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INDUCTION OF MITOCHONDRIAL PHOSPHO*ENOL*PYRUVATE CARBOXYKINASE IN THE LIVER OF *RANA CATESBEIANA* TADPOLE TREATED WITH 3,5,3′-TRIIODOTHYRONINE AND UNDERGOING NATURAL METAMORPHOSIS

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The rates of synthesis and degradation of mitochondrial phospho*enol*pyruvate carboxykinase (GTP : oxaloacetate carboxy-lyase (transphosphorylating), EC 4.1.1.32) in the liver of tadpoles in the two developmental stages (stages VIII and XXII), and in those treated with 3,5,3′-triiodothyronine, were studied by immunochemical techniques. The rate of synthesis of the enzyme was found to be accelerated at 9 h and 6 days after triiodothyronine administration and also during natural metamorphic climax. No difference was observed in the degradation rate of the enzyme between the tadpoles in the two stages, VIII and XXII.

Introduction

The distribution of the phospho*enol*pyruvate carboxykinase (GTP : oxaloacetate carboxy-lyase (transphosphorylating), EC 4.1.1.32) in the cytosolic and mitochondrial compartments of the liver depends upon the species [1–6]. Although there are many reports concerning the intracellular concentrations and distribution of phospho*enol*pyruvate carboxykinase [1–6], the functional, structural and genetic relationships between the enzymes localizing to the cytosolic and mitochondrial compartments are obscure. A significant difference between the two varieties of the enzyme is their response to nutritional and hormonal stimuli; the cytosolic enzyme increases its activity in response to a variety of stimuli, whereas the mitochondrial one does not [7–11].

We reported that the phospho*enol*pyruvate carboxykinase occurring in both the cytosolic and mitochondrial fractions of the liver of bullfrog and tadpole, *Rana catesbeiana*, are immunologically identical, and that the mitochondrial enzyme activity increases markedly during natural and triiodothyronine-induced metamorphose [12,13]. These findings led us to the view that the mitochondrial enzyme is of importance in the formation of phospho*enol*pyruvate from oxaloacetate, and thyroid hormone may be involved in the regulation of the mitochondrial enzyme level in the bullfrog liver. To examine the mechanism by which the phospho*enol*pyruvate carboxykinase level is regulated, we have investigated the rates of synthesis and degradation of the mitochondrial phospho*enol*pyruvate carboxykinase in the liver of *R. catesbeiana* tadpole treated with thyronine and undergoing natural metamorphosis.

Materials

Bullfrog (*R. catesbeiana*) tadpoles were obtained commercially in Tokyo and maintained at room temperature on a diet of boiled spinach. Their developmental stages were classified according to the criteria defined by Taylor and Kollros [14].

The following materials were purchased from the sources indicated. L-[4,5-^{3}H]leucine (specific activity

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

50 Ci/mmol) (RCC Amersham); Aquasol-2 (New England Nuclear); 3,5,3′-triiodothyronine (Fluka AG); Triton X-100 (Wako Junyaku); Non-Idet P40 (Nakarai); Whatman glass fiber disk, GF/C (Whatman). *Staphylococcus aureus* Cowan type I cell was a gift from Dr. Takeuchi at Department of Serology, Nippon Medical School.

Methods

Preparation of mitochondrial phosphoenolpyruvate carboxykinase and its antibody. The mitochondrial phospho*enol*pyruvate carboxykinase was purified to electrophoretical homogeneity from adult bullfrog liver as previously described [12]. The immunoglobulin G (IgG) fraction against the purified enzyme was prepared according to the method of Tomita et al. [15].

Determination of phosphoenolpyruvate carboxykinase. The activity of phospho*enol*pyruvate carboxykinase was assayed for phospho*enol*pyruvate carboxylation as previously described [16]. The amount of the enzyme in the cytosolic and mitochondrial fractions was immunologically determined by the methods of Weinberg and Utter [17] using staphylococcal cells which had been inactivated with formaldehyde [18]. The enzyme amount was expressed as μg/mg of nuclear DNA.

Treatment of tadpoles with thyroid hormone. Tadpoles in stage VIII (premetamorphic stage) were intraperitoneally injected with a single dose of 0.5 μg 3,5,3′-triiodothyronine/g body weight. For a control experiment only the vehicle solution (140 mM NaCl/5 mM NaOH) was injected. After the injections the tadpoles were maintained at room temperature and killed at intervals up to about 7 days. The livers of three to four tadpoles were used for the immunochemical analysis. Three groups of tadpoles, in stage VIII, stage XXII (metamorphic climax) and treated with triiodothyronine, were used for the determination of the rate of synthesis of the enzyme.

Rate of synthesis in vivo of mitochondrial phosphoenolpyruvate carboxykinase. Tadpoles were intraperitoneally injected with L-[4,5-^{3}H]leucine, a single dose of 2.5 μCi/g body weight. The livers were removed 90 min later and rapidly chilled in a cold homogenizing buffer (70 mM sucrose/220 mM mannitol/2 mM Hepes/10 mM EDTA/5 mM β-mercaptoethanol, pH 7.4) containing 100 mM leucine. After homogenization the mitochondrial fraction was prepared by the method of Bustamante et al. [19]. Mitochondrial matrix fraction was obtained by mitochondriolysis with 1% Triton X-100. Mitochondrial lysate from a known mass of tissue was incubated at 37°C for 15 min with 2.5-fold equivalent amount of anti-phospho*enol*pyruvate carboxykinase-IgG and left to stand overnight at 4°C. The antigen-antibody complex formed was quantitatively precipitated by use of staphylococcal cells according to the method of Ivarie and Jones [20]. The phospho*enol*pyruvate carboxykinase was released from the immunoprecipitate by SDS and was subjected to a SDS-polyacrylamide gel electrophoresis as described previously [13]. The gels were stained with Coomassie brilliant blue R-250 for 4 h and destained by soaking in several changes of destaining solution (25% methanol/7.5% acetic acid) until the background became clear. The gels were then sliced. The sliced gels corresponding to the enzyme were solubilized by incubation with 0.5 ml 35% H_2O_2 at 80°C. After cooling in a dark room, the radioactivity was determined with a scintillation counter. Background radioactivity (the average of 20 slices not associated with protein) was subtracted. For the determination of radioactivity incorporated into total cytoplasmic protein, 50 μl cytoplasmic fraction from a known mass of tissue were used. The protein was precipitated with 10% (w/v) trichloroacetic acid containing 10 mM leucine. After heating in a boiling water bath for 15 min, the precipitate formed was trapped on a glass fiber disk and washed with the trichloroacetic acid solution. After drying the radioactivity remained on the disk was counted.

Determination of protein and nuclear DNA. Protein was determined by the method of Lowry et al. [21] and when detergent was used for mitochondriolysis, by the method of Bradford [22]. Nuclear DNA was extracted by the method of Schneider [23] and determined colorimetrically as described by Burton [24].

Results and Discussion

Changes in the amount of phosphoenolpyruvate carboxykinase after triiodothyronine treatment. To examine the most effective period for studying regulatory mechanisms, changes in the amount of cyto-

solic and mitochondrial phospho*enol*pyruvate carboxykinase in the tadpole liver were determined during the 7 days following the injection of triiodothyronine. Since the phospho*enol*pyruvate carboxykinase of tadpole liver was immunologically identical to that of adult frog liver as previously reported [12], we used an immunoprecipitation with the IgG against the enzyme from adult bullfrog mitochondria to isolate the enzyme in the cytosolic and mitochondrial fractions of the tadpole liver. As shown in Fig. 1, no change was observed in the amount of cytosolic enzyme expressed as μg/mg nuclear DNA, but that of mitochondrial enzyme was found to increase to about 2-fold that of control level 9 h after the injection and again about 3-fold 7 days later at which time morphological changes in the tadpole appeared. It is noted that only the amount of mitochondrial enzyme increases in response to thyroid hormone. This finding supports the idea that the mitochondrial but not the cytosolic phospho*enol*pyruvate carboxykinase may be subject to regulation of its rate of synthesis in the tadpole liver by thyroid hormone.

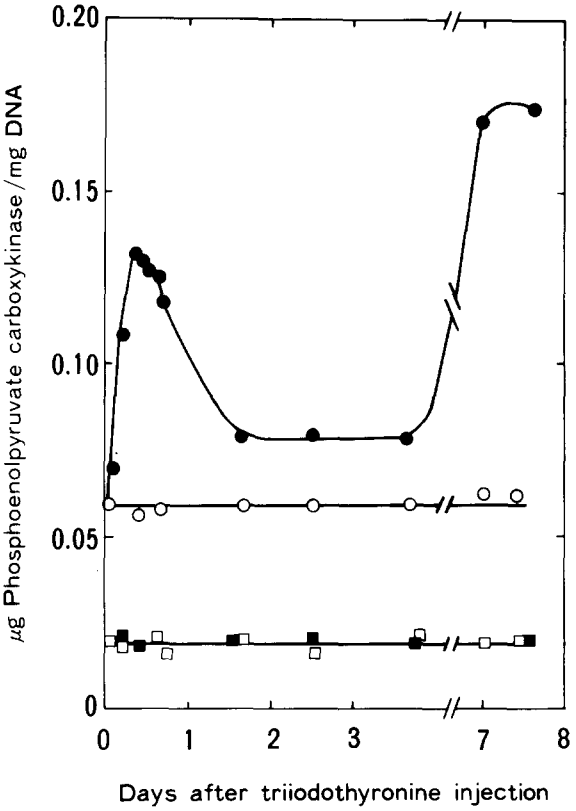

Fig. 1. Changes in the amount of phospho*enol*pyruvate carboxykinase after 3,5,3'-triiodothyronine injection. Tadpoles (stage VIII) were intraperitoneally injected with 10 μg triiodothyronine. Changes in the amount of enzyme in both cytosolic (■, □) and mitochondrial (●, ○) fractions were followed until metamorphosis was induced. The amount of enzyme was determined as described in Methods. Filled and open symbols represent the amount of enzyme in the liver of tadpole treated with and without triiodothyronine, respectively.

Relative rate of synthesis of mitochondrial phosphoenolpyruvate carboxykinase of the tadpole liver. The relative rate of synthesis of the enzyme was determined by measuring the incorporation of isotope into the enzyme following the injection of L-[4,5-^{3}H]leucine. Three to four tadpoles were injected with L-[4,5-^{3}H]leucine. The liver was removed 90 min after the injection. The radioactivities in the total cytoplasmic protein and the amount of the enzyme in the mitochondrial fraction expressed cpm/g liver tissue are listed in column A and B of Table I, respectively. The relative rate of enzyme synthesis (ratio (B/A)) is expressed as the amount of L-[4,5-^{3}H]leucine incorporated into mitochondrial phospho*enol*pyruvate carboxykinase per 10^3 cpm incorporated into the total cytoplasmic protein. This calculation corrects for any changes in the size of the leucine pool in the liver [17,25]. As shown in Table I, the relative rate of synthesis of the mitochondrial enzyme was increased 4.7-fold and 2.2-fold in the tadpole liver at 9 h and 7 days after triiodothyronine injection, respectively. Morphological changes set in at about 6 days following triiodothyronine injection. The relative rate of the enzyme synthesis in the liver of tadpole undergoing natural metamorphosis (stage XXII) was also 2.9-times higher than that of normal tadpole (stage VIII). These results indicate that the rate of synthesis of the enzyme is accelerated in response to thyroid hormone and during natural metamorphosis.

The degradation rate of mitochondrial enzyme. The results of the pulse labelling experiments described above indicate that the alterations in the amount of mitochondrial phospho*enol*pyruvate carboxykinase observed in the tadpoles treated with triiodothyronine and at metamorphic climax reflect, in part, changes in the rate of its synthesis. According to the method of Schimke [26], the absolute rate of

TABLE I

RELATIVE RATES OF SYNTHESIS OF MITOCHONDRIAL PHOSPHO*ENOL*PYRUVATE CARBOXYKINASE IN THE LIVER OF TADPOLES TREATED WITH TRIIODOTHYRONINE AND UNDERGOING NATURAL METAMORPHOSIS

Two groups of tadpoles treated with 3, 5, 3,-triiodothyronine, and two groups of tadpoles at stage VIII and XXII were used. Three to four tadpoles in each group were injected with 2.5 μCi L-[4,5-^{3}H]leucine/g body weight and were hepatectomized 90 min later. The cytoplasmic and mitochondrial fractions were obtained by the method of Bustamante et al. [19]. The radioactivities incorporated into total protein and mitochondrial phospho*enol*pyruvate carboxykinase were determined as described in Methods and expressed as per g liver tissue.

	[^{3}H]Leucine incorporated (cpm/g liver)		Relative rate of synthesis (B/A) · 10^3
	Total protein (A) (cpm)	Mitochondrial phospho*enol*-pyruvate carboxykinase (B) (cpm)	
Time of T_3 treatment			
0 h	$1.82 \cdot 10^6$	$1.96 \cdot 10^3$	1.37
9 h	$0.69 \cdot 10^6$	$4.53 \cdot 10^3$	6.56
7 days	$0.79 \cdot 10^6$	$2.36 \cdot 10^3$	2.98
Metamorphic stage			
stage VIII	$1.62 \cdot 10^6$	$2.26 \cdot 10^3$	1.46
stage XXII	$0.51 \cdot 10^6$	$2.16 \cdot 10^3$	4.22

synthesis of the enzyme is calculated from the degradation rate during steady-state conditions. Under these conditions, $E = k_s/k_d$, where E is the amount of the enzyme/g tissue, k_s is an apparent zero-order rate constant of synthesis per mass of tissue and k_d is an apparent first-order rate constant of degradation expressed in t^{-1}. The requirement for steady-state conditions may be met for phospho*enol*pyruvate carboxykinase in the liver of tadpoles in both premetamorphic stage and metamorphic climax where the alteration in the enzyme level was small enough to neglect. Thus, we used the tadpoles in the two stages (VIII and XXII) for the determination of the degradation rate of the enzyme.

Tadpoles were pulse labelled with L-[4,5-^{3}H]leucine for 90 min and chased with an excess amount of leucine until they were killed. The livers were removed at 12 h intervals up to 48 h. The mitochondrial phospho*enol*pyruvate carboxykinase was immunochemically analyzed for its radioactivity. The pro-

TABLE II

RATES OF DEGRADATION OF MITOCHONDRIAL PHOSPHO*ENOL*PYRUVATE CARBOXYKINASE OF THE LIVER OF TADPOLES IN STAGE VIII AND XXII

Tadpoles in two developmental stages, VIII and XXII, were pulse labelled for 90 min with 2.5 μCi L-[4,5-^{3}G]leucine/g body weight and were chased for 0, 12, 24, 36 and 48 h with an excess amount of leucine. The amount of enzyme and the radioactivity incorporated into the enzyme were immunologically determined as described in Methods. The apparent first-order rate constant of the enzyme degradation (k_d) was calculated from the slope of the straight line which was obtained by plotting the progressive loss of radioactivity of the enzyme as a function of time. The half-life and the apparent zero-order rate constant of the synthesis were calculated as described in the text.

Stage	Phospho*enol*pyruvate carboxykinase (μg/mg DNA)	k_d (day^{-1})	Half-life (days)	k_s (μg/mg DNA per day)
VIII	0.065	0.116	6.0	$0.76 \cdot 10^{-2}$
XXII	0.158	0.121	5.7	$1.91 \cdot 10^{-2}$

gressive loss of the radioactivity specific for the enzyme was plotted semilogarithmically versus time. Data points were on a straight line (data not shown), from which an apparent first-order rate constant of the enzyme degradation (k_d) expressed in t^{-1} was calculated. The apparent half-life ($1/k_d \cdot \ln 2$) of the mitochondrial enzyme was obtained as being 6.0 and 5.7 days for the tadpoles at stage VIII and XXII, respectively. As seen in Table II, the rate of enzyme synthesis is higher in the liver of tadpole at metamorphic climax (stage XXII) than at premetamorphic stage (stage VIII). This result is similar to that shown in Table I. The increase in the amount of mitochondrial phospho*enol*pyruvate carboxykinase in response to triiodothyronine seems to be unique for the tadpole liver, since it is not observed in the adult frog liver. In mammalian liver, it is known that cytosolic phospho*enol*pyruvate carboxykinase is induced by nutritional and hormonal stimuli but the mitochondrial enzyme is not [1–11]. Increase in the amount of the enzyme occurred firstly at 9 h and secondly at 6 days following the triiodothyronine injection. The former was independent of metamorphosis and the latter was brought about in parallel with other biochemical metamorphic events so far studied [27]. The second response may be dependent upon endogenous glucagon according to Greengard [28] who reported that the level of glucose-6-phosphatase in fetal and perinatal rats is regulated by thyroid hormone and glucagon, respectively. Fischer and Cohen [29] have also reported a similar dual increase in the activity of ornithine decarboxylase in the liver of tadpoles treated with triiodothyronine.

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