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CYTOSOLIC AND MITOCHONDRIAL PHOSPHO*ENOL*PYRUVATE CARBOXYKINASE OF THE BULLFROG, *RANA CATESBEIANA*, LIVER

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Summary

The mitochondrial and cytosolic phospho*enol*pyruvate carboxykinase (GTP: oxaloacetate carboxy-lyase(transphosphorylating), EC 4.1.1.32) occurring in the bullfrog (*Rana catesbeiana*) liver were studied. The enzymes in the two intracellular compartments of both tadpole and adult frog liver were immunologically identical. Both radioactively-labelled forms of the mitochondrial and cytosolic phospho*enol*pyruvate carboxykinase from bullfrog liver were imported at the same rate into intact mitochondria in vitro. The mitochondrial and cytosolic enzyme activities did not respond to the administration of glucagon, glucocorticoid, quinolinate and D-mannoheptulose which are known as enhancers of phospho*enol*pyruvate carboxykinase, but were found to increase during natural metamorphosis. The former activity was markedly increased in the tadpoles treated with 3,5,3′-triiodothyronine. It was supposed that in the bullfrog liver the phospho*enol*pyruvate carboxykinase localized in the mitochondria is of central importance in phospho*enol*pyruvate synthesis from oxaloacetate.

Many species-dependent factors are known to be involved in gluconeogenesis and its regulation. One of them is that of the intracellular location of phospho*enol*pyruvate carboxykinase (GTP:oxaloacetate carboxy-lyase (transphosphorylating), EC 4.1.1.32) [1—6]. Most animals, including guinea pig [1,2], rabbit [3], pig [4] and man [2,5] have substantial activities of both the cytosolic and the mitochondrial phospho*enol*pyruvate carboxykinases, whereas

Abbreviation: Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

the enzyme of pigeon liver is located entirely within the mitochondrial matrix [6] and that of rat, mouse and hamster [5] is predominantly found in the cytosolic fraction. However, the functional and structural relationships between the two types of phospho*enol*pyruvate carboxykinase are still obscure.

In the previous report we have reported the purification of mitochondrial and cytosolic phospho*enol*pyruvate carboxykinases from bullfrog liver and found that the two enzymes were different from each other in their isoelectric points, but were immunologically indistinguishable [7,8]. In order to determine the role of the two compartmentalized enzymes in the regulation of gluconeogenesis in the bullfrog liver, we have studied the possible relationship between the two enzymes and the changes in their activities during natural or 3,5,3′-triiodothyronine-induced metamorphosis.

Materials

Adult bullfrogs and tadpoles were obtained commercially in Tokyo. Tadpoles were maintained at room temperature in winter on a diet of boiled spinach. Experiments presented here were carried out using animals in summer and autumn.

The following materials were purchased from the sources indicated. Iodo [1-^{14}C]acetamide, L-[4,5-^{3}H]leucine (5.0 Ci/mmol) and [^{14}C]$NaHCO_3$ (RCC Amersham); Aquasol-2 (New England Nuclear), 3,5,3′-triiodothyronine (Fluka AG), Triton X-100 and Non-Idet P40 (Wako Junyaku); phospho*enol*pyruvate, NADH, GTP, pig heart malate dehydrogenase and oxaloacetate (Boehringer Mannheim); iodocetamide (Eastman); kynurenine sulfate (Sigma); Sephadex G-25 (Pharmacia).

Methods

Preparation of enzymes. The mitochondrial and cytosolic phospho*enol*pyruvate carboxykinases from bullfrog liver were prepared to electrophoretical and immunological homogeneity according to the method described previously [7,8].

Preparation of antibody against mitochondrial phosphoenolpyruvate carboxykinase. Mitochondrial phospho*enol*pyruvate carboxykinase (0.2 mg in 0.2 ml) purified from bullfrog liver was emulsified with an equal volume of Freund's complete adjuvant (Difco). The emulsion was injected into a female white rabbit once a week for 4 weeks. After the last injection 1 mg enzyme was boosted. The rabbit was bled 1 week after the boost. The immunoglobulin G (IgG) fraction was purified by repeated fractionation with 50% saturated $(NH_4)_2SO_4$ and then by DEAE-cellulose column chromatography as previously described [9]. The IgG fraction from a female rabbit without immunization was used for control experiments. Quantitative immunoprecipitation was carried out by the method of Ivarie and Jones [10].

Incorporation of radioactively labelled phosphoenolpyruvate carboxykinase into mitochondria. The mitochondrial and cytosolic phospho*enol*pyruvate carboxykinases purified from the liver were separately alkylated with iodo[1-^{14}C]acetamide as described previously [7] and freed from the excess

iodoacetamide with a Sephadex G-25 column. Incorporation of the labelled enzyme into mitochondria was measured as follows. Mitochondria (1 mg protein) were incubated with 40 pmol labelled enzyme in a total volume of 0.5 ml at 37°C. Buffer used was 220 mM mannitol/70 mM sucrose/2 mM Hepes/1 mM 2-β-mercaptoethanol, pH 7.4, (buffer A) containing 50 mM NaCl. After the incubation the mitochondria were repeatedly washed with buffer A containing 50 mM NaCl by means of centrifugation with a Beckman microfuge B. Finally the mitochondrial pellet was solubilized with 1.0% sodium dodecyl sulfate (SDS) and the radioactivity was counted using Aquasol-2 as a scintillator.

Determination of protein and nuclear DNA. Protein was determined by the method of Lowry [11] and when detergent was used for mitochondriolysis, by the method of Bradford [12]. Nuclear DNA was extracted by the method of Schneider [13] and determined colorimetrically as described by Burton [14].

Results and Discussion

Immunological identity between mitochondrial and cytosolic phosphoenolpyruvate carboxykinases. We have previously shown that the phospho*enol*pyruvate carboxykinases purified from mitochondrial and cytosolic fractions of the adult frog liver are immunologically identical [7]. To examine further whether the mitochondrial and cytosolic enzymes of tadpole liver are immunologically the same as those of the adult frog liver, an immunoprecipitation test and Ouchterlony double immuno-diffusion were carried out with the IgG against the mitochondrial enzyme from the adult frog liver. Fig. 1 shows the immunoprecipitation of the IgG with the cytosolic and mitochondrial enzymes from the tadpole liver. The results indicate that the two phospho*enol*pyruvate carboxykinases, which are compartmentalized into the mitochondria and cytoplasm of the tadpole liver, are immunologically identical and are also identical to those of the adult frog. In the Ouchterlony test a single precipitation line was detected between the IgG and phospho*enol*pyruvate carboxykinase of the cytosolic and mitochondrial extracts from the tadpole liver (data not shown).

To obtain further evidence for the structural co-identity between the mitochondrial and cytosolic enzymes, the rate of entry of the radioactively-labelled enzymes into mitochondria was measured in vitro. Intact mitochondria prepared from the frog liver were incubated with the labelled enzyme. The mitochondria were collected at intervals and extensively washed with buffer A containing 50 mM NaCl. The radioactivity of the mitochondrial pellet was determined after solubilization. Fig. 2 shows a typical time course for the incorporation of radioactivity into the mitochondrial pellet. No difference was observed in the incorporation rate between the mitochondrial and cytosolic enzymes. The incorporation increased linearly for about 15 min and reached a maximal value at 30 min incubation, where 18% of the labelled enzyme added to the incubation medium was recovered from the mitochondrial pellet. When the mitochondria treated with digitonin to remove the outer-membrane were used, the incorporation rate of the radioactivity decreased extremely, indicating that intact mitochondria are required for the transport of the enzyme.

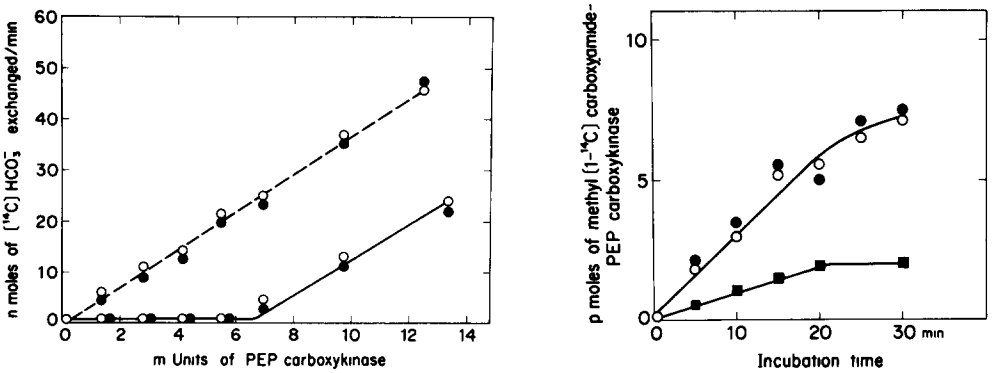

Fig. 1. Immunoprecipitation curves of antibody against mitochondrial phospho*enol*pyruvate (PEP) carboxykinase from adult frog liver as titrated with cytosolic and mitochondrial antigens from tadpole liver. Varying amounts of mitochondrial or cytosolic enzyme from tadpole liver were mixed with a constant amount of antibody against the purified mitochondrial enzyme from adult frog liver. The reaction mixture in a total volume of 0.1 ml was composed of 25 mM potassium phosphate, pH 7.6/100 mM NaCl/1 mM EDTA/0.25% Nonidet P40/12 μg IgG and cytosolic or mitochondrial enzymes, and was incubated for 15 min at 37° C. After that the reaction mixture was kept at 34° C overnight and then 20 μl of 10% suspension of staphylococcal cells was added. The precipitate which appeared was discarded by centrifugation with a Beckman microfuge B. The remaining enzyme activity in the supernatant was assayed for [^{14}C]HCO_3^--oxaloacetate exchange reaction [7]. The activity was expressed as nmol [^{14}C] HCO_3^- exchanged with β-carboxyl group of oxaloacetate. Symbols (●———●) and (○———○) represent the activity of the mitochondrial and cytosolic enzymes from tadpole liver, respectively. Solid line indicates the titrations of anti-mitochondrial phospho*enol*pyruvate carboxykinase-IgG with cytosolic and mitochondrial enzymes, and the dotted line the titrations of control IgG with each enzyme.

Fig. 2. Mitochondrial uptake in vitro of phospho*enol*pyruvate (PEP) carboxykinase. Mitochondria were incubated with radioactively labelled cytosolic and mitochondrial enzymes. Symbols (●———●) and (○———○) represent uptake of mitochondrial and cytosolic enzymes by the mitochondria, respectively. ■———■, indicates uptake of mitochondrial enzyme by mitoplast.

The localization of the labelled enzyme incorporated into the mitochondria was examined by submitochondrial fractionation according to the method of Greenawalt [15]. The identity of specific submitochondrial components was confirmed following subfractionation by the determination of relevant enzyme activities. The value obtained with the whole mitochondria was taken as 100%. As shown in Table I, 70% or more of the radioactivity incorporated into the mitochondria was recovered from the mitoplast, indicating that both the labelled enzymes were imported into the mitochondria.

The incorporation of [^{3}H]leucine into the cytosolic and mitochondrial phospho*enol*pyruvate carboxykinases in vivo was also studied to establish whether there is any species-specific process for the compartmentalization of phospho*enol*pyruvate carboxykinase in bullfrog liver. Tadpoles were injected intraperitoneally with [^{3}H]leucine and the appearance of newly labelled phospho*enol*pyruvate carboxykinase in the whole homogenate and the cytosolic and mitochondrial fractions was traced by immunoprecipitation with the IgG against the mitochondrial phospho*enol*pyruvate carboxykinase from bullfrog liver and *Staphylococcus aureus* Cowan type I cells. The radioactivity of phospho*enol*pyruvate carboxykinase in the three fractions were analyzed by SDS-polyacrylamide gel electrophoresis as described by Weinberg and Utter [16]. As shown in Fig. 3, the radioactivity of phospho*enol*pyruvate carboxy-

TABLE I

SUBMITOCHONDRIAL LOCALIZATION OF THE LABELLED PHOSPHO*ENOL*PYRUVATE CARBOXYKINASE

Mitochondria isolated from adult frog liver were incubated with the labelled cytosolic or mitochondrial phospho*enol*pyruvate carboxykinase for 30 min at 37°C as described in Methods. Submitochondrial fractionation was carried out according to the method of Greenawalt [15]. The mitochondrial subfractions were specified with the following enzymes; Kynurenine hydroxylase assayed by the method of Hayaishi [24] for the outer-membrane, sulfite oxidase assayed by the method of Cohen et al. [25] for the intermembrane space fraction, cytochrome *c* oxidase assayed by the method of Wharton and Tzagoloff [26] for the innermembrane and glutamate dehydrogenase assayed by the method by Schmidt [27] for the matrix fraction. The values were represented with percent activity when the value of the whole mitochondria was 100%.

	Outer membrane (%)	Inter membrane space component (%)	Mitoplast (%)	Recovery (%)
Protein	24.3	42.6	39.7	106.6
Cytochrome *c* oxidase	15.9	trace	84.2	100.1
Kynurenine hydroxylase	56.9	21.6	6.3	84.8
Sulfite oxidase	13.9	87.4	18.2	119.5
Glutamate dehydrogenase	8.8	17.3	68.9	95.0
Labelled cytosolic phospho*enol*pyruvate carboxykinase	18.0	5.0	71.4	94.4
Labelled mitochondrial phospho*enol*pyruvate carboxykinase	17.3	5.9	73.5	96.7

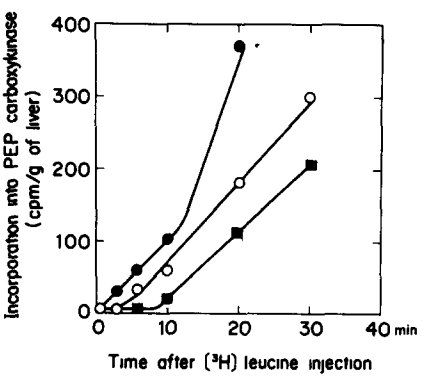

Fig. 3. Labelling of phospho*enol*pyruvate carboxykinase with [^{3}H]leucine in vivo. Tadpoles at premetamorphic stage were injected intraperitoneally with [^{3}H]leucine, a single dose of 2.5 μCi/g body weight, and hepatectomized at the indicated time. Livers were rapidly chilled in cold homogenizing buffer A containing 100 mM leucine and homogenized. Cytosolic and mitochondrial fractions were obtained by the method of Bustamante [28]. The phospho*enol*pyruvate (PEP) carboxykinase in each subcellular fraction was incubated with IgG against mitochondrial phospho*enol*pyruvate carboxykinase from bullfrog liver to form antigen-antibody complex as described in the legend of Fig. 1. The complex was quantitatively precipitated by the addition of staphylococcal cells. The phospho*enol*pyruvate carboxykinase was released from the immunoprecipitate in 70 mM Tris-HCl buffer, pH 6.8/2.5% SDS/6.25% 2-β-mercaptoethanol, by heating for 3 min in a boiling water-bath. After centrifugation the supernatant was subjected to a SDS-polyacrylamide gel electrophoresis according to the method of Laemmli [29] and the phospho*enol*pyruvate carboxykinase was determined by the method of Weinberg and Utter [16]. The yield of the enzyme was 65% after electrophoresis. Symbols (●———●), (○———○) and (■———■) represent the radioactivities of immunoprecipitate in the whole homogenate and the cytosolic and mitochondrial fractions, respectively.

TABLE II

MITOCHONDRIAL AND CYTOSOLIC PHOSPHO*ENOL*PYRUVATE CARBOXYKINASE ACTIVITIES OF TADPOLE AND ADULT FROG LIVERS AND THEIR CHANGE DURING NATURAL AND INDUCED METAMORPHOSIS

Tadpoles undergoing natural metamorphosis were divided into three groups of the developmental stages X—XI, XV—XVIII and XXII—XXIV, according to the criteria established by Taylor and Kollros [30]. Tadpoles at stage VII—VIII were injected intraperitoneally with a single dose of 0.5 μg 3,5,3′-triiodothyronine (T_3) dissolved in 0.2 ml 10 mM NaOH/140 mM NaCl per g body weight and were hepatectomized after 3 and 7 days. Cytosolic fraction was obtained by centrifugation using a Beckman microfuge B. The pellet was washed with buffer A and washings were combined to the cytosolic fraction. The difference between the enzyme activities in the pellet with and without 1% Triton X-100 was defined as the mitochondrial activity. The phospho*enol*pyruvate carboxykinase activity was assayed for the phospho*enol*pyruvate carboxylation and represented as unit/mg nuclear DNA.

Developmental stages	Activity unit/mg DNA			Ratio $\left(\frac{\text{mitochondrial}}{\text{cytosolic}}\right)$
	Whole liver homogenate	Cytosolic fraction	Mitochondrial fraction	
Spontaneous metamorphosis				
stage X—XI	1.90 ± 0.21 *	0.39 ± 0.05	1.15 ± 0.12	2.95
stage XV—XVIII	2.20 ± 0.32	0.50 ± 0.10	1.62 ± 0.21	3.24
stage XXII—XXIV	3.50 ± 0.20	0.90 ± 0.17	2.79 ± 0.40	3.10
3 Days after T_3 treatment	2.19 ± 0.25	0.38 ± 0.10	1.60 ± 0.13	4.21
7 Days after T_3 treatment	3.17 ± 0.20	0.39 ± 0.06	2.47 ± 0.20	6.33
Adult frog	3.43 ± 0.30	0.81 ± 0.10	2.23 ± 0.23	2.75

* Standard deviation.

kinase in the cytosolic fraction increased almost linearly until 30 min, while that in the mitochondrial fraction started to increase after 6 min lag period. The radioactivity of phospho*enol*pyruvate carboxykinase in the whole homogenate was close to the sum of the radioactivities in the cytosolic and mitochondrial fractions. [^{3}H]Leucine was not incorporated into either fractions when the tadpoles were pretreated with cycloheximide at a dose of 0.2 mg/g body weight (data not shown here). These results support the idea that in the bullfrog liver the phospho*enol*pyruvate carboxykinase cytoplasmically synthesized is imported into the mitochondria.

Although we know little as yet about the process by which cytoplasmically synthesized proteins are transported into mitochondria, it is supposed from all the results mentioned above that the phospho*enol*pyruvate carboxykinases occurring in the cytoplasm and mitochondria of the bullfrog liver are genetically the same protein.

Mitochondrial and cytosolic phosphoenolpyruvate carboxykinase activities in tadpole and adult frog livers and their change during metamorphosis. A number of lines of evidence are available to show that the cytosolic phospho*enol*pyruvate carboxykinase responds to a variety of stimuli to increase its amount [17—21], but the situation with the mitochondrial enzyme is less clear [5]. We studied the response of the two compartmentalized enzymes of bullfrog to triiodothyronine and several compounds known as enhancers of phospho*enol*pyruvate carboxykinase. The phospho*enol*pyruvate carboxykinase activities in the mitochondrial and cytosolic fractions from the tadpole and bullfrog livers and their changes during natural and triiodothyronine-induced

metamorphosis are summarized in Table II. Both activities of the adult frog liver are about 2-times higher than corresponding activities of the tadpole liver, indicating that the latter activities increase to the levels of adult frog liver during metamorphosis. The activity of the mitochondrial enzyme is about 3-times higher than that of the cytosolic fraction in both the tadpole and bullfrog livers and is found to increase during natural and triiodothyronine-induced metamorphosis. As seen from the ratio of mitochondrial to cytosolic enzyme activity, the mitochondrial enzyme activity markedly increases in response to the thyronine treatment when compared with the cytosolic enzyme activity. The increase in the activity of the mitochondrial enzyme suggests that in the bullfrog liver the mitochondrial phospho*enol*pyruvate carboxykinase plays a central role in the regulation of gluconeogenesis rather than the cytosolic enzyme. This does not contradict the finding [22,23] that an increase in the activities of glucose-6-phosphatase and glycogen synthetase of bullfrog liver occurs during metamorphosis.

Although several compounds, known as enhancers of phospho*enol*pyruvate carboxykinase, such as glucocorticoid, glucagon, D-mannoheptulose and quinolinate were tested in vivo, but no increase was found in the enzyme activities of both compartments. In this respect, the phospho*enol*pyruvate carboxykinase of bullfrog liver seems to be different from that of animals so far studied [17—21], in which the cytosolic phospho*enol*pyruvate carboxykinase is subject to complex regulation of its rate of synthesis through a variety of stimuli.

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