Phosphoenolpyruvate Carboxylase from the Crassulacean Plant *Bryophyllum fedtschenkoi* Hamet et Perrier

PURIFICATION, MOLECULAR AND KINETIC PROPERTIES

By ROBERT JONES,* MALCOLM B. WILKINS, JOHN R. COGGINS,
CHARLES A. FEWSON and ALAN D. B. MALCOLM†

Departments of Botany and Biochemistry, University of Glasgow, Glasgow G12 8QQ,
Scotland, U.K.

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Phosphoenolpyruvate carboxylase from the Crassulacean plant *Bryophyllum fedtschenkoi* has been purified to homogeneity by DEAE-cellulose treatment, $(\text{NH}_4)_2\text{SO}_4$ fractionation, and chromatography on DEAE-cellulose and hydroxyapatite. Poly(ethylene glycol) is required in the extraction medium to obtain maximum enzyme activity. The purified enzyme has a specific activity of about 26 units/mg of protein at 25°C. It gives a single band on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, corresponding to a mol.wt. of 105000, and gives a single band on non-denaturing gel electrophoresis at pH 8.4. Cross-linking studies at pH 8.0 indicate that the subunit structure is tetrameric but that the dimer may also be an important unit of polymerization. Gel filtration results at pH 6.7 confirm that the native enzyme is tetrameric with a concentration-dependent dissociation to a dimer. The kinetic behaviour is characterized by (i) relatively small variations in maximum velocity between pH 5.5 and 9.0 with a double optimum, (ii) a reversible temperature-dependent inactivation between 30 and 45°C, (iii) inhibition by malate, which is pH-sensitive, and (iv) almost Michaelis–Menten behaviour with phosphoenolpyruvate as the varied ligand but sigmoidal behaviour under suitable conditions with malate as the varied ligand. The findings are related to other studies and to the possible role of phosphoenolpyruvate carboxylase in controlling a circadian rhythm of CO_2 fixation.

The circadian rhythm of CO_2 output from leaves of the Crassulacean plant *Bryophyllum fedtschenkoi* Hamet et Perrier, maintained in continuous darkness in CO_2 -free air, is well characterized with regard to period, phase-resetting, damping and re-initiation, and entrainment in response to light and other stimuli (Wilkins, 1960). The rhythm appears to originate from a periodic CO_2 fixation in the mesophyll cells via the Crassulacean acid metabolic pathway (Warren & Wilkins, 1961), the initial reaction of which is catalysed by phosphoenolpyruvate carboxylase (EC 4.1.1.31). Early attempts to elucidate the biochemical mechanism of this periodic activity were made by Warren (1964), but success was limited by the inadequate methods then available for extracting active enzymes from plant sources.

The periodic activity of phosphoenolpyruvate carboxylase might arise in several ways. Rhythms of

enzyme capacity, i.e. the total extractable activity assayed under non-limiting conditions, have been demonstrated, particularly in *Kalanchoë blossfeldiana* (another Crassulacean plant) (Queiroz & Morel, 1974; Brulfert *et al.*, 1975), but these appear to be inadequate to account for the very large activity changes *in vivo*. A second possible way is by a cyclic structural modification of the enzyme to give different molecular forms. The most widely accepted control hypothesis is that of allosteric control by metabolites and in particular feedback inhibition by malate (Kluge & Osmond, 1972). These possibilities can only be resolved when the molecular and regulatory properties of the enzyme have been determined; the information available to date is fragmentary and has not been obtained with purified enzymes.

Phosphoenolpyruvate has been purified to homogeneity from bacteria (Maeba & Sanwal, 1969; Smith, 1971), from peanut cotyledons (Maruyama *et al.*, 1966), from leaves of spinach, a C_3 -pathway plant (Miziorko *et al.*, 1974) and from maize, a C_4 -pathway plant (Uedan & Sugiyama, 1976). Phosphoenolpyruvate carboxylase has quite different metabolic functions in different groups of organisms, but in all cases, the enzyme is thought to have a regulatory

Abbreviations used: SDS, sodium dodecyl sulphate; Mes, 4-morpholine-ethanesulphonic acid.

* Present address: Department of Physics, University of Lancaster, Lancaster LA1 4YB, U.K.

† Present address: Department of Biochemistry, St. Mary's Hospital Medical School, University of London, Paddington, London W2 1PG, U.K.

role, and evidence for this is best in C_4 -pathway plants, where both allosteric regulation (Coombs *et al.*, 1973) and molecular modification in response to environmental changes (Goatly *et al.*, 1975; Vidal *et al.*, 1976) appear to occur. By contrast, the studies on the regulation of the enzyme from plants having Crassulacean acid metabolism have been fewer in number (Kluge & Osmond, 1972; Ting & Osmond, 1973a,b; von Willert *et al.*, 1976) despite the interest of its role in the temporal regulation of the pathway in these plants. Wilkinson & Smith (1976) have partially purified phosphoenolpyruvate carboxylase from *B. fedtschenkoi* by using DEAE-cellulose chromatography. In a preliminary communication (Jones *et al.*, 1976) we outlined a procedure for obtaining highly purified phosphoenolpyruvate carboxylase from *B. fedtschenkoi* and described some properties of the purified enzyme. In the present paper an improved purification scheme is described, together with some further molecular and kinetic characteristics of the purified enzyme that may be relevant to the mechanism controlling the circadian rhythm of CO_2 fixation.

Experimental

Materials

Phosphoenolpyruvate (monosodium salt), NADH, rabbit muscle aldolase, ox liver glutamate dehydrogenase, pig heart malate dehydrogenase, jack-bean urease, and cow's milk xanthine oxidase were from Boehringer Corp. (London) Ltd., Lewes, Sussex, U.K. Sodium [^{14}C]bicarbonate was from the Radiochemical Centre, Amersham, Bucks., U.K. L-Malic acid was from Fluorochem Ltd., Glossop, Derbyshire, U.K., DEAE-cellulose (DE 52) from Whatman Biochemicals, Maidstone, Kent, U.K., hydroxyapatite (Bio-Gel HT) from Bio-Rad Laboratories (England) Ltd., Bromley, Kent, U.K. and Sepharose CL-6B from Pharmacia (G.B.) Ltd., London W.5, U.K. Tris base (reagent grade), poly(ethylene glycol) 20000, bovine serum albumin, ovalbumin and ox liver catalase were from Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K. Imido ester dihydrochlorides, cross-linked rabbit muscle aldolase, and cross-linked RNA polymerase from *Escherichia coli* MRE 600 were prepared as described by Coggins *et al.* (1977). $(NH_4)_2SO_4$ (enzyme grade) and all other chemicals were from BDH Chemicals, Poole, Dorset, U.K.

Solutions

Solutions of oxaloacetic acid, L-malic acid and L-ascorbic acid were titrated to pH 5–6 by adding NaOH. Diluted enzyme solutions were stored at 4°C and used the same day. Column buffers were

prepared by adding 0.5 mM-dithiothreitol immediately before use.

Plant material

Bryophyllum fedtschenkoi Hamet et Perrier cuttings from the clone used in previous investigations (Wilkins, 1960) were grown for several weeks in a greenhouse with supplementary illumination to maintain a photoperiod of at least 16 h, then transferred to either a long-day (photoperiod 16 h) or short-day (photoperiod 8 h) growth room. Illumination ($0.45 W/cm^2$ in the visible waveband) was provided by fluorescent tubes. The temperature regime was 23°C (day)/16°C (night). For routine extraction of phosphoenolpyruvate carboxylase, healthy leaves were removed during the light period from the top two-thirds of shoots grown in long days or no more than 20 short days.

Extraction and purification of phosphoenolpyruvate carboxylase

The following procedure (Table 1) was used for 1–2 kg of starting material. All operations were carried out at 4°C.

(1) *Extraction.* Leaves were homogenized in batches (150 g) in a 3-litre capacity Waring blender for 15 s at low speed with 450 ml of a medium containing 50 mM-Tris base, 2 mM-EDTA, 6 mM-sodium diethyl-dithiocarbamate, 25 mM-sodium ascorbate and 20 g of poly(ethylene glycol) 20000/litre. The extract was shaken with a few drops of octan-1-ol to destroy the foam and filtered through muslin to remove the coarse debris. It was then centrifuged for 30 min at 6300 g to give a clear supernatant (fraction 1).

(2) *DEAE-cellulose adsorption.* Fraction 1 was diluted with an equal volume of water, the pH adjusted to 7.8 by adding 5 M-NaOH or 5 M-HCl as required, and the solution stirred for 10 min with 100 g of DEAE-cellulose/kg of starting material. The cellulose was filtered off and washed twice with 50 mM-Tris/HCl (pH 7.8)/20 mM-KCl. It was then resuspended in a small volume of 50 mM-Tris/HCl (pH 7.8)/0.3 M-KCl, filtered again, and washed with a small volume of the same buffer. These filtrates were combined, giving a total volume of approx. 300 ml/kg of starting material (fraction 2). The DEAE-cellulose retained a large amount of pigments and was discarded.

(3) *$(NH_4)_2SO_4$ fractionation.* Solid $(NH_4)_2SO_4$ (final concn. 180 g/litre) was slowly added to fraction 2 with stirring, and the stirring was continued for 20 min. A light precipitate was removed by centrifuging at 3200 g for 20 min. More solid $(NH_4)_2SO_4$ (final concn. 260 g/litre) was then slowly added. The solution was stirred and centrifuged as before, and the precipitate was redissolved in a

Table 1. *Purification of phosphoenolpyruvate carboxylase*
The enzyme was purified from 1600g of *B. fedtschenkoi* leaves as described in the Experimental section.

	Volume (ml)	Enzyme activity (units/ml)	Total activity (units)	Protein (mg/ml)	Specific activity (units/mg)	Yield (%)
1. Crude extract	7500	0.32	2380	*	*	100
2. DEAE-cellulose fraction	467	3.2	1490	*	*	63
3. (NH ₄) ₂ SO ₄ fraction	64	19.5	1250	8.4	2.3	53
After DE-52 column			650			27
Pooled fractions			500			21
4. After (NH ₄) ₂ SO ₄ and dialysis	9.6	42	403	12.0	3.5	17
After Bio-Gel HT column			335			14
Pooled fractions			298			12.5
5. Final suspension	3.1	72	224	2.7	26	9.5

* Poly(ethylene glycol) interferes with protein determinations.

small volume (approx. 30ml/kg of starting material) of 50mM-Tris/HCl (pH 7.8)/20mM-KCl/0.5mM-dithiothreitol, and dialysed overnight against the same buffer (fraction 3).

(4) *DEAE-cellulose chromatography*. The solution was applied to a column (21cm×3.8cm) of DEAE-cellulose previously equilibrated with 50mM-Tris/HCl (pH 7.8)/20mM-KCl/0.5mM-dithiothreitol and the column washed with the buffer for 3h at a flow rate of 80ml/h while the A_{280} was monitored in a 3mm path-length cell. The KCl concentration was then increased in a linear gradient from 20mM to 0.3M (total volume 1 litre) and 12-min fractions were collected at a flow rate of 60ml/h. Fractions with phosphoenolpyruvate carboxylase specific activity >2 units (see below)/mg were pooled and solid (NH₄)₂SO₄ was added with stirring to a final concentration of 300g/litre. The precipitate was collected by centrifuging for 15min at 12000g, dissolved in approx. 10ml of a buffer containing 50mM-KH₂PO₄ (adjusted to pH6.5 by adding NaOH)/0.5mM-dithiothreitol, and dialysed for 3h against the same buffer (fraction 4).

(5) *Hydroxyapatite chromatography*. The solution was applied to a column (12cm×2.0cm) of hydroxyapatite previously equilibrated with the same buffer, and the column washed with the buffer for 2h at 16ml/h. The phosphate concentration was then increased to 0.6M in a linear gradient of total volume 100ml, and 12 min fractions were collected at the same flow rate. The phosphoenolpyruvate carboxylase fraction with activity exceeding approx. 5 units/ml were pooled and concentrated by precipitation with (NH₄)₂SO₄ as before. The purified enzyme was stored at 4°C as a suspension (approx. 2mg/ml) in 0.2M-KH₂PO₄ (pH6.5)/(NH₄)₂SO₄ (300g/litre)/2mM-EDTA/2mM-dithiothreitol.

Enzyme assays

Phosphoenolpyruvate was routinely assayed at 25°C in a coupled spectrophotometric assay by following the oxidation of NADH ($\epsilon = 6200 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$) at 340nm in a 1cm-path-length cuvette in a Unicam SP.8000 spectrophotometer. The standard reaction mixture (1.0ml) contained 50mM-Tris/HCl(pH7.8), 1mM-dithiothreitol, 5mM-MgCl₂, 10mM-NaHCO₃, 2mM-phosphoenolpyruvate, 0.15mM-NADH, and 5 μ g of malate dehydrogenase (undialysed). The reaction was initiated by adding phosphoenolpyruvate carboxylase diluted in the first two components, generally sufficient to give $\Delta A_{340}/\text{min}$ in the range 0.01–0.2 (final concn. approx. 0.1–1 μ g/ml). Reaction mixtures below pH7.0 were buffered by 50mM-Mes adjusted to the required pH by NaOH, and at pH7.0 and above by 50mM-Tris/HCl. In pH-dependence studies, the pH values of individual reaction mixtures were checked after assay.

Other assays used for phosphoenolpyruvate carboxylase were: (i) A radiochemical assay carried out under conditions identical with those in the coupled assay except for the omission of NADH and the coupling enzyme in some cases, and the substitution of 10mM-[¹⁴C]bicarbonate (specific activity 0.1mCi/mmol) for unlabelled bicarbonate. 100 μ l portions of the reaction mixture were taken at intervals and added to 100 μ l of 0.1M-HCl/saturated phenylhydrazine in scintillation vials to stop the reaction and fix the product as the phenylhydrazone of [¹⁴C]oxaloacetate or as [¹⁴C]malate. Samples were gassed at room temperature with N₂ for a few minutes to remove unchanged ¹⁴CO₂, dissolved in 3.0ml of ethanol followed by 10ml of toluene/2,5-diphenyloxazole (5g/litre)/1,4-bis-(5-phenyloxazol-2-yl)benzene (0.3g/

litre), and counted in a liquid-scintillation counter. Internal standards contained known amounts of [^{14}C]bicarbonate in the same mixture and were counted immediately after preparation. (ii) A spectrophotometric assay for oxaloacetate ($\epsilon = 910 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ at 280nm in the presence of excess Mg^{2+}) carried out under conditions identical with those used in the coupled assay except for the omission of NADH and coupling enzyme. Above pH 7.2, a correction for the large absorption increase in oxaloacetic acid in the presence of Mg^{2+} (approx. 7-fold at pH 9.3) was necessary.

Malate dehydrogenase was assayed as for phosphoenolpyruvate carboxylase in the standard coupled assay except for the omission of phosphoenolpyruvate and exogenous malate dehydrogenase and the substitution of 2mM-oxaloacetate. The initial linear portion of progress curves was measured, before the effects of substrate depletion were apparent.

A unit of enzyme activity is defined as the amount catalysing the formation of 1 μmol of product per minute under standard conditions.

Analytical methods

Protein concentrations were initially determined with the Amido Black dye-precipitation method (Schaffner & Weissmann, 1973) with crystalline bovine serum albumin as a standard and samples containing between 2 and 10 μg of protein. Subsequent determinations on the purified enzyme were made with a determined value of the specific absorption coefficient at 280nm ($A_{1\text{cm}}^{1\%} = 11.6$). Salt concentrations in column fractions were determined conductimetrically, from a comparison with standard solutions.

Electrophoresis

Sample preparation and SDS/polyacrylamide-gel electrophoresis was carried out as described by Shapiro & Maizel (1969). Gels were stained, destained, and scanned as described by Lumsden & Coggins (1977). For determining the subunit molecular weight of phosphoenolpyruvate carboxylase, 3% (w/v) and 5% gels were used and the standards were myoglobin, bovine serum albumin, ovalbumin, glutamate dehydrogenase, and aldolase cross-linked with dimethyl suberimide (Lumsden & Coggins, 1977). For analysing the products of cross-linking, 3% gels containing 6M-urea were used and the standards were cross-linked aldolase and cross-linked RNA polymerase (Coggins *et al.*, 1977).

Non-denaturing gel electrophoresis in the absence of SDS was by the method of Davis (1964) as modified by Hayes & Wellner (1969). Samples were dialysed for 3h against buffer containing 25mM-Tris base,

0.19M-glycine, 0.5mM-EDTA and 0.5mM-dithiothreitol, pH 8.4, before electrophoresis. 5% gels were used and sample loading was in the range 5–20 μg . Pre-electrophoresis (1h) and electrophoresis were carried out at 4°C at 2mA/tube. Gels were stained either for protein by Coomassie Brilliant Blue G250 or for phosphoenolpyruvate carboxylase activity by incubating for 1h at 20°C in a medium containing phosphoenolpyruvate, NaHCO_3 , MgCl_2 and Fast Violet B, a stain specific for oxaloacetic acid, as described by Vidal *et al.* (1976).

Gel filtration

This was carried out at 4°C on a column (67cm $\times$ 2.5cm) of Sepharose CL-6B in 50mM- Na_2HPO_4 / NaH_2PO_4 (pH 6.7)/0.5mM-EDTA/0.5mM-dithiothreitol. Enzyme samples (0.5–1.5mg) were dialysed overnight against the same buffer before applying to the column in a volume of 0.5–1.5ml. The molecular-weight standards were urease, xanthine oxidase, catalase and aldolase.

Cross-linking

In the studies on the effect of chain length on cross-linking, the reagents used were dimethyl malonimide, dimethyl succinimide, dimethyl glutarimide, dimethyl adipimide and dimethyl suberimide. Phosphoenolpyruvate carboxylase (0.48mg/ml), previously dialysed overnight, was incubated for 2h at 20°C with freshly prepared 20mM solutions of reagents (Coggins *et al.*, 1977) in 0.1M-triethanolamine/HCl (pH 8.0)/40mM-NaCl/0.5mM-EDTA/0.5mM-dithiothreitol. The reaction was terminated by adding NH_4HCO_3 to a concentration of 0.14M, samples were removed for assay and the products were analysed on 3% gels. A control solution treated identically except for the absence of reagents was included. Similar conditions were used in studies on the effect of temperature and effector molecules on cross-linking, except phosphoenolpyruvate carboxylase concentrations were 0.35mg/ml and 0.25mg/ml respectively and the incubation time was 1h.

Results

Extraction and purification of phosphoenolpyruvate carboxylase from B. fedtschenkoi

(1) *Phosphoenolpyruvate activity in crude extracts.* Extracts made as described in the Experimental section usually have activity in the range 1.4–2.0 units/g fresh weight, declining approx. 30% if stored overnight. The pH is generally in the range 7.5–8.5 and must not be allowed to fall below 6.5. The activity on a fresh-weight basis is greatest

(approx. 4 units/g) in long-day plants and in leaves taken from approximately one-third of the way down the shoot, declining to about 30% of this value in very old or very young leaves. Under short-day conditions a general decrease in overall activity relative to comparable long-day plants occurs over several weeks. Diurnal variations in extractable activity are rather small (total range up to 40% of the maximum value). This contrasts with the more striking changes observed in the closely related plant *Kalanchoë blossfeldiana* (Queiroz & Morel, 1974). Both plants flower under prolonged short-day conditions and in both there is ultimately a decline in phosphoenolpyruvate carboxylase activity to low values (to approx. 0.5 unit/g in *B. fedtschenkoi*). The malate dehydrogenase activity in crude extracts is somewhat less sensitive to growth conditions. If crude extracts are desalted with Sephadex G25M to remove endogenous inhibitors such as malate before assaying, phosphoenolpyruvate carboxylase activities are increased by 10–30% but the results obtained are essentially the same.

If poly(ethylene glycol) 20000 is omitted from the extraction medium, or replaced by 50g of finely powdered insoluble polyvinylpyrrolidone (Polyclar AT)/l the phosphoenolpyruvate carboxylase activities measured are more variable and usually less than in the presence of poly(ethylene glycol), particularly in the case of plants from prolonged short-day conditions (approx. 0.1 unit/g, compared with 0.5 unit/g with poly(ethylene glycol) present). Concentrations of poly(ethylene glycol) less than 20g/litre, and molecular weights of poly(ethylene glycol) less than 20000 are significantly less effective in protecting phosphoenolpyruvate carboxylase activity, but other

factors in the reaction medium apart from pH are less critical.

(2) *DEAE-cellulose adsorption*. This step removes the bulk of the poly(ethylene glycol), which would otherwise interfere with $(\text{NH}_4)_2\text{SO}_4$ fractionation, decreases the volume and removes some protein and the bulk of the pigments. At the ionic strengths used, phosphoenolpyruvate activity is almost completely recovered.

(3) *$(\text{NH}_4)_2\text{SO}_4$ fractionation*. Phosphoenolpyruvate carboxylase precipitates at an $(\text{NH}_4)_2\text{SO}_4$ concentration of approx. 220g/litre, with a high recovery at the enzyme concentration involved. Fraction 3 and subsequent fractions have relatively stable activity with a decrease of only 20% after several days at 4°C.

(4) *DEAE-cellulose chromatography*. The elution profile of phosphoenolpyruvate carboxylase and malate dehydrogenase activity is shown in Fig. 1. Peak activities occur in 70mM-KCl and 120mM-KCl respectively, with a 60% recovery of phosphoenolpyruvate carboxylase. The malate dehydrogenase/phosphoenolpyruvate carboxylase activity ratio from the crude extract up to this stage is of the order 10–40, depending on the starting material. The peak phosphoenolpyruvate carboxylase specific activity is 4–6 units/mg of protein, representing about 20% pure enzyme, and SDS/polyacrylamide-gel electrophoresis on this fraction shows only four major protein bands, with several minor contaminants (see Fig. 3*b* below).

Fig. 1. Chromatography of phosphoenolpyruvate carboxylase and malate dehydrogenase on DEAE-cellulose (step 4 of the purification scheme)

The column (21 cm $\times$ 3.8 cm) was eluted with a linear gradient of KCl (----) at a flow rate of 60 ml/h after washing for 3 h at the starting concentration (20 mM-KCl). A_{280} (—) was monitored, and fractions (12 min) were collected and assayed for phosphoenolpyruvate carboxylase (●) and malate dehydrogenase (▲). The elution buffer also contained 50 mM-Tris/HCl (pH 7.8)/0.5 mM-dithiothreitol.

Fig. 2. Chromatography of phosphoenolpyruvate carboxylase on hydroxyapatite (step 5 of the purification scheme)

Phosphoenolpyruvate activity (●) was eluted from the column (12 cm $\times$ 2.0 cm) in a linear gradient (----) of $\text{KH}_2\text{PO}_4/\text{NaOH}$ (pH 6.5)/0.5 mM-dithiothreitol, after washing for 2 h at the starting concentration (50 mM- KH_2PO_4). Fractions (12 min) were collected at a flow rate of 17 ml/h. The first component had a peak A_{280} (—) of 1.3. The values on the graph are estimates of the specific activity of phosphoenolpyruvate carboxylase in some fractions from the second peak, based on A_{280} .

(5) *Hydroxyapatite chromatography*. Protein peaks are eluted in 90mM-phosphate and approx. 0.30M-phosphate; the ionic strength of elution of the second peak was found to vary slightly and is pH-sensitive. Phosphoenolpyruvate carboxylase activity (recovery approx. 80%) is almost coincident with the second peak (Fig. 2) and the specific activity is in the range 23–26 units/mg over the peak in most preparations, suggesting that the enzyme is essentially homogeneous. This is confirmed by SDS/polyacrylamide-gel electrophoresis on several fractions over the peak, all of which show one band (Fig. 3c,d) with in some preparations a minor contaminant (Fig. 3c). The first peak shows two major components on SDS/polyacrylamide gels, with mol.wts. of approx. 56000 and 15000–20000. These are probably the two subunits of ribulose biphosphate carboxylase.

Table 1 gives a summary of the purification steps for a single preparation. The phosphoenolpyruvate carboxylase band can be readily detected in SDS/polyacrylamide gels of all fractions including the crude extract (Fig. 3a) if the sample preparation is done rapidly. A gel-filtration step in the purification, used in most other published purification procedures for phosphoenolpyruvate carboxylase, was found to be unnecessary.

Stability of the purified enzyme

No loss of activity can be detected in the $(\text{NH}_4)_2\text{SO}_4$ suspension stored at 4°C for 1 month. Dialysed and diluted solutions in Tris or phosphate buffers (pH 7.8 and 6.7) in the presence of 0.5mM-EDTA and 0.5mM-dithiothreitol, lose activity at 4°C to an extent determined mainly by enzyme concentration (30% loss in 1 month at 0.2mg/ml; 70% loss in 1 week at 10 µg/ml). Losses are greater in the absence of a thiol-protecting reagent. Preparations up to 6 months old are indistinguishable from recent preparations on SDS/polyacrylamide-gel electrophoresis, with no evidence of degradation.

U.v. spectrum and specific activity

The ratio A_{280}/A_{260} is 1.6–1.7:1.0 in neutral solutions, and $A_{1\text{cm}}^{1\%}$ at 270nm is estimated to be 11.6 with the Amido Black method for protein determination. The specific activities of four different preparations of the purified enzyme were in the range 23–28 units/mg, based on the standard assay and the Amido Black protein method. This compares closely with the specific activity of 20 units/mg reported for the spinach enzyme (Mizioro *et al.*, 1974) and the specific activity of 24.8 units/mg reported for the maize enzyme (Uedan & Sugiyama, 1976) under similar conditions.

Fig. 3. Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate of fractions from the purification of phosphoenolpyruvate carboxylase

Densitometric scans are shown for 5% (w/v) acrylamide gels run as indicated in the experimental section: (a) crude extract; (b) peak of phosphoenolpyruvate carboxylase activity from DEAE-cellulose column; (c) and (d) two different preparations of purified enzyme. (1), top of gel; (2), phosphoenolpyruvate carboxylase band; (3) position of marker dye; (4) bottom of gel.

Homogeneity and subunit molecular weight of the purified enzyme

The elution profiles of phosphoenolpyruvate carboxylase activity on the preparative columns show no evidence for heterogeneity (Figs. 1 and 2). In about 50% of preparations, only one band can be detected after gel electrophoresis in the presence of SDS (Fig. 3d). In the remaining cases a minor contaminant (~5% of total protein) of unknown nature is seen migrating slightly ahead of the main band (Fig. 3c). Non-denaturing gels run at pH 8.4 and stained for either protein or phosphoenolpyruvate carboxylase activity show only one band, with $R_F = 0.26$ relative to the Bromophenol Blue marker. Thus there is no evidence in the purified enzyme for major contamination, for heterogeneity of subunits with regard to molecular weight, or for isoenzymes.

Fig. 4. Determination of the molecular weight of cross-linked phosphoenolpyruvate carboxylase

The log(molecular weight) is plotted against the R_F value on electrophoresis in 3% (w/v) acrylamide/SDS gels for various species of the cross-linked marker proteins aldolase ($\square$) and RNA polymerase ($\bullet$) (Lumsden & Coggins, 1977). The vertical lines indicate the positions of the products after cross-linking phosphoenolpyruvate carboxylase with dimethyl suberimide as indicated in the Experimental section. Gel scans showing the products of cross-linking with dimethyl suberimide ($x=6$), dimethyl adipimide ($x=4$) and dimethyl glutarimide ($x=3$) are also included (alignment along the horizontal axis is only approximate) together with the percentage inactivation of each product as measured in a standard assay.

The subunit molecular weight of phosphoenolpyruvate carboxylase was determined by electrophoresis on both 3% and 5% polyacrylamide gels in the presence of SDS. Plots of the log(molecular weight) against the R_F value (relative to Bromophenol Blue) for the marker proteins showed the usual linear relationship and the R_F value for phosphoenolpyruvate carboxylase gave a subunit mol.wt. of $105\,000 \pm 3000$ (s.d.) in six independent experiments.

Molecular properties of the native enzyme

(a) *Cross-linking.* The results obtained when phosphoenolpyruvate carboxylase was treated with a

series of bis-imido esters of increasing length ($H_2N=C(OCH_3)-[CH_2]_x-C(OCH_3)=NH_2$, $x=1$ to $x=4$, and $x=6$) are presented in Fig. 4. The major product with dimethyl suberimide ($x=6$) has a mol.wt. of 385000 and is undoubtedly the tetramer. The fainter bands, which have mol.wts. corresponding to 97000, 190000 and 300000 are the monomer, dimer and trimer respectively. With the shorter reagent dimethyl adipimide ($x=4$) the dimer was the major product. Still shorter reagents ($x=1, 2$ and 3) caused very little cross-linking. The longer reagents ($x=4$ and 6), which gave substantial amounts of cross-linking, caused more than 70% inactivation, whereas the shorter reagents had little effect on enzyme activity (Fig. 4). The results for $x=1$ and 2 (not shown) only differed from the result for $x=3$ (Fig. 4) in that slightly less dimer was present and the activity losses were smaller (approx. 10%). The longer reagents ($x=4$ and 6) gave apparent molecular weights on 3% polyacrylamide gels in the presence of SDS for the monomer and dimer significantly lower (97000 and 190000 respectively) than the corresponding molecular weights in the untreated control (103000; monomer only) and after reaction with the shorter reagents (102000 and 210000 respectively).

Varying the temperature between 20 and 40°C had no effect on the cross-linking pattern observed with dimethyl glutarimide ($x=3$), dimethyl adipimide and dimethyl suberimide. Malate (10 or 50mM) had no significant effect on the cross-linking pattern observed with dimethyl adipimide and dimethyl suberimide, either at 20 or 40°C, nor did it afford any protection against inactivation. The addition of $MgCl_2$ (10mM) afforded limited protection against inactivation by cross-linking with the two longest reagents ($x=4$ and 6) at 20°C, but there was no protection at 40°C. There was, however, a very striking effect on the molecular weight of the cross-linked enzyme. It was polymerized to such an extent that it could no longer enter a 3% polyacrylamide gel.

Cross-linking of the enzyme with dimethyl suberimide and dimethyl adipimide in the presence of phosphoenolpyruvate (20mM) and EDTA (2mM) together gave very different results from those obtained in the absence of these substances. At both 20 and 40°C the major product with dimethyl suberimide was the dimer and not the tetramer, which is the major product in the absence of phosphoenolpyruvate/EDTA. Similarly, with dimethyl adipimide the major product at both temperatures was the monomer and not as expected from experiments in the absence of phosphoenolpyruvate/EDTA the dimer. With both reagents and at both temperatures, phosphoenolpyruvate/EDTA had a strong protective effect on enzyme activity. Approximately 70% of the enzyme activity remained after cross-linking compared with the 15–25% that remained after

Fig. 5. Determination of the native molecular weight of purified phosphoenolpyruvate carboxylase

The elution of phosphoenolpyruvate carboxylase activity (Δ) from a column (67 cm $\times$ 2.5 cm) of Sepharose CL-6B at 4°C is compared with marker enzymes urease (●), xanthine oxidase (▲), catalase (○) and aldolase (■) run separately under identical conditions. The buffer contained 50 mM- $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ (pH 6.7) / 0.5 mM-EDTA / 0.5 mM-dithiothreitol. Phosphoenolpyruvate carboxylase (1.2 mg) was dialysed overnight against the buffer before loading. The specific activity of fractions determined by the Amido Black protein method is given. The inset shows four independent molecular-weight determinations using the partition coefficient $K_{av} = (V_e - V_0)/(V_i - V_0)$, where V_e is the peak elution volume, V_0 is the exclusion volume and V_i is the elution volume for small molecules. The horizontal bars indicate the partition coefficient of phosphoenolpyruvate carboxylase.

cross-linking with the two reagents in the absence of phosphoenolpyruvate/EDTA. The detailed mechanism of these protective effects is not known.

(b) *Gel filtration.* At high-protein loadings (0.5–1.5 ml of a solution of approx. 1.0 mg/ml), phosphoenolpyruvate carboxylase activity is eluted from a column of Sepharose CL-6B as a single, symmetrical peak homogeneous with respect to specific activity and corresponding to a mol.wt. of 500000 ± 35000 (s.d.; $n = 4$) (Fig. 5). Rechromatography of a single fraction from near the peak of activity again gives a single, nearly symmetrical peak, but its position is concentration-dependent (Fig. 6); as the loading is decreased from 500 to 20 μg , the elution position progressively alters and the apparent mol.wt decreases from 490000 to 310000. The smallest of these loadings corresponds to a peak eluted enzyme

concn. of about 2 $\mu\text{g}/\text{ml}$. If phosphoenolpyruvate carboxylase (0.5 mg) and the dialysed marker enzymes are run simultaneously, two components are clearly evident in the eluted activity profile, corresponding to mol.wts. of approx. 520000 and 260000.

A comparison with the studies on subunit mol.wt. strongly suggests that the component with an apparent mol.wt. of about 500000 is the tetramer and that the 260000 mol.wt. component observed under some conditions is an active dimer. The reason for the increased prominence of the dimer in the presence of marker enzymes is not understood. It has been pointed out (Ackers, 1970) that interacting systems subjected to gel filtration may, under the appropriate conditions, give either separately resolved peaks in the elution profile or a single peak intermediate in apparent molecular

Fig. 6. Concentration-dependence of the apparent molecular weight of phosphoenolpyruvate carboxylase

A series of gel-filtration experiments similar to those of Fig. 5 was carried out. The main diagram shows the elution profile of phosphoenolpyruvate carboxylase activity (normalized on a basis of loading) at total protein loadings of 200 μg (●), 80 μg (Δ), 40 μg (○) and 20 μg (▲). The inset shows the dependence of the apparent molecular weight on loading for this experiment (■) and for several independent experiments (□).

weight between the two forms. The data of Fig. 6 fit the latter case quite closely; the peak elution position corresponds to a dimer (apparent mol.wt. 260000) in the limit of infinite dilution, suggesting that a concentration-dependent dissociation is occurring. There is no evidence for active species other than the dimer and tetramer under the conditions studied, but it may be noted that there is a loss in area under the activity profiles shown in Fig. 6, and this loss appears to be stabilizing at about 50% for the most dilute samples. This suggests that the dimer has about 50% of the specific activity of the tetramer, but other, non-specific inactivation processes cannot entirely be ruled out. Since the differences were detected by assaying all samples at similar final enzyme concentrations (approx. 0.1 $\mu\text{g}/\text{ml}$), it appears that the changes responsible are not readily reversible at high dilutions. At the present time it is not clear whether the enzyme is present as a dimer or tetramer under standard assay conditions.

Kinetic properties of purified phosphoenolpyruvate carboxylase

All kinetic studies used the standard coupled assay unless otherwise stated. Under the standard assay conditions (pH 7.8), progress curves are linear with time for several minutes, and the reaction rate is linear with enzyme concentration and is nearly

maximal with respect to variations in phosphoenolpyruvate, Mg^{2+} and HCO_3^- concentrations, and to variations in pH. The reaction has an absolute requirement for phosphoenolpyruvate under all conditions of pH. Substitution of 2mM-pyruvate for phosphoenolpyruvate results in negligible activity at both pH 5.5 and 7.0, indicating that none of the apparent activity is due to a combination of phosphoenolpyruvate hydrolysis and contaminating lactate dehydrogenase activity. The phosphoenolpyruvate kinetics are Michaelis-Menten with an apparent K_m of 0.7mM in the absence of added effectors (e.g. Fig. 8). HCO_3^- is saturating at the residual concentration produced by dissolved atmospheric CO_2 alone at pH 7.8, but is inhibitory at 30mM. P_i is inhibitory; at pH 7.0 with 4mM-phosphoenolpyruvate/5mM- MgCl_2 present, the maximum inhibition achieved at high concentrations of P_i is 50%, with half-maximal inhibition occurring at 35mM- P_i . $(\text{NH}_4)_2\text{SO}_4$ is inhibitory at high concentrations, but effects due to the use of undialysed enzymes in the assay are negligible.

The maximum velocity (V_{max}) shows relatively small variations with pH between 5.5 and 9.0 at moderately high enzyme concentrations (total variation less than 30% of the maximum), but there is a distinct double optimum at pH values 5.8 and 7.8, with a minimum at pH 6.6 (see Fig. 10a below). There is a sharp fall in activity to zero between pH 5.2 and

4.8. Reaction rates were measured under standard conditions at 25°C but with phosphoenolpyruvate and Mg^{2+} concentrations increased from 2 to 6mM and from 5 to 20mM respectively to ensure saturating conditions throughout the range. The increased Mg^{2+} concentration was essential at $\text{pH} < 6.0$ to observe the maximum rate, presumably because of ionizations in the enzyme or substrate, but the apparent K_m for phosphoenolpyruvate was less pH sensitive. The reaction rate at $\text{pH} 5.5$ was almost unaffected by the addition of a further 10mM- NaHCO_3 .

The double maximum, for which there is also evidence in crude extracts, cannot be fully explained at present. The same pH-activity profile is obtained by using the oxaloacetate assay instead of the coupled assay (see Fig. 10a below), at least in the pH range 4.8–8.0. Above pH 8, small deviations occur and may be due to errors in applying the very large correction factor to the absorption coefficient of oxaloacetate (see the Experimental section). The pH-activity profile is somewhat dependent on enzyme concentration; at pH 7.8 the measured specific activity is independent of enzyme concentration over a large range, whereas at pH 5.8 it decreases to about

50% of the original value on diluting to below about 0.4 $\mu\text{g}/\text{ml}$.

Effects of temperature

The apparent K_m for phosphoenolpyruvate under standard assay conditions is somewhat temperature-dependent (1.7mM at 11°C; 0.7mM at 25°C; 2.4mM at 40°C). In all cases the kinetics are hyperbolic. The temperature-dependence of V_{max} was studied in the range 0–45°C at pH 7.8 by using in the assay mixture 4–6mM-phosphoenolpyruvate, 0.16–1.6 μg of phosphoenolpyruvate carboxylase/ml, and the usual concentrations of NaHCO_3 , MgCl_2 and coupling enzyme, which were non-limiting. The Arrhenius plot of the results is shown in Fig. 7. Between 0 and 20°C there is a linear region from which an activation energy of 81 kJ/mol for the overall reaction can be calculated. Above 20°C the reaction rate falls progressively below the expected value and a maximum occurs in the curve at 35–40°C. The reaction cannot be followed above 50°C because the measured rates decrease with time, suggesting that the enzyme is irreversibly denatured during the course of the assay. To determine whether the loss of activity below 50°C

Fig. 7. Temperature-dependence of the phosphoenolpyruvate carboxylase reaction

Reaction rates were measured at various temperatures in a standard assay (pH 7.8) with the phosphoenolpyruvate concentration increased to 4–6mM and enzyme concentrations of 1.6 $\mu\text{g}/\text{ml}$ (●), 0.4 $\mu\text{g}/\text{ml}$ (▲) and 0.16 $\mu\text{g}/\text{ml}$ (■). The activity scale is that of the highest enzyme concentration; the other data are normalized to this. The data (■) were obtained by cooling a single reaction mixture from 45 to 15°C over a 1 h period. The inset shows the temperature-dependence of the assumed equilibrium, calculated from the deviation of the activity from the extrapolated low-temperature data.

represents denaturation or a reversible change in the enzyme, further studies on heat stability were carried out.

Firstly, solutions of phosphoenolpyruvate carboxylase (0.6, 1.5 and 7.5 $\mu\text{g/ml}$) were preincubated for 10 min at several temperatures in the range 30–60°C in 50 mM-Tris/HCl (pH 7.8) / 10 mM- NaHCO_3 / 6 mM-phosphoenolpyruvate / 2 mM-dithiothreitol / 2 mM-EDTA. They were then assayed in the same mixture at 25°C in the presence of 20 mM- MgCl_2 and the usual concentrations of NADH and malate dehydrogenase. Only small activity losses occurred below 50°C. The temperature for 50% denaturation was 55–57°C, and was almost independent of enzyme concentration over the range studied. Denaturation was almost complete at 59°C. Since the preincubation conditions used were very similar to the assay conditions for studying the effect of temperature on the reaction rate, except for the absence of Mg^{2+} , it appears very unlikely that denaturation is involved in the activity loss below 50°C to any significant extent. If the preincubation experiment is carried out in the absence of substrates with only Tris/HCl and dithiothreitol present, denaturation occurs at lower temperatures and is much more concentration-dependent; several times the usual assay concentration (>7.5 $\mu\text{g/ml}$) is required to stabilize activity between 40 and 50°C. Secondly, the reversibility of the activity loss in assays at 30–50°C is confirmed by cooling a single reaction mixture from 45°C to 15°C over a 1 h period; there is an initial increase in activity and then a fall, almost exactly reproducing the original experiment (Fig. 7). The reversibility is also supported by the observation that all progress curves are linear with time below 50°C.

The simplest plausible explanation is that there is an equilibrium between two forms of the enzyme (E , E'), one of which (E') is completely inactive. On this basis, the deviation of the data in the region 30–50°C from the extrapolated straight-line relation-

ship can be used to calculate the equilibrium ($K = [E']/[E]$) at different temperatures. The temperature-dependence of the equilibrium constant (inset of Fig. 7) then gives an estimate of +221 kJ/mol for the enthalpy change (ΔH) for the conversion $E \rightarrow E'$.

Malate inhibition

Inhibition of phosphoenolpyruvate carboxylase by malate is significant (50% at 13 mM-malate) under standard assay conditions (pH 7.8) and increases sharply as the pH is decreased. With suitable concentrations of malate, progress curves are non-linear with time, the reaction only reaching a steady final rate (typically about 50% of the initial rate) after 2–5 min. The final rate was measured in all cases. Because of this non-linearity and because inhibition of malate dehydrogenase (an ordered-sequence enzyme) might occur in the presence of malate when some NAD^+ has been formed, the possibility that the measured reaction rate in the coupled assay may not accurately reflect phosphoenolpyruvate carboxylase activity has to be considered. Thus the coupled-assay system was tested at pH 5.5 and 6.9, both in the presence and absence of a malate concentration sufficient to cause approx. 80% inhibition (4 mM at pH 5.5; 20 mM at pH 6.9). The following results indicate that the coupled assay is valid under all these conditions: (i) the reaction rate was proportional to phosphoenolpyruvate carboxylase concentration in the range 0.4–4.0 $\mu\text{g/ml}$ under all conditions; (ii) varying the concentration of malate dehydrogenase in the range 5–30 $\mu\text{g/ml}$ caused less than 10% change in all measured rates; (iii) no effect of NADH concentration in the range 0.01–0.15 mM could be detected; (iv) the rate was altered less than 20% by adding up to 0.3 mM- NAD^+ (the concentration that accumulates during normal assays never exceeds 0.1 mM); (v) progress curves all become linear with time when only a very small amount of product is formed

Table 2. Comparison of assay methods for phosphoenolpyruvate carboxylase

The enzyme (concn. approx. 3 $\mu\text{g/ml}$) was assayed at 25°C in a solution containing 50 mM buffer, 1 mM-dithiothreitol, 20 mM- MgCl_2 , 10 mM- NaHCO_3 , 2 mM-phosphoenolpyruvate at the indicated pH values in the presence or absence of malate and the presence or absence of the coupling system [malate dehydrogenase (5 $\mu\text{g/ml}$) plus 0.15 mM-NADH (standard assay) or 0.3 mM-NADH (^{14}C assay)]. The ^{14}C assay was sampled at 0, 1 and 2 min (no malate) or at 0, 5 and 10 min (with malate present). Activities are expressed as $\text{nmol} \cdot \text{min}^{-1} \cdot \text{ml}^{-1}$.

pH value	[Malate] (mM)	Standard assay (mean of four determinations) (Coupled)	Direct assay of oxaloacetate formation (Uncoupled)	^{14}C assay	
				(Coupled)	(Uncoupled)
6.9	0	52	54	50	55
6.9	20	8.5	8.4	13	11
5.5	0	70	84	59	71
5.5	4	17	14	18	7.2

(<0.03mm); (vi) rates in the coupled assay are generally in good agreement with rates measured in the spectrophotometric assay for oxaloacetate and in the radiochemical assay (both with and without the coupling system) under all four conditions (Table 2); we doubt whether too much significance should be attached to the single exceptionally low activity in the presence of malate at pH 5.5 (7.2nmol/min per ml), since in two other experiments values of 12 and 13 were obtained; (vii) the transient ('burst') effect is clearly seen in the oxaloacetate assay, and less clearly, because of difficulties in taking sufficient samples, in the radiochemical assay. The burst effect can be partially abolished by preincubating phosphoenolpyruvate carboxylase with malate. These observations suggest that a hysteretic effect (Frieden, 1970) in phosphoenolpyruvate carboxylase, rather than an artifact of the assay methods, is responsible.

The coupled assay is superior to the radiochemical assay, at least for the purified enzyme, because of its convenience and the ease of detection of non-linearity. The oxaloacetate assay has the advantages of convenience and directness, but suffers from the minor disadvantage of lower sensitivity than the coupled assay and is difficult to correct for absorb-

ance changes at pH > 8. The coupled assay was thus the method of choice for all routine kinetic studies, both with and without malate present.

The phosphoenolpyruvate kinetics were investigated at two fixed concentrations of malate at pH 7.8 (Fig. 8). The kinetics remain hyperbolic at high malate concentrations and the inhibition appears to be purely competitive. By contrast, the kinetics of malate inhibition at a fixed concentration (2mm) of phosphoenolpyruvate are sigmoidal. The sigmoidicity and the malate concentration for half-maximal inhibition ($[malate]_{0.5}$) are strongly pH-dependent (Fig. 9), with the largest changes occurring around pH 6.5. The partially inhibited activity is relatively insensitive to the addition of a high concentration (20mm) of Mg^{2+} . The co-operativity was analysed by Hill plots $\{\log [v/(V-v)]\}$ against $\log [malate]$ that were linear, permitting Hill coefficients (h) for the malate inhibition to be determined. The effects of pH on V , $[malate]_{0.5}$ and h are summarized in Fig. 10. $[Malate]_{0.5}$ has distinct pH-dependent (pH 5–7) and pH-independent (pH 7–9) regions (Fig. 10b). The co-operativity (Fig. 10c) shows rather different behaviour, with a sharp maximum ($h \sim 2.1$) near pH 7.0. Although this behaviour is obviously consistent with an oligomeric structure, no detailed explanation of the behaviour in terms of the molecular properties is possible at this stage.

Discussion

Results from crude extracts obtained under a variety of conditions indicate that circadian and

Fig. 8. Double-reciprocal plots showing the effect of malate on phosphoenolpyruvate kinetics of purified phosphoenolpyruvate carboxylase

Activities were measured under standard assay conditions (pH 7.8, 25°C) except for the indicated variations in total malate and phosphoenolpyruvate added. Malate concentrations were 0mm (■), 10mm (●) and 19mm (▲).

Fig. 9. Inhibition of phosphoenolpyruvate carboxylase activity as a function of malate concentration and pH. Assay conditions were standard except for variations in the buffer, pH, and total malate concentration. Reaction mixtures were buffered below pH 7.0 by 50mm-Mes/NaOH, and at pH 7.0 and above by 50mm-Tris/HCl. The data are normalized to the rate in the absence of the inhibitor to allow for variations in V with pH and in enzyme concentration (approx. 1 μ g/ml). The pH values were 5.5 (●), 6.0 (□), 6.5 (▲), 7.0 (△), 7.8 (■) and 9.1 (○).

Fig. 10. Summary of pH-dependence of some kinetic properties of phosphoenolpyruvate carboxylase

Assay conditions were standard (25°C) except for variations in buffer, pH, and malate concentration as for Fig. 9 and for increased phosphoenolpyruvate and Mg^{2+} concentrations for determining the maximum velocity as given in the text. (a) Maximum velocity (V); (b) malate concentration for half-maximal inhibition ($[\text{malate}]_{0.5}$); (c) Hill coefficient for malate inhibition (h). Enzyme concentrations were approx. $1 \mu\text{g/ml}$. Some maximum-velocity data obtained in a parallel oxaloacetate assay under identical conditions are included ($\square$).

diurnal changes of phosphoenolpyruvate carboxylase capacity in *B. fedtschenkoi* are invariably small, and they are thus inadequate to explain the very large changes in phosphoenolpyruvate carboxylase activity *in vivo*. The changes are smaller than those reported for the closely related plant *Kalanchoë blossfeldiana* (Queiroz & Morel, 1974), where they are also considered to be secondary to the main control mechanism for the circadian rhythm. The protective effects of poly(ethylene glycol) during extraction are noteworthy in that they vary with the plant material, and are not obtained with polyvinylpyrrolidone, which is widely used as a protecting agent for enzyme activity in plant extracts. It is possible that a specific inhibitor of phosphoenolpyruvate carboxylase, similar to that from long-day *Kalanchoë* plants described by Brulfert *et al.* (1973, 1975) and present in variable amounts, could be responsible for the effects.

The procedure we have described for purifying phosphoenolpyruvate carboxylase from *B. fedtschenkoi* is simple and reproducible and yields enzyme (which is homogeneous and highly stable) in milligram quantities. No evidence has been found for isoenzymes nor for subunit heterogeneity. Polyacrylamide-gel electrophoresis in the presence of SDS indicates a mol.wt. of 105000. Gel filtration and cross-linking studies with dimethyl suberimide suggest that the native enzyme is a tetramer. In this respect and also in specific activity it closely resembles all the other plant phosphoenolpyruvate carboxylases that have been purified.

The cross-linking patterns obtained with bis-imido esters of increasing chain length provide useful evidence that subunit interactions in the tetramer are not all equivalent and that the tetramer behaves as a dimer of dimers. This behaviour is by no means unusual in oligomeric enzymes (Hucho *et al.*, 1975; Hajdu *et al.*, 1976), and is consistent with the dimer behaving as an independent, stable and active species during gel filtration. The apparent molecular weights from gel filtration are about 20% higher than those from polyacrylamide-gel electrophoresis in the presence of SDS. We have taken the latter as the definitive molecular-weight estimate, since it is well known that gel-filtration results can be significantly dependent on shape and charge differences between native proteins. Nevertheless, the gel-filtration results are invaluable in confirming the tetrameric structure and in showing the existence of a dimer-tetramer equilibrium. The smaller discrepancy between the apparent subunit mol.wt. of the unmodified and slightly cross-linked enzymes (102000–105000) and the more highly cross-linked enzymes (97000) on 3% gels in the presence of SDS is probably also significant; the most likely explanation is that intrasubunit cross-links constrain protein unfolding in SDS and alter the hydrodynamic properties slightly compared with the unmodified subunit. This problem has not been systematically investigated, but there is evidence that some other cross-linked proteins deviate in a regular fashion from the expected mobility in the presence of SDS [see the data of Carpenter & Harrington (1972) and Hucho *et al.* (1975)].

The native enzyme is homogeneous by several criteria (electrophoresis under non-denaturing conditions, ion-exchange and adsorption chromatography, and gel filtration at high concentration), but other results (gel filtration at low concentrations, the pH-maximum velocity profile and the kinetic effects of temperature and malate) suggest that the enzyme might exist in different molecular forms. Most of the effects observed are undoubtedly due to reversible equilibria with in some cases dissociation occurring; thus a concentration-dependent dissociation to a dimeric species with about 50% of the specific activity of the tetramer can fully explain

the gel-filtration results and can explain the concentration-dependence of the pH profile at low enzyme concentrations. The dissociation must be favoured by low pH, since activity losses are only seen at low pH and low enzyme concentrations. It also appears to be favoured by the absence of substrates since the changes occur over a higher concentration range in the gel-filtration studies (2–20 µg/ml approx.) than in kinetic studies (0.1–0.4 µg/ml approx.). The heat-denaturation results also suggest that substrates both protect activity and prevent dissociation, since denaturation occurs at lower temperatures and is also much more concentration-dependent in their absence. At first sight, the cross-linking studies in the presence of phosphoenolpyruvate seem to show the opposite effect, since phosphoenolpyruvate significantly inhibits the formation of cross-linked enzyme. This could be due to dissociation, but an alternative explanation that is consistent with all the other data is that phosphoenolpyruvate binding causes a conformational change that results in a rearrangement of the lysine side chains involved in the cross-linking reaction. The protection against inactivation by cross-linking afforded by phosphoenolpyruvate suggests that there may be a lysine side chain in or near the active site of phosphoenolpyruvate carboxylase.

The reversible temperature-dependent loss of activity, and the co-operativity and hysteretic effects with malate, have not been investigated sufficiently to indicate whether dissociation or simply isomerization is involved. Hysteretic effects have also been observed in phosphoenolpyruvate carboxylase from *Escherichia coli* (Wohl & Markus, 1972), and in that case were shown to be due to a substrate-induced association from the dimer to the tetramer. The absence of an effect of temperature or malate on the products of cross-linking is inconclusive, since it could mean that the changes responsible for the kinetic behaviour either do not occur at the higher concentrations of the cross-linking experiments or are too small to be detected by cross-linking. However, the kinetic temperature effects are essentially independent of concentration; this suggests that dissociation is not a limiting factor over the range studied.

We do not at present understand the double maximum in the pH-activity profile (Fig. 10a); the observed behaviour undoubtedly results from the interplay of a number of factors. One very likely possibility is that, at low enzyme concentrations, the observed activity is modified by a dimer-tetramer equilibrium as already mentioned, but since the double maximum persists at high concentrations where dissociation is probably not significant, effects other than simple reversible equilibria or ionizations in the enzyme must also be considered. Although most investigations have suggested that phosphoenol-

pyruvate carboxylase utilizes HCO_3^- and has an alkaline optimum (e.g. Coombs *et al.*, 1973; Coombs *et al.*, 1975; Maruyama *et al.*, 1966), Waygood *et al.* (1969) have argued for a CO_2 -utilizing enzyme, and Pan & Waygood (1971) have described an enzyme effecting the carboxylation of phosphoenolpyruvate under acidic conditions. Thus another possibility is that there could be two forms of the enzyme, one utilizing CO_2 with an apparent optimum at pH 5.8 and the other utilizing HCO_3^- with an optimum at pH 7.8. Finally, modifications of the enzyme to give forms that also differ in malate-sensitivity may be involved. These forms might be interconvertible by pH changes (Fig. 10b), but the presence of non-interconvertible forms undetected by the other methods cannot be ruled out.

Multiple molecular forms of phosphoenolpyruvate carboxylase have frequently been reported in other plant systems, usually in crude or partially-purified extracts or after drastic environmental changes. None has been fully characterized, and thus some might only be different polymerization states of the same enzyme (as found in the present work) with slightly different electrophoretic or chromatographic properties, rather than true isoenzymes. The single component we detect by gel electrophoresis under non-denaturing conditions at pH 8.4 is similar in mobility ($R_F = 0.26$) to a second component, induced by greening, in phosphoenolpyruvate carboxylase from bean and sorghum (Vidal *et al.*, 1976) and to a third component, associated with salt-induced Crassulacean acid metabolism, in phosphoenolpyruvate carboxylase from *Mesembryanthemum* (von Willert *et al.*, 1976). There is little clear evidence from other work that multiple forms occur in purified phosphoenolpyruvate carboxylase or in the enzyme from plants grown under uniform conditions. Thus our failure to show clear evidence for such forms is not surprising, but it is noteworthy that in other plant systems there are reports of systematic changes in kinetic properties of phosphoenolpyruvate carboxylase after the initial extraction (Bonugli & Davies, 1977; Coombs *et al.*, 1973), suggesting that extraction and purification procedures may modify the enzyme or select for specific forms. Nevertheless, we have no direct evidence for modification or for isoenzymes in the purified enzyme.

The kinetic behaviour with phosphoenolpyruvate as the varied substrate is similar to that reported in studies on partially purified enzyme from Crassulacean plants (Kluge & Osmond, 1972; Ting & Osmond, 1973a; Wilkinson & Smith, 1976). The competitive inhibition we observe with malate differs from the non-competitive or mixed-inhibition patterns observed in these studies, although the malate concentrations for half-maximal inhibition are similar under comparable conditions of pH, and the studies agree in that the phosphoenolpyruvate kinetics

are hyperbolic in both the presence and absence of malate. The results with malate as the varied ligand are noteworthy in that (i) positive co-operativity is present with a maximum Hill number at about pH 7, (ii) between pH 7 and 5 there is a 20-fold increase in malate sensitivity and (iii) hysteretic effects occur when the enzyme is transferred to an assay medium containing an inhibitory concentration of malate. A simple explanation might be as follows. Phosphoenolpyruvate carboxylase exists in two states, with high malate affinity (pH 5) and low malate affinity (pH ≥ 7). Malate and H^+ are both negative effectors. Co-operativity is only present at intermediate pH values where interconversion most readily occurs. The two states cannot be further characterized at present, although they may be related to the dimer-tetramer equilibrium and to the two pH optima as discussed above.

The availability of purified phosphoenolpyruvate carboxylase and its partial characterization now allows an examination of its possible physiological significance in controlling the circadian rhythm of CO_2 fixation. With the reservations already mentioned, we have little evidence that variations in the amount of enzyme or structural modifications play a significant part in regulation, and the overriding type of control must therefore involve reversible conformation changes. Of the properties investigated in the present work the inhibition by malate, the temperature-dependent inactivation, and the reversible dissociation seem most likely to have some physiological role. The kinetic results with malate are consistent with the feedback-inhibition hypothesis of Kluge & Osmond (1972) for regulating Crassulacean acid metabolism. The mesophyll cells accumulate very large concentrations of malate (approx. 50 mM) towards the end of the CO_2 -fixation period, mostly in the large vacuole. This concentration would be sufficient to largely inhibit phosphoenolpyruvate carboxylase under a wide range of conditions, and can readily be synthesized by the enzyme operating at only a small fraction of its total capacity as measured in crude extracts. Thus the enzyme could be highly inhibited by malate over much of the cycle. However, a complete understanding of regulation by malate depends on as yet unknown factors such as the degree of partitioning between cytoplasm and vacuole as a result of active-transport processes across the tonoplast membrane (Lüttge *et al.*, 1975) and the concentration of substrates, other effectors, and H^+ in the cytoplasm. Similar considerations apply to the regulatory significance of dissociation; cytoplasmic concentrations of phosphoenolpyruvate carboxylase may be such that the enzyme is entirely tetrameric, but this will also depend on enzyme localization and the metabolites present.

A sensitive control mechanism could be provided

by the combined effects of malate and H^+ concentrations on phosphoenolpyruvate carboxylase activity, since it is quite likely that both vary in parallel in the cytoplasm. By contrast, it is difficult to see how the pH-maximum velocity profile, which is almost a plateau in the normal physiological range, could be the basis of a control mechanism. A similar conclusion, that pH only exerts control indirectly through the effects of metabolites, was also reached in the case of the potato enzyme (Bonugli & Davies, 1977). The significance of the observation by Kluge & Osmond (1972) that the enzyme from succulent plants usually has a more acidic pH optimum (6.5–7.0) than the enzyme from non-succulents (pH ~ 8) is thus unclear; it is only supported by limited data, and many pH studies also appear to overlook the possibility of increasing Mg^{2+} -dependence and enzyme concentration-dependence at low pH, which we have found important factors. Likewise, the results of Waygood *et al.* (1969) and Pan & Waygood (1971), which contradict virtually all other findings, lead us to conclude that the pH characteristics of phosphoenolpyruvate carboxylases in general, but especially at acidic pH, may be quite complex and are not adequately investigated.

Finally, the reversible loss of activity of phosphoenolpyruvate carboxylase at high temperatures could be of particular significance in explaining some of the properties of circadian rhythms of CO_2 fixation. Several other plant phosphoenolpyruvate carboxylases also show somewhat unusual temperature features, i.e. slope changes in Arrhenius plots (Uedan & Sugiyama, 1976) or low optima, which can account for the observed inhibition of malic acid synthesis by high temperatures in both intact tissues and crude extracts (Brandon, 1967; Lakso & Kliewer, 1975). Our results might similarly explain why CO_2 fixation is abolished by high temperatures during the circadian rhythm (Wilkins, 1962). Of even greater interest is the fact that the period of the rhythm is highly temperature-compensated (Wilkins, 1962), as in all circadian rhythms (Hastings & Schweiger, 1975). The detailed molecular mechanism is not known for any circadian rhythm, but the reversible inactivation of enzymes by high temperatures, as occurs with phosphoenolpyruvate carboxylase, has been put forward as one of the most likely control features.

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