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THE SUBUNIT STRUCTURE OF RAT LIVER PYRUVATE KINASE

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SUMMARY

The amino acid composition for rat liver pyruvate kinase is reported. Thin layer peptide mapping of the tryptic digests yields 44 ninhydrin-reactive peptides, which is one-quarter the total number of lysyl and arginyl residues. No amino-terminal residue has been detected using the dansyl chloride procedure. Acid urea disc gel electrophoresis of the protein subunits yields only one protein band; yet, isoelectric focusing of the subunits in urea yields two protein bands. These results suggest that pyruvate kinase (L-type isozyme) consists of four subunits of similar primary structure, but with sufficient microheterogeniety to be able to resolve two types of subunits upon isoelectric focusing.

The L-type isozyme of pyruvate kinase (EC 2.7.1.40) found in rat liver is an allosteric enzyme whose specific activity is decreased during fasting and increased upon refeeding a high carbohydrate, low protein diet (1,2,3,4). Our recent investigations have been directed toward the elucidation of the *in vivo* modifications of liver pyruvate kinase that bring about this change in specific activity. Our approach has been to characterize and compare homogeneous preparations of the enzyme from rats in various dietary states. In this manuscript the amino acid composition and subunit structure of the liver pyruvate kinase isolated from rats fed a high carbohydrate, low protein diet are reported.

MATERIALS AND METHODS

Rat liver pyruvate kinase was purified from mature male rats fasted for 2 days then fed a high carbohydrate diet for 4 days. The high carbohydrate diet was 85% sucrose, 10% casein, 4% Rogers and Harper inorganic salt mix, and 1% B vitamin complex dry mix (all but sucrose from Tekland Mills, Madison, Wisconsin). The enzyme was purified by a slightly modified version (3) of the Ljungström, *et al* procedure (5). Electrophoresis of the isolated L-type pyruvate kinase yielded a single protein band in both 7% polyacrylamide gels at pH 9.5 and 5.6% polyacrylamide gels in the presence of 0.1 gm % sodium dodecyl sulfate (3).

Pyruvate kinase activity was assayed at 30° by the spectrophotometric procedure of Bücher and Pfleiderer (6) as modified by Tietz and Ochoa (7). The standard reaction mixture employed contained 1 millimolar fructose-1,6-bisphosphate (3). Protein concentrations were determined by the method of Lowry, *et al* (8) with bovine serum albumin as a standard. Total sugar was estimated by the phenol-sulfuric acid reaction of Dubois, *et al* (9).

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Amino acid analysis was carried out according to the procedure described by Moore and Stein (10). Purified enzyme was dialyzed against deionized water, lyophilized, and then hydrolyzed under nitrogen atmosphere in 6.1 N HCl for 24, 48, and 72 hours at 100°. The protein concentration was 1 mg/ml. The HCl was removed on a rotary evaporator and the dried hydrolyzate was dissolved in citrate buffer, pH 2.2, and stored at -20°. The amino acid analysis was carried out with a Durrum Rapid Sequence Analyzer and an Amino "Aminalyzer". Tryptophan and tryosine were determined spectrophotometrically by the method of Edelhoch (11). Disulfide content was determined by the method of Cavallini, et al (12). The cysteine content in the native and sodium dodecyl sulfate treated protein were each determined by titrating the protein with 5,5'dithiobis-(2-nitrobenzoic acid) as described by Torchinskii (13).

The general techniques for tryptic digestion and thin layer peptide mapping were described by Tarui, et al (14). Rat liver pyruvate kinase was dialyzed versus deionized water and denatured by heating for 5 minutes in boiling water. The denatured protein was then suspended in 0.2 M ammonium bicarbonate, pH 8.5, so that the final protein concentration was 10 mg/ml. Trypsin as a 2% (w/v) solution in 1 mM HCl was added so that the final ratio of liver pyruvate kinase to trypsin (w/w) was 50:1. The solution was incubated for 1 hour at 37°; then, additional trypsin (half the previous amount) was added, and the incubation was carried out for an additional 2 hours. At the end of the 3 hour incubation, the solution was essentially clear. The solution was centrifuged and the supernatant fraction was subjected to electrophoresis. Approximately 150 μg of peptides were applied to a thin layer chromatography plate coated with cellulose (MN 300 Brinkman Instrument, New York). The plate was sprayed with a buffer mixture of pyridine:glacial acetic acid:water (100:30:3000, pH 5.5). Electrophoresis was carried out in the same buffer at 5° for 2 1/2 hours at 300 volts on a DESAGA-Brinkman Thin Layer Electrophoresis apparatus. The plate was dried at room temperature overnight, equilibrated for 2 hours with 1-butanol:pyridine:glacial acetic acid:water (150:100:30:120), and developed in this solvent system for 10 hours at room temperature. The chromatographic plate was again dried overnight and the peptide spots developed by spraying the plate with 0.2% ninhydrin in 95% ethanol and heating it for 15-30 min at 110°.

Performic acid oxidized (15) L-type pyruvate kinase and bovine serum albumin were dissolved in 1% sodium dodecyl sulfate (w/v) and reacted for 2 hours with fresh dansyl chloride (25 mg/ml) via the procedure of Gray (16). The labeled proteins were hydrolyzed in 6.1 N HCl for 18 hours at 103°. After hydrolysis, the HCl was removed and the hydrolyzates extracted with water-saturated ethyl acetate, dried, and redissolved in 50% pyridine (v/v). The labeled amino acids were identified using the two-dimensional procedure of Arnott and Ward (17) which involves ascending chromatography on cellulose thin layer plates in 0.4% pyridine, 0.8% acetic acid, pH 4.4 and high voltage electrophoresis in the same buffer. In addition, the hydrolyzates were chromatographed on polyamide sheets in water:88% formic acid (200:3) and ethyl acetate:methanol:acetic acid (20:1:1) (16). Dansyl chloride and various dansyl amino acid standards were purchased from Sigma Chemical Company.

Disc gel electorphoresis in 7.5 M urea and 6 mM Triton X-100 was carried out as described by Borun et al (18). In addition to the 12% acrylamide and 0.08% bisacrylamide gels described, disc gels containing 7.5% acrylamide and 0.05% bisacrylamide were prepared and run. The gels were then placed in numbered, screw-top, perforated plastic tubes, and stained while being stirred for 2 hours at 60° with 0.4% Amido black in 50% methanol-10% acetic acid. Destaining was done at room temperature by stirring overnight in 50% methanol-10% acetic acid or electrophoretically with the Cannalco Quick Gel Destainer using 7% acetic acid.

Isoelectric focusing gels were made and run according to the procedure of O'Farrell (19). The final concentration of reagents in the gels were as follows: 9 M urea, 2% Nonidet P-40, 3.8% acrylamide, 0.22% bisacrylamide, and 2% Ampholines, pH 3.5-10. Upon completion of the run, the gels were placed into 50% methanol-10% acetic acid for 2 hours. The protein bands were then visualized by fluorescent staining in 0.003% 8-anilino-1-naphthalene-sulfonic acid magnesium salt in 0.1 M sodium phosphate buffer at pH 7.0 (20). For determination of the pH gradient, an unfixed gel was sliced into 5 mm sections. Each section was equilibrated overnight at 4° in 0.5 ml of distilled water before measuring the pH values.

RESULTS AND DISCUSSION

Liver pyruvate kinase is comprised of four subunits that are inseparable by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (3,21) or by sedimentation equilibrium in guanidine hydrochloride (22). Based on this data, it has been suggested that the enzyme consists of very similar, if not identical, monomeric units. Such data indicates a similarity in the mass of the enzymatic subunits, but does not address the question of identity in the primary structure of the subunits. As dissimilarity is often easier to illustrate than identity, our approach to the question of subunit identity has been to employ a variety of techniques known to separate closely related molecules and observe whether or not any differences among the subunits could be demonstrated.

Amino Acid and Carbohydrate Composition

The amino acid composition of purified rat liver pyruvate kinase is reported in Table I. The values represent, except for serine and threonine, the average values for 24, 48, and 72 hours hydrolyses. The values for serine and threonine are extrapolated from the three experimental values to zero hydrolysis time. The yield of amino acids from the protein hydrolysis was high, as evidenced by the mass of the amino acid residues accounting for more than 90% of the mass of the protein. Tryosine determined spectrophotometrically (11) yielded a value of 30.2 mol per 220,000 g of enzyme, which is in good agreement with results obtained from the amino acid analyzer. The total cysteine content was determined by titration of the sodium dodecyl sulfate treated enzyme with 5,5'dithiobis (2-nitrobenzoic acid). Treatment of the enzyme with 8 M urea and sodium borohydride (12) prior to titration with 5,5'-dithiobis (2-nitrobenzoic acid) resulted in no further increase in titratable sulfhydryl groups. This suggests that the purified native enzyme contains no disulfide linkages.

The amino acid composition for rat liver pyruvate kinase reported in Table I is remarkably similar to that reported for pig liver pyruvate kinase (23,24), yet considerably different from enzyme isolated from the kidney (24), or skeletal muscle (24). A sugar analysis of liver pyruvate kinase resulted in a total carbohydrate content of less than 1%.

TABLE I

Amino Acid Content of Rat Liver Pyruvate Kinase Purified from Animals Fed a High Carbohydrate, Low Protein Diet

Residue	Molar ratio relative to aspartate				moles per 220,000 g of protein
	24 hr	48 hr	72 hr	average	
aspartate	1.00	1.00	1.00	1.00	140
threonine[a]	.77	.70	.66	.81	113
serine[a]	.86	.75	.66	.95	133
glutamate	1.73	1.66	1.64	1.68	234
proline	.96	.90	.84	.90	126
glycine	1.31	1.24	1.24	1.26	176
alanine	1.60	1.54	1.52	1.55	216
valine	1.42	1.38	1.35	1.38	193
methionine	.37	.32	.33	.34	48
isoleucine	1.06	1.02	1.00	1.03	144
leucine	1.31	1.28	1.25	1.28	179
tyrosine	.27	.20	.22	.23	32
phenylalanine	.42	.44	.43	.43	60
histidine	.33	.30	.30	.31	43
lysine	.55	.54	.53	.54	75
arginine	.77	.70	.74	.74	103
cysteine[b]					19
tryptophan[c]					14
NH_3	.47	.52	.54	.51	71

[a] Extrapolated to zero hours of hydrolysis

[b] Determined by titrations of protein with 5,5'-dithiobis(2-nitrobenzoic acid) in sodium dodecyl sulfate or in 8 M urea following treatment of the protein with sodium borohydride (12,13).

[c] Determined spectrophotometrically by the method of Edelhoch (11).

Tryptic Peptide Mapping

The peptide map obtained using the tryptic digest of heat denatured liver pyruvate kinase is shown in Fig. 1. Since the enzyme contains a total of 178 lysyl and arginyl residues, about 178 individual peptides would be expected upon tryptic hydrolysis if each subunit contained a unique primary sequence. However, the total number of ninhydrin-sensitive spots observed is 44, with virtually no staining at the origin. The fact that the number of peptide spots is almost exactly one-quarter of the sum of lysyl and arginyl residues suggests that the native enzyme consists of four subunits of similar primary structure.

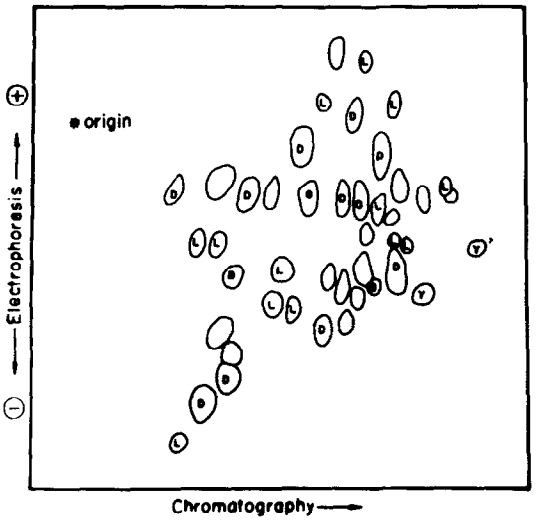

Fig. 1: Thin layer peptide map of the tryptic digest of liver pyruvate kinase purified from rats fed a high carbohydrate, low protein diet. Ninhydrin positive spots are encircled. Letter designation within the circled spots indicates a distinctive character for that spot: D-dark purple; L-light purple; B-brown; G-gray; Y-yellow.

Amino Terminal Analysis with Dansyl Chloride

After reaction of liver pyruvate kinase with dansyl chloride the acid hydrolyzates were examined for the presence of α-amino derivatives. Fluorescent spots corresponding to dansyl-ε-amino lysine, dansyl-amine, and dansyl-hydroxide were easily identified. The only derivative of an α-amino acid observed in the hydrolyzate of liver pyruvate kinase was a trace of aspartic acid. However, the aspartic acid amino-terminus (25) of albumin was easily detected by this same procedure.

Acid-Urea Electrophoresis

Since the four subunits of liver pyruvate kinase are similar in mass as determined by sodium dodecyl sulfate-polyacrylamide electrophoresis (3,21), attempts have been made to detect charge differences among the subunits when dissociated in urea. Polyacrylamide gel electrophoresis in 7.5 M urea containing 6 mM Triton X-100 has proved to be very sensitive in detecting slight differences in charge to mass ratios in histones (18). When liver pyruvate kinase is dissociated and electrophoresed by this procedure, one major protein band is detected which contains greater than 95% of the protein. Similar results are observed with 12% polyacrylamide gels run for 10 hours and 7.5% polyacrylamide gels run for 3.5 hours (Fig. 2). When a sample of hemoglobin is run under similar conditions the alpha and beta

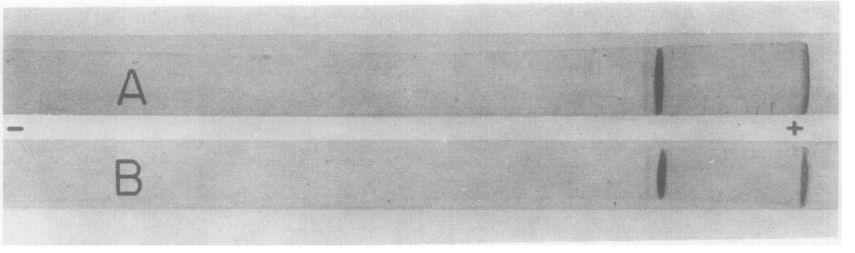

Fig. 2: Disc gel electrophoresis of liver pyurvate kinase in the presence of 7.5 M urea and 6 mM Triton X-100. Liver pyruvate kinase was run in (A) 12% acrylamide at 120 volts for 10 hours and (B) 7.5% acrylamide at 120 volts for 3.5 hours. Staining and destaining was carried out as described in Materials and Methods.

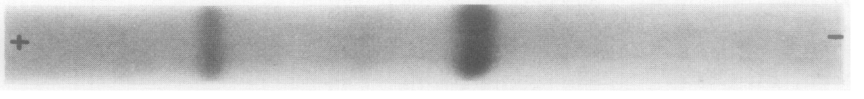

Fig. 3: Isoelectric focusing of liver pyruvate kinase in the presence of 9 M urea and 2% Nonidet P-40. Isoelectric focusing of liver pyruvate kinase yields two protein bands. The major band represents 70 to 74% of the protein and has a pI of 6.6. The minor band represents 26 to 30% of the protein and has a pI of 6.3. The protein bands were visualized by fluorescent staining with the magnesium salt of 8-anilino-1-napthalenesulfonic acid (20).

chains are easily separated. These results suggest that the four subunits of liver pyruvate kinase isolated from rats fed a high carbohydrate, low protein diet have very similar charge to mass ratios.

Isoelectric Focusing in Urea

Isoelectric focusing of the liver pyruvate kinase subunits in 9 M urea containing 2% Nonidet P-40 yields two protein bands as seen in Fig. 3. Scanning isoelectric focusing gels of several different protein preparations revealed a major band (pI = 6.6) containing 70 to 74% of the protein and a minor band (pI = 6.3) containing 26 to 40% of the protein. Both of these pI values are larger than those reported for native liver pyruvate kinase (4,26). The two protein bands observed upon isoelectric focusing of liver pyruvate kinase in urea and nonionic detergent could result from either an $\alpha_3\beta_1$ subunit structure, or more likely from an undetermined microheterogeneity among the protein subunits. Microheterogeneity for the native enzyme has previously been discussed by Hess and Kutzbach (26).

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