A REINVESTIGATION OF THE KINETIC PARAMETERS OF PHOSPHOENOLPYRUVATE CARBOXYKINASE

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SUMMARY

A new assay for phosphoenolpyruvate carboxykinase has been devised whose sensitivity is sufficient to detect the formation by crude hepatic extracts of 1×10^{-11} moles of phosphoenolpyruvate. By the utilization of this assay it has been determined that the apparent Km of oxaloacetate is in the range of 1 to 5×10^{-6} M for the hepatic enzymes from rat and guinea pig. These values are approximately two orders of magnitude lower than all previous data and indicate that the enzyme is sensitive to modulations of oxaloacetate concentrations that occur physiologically.

INTRODUCTION

Phosphoenolpyruvate carboxykinase (PEPCK) was first described by Utter and Kurahashi (1) utilizing an assay based on the incorporation of $^{14}CO_2$ into oxaloacetate. Assays for the enzyme, measured in what is considered to be the physiological direction of phosphoenolpyruvate formation (2) have been described that are based on either the cleavage of phosphoenolpyruvate in the presence of mercuric chloride (3) or the utilization of DPNH in the presence of pyruvate kinase and lactate dehydrogenase (4). The latter assay can only be utilized for the measurement of PEPCK preparations that have been partially purified to remove contaminating enzymes that compete for the utilization of the various intermediates. The former method, as based on the colorimetric determination of phosphate, although suitable for the determination of PEPCK in crude cellular fractions, has a sensitivity that is extensively below that needed to examine the reaction in the concentration ranges of oxaloacetate that occur physiologically. By the utilization of a new procedure that is suitable for the determination of this enzyme in unfractionated hepatic extracts at physio-

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logical concentrations of oxaloacetate, it is demonstrated in this communication that the apparent Km of oxaloacetate is 50 to 100 times lower than any previous determination (2,5).

MATERIALS AND METHODS

PEPCK was determined by a method based on the incorporation of ^{32}P into phosphoenolpyruvate from either γ-^{32}P-GTP or γ-^{32}P-ITP. The ^{32}P-phosphoenolpyruvate was determined as $^{32}PO_4$, following cleavage with mercuric chloride as described by Lehmann and Meyerhof (6), by precipitation of the triethylamine-molybdate-phosphate complex according to the procedure of Sugino and Miyoshi (7). When using crude cellular extracts it was necessary to precipitate any phosphate formed from hydrolysis of the trinucleotide, prior to the cleavage of phosphoenolpyruvate with mercuric chloride.

The incubation mixture for the PEPCK reaction contained 20 mM Tris chloride, pH 8.0; 4 mM sodium fluoride; 5 mM magnesium chloride; 0.1 mM glutathione; 0.1 mM γ-^{32}P-nucleotide triphosphate; 1 mM oxaloacetate (unless otherwise indicated); and a source of the enzyme in a total volume of 0.2 ml. The reaction was initiated by the addition of enzyme and incubated at 30° for the periods indicated. The reaction was terminated by the addition of 1.0 ml of 0.2 N perchloric acid containing 0.2 mM sodium phosphate. Phosphate was precipitated from this solution by the addition of 1.0 ml of a solution of 2% (w/v) of ammonium molybdate containing 50 mM triethylamine hydrochloride. Complete precipitation of phosphate was insured by allowing the reaction mixture to stand at 0° for 10 min. All other operations were performed at 0°. The precipitate containing denatured protein and triethylamine-molybdate-phosphate complex was removed by centrifugation, and to a 1.5 ml-aliquot of the supernatant solution was added 1.5 ml of 0.25 M mercuric chloride. The solution was maintained at 0° for 45 min followed by the addition of 20 µl of 0.16 M triethylamine hydrochloride-10 mM sodium phosphate and by an additional 30 min incubation at 0°. The supernatant solution was removed by centrifugation and the precipitate was washed by the addition of 2 ml of 0.2 N perchloric acid con-

taining 0.5% (w/v) ammonium molybdate and 50 mM triethylamine hydrochloride. This washing procedure was repeated three times. The final precipitate was dissolved in 2 ml of Aquasol (New England Nuclear) and counted in a Packard 3375 Scintillation Counter.

γ-^{32}P-GTP and γ-^{32}P-ITP were prepared by an adaptation of the procedure of Glynn and Chappell (8) according to a suggestion of Dr. Earl Davie, University of Washington, Seattle. This procedure yields a product of specific activity of 1×10^9 dpm per µmole of nucleotide.

RESULTS

The results of a typical assay of PEPCK utilizing rat liver cytosol as the source of the enzyme are indicated in Fig. 1. The extent of formation of phosphoenolpyruvate is linear with respect to both enzyme concentration (Fig. 1A) and incubation time (Fig. 1B). Fig. 1 illustrates that the determination is sensitive at a minimal production of 10 µµmoles of phosphoenolpyruvate and that even at an oxaloacetate concentration of 1×10^{-6} M the rate of formation of product is linear for at least 4 mins. The assay is extremely

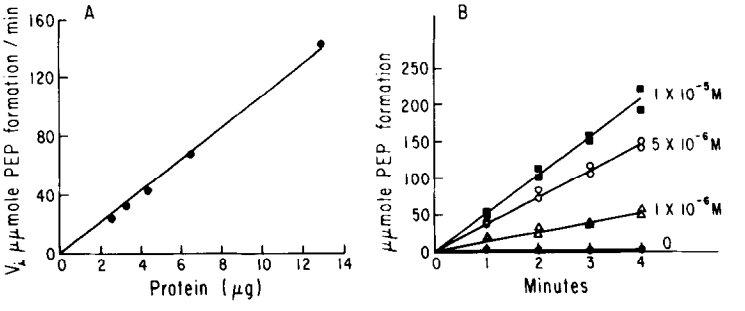

Fig. 1 A - Variation of the initial rate of phosphoenolpyruvate formation with varying concentrations of rat liver cytosol. Initial reaction velocities were determined from the linear production of phosphoenolpyruvate over a 4 min period
B - Variation of phosphoenolpyruvate formation with incubation time at 0 (●——●), 1 x 10-6 M (Δ——Δ), 5 x 10-6 M (o——o) and 1 x 10^{-5} M (■——■) oxaloacetate. The concentration of rat liver cytosol protein used was 5.5 µg per incubation. In both A and B the enzyme was prepared from rats fed chow ad libitum. The livers were homogenized in 5 volumes of 0.25 M sucrose - 5 mM Tris chloride, pH 7.4 - 1 mM β-mercaptoethanol. The homogenate was centrifuged at 105,000 x g for 1 h. The supernatant solution obtained by this procedure was dialyzed for 1.5 h in 0.25 M sucrose - 5 mM Tris chloride, pH 7.4 - 0.1 mM β-mercaptoethanol and diluted in this buffer immediately prior to assay. All other conditions are described in the text.

sensitive and a significant phosphoenolpyruvate formation can be detected with a protein concentration of 2 μg of unfractionated cytoplasmic protein per 0.2 ml reaction mixture. Reactions which could potentially utilize phosphoenolpyruvate prior to its determination are not of significance under the assay conditions. Enolase is inhibited by the presence of fluoride. Pyruvate kinase is of insignificant activity under the conditions of the assay because of the low amounts of cytoplasmic protein used in the assay and since the total phosphoenolpyruvate concentration produced during the incubation is less than 1×10^{-6} M; a concentration that is at least two orders of magnitude lower than the Km of phosphoenolpyruvate for pyruvate kinase (10).

The initial reaction velocities, assayed as indicated in Fig. 1B, were determined at varying oxaloacetate concentrations for extracts of either rat liver cytosol or guinea pig liver mitochondria. Typical saturation plots were obtained for each of these (Fig. 2 and 3, respectively). Lineweaver Burk plots of these data indicated an apparent Km for oxaloacetate of 5×10^{-6} and 1.5×10^{-6} M for the rat liver cytoplasmic and guinea pig liver mitochondria enzymes, respectively.

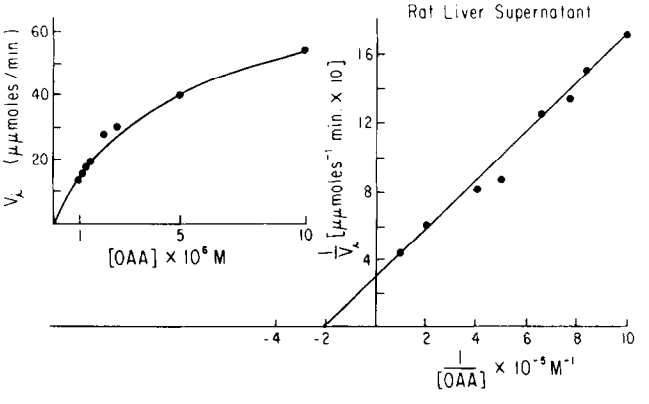

Fig. 2 - Double reciprocal plot of the initial reaction velocity of rat liver PEPCK against oxaloacetate concentration. Conditions are identical to those of the experiment depicted in Fig. 1B. Insert indicates plot of initial reaction velocity versus substrate concentration.

TABLE I

Apparent Michealis Constants for Oxaloacetate

Enzyme Source	Michaelis Constant[a]	
	Phosphate donor	
	GTP	ITP
Rat liver cytosol	5×10^{-6} M	5×10^{-6} M
Guinea pig liver cytosol[b]	5×10^{-6} M	4×10^{-6} M
Guinea pig liver mitochondria	1.5×10^{-6} M	1.5×10^{-6} M

a Determined as depicted in Fig. 2 and 3.

b Prepared as indicated in the legend of Fig. 1 for rat liver with the exception that the guinea pigs were starved for 48 hr prior to sacrifice.

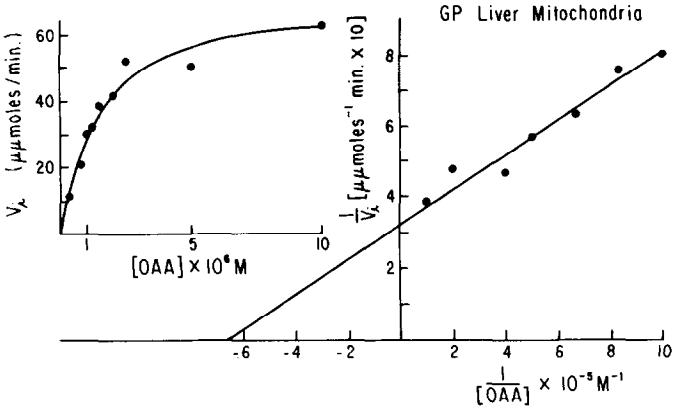

Fig. 3 - Double reciprocal plot of the initial reaction velocity of guinea pig liver mitochondrial PEPCK against oxaloacetate concentration. All conditions are identical to those of the experiment depicted in Fig. 1B with the exception that the enzyme was prepared as follows: Liver was homogenized in 5 volumes of 0.25 M sucrose - 5 mM Tris chloride, pH 7.4 - 1 mM β-mercaptoethanol and the mitochondria separated according to the procedure of Schneider (18). Mitochondria were suspended in 5 mM Tris chloride, pH 7.4, -1 mM β-mercaptoethanol, frozen and thawed three times, and the insoluble material removed by centrifugation. The enzyme was dialyzed as described for the rat liver protein (Fig. 1B). The reaction mixture contained 1.1 μg of protein.

The apparent Km values[1] for oxaloacetate utilizing either GTP or ITP as

[1]The kinetic mechanism of phosphoenolpyruvate carboxykinase is either Random or Ordered (unpublished observations, Walsh, D. A. and Chen, L.), thus the true Km values will be slightly lower than those obtained in this study.

the phosphate donor were determined for the enzyme in crude hepatic extracts of cytosol from either rat or guinea pig and of mitochondria from guinea pig. These are presented in Table I. For each of these the apparent Km for oxaloacetate was in the range of 1.5 to 5 x 10^{-6} M and was independent of the phosphate donor utilized.

DISCUSSION

The physiological concentration of oxaloacetate in the cytosol varies in the range of 2 to 10 x 10^{-6} M whereas that in the mitochondria is between 1 to 20 x 10^{-7} M; each depending on the metabolic status of the animal (11,12). The Km constants determined in this study would indicate that the flux of metabolites to phosphoenolpyruvate would be markedly sensitive to physiological modulations of oxaloacetate concentration. In addition, on the basis of the Michaelis constants of oxaloacetate for the various enzymes utilizing or producing oxaloacetate [citrate synthetase, 4 x 10^{-6} M (13); mitochondrial malate dehydrogenase, 2 x 10^{-6} M (14); mitochondrial glutamate: oxaloacetate transaminase, 2.5 x 10^{-5} M (14); pyruvate carboxylase, 5 x 10^{-5} M (15); cytoplasmic malate dehydrogenase 9 x 10^{-6} M (16); cytoplasmic glutamate: oxaloacetate transaminase, 4.0 x 10^{-5} M (17)] it would appear that the flux to phosphoenolpyruvate could adequately compete with each of these reactions. Previous determinations of the Km for oxaloacetate indicated the values to be 3 x 10^{-3} M for the guinea pig liver mitochondrial enzyme (2), 1.5 x 10^{-4} M for the pig liver mitochondrial enzyme (5), and 1.5 x 10^{-4} M for the rat liver cytoplasmic enzyme (14). The divergence of results between the previous studies and this communication may be due to the denaturation of the enzyme occurring during the various purification procedures used (2,5,14) or the removal of an essential cofactor. Alternatively, the enzyme may exist in two different forms. If the latter were true, it could serve as a potential mechanism for the control of gluconeogenesis.

ACKNOWLEDGEMENTS

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