Phosphoenolpyruvate carboxykinase from the moderate halophile *Vibrio costicola*

Purification, physicochemical properties and the effect of univalent-cation salts

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Phosphoenolpyruvate carboxykinase (PEPCK) was purified to homogeneity from the moderately halophilic bacterium *Vibrio costicola*. The enzyme is monomeric, with an M_r of 62000, as determined by the Svedberg equation, by using values of $s_{20,w}^0$ 4.4×10^{-13} s, $D_{20,w}$ 6.13×10^{-7} cm²·s⁻¹ and $\bar{v}$ 0.719 cm³·g⁻¹. Compared with other, non-halophilic, PEPCKs, the enzyme from *V. costicola* had a significantly lower total content of hydrophobic amino acids. The contents of glycine and serine were higher in the *V. costicola* enzyme (16.7 and 10.22% respectively) than in the non-halophilic PEPCKs (6.8–9.6% and 4.67–6.28% respectively). These results resemble those obtained by De Médicis & Rossignol [(1979) *Experientia* **35**, 1546–1547] with the pyruvate kinase from *V. costicola*, and agree with the proposal by Lanyi [(1974) *Bacteriol. Rev.* **38**, 272–290] of partial replacement of hydrophobic amino acids by glycine and serine to maintain the balance between hydrophobic and hydrophilic forces in halophilic enzymes. In agreement with this ‘halophilic’ characteristic, the PEPCK was somewhat stabilized by 1 M-KCl or -NaCl and by 20% (v/v) glycerol, and its oxaloacetate-decarboxylation and ¹⁴CO₂-oxaloacetate-exchange reactions were activated by KCl and NaCl up to 1 M, whereas the fixation of CO₂ on PEP had a maximum at 0.025–0.05 M salt. These facts suggest that the salts, at concentrations probably physiological for the bacterium, increase the formation of the complex of oxaloacetate and ATP with the enzyme, and the liberation of the products, PEP and ADP, thus favouring PEP synthesis.

INTRODUCTION

Moderately halophilic bacteria can grow over a wide range of salt concentrations intermediate between those for marine bacteria and the extreme halophiles (Larsen, 1962). *Vibrio costicola* is able to grow in a complex culture medium between 0.5 M- and 3.5 M-NaCl (Forsyth & Kushner, 1970). The intracellular salt concentration is also intermediate between those for marine and extremely halophilic bacteria, and has been shown to depend to some extent on the salt concentration in the medium (Christian & Waltho, 1962; Masui & Wada, 1973; Shindler *et al.*, 1977; Kamekura & Ohnishi, 1982) and on the age of the culture (Sadler *et al.*, 1980). Since there is a general agreement on the fact that the cells of moderately halophilic bacteria have associated cation concentrations that are considerably higher than those shown by non-halophiles, it could be expected that the enzyme systems of these organisms might be adapted, as are those of extreme halophiles (Lanyi, 1974), to function best in the presence of fairly high salt concentrations that are able to inhibit the activity of most of their non-halophilic counterparts. Although there are some cases that seem to fulfil this prediction (Higa & Cazzulo, 1978; Sadler *et al.*, 1980), most of the enzymes studied in these organisms present their optimal activity in the absence of

added salt, or at least in the presence of salt concentrations much lower than those supposed to be physiological (Larsen, 1962; Shindler *et al.*, 1977; Wydro *et al.*, 1977; De Médicis & Rossignol, 1977; Salvarrey & Cazzulo, 1980; Sadler *et al.*, 1980). This fact has led Kushner and co-workers to suggest that the actual intracellular salt concentrations in moderate halophiles might be considerably lower than those reported, the salt being associated mostly with the cell envelope (Wydro *et al.*, 1977).

Phosphoenolpyruvate carboxykinase [PEPCK; EC 4.1.1.32 (GTP-dependent) or EC 4.1.1.49 (ATP-dependent)] is one of the key enzymes in the gluconeogenic pathway in most organisms from bacteria to higher animals (Utter & Kolenbrander, 1972). This enzyme requires a bivalent-cation-nucleotide complex as substrate and free bivalent cation as an activator (Foster *et al.*, 1967; Cannata & Flombaum, 1974; Lee *et al.*, 1982); high concentrations of univalent-cation salts might interfere with its activity through interference with complex-formation, as proposed by De Médicis & Rossignol (1977, 1979) for the pyruvate kinase from *V. costicola*. PEPCK has been thoroughly studied in several non-halophilic organisms (Utter & Kolenbrander, 1972), and thus a considerable amount of data is available for comparisons.

Abbreviations used: PEP, phosphoenolpyruvate; PEPCK, phosphoenolpyruvate carboxykinase; PAGE, polyacrylamide-gel electrophoresis.

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We report in the present paper a study of some properties of the homogeneous PEPCK purified from *V. costicola* that suggests that the enzyme has some molecular and kinetic properties similar to those shown by its non-halophilic counterparts, whereas it is maximally active and stable at salt concentrations higher than those reported for non-halophilic PEPCK, and presents some differences in amino acid composition as compared with the PEPCKs from non-halophiles similar to those shown for the only other enzyme studied in this respect, the pyruvate kinase from *V. costicola* (De Médicis & Rossignol, 1979).

MATERIALS AND METHODS

Organism and growth

Vibrio costicola (N.R.C. 37001) was kindly given by Dr. D. J. Kushner, University of Ottawa, Ottawa, Ont., Canada. Cultures were grown in a complex medium without glucose [Oxoid Nutrient Broth (1.3 g/l/1 M-NaCl; 300 ml in 1-litre Erlenmeyer flasks), with good aeration, provided by shaking in a NBS Gyrotory Shaker, at 30 °C. When the A_{680} value reached 1.0–1.3 (early stationary phase), the cells were harvested by centrifugation at 6000 *g* for 20 min at 0 °C and washed twice with 50 mM-Tris/HCl buffer, pH 7.6, containing 1 mM-EDTA and 1 M-NaCl.

Enzyme purification

The washed cells were suspended (2 ml/g wet wt.) in 50 mM-Tris/HCl buffer, pH 7.6, containing 1 mM-EDTA, 5 mM-MgCl₂, 0.5 M-NaCl, 0.5 M-KCl and DNAase I (final concn. 50 µg/ml), and disrupted by four sonication treatments (30 s each) at 3 °C at approx. 50 W in a Heat System W185 sonifier. The homogenate was incubated at 13–15 °C for 20 min, and then centrifuged at 27000 *g* for 30 min at 0 °C; the precipitate was suspended in half the initial volume of the same buffer solution, subjected to two sonication treatments (15 s each) and centrifuged again. The precipitate was discarded, and the two supernatants were combined to give the cell-free extract.

The cell-free extract was 60% saturated with (NH₄)₂SO₄ (36.1 g/100 ml) at 0 °C, the pH being maintained at 7.0 by addition of 2 M-NH₃. The suspension was stirred overnight and then centrifuged at 27000 *g* for 90 min at 0 °C. The precipitate was discarded, and the supernatant, containing most of the PEPCK activity, was dialysed at 4 °C with stirring against 20 mM-potassium phosphate buffer, pH 6.8, containing 0.06 M-KCl, 1 mM-EDTA and 20% (v/v) glycerol for 45 h with three buffer changes, 45 vol. each. The dialysed enzyme solution was percolated through a DEAE-cellulose column (2.5 cm × 31 cm) equilibrated with the same buffer at 3 °C. Elution was performed with a linear gradient of 0.08–0.25 M-KCl in the phosphate/EDTA/glycerol solution described above; 6 ml fractions were collected, at a rate of 10 ml/h. The peak of enzyme activity was eluted at 0.150–0.155 M-KCl, as determined by flame photometry. The more active fractions were pooled and dialysed for 15 h at 3 °C, with stirring, against 50 vol. of 5 mM-potassium phosphate buffer, pH 6.8, containing 0.1 mM-EDTA, 1 M-KCl and 10% (v/v) glycerol. The dialysed enzyme solution was then applied to the top of a small hydroxyapatite column (4.4 cm × 2 cm), equilibrated with the same buffer at 3 °C,

which was successively washed with 50 ml volumes of 15 mM-, 30 mM- and 50 mM-potassium phosphate buffer, pH 6.8, containing 0.1 mM-EDTA, 1 M-KCl and 10% (v/v) glycerol; 3 ml fractions were collected, at a rate of 12 ml/h. The enzyme was eluted with the buffer containing 30 mM-potassium phosphate; raising the phosphate concentration to 50 mM did not result in any further recovery of enzyme activity. The more active fractions were pooled and dialysed for 20 h at 3 °C with stirring against 170 vol. of 20 mM-Tris/HCl buffer, pH 7.6, containing 0.1 mM-EDTA, 0.1 mM-dithiothreitol, 2 mM-MnCl₂, 1 mM-MgCl₂ and 20% (v/v) glycerol. The dialysed enzyme solution was percolated through an ATP-agarose column (0.8 cm × 7 cm) equilibrated with a similar buffer, but lacking MgCl₂, at 3 °C; 1.5 ml fractions were collected, at a rate of 9 ml/h. The column was washed with 2 column vol. of the equilibration buffer, and then the PEPCK activity was eluted with the same buffer also containing 1 mM-ATP. The more active fractions were pooled, and kept frozen at –30 °C. Under these conditions the enzyme was stable for at least 2 years. Before use for the experiments described, samples were dialysed overnight against 50 vol. of 20 mM-Tris/HCl buffer, pH 7.6, containing 1 mM-EDTA and 1 M-KCl.

Enzyme assays

1. CO₂ fixation on PEP. (a) Radiochemical method. The PEPCK activity was assayed in a reaction mixture containing, in a final volume of 0.5 ml, 10 µmol of Tris/acetate buffer, pH 6.8, 1 µmol of MnCl₂, 1.5 µmol of PEP, 1.5 µmol of ADP, 1.5 µmol (1 µCi) of NaH¹⁴CO₃, 0.7 µmol of NADH, 1 unit of malate dehydrogenase, and enzyme, which was added last to start the reaction. After incubation for 5 min at 30 °C, the reaction was stopped by addition of 0.05 ml of 2 M-HClO₄. The remaining ¹⁴CO₂ was removed by aeration, the samples were centrifuged, and 0.2 ml portions of the supernatant were placed on to filter-paper discs held in counting vials. After they had been dried in an oven at 80–90 °C, 10 ml of scintillation fluid [2.5 g of 2,5-diphenyloxazole and 0.05 g of 1,4-bis-(5-phenyloxazol-2-yl)benzene per 1 of toluene] was added, and the radioactivity was measured in a Beckman LS 233 scintillation counter. One unit of PEPCK is defined as the amount of enzyme that catalyses the carboxylation of 1.0 µmol of PEP/min under the assay conditions described.

(b) Spectrophotometric method. The PEPCK activity was assayed at 30 °C by monitoring the decrease in A_{340} in a Beckman DU spectrophotometer equipped with a Gilford converter and recorder, in reaction mixtures (1 ml final volume) containing, unless stated otherwise, 50 µmol of Tris/acetate buffer, pH 6.8, 2 µmol of MnCl₂, 2.5 µmol of PEP, 2 µmol of ADP, 15 µmol of NaHCO₃, 0.15 µmol of NADH, 1 unit of malate dehydrogenase, and enzyme, which was added last to start the reaction. One unit of PEPCK is defined as the amount of enzyme that leads to the oxidation of 1 µmol of NADH/min under the assay conditions described.

2. Oxaloacetate decarboxylation to PEP. PEP formation was determined by a colorimetric method measuring the production of P_i from PEP after treatment with alkaline I₂ (Lohmann & Meyerhof, 1934). The reaction was carried out in glass-stoppered tubes (15 mm × 100 mm) containing, in a final volume of 1 ml,

50 μmol of Tris/acetate buffer, pH 6.7, 11.6 μmol of Tris/oxaloacetate, 2 μmol of MnCl_2 , 2 μmol of ATP, and enzyme, which was added last to start the reaction. After incubation for 10 min at 30 °C, the reaction was stopped by addition of 0.1 ml of 2 M-KOH. Then 0.4 ml of 0.1 M- I_2 in KI was added, the samples were incubated for 15 min, and then 0.5 ml of 5 M- H_2SO_4 was added, and the excess I_2 was discharged with 0.20 ml of 0.1 M- NaHSO_3 . The volume was adjusted to 3 ml with distilled water, and the P_i liberated was determined in the same tube by the 2-methylpropan-1-ol/benzene extraction method of Ernster *et al.* (1950). Controls without enzyme were subtracted from the values obtained. One unit of PEPCK is defined as the amount of enzyme that catalyses the formation of 1 μmol of PEP/min under the assay conditions described.

It is worth noting that the $\text{Hg}(\text{NO}_3)_2$ method for the liberation of P_i from PEP (Lohmann & Meyerhof, 1934) previously used for this assay (Cannata & Flombaum, 1974) could not be applied to samples containing high salt concentrations.

3. $^{14}\text{CO}_2$ exchange into oxaloacetate. The reaction mixture contained, in a final volume of 0.5 ml, 25 μmol of Tris/acetate buffer, pH 6.8, 1 μmol of MnCl_2 , 1 μmol of ATP, 12 μmol of Tris/oxaloacetate, 0.96 μmol (1.2 μCi) of $\text{NaH}^{14}\text{CO}_3$, and enzyme, which was added last to start the reaction. After incubation for 5 min at 30 °C, the reaction was stopped by addition of 0.05 ml of 2 M- HClO_4 . The samples were processed for counting of radioactivity by a similar procedure to that used for method 1(a), except that drying was performed overnight at 0 °C in a desiccator over conc. H_2SO_4 and that counting was performed immediately after opening the desiccator, to minimize spontaneous decarboxylation of oxaloacetate. One unit of PEPCK is defined as the amount of enzyme that catalyses the exchange of 1 μmol of $^{14}\text{CO}_2$ into oxaloacetate/min under the assay conditions described.

Protein determination

Protein was determined by the spectrophotometric method of Warburg & Christian (1941), except in the cell-free extracts and in the active fractions of the $(\text{NH}_4)_2\text{SO}_4$ and ATP-agarose steps, where the method of Bensadoun & Weinstein (1976) was employed, with bovine serum albumin as standard.

PAGE

Enzyme purity was assessed by PAGE, either in the native state, by the procedure described by Davis (1964), in a small-pore gel containing 7.5% (w/v) acrylamide, or in the denatured state, by the method of Weber & Osborn (1969) (SDS/PAGE); in the latter case the sample was denatured by boiling for 2 min in a solution containing 1% (w/v) SDS and 1% (v/v) 2-mercaptoethanol. Electrophoretic runs were performed until the tracking dye, Bromophenol Blue, migrated almost to the bottom of the gel column. Runs were performed at room temperature, except in the experiments where PEPCK was to be recovered from the gel by elution; in the latter case PAGE was performed at 3 °C, to minimize enzyme inactivation. Except in the latter case, the gels were fixed and stained with 0.25% Coomassie Brilliant Blue R in aq. 9% (v/v) acetic acid/45% (v/v) methanol in water,

and destained by repeated washings in aq. 7% (v/v) acetic acid/5% (v/v) methanol.

When the enzyme was to be eluted from the gels, they were cut into 2 mm-thick discs, which were incubated overnight at 0 °C in 0.3 ml of 1 M-KCl; the enzyme activity was assayed in the elution fluids by method 3.

When PEPCK was carbamoylated before PAGE, 10 μg of purified enzyme dissolved in 0.2 ml of 25 mM-potassium phosphate buffer, pH 7.4, containing 15% (v/v) glycerol was treated with KCNO (final concn. 0.2 M) for 1 h at 30 °C (Nowicki & Santomé, 1981). Controls were incubated in parallel in a similar solution lacking KCNO.

Determination of the sedimentation coefficient ($s_{20,w}^0$)

The $s_{20,w}^0$ of the purified PEPCK was determined by ultracentrifugation in sucrose gradients, by the method of Martin & Ames (1961). Sucrose gradients [5–20% (w/v) in 50 mM-Tris/HCl buffer, pH 7.6] were prepared in cellulose acetate tubes (13 ml) of the Beckman SW40T rotor. After 4 h at 3 °C, 0.3 ml of a mixture containing PEPCK and the protein markers catalase (5 mg/ml), malate dehydrogenase (0.7 mg/ml) and cytochrome *c* (2 mg/ml) was applied to the top of the gradient, and ultracentrifugation was performed at 38000 rev./min for 14 h at 4 °C. Fractions (0.3 ml each) were collected by injection of 2.5 M-sucrose in the buffer into the bottom of the tube, at a rate of 0.5 ml/min. Catalase (Chance, 1954) and malate dehydrogenase (Ochoa, 1955) were assayed as described in the respective references, and cytochrome *c* was determined by its A_{410} value; their respective distances to the meniscus were 5.0, 2.1 and 1.0 cm.

Determination of the Stokes radius (a) and the free diffusion coefficient (D)

The Stokes radius of the purified PEPCK was determined by gel filtration on Sephacryl S-200 (Ackers, 1964). Gel filtration was performed at 4 °C in a column (2.5 cm $\times$ 94.5 cm) of Sephacryl S-200, equilibrated with 50 mM-Tris/HCl buffer, pH 7.6, containing 1 mM-EDTA and 1 M-KCl. The enzyme and the protein markers at concentrations of 2–3 mg/ml, as well as $^3\text{H}_2\text{O}$ and Blue Dextran 2000, were applied (in separate runs) to the bottom of the column, which was eluted upward with the equilibrating buffer, at a rate of 8 ml/h. Fractions (3.5 ml) were collected, and protein concentrations were determined by measuring the A_{230} . The elution volumes of the protein markers (human γ -globulin, bovine serum albumin, ovalbumin, bovine pancreas chymotrypsinogen and horse heart cytochrome *c*) were 206, 250, 276, 320 and 332 ml respectively; the elution volumes of Blue Dextran and $^3\text{H}_2\text{O}$ were 173 and 454 ml respectively. Stokes radii of the marker proteins were calculated from the diffusion coefficients reported in the literature: human γ -globulin and bovine serum albumin (Phelps & Putnam, 1960), ovalbumin and chymotrypsinogen A (Pharmacia Gel Filtration Calibration Kit Instruction Manual), and cytochrome *c* (Haga *et al.*, 1977). PEPCK was assayed by method 3. The results were analysed by the method of Ackers (1964), which permits the calculation of the Stokes radius (a) of an effluent molecule from its elution volume (V_e) and the inner volume (V_i), the void volume (V_0) and effective pore radius (r) of the calibrated column. The free diffusion coefficient (D) was calculated from the Stokes radius by use of the Stokes-Einstein equation $D = kT/6\pi\eta a$, where k is the Boltzmann constant, T is

the absolute temperature and η is the viscosity of the solution. A temperature of 293 K and the viscosity of water at 20 °C were used in the calculation of $D_{20,w}$.

Amino acid analysis

A sample of the homogeneous PEPCK was prepared for amino acid analysis by exhaustive dialysis against 1000 vol. of 25 mM-pyridine/acetate buffer, pH 6.5, for 3 days at 3 °C, with shaking. Samples were transferred to hydrolysis tubes, evaporated and, after sealing under vacuum, hydrolysed in 6 M-HCl/phenol (1 mg/ml) for 24, 48 or 72 h. Hydrolysates were dried under vacuum, dissolved in 0.12 M-HCl and analysed in a Beckman 119CL automatic amino acid analyser by the procedure of González Cadavid & Paladini (1964). Unless stated otherwise, the values given are the integral numbers corresponding to averages of values obtained for each period of hydrolysis. For threonine and serine the values were extrapolated to zero time. For valine and isoleucine the highest values obtained were used. The total number of half-cystine residues was determined on the amino acid analyser as cysteic acid, after performic acid oxidation and hydrolysis of the protein (Moore, 1963). The tyrosine/tryptophan ratio was obtained from the u.v. spectrum of the enzyme in 0.1 M-NaOH (Beaven & Holiday, 1952). This was converted into tryptophan contents by assuming that the tyrosine residue value obtained by amino acid analysis was correct.

Partial specific volume ($\bar{v}$)

The value of $\bar{v}$ for the purified PEPCK was calculated from its amino acid composition and partial specific volumes of amino acid residues by the method of Cohn & Edsall (1943).

Determination of M_r

The M_r of the purified PEPCK was determined under (a) non-denaturing conditions and (b) dissociating conditions (subunit M_r). (a) Non-dissociating conditions: an approximate value of M_r was determined by the method of Andrews (1965), by using the data of Sephacryl experiments, as described above. A more accurate value was calculated from the $s_{20,w}^0$, $D_{20,w}$ and $\bar{v}$ values, by applying the Svedberg equation

$$M_r = \frac{s}{D} \cdot \frac{RT}{(1 - \bar{v}\rho)}$$

where R is the gas constant and ρ is the solvent density.

(b) Dissociating conditions: the subunit M_r was determined by SDS/PAGE by the method of Weber & Osborn (1969). The experiments were performed as described in the section on PAGE (above), with as protein markers bovine serum albumin, pyruvate kinase, ovalbumin, lactate dehydrogenase, chymotrypsinogen and myoglobin, with subunit M_r values of 67 000, 57 000, 45 000, 36 000, 25 000 and 17 800 respectively.

Chemicals

PEP, ATP, ADP, NADH, avidin, pyruvate kinase from rabbit muscle, lactate dehydrogenase from rabbit muscle, catalase from bovine liver, Tris base, *p*-chloromercuribenzoic acid, *p*-chloromercuribenzenesulphonic acid, 5,5'-dithiobis-(2-nitrobenzoic acid), fluorescein mercuric acetate, *o*-iodosobenzoic acid, *N*-ethylmaleimide, iodoacetamide, dithiothreitol and agarose-*N*⁶-(aminoethyl)carbamoylmethyl-ATP were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. DEAE-cellulose (0.66 mequiv./g) was obtained from Serva Entwicklungslabor, Heidelberg, West Germany. Crystalline bovine serum albumin was obtained from Armour. Ovalbumin, chymotrypsinogen A from bovine pancreas, Blue Dextran 2000 and Sephacryl S-200 were reagents from Pharmacia, Uppsala, Sweden. Human γ -globulin, horse heart cytochrome *c* and sperm-whale myoglobin were obtained from Mann Research Laboratories, New York, NY, U.S.A. $(\text{NH}_4)_2\text{SO}_4$ (analytical reagent, pyridine-free) was obtained from Mallinckrodt, St. Louis, MO, U.S.A. $\text{NaH}^{14}\text{CO}_3$ and $^3\text{H}_2\text{O}$ were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Hydroxyapatite was prepared as described by Tiselius *et al.* (1956). Solutions of PEP and oxaloacetate were prepared and assayed as previously described (Cannata & Flombaum, 1974).

RESULTS

Enzyme purification

The PEPCK from *V. costicola* was purified about 44-fold, with a yield of 4%. Table 1 shows the results obtained with a representative preparation, starting from 30 g wet wt. of cells. The purified enzyme was homogeneous on SDS/PAGE (Fig. 1a); however, when PAGE was performed under non-denaturing conditions (Fig. 1b) two bands were observed. The relative intensity of both bands changed in different electrophoretic runs,

Table 1. Purification of the PEPCK from *V. costicola*

The enzyme was purified as described in the Materials and methods section, starting from 30 g wet wt. of cells. The enzyme activity was assayed by method 3 ($^{14}\text{CO}_2$ exchange into oxaloacetate).

Step	Volume (ml)	Protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification (fold)	Yield (%)
1. Cell-free extract	121	907.5	86.6	0.095	1	100
2. $(\text{NH}_4)_2\text{SO}_4$ fractionation	134	281.4	65.5	0.233	2.4	75.6
3. DEAE-cellulose chromatography	37	56.5	26.7	0.481	5.0	30.8
4. Hydroxyapatite chromatography	5.8	10.9	19.1	1.75	18.4	22.1
5. ATP-agarose chromatography	4.4	0.79	3.3	4.18	43.8	3.8

Fig. 1. PAGE of native, modified and denatured purified PEPCK from *V. costicola*

(a) SDS/PAGE, 23.4 μg of purified enzyme; (b) PAGE under non-denaturing conditions, 20 μg of purified enzyme; (c) PAGE under non-denaturing conditions, 20 μg of carbamoylated enzyme; (d) and (e) PEPCK activity in the gel slices obtained from a duplicate gel identical with (b) and (c) respectively. The enzyme activity was assayed by method 3.

even with different samples of the same preparation; this fact, together with the homogeneity suggested by SDS/PAGE, indicated the possibility of the presence of two interconvertible PEPCK forms. When the enzyme was treated with KCNO, as described in the Materials and methods section, only the form with the higher electrophoretic mobility was present (Fig. 1c). Duplicate gels of those shown in Figs. 1(b) and 1(c) were cut and

the enzyme was eluted as described in the Materials and methods section (Figs. 1d and 1e). The enzyme activity was clearly associated with the enzyme form with the higher electrophoretic mobility; the enzyme form with the lower mobility, which disappeared as a result of the carbamoylation, was devoid of enzyme activity (Fig. 1d).

Molecular and hydrodynamic parameters of the PEPCK from *V. costicola*

All determinations were performed with homogeneous enzyme from step 5 (Table 1) of the purification method.

(a) Ultracentrifugation in sucrose gradient. When the purified PEPCK was ultracentrifuged in a sucrose gradient, as described in the Materials and methods section, its position in the gradient (2.15 cm from the meniscus), compared with those of the protein markers, indicates that the sedimentation coefficient of the enzyme is about 4.4×10^{-13} s, close to the value for one of the markers, malate dehydrogenase (4.3×10^{-13} s).

(b) Gel filtration. Chromatography of the purified PEPCK on a Sephacryl S-200 column, as described in the Materials and methods section, showed a single symmetrical protein peak corresponding to the enzyme activity. From its elution pattern the Stokes radius, a , was determined by the method of Ackers (1964). The gel pore radius, r , of the Sephacryl S-200 column, was determined from the elution volumes and calculated Stokes radii of human γ -globulin, bovine serum albumin, ovalbumin, chymotrypsinogen A and cytochrome *c*. The elution volume of the purified PEPCK was 260 ml. A value of 3.46 nm (34.6 Å) was obtained for the Stokes radius of the latter; this value was subsequently used to calculate a value of 6.13×10^{-7} cm²·s⁻¹ for the diffusion coefficient ($D_{20,w}$) from the Stokes-Einstein equation. When the elution volumes of the marker proteins were plotted as a function of $\log M_r$, in accordance with Andrews (1965), an approximate M_r of 57000 was obtained for the PEPCK.

Fig. 2. Determination of the subunit M_r of the PEPCK from *V. costicola* by SDS/PAGE

The experiment was performed as described in the Materials and methods section, with 23.4 μg of purified enzyme.

(c) **Subunit M_r .** When the purified PEPCK from *V. costicola* was subjected to SDS/PAGE, in parallel with protein markers, as described by Weber & Osborn (1969), and the results were plotted as relative electrophoretic mobility versus $\log M_r$, the subunit M_r of the enzyme was estimated to be about 60000 (Fig. 2).

(d) **Amino acid analysis and partial specific volume.** The amino acid composition of the PEPCK from *V. costicola* is given in Table 2. From its amino acid composition a partial specific volume, $\bar{v}$, of $0.719 \text{ cm}^3 \cdot \text{g}^{-1}$ was calculated.

(e) **M_r and frictional ratio.** The M_r , calculated from the $s_{20,w}^0$, $D_{20,w}$ and $\bar{v}$ values by using the Svedberg equation,

was 62000, in good agreement with the approximate value determined by the gel-filtration method of Andrews (1965). A frictional ratio $f/f_0 = 1.34$ was obtained from the expression

$$f/f_0 = a \left/ \left(\frac{3\bar{v} \cdot M_r}{4\pi \cdot N} \right)^{\frac{1}{3}} \right.$$

where N is Avogadro's number. This value suggests that the molecule of the PEPCK from *V. costicola* is slightly asymmetrical.

The physical parameters determined for the PEPCK from *V. costicola* are summarized in Table 3.

Requirements for enzyme activity

When the PEPCK from *V. costicola*, either partially

Table 2. Amino acid composition of the PEPCK from *V. costicola*, compared with those of the similar enzymes from other sources

Experimental conditions for the PEPCK from *V. costicola* are as described in the Materials and methods section. In each case the corresponding M_r is indicated in parenthesis. Key to references: ^aChang & Lane (1966); ^bBarns & Keech (1972); ^cColombo *et al.* (1978); ^dHebda & Nowak (1982); ^eCannata (1970).

Amino acid	Composition (residues per molecule of enzyme)					
	<i>V. costicola</i> (62000)	Pig liver ^a (73300)	Sheep kidney ^b (71000)	Rat liver ^c cytosol (72000)	Chicken liver ^d mitochondria (73000)	Baker's yeast ^e (252000)
Lysine	33	26	34	44	20	155
Arginine	21	48	38	28	52	95
Histidine	11	12	13	12	15	69
Tyrosine	13	12	12	15	14	90
Tryptophan	13	13	13	19	15	30
Half-cystine	4	15	13	13	9	41
Methionine	10	20	14	18	19	16
Aspartic acid	68	53	57	56	55	224
Glutamic acid	62	70	79	76	56	231
Threonine	33	31	38	23	27	147
Serine	63	31	39	34	40	142
Proline	20	56	54	41	66	116
Glycine	103	64	63	58	63	155
Alanine	40	55	65	51	54	180
Valine	38	44	42	28	45	145
Leucine	36	58	52	55	60	181
Isoleucine	28	28	29	36	21	150
Phenylalanine	20	27	29	28	29	95
Glycine (%)	16.7	9.6	9.3	9.1	9.5	6.8
Proline (%)	3.2	8.4	8.0	6.5	10.0	5.1
Basic amino acids (%)	10.5	13.0	12.6	13.2	13.2	14.1
Acidic amino acids (%)	21.1	18.5	20.2	20.8	16.8	20.1
Hydrophobic amino acids (%)	33.3	45.4	42.7	43.5	46.8	40.3
Aromatic amino acids (%)	7.5	7.8	8.0	9.8	8.8	9.5

Table 3. Summary of physicochemical parameters of the PEPCK from *V. costicola*

Physical parameter	Method	Value
Sedimentation coefficient ($s_{20,w}^0$)	Velocity sedimentation	$4.4 \times 10^{-13} \text{ s}$
Diffusion coefficient ($D_{20,w}$)	Gel filtration	$6.13 \times 10^{-7} \text{ cm}^2 \cdot \text{s}^{-1}$
Stokes radius (a)	Gel filtration	3.46 nm (34.6 Å)
Partial specific volume ($\bar{v}$)	From amino acid composition	$0.719 \text{ cm}^3 \cdot \text{g}^{-1}$
M_r	From $D_{20,w}$, $s_{20,w}^0$ and $\bar{v}$ data	62000
	From gel filtration (Andrews method)	57000
Subunit M_r	From SDS/PAGE	60000
Frictional coefficient (f/f_0)	From $\bar{v}$ and M_r data	1.34

purified (step 3) or homogeneous (step 5), was assayed in the direction of CO_2 fixation on PEP by the radiochemical method (1a), the activity required PEP and ADP, and bivalent cation; Mn^{2+} (1 mM) was much more effective than Mg^{2+} (3 mM), and the activity increased by 16% in the presence of both cations together. Avidin did not inhibit the reaction, thus ruling out the possibility that the observed activity might be due to pyruvate kinase and pyruvate carboxylase acting together. The requirement for ADP ruled out the possibility that the activity might be due to PEP carboxylase; the latter enzyme was also present in the cell-free extract, at a lower activity, but was eliminated during steps 2 and 3 of the purification procedure (results not shown). The identity of the enzyme as a PEPCK was confirmed by the demonstration of an ATP-dependent $^{14}\text{CO}_2$ -oxaloacetate exchange, which is strictly specific for PEPCK. When this assay (method 3) was used, again the activity was unaffected by avidin, strictly required ATP and bivalent cation, Mg^{2+} was less effective than Mn^{2+} , and the presence of both cations together increased the activity by 76%.

Effects of NaCl and KCl on enzyme activity

Fig. 3 shows the effects of increasing concentrations of KCl and NaCl, up to 1 M, on the CO_2 -fixation, PEP-formation and $^{14}\text{CO}_2$ -oxaloacetate-exchange reactions catalysed by the PEPCK from *V. costicola*. The CO_2 -fixation reaction was maximally activated (about 130%) by KCl at 0.025–0.050 M, NaCl being less effective; higher concentrations of the salts caused a relative inhibition, the activity in the presence of 1 M salt being nearly the same as that determined in the absence of added salts. When the $^{14}\text{CO}_2$ -oxaloacetate-exchange activity was determined, on the other hand, the enzyme was activated by both salts to a similar extent, and the activity increased up to 1 M salt. When PEP formation

Fig. 3. Effects of KCl and NaCl on the activity of the PEPCK from *V. costicola*

The enzyme activity was determined by method 1(b) (CO_2 fixation, spectrophotometric), with 2.6 μg of purified enzyme; (Δ , NaCl; $\blacktriangle$, KCl), by method 2 (PEP formation), with 2.6 μg of purified enzyme ($\circ$, NaCl; $\bullet$, KCl), or by method 3 ($^{14}\text{CO}_2$ -oxaloacetate exchange), with 1.1 μg of purified enzyme ($\square$, NaCl; $\blacksquare$, KCl).

Fig. 4. Effects of KCl ($\circ$ and Δ) and NaCl ($\bullet$ and $\blacktriangle$) on the apparent K_m ($\circ$ and $\bullet$) and $V_{\max}$ (Δ and $\blacktriangle$) of the PEPCK from *V. costicola*, with PEP (a) or ADP (b) as the varied substrate

Enzyme activity was assayed spectrophotometrically [method 1(b)], at a fixed concentration (2 mM) of ADP (a) or PEP (b).

from oxaloacetate, namely the direction of the reaction physiologically important for gluconeogenesis, was determined, curves similar to those for the $^{14}\text{CO}_2$ -oxaloacetate exchange were obtained, with maximal activation of 2-fold at 1 M salt.

Fig. 4 shows the effects of increasing concentrations of KCl and NaCl on the apparent kinetic constants for the substrates of the CO_2 -fixation reaction, PEP (Fig. 4a) and ADP (Fig. 4b). The apparent K_m value for PEP increased from 0.04 mM in the absence of salt to a maximum of 0.67 mM or 2.17 mM in the presence of 0.5 M-KCl or -NaCl respectively, whereas the $V_{\max}$ values had a maximum at 0.025–0.050 mM salt, and then decreased to values lower than those obtained in the absence of salts (Fig. 4a). Similar results were obtained when the varied substrate was ADP (Fig. 4b), but in this case there was much less variation in the apparent K_m values in the absence of salts (about 20 μM) and in the presence of 0.5 M-NaCl or -KCl (28 and 50 μM respectively).

Effects of NaCl, KCl and glycerol on enzyme stability

Fig. 5 shows the enzyme behaviour at 30 $^{\circ}\text{C}$ when it was preincubated, at a concentration of 10.5 $\mu\text{g}/\text{ml}$, in the absence of protectors, or in the presence of 1 M-NaCl, 1 M-KCl or 20% (v/v) glycerol. When the enzyme was diluted (50-fold) into the solutions containing NaCl or KCl, or without salt addition, a considerable increase (230–300%) in the enzyme activity was observed within the first 4 h. This increase was much less (40%) in the presence of glycerol. After 4 h enzyme decay was

Fig. 5. Effects of KCl, NaCl and glycerol on the stability of the PEPCK from *V. costicola*

The enzyme (10.5 $\mu\text{g}/\text{ml}$) was incubated at 30 °C in the absence of protectors (○) or in the presence of 1 M-KCl (□), 1 M-NaCl (●) or 20% (v/v) glycerol (△). The enzyme activity was assayed by method 1(b).

observed; this decay was biphasic, except with glycerol. The effects of the salts varied with time; in the first phase of decay the enzyme half-life was shorter in the presence of 1 M-NaCl (43 h) or 1 M-KCl (22 h) than in the absence of salts (192 h). In the second phase of decay, on the other hand, the respective values were 86, 206 and 48 h, thus showing a protective effect of the salts, KCl being the most effective. Glycerol was the best protector, since the decay curve in its presence was monophasic, with a half-life of 155 h; as a result, the remaining enzyme activity at the end of the experiment (214 h) was 57, 37, 31 and 28% in the presence of glycerol, NaCl or KCl or in the absence of salts respectively.

Inhibition by thiol-blocking reagents

The PEPCK from *V. costicola* was inhibited to different extents by several thiol-blocking reagents; the $^{14}\text{CO}_2$ -oxaloacetate-exchange method (method 3) was used for the determination of the enzyme activity, in order to avoid the use of coupled enzymes, whose inhibition by the reagents might give doubtful results. Fluorescein mercuric acetate was the most effective inhibitor, giving 80% inhibition at 0.1 mM; 50% inhibition was attained at 0.8 μM . *p*-Chloromercuribenzenesulphonic acid nearly abolished the enzyme activity at 1 mM, with 50% inhibition at 2.5 μM . *p*-Chloromercuribenzoic acid inhibited by about 70% at 1 mM, with 50% inhibition at 7 μM . *o*-Iodosobenzoic acid was less effective, giving about 60% inhibition at 1 mM, with 50% inhibition at about 0.2 mM; iodoacetamide and *N*-ethylmaleimide had little effect, with maximal inhibitions of about 10% at 1 mM. 5,5'-Dithiobis-(2-nitrobenzoic acid) was ineffective, with 6% inhibition at 5 mM.

The purified PEPCK was activated by about 60% by preincubation with 1.6 mM-dithiothreitol. In addition, the enzyme inhibited by 70% or by 88% respectively by

preincubation (5 min) with fluorescein mercuric acetate or *p*-chloromercuribenzenesulphonic acid could be re-activated to about 60% of the activity in the presence of dithiothreitol by further preincubation (2 min) with the latter reagent.

DISCUSSION

The moderately halophilic bacterium *V. costicola*, grown in complex medium without glucose, showed activity of three enzymes potentially able to fix CO_2 , namely PEPCK, PEP carboxylase [assayed radiochemically by method 1(a), with ADP omitted from the reaction mixture] and NADP-linked malic enzyme (Salvarrey & Cazzulo, 1980). The specific activity of PEPCK in the cell-free extracts was 10-fold higher than that of PEP carboxylase; the latter showed maximal activity in the presence of 0.1 M-KCl or -NaCl, but was strongly inhibited by higher salt concentrations, which at 1 M almost abolished its activity (M. S. Salvarrey & J. J. Cazzulo, unpublished work).

We have now purified the PEPCK 44-fold, to protein homogeneity; this fact might suggest that a considerable amount of the protein in the initial crude extract was PEPCK. The enzyme is monomeric, with an M_r of about 62000. This value is almost identical with that of the subunit of the PEPCK from baker's yeast (Müller *et al.*, 1981), and slightly lower than those of mammalian PEPCKs (Table 2). An association process seems to occur, however, leading to an inactive enzyme form, as suggested by the results in Fig. 1; this association is prevented by carbamylation, which stabilizes the enzyme form with the higher electrophoretic mobility, without effect on the enzyme activity. Under the experimental conditions used, carbamylation of the *N*-terminal group is predominant, although some reaction

with lysine ϵ -amino groups may occur (Means & Feeney, 1971).

The amino acid composition of the PEPCK from *V. costicola* has some clear differences from those determined for other, non-halophilic, PEPCKs (Table 2). The enzyme from *V. costicola* had a slightly lower content of basic amino acids, and had a content of acidic amino acids at the upper limit, as compared with its non-halophilic counterparts. The content of aromatic amino acids was at the lower limit, whereas the total content of hydrophobic amino acids was significantly lower than that of the non-halophilic PEPCKs. The main difference found was in the contents of glycine (16.7% in the PEPCK from *V. costicola*, as compared with 6.8–9.6% for the other PEPCKs) and serine (10.22% for the halophilic PEPCK, as compared with 4.67–6.28% for the other enzymes). It is noteworthy that in the only other enzyme from *V. costicola* whose amino acid composition has been determined, pyruvate kinase (De Médicis & Rossignol, 1979), a significant decrease in hydrophobic residues and an increase in serine and glycine residues were found. Lanyi (1974) has proposed that in halophilic proteins hydrophobic residues could be replaced by mildly hydrophobic residues such as glycine and serine in order to maintain the balance between hydrophobic and hydrophilic forces. Our results, like those obtained by De Médicis & Rossignol (1979), agree with the proposal.

The half-cystine content of the *V. costicola* PEPCK (four residues per enzyme molecule) is much lower than that for the baker's-yeast enzyme (ten residues per subunit of similar M_r). The inhibition by thiol-blocking reagents suggests that the PEPCK from the moderate halophile requires at least one free thiol group for activity, as also shown by the activation by dithiothreitol. Therefore the PEPCK from *V. costicola* has at most one disulphide bridge in its structure. Other PEPCKs have also been shown to be inhibited by thiol-blocking reagents (Utter & Kolenbrander, 1972).

Previous kinetic studies with the pyruvate kinase (De Médicis & Rossignol, 1977) and the malic enzyme (Salvarrey & Cazzulo, 1980) from *V. costicola* indicate that these enzymes are able to function best at salt concentrations (0.1–0.2 M) that, although higher than those optimal for their non-halophilic counterparts, are still considerably lower than the salt concentrations reported to be associated with living cells of the bacterium. The present results show a more complex situation for PEPCK. The CO_2 -fixation reaction followed a pattern of response to salt concentration similar to those shown by pyruvate kinase and malic enzyme, although maximal activity was reached at lower salt concentrations (0.025–0.050 M). The oxaloacetate-decarboxylation and the $^{14}\text{CO}_2$ -oxaloacetate-exchange reactions, on the other hand, were activated by the salts at higher concentrations, maximal activity being reached at about 1 M. These results suggest that salts favour the formation of the complex of oxaloacetate and ATP with the enzyme, in which the exchange reaction would take place (Utter & Kolenbrander, 1972; Hebda & Nowak, 1982), and the liberation of the products, PEP and ADP, the affinity for which decreases at the higher salt concentrations (Fig. 4). Therefore the physiological direction of the enzyme reaction, namely PEP formation, would be favoured at salt concentrations similar to those reported for moderate halophiles (see the Introduction).

The effects of salts on the stability of the PEPCK from *V. costicola* were also complex. First, the enzyme was strongly activated by 50-fold dilution to a final concentration of 10 $\mu\text{g/ml}$; this activation might be due to dissociation of inactive complexes present in the concentrated enzyme solution to the monomeric active form (suggested by the experiment in Fig. 1). Secondly, except with the addition of 20% (v/v) glycerol, the decay curve was biphasic. In the first phase, up to 60–70 h at 30 °C, the salts actually decreased enzyme stability; in the second phase, on the other hand, KCl, and to a lesser extent NaCl, increased the half-life of the enzyme. On the basis of these results, glycerol was added as a protector for the latter steps of the purification procedure. In any case, this PEPCK proved to be remarkably stable in very dilute solutions. The present results indicate that the PEPCK from *V. costicola* shares some properties common to other enzymes purified from the same organism, namely some peculiarities in amino acid composition, and salt activation and stabilization at concentrations higher than those for their non-halophilic counterparts.

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REFERENCES

- Ackers, G. K. (1964) *Biochemistry* **3**, 723–730
- Andrews, P. (1965) *Biochem. J.* **96**, 596–606
- Barns, R. J. & Keech, D. B. (1972) *Biochim. Biophys. Acta* **276**, 284–296
- Beaven, G. H. & Holiday, E. R. (1952) *Adv. Protein Chem.* **7**, 319–386
- Bensadoun, A. & Weinstein, D. (1976) *Anal. Biochem.* **70**, 241–250
- Cannata, J. J. B. (1970) *J. Biol. Chem.* **245**, 792–798
- Cannata, J. J. B. & Flombaum, M. A. C. (1974) *J. Biol. Chem.* **249**, 3356–3365
- Chance, B. (1954) *Methods Biochem. Anal.* **1**, 410–424
- Chang, H. C. & Lane, M. D. (1966) *J. Biol. Chem.* **241**, 2413–2420
- Christian, J. H. B. & Waltho, J. A. (1962) *Biochim. Biophys. Acta* **65**, 506–508
- Cohn, E. I. & Edsall, J. T. (1943) *Proteins, Aminoacids and Peptides as Ions and Dipolar Ions*, pp. 370–381, Reinhold Publishing Corp., New York
- Colombo, G., Carlson, G. M. & Lardy, H. A. (1978) *Biochemistry* **17**, 5321–5329
- Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.* **121**, 404–427
- De Médicis, E. & Rossignol, B. (1977) *Can. J. Biochem.* **55**, 825–833
- De Médicis, E. & Rossignol, B. (1979) *Experientia* **35**, 1546–1547
- Ernster, L., Zetterström, R. & Lindberg, R. (1950) *Acta Chem. Scand.* **4**, 942–958
- Forsyth, M. P. & Kushner, D. J. (1970) *Can. J. Microbiol.* **16**, 253–261
- Foster, D. O., Lardy, H. A., Ray, P. D. & Johnston, J. B. (1967) *Biochemistry* **6**, 2120–2128
- González Cadavid, N. & Paladini, A. C. (1964) *Anal. Biochem.* **9**, 170–174

- Haga, T., Haga, K. & Gilman, A. C. (1977) *J. Biol. Chem.* **252**, 5776–5780
- Hebda, C. A. & Nowak, T. (1982) *J. Biol. Chem.* **257**, 5512–5522
- Higa, A. I. & Cazzulo, J. J. (1978) *FEMS Microbiol. Lett.* **3**, 157–160
- Kamekura, M. & Ohnishi, H. (1982) *Can. J. Microbiol.* **28**, 155–161
- Lanyi, J. K. (1974) *Bacteriol. Rev.* **38**, 272–290
- Larsen, H. (1962) in *The Bacteria* (Gunsalus, I. C. & Stanier, R. Y., eds.), vol. 4, pp. 297–342, Academic Press, New York and London
- Lee, M. H., Hebda, C. A. & Nowak, T. (1982) *J. Biol. Chem.* **256**, 12793–12801
- Lohmann, K. & Meyerhof, O. (1934) *Biochem. Z.* **273**, 60–81
- Martin, R. G. & Ames, B. C. (1961) *J. Biol. Chem.* **236**, 1372–1379
- Masui, M. & Wada, S. (1973) *Can. J. Microbiol.* **19**, 1181–1186
- Means, G. E. & Feeney, R. E. (1971) *Chemical Modification of Proteins*, pp. 84–89, Holden-Day, San Francisco
- Moore, S. (1963) *J. Biol. Chem.* **238**, 235–237
- Müller, M., Müller, H. & Holzer, H. (1981) *J. Biol. Chem.* **256**, 723–727
- Nowicki, C. & Santomé, J. A. (1981) *Int. J. Pept. Protein Res.* **18**, 52–60
- Ochoa, S. (1955) *Methods Enzymol.* **1**, 735–739
- Phelps, R. A. & Putnam, F. W. (1960) in *The Plasma Proteins* (Putnam, F. W., ed.), vol. 1, pp. 143–148, Academic Press, New York
- Sadler, M., McAninch, M., Alico, R. & Hochstein, L. I. (1980) *Can. J. Microbiol.* **26**, 496–502
- Salvarrey, M. S. & Cazzulo, J. J. (1980) *Can. J. Microbiol.* **26**, 50–57
- Shindler, D. B., Wydro, R. M. & Kushner, D. J. (1977) *J. Bacteriol.* **130**, 698–703
- Tiselius, A., Hjertén, S. & Levin, O. (1956) *Arch. Biochem. Biophys.* **65**, 132–155
- Utter, M. F. & Kolenbrander, H. M. (1972) *Enzymes* 3rd Ed. **6**, 117–168
- Warburg, O. & Christian, W. (1941) *Biochem. Z.* **310**, 384–421
- Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4412
- Wydro, R. M., Madira, M., Hiramatsu, T., Kogut, M. & Kushner, D. J. (1977) *Nature (London)* **269**, 824–825

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