The Anaplerotic Phosphoenolpyruvate Carboxylase of the Tricarboxylic Acid Cycle Deficient *Acholeplasma laidlawii* B-PG9

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Phosphoenolpyruvate carboxylase (EC 4.1.1.31) (PEP-C) was purified approximately 770-fold from the mollicute *Acholeplasma laidlawii* B-PG9. The partially purified PEP-C required phosphoenolpyruvate (PEP) and MnCl_2 at pH 7.4 or MgCl_2 at pH 8.6 for optimal activity. The product is oxaloacetate as detected by a malate dehydrogenase indicator system. The K_{m_A} (PEP variable) was 0.66 mM and the K_{m_B} (bicarbonate variable) was 1.02 mM. At low bicarbonate concentrations (0.5 mM), PEP-C activity was stimulated approximately 240% by fructose 1,6-bisphosphate. Aspartate was a non-competitive inhibitor of PEP-C activity. The K_{i_A} (PEP variable) for aspartate was 0.69 mM and the K_{i_B} (bicarbonate variable) was 0.99 mM. Malate, citrate, isocitrate, 2-oxoglutarate, acetyl-CoA, CMP, CDP, GDP, GTP, ADP and ATP had no effect on the PEP-C reaction. The Hill interaction coefficient was 0.98–1.11. The molecular mass by sucrose density gradient analysis was 353 kDa; by gel filtration chromatography it was 384 kDa. The Stokes radius was about 7.4 nm. PEP-C activity and its inhibition by aspartate in *Acholeplasma laidlawii* B-PG-9 extracts may reflect an involvement of this enzyme in the interdependent regulation of protein, lipid and nucleic acid precursor metabolism of this TCA-cycle-deficient and cytochrome-less mollicute.

INTRODUCTION

Phosphoenolpyruvate carboxylase (PEP-C) (EC 4.1.1.31) catalyses the irreversible carboxylation of PEP to oxaloacetate (Ashworth & Kornberg, 1963). PEP-C is generally considered to be an anaplerotic enzyme, an enzyme with C_4 -acid-replenishing activity, that is associated with the tricarboxylic acid (TCA) cycle (Gottschalk, 1986). PEP-C deficient mutants of *Escherichia coli* (Amarasingham, 1959; Ashworth & Kornberg, 1963) and *Salmonella typhimurium* (Theodore & Englesberg, 1964), with functional 'malic' enzyme and PEP-carboxykinase activities, require additions of TCA cycle intermediates to their culture medium for growth. These studies indicate that in these organisms, PEP-C is the major enzyme activity responsible for replenishment of carbon drained from the TCA cycle for biosynthetic purposes.

Evidence for the presence of PEP-C in any mollicute, specifically, *Acholeplasma laidlawii* B-PG9, was first obtained by Beaman & Pollack (1984). Recently, we reported the presence of PEP-C in *Acholeplasma morum* S2, but not in any species of the related genus *Mycoplasma* (Manolukas *et al.*, 1988). We found no evidence for a functional TCA cycle in any mollicute, and only detected malate dehydrogenase (MDH) (EC 1.1.1.37) activity in all the *Mycoplasma* species, but no other TCA cycle enzyme activity. In unpublished studies with *Ureaplasma urealyticum* T960(CX8) we also failed to detect PEP-C activity and found the identical anaplerotic-TCA-cycle-deficient metabolic pattern we have described for all *Mycoplasma* species (Manolukas *et al.*, 1988).

Abbreviations: PEP-C, phosphoenolpyruvate carboxylase; PEP, phosphoenolpyruvate; TCA, tricarboxylic acid; MDH, malate dehydrogenase.

In order to study the properties of PEP-C in an organism without a functional TCA cycle (Manolukas *et al.*, 1988), nor cytochrome pigments (Pollack *et al.*, 1981), we have purified the enzyme present in *Acholeplasma laidlawii* B-PG9. To determine the role of this enzyme in mollicute metabolism, the sensitivity of the purified enzyme to various activators and inhibitors of PEP-C in other organisms was tested.

METHODS

Materials. Phosphoenolpyruvate (PEP) was from Sigma. Matrix Gel Green A was from Amicon. DEAE Sephacel, Sephadex (superfine) G-200 particle size 10–40 μm and Phenyl-Sepharose CL-6B were from Pharmacia.

Preparation of cell extract. *Acholeplasma laidlawii* B-PG9 was grown at 37 °C in modified Edward medium (Beaman & Pollack, 1981). Cytoplasmic extracts were prepared from washed cells after lysis by explosive decompression in a Parr-Bomb. The lysate was centrifuged at 260 000 *g* for 1.5 h at 4 °C and the supernatant was dialysed overnight in EQ-buffer (20 mM-Tris/HCl, pH 7.4, 1 mM-2-mercaptoethanol, 1 mM-MgCl₂ and 20%, v/v, glycerol) at 4 °C. The dialysed supernatant (cytoplasmic extract) was the starting material for all assays and purification studies.

Assay for PEP-C. This was a modification of the procedure described by T. E. Smith (1968). The basic reaction mixture (1.0 ml) contained 100 mM-HEPES (pH 7.4), 6 mM-PEP, 5 mM-MnCl₂ (or 5 mM-MgCl₂ as indicated) and as an indicator system 0.1 U MDH and 0.15 mM-NADH and various amounts of enzyme (0.02–910 μg protein). The effects of pH, metal ions, inhibitors and activators on 0.13 μg of partially purified PEP-C protein was determined. Reactions were started with the addition of 10 mM, or 0.5 mM, sodium bicarbonate. The specific activity values were determined from the change in absorbance in time, measured spectrophotometrically at 340 nm at 22–25 °C. A unit of PEP-C activity was the amount of enzyme which produced 1 μmol oxaloacetate min^{-1} from PEP at 22–25 °C.

Purification of *A. laidlawii* B-PG9 PEP-C. All purification procedures were done at 4 °C. The cytoplasmic extract was loaded onto a Green A column (2.5 $\times$ 20 cm, flow rate 0.5 ml min^{-1}) equilibrated in EQ-buffer. The column was washed with EQ-buffer and PEP-C activity was eluted from the column by the addition of a 6 mM solution of PEP in EQ-buffer. After dialysis against EQ-buffer, fractions containing PEP activity were pooled and ammonium sulphate was added to a final concentration of 3 M. The mixture was centrifuged at 30 000 *g* for 30 min and the precipitate, which contained PEP-C activity, was dissolved in about 1.5 ml EQ-buffer with 0.8 M-ammonium sulphate (EQA-buffer). The solution was applied to a Phenyl-Sepharose column (1.5 $\times$ 28 cm, flow rate 0.5 ml min^{-1}) equilibrated in EQA-buffer. The column was eluted with EQA-buffer + 10 mM-aspartate following the technique of Izui *et al.* (1982). Fractions (1 ml) were collected, dialysed against EQ-buffer and assayed for activity. The pooled active fractions were chromatographed on a DEAE-Sephacel column (2.5 $\times$ 30 cm, flow rate 0.5 ml min^{-1}) equilibrated in EQ-buffer. PEP-C activity was eluted with a linear gradient of increasing concentration of KCl in EQ-buffer. The PEP-C activity eluted at 0.41 M-KCl. Fractions containing PEP-C activity were dialysed against EQ-buffer.

The dialysed active fractions were pooled and 2.0 ml were chromatographed on a Sephadex G-200 gel filtration column (2.0 $\times$ 53 cm, flow rate 0.2 ml min^{-1}) equilibrated in EQ-buffer. PEP-C activity was eluted with EQ-buffer. Active fractions were pooled and this sample was used for further studies.

Optimal pH determination. pH optimum was determined from 3.4 to 10.3, using different buffers in the PEP-C assay (final concentration 50 mM): dimethylglutaric acid/NaOH, pH 3.4 to 7.6; imidazoleacetic acid, pH 7.1 to 7.8; Tris/acetic acid, pH 7.4 to 8.9; and glycine/NaOH, pH 8.6 to 10.3.

Determination of divalent cation requirement. To determine the divalent cation requirement and concentration yielding the maximum specific activity of PEP-C, MgCl₂ or MnCl₂ were added individually or together to the PEP-C reaction mixture at final concentrations ranging from 0 to 10 mM. Divalent cation requirements were examined over the range of pH values listed above.

Inhibition and activation studies. Under standard assay conditions for PEP-C, known inhibitors and activators of PEP-C (Maeba & Sanwall, 1969; T. E. Smith 1970; Morse *et al.*, 1974; Cox & Baugh, 1976) were tested. Aspartate, malate, citrate, isocitrate, 2-oxoglutarate, succinate and fumarate were tested at 10 mM, ATP, ADP, GTP, GDP, CDP and CMP at 4 mM, fructose 1,6-bisphosphate at 8 mM and acetyl-CoA at 0.1 mM. These studies were done at both 0.5 mM- and 10 mM-bicarbonate levels.

Molecular mass determination. The molecular mass of the PEP-C was determined by sedimentation analysis and by gel filtration chromatography. Sucrose gradient centrifugation was done in a linear (5–20%, w/v) sucrose gradient (50 mM-Tris/HCl, pH 7.5, 2 mM-2-mercaptoethanol, 100 mM-NaCl, 2 mM-MgCl₂) at 35 000 r.p.m for 15 h in a SW41 Ti rotor (Beckman) at 4 °C. Approximately 100 μg protein was loaded onto each gradient. After centrifugation, fractions (0.5 ml) were collected and assayed for protein and PEP-C activity. The protein standards (Sigma) used were PEP-C (402 kDa), α -amylase (200 kDa), alcohol dehydrogenase (141 kDa), creatine phosphokinase (81 kDa) and bovine serum albumin (BSA) (67 kDa). The sedimentation coefficient was estimated

by the method of Martin & Ames (1961). Gel filtration chromatography was done using a Sephadex G-200 column (2.0 × 53 cm) equilibrated in EQ-buffer. Partially purified enzyme (100 µg) was added to the column and eluted with EQ-buffer. Fractions (2.5 ml) were collected and assayed for PEP-C activity and protein. The Stokes radius was determined by the procedure of Siegel & Monty (1966).

Gel electrophoresis. Polyacrylamide tube gel electrophoresis was done on 50 µg protein of the partially purified PEP-C as described previously (Williams & Pollack, 1984). After electrophoresis, the gels were either stained with Coomassie blue or cut into 3 mm slices for assaying PEP-C activity. Each slice was immersed in 1 ml of the standard reaction mixture without the indicator system (0.1 U MDH and 0.15 mM-NADH) and incubated at 25 °C for 10 h. The indicator system was then added and the reaction monitored at A_{340} nm.

Kinetic analysis. To calculate K_m values, initial reaction velocities were determined at various concentrations of bicarbonate (0.2–2 mM) and fixed PEP concentrations (0.5, 5, 10 mM; K_{m_A} for PEP), and at various concentrations of PEP (0.2–3 mM) and fixed bicarbonate concentrations (0.5, 2, 4 mM; K_{m_B} for bicarbonate). Besides PEP and bicarbonate, the reaction mixtures also contained in 1 ml 0.1 U partially purified PEP-C, 0.1 U MDH, 0.15 mM-NADH, 5 mM-MnCl₂, and 50 mM-HEPES.

The inhibition constants for aspartate were determined in 1.0 ml with 0.1 IU partially purified PEP-C, at a fixed concentration of PEP (0.66 mM) and varying bicarbonate concentrations (0.1–1 mM; K_{i_A} for aspartate), and at a fixed concentration of bicarbonate (1 mM) and varying PEP concentrations (0.1–1 mM; K_{i_B} for aspartate), using three different aspartate concentrations (2, 4, 8 mM). The Hill coefficient (n) was determined as described by Suelter (1985).

Other assays. Protein concentrations were determined by their direct relationship to the absorbance difference A_{215} minus A_{225} (Waddell, 1956) using BSA as the standard.

Pyrophosphate formation was assayed using the PP_i assay reagent kit (Sigma).

RESULTS

Purification of *A. laidlawii* B-PG9 PEP-C. The results of the purification of PEP-C from *A. laidlawii* B-PG9 are summarized in Table 1. Purification of 600–700-fold was routinely achieved. There was no loss of activity after storage at –20 °C for 2 months. The enzyme activity was higher after passage through the Green A dye affinity column, presumably due to the removal of an inactivator of the enzyme or to a competing enzyme activity. Therefore, enzyme recovery was calculated relative either to the cytoplasmic fraction or to the eluate of the Green A column. Using the eluate from the Green A column, the average recovery was 15%.

Electrophoretic analysis of the enzyme preparation following Sephadex G-200 chromatography demonstrated that the enzyme preparation contained two protein species. Based upon the ability to detect bands containing 0.8 µg protein, we calculated that these two proteins composed at least 99% of the protein in the preparation. Compared to a series of BSA standards, we estimate that the minor band was about 2% of the total protein. Following electrophoresis, all PEP-C activity was associated with the major protein species (R_f 0.36).

pH and divalent cation requirements. We found that either MgCl₂ or MnCl₂ was required for enzyme activity. CaCl₂, CoCl₂ and ZnCl₂ could not replace MgCl₂ or MnCl₂. Maximal enzyme activity occurred at 5 mM at pH 7.5 with MnCl₂ and at 5 mM at pH 8.5 with MgCl₂. At pH 7.9, the activity of the enzyme with either chloride was approximately equal. At pH 7.9 the maximal

Table 1. Purification of PEP-C activity from *A. laidlawii* B-PG9

Data shown are representative. The average purification was 770 ± 71 ($n = 3$).

Purification step	Total volume (ml)	Total activity (U)	Protein (mg ml ⁻¹)	Specific activity*	Recovery (%) compared with recovery in:		Purification (fold)
					Cytoplasmic extract	Green A	
Cytoplasmic extract	40.5	108.26	2.97	0.9	100		1
Green dye A	24.5	434.58	0.49	36.2	401	100	40
Phenyl-Sepharose	12.0	192.84	0.05	321.4	178	44	357
DEAE-Sepharcel	3.0	104.79	0.09	388.1	97	24	431
Sephadex G-200	7.4	65.62	0.02	591.2	61	15	657

* Expressed in µmol product synthesized min⁻¹ (mg protein)⁻¹ in standard assay.

activity was detected at 5 mM of either chloride or a combination of both to a total concentration of 5 mM.

Inhibitors and activators. Using three different batches of partially purified PEP-C, we found that aspartate (10 mM) at a concentration of either 0.5 or 10 mM-bicarbonate inhibited the PEP-C reaction to 85–86% of the control value. Neither malate, citrate, isocitrate, 2-oxoglutarate, succinate nor ATP inhibited PEP-C activity, i.e. activity was within 2% of that found in control reactions lacking inhibitor. In parallel experiments we similarly tested known activators of PEP-C. Fructose 1,6-bisphosphate at 0.5 mM-bicarbonate stimulated the PEP-C reaction to 236–239% of the control value and at 10 mM-bicarbonate, 2–4%. At 0.5 mM- or 10 mM-bicarbonate, none of ADP, acetyl-CoA, CDP, CMP, GDP and GTP had any detectable effect on PEP-C activity, i.e. activity was within 2% of that found in control reactions lacking activator.

Molecular mass determination. In sucrose density gradient studies, an approximate molecular mass of 353 kDa was determined for the partially purified PEP-C from *A. laidlawii* B-PG9. The sedimentation coefficient of this material was approximately 10.8 S. The molecular mass of partially purified PEP-C from *A. laidlawii* B-PG9 was also determined by gel filtration chromatography. The molecular mass was determined by comparing the partition coefficient (K_{av}) of the PEP-C from *A. laidlawii* B-PG9 to standards and was calculated to be about 384 kDa. The Stokes radius was about 7.4 nm.

Kinetic analysis. When the bicarbonate concentration was fixed and the PEP concentration was varied, a K_{m_A} of 0.66 mM could be calculated. In the presence of aspartate the K_{i_A} was 0.69 mM, which is essentially the same value. However, the maximum velocities for these reactions were different: V_{max_A} 1.06 $\mu\text{mol min}^{-1}$ and $V_{max_{i_A}}$ 0.86 $\mu\text{mol min}^{-1}$, respectively. When the PEP concentration was fixed and the bicarbonate concentration was varied a K_{m_B} of 1.02 mM could be calculated. In the presence of aspartate, the K_{i_B} was 0.99 mM, again essentially the same values. The maximum velocities for these reactions were again different: V_{max_B} 1.13 $\mu\text{mol min}^{-1}$ and $V_{max_{i_B}}$ 0.54 $\mu\text{mol min}^{-1}$, respectively. This pattern is compatible with the interpretation that aspartate is a non-competitive inhibitor of the PEP-C reaction irrespective of which reactant concentration is varied. The Hill interaction coefficient was calculated to be 0.98–1.11.

DISCUSSION

PEP carboxylase is regulated by a variety of modulators (Scrutton, 1978). As in *Neisseria gonorrhoeae* (Cox & Baugh, 1976), the PEP-C from *Acholeplasma laidlawii* B-PG9 is activated by fructose 1,6-bisphosphate at low (0.5 mM) bicarbonate levels, but not at higher (10 mM) levels. Fructose 1,6-bisphosphate is an allosteric activator of the NAD-dependent L(+)-lactate-specific lactic dehydrogenase of *A. laidlawii* (Neimark & Lemcke, 1972), and is also the product of the rate-limiting step of glycolysis in this organism which is mediated by a pyrophosphate-dependent phosphofructokinase (Pollack & Williams, 1986). Accumulation of fructose 1,6-bisphosphate in intact and metabolically active cells of *Mycoplasma gallisepticum* has been demonstrated (Egan *et al.*, 1986). Unlike most other PEP-C, our samples were not stimulated by acetyl-CoA (Scrutton, 1978). Other known activators, CDP, CMP and dCTP of prokaryotic PEP-C were tested but they had no effect on the PEP-C activity from *A. laidlawii* B-PG9.

The PEP-C from *A. laidlawii* B-PG9 is non-competitively inhibited by aspartate as has been observed with the enzyme from other prokaryotes (Scrutton, 1978). Therefore, accumulation of aspartate, either by increased transamination of oxaloacetate (Manolukas *et al.*, 1988) or its reduced use in the synthesis of AMP from IMP (Tryon & Pollack, 1985) or reduced consumption in other reactions, such as protein synthesis, might inhibit PEP-C activity. Inhibition of *A. laidlawii* PEP-C by aspartate might thus cause an increased rate of conversion of PEP to pyruvate and ATP, the pyruvate then being converted to acetyl-CoA (Constantopoulos & McGarrity, 1987) and lipids (Smith & Henrikson, 1965). Regulation of PEP-C activity by aspartate may thus be effective in modulating protein, lipid and nucleic acid precursor metabolism in *A. laidlawii* B-PG9.

Other known inhibitors of PEP-C (citrate, isocitrate, 2-oxoglutarate, succinate, fumarate and ATP) were tested, but in our assays these inhibitors had no effect on the activity of partially purified PEP-C of *A. laidlawii* B-PG9. It is interesting that TCA-cycle components which are known inhibitors of PEP-C activity isolated from prokaryotes with a functional TCA cycle have no effect on the PEP-C activity isolated from a prokaryote which apparently has no functional TCA cycle (Manolukas *et al.*, 1988).

The molecular masses for native PEP-C range from 560 kDa in *Coccochloris penicystis* (Owtrim & Colman, 1986) to a relatively low 105 kDa in *Bryophyllum fedtschenkoi* (Jones *et al.*, 1978). Wolpert & Ernst-Fonberg (1975) reported a multienzyme complex of PEP-C, malate dehydrogenase and acetyl-CoA carboxylase of about 360 kDa. They separated the PEP-C and determined that it was 183 kDa, and was stimulated by either ATP or acetyl-CoA. Our PEP-C of 353–384 kDa has no malate dehydrogenase activity and is not stimulated by either ATP or acetyl-CoA.

Millay *et al.* (1978) reported that *Pseudomonas* MA, grown on methylamine as the sole carbon source, produces a tetrameric PEP-C of about 12 S that is converted by NADH to a more active dimeric form of about 9 S. This NADH-activated conversion is inhibited by ADP. Although ADP has no detectable effect in our experiments, we cannot exclude the possibility that this conversion takes place, because NADH is present in our assay system. Millay & Hersh (1976) proposed that the flow of carbon into energy-producing pathways is affected by the response of PEP-C to the energy state of the organism, the energy state being reflected by the level of cellular NADH. The availability of NADH at the same PEP-pyruvate locus is obviously also modulating the reduction of pyruvate to lactate, a major end-product of glucose metabolism in fermentative Mollicutes like *A. laidlawii* (P. F. Smith, 1971).

Our calculation of 7.4 nm for the Stokes radius of *A. laidlawii* PEP-C is close to the value of 7.52 nm reported for the PEP-C of *E. coli* (T. E. Smith, 1971).

Our *in vitro* studies suggest that PEP-C activity may serve *A. laidlawii* B-PG9 by shunting glycolytically derived PEP away from pyruvate, acetyl-CoA and lactate and lipid formation towards amino acids and nucleic acids. This may occur during periods when cellular ATP is not limiting, since by circumventing the synthesis of pyruvate from PEP by pyruvate kinase the concomitant synthesis of ATP may not occur. These data also suggest that fructose 1,6-bisphosphate, NADH and aspartate by affecting pyruvate and PEP utilization have an important role in regulating metabolism in *A. laidlawii*.

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