Kinetic Studies with Cytosol and Mitochondrial Phosphoenolpyruvate Carboxykinases

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1. Measurements of Michaelis constants for oxaloacetate in the reaction catalysed by liver phosphoenolpyruvate carboxykinase give values much lower than previously reported. With Mg^{2+} as bivalent cation, the Michaelis constant was approx. $2.5 \times 10^{-5} M$ whether the enzyme used was the mitochondrial phosphoenolpyruvate carboxykinase purified from sheep liver or chicken liver or the cytosol enzyme purified from rat liver or sheep liver. 2. When Mn^{2+} replaced Mg^{2+} in the reaction a lower Michaelis constant of $9 \times 10^{-6} M$ was found, but only with the mitochondrial enzymes. 3. With all enzymes malate at high concentration was a competitive inhibitor with respect to oxaloacetate when Mn^{2+} was the added cation. With Mg^{2+} the inhibition by malate was competitive with the mitochondrial enzymes and non-competitive with the cytosol enzymes.

Although phosphoenolpyruvate carboxykinase (EC 4.1.1.32) is considered to be an obligatory enzyme in the synthesis of glucose from lactate, pyruvate, propionate and most amino acids, a marked discrepancy exists between the kinetic properties of the phosphoenolpyruvate carboxykinase-catalysed reaction and the hepatic concentrations of oxaloacetate that are available as substrate for this enzyme. Michaelis constants for oxaloacetate have been reported for phosphoenolpyruvate carboxykinase isolated from the livers of several mammals. Values in the range $9 \times 10^{-4} M$ to $4 \times 10^{-3} M$ were obtained for the enzymes purified from guinea-pig liver mitochondria or cytosol (Holten & Nordlie, 1965) and chicken liver mitochondria (Felicoli, Barsacchi & Ipata, 1970), and values of approx. $1.4 \times 10^{-4} M$ were reported for the enzymes from pig liver mitochondria (Chang, Maruyama, Miller & Lane, 1966) or the cytosol fraction of rat liver (Foster, Lardy, Ray & Johnston, 1967). The concentrations of oxaloacetate in the cytosol and mitochondrial compartments of rat liver are much lower, and have been calculated as $5\text{--}10 \mu M$ and $0.1\text{--}0.7 \mu M$ respectively (Williamson, Lund & Krebs, 1967; Williamson, Veloso, Ellington & Krebs, 1969; Williamson, Scholz & Browning, 1969). It is also clear from measurements of gluconeogenic flux that phosphoenolpyruvate carboxykinase must function *in vivo* at rates at least tenfold and possibly 1000-fold greater than could be predicted from the kinetic measurements (Greville & Tubbs, 1968; Scrutton & Utter, 1968). In the present work assays for phosphoenolpyruvate

carboxykinase have been used that are applicable to low oxaloacetate concentrations so that an attempt can be made to resolve the discrepancy between kinetic studies and measurements of the situation *in vivo*.

MATERIALS AND METHODS

Animals. Rats were hooded Wistar animals from the Divisional colony. Livers from sheep or chickens were obtained immediately after slaughterhouse killing. Mitochondria were isolated from sheep or chicken liver by differential centrifugation of liver homogenates in 0.25 M-sucrose (Schneider & Hogeboom, 1950) and were freeze-dried.

Chemicals. Lactate dehydrogenase, pyruvate kinase, malate dehydrogenase and oxaloacetate were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany; NAD^+ , NADH, ITP and IDP were from P-L Biochemicals Inc., Milwaukee, Wis., U.S.A.; malic acid was from the Sigma Chemical Co., St. Louis, Mo., U.S.A.; $NaH^{14}CO_3$ was from The Radiochemical Centre, Amersham, Bucks., U.K.

Assays of phosphoenolpyruvate carboxykinase. Three methods were used. (a) The carboxylation assay as described by Chang & Lane (1966).

(b) An assay in which the oxaloacetate is regenerated from a large pool of malate. This is possible because the equilibrium constant of the malate dehydrogenase reaction requires that under conditions of low NADH and high NAD^+ concentration the amount of oxaloacetate present at equilibrium will be very small but predictable from measurements of the equilibrium constant and the concentrations of malate, NAD^+ and NADH. Spectrophotometer cuvettes with 5 cm light-paths contained $200 \mu mol$ of imidazole, $2 \mu mol$ of NAD^+ , $2 \mu mol$ of $MnCl_2$,

2 μ mol of ITP and various concentrations of sodium malate at pH 7.0 and 37°C. The total volume was 2.0 ml. After addition of malate dehydrogenase (2 units) to generate oxaloacetate at concentrations dependent on the initial malate concentration, a slow increase in NADH was measured at 340 nm as the oxaloacetate was degraded non-enzymically and replaced from the large malate pool. The rate of this blank reaction was followed for 3 min before the addition of approx. 0.002 unit of phosphoenolpyruvate carboxykinase (determined by the carboxylation assay method of Chang & Lane, 1966). The additional increase in NADH was followed for a further 3 min. The rate of NADH formation slowly decreased as the equilibrium concentration of oxaloacetate fell according to the relationship in eqn. (1).

$$K_{eq.} = \frac{[\text{oxaloacetate}][\text{NADH}][\text{H}^+]}{[\text{malate}][\text{NAD}^+]} \quad (1)$$

The exact concentration of oxaloacetate could be calculated at known concentrations of NADH, malate and NAD^+ by the use of eqn. (1) and the equilibrium constant that was determined under conditions of pH and ionic

strength similar to those used in the assay. The velocity of oxaloacetate removal was corrected for the non-enzymic breakdown of oxaloacetate.

(c) The assay method developed by Seubert & Huth (1965). Tubes contained 100 μ mol of imidazole, 1 μ mol of MnCl_2 , 0.8 μ mol of ITP, 1 μ mol of GSH and oxaloacetate at the concentrations indicated. The pH was 7.0. Phosphoenolpyruvate carboxykinase (0.0002–0.0005 unit was measured by the carboxylation assay) was added to give a total volume of 1.0 ml and the tubes were incubated for 5 or 10 min at 37°C. The reaction was stopped by the addition of approx. 10 mg of solid KBH_4 , and after 2 min in an ice bath the excess of KBH_4 was destroyed by 0.2 ml of 15% HClO_4 . After neutralization with 3 M- KHCO_3 and centrifugation at 2°C, the phosphoenolpyruvate formed was measured in 5 cm cuvettes as described by Czok & Eckert (1965). To calculate the true concentration of oxaloacetate available as substrate for phosphoenolpyruvate carboxykinase, allowance was made for the non-enzymic breakdown of oxaloacetate in parallel reactions that did not contain phosphoenolpyruvate carboxykinase. The average of the initial and final oxaloacetate concentrations (Hohorst & Reim, 1965) was taken as the oxaloacetate concentration available for phosphoenolpyruvate carboxylase reaction.

Fig. 1. Lineweaver-Burk plots of the reciprocal of velocity against the reciprocal of the oxaloacetate concentration, with the oxaloacetate-generation assay method. (a) IDP-dependent decarboxylation activity of phosphoenolpyruvate carboxykinase. (b) ITP-dependent phosphorylation activity. Velocity is expressed as nmol/min. The enzyme used was purified from the cytosol fraction of rat liver through the DEAE-cellulose chromatography stage and had a specific activity of 2.5 units/mg of protein as measured by the carboxylation assay method (Ballard & Hanson, 1969).

RESULTS

One difficulty in measuring the Michaelis constant of phosphoenolpyruvate carboxykinase for oxaloacetate is the sensitivity limit of spectrophotometric methods that depend on changes in the concentration of NAD^+ or NADH. This problem is partially overcome in the following studies by the use of a regeneration system for oxaloacetate (assay b). Lineweaver-Burk plots (Lineweaver & Burk, 1934) of phosphoenolpyruvate carboxykinase-dependent oxaloacetate utilization are shown in Fig. 1. For the reaction:

the Michaelis constant for oxaloacetate was between 6×10^{-6} M and 10^{-5} M (Fig. 1b) and in the IDP-dependent decarboxylation reaction:

the K_m for oxaloacetate was between 2×10^{-6} M and 3×10^{-6} M (Fig. 1a). The ratios of extrapolated maximum velocities showed that reaction (2) was catalysed at about ten times the rate of reaction (3). Fig. 1 shows that both reactions are inhibited at high substrate concentrations. This inhibition could be caused by either the large amount of malate present or the high concentration of oxaloacetate generated. These possibilities cannot be distinguished with the present assay.

Fig. 2. Lineweaver-Burk plots of the reciprocal of velocity against the reciprocal of the oxaloacetate concentration, with the phosphoenolpyruvate-formation assay method of Seubert & Huth (1965). Values shown are in the absence (●) or the presence (○) of 40 mM-malate. Velocity is expressed as nmol/min. The enzyme used was the same preparation as for the experiments in Fig. 1. (a) Mn^{2+} (1 mM) was the added cation; the amount of phosphoenolpyruvate carboxykinase used was 0.0004 unit as measured by the carboxylation assay method of Chang & Lane (1966). (b) Mg^{2+} (1 mM) was the added cation, and 0.0012 unit of enzyme was used.

Chang & Lane (1966) have developed a continuous spectrophotometric assay method for phosphoenolpyruvate carboxykinase in which NADH oxidation is measured in the presence of manganese chloride, ITP, oxaloacetate, pyruvate kinase, lactate dehydrogenase and phosphoenolpyruvate carboxykinase. This method was not suitable because the rate of non-enzymic formation of pyruvate was substantial. Further, when ITP was omitted from the reaction system an even larger blank was found, presumably as more Mn^{2+} was then available to stimulate the non-enzymic breakdown of oxaloacetate.

The assay method developed by Seubert & Huth (1965) avoids the errors of non-enzymic breakdown of oxaloacetate since the oxaloacetate remaining after phosphoenolpyruvate carboxykinase action is reduced by potassium borohydride before the measurement of phosphoenolpyruvate. A reinvestigation of the apparent substrate inhibition shown in Fig. 1 was carried out by using this assay method. With either Mn^{2+} (Fig. 2a) or Mg^{2+} (Fig. 2b) as

added cation there was no indication of inhibition by high oxaloacetate concentrations. The K_m for oxaloacetate with added Mn^{2+} was $2.7 \times 10^{-5} \text{ M}$, considerably higher than that calculated from oxaloacetate-disappearance assay of Fig. 1(b). The difference in K_m for oxaloacetate between the two assays is explained by the competitive nature of the malate inhibition (Fig. 2a). Thus the higher [malate]/[oxaloacetate] ratio at high oxaloacetate concentrations (Fig. 1) produces a relatively greater inhibition of phosphoenolpyruvate carboxykinase activity than is found at low oxaloacetate concentration. This has the effect of displacing the double-reciprocal plot from its true position and results in an anomalous low K_m for oxaloacetate. When Mg^{2+} replaces Mn^{2+} in the assay system (Fig. 2b) the maximum velocity is lowered to one-quarter, but the K_m for oxaloacetate is not markedly altered. However, the inhibition by malate becomes non-competitive.

Holten & Nordlie (1965) reported differences in the Mn^{2+} - and Mg^{2+} -catalysed reactions between cytosol and mitochondrial phosphoenolpyruvate carboxykinases. To investigate whether the malate inhibition pattern was different with different sources of phosphoenolpyruvate carboxykinase, the enzymes were purified from the cytosol of sheep liver, the mitochondria of sheep liver and from chicken liver mitochondria, and all were compared with the enzyme from the cytosol of rat liver. These four sources were chosen since rat liver contains principally the cytosol enzyme (Nordlie & Lardy, 1963), chicken liver contains only the mitochondrial enzyme and sheep liver contains both enzymes in approximately equal activity (Scrutton & Utter, 1968). The cytosol enzymes were purified from liver homogenates by differential centrifugation, ammonium sulphate fractionation, Sephadex G-100 chromatography and DEAE-cellulose chromatography by methods described in detail by Ballard & Hanson (1969). Mitochondria were isolated by differential centrifugation of liver homogenates (Schneider & Hogeboom, 1950) and were freeze-dried. The dry powder was extracted by homogenization in 15 vol. of 0.05 M-tris-hydrochloric acid buffer, pH 8.0, centrifuged at 100 000g for 30 min, and the supernatant fractionated by methods identical with those described for the cytosol enzymes (Ballard & Hanson, 1969). No differences were found between the four sources of enzyme with respect to ammonium sulphate fractionation or the separation on Sephadex G-100 and DEAE-cellulose. Representative specific activities of enzymes in initial extracts and in combined fractions from the DEAE-cellulose chromatography are shown in Table 1. All activities were stable for at least 3 months at 2°C when enzymes eluted from DEAE-cellulose columns were precipitated with

ammonium sulphate and stored in the presence of EDTA (0.5mM) and GSH (0.5mM) at pH 7.0.

Kinetic studies with all four enzymes are shown in Tables 2 and 3. The assay method of Seubert & Huth (1965) was used. A marked difference between

phosphoenolpyruvate carboxykinases from cytosol and mitochondria is the K_m for oxaloacetate with Mn^{2+} as bivalent cation. Both cytosol enzymes have a K_m under these conditions of approx. $2.5 \times 10^5 M$, whereas the mitochondrial enzymes have a K_m for

Table 1. *Purification of phosphoenolpyruvate carboxykinases*

Phosphoenolpyruvate carboxykinase was purified from mitochondria of sheep liver or chicken liver and from the cytosol fraction of sheep liver or rat liver by methods outlined in the Materials and Methods section and described in detail by Ballard & Hanson (1969). Rats were hooded Wistar animals from the Divisional colony. Sheep were Merino wethers and chickens were obtained from the abattoirs. Activity was measured by the carboxylation assay method (Chang & Lane, 1966) and expressed as μmol of bicarbonate fixed/min at 37°C . Fractions after chromatography on DEAE-cellulose were combined and $(\text{NH}_4)_2\text{SO}_4$ was added to give 75% saturation. After standing for 2 h this solution was centrifuged and the $(\text{NH}_4)_2\text{SO}_4$ precipitate suspended in a small volume of 75%-saturated $(\text{NH}_4)_2\text{SO}_4$ containing 0.5mM-EDTA and 0.5mM-GSH at pH 7.0. Values are representative of at least two purifications.

Source of enzyme	Specific activity (units/mg of protein)	
	Initial extract	DEAE-cellulose eluate
Rat liver cytosol	0.055	1.85
Sheep liver cytosol	0.034	1.80
Sheep liver mitochondria	0.120	2.40
Chicken liver mitochondria	0.64	13.0

Table 2. *Michaelis constants for oxaloacetate in the phosphoenolpyruvate carboxykinase reaction*

The K_m for oxaloacetate was determined from Lineweaver-Burk plots of the reciprocal of velocity against the reciprocal of oxaloacetate concentration as described in Fig. 2. The bivalent cations were present at a concentration of 1 mM. Values are means $\pm$ S.E.M. of four determinations with the purified enzymes.

Source of enzyme	Added cation	...	$10^5 K_m (M)$	
			Mn^{2+}	Mg^{2+}
Rat liver cytosol			2.6 ± 0.1	2.9 ± 0.4
Sheep liver cytosol			2.2 ± 0.3	2.0 ± 0.2
Sheep liver mitochondria			0.85 ± 0.08	2.8 ± 0.3
Chicken liver mitochondria			0.91 ± 0.05	2.2 ± 0.2

Table 3. *Inhibition by 40mM-L-malate of phosphoenolpyruvate carboxykinase activity*

The inhibition was measured with purified phosphoenolpyruvate carboxykinase in the presence of either Mn^{2+} or Mg^{2+} (see Fig. 2). The inhibited activity is expressed as the percentage of the activity in the absence of malate and is the mean of four determinations at oxaloacetate concentrations of approx. 1 mM and 15 μM .

Source of enzyme	Cation	Activity with malate (%)	
		At 1 mM-oxaloacetate	At 15 μM -oxaloacetate
Rat liver cytosol	Mg^{2+}	65	61
	Mn^{2+}	96	60
Sheep liver cytosol	Mg^{2+}	63	55
	Mn^{2+}	97	69
Sheep liver mitochondria	Mg^{2+}	98	68
	Mn^{2+}	102	77
Chicken liver mitochondria	Mg^{2+}	95	57
	Mn^{2+}	96	66

oxaloacetate of 0.9×10^{-5} M (Table 2). This difference has been noted between 12 different enzyme purifications from the various sources. With Mg^{2+} replacing Mn^{2+} in the reaction the K_m for oxaloacetate is similar for all four enzymes, being between 2×10^{-5} and 3×10^{-5} M. Other experiments showed that the maximum velocity with Mn^{2+} was about four times the velocity with Mg^{2+} and was twice the velocity measured in the direction of phosphoenolpyruvate carboxylation (Chang & Lane, 1966).

Inhibitor studies with added malate show another constant difference between the cytosol and the mitochondrial enzymes. Although malate inhibits all four enzymes to about the same extent at low oxaloacetate concentrations, the inhibition is not evident at high oxaloacetate concentrations with the mitochondrial enzymes, and when Mn^{2+} is used as cation for the cytosol enzymes (Table 3). The cytosol enzymes are inhibited non-competitively by malate when oxaloacetate is the variable substrate and Mg^{2+} is the added cation.

DISCUSSION

Michaelis constants for oxaloacetate. The K_m for oxaloacetate reported in this paper is much lower than that found by other investigators. For example, Felicioli *et al.* (1970) report a K_m for oxaloacetate of 4 mM with chicken mitochondrial phosphoenolpyruvate carboxykinase that had been purified to a specific activity of 1.95 units/mg of protein. This value is 180-fold that found in the present study. It is unlikely that the differences in pH and $[Mg^{2+}]/[ITP]$ ratio used in the two investigations can account for the discrepancy (Holten & Nordlie, 1965; Chang *et al.* 1966). One possible explanation is that phosphoenolpyruvate carboxykinase could exhibit co-operative behaviour with respect to oxaloacetate and give non-linear double-reciprocal plots. For this reason the Michaelis constants for all four enzymes were determined from measurements carried out over a wide range of oxaloacetate concentrations (Fig. 2). No deviation from linearity was apparent at least between $10 \mu M$ - and $3 mM$ -oxaloacetate and under the conditions used in these experiments. Another factor that could account in part for the differences in the reported Michaelis constants is the problems caused by non-enzymic breakdown of oxaloacetate. In the present study care has been taken to prepare oxaloacetate solutions immediately before each experiment and to use true concentrations of oxaloacetate for calculations of Michaelis constants.

Differences between the cytosol and the mitochondrial phosphoenolpyruvate carboxykinases. In an attempt to resolve different metabolic functions of cytosol and mitochondrial phosphoenolpyruvate carboxy-

kinases, Holten & Nordlie (1965) undertook a comparative study between the two enzymes from guinea-pig liver. Although most kinetic properties were similar, they found that only the mitochondrial enzyme was inhibited by 1.33 mM-AMP and that the mitochondrial enzyme was more active with Mg^{2+} than with Mn^{2+} at pH 8. The cytosol enzyme had more activity with Mn^{2+} than with Mg^{2+} under all conditions tested. To these differences are now added the lower K_m for oxaloacetate exhibited by the mitochondrial enzyme in the presence of Mn^{2+} and the differences in malate inhibition. Further, the cytosol and the mitochondrial enzymes are immunochemically distinct (Ballard & Hanson, 1969).

Although the animals chosen in the present study have different hepatic patterns of phosphoenolpyruvate carboxykinase distribution, it is presumed that the enzyme has an obligatory role in gluconeogenesis in the three species. One might therefore expect that the kinetic differences between the cytosol and the mitochondrial phosphoenolpyruvate carboxykinases may be important in the particular nutritional situation of each animal. Unfortunately it is difficult to apply this type of teleological argument to the available information. The malate inhibition, although of interest from a kinetic standpoint, is unlikely to be of physiological importance, since the K_i found for malate is about 2.5×10^{-2} M. This is well in excess of malate concentrations *in vivo* (J. R. Williamson *et al.* 1969).

The lower K_m for oxaloacetate with the mitochondrial enzyme is consistent with the lower oxaloacetate concentration found in mitochondria as compared with the cytosol (J. R. Williamson *et al.* 1969; Williamson *et al.* 1967). However, this low K_m was only found with Mn^{2+} as the added cation, and Mn^{2+} is known to be present in mitochondria at low concentrations (Thiers & Vallee, 1957). Perhaps the higher stability constants for Mn^{2+} -ITP and Mn^{2+} -GTP as compared with the respective Mg^{2+} complexes indicate that a significant proportion of these nucleotides are present as Mn^{2+} complexes (Walaas, 1958). If this is the case the low K_m for oxaloacetate of mitochondrial phosphoenolpyruvate carboxykinase may be of physiological significance.

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