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 62,000, as determined by the Svedberg equation, by using values of $\varepsilon^{19,5}_{280}, 4.4 \times 10^{13}$ s, $D_{a0}, 6.13 \times 10^{-7}$ cm$^2$·s$^{-1}$ and $b, 0.719$ cm$^3$·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% (w/v) glycerol, and its oxaloacetate-decarboxylation and $^{14}$CO$_2$-oxaloacetate-exchange reactions were activated by KCl and NaCl up to 1 m, whereas the fixation of CO$_2$ 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 fulfill 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 Gyratory Shaker, at 30°C. When the A_{490} 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 mM-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 mM-NaCl, 0.5 mM-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 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 mM-Na₂HPO₄. 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 mM-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 x 31 cm) equilibrated with the same buffer at 3°C. Elution was performed with a linear gradient of 0.08–0.25 mM-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 mM-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 mM-KCl and 10% (v/v) glycerol. The dialysed enzyme solution was then applied to the top of a small hydroxyapatite column (4.4 cm x 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 mM-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 x 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 mM-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 A₄₅⁴ 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, from PEP after treatment with alkaline I₂ (Lohmann & Meyerhof, 1934). The reaction was carried out in glass-stoppered tubes (15 mm x 100 mm) containing, in a final volume of 1 ml,
Phosphoenolpyruvate carboxykinase from a moderate halophile

50 μmol of Tris/acetate buffer, pH 6.7, 11.6 μmol of Tris/oxaloacetate, 2 μmol of MnCl₂, 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-KI in KI was added, the samples were incubated for 15 min, and then 0.5 ml of 5 M-H₂SO₄ was added, and the excess I₂ was discharged with 0.20 ml of 0.1 M-NaHSO₃. The volume was adjusted to 3 ml with distilled water, and the P, 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 results 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 Hg(NO₃)₂ method for the liberation of P, from PEP (Lohmann & Meyerhof, 1934) previously used for this assay (Cannata & Fliombau, 1974) could not be applied to samples containing high salt concentrations.

3. ¹⁴CO₂ 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 MnCl₂, 1 μmol of ATP, 12 μmol of Tris/oxaloacetate, 0.96 μmol (1.2 μC) of NaH¹⁴CO₃, 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 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. H₂SO₄ 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 ¹⁴CO₂ 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 extract and in the active fractions of the (NH₄)₂SO₄ 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 inaq. 9% (v/v) acetic acid/45% (v/v) methanol in water, and destained by repeated washings inaq. 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 carbamylated 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 & Santome, 1981). Controls were incubated in parallel in a similar solution lacking KCNO.

Determination of the sedimentation coefficient (S₂₀,₅₀)

The S₂₀,₅₀ 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 ml-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 A₅₅₅ 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 (Akers, 1964). Gel filtration was performed at 4 °C in a column (2.5 cm x 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 H₂O 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 A₃₉₀. 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 ³²H₂O 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 (Vᵣ) and the inner volume (Vᵢ), the void volume (Vᵥ) 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

\[ D = kT/6πηa \]

where k is the Boltzmann constant, T is
the absolute temperature and \( \eta \) 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 (\( \bar{\rho} \))**

The value of \( \bar{\rho} \) 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 \( M_s \)). (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 \( s^2_{20,w} \), \( D_{20,w} \) and \( \bar{\rho} \) values, by applying the Svedberg equation

\[
M_r = s^2 \frac{RT}{D(1-\bar{\rho})}
\]

where \( R \) is the gas constant and \( \rho \) is the solvent density.

(b) Dissociating conditions: the subunit \( M_s \) 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 \( M_s \) values of 67000, 57000, 45000, 36000, 25000 and 17800 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, \( \alpha \)-iodosobenzoic acid, \( \alpha 

**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>
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<th>Step</th>
<th>Volume (ml)</th>
<th>Protein (mg)</th>
<th>Total activity (units)</th>
<th>Specific activity (units/mg)</th>
<th>Purification (fold)</th>
<th>Yield (%)</th>
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<tr>
<td>1. Cell-free extract</td>
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<td>5. ATP-agarose chromatography</td>
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<td>4.18</td>
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**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 (\(^{14}\)CO₂ exchange into oxaloacetate).
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 carboxamoylation, 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 $4.4 \times 10^{-12}$ s, close to the value for one of the markers, malate dehydrogenase ($4.3 \times 10^{-12}$ 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 $\gamma$-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 $6.13 \times 10^{-7} \text{cm}^2 \cdot \text{s}^{-1}$ 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. 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 carboxamylated 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.

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 cm$^3$·g$^{-1}$ was calculated.

(e) $M_r$ and frictional ratio. The $M_r$, calculated from the $s_{20,w}^2$, $D_{20,w}$ 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 $f/f_o = 1.34$ was obtained from the expression

$$f/f_o = a \left( \frac{3 \bar{v} \cdot M_r}{4 \pi N} \right)^{\frac{1}{2}}$$

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>
<thead>
<tr>
<th>Amino acid</th>
<th>V. costicola (62000)</th>
<th>Pig liver$^a$ (73000)</th>
<th>Sheep kidney$^b$ (71000)</th>
<th>Rat liver$^c$ cytosol (72000)</th>
<th>Chicken liver$^d$ mitochondria (73000)</th>
<th>Baker’s yeast$^e$ (252000)</th>
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<td>34</td>
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<td>Aspartic acid</td>
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<td>Glutamic acid</td>
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<tr>
<td>Threonine</td>
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<td>31</td>
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<td>23</td>
<td>27</td>
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<tr>
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<td>39</td>
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<tr>
<td>Proline</td>
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<td>56</td>
<td>54</td>
<td>41</td>
<td>66</td>
<td>116</td>
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<tr>
<td>Glycine</td>
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<td>63</td>
<td>58</td>
<td>63</td>
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<td>Alanine</td>
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<td>Leucine</td>
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<td>58</td>
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<td>Isoleucine</td>
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<td>28</td>
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<td>Phenylalanine</td>
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<td>29</td>
<td>28</td>
<td>29</td>
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<tr>
<td>Glycine (%)</td>
<td>16.7</td>
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<td>9.3</td>
<td>9.1</td>
<td>9.5</td>
<td>6.8</td>
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<tr>
<td>Proline (%)</td>
<td>3.2</td>
<td>8.4</td>
<td>8.0</td>
<td>6.5</td>
<td>10.0</td>
<td>5.1</td>
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<tr>
<td>Basic amino acids (%)</td>
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<td>13.0</td>
<td>12.6</td>
<td>13.2</td>
<td>13.2</td>
<td>14.1</td>
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<tr>
<td>Acidic amino acids (%)</td>
<td>21.1</td>
<td>18.5</td>
<td>20.2</td>
<td>20.8</td>
<td>16.8</td>
<td>20.1</td>
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<td>Hydrophobic amino acids (%)</td>
<td>33.3</td>
<td>45.4</td>
<td>42.7</td>
<td>43.5</td>
<td>46.8</td>
<td>40.3</td>
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<td>Aromatic amino acids (%)</td>
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<td>7.8</td>
<td>8.0</td>
<td>9.8</td>
<td>8.8</td>
<td>9.5</td>
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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: $^a$Chang & Lane (1966); $^b$Barns & Keech (1972); $^c$Colombo et al. (1978); $^d$Hebda & Nowak (1982); $^e$Cannata (1970).

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

<table>
<thead>
<tr>
<th>Physical parameter</th>
<th>Method</th>
<th>Value</th>
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<tbody>
<tr>
<td>Sedimentation coefficient ($s_{20,w}^2$)</td>
<td>Velocity sedimentation</td>
<td>$4.4 \times 10^{-13}$ s</td>
</tr>
<tr>
<td>Diffusion coefficient ($D_{20,w}$)</td>
<td>Gel filtration</td>
<td>$6.13 \times 10^{-7}$ cm$^2$.s$^{-1}$</td>
</tr>
<tr>
<td>Stokes radius ($a$)</td>
<td>Gel filtration</td>
<td>$3.46$ nm (34.6 Å)</td>
</tr>
<tr>
<td>Partial specific volume ($\bar{v}$)</td>
<td>From amino acid composition</td>
<td>$0.719$ cm$^3$.g$^{-1}$</td>
</tr>
<tr>
<td>$M_r$</td>
<td>From $D_{20,w}$, $s_{20,w}$</td>
<td>62000</td>
</tr>
<tr>
<td>Subunit $M_r$</td>
<td>From gel filtration (Andrews method)</td>
<td>57000</td>
</tr>
<tr>
<td>Frictional coefficient ($f/f_o$)</td>
<td>From SDS/PAGE</td>
<td>60000</td>
</tr>
</tbody>
</table>

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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 $^{14}$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 $^{14}$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 $^{14}$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 from oxaloacetate, namely the direction of the reaction physiologically important for gluconeogenesis, was determined, curves similar to those for the $^{14}$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 mM) and in the presence of 0.5 M-NaCl or -KCl (28 and 50 mM respectively).

**Effects of NaCl, KCl and glycerol on enzyme stability**

Fig. 5 shows the enzyme behaviour at 30°C when it was preincubated, at a concentration of 10.5 μg/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
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 $^{14}$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 mM. p-Chloromercuribenzenesulphonic acid nearly abolished the enzyme activity at 1 mM, with 50% inhibition at 2.5 mM. p-Chloromercuribenzoic acid inhibited by about 70% at 1 mM, with 50% inhibition at 7 mM. d-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 reactivated 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 carbamoylation, which stabilizes the enzyme form with the higher electrophoretic mobility, without effect on the enzyme activity. Under the experimental conditions used, carbamoylation of the N-terminal group is predominant, although some reaction
Phosphoenolpyruvate carboxykinase from a moderate halophile

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 μg/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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