESULTS AND DISCUSSION

Purities and molecular weights of M-type and S-type isoenzymes of pyruvate kinase. The purification of the M-type is summarized in Table I. In SDS-polyacrylamide gel electrophoresis, the respective preparations of the M-type and the S-type migrated, forming a single protein zone at a rate corresponding to approximately 60,000 daltons (see also ref. 1). With the mixture of these two isoenzymes, the zones were not separable, migrating at practically the same rate. In molecular-sieve chromatography on a Sephadex G-200 column, the M-type was eluted, forming an activity peak centered at a fraction of approximately 240,000 daltons. With mixtures of these two isoenzymes, their activity peaks were not separable when the activities were measured in the presence and absence of FDP, provided that the concentration of the S-type was appropriately high (see below). With the M-type, the location of the activity peak was not changed either by changing the enzyme concentration or by the presence and absence of FDP. It was previously reported that in the presence of FDP, the S-type has a molecular weight of approximately 240,000 (tetramer), regardless of the enzyme concentrations, whereas in the absence of the substrate the tetramer is dissociated into dimers of 120,000 daltons and then into monomers of 60,000 daltons as the enzyme concentrations are lowered (1).

FDP-binding properties and pI values of M-type and S-type isoenzymes of pyruvate kinase. When the M-type was incubated at 24°C for 30 min with 1 mM

Table I. Summary for purification of muscle-type isoenzyme of pyruvate kinase from skeletal muscles of rats

Steps	Total activity (units)	Total protein (A_{280nm})	Specific activity (units/A_{280nm})	Purification fold	Yield (%)
Extract	217,000	45,000	4.82	(1.00)	(100)
50-70% ammonium sulfate	187,000	13,000	14.4	2.99	86
Heat-treatment	159,000	2,190	72.6	15.1	73
DEAE-cellulose non-adsorbed	96,000	1,020	94.1	19.5	44
CM-cellulose eluate	88,400	231	383	79.5	41
P-cellulose eluate*	61,000	124	491	102	28
Isoelectric electrophoresis	42,600	92	493	102	20

* A_{280nm} was measured after dialysis.

[^{14}C]FDP (1 Ci/mol) and subjected to isoelectric electrophoresis, the activity and the protein formed peaks at pI 7.4, whereas all the radioactivity migrated into fractions close to the anode, different from the case with the S-type. It was previously found that in isoelectric electrophoresis, the S-type at the tetramer shows three different pI values; pI 7.8 at the FDP-free form, pI 6.6 at the form binding 2 mol FDP/tetramer and pI 6.2 at the form binding 4 mol FDP/tetramer (1). It was found that both isoenzymes were completely dissociated into monomers in the presence of 1% Nonidet P40 and 2 M urea, losing their activities. In isoelectric electrophoresis in the presence of those reagents, the pI values of the monomers were approximately 7.4 with the M-type and approximately 7.8 with the S-type.

Amino acid compositions of M-type and S-type isoenzymes of pyruvate kinase. The amino acid compositions of the M-type and the S-type are shown in Table II, in which the numbers of amino acid residues were estimated on the basis of a molecular weight of the subunits of 590,000 with both isoenzymes. The data with the M-type are well consistent with those with rabbit muscle (14) and human muscle (16). The amino acid compositions of the M-type and the S-type were significantly similar to each other. The total numbers of basic amino acid residues (aspartate and glutamate) and those of dicarboxylic amino acid residues (lysine, histidine and arginine) are 102 and 80 with the M-type, respectively, and 100 and 83 with the S-type, respectively. This accords with

Table II. Amino acid compositions of M-type and S-type isoenzymes of pyruvate kinate

Amino acids	M-type/S-type (molar ratio relative to phenylalanine)				M-type/S-type (mol per 59,000 g protein)[b]
	24 h	48 h	72 h	Corrected[a]	
Aspartate	3.33/3.26	3.34/3.30	3.35/3.22	3.34/3.26	50/49
Threonine	1.53/1.60	1.30/1.63	1.29/1.52	1.67/1.64	25/25
Serine	1.49/1.56	0.99/1.45	0.96/1.23	1.75/1.76	26/26
Glutamate	3.40/3.36	3.44/3.42	3.52/3.35	3.45/3.38	52/51
Proline	1.50/1.57	1.50/1.60	1.54/1.53	1.51/1.57	23/24
Glycine	2.70/2.82	2.75/2.74	2.70/2.74	2.72/2.77	41/42
Alanine	4.02/3.98	4.06/3.90	4.11/3.91	4.06/3.93	61/59
Half-cystine[c]	0.64/0.67				10/10
Valine	3.05/2.96	3.09/3.02	3.01/3.28	3.01/3.28	45/49
Methionine	1.20/1.11	1.22/1.03	1.20/1.01	1.21/1.05	18/16
Isoleucine	2.30/2.44	2.31/2.44	2.26/2.47	2.26/2.47	34/37
Leucine	2.85/2.68	2.89/2.68	2.82/2.67	2.85/2.68	43/40
Tyrosine	0.69/0.67	0.70/0.69	0.69/0.67	0.70/0.68	10/10
Phenylalanine	1.00/1.00	1.00/1.00	1.00/1.00	1.00/1.00	15/15
Tryptophan[d]					5/5
Lysine	2.43/2.52	2.49/2.58	2.44/2.60	2.45/2.57	37/39
Histidine	0.80/0.75	0.81/0.79	0.78/0.77	0.80/0.77	12/12
Arginine	2.06/2.03	2.09/2.14	2.07/2.13	2.07/2.10	31/32

[a]The values for threonine and serine were obtained by extrapolation to zero time of hydrolysis. For valine and isoleucine, the values at 72 h were taken. For other amino acids, average values were taken.
[b]The number of phenylalanine was taken as 15 residues.
[c]Determined as S-carboxymethylcysteine.
[d]Determined spectrophotometrically by the method of Bencze and Schmid (30).

the facts that at the FDP-free form, both isoenzymes were slightly alkaline, the pI of the M-type lower than that of the S-type.

Peptide maps of M-type and S-type isoenzymes of pyruvate kinase. The preparations of the M-type and the S-type were analyzed by peptide mapping of their tryptic digests (Fig. 1). The numbers of spots detectable were 65 with the M-type and 66 with the S-type, which were slightly lower than the total numbers of the lysine and arginine residues; 68 with the M-type and 71 with the S-type. Together with the findings on the molecular weights and the pI values of the subunits, these facts indicate that both isoenzymes were respectively composed of identical subunits. Of the spots in the peptide maps, 61 spots were common for both isoenzymes; 4 spots were specific to the M-type and 5 spots to the S-type.

TABLE 2

The phenotype and rRNA gene number of Y-NO and X-NO bb mutants

GENOTYPE	PHENOTYPE	NUMBER rRNA GENES PER NO
C(1)DX/$B^{s}Y$ $bb^{1}y^{+}$	extreme bb adult	120
C(1)DX/$B^{s}Y$ $bb^{2}y^{+}$	extreme bb adult	109
C(1)DX/$B^{s}Y$ $bb^{1-3}y^{+}$	early-late pupal lethal	63
C(1)DX/$B^{s}Y$ $bb^{1-4}y^{+}$	egg-early larvae lethal	80
C(1)DX/$B^{s}Y$ $bb^{1-5}y^{+}$	late pupal lethal	97
bb^{2rl}/0	egg-larvae lethal	46*
bb^{8}/0	extreme bb adult	101*

* The bb^{2rl} and bb^{8} gene numbers measured under non-compensating conditions (6,7)

tion (6,11). Thus when determining rDNA content of a particular sex chromosome, one must place this chromosome opposite a non-compensating or stable sex chromosome. For measuring the gene number of particular Y chromosomes, one can use an $\overline{XY}$ or C (1) R M chromosome in which both are refractory to compensation (Table 1). The rRNA gene numbers of the five mutant Y-NO chromosomes was measured by placing these chromosomes opposite both the $\overline{XY}$ and C (1) R M chromosomes. The rDNA content for each of the five Y-NO mutants was virtually the same whether measured in $\overline{XY}$ males or C (1) R M females. The two values were averaged to give 120 genes for the bb^{1} allele, 109 for bb^{2}, 63 for bb^{1-3}, 80 for bb^{1-4} and 97 for bb^{1-5} (Table 2).

Lethality tests of the Y-NO mutants:

The bobbed phenotype can range from an egg lethal (12) to a mild bobbed adult fly depending upon the number of rRNA genes present in the genome. The classifications used in this study were: (1) mild bobbed as adult flies with no abdominal etching, thin bristles (2) extreme bobbed as adult flies with severe abdominal etching, short, thin bristles and (3) bobbed lethal as the absence of adult flies, the lethal phase occurring sometime during the egg, larval, and pupal stages. The bb^{1} and bb^{2} alleles have 120 and 109 genes, respectively and their phenotypes are both extreme bobbed (Table 2). Thus there is enough rRNA genes in these Y-NO mutants for the organism to reach the adult stage. The bb^{1-3} allele (63 genes)

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