BBA 66104

DISTRIBUTION OF PHOSPHOPYRUVATE CARBOXYLASE IN PIG LIVER

KENNETH R. SWIATEK, KUEN LAN CHAO, HSIANG LIN CHAO, MARVIN CORNBLATH AND TYSON TILDON

Illinois State Pediatric Institute, Department of Biological Chemistry, University of Illinois College of Medicine, Chicago, Ill. and the Department of Pediatrics, School of Medicine, University of Maryland, Baltimore, Md. (U.S.A.)

(Received December 16th, 1969)

SUMMARY

Phosphopyruvate carboxylase (GTP:oxaloacetate carboxylase (transphorylating), EC 4.1.1.32) activity has been found in pig and lamb liver mitochondria and in the soluble cell fraction in rat, hamster, and mouse livers. In pig liver, the cellular distribution was found to be 60–70% soluble and 15–25% mitochondrial. This cellular distribution was similar in both fed and 72-h fasted pigs. The specific activity increased 2 fold in the mitochondria and 3 fold in the soluble fraction prior to sacrifice.

The K_m constants for pig phosphopyruvate carboxylase at pH 6.4 obtained from the 100 000 $\times g$ cell supernatant were phosphoenolpyruvate, $6.65 \cdot 10^{-4}$ M; Mn^{2+}, $8.33 \cdot 10^{-4}$ M; $NaHCO_3$, $1.47 \cdot 10^{-2}$ M; and IDP, $5.4 \cdot 10^{-5}$ M.

INTRODUCTION

Phosphopyruvate carboxylase (GTP:oxaloacetate carboxylase (transphosphorylating), EC 4.1.1.32) has been shown to be involved in the over-all formation of phosphoenolpyruvate (phosphopyruvate) in the livers of many mammalian species[1]. Recent studies[2–5] have shown phosphopyruvate carboxylase to be absent in fetal rat liver and have established some of the factors which influence its appearance after birth. The ability of rat liver to perform gluconeogenesis has been directly correlated to the level of this hepatic enzyme[3].

Phosphopyruvate carboxylase activity has been demonstrated in the liver mitochondria of both pig[6] and lamb[7]. It also has been found in the soluble and mitochondrial fractions of several other mammalian species[1]. In the rat, hamster and mouse livers more than 90% of the activity resides in the soluble cell fraction, while in rabbit and guinea pig liver, 50–60% is in the mitochondrial fractions[1]. CHANG AND LANE[6] have purified phosphopyruvate carboxylase from pig liver mitochondria and in addition have reported that a large portion exists in the mitochondrial cell fraction (35–100%).

In the course of our studies into the mechanism of hypoglycemia[8] and the

development of gluconeogenic enzymes in the pig, we have observed several unique aspects of the phosphopyruvate carboxylase enzyme. It is the purpose of this paper to report the cellular distribution of phosphopyruvate carboxylase in the pig liver, to report the kinetic properties of the enzyme obtained from the soluble cell fraction and to describe the assay procedure utilized for its assay.

METHODS

Materials

Imidazole–HCl, the trisodium salt of phosphoenolpyruvate, the dilithium salt of IDP, and malate dehydrogenase (porcine heart) were obtained from Calbiochem., Los Angles, Calif. NADH was purchased from Boehringer–Mannheim Co., New York, N.Y.

Chemical analyses

Protein was determined by the method of LOWRY *et al.*[9]. Commercial IDP was purified prior to its use in kinetic studies.

The dilithium salt of IDP (250 mg) was dissolved in water and applied to a DEAE-cellulose column (2 cm × 30 cm) in the bicarbonate form previously equilibrated with 0.5 M triethylammonium bicarbonate buffer (pH 7.5). The column was washed with water until the absorbance of the readings at 260 nm was negligible. The column was then eluted with a linear gradient from 1 l of 0.4 M triethylammonium bicarbonate in the reservoir and 1 l of water in the mixing chamber. The flow rate was 2 ml/min and 10-ml fractions were collected. The main peak containing IDP was eluted at a triethylammonium bicarbonate concentration of 0.08–0.18 M. Two smaller peaks were also eluted which were identified as IMP and ITP. The desired peak was evaporated to dryness on a rotary evaporator at 30° and the residue triethylammonium bicarbonate was removed by repeated evaporations with methanol. The residue was dissolved in water and was converted to the lithium salt by passage through a Dowex 50 (Li^+) column (1 cm × 25 cm). The eluate was collected and 196 mg of IDP was obtained after lyophilization. It migrated as a single spot on silica gel thin-layer chromatograms in *n*-propanol–conc. NH_4OH–water (6:3:1, by vol.).

Homogenate preparation

Three-week-old pigs were anesthetized with sodium amytal (10 mg/100 g body wt.) and their livers rapidly removed. A portion of each liver was frozen in liquid nitrogen and stored at −60° to determine phosphopyruvate carboxylase stability in frozen tissue. All livers were homogenized for approx. 1 min with 9 ml of 0.25 M sucrose per g tissue in a motor-driven Potter–Elvehjem type grinder fitted with a Teflon pestle. A portion of the liver homogenate was centrifuged directly at 100 000 × *g* for 30 min at 4° to obtain particle-free supernatant which was used for kinetic determinations as well as to determine enzyme stability in this supernatant fraction. The particle-free fraction was divided into three portions; one portion assayed initially and after 10, 24, and 35 days of storage in a cold room at 4°, the second portion was frozen and stored in one tube. This sample was thawed, assayed and refrozen on days 10, 24, and 35. The third portion was frozen in separate tubes, and one tube thawed and assayed on each of the same days indicated above. In addition, 100 000 × *g*

particle-free supernatants were prepared from frozen pig liver and assayed after 24 and 35 days of storage at −60°. Nuclei, mitochondria, microsomes, and particle-free fractions were prepared by the method of SCHNEIDER[10]. Particle material was resuspended in 0.25 M sucrose prior to assay.

Phosphopyruvate carboxylase assay

The assay procedure determines the amount of oxaloacetic acid formed from phosphoenolpyruvate by measuring the rate of NADH oxidation in the presence of excess malate dehydrogenase in a Gilford 2400 multiple sample spectrophotometer at 340 nm and at 37°.

The assay mixture contained in micromoles unless otherwise specified: imdazole–HCl buffer, 110, pH 5.8; $MnCl_2$, 6.6; NADH, 0.31; IDP, 2.7; malate dehydrogenase, 50 μg; supernatant or cellular fractions, 0.02 ml; $NaHCO_3$, 75. The reaction was initiated by the addition of 6.8 μmoles of phosphoenolpyruvate. The final pH of this mixture was 6.3. The assay mixture maintained a constant pH during the course of the reaction and the reaction was linear for at least 5 min. $NaHCO_3$ was omitted from the reaction and served as the reaction blank. The total volume was 1 ml.

RESULTS

Subcellular distribution of pig liver phosphopyruvate carboxylase

Results of studies of phosphopyruvate carboxylase activity levels in various subcellular portions of pig liver are summarized in Table I. The data were obtained from five 72-h fasted and three fed control pigs and represent the mean activity in various fractions assayed under identical conditions. The tissue distributional pattern was essentially unchanged in both groups of animals although the total activity of the fasted pigs was twice as high as the fed animals. In both groups of animals, 60–70% of phosphopyruvate carboxylase activity was located in the soluble cell fraction and 15–25% was located in the mitochondrial cell component. The specific activity (μmoles oxaloacetate per mg protein per min) of the soluble and mitochondrial cell

TABLE I

SUBCELLULAR DISTRIBUTION OF PHOSPHOPYRUVATE CARBOXYLASE ACTIVITY IN FED AND 72-h FASTED PIG LIVER

State of animal	*Cell fraction*	*Total activity**	*Specific activity***	*Total protein in each fraction (mg)*
Fed	Nuclei	1.30	0.030	43.1
	Mitochondria	2.66	0.096	27.6
	Microsomes	0.07	0.002	31.0
	Soluble	6.34	0.065	97.1
Fasted	Nuclei	1.28	0.066	18.6
	Mitochondria	4.30	0.181	24.1
	Microsomes	1.01	0.032	31.0
	Soluble	18.06	0.176	101.4

* Total activity expressed as μmoles oxaloacetate produced per min per g wet liver.
** Specific activity expressed as μmoles oxaloacetate produced per min per mg protein.

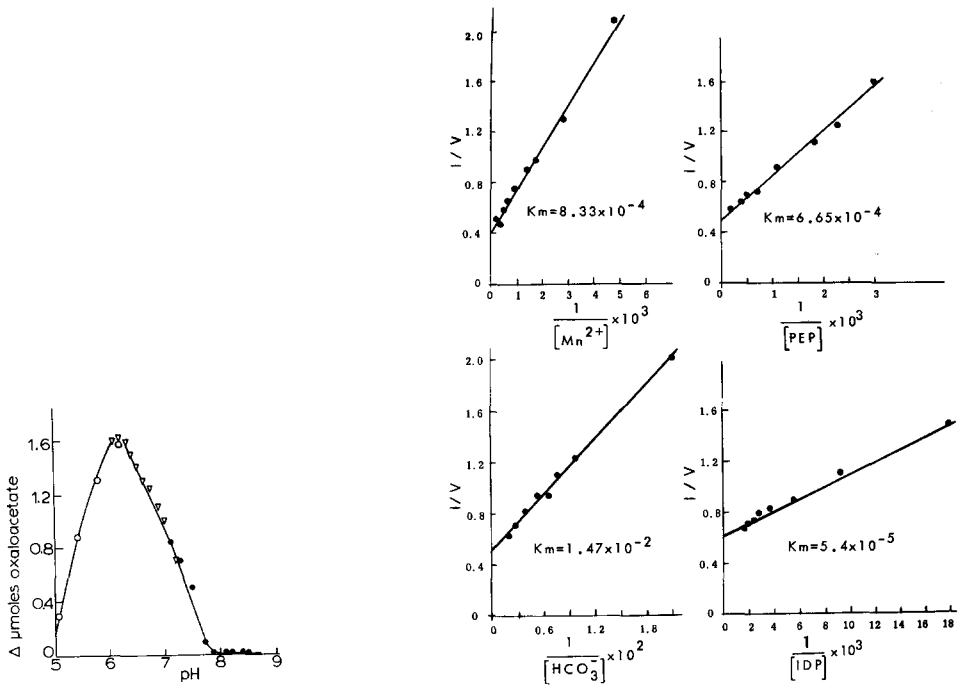

Fig. 1. Phosphopyruvate carboxylase activity as a function of pH. The reaction mixtures were identical with those in methods except that 100 μmoles of acetate buffer (○) and 100 μmoles of Tris–HCl (●) were used in addition to 100 μmoles of imidazole–HCl (△) to obtain proper pH ranges. Assay mixtures were prepared in duplicate, and pH measurements made on one series while activity measurements were performed with the duplicate series.

Fig. 2. Lineweaver–Burk plots of the effect of Mn^{2+}, phosphoenolpyruvate (PEP), HCO_3^-, and IDP on oxaloacetate formation. The composition of the reaction mixture was as described under phosphopyruvate carboxylase assay except for the substrate indicated. The final pH was 6.4.

fractions were similar. Two-fold increases in the specific activity occurred in cell fractions prepared from livers of fasted animals. It is interesting to note that the total protein content of both the soluble and mitochondrial cellular fractions were essentially identical in livers obtained from fed and starved animals.

Our studies indicate that oxaloacetate formation from phosphoenolpyruvate and IDP in the soluble cellular portion from pig liver proceeded optimally at pH 6.2–6.4 in imidazole–HCl buffer (Fig. 1). This value is very similar to the pH optimum of 6.4–6.7 obtained using the same buffer system for phosphopyruvate carboxylase from pig liver mitochondria[6]. This was far removed from the pH 8.0 value in Tris–HCl or Tris–maleate buffers reported for the guinea pig mitochondrial enzyme[1].

To insure that future determinations of phosphopyruvate carboxylase activity in developing pig liver were assayed under optimal conditions but not at inhibitory concentrations, the kinetic constants for substrates and cofactors were determined and are summarized in Fig. 2.

The K_m value for $NaHCO_3$ (0.0147 M) at pH 6.4 indicates that the enzyme would be only half-saturated with HCO_3^- at physiological concentrations of HCO_3^- (0.02 M) for mammalian plasma.

The K_m values determined for phosphoenolpyruvate, IDP, and Mn^{2+} are quite similar to those reported for the pig mitochondrial enzyme[6]. GDP was as active as was IDP in the reaction, but ADP and IMP were completely inactive.

While a Mn^{2+} concentration of $8.8 \cdot 10^{-3}$ M produced optimal activity, the same concentration of Mg^{2+} was only 30% as active.

Stability of phosphopyruvate carboxylase in pig liver and 0.25 M sucrose homogenates

Enzyme activity measured in 100 000 $\times g$ supernatant fractions prepared from livers stored frozen at −60° for up to 35 days remained essentially unchanged (Table II). Particle-free supernatants frozen and thawed several times during the 35 days lost about 27% of their activity, while supernatants kept at 4° lost almost half of their original activity after 35 days. In contrast, supernatants kept frozen for as long as 35 days did not lose any significant activity.

Attempts to solubilize the pig mitochondrial carboxylase enzyme by the procedure used by BANDURSKI AND LIPMANN[7] for the solubilization of lamb mitochondrial

TABLE II

THE EFFECT OF STORAGE ON THE STABILITY OF PIG PHOSPHOPYRUVATE CARBOXYLASE ACTIVITY IN 100 000 $\times$ g SUPERNATANT FRACTION AND FROZEN LIVER

Length (days)	*Method of storage**			*Frozen liver*
	4°	*Frozen and thawed*	*Frozen*	
Initial	1.66**	1.66	1.66	1.42
10	1.54	1.40	1.56	—
24	1.03	1.29	1.57	1.39
35	0.87	1.22	1.49	1.46

* Conditions of storage as indicated in METHODS under homogenate preparation.
** All activities expressed as μmoles oxaloacetate per min per ml supern ıtant.

enzyme or employed by NORDLIE AND LARDY[1] for guinea pig liver mitochondrial enzyme were unsuccessful. While approx. 5% of the pig mitochondrial enzyme could be solubilized by repeated freezing and thawing, over 50% of the total mitochondrial activity was lost by this procedure.

DISCUSSION

In unpublished results CHANG AND LANE[6] state that 35–100% of pig phosphopyruvate carboxylase activity was found in the mitochondrial cell fractions. Results reported in this study demonstrate that the enzyme distribution is approx. 70% soluble and 20% mitochondria. In addition, the nutritional state of pigs prior to sacrifice did not influence the distributional pattern (Table I). Starvation, however, did increase the total activity almost 3 fold. Furthermore, the specific activity in both the mitochondrial and in the 100 000 $\times g$ cell fractions were similar under fed

and fasted conditions. Although there was no significant change in the protein content between fed and fasted mitochondrial and soluble cell fractions, there was an increase in activity which would indicate a shift in protein synthesis towards gluconeogenic reactions.

In the rat liver, phosphopyruvate carboxylase has been suggested as one of the enzymes controlling the rate of gluconeogenesis from lactate and other gluconeogenetic substrates[11,12]. The responsiveness of the pig enzyme to changes in nutritional state indicates its involvement as one of the controlling factors of gluconeogenesis in this animal as well.

From our data obtained at 37° and under standard assay conditions, rates for oxaloacetate conversion to phosphoenolpyruvate of 2–5 μmoles/min per g liver was calculated for the mitochondrial cell fraction. A rate of 5–9 μmoles/min per g liver was found for the soluble cell fraction from livers of fed animals and 15–21 μmoles/min per g liver in the fasted pigs. The data and conclusions reported here are consistent with the recent work of WILLIAMSON *et al.*[13,14] showing the importance of compartmentalization in hepatic gluconeogenesis. It would certainly appear that the capacity of the mitochondrial and soluble cell fractions would account for the major portion of liver gluconeogenesis.

ACKNOWLEDGMENT

This investigation was supported in part by Public Health Research Grants HD-03953-01 and HD-03959-01.

REFERENCES

1 R. C. NORDLIE AND H. A. LARDY, *J. Biol. Chem.*, 238 (1963) 2259.
2 D. YEUNG, R. S. STANLEY AND I. T. OLIVER, *Biochem. J.*, 105 (1967) 1219.
3 D. YEUNG AND I. T. OLIVER, *Biochem. J.*, 105 (1967) 1229.
4 D. YEUNG AND I. T. OLIVER, *Biochem. J.*, 108 (1968) 325.
5 D. YEUNG AND I. T. OLIVER, *Biochemistry*, 7 (1968) 3231.
6 H. C. CHANG AND M. D. LANE, *J. Biol. Chem.*, 241 (1966) 2413.
7 R. S. BANDURSKI AND F. LIPMANN, *J. Biol. Chem.*, 219 (1956) 741.
8 K. R. SWIATEK, D. M. KIPNIS, G. MASON, K. L. CHAO AND M. CORNBLATH, *Am. J. Physiol.*, 214 (1968) 400.
9 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
10 W. C. SCHNEIDER, *J. Biol. Chem.*, 176 (1948) 259.
11 J. H. EXTON AND C. R. PARK, *J. Biol. Chem.*, 242 (1967) 2622.
12 J. H. EXTON AND C. R. PARK, *J. Biol. Chem.*, 243 (1968) 4189.
13 J. R. WILLIAMSON, E. T. BROWNING AND R. SCHOLZ, *J. Biol. Chem.*, 244 (1969) 4607.
14 J. R. WILLIAMSON, R. SCHOLZ AND E. T. BROWNING, *J. Biol. Chem.*, 244 (1969) 4617.