Purification and characterization of phosphoenolpyruvate carboxykinase, a catabolic CO₂-fixing enzyme, from *Anaerobiospirillum succiniciproducens*

SERGEY M. PODKOVYROV¹ and J. GREGORY ZEIKUS^{1,2*}

¹Department of Biochemistry, and ²Department of Microbiology and Public Health, Michigan State University, East Lansing, Michigan 48824, USA

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Phosphoenolpyruvate (PEP) carboxykinase (EC 4.1.1.49) from the obligate anaerobe *Anaerobiospirillum succiniciproducens* was purified 18-fold. The enzyme was monomeric, with an M_r of $57\,000 \pm 2\,000$. The enzyme was oxygen stable, had a pH optimum of 6.5–7.1, and was stable from pH 5.0 to 9.0. The enzyme displayed Michaelis–Menten kinetics for the substrate PEP and the cosubstrates bicarbonate and ADP with a K_m of 0.54 mM, 17 mM and 0.42 mM, respectively. The enzyme required Mn²⁺ or Co²⁺ in addition to Mg²⁺ to exhibit maximum activity. *p*-Chloromercuribenzoate inhibited activity and phosphoenolpyruvate protected the enzyme against inactivation, suggesting that an essential cysteine may be in the active site.

Introduction

A number of anaerobic bacteria produce succinate as a fermentation product as a result of glucose metabolism (Zeikus, 1980). *Anaerobiospirillum succiniciproducens* forms succinate in high concentrations, in addition to acetate, lactate and ethanol (Davis *et al.*, 1976). Recently our laboratory has reported the proposed catabolic pathway for glucose fermentation by *A. succiniciproducens* (Samuelov *et al.*, 1991). Fermentation studies indicated that succinate versus lactate and ethanol production in this organism was regulated by the level of available CO₂ and culture pH. Enzymic studies using crude cell extracts indicated a branch point for glucose fermentation occurring at phosphoenolpyruvate (PEP). If PEP is converted to pyruvate through the action of pyruvate kinase, lactate and ethanol formation takes place. Alternatively phosphoenolpyruvate carboxykinase (PEPCK, EC 4.1.1.49) catalyses carboxylation of PEP to oxaloacetate (OAA) and is the first enzyme in the pathway leading to succinate production. Control of carbon and electron flow appears to occur at the branch point for PEP metabolism with levels of PEPCK increasing in cells producing high levels of succinate (Samuelov *et al.*, 1991).

PEPCK catalyses the following reaction:

where M²⁺ represents an absolute requirement for a divalent metal ion. Most animal PEPCKs are GDP-dependent, whereas the enzymes from most plants, yeasts and bacteria are ADP-dependent (Utter & Kolenbrander, 1972). Since the discovery of PEPCK (Utter & Kurahashi, 1953) a great deal of attention has been focused on PEPCKs from eukaryotic organisms, while only a few studies have concerned the purification of prokaryotic PEPCKs (Goldie & Sanwal, 1980; Salvarrey *et al.*, 1989).

Most previous studies on PEPCK have dealt with the anabolic function of this enzyme. It is recognized as being a key enzyme in gluconeogenesis in different organisms from bacteria to vertebrates (Utter & Kolenbrander, 1972). The same enzyme has been identified in some invertebrates and eukaryotic microorganisms as a CO₂-fixing enzyme (Rohrer *et al.*, 1986; de Flombaum *et al.*, 1977). In anaerobic bacteria catabolic CO₂ fixation studies have recently focused on CO₂ reduction to methane or acetic acid by lithotrophic bacteria (Zeikus *et al.*, 1985). In this paper we report the purification and biochemical characterization of PEPCK from *A. succiniciproducens*, which functions in catabolic CO₂ fixation.

Methods

Organism and growth conditions. *Anaerobiospirillum succiniciproducens* (ATCC 29305) was grown at 37°C in a rotary shaker in the

*Author for correspondence. Tel (517) 336 4614; fax (517) 337 2122.

Abbreviations: PEP, phosphoenolpyruvate; PEPCK, phosphoenolpyruvate carboxykinase; OAA, oxaloacetate; pCMB, *p*-chloromercuribenzoate.

following medium (g per litre of distilled water): glucose 20; peptone 10; yeast extract 5; K_2HPO_4 3; NaCl 1; $(NH_4)_2SO_4$ 1; $MgCl_2 \cdot 6H_2O$ 0.2; $CaCl_2 \cdot 2H_2O$ 0.2; $MgCO_3$ 15. The medium was heat-sterilized in anaerobic vials with a nitrogen headspace. Sulphuric acid was added to the sterile medium to reduce the pH to 6.3 ± 0.1 . The nitrogen headspace was replaced by carbon dioxide and $Na_2S \cdot 9H_2O$ was added to a final concentration of 0.05% in order to establish strictly anaerobic conditions. For large-scale enzyme preparations the organism was grown in stirred, pH controlled, 4 litre Kimax jars (Baxter Scientific Products) or 16 litre glass carboys as described previously (Samuelov *et al.*, 1991). The pH was maintained at 6.3, and the vessel was continuously sparged with 100% CO_2 .

Enzyme assays. PEPCK activities were measured aerobically by monitoring NADH oxidation at 340 nm at 37°C. The assay in the direction of PEP carboxylation was conducted in a 1.0 ml reaction mixture containing: 100 mM-MES buffer (pH 6.2), 75 mM- $NaHCO_3$, 10 mM-PEP, 10 mM- $MgCl_2$, 10 mM-ADP, 0.3 mM-NADH, 2 units of malate dehydrogenase, and enzyme. The reaction was started by the addition of PEPCK. In the decarboxylation direction the activity was determined in the reaction mixture contained 100 mM-MES buffer (pH 6.2), 5 mM-OAA, 10 mM- $MgCl_2$, 1 mM- $MnCl_2$, 4 mM-ATP, 0.3 mM-NADH, 2 units ml^{-1} of both pyruvate kinase and lactic dehydrogenase, and enzyme. The reaction was started by the addition of OAA. One unit (U) of activity is defined as the amount of enzyme that catalyses the oxidation of 1 μ mol of NADH min^{-1} under the assay condition. Protein was determined by the method of Bradford (1976), using bovine serum albumin as the standard.

Purification of PEPCK. All the procedures were carried out under aerobic conditions at 4°C.

Step 1. Extraction and centrifugation. Cells of *A. succiniciproducens* (40 g) were suspended in 150 ml of 50 mM-MES buffer (pH 6.2) containing 1 mM-DTT. The cells were disrupted by one passage through a French pressure cell at 124 MPa and then centrifuged at 15000 g for 20 min. The supernatant was used as the cell-free extract.

Step 2. Ammonium sulphate precipitation. Solid $(NH_4)_2SO_4$ was added to the supernatant solution to give 70% saturation and the mixture was left overnight. The precipitate formed was collected by centrifugation at 10000 g for 30 min, dissolved in 50 mM-MES buffer (pH 6.2) containing 1 mM-DTT and washed and concentrated by ultrafiltration (YM 30 membrane; Amicon).

Step 3. Column chromatography. The enzyme solution was applied to a DEAE-Sepharose CL-6B column (2.8 cm $\times$ 50 cm) equilibrated with 50 mM-MES buffer (pH 6.2) containing 1 mM-DTT. The column was washed with the same buffer and then eluted with a linear NaCl gradient (0–0.5 M) in the same buffer. The active fractions were pooled, concentrated and dialysed with the same buffer by ultrafiltration. The enzyme solution was loaded onto an anion exchange Mono Q HR 5/5 column (Pharmacia) pre-equilibrated with 50 mM-MES buffer (pH 6.2) containing 1 mM-DTT and eluted with a 0–0.5 M linear gradient of NaCl by using a Waters 650E FPLC system. The fractions containing PEPCK were pooled and concentrated using ultrafiltration.

Electrophoresis, gel scanning and M_r determination. SDS-PAGE was performed as described by Laemmli (1970). Protein bands were visualized by Coomassie Brilliant Blue G-250 staining. The lane corresponding to the crude extract protein was scanned with a BioImage densitometer (Kodak). The M_r of the denaturated protein was estimated using 12% SDS-PAGE with low-range M_r standards. The M_r of the purified PEPCK was determined by gel filtration on a G3000SW TSK-GEL column (TosoHaas) in a Waters FPLC system, using apoferritin (443000), β -amylase (200000), alcohol dehydrogenase (150000) and BSA (66000) as M_r standards.

Isoelectric point determination. The isoelectric point of the enzyme was determined with a Servalyt-Precotes isoelectric focusing gel (pH

3–10; Serva), using an Ultrapore isoelectric focusing apparatus (Pharmacia LKB Biotechnology).

Amino acid composition and sequence determination. The protein was prepared by washing the purified PEPCK with double-distilled water five times with a Centricon-30 (Amicon) filtration device to remove contaminating salts and buffer from the solution. Amino acid composition analysis was performed with a Pico-Tag amino acid analyser (Waters Associates) and the N-terminal amino acid sequence was identified by a protein sequencer model 477A (Applied Biosystems) with an on-line phenylthiohydantoin analyser (Applied Biosystems) in the Macromolecular Structure Facility, Department of Biochemistry, Michigan State University. The repetitive yield for sequenator runs with 12 pmol of PEPCK was around 89%. The N-terminal sequence was compared with known PEPCK sequences by GCG sequence analysis software package (Devereux *et al.*, 1984).

Reproducibility of results. Unless otherwise stated, all experiments were carried out at least in triplicate and the results given are the average of these data.

Results and Discussion

Purification, and molecular and physicochemical properties

PEPCK from *A. succiniciproducens* was purified about 18-fold with a yield of 10% (Table 1). The presence of DTT in all buffers used during purification was essential for enzyme stability. PEPCK from other sources also required DTT for stability or optimum enzyme activity (Ballard & Hanson, 1969; Hebda & Nowak, 1982; Bentle & Lardy, 1976).

PEPCK appeared to be one of the major proteins in cells of *A. succiniciproducens* producing succinate (Fig. 1). Using scanning densitometry of the SDS gel, approximately 10% of the crude extract protein could be attributed to PEPCK, and this high level supports the key catabolic role of this enzyme in CO_2 fixation by this micro-organism. The purified enzyme was homogeneous on SDS-PAGE and revealed an M_r of 58000. The M_r of

Table 1. Purification of PEPCK from *A. succiniciproducens*

All steps were carried out at least three times. The data shown are from a representative purification run.

Step	Protein (mg)	Total activity (U)	Specific activity [U (mg protein) ⁻¹]	Purification (-fold)
Cell-free extract	920	450	0.49	1
$(NH_4)_2SO_4$ fractionation	270	183	0.68	1.3
DEAE Sepharose CL-6B	12	85	7.1	14.4
anion exchange				
Mono Q HR 5/5	5	45	9.1	18.5
anion exchange

Fig. 1. SDS-PAGE of purified PEPCK (lane 1), cell extract of *A. succiniciproducens* (lane 2) and standard M_r markers (lane 3). Sample loading was 1.5 μ g for purified enzyme and 20 μ g for crude enzyme preparation.

the native PEPCK as determined by gel filtration chromatography using FPLC was 56000, indicating that the enzyme possesses a monomeric structure. The M_r of PEPCK from *A. succiniciproducens* was similar to those reported for the same enzyme from bacteria (Goldie & Sanwal, 1980; Salvarrey *et al.*, 1989), but lower than the 70000–80000 M_r of PEPCK from vertebrates and helminths (Utter & Kolenbrander, 1972; Rohrer *et al.*, 1986) and significantly lower than M_r of the same enzyme from lower eukaryotes and plants (Cannata, 1970; Urbina & Avilan, 1989). Furthermore, the enzyme from bakers' yeast is a tetramer (Cannata, 1970) and PEPCK from *Trypanosoma cruzi* is a dimer (Urbina, 1987).

The amino acid composition of the purified enzyme is given in Table 2. The total amount of hydrophobic residues was similar to the PEPCK from pig liver (Chang & Lane, 1966), rat liver (Colombo *et al.*, 1978), chicken liver mitochondria (Hebda & Nowak, 1982) and bakers' yeast (Cannata, 1970), but was higher than that of *Vibrio costicola* (Salvarrey *et al.*, 1989) and *Escherichia coli* (Medina *et al.*, 1990). Glycine was the most abundant residue (14.9%) and in this respect *A. succiniciproducens* PEPCK is similar to PEPCK from *V. costicola*.

Table 2. Amino acid composition of PEPCK from *A. succiniciproducens*

Amino acid	Composition (mol %)
Asx	5.0
Glx	6.4
Ser	7.4
Gly	14.9
His	1.5
Arg	4.8
Thr	9.4
Ala	13.6
Pro	7.1
Tyr	0.0
Val	7.7
Met	0.0
Ile	5.1
Leu	7.6
Phe	4.4
Lys	5.1
Thr	ND
Cys	ND
Total	100
Hydrophobic	45.6
Hydrophilic	39.5

ND, Not determined.

The N-terminal sequence of the first eighteen residues of PEPCK from *A. succiniciproducens* was determined (Ser-Leu-Ser-Glu-Ser-Leu-Ala-Asn-Tyr-Gly-Ile-Thr-Gly-Ala-Thr-Asn-Ile-Val) and compared with other N-terminal sequences of ADP-dependent PEPCKs from *Trypanosoma brucei*, *Saccharomyces cerevisiae* and *E. coli* (Medina *et al.*, 1990). There was very low homology between the N-terminal sequences of PEPCK from the eukaryotes, and only 33.3% identity with the N-terminal sequence of the PEPCK from *E. coli*. However, as the conserved regions occurred in areas other than the N-terminal region (Medina *et al.*, 1990), conclusive similarity between *A. succiniciproducens* PEPCK and PEPCKs from other sources may only be known when the whole amino acid sequence of the enzyme is available.

It is of interest to note that the first amino acid of the purified enzyme from *A. succiniciproducens* was Ser, indicating N-terminal post-translation modification of this enzyme. We may only speculate that a process operates which is similar to the removal of the initiator Met by methyl aminopeptidase in *E. coli*, where cleavage is dependent on the nature of the second amino acid and usually occurs when Ser is in this position (Lapidus *et al.*, 1990).

The pI values for PEPCKs from different sources ranged from approximately 5.0 for the rat liver cytosolic enzyme (Ballard & Hanson, 1969) to 8.0 for the *T. brucei* enzyme (Parsons & Smith, 1989). The pI of PEPCK from *A. succiniciproducens* was estimated to be 4.9, and was

very similar to the rat liver cytosolic PEPCK. The pH optimum of PEPCK from *A. succiniciproducens* was between 6.5 and 7.1 and is similar to the pH optimum reported for PEPCKs from some different species (Chang *et al.*, 1966; Urbina & Avilan, 1989; Cannata & de Flombaum, 1974). The enzyme was stable in the pH range 5.0–9.0, completely losing activity at pH values lower than 4.5 and retaining some activity in the pH range 9–12.

Catalytic properties

The kinetic features of the purified PEPCK were obtained from Lineweaver–Burk plots of specific activities at various substrate concentrations. The apparent K_m values for PEP, HCO_3^- and ADP were 0.54 mM, 17 mM and 0.42 mM, respectively. The apparent $V_{\max}$ and k_{cat} values were 10 U (mg protein) $^{-1}$ and 580 min $^{-1}$, respectively. The apparent K_m values for OAA and ATP were 1.2 mM and 2.3 mM, respectively. The K_m for OAA was approximately twice as high as that for PEP, and the K_m for ADP was approximately one-sixth of that for ATP. No activity was observed when inosine or guanosine nucleotides were used as co-enzymes. This specificity for ADP was typical of PEPCKs from bacterial species, and only PEPCK from *Bacillus stearothermophilus* was active with both ADP and GDP (Uter & Kolenbrander, 1972). The kinetic constants of *A. succiniciproducens* PEPCK are generally similar to those of the same enzyme isolated from other species (Chang *et al.*, 1966; Johnson *et al.*, 1990), although the K_m values for PEP reported for PEPCK from *Ascaris suum* and chicken liver were four-to five-fold lower (Rohrer *et al.*, 1986; Hebda & Nowak, 1982). The kinetic constants for these enzymes were determined under different conditions to those used in this study. In particular, all measurements were carried out in the presence of Mn^{2+} ions and it was shown that the K_m for PEP decreased and the K_m for HCO_3^- was not changed by increasing the concentration of Mn^{2+} .

The K_m value for HCO_3^- for *A. succiniciproducens* PEPCK was indistinguishable from that reported for vertebrates (Uter & Kolenbrander, 1972; Colombo *et al.*, 1978; Schramm *et al.*, 1981) and approximately fivefold higher than that found in plants and helminths (Daley *et al.*, 1977; Urbina & Avilan, 1989). It has been proposed that the high K_m for HCO_3^- (15–20 mM) of PEPCK from animal sources is responsible for the reaction proceeding in the direction of PEP formation *in vivo* (Hebda & Nowak, 1982). To our knowledge this is the first report which presents purification of a CO_2 -fixing PEPCK from a bacterium. Interestingly, although PEPCK from *A. succiniciproducens* also has a high K_m for HCO_3^- , carboxylation of PEP effectively occurs *in*

Table 3. Effect of metal ions on activity of PEPCK from *A. succiniciproducens*

Enzyme activity was measured as described in Methods; addition of substrate was made after incubation of the enzyme with the metal ion for 5 min.

Addition	10 mM-MgCl ₂	Activity (%)
None	—	0
None	+	100
CoCl ₂ (5 mM)	+	1080
MnCl ₂ (5 mM)	+	1013
KCl (5 mM)	+	66
CaCl ₂ (5 mM)	+	71
ZnCl ₂ (1 mM)	+	32
HgCl ₂ (5 mM)	+	0
MnCl ₂ (5 mM)	—	773
CoCl ₂ (5 mM)	—	864

vivo, suggesting that many factors in addition to substrate affinity may play a role in influencing the direction of a reaction. Rohrer *et al.* (1986) proposed that in the case of PEPCK from *Ascaris suum*, which has a K_m for HCO_3^- of 7 mM, but which functions as a CO_2 -fixing enzyme, the 'physiological' direction was determined by a high concentration of HCO_3^- in the small intestine of the vertebrate host. *A. succiniciproducens* is a gastrointestinal anaerobe and requires high concentrations of HCO_3^- for optimal growth (Samuelov *et al.*, 1991). It is possible that PEPCK from *A. succiniciproducens* functions as a CO_2 fixing enzyme due to the high concentration of HCO_3^- within its external milieu.

The effect of various metal ions on the activity of PEPCK from *A. succiniciproducens* was investigated (Table 3). The enzyme did not exhibit any activity in the absence of divalent metal ions, confirming the common property of PEPCK of an absolute requirement for a divalent cation activator. Addition of Mn^{2+} (5 mM) or Co^{2+} (5 mM) in the presence of Mg^{2+} (10 mM) resulted in considerable increases in PEPCK activity. Also the purified enzyme was activated by either Mn^{2+} or Co^{2+} alone, but combinations of Mg^{2+} and Mn^{2+} or Mg^{2+} and Co^{2+} demonstrated a synergistic effect on activity. This synergistic phenomenon was observed with PEPCK from other sources (Rohrer *et al.*, 1986; Bentle & Lardy, 1976), and was interpreted on the basis of a dual-cation role in PEPCK-catalysed OAA formation. It was suggested that Mn^{2+} binds directly and activates the enzyme, while either Mn^{2+} or Mg^{2+} binds to the nucleotide to give the nucleotide–metal complex which is required for activity (Colombo *et al.*, 1981). This mechanism is attractive in explaining the activation of PEPCK from *A. succiniciproducens* by Mn^{2+} or Co^{2+} , but at present it is not known if this is common to all PEPCKs. Other metal ions had very little effect on

PEPCK activity, Zn^{2+} (1 mM) caused 68% reduction in activity and Hg^{2+} (5 mM) totally inhibited enzyme activity.

A. succiniciproducens PEPCK was sensitive to thiol-blocking reagents. Incubation of the enzyme with pCMB (20 μM) for 20 min gave 94% inhibition of the activity in the absence of PEP, whereas in the presence of PEP the inactivation of the enzyme was substantially diminished (16% reduction in activity). The action of pCMB could be reversed by the addition of 5 mM-DTT, indicating the existence of at least one cysteine residue which is essential for catalytic activity and probably involved in the binding and/or activation of PEP in the active site. In this respect PEPCK from *A. succiniciproducens* is similar to many PEPCKs that have cysteine residue(s) essential for catalysis (Lewis *et al.*, 1989; Goto *et al.*, 1980).

The kinetic properties of purified PEPCK from *A. succiniciproducens* are consistent with the important role of this enzyme in succinate formation. V_{max} and k_{cat} were shown to increase significantly in the presence of both Mn^{2+} and Mg^{2+} or Co^{2+} and Mg^{2+} and it is possible that *in vivo* combination of these metals contributes to the catalytic activity of PEPCK. Further studies are required to define the amino acids that may participate in catalysis and to propose a structural model of the active site and the molecular mechanism of enzymic activity.

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