Phosphoenolpyruvate carboxykinase from guinea-pig liver mitochondria

Immunological evidence for increase in enzyme amount during neonatal development

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Phosphoenolpyruvate carboxykinase was purified from mitochondria of guinea-pig liver by affinity chromatography on GMP–Sephrose. The enzyme was purified 100-fold to a high degree of electrophoretic homogeneity as judged by detection of a single protein band on sodium dodecyl sulphate/polyacrylamide gels. The yield was about 16%. The M_r of the purified enzyme was estimated to be $68\,500 \pm 680$ by analysis on sodium dodecyl sulphate/polyacrylamide gels. Antibodies raised in rabbits against the purified enzyme were highly specific for mitochondrial phosphoenolpyruvate carboxykinase and did not precipitate the cytosolic form of this enzyme from either rat or guinea-pig liver cytosol. The use of this antibody showed that starvation does not increase the amount of the enzyme. However, neonatal-development-dependent increase in its activity is shown to be mediated by accumulation of phosphoenolpyruvate carboxykinase-specific protein.

Phosphoenolpyruvate carboxykinase (EC 4.1.1.32) is one of the key regulatory enzymes in gluconeogenesis (Scrutton & Utter, 1968; Tilghman *et al.*, 1976). The mitochondrial form of this enzyme has generally been thought to be non-inducible, since its activity does not appear to be altered by starvation or hormonal manipulations which lead to changes in the overall rate of gluconeogenesis (Nordlie *et al.*, 1965; Ballard & Hanson, 1969; Söling & Kleineke, 1976). However, Elliott & Pogson (1977) have suggested that this enzyme in guinea-pig liver might in fact be 'induced' under starvation conditions. In their study the activity of the enzyme in liver mitochondria, but not in kidney mitochondria, increased almost 2-fold in diabetic animals and in animals starved for 48 h. We have also demonstrated that in cultured human fibroblasts the activity of the enzyme can be increased 2-fold by addition of N^6, O^2 -dibutyryl cyclic AMP or cortisol in a manner suggesting new protein synthesis (Arinze *et al.*, 1978). These fibroblasts lack the cytosolic form of the enzyme. Changes in the activity of mitochondrial phosphoenolpyruvate carboxykinase in neonatal guinea-pig liver (Raghunathan & Arinze, 1977) also suggest inducibility of the

enzyme. In all of these studies, however, measurements were restricted to enzyme activity only; the authors made no efforts to quantify the amount of phosphoenolpyruvate carboxykinase-specific protein under the 'induced' conditions. The regulation of the amounts of mitochondrial phosphoenolpyruvate carboxykinase is poorly understood. Since understanding of this regulation may be aided by the availability of purified enzyme or antibodies to it, we decided to purify the enzyme from guinea-pig liver mitochondria. This paper reports purification of mitochondrial phosphoenolpyruvate carboxykinase by a modification of the combination of hydrophobic- and affinity-chromatographic steps suggested for the hog and human liver enzymes (Hung & Silverstein, 1978). This procedure resulted in an electrophoretically homogeneous enzyme with a 5-fold higher specific activity than that of a previous preparation (Holten & Nordlie, 1965) carried out before the advent of affinity chromatography. Antibodies produced in rabbits against the purified enzyme do not react with phosphoenolpyruvate carboxykinase from either rat or guinea-pig liver cytosol. With this antibody we have demonstrated that the neonatal-development-dependent increase in mitochondrial

phosphoenolpyruvate carboxykinase in guinea-pig liver is mediated by accumulation of enzyme-specific protein.

Experimental

Animals and materials

Hartley-strain guinea pigs (500–600g) were purchased from Williams Kentucky Cavies, Fern Creek, KY, U.S.A. New Zealand White rabbits were purchased from Camm Laboratory Animals, Wayne, NJ, U.S.A. Unless otherwise indicated, all animals were fed *ad libitum*. $\text{NaH}^{14}\text{CO}_3$ (9.2Ci/mol) was purchased from New England Nuclear Corp., Boston, MA, U.S.A. Hydrofluor was purchased from National Diagnostics, Somerville, NJ, U.S.A. Enzyme-grade $(\text{NH}_4)_2\text{SO}_4$ was obtained from Schwartz/Mann. The following chemicals were products of Boehringer Mannheim: phosphoenolpyruvate (potassium salt), ADP, AMP, CDP, GMP, GTP, IDP, UDP and malate dehydrogenase. Sepharose 4B, norleucine, adipic acid dihydrazide, reduced glutathione and sucrose were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. All other chemicals were of the highest grade commercially available.

Assay of phosphoenolpyruvate carboxykinase

The activity of the enzyme was routinely measured in the direction of oxaloacetate formation by using the $[^{14}\text{C}]$ bicarbonate fixation method (Ballard & Hanson, 1969). The reaction mixture contained 100mM-imidazole/HCl buffer, pH 6.6, 2mM- MnCl_2 , 1.5mM-potassium phosphoenolpyruvate, 1.25mM-IDP, 50mM- $\text{NaH}^{14}\text{CO}_3$, 2mM-reduced glutathione, 2.5mM-NADH and 2 units of malate dehydrogenase, in a total volume of 1.0ml. The final pH was 6.9–7.2. One unit of activity represents the incorporation of 1 μmol of $\text{NaH}^{14}\text{CO}_3$ /min at 37°C. During purification, the activity of enzyme preparations was also measured spectrophotometrically at 340nm (Jomain-Baum *et al.*, 1976) in a reaction mixture containing 50mM-Tris/HCl buffer, pH 8.0, 0.75mM- MnCl_2 , 1mM- NAD^+ , 3 units of malate dehydrogenase, 20mM-malate and 10–100 μl of purified or partially purified enzyme sample. After equilibration of the reaction mixture for 4min at 37°C, 100 μl of 0.75mM-GTP was added to start the reaction. The total volume of the assay mixture was 1.0ml. Initial velocity was calculated from the linear portion of the recording. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Enzyme purification procedures

All steps in the purification protocol were performed at 0–4°C.

Preparation of mitochondrial extract. About 100g of liver from adult guinea pigs was minced and suspended in 200ml of 0.25M-sucrose solution containing 0.5mM-EDTA and 1mM-reduced glutathione. The liver was homogenized with a motor-driven Potter–Elvehjem homogenizer; a final 10% (w/v) homogenate was prepared. Mitochondrial fractions were obtained by differential centrifugation (10500rev./min for 10min; Sorvall SS-34 rotor) and washed three times. The washed mitochondrial pellet was disrupted by sonication with a Branson Cell Disrupter (model W 185) five times at 15s intervals. The temperature was maintained near 0°C.

$(\text{NH}_4)_2\text{SO}_4$ precipitation. The enzyme was precipitated in two steps (Hung & Silverstein, 1978). The second $(\text{NH}_4)_2\text{SO}_4$ precipitate (45–70% saturation) was suspended in 60mM-potassium phosphate buffer containing 0.5mM-EDTA and 2mM-mercaptoethanol, in a total volume of 20–40ml. This sample could be stored at –20°C without much loss of activity.

Norleucine–Sepharose chromatography. Hydrophobic chromatography on norleucine–Sepharose was performed as described by Hung & Silverstein (1978), except that the enzyme sample, before application to the column, was mixed with 1 vol. of 60mM-potassium phosphate buffer, pH 7.0, containing 1.5M- $(\text{NH}_4)_2\text{SO}_4$, 0.5mM-EDTA and 2mM-mercaptoethanol. Norleucine–Sepharose was prepared by mixing CNBr-activated Sepharose with norleucine and allowing the reaction to proceed overnight at 4°C (March *et al.*, 1974).

GMP–Sepharose chromatography. GMP–Sepharose was prepared by the method of Lamed *et al.* (1973). The procedure resulted in the incorporation of 2–4 μmol of periodate-oxidized GMP/ml of gel. The pooled fraction containing the enzyme activity eluted from the norleucine–Sepharose column was precipitated by $(\text{NH}_4)_2\text{SO}_4$ (36g/100ml) and centrifuged (22000g) for 10min. The precipitate was suspended in 5mM-phosphate buffer containing 0.5mM-EDTA and 2mM-mercaptoethanol, pH 6.1, in a volume of 10–15ml, and immediately applied to the GMP–Sepharose.

In initial experiments, GMP–Sepharose chromatography was performed by the elution schedule devised by Hung & Silverstein (1978), which involves a buffer wash followed by another wash with a mixture of nucleotides before eluting the enzyme with 0.025mM-IDP. We have since changed this procedure such that the wash with a mixture of nucleotides is omitted. In our procedure, the column is initially thoroughly washed with 2–3 column vol. of equilibrating buffer. The enzyme is then eluted with 0.2mM-IDP (instead of 0.025mM). With this modification, elution profiles similar to that shown in Fig. 2 can be obtained.

Fractions enriched in enzyme activity were concentrated with a Pro-DiCon membrane apparatus (Bio-Molecular Dynamics, Beverton, OR, U.S.A.), and 1 vol. of 5mM-phosphate buffer, pH 7.0, containing 50% (v/v) glycerol, 0.5mM-EDTA and 2mM-mercaptoethanol was added to stabilize the enzyme.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

This analysis was performed by the Weber & Osborn (1969) method. In some experiments this analysis was also performed as described by Laemmli (1970). The gels were fixed, stained with Coomassie Blue and destained by the method of Fairbanks *et al.* (1971).

Antibody preparation

Purified mitochondrial phosphoenolpyruvate carboxykinase (2mg in 5mM-phosphate buffer, pH 7.0) was mixed thoroughly with an equal volume of complete Freund's adjuvant, and then injected subcutaneously on the back and ear lobe of New Zealand White rabbits (1.5–2.0kg). The injections were repeated every 2 weeks. A high titre of antibody was usually attained 6 weeks after the first injection. The animal was bled from the marginal ear vein to obtain approx. 30ml of blood. The blood was left to clot at 4°C overnight, and the serum was collected by centrifugation and heated (56°C) for 1 h in the presence of 1mM-phenylmethanesulphonyl fluoride and 1mM-EDTA. The γ -globulin-enriched fractions were prepared by adding $(\text{NH}_4)_2\text{SO}_4$ (0–50% satn.), and the precipitate was washed twice with 40%-satd. $(\text{NH}_4)_2\text{SO}_4$. The washed antibody preparation was dissolved in 1 vol. of 0.9% NaCl and exhaustively dialysed against 0.9% NaCl at 4°C overnight and stored at –80°C.

Quantitative immunotitration

To assess the immunological potency of the antibody preparation, various amounts of liver mitochondrial and cytosolic extracts and purified enzyme were mixed with 20 μ l of a diluted (1:25,

v/v) antibody preparation in a total volume of 0.4, 0.85 and 0.30ml respectively, and incubated at 37°C for 15 min and then overnight at 0–4°C. Non-immune serum (control serum) was used to assess non-specific precipitation. After centrifugation, a 100 μ l sample of supernatant was taken to determine the enzyme activity in each tube.

Ouchterlony double-diffusion analysis

A 0.75% suspension of agarose powder (Bio-Rad) in 0.15M-NaCl was layered on to a standard microscope slide. The antibody preparation was added in the centre well, and enzyme or extract from various sources was added in the other wells. The microscope slide was incubated at 37°C in a moisture chamber for at least 24h.

Results and discussion

Table 1 summarizes results obtained at several stages of purification in a representative experiment. A 100-fold purification of phosphoenolpyruvate carboxykinase with 15–20% yield was achieved routinely. The final enzyme preparation had a specific activity of 21–30 units/mg of protein as determined by the [^{14}C]bicarbonate fixation assay. The yield of enzyme protein was usually 2–4mg/100g starting wt. of liver.

The elution profiles from the hydrophobic and affinity-chromatography steps are shown in Figs. 1 and 2 respectively. It was necessary in the initial experiments, where we used the elution schedule devised by Hung & Silverstein (1978), to re-chromatograph the enzyme (twice) on the GMP-Sepharose column in order to remove a contaminating high- M_r protein which appeared to have a mobility, on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, much slower than that of the enzyme. This second pass resulted in a symmetrical elution profile with constant specific activity across the peak, indicating a high degree of purity. We found that the second pass on the GMP-Sepharose column can be completely eliminated by eluting the enzyme, during the first pass, with a high concentration (0.2mM) of IDP (see Fig.

Table 1. *Summary of purification of guinea-pig liver mitochondrial phosphoenolpyruvate carboxykinase*
Enzyme was purified from 100g of guinea-pig liver. One unit of enzyme activity equals 1 μ mol of [^{14}C]bicarbonate fixed/min.

Procedure	Total protein (mg)	Volume (ml)	Total activity (units)	Specific activity (units/mg of protein)	Yield (%)	Purification (fold)
Mitochondrial extract	2275	200	682.5	0.30	100	1
$(\text{NH}_4)_2\text{SO}_4$ precipitation (45–70%-satn.)	549	36	488.6	0.89	72	3.0
Norleucine-Sepharose	205	11	471.5	2.30	69	7.7
GMP-Sepharose	3.6	0.9	107.3	29.8	16	99.3

Fig. 1. Elution profile of phosphoenolpyruvate carboxykinase activity on norleucine-Sepharose

The column (2.5 cm $\times$ 25 cm) was equilibrated with 30 mM-phosphate buffer, pH 7.0, containing 1.5 M-(NH₄)₂SO₄, 0.5 mM-EDTA and 2 mM-mercaptoethanol. The initial elution of the column was started with 150 ml of 30 mM-phosphate/1.5 M-(NH₄)₂SO₄ (containing 0.5 mM-EDTA and 2 mM-mercaptoethanol), pH 7.0, and then followed with a decreasing linear concentration gradient (300 ml) of 30 mM-phosphate/1.5 M-(NH₄)₂SO₄ 20 mM-phosphate/0.5 M-(NH₄)₂SO₄, pH 7.0 (the starting point is indicated by an arrow). Fractions (4.7 ml) were collected at a flow rate of about 50 ml/h. Enzyme activity (●) was assayed spectrophotometrically as described in the Experimental section, and protein (○) was estimated on the basis of the A₂₈₀.

2). In the initial paper by Hung & Silverstein (1978) they eluted the hog and human liver enzyme with 0.025 mM-IDP after a preliminary wash with a mixture of nucleotides. We also found that the preliminary wash-out step with a mixture of nucleotides can also be omitted with no significant change in the purity of the preparation. A typical elution profile from the GMP-Sepharose column, by using this technique, is shown in Fig. 2.

In sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, the purified enzyme migrated as a single protein band (Fig. 3) with M_r 68 500 $\pm$ 680 (four experiments). Spectrophotometric scans of such gels usually indicated a single peak of absorbance. The M_r marker proteins were phosphorylase *b* (92 500), bovine serum albumin (66 200), ovalbumin (45 000), carbonic anhydrase (31 000), soya-bean trypsin inhibitor (21 500) and lysozyme (14 400).

These results show that phosphoenolpyruvate carboxykinase from guinea-pig liver mitochondria has been purified to a high degree of electrophoretic homogeneity. While these studies were in progress, improvements in the Hung & Silverstein (1978) procedure were published by Colombo *et al.*

(1978) and Brinkworth *et al.* (1981), who purified the enzyme from rat liver cytosol. In their studies 1,6-diaminohexane (instead of norleucine) was linked to Sepharose, and the rat liver enzyme was eluted in the affinity step with a salt gradient (0–500 mM-KCl) instead of with IDP.

To our knowledge there has been one previous purification of mitochondrial phosphoenolpyruvate carboxykinase from guinea-pig liver. In that study (Holten & Nordlie, 1965) the enzyme was purified about 50-fold to a final specific activity of 6–9 units/mg of protein; no degree of purity was indicated. In any case that study was performed long before the advent of affinity chromatography. Our present study shows that this enzyme can be purified at least 100-fold to a high degree of homogeneity and specific activity. The whole procedure can be completed in less than 3 days.

Antibodies were raised in rabbits against the purified enzyme, and their purity and specificity were verified by the Ouchterlony double-immunodiffusion analysis and by immunotitration. In the immunodiffusion analysis, a single precipitin line (with no spur formation) was observed in the presence of crude mitochondrial extract from

Fig. 2. Elution profile of phosphoenolpyruvate carboxykinase activity on GMP-Sepharose

The column (2.5 cm $\times$ 15 cm) was equilibrated with 5 mM-phosphate buffer containing 0.5 M-EDTA and 2 mM-mercaptoethanol, pH 6.1. The elution schedule was as follows: (A) 315 ml of equilibrating buffer; (B) 100 ml of 0.2 mM-IDP in 5 mM-phosphate buffer containing 0.5 mM-EDTA and 2 mM-mercaptoethanol, pH 7.0. Fractions 2–21 (4.3 ml each) were collected at a flow rate of about 20 ml/h. Enzyme activity (●) was determined spectrophotometrically as described in the Experimental section and protein (○) was determined by the method of Lowry *et al.* (1951).

guinea-pig liver and highly purified enzyme (Fig. 4). No precipitation was observed in the presence of extracts from guinea-pig liver cytosol or rat liver cytosol. In the quantitative immunoprecipitation test, various amounts of enzyme were incubated with a constant amount of antibody, and the amount of enzyme units recovered in the supernatant after centrifugation was corrected for non-specific precipitation with non-immune serum. In Fig. 5 the recovered activities are plotted against the amounts of enzyme units added initially. The results indicated a high degree of specificity of the antibody preparation in that (i) the antibody did not inhibit phosphoenolpyruvate carboxykinase activity in the cytosol extract and (ii) the antibody inhibited the activity of the mitochondrial enzyme. With both crude mitochondrial extracts and highly purified enzyme, the equivalence point was the same (60.9 ± 0.3 munits) irrespective of the purity of the enzyme added. Beyond the equivalence point the recovered activity was proportional to the units of enzyme activity added.

The availability of a highly specific antibody to mitochondrial phosphoenolpyruvate carboxykinase should now allow resolution of the question of whether the amount of this enzyme is altered by starvation, growth and development, as suggested by previous studies (Elliott & Pogson, 1977;

Fig. 3. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of purified guinea-pig liver mitochondrial phosphoenolpyruvate carboxykinase

A sample of mitochondrial phosphoenolpyruvate carboxykinase purified as described in Table 1 was electrophoresed as described by Laemmli (1970). Electrophoresis was performed with a 3%-acrylamide stacking gel and 10%-acrylamide separating gel (1.5 mm-thick slab gel) at a current of 20 mA/slab gel for 4 h at room temperature. After electrophoresis, slab gels were stained for 1 h in 0.05% Coomassie Brilliant Blue R-250 in 25% propan-2-ol/10% acetic acid and then destained overnight in 10% propan-2-ol/10% acetic acid. Left lane, 25 μ g of purified protein; right lane, 5 μ g of purified protein.

Raghunathan & Arinze, 1977) relying solely on enzyme-activity measurements. Therefore, using this antibody, we measured the amounts of mitochondrial phosphoenolpyruvate carboxykinase in liver during neonatal development as well as in livers of 48 h-starved guinea pigs. Fig. 6 shows that in the livers of neonatal animals the amount of immunoprecipitable phosphoenolpyruvate carboxykinase-specific protein is least at term and increases subsequently up to day 7. In this series of immunotitrations, the equivalence points are different for the various age groups; thus smaller amounts of antibody are required to precipitate the same amount of enzyme protein until 7–14 days of

Fig. 4. Analysis of purified phosphoenolpyruvate carboxykinase by Ouchterlony double immunodiffusion

The antibody preparation was diluted 1:10 with 0.9% NaCl and a 20 μ l portion was added to the centre well; the outer wells contained 20 μ l each of the following: (1) guinea-pig liver mitochondrial extract, (2) guinea-pig liver cytosol, (3) rat liver cytosol, (4) rat liver mitochondrial extract, (5) 0.9% NaCl and (6) purified guinea-pig liver mitochondrial enzyme. The crude extracts were prepared from 10% liver homogenates. Rat liver mitochondrial pellets were resuspended in 1 volume equivalent of the original tissue weight.

Table 2. Developmental profile of mitochondrial phosphoenolpyruvate carboxykinase activity in neonatal guinea-pig liver

Phosphoenolpyruvate carboxykinase activity was measured in the same extracts used for the immunotitration data displayed in Fig. 6. The values are means $\pm$ S.E.M. for the numbers of animals indicated in parentheses.

Time after birth (days)	Enzyme activity (μ mol/min per g wet wt. of liver)
0	6.2 $\pm$ 0.2 (8)
3	8.0 $\pm$ 0.6 (7)
7	11.9 $\pm$ 0.9 (6)
14	11.0 $\pm$ 0.4 (6)
21	11.7 $\pm$ 0.5 (9)

age, indicating an increase in immunoprecipitable enzyme during development. These data parallel the 2-fold increase in enzyme activity during neonatal development (Table 2; see also Raghunathan & Arinze, 1977) and support the conclusion that the development-dependent increase in activ-

Fig. 5. Immunotitration curves for phosphoenolpyruvate carboxykinase from cytosol and mitochondria

Diluted (1:25, v/v) antibody (20 μ l) prepared against highly purified mitochondrial phosphoenolpyruvate carboxykinase was mixed with various amounts of enzyme from liver cytosol ($\square$), liver mitochondrial extract ($\bullet$) or purified enzyme (Δ) and incubated at 37°C for 15 min, and then at 0°C overnight. After centrifugation at 1700g for 5 min, the activity of phosphoenolpyruvate carboxykinase was determined in the supernatants. The recovered activity in each supernatant was corrected for non-specific precipitation by using non-immune serum.

Fig. 6. Quantitative immunotitration of mitochondrial phosphoenolpyruvate carboxykinase in neonatal guinea-pig liver

Mitochondrial extracts were prepared from livers of guinea pigs at term ($\blacktriangle$), 3–4 days ($\bullet$), 7 days ($\circ$), 14 days ($\square$) and 21 days ($\blacksquare$) after birth. To 40 μ l of antibody (1:10 dilution) were added the amounts of enzyme extracts indicated. The total volume was 0.6 ml, made up with 5 mM-phosphate buffer, pH 7.4, containing 0.14 M-NaCl, 1 mM-EDTA and 2 mM-mercaptoethanol. The mixture was incubated at 37°C for 15 min, then overnight at 0°C. Enzyme activity remaining after neutralization by antibody was determined. The recovered activity in each supernatant was corrected for non-specific precipitation by using non-immune serum.

Fig. 7. Immunotitration of mitochondrial phosphoenolpyruvate carboxykinase in livers of fed and starved guinea pigs

Animals weighing 350–400g were used. Liver mitochondrial extracts were prepared from 10% homogenates made up with 0.32M-sucrose/20mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] buffer, pH7.4, containing 2mM-EDTA. Increasing amounts of antibody were added to 200μl of mitochondrial extract to a final volume of 0.4ml, made up with 5mM-potassium phosphate buffer, pH7.4. The enzyme activity remaining in the supernatant after neutralization with antibody was assayed as described in Fig. 6: ○, fed animals; △, 48h-starved animals, □, 72h-starved animals.

ity is mediated by accumulation of phosphoenolpyruvate carboxykinase-specific protein.

On the other hand, starvation for 48 or 72 h does not lead to any alterations in the amounts of immunoprecipitable enzyme (Fig. 7). With mitochondrial extracts from both fed (control) and starved adult animals, the same amount of enzyme activity is recovered in the supernatant at any given dose of antibody added, i.e. no change in equivalence point. These data, which indicate that starvation does not lead to induction of mitochondrial phosphoenolpyruvate carboxykinase, do not support the conclusions of Elliott & Pogson (1977), which were based on measurements of enzyme activity only. In the experiments summarized in Fig. 7, we could not detect any significant changes in enzyme activity whether the activity was expressed per mg of protein or per g of liver. As indicated in the introduction, there appears to be some uncertainty as to whether mitochondrial phosphoenolpyruvate carboxykinase is induced under starvation conditions. Unlike previous experiments, which were based on enzyme-activity measurements only, our present studies have

quantified the amount of mitochondrial phosphoenolpyruvate carboxykinase by using antibodies specific to the enzyme. The data indicate that starvation does not increase the amount of this enzyme in guinea-pig liver.

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