Purification of the phosphorylated night form and dephosphorylated day form of phosphoenolpyruvate carboxylase from *Bryophyllum fedtschenkoi*

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1. Phosphoenolpyruvate carboxylase of *Bryophyllum fedtschenkoi* was shown to exist in two forms: a night form, which is phosphorylated and has low sensitivity to inhibition by malate, and a day form, which is dephosphorylated and 10 times more sensitive to malate. 2. The day and night forms of the enzyme were purified retaining their distinct malate sensitivities and phosphorylation states. The purified enzymes contained a major protein (subunit M_r 112000) and a minor protein (subunit M_r 123000). The two polypeptides appeared to have closely related amino acid sequences and were present in a similar ratio in extracts that had been prepared rapidly. 3. The phosphate present in the night form of the enzyme was covalently bound to serine. It was not a catalytic intermediate. Alkaline phosphatase removed the phosphate group *in vitro* and increased the malate sensitivity of the enzyme to that observed for the day form. 4. Both the day and night forms of the enzyme were probably tetramers, and their apparent M_r was lowered by the presence of malate, but was unaffected by Mg^{2+} ions, EDTA, a rise in pH or a 10-fold change in enzyme concentration. 5. The rapid loss of malate sensitivity, observed in extracts of leaves prepared during the day and at night, was shown to be due to proteolysis of the enzyme. It was slowed in the presence of malate and by phosphorylation of the enzyme.

INTRODUCTION

Bryophyllum fedtschenkoi is a Crassulacean plant. During Crassulacean acid metabolism, CO_2 is fixed at night into malate via phosphoenolpyruvate carboxylase (EC 4.1.1.31) and is then released intracellularly during the day and re-fixed photosynthetically. Phosphoenolpyruvate carboxylase is an important site for the regulation of this process. To avoid futile cycling, the enzyme must be active at night and inactive during the day. Phosphoenolpyruvate carboxylase is subject to feedback inhibition by malate, and it is widely believed that periodic accumulation of malate in the cytoplasm may regulate the enzyme (for a review, see [1]). Furthermore, there is considerable evidence that the kinetic properties of phosphoenolpyruvate carboxylase, in particular its sensitivity to inhibition by malate, change reversibly during the diurnal cycle and also during the circadian rhythms of CO_2 fixation, which are exhibited by many Crassulacean plants when maintained under constant environmental conditions [1–11]. In a previous paper we reported a diurnal variation in the malate sensitivity of phosphoenolpyruvate carboxylase measured in extracts of *B. fedtschenkoi* and showed that this was accompanied by a reversible covalent modification involving the incorporation of a phosphorus-containing group to give the less-sensitive night form of the enzyme [9].

The malate sensitivity of phosphoenolpyruvate carboxylase decreases fairly rapidly in crude extracts prepared from Crassulacean plants, and this effect has been ascribed to conversion of the more malate-sensitive day form into the less-sensitive night form [8,12–14]. An early purification of the enzyme from *B. fedtschenkoi* resulted in considerable loss of malate-sensitivity [13,15]. However, this decline in malate sensitivity was shown to be largely

prevented by malate [12] and, more recently, day and night forms of the enzyme have been purified from *Crassula argentea* [14]. In the present paper we report the purification from *B. fedtschenkoi* leaves of both the night and day forms of phosphoenolpyruvate carboxylase from *B. fedtschenkoi*, retaining their different malate sensitivities, and the characterization of the covalent modification of the night form.

EXPERIMENTAL

Materials

Calf intestinal alkaline phosphatase, rabbit muscle pyruvate kinase and phenylmethanesulphonyl fluoride were from Boehringer Corp. (London) Ltd., Lewes, Sussex, U.K. Thyroglobulin, benzamide, polyoxyethylenesorbitan monolaurate (Tween 20), horse spleen apoferritin, antipain and 1,3-bis[tris(hydroxymethyl)methylamino]propane were from Sigma (London) Chemical Co., Poole, Dorset, U.K. *Crotalus adamanteus* phosphodiesterase was from Lorne Diagnostics, Reading, Berks., U.K. Phosphorylase was purified from rabbit muscle [16]. Freund's complete and incomplete adjuvant were from Miles Laboratories, East Molesey, Surrey, U.K. Normal goat serum and horseradish peroxidase conjugated to goat anti-(rabbit IgG) antiserum were from Scottish Antibody Production Unit, Carlisle, Lanarkshire, Scotland, U.K. Nitrocellulose paper (0.45 μ m pore size) was from Anderman, Kingston-upon-Thames, Surrey, U.K. Polygram SIL G 0.25 mm silica-gel thin layers were from CamLab, Cambridge, U.K. Centricon 30 micro-concentrators were from Amicon Corp., Stonehouse, Glos., U.K. Mono Q, Mono P and Superose 6 Fast Protein Liquid Chromatography columns were

from Pharmacia, Milton Keynes, Bucks., U.K. The sources of other materials were as given in references [9], [13] and [15].

Plants

Bryophyllum (Kalanchoë) fedtschenkoi Hamet et Perrier plants were maintained and harvested as described in [9]. Day extracts were prepared between 09:00 and 10:00 h and night extracts between 01:00 and 02:00 h.

Purification of phosphoenolpyruvate carboxylase

All steps were carried out at 5 °C except where indicated. The pH of buffers was adjusted at the temperature at which they were to be used.

Extraction. *B. fedtschenkoi* leaves (30 g) were homogenized in a Waring Blender for 30 s at low speed with 150 ml of 100 mM-Tris/HCl, pH 8.0, containing 2% (w/v) poly(ethylene glycol) 20000, 2 mM-EDTA, 10 mM-malate, 1 mM-benzamidine, 0.05% (v/v) 2-mercaptoethanol, 2% (w/v) insoluble polyvinylpyrrolidone, 1.2 mM-phenylmethanesulphonyl fluoride and a few drops of octan-1-ol. The pH of the extract was adjusted to 7.5. The extract was filtered through two layers of muslin and centrifuged for 20 min at 20000 g.

Hydroxyapatite chromatography. The supernatant was mixed with hydroxyapatite Bio-Gel HTP (10 ml bed volume), pre-equilibrated in 100 mM-Tris/HCl, pH 7.5, and swirled gently for 10 min. The mixture was centrifuged briefly and the supernatant was discarded. The hydroxyapatite was resuspended gently in extraction buffer, without octan-1-ol or polyvinylpyrrolidone, and poured into a 1.2 cm × 3.3 cm (10 ml) column. The column was washed with 50 ml of extraction buffer, without octan-1-ol or polyvinylpyrrolidone, and then with 100 mM-Tris/HCl, pH 7.5, containing 40 mM-potassium phosphate, 0.1 mM-EDTA, 1 mM-benzamidine, 1 mM-dithiothreitol and 10 mM-malate, until the A_{280} of the effluent fell to zero. Phosphoenolpyruvate carboxylase was eluted by increasing the potassium phosphate concentration to 150 mM. The flow rate was 1.5 ml/min, and 1.5 min fractions were collected.

Sephadex G-25 chromatography. The active fractions from the hydroxyapatite column were pooled and desalted into 50 mM-Tris/HCl, pH 7.5, containing 0.1 mM-EDTA, 1 mM-dithiothreitol and 1 mM-benzamidine on a 56 cm × 2.2 cm (200 ml) column of Sephadex G-25M. The flow rate was 7 ml/min, and 0.5 min fractions were collected.

DEAE-cellulose chromatography. The active fractions from the Sephadex column were pooled and loaded on a 1 cm × 1.1 cm (1 ml) column of DEAE-cellulose pre-equilibrated in the same buffer as the Sephadex column. The column was washed with the same buffer until the A_{280} of the effluent fell to zero, and the phosphoenolpyruvate carboxylase was eluted with the same buffer containing 100 mM-NaCl. The flow rate was 0.5 ml/min, and 2 min fractions were collected. The active fractions were pooled and dialysed overnight against 50 mM-1,3-bis[tris(hydroxymethyl)methylamino]propane/HCl, pH 7.5, containing 50 mM-KCl, 0.1 mM-EDTA, 1 mM-dithiothreitol and 1 mM-benzamidine.

Superose 6 chromatography. The phosphoenolpyruvate carboxylase was concentrated to 0.5 ml with a Centricon 30 micro-concentrator and was chromatographed at room temperature on a 30 cm × 1.0 cm column of Superose 6 equilibrated in the dialysis buffer and connected to a Pharmacia Fast Protein Liquid Chromatography system. The flow rate was 0.3 ml/min, and 0.5 min fractions were collected. The active fractions were pooled and dialysed overnight into 50 mM-1,3-bis[tris(hydroxymethyl)methylamino]propane/HCl, pH 7.5, containing 0.1 mM-EDTA, 1 mM-dithiothreitol, 1 mM-benzamidine and 50% (v/v) glycerol, and stored at -15 °C. For long-term storage 10 mM-malate was included.

Phosphoenolpyruvate carboxylase assay

The standard spectrophotometric assay mixture [15] contained, in 1 ml, 50 mM-Tris/HCl, pH 7.8, 5 mM-MgCl₂, 2 mM-phosphoenolpyruvate, 0.2 mM-NADH, 10 mM-NaHCO₃, 5 units of malate dehydrogenase and the enzyme sample. No more than 10 µl of the early fractions of the purification was used for assay, in order to dilute out the high concentrations of malate present in these fractions. Since the sensitivity of the enzyme to inhibition by malate decreased fairly rapidly in crude extracts (see the Results section), the early fractions of the purification were assayed immediately. One unit of enzyme activity is the amount required to catalyse the production of 1 µmol of oxaloacetate/min at 25 °C.

Protein assay

Protein was measured as in [17]. Samples containing polyethylene glycol were precipitated and washed with trichloroacetic acid to decrease interference.

Isolation and analysis of ³²P-labelled phosphoenolpyruvate carboxylase

B. fedtschenkoi leaves (3 g) were labelled with 1.5 mCi of carrier-free [³²P]P_i as in [9]. The leaves were mixed with 27 g of unlabelled leaves and extracted at 02:00 h, and the phosphoenolpyruvate carboxylase was purified up to and including the chromatography on DEAE-cellulose (see above). Enzyme (100 µg) was electrophoresed on an SDS/polyacrylamide (8%, w/v) gel. The enzyme band was extracted, hydrolysed in 5 M-HCl for 2 h at 110 °C and analysed for ³²P-labelled amino acids by two-dimensional thin-layer electrophoresis on silica gel and autoradiography as in [18]. The standard markers were detected by staining for phosphate and amino acids as in [18].

Preparation of antiserum to phosphoenolpyruvate carboxylase

Antiserum was raised in a New Zealand White rabbit. Pre-immune serum was first collected from the rabbit. Subsequently 0.5 mg of phosphoenolpyruvate carboxylase, purified as in [15], was emulsified in 50% Freund's complete adjuvant and injected subcutaneously at many sites. The animal was injected subcutaneously 4 weeks later with 0.25 mg of purified enzyme emulsified in 50% Freund's incomplete adjuvant; 14 days later blood was removed and allowed to coagulate, and antiserum was prepared. Before the preparation of further antiserum, the animal was again inoculated with a further 0.25 mg of enzyme emulsified in 50% Freund's incomplete

Table 1. Purification of (a) the day form and (b) the night form of phosphoenolpyruvate carboxylase

Inhibition is by 0.5 mM-malate in (a) and by 5 mM-malate in (b)

Step	Volume (ml)	Total protein (mg)	Total enzyme activity (units)	Specific activity (units/mg)	Yield (%)	Purification (fold)	Inhibition by malate (%)
(a) Day form							
Homogenate	180	27	48	1.8	100	1	73
20000 g supernatant	180	31	50	1.6	105	1	61
Hydroxyapatite pool	16	4.1	23	5.6	47	3	61
Sephadex G-25 pool	25	1.4	26	19	55	11	74
DEAE-cellulose pool	4.0	0.64	14	22	29	12	74
Superose 6 pool	1.5	0.36*	7.2	20	15	11	72
(b) Night form							
Homogenate	180	28	50	1.8	100	1	70
20000 g supernatant	180	36	57	1.6	114	1	64
Hydroxyapatite pool	14	3.4	26	7.6	52	4	77
Sephadex G-25 pool	25	0.83	19	23	38	13	78
DEAE-cellulose pool	9.2	0.50	13	26	26	14	77
Superose 6 pool	1.5	0.24*	5.1	21	10	12	74

* Measured by A_{280} .

adjuvant, and then blood was removed over the next 6–8 weeks.

Immunoprecipitation of phosphoenolpyruvate carboxylase from crude extracts

B. fedtschenkoi leaves were extracted as described above for the purification of phosphoenolpyruvate carboxylase. The extract was centrifuged for 2 min at 11600 g, and the supernatant was rapidly assayed for phosphoenolpyruvate carboxylase. Extract containing 0.07 unit of enzyme was mixed with 15 μ l of antiserum and left at 0 °C for 30 min. The mixture was centrifuged for 2 min at 11600 g, and the pellet was washed with 1 ml of 1.5 M-NaCl/2 mM-EDTA, pH 7. The pellet was resuspended in SDS sample buffer, boiled for 3 min and analysed by SDS/polyacrylamide-gel electrophoresis [19]. In controls the antiserum was replaced by 15 μ l of pre-immune serum.

Polyacrylamide-gel electrophoresis

SDS/polyacrylamide-slab gels were run as in [19]. The M_r marker proteins were β -galactosidase (125000), phosphorylase (100000), serum albumin (68000) and lactate dehydrogenase (35000). Limited proteolysis and peptide mapping ('Cleveland mapping') were carried out as in [20] by using chymotrypsin and V8 protease.

Electrophoretic transfer to nitrocellulose paper was carried out as in [21]. The unoccupied binding sites were blocked with Tween 20 [22] at a concentration of 0.5% (v/v). To detect phosphoenolpyruvate carboxylase, the nitrocellulose paper was incubated for 90 min in 20 mM-Tris/HCl, pH 7.2, containing 150 mM-NaCl, 0.5% (v/v) Tween 20, 5% (v/v) normal goat serum and 0.5% (v/v) anti-(phosphoenolpyruvate carboxylase) serum. The paper was washed for 4 $\times$ 12 min in 20 mM-Tris/HCl (pH 7.2)/15 mM-NaCl/0.5% Tween 20 and for 12 min in buffer without Tween 20. The paper was incubated for 90 min in 20 mM-Tris/HCl, pH 7.2, containing 150 mM-NaCl, 5% normal goat serum and 0.1% (v/v) goat anti-rabbit peroxidase conjugate, and then washed for 5 $\times$ 12 min in 20 mM-Tris/HCl (pH 7.2)/

150 mM-NaCl. The peroxidase was detected by immersing the paper in 50 ml of 10 mM-Tris/HCl, pH 7.4, 30 mg of chloronaphthol in 10 ml of methanol, and 150 μ l of 4% (v/v) H_2O_2 .

Non-denaturing polyacrylamide (7%, w/v) tube gels were run as in [23]. Gels were stained for phosphoenolpyruvate carboxylase activity as in [24]. The staining mixture contained 50 mM-Tris/HCl, pH 8.0, 5 mM-MgCl₂, 2 mM-phosphoenolpyruvate, 10 mM-NaHCO₃, 0.1% (v/v) 2-mercaptoethanol and 10 mM-CaCl₂ at 30 °C. Glycine was removed from the gels by presoaking them in 50 mM-Tris/HCl (pH 8.0)/0.1% 2-mercaptoethanol/10 mM-CaCl₂, as it inhibited phosphoenolpyruvate carboxylase.

Determination of apparent M_r by gel filtration

This was carried out on the Superose 6 column used for the purification of phosphoenolpyruvate carboxylase. The flow rate and fraction size were as described above. All buffers contained 50 mM-1,3-bis[tris(hydroxymethyl)methylamino]propane/HCl, pH 7.5 or 8.5, 50 mM-KCl, 0.1 mM-EDTA, 1 mM-dithiothreitol, 1 mM-benzamidine, and 5–50 μ g of purified phosphoenolpyruvate carboxylase was loaded. The column was calibrated with the following M_r markers: thyroglobulin (669000), apoferritin (440000), pyruvate kinase (237000), lactate dehydrogenase (144000). Where additions were made to the column buffer, the enzyme was preincubated for 30 min at room temperature in the presence of the additions before it was loaded on the column.

RESULTS

Purification of the day and night forms of phosphoenolpyruvate carboxylase

The purifications of the day and night forms of phosphoenolpyruvate carboxylase from *B. fedtschenkoi* as described in the Experimental section are summarized in Table 1. The same method was used for both forms of the enzyme; this was possible because they were not resolved by chromatography on hydroxyapatite, DEAE cellulose or Superose 6. This procedure represents a

Fig. 1. Non-denaturing polyacrylamide-gel electrophoresis of phosphoenolpyruvate carboxylase

Gels 1–3, purified day form (4, 1, 1 μ g). Gels 4–6, purified night form (4, 1, 1 μ g). Gel 7, purified day form (1 μ g)+purified night form (1 μ g). Gel 8, crude night extract (from 0.01 g of leaf). Gel 9, crude day extract (from 0.01 g of leaf). Gels 1, 2, 4, 5 and 7 were stained with Coomassie Brilliant Blue. Gels 3, 6, 8 and 9 were stained for phosphoenolpyruvate carboxylase activity (see the Experimental section). The position of the tracking dye was marked with wire.

considerable improvement over the previous method [15], because the malate sensitivities of the two forms of the enzyme were preserved (Table 1). The apparent K_i values for malate under the standard assay conditions, determined as in [9], were 2.75 ± 0.25 mM (mean $\pm$ S.D., $n = 9$) for the night enzyme and 0.30 ± 0.03 mM (mean $\pm$ S.D., $n = 5$) for the day enzyme, which compare well with the values of 2.5–3.0 mM and 0.3 mM previously measured in rapidly prepared crude extracts [9]. The malate sensitivity of phosphoenolpyruvate carboxylase decreased fairly rapidly after extraction, but this was slowed by the inclusion of 10 mM-malate in the buffers and by chromatography on hydroxyapatite. At this point the malate sensitivity of the night form of the enzyme was fairly stable but that of the day form was not. After chromatography on DEAE-cellulose, both forms were fairly stable. Accordingly, the purification was carried out as rapidly as possible up to this stage (~ 7 h). The malate sensitivity of the purified night form of the enzyme was stable for several weeks, but that of the day form was stable for only 1–2 weeks. Although the chromatography on DEAE-cellulose and Superose 6 had little effect on the specific activity of the enzyme (Table 1), they removed several minor proteins which were seen on heavily loaded SDS/polyacrylamide gels, and they stabilized the enzyme for long-term storage. The estimates of protein in the early stages of the purification were reproducible, but were probably inaccurate, since several components of the extract interfered with the protein assay.

Non-denaturing polyacrylamide-gel electrophoresis

When examined by non-denaturing polyacrylamide-gel electrophoresis, both preparations, even at high loadings,

Fig. 2. SDS/polyacrylamide (8%, w/v)-gel electrophoresis of purified phosphoenolpyruvate carboxylase

Tracks 1 and 2, day form (2, 0.5 μ g). Tracks 3 and 4, night form (2, 0.5 μ g). Track 5, day form (0.5 μ g). Track 6, day form (0.5 μ g) purified without added malate. The numbers on the left indicate the M_r values ($\times 10^{-3}$) of the marker proteins for tracks 1–4.

gave a single band of protein coincident with a single band of phosphoenolpyruvate carboxylase activity (Fig. 1). These bands had the same mobility as the bands of activity observed when crude day- and night-time extracts were electrophoresed under the same conditions (Fig. 1). Only one band was seen when the day and night forms of the enzyme were electrophoresed together on one gel (Fig. 1).

SDS/polyacrylamide-gel electrophoresis

After polyacrylamide-gel electrophoresis in the presence of SDS, both preparations gave two protein bands, a minor band of subunit M_r 123000 ± 3000 (mean $\pm$ S.D., $n = 7$) and a major band of subunit M_r 112000 ± 3000 (mean $\pm$ S.D., $n = 9$) (Fig. 2). These two bands were not resolved at all by gradient elution from Mono Q at pH 7.5 or 9.0, by chromatofocusing on Mono P or by gel filtration on Superose 6 in the presence or absence of 10 mM-malate. The two proteins appeared to have closely related amino acid sequence as judged by 'Cleveland mapping' (Fig. 3). Leaf extracts that were prepared as rapidly as possible and then analysed by SDS/polyacrylamide-gel electrophoresis and immunoblotting showed the two bands in the same ratio as that found in purified enzyme (Fig. 4). The 60000- M_r -subunit band observed in crude extracts probably represents a minor, highly antigenic, contaminant in the enzyme used to raise the antiserum. The minor bands observed in the

Fig. 3. 'Cleveland mapping' of the two components of purified phosphoenolpyruvate carboxylase

Digestion was for 30 min with 25 ng of V8 protease per track. Track 1, 123000- M_r -subunit protein, and track 2, 112000- M_r -subunit protein, of the night form of the enzyme. The day form gave an identical result.

purified preparation (Fig. 4, tracks 4 and 5) are proteolysed phosphoenolpyruvate carboxylase and were not observed with freshly purified enzyme.

Heavily loaded gels showed no other protein staining (Fig. 2) or immunoreactive (Fig. 4) bands in the purified enzymes. Phosphoenolpyruvate carboxylase that had been rapidly immunoprecipitated from day and night extracts of leaves (see the Experimental section) appeared to be identical with the purified enzymes as judged by immunoblotting and protein staining of SDS/polyacrylamide gels and by 'Cleveland mapping'. No differences between the day and night forms of the enzyme were observed by these methods.

When the same purification procedure was carried out more slowly, or in the absence of malate, or with old leaves, purified enzyme was obtained from day and night extracts with a much lower sensitivity to malate inhibition, comparable with that of the earlier preparation of the enzyme [15]. These enzyme preparations had a slightly lower subunit M_r of 107000 ± 3000 (mean $\pm$ s.d., $n = 5$) as measured by SDS/polyacrylamide-gel electrophoresis (Fig. 2), but were closely related to the 112000- M_r -subunit enzyme as judged by 'Cleveland mapping'. Therefore at least one factor in the decline in the malate sensitivity of phosphoenolpyruvate carboxylase after extraction is a proteolytic cleavage of the protein chain near one end. The proteolysis was not prevented by EDTA, phenylmethanesulphonyl fluoride, benzamidine, antipain, leupeptin or neutral pH, but was

Fig. 4. Comparison by immunoblotting of phosphoenolpyruvate carboxylase in crude extracts and after purification

A 1:3 extract of *B. fedtschenkoi* leaves was prepared by homogenization at low speed for 1 min at -15°C in 100 mM-Tris/HCl, pH 8.5, containing 5 mM-EDTA, 40% (v/v) ethanediol, 1 mg of phenylmethanesulphonyl fluoride/ml, 5 μg of leupeptin/ml, 5 μg of antipain/ml, 1% (v/v) 2-mercaptoethanol, 5 mM-benzamidine and a few drops of octan-1-ol. Extract (1 ml) was rapidly added to 1 ml of SDS sample buffer [19] in a wide flask in a boiling-water bath and boiled for 5 min. The pH was adjusted to 6.8 and insoluble material was removed by centrifugation. The samples were separated on an SDS/polyacrylamide (8%, w/v) gel and immunoblotted and stained for phosphoenolpyruvate carboxylase (see the Experimental section). Tracks 1–5, purified day form of the enzyme (25, 50, 125, 250, 500 ng). Tracks 6–9, day extract (2.5, 1.25, 0.625, 0.25 μl). The numbers on the left indicate the subunit M_r values ($\times 10^{-3}$) of the immunoreactive proteins.

slowed in the presence of 10 mM-malate. The night form of the enzyme was less susceptible to proteolysis than the day form. The proteolysis had little effect on the V_{max} of the enzyme.

Gel filtration of purified phosphoenolpyruvate carboxylase

The day and night forms of the enzyme were eluted from Superose 6 (see the Experimental section) at pH 7.5 with an apparent M_r of 370000 ± 20000 (mean $\pm$ s.d., $n = 11$). In the presence of 10 mM-malate both forms of the enzyme appeared to have a lower M_r of 310000 ± 20000 (mean $\pm$ s.d., $n = 7$). The elution volume was unaltered by increasing the pH from 7.5 to 8.5, or by the addition of 5 mM-EDTA or 5 mM- MgCl_2 , or over a 10-fold range of enzyme concentration. No differences in the elution profiles of the two forms of the enzyme were observed. The sensitivity to malate and specific activity of the enzymes were constant across the peaks of phosphoenolpyruvate carboxylase activity (results not shown).

Analysis of the covalent modification of the night enzyme

We have previously shown, by immunoprecipitation from ^{32}P -labelled leaves, that the night form of phosphoenolpyruvate carboxylase contains a covalently bound phosphorus-containing group, whereas the day form does not [9]. To investigate the nature of this group further, the enzyme was purified from leaves which were labelled with $^{32}\text{P}[\text{P}_i]$ and extracted at night. It was then

Fig. 5. Identification of the ^{32}P -containing residue in the night form of phosphoenolpyruvate carboxylase

Two-dimensional thin-layer electrophoresis of hydrolysed ^{32}P -labelled night enzyme was carried out (see the Experimental section). The positions of the standard markers ($0.5\ \mu\text{g}$) are indicated on the autoradiograph.

subjected to acid hydrolysis and the ^{32}P -labelled amino acids were analysed (see the Experimental section). The results (Fig. 5) showed three radioactive spots corresponding to P_i , phosphoserine and an unknown, assumed to be a small phospho-peptide. ^{32}P was not released from the purified ^{32}P -labelled enzyme by 1 M-HCl (1 h, 37°C) or 0.8 M-hydroxylamine in 0.15 M-sodium acetate, pH 6 (30 min, 37°C), and was $\sim 50\%$ hydrolysed by 0.1 M-NaOH (1 h, 37°C). These effects were consistent with the properties of a phosphoserine group.

No ^{32}P was released from the enzyme by incubation with non-radioactive substrates under conditions where the enzyme turned over many times (Fig. 6). Therefore the phosphoserine is not a covalent intermediate in the catalytic mechanism.

Alkaline phosphatase catalysed the removal of ^{32}P from phosphoenolpyruvate carboxylase without any proteolysis, as judged by SDS/polyacrylamide-gel electrophoresis (Fig. 6), and also lowered the apparent K_i of the enzyme for malate from 3.0 mM to 0.3 mM. Phosphodiesterase (100 munits/ml) did not release any ^{32}P from phosphoenolpyruvate carboxylase ($60\ \mu\text{g}/\text{ml}$) in 1 h at 37°C . Incubation of the purified ^{32}P -labelled enzyme with a 1:25 extract of *B. fedtschenkoi* leaves for 1 h at 37°C raised the apparent K_i for malate from 3 mM to 8 mM and decreased the subunit M_r to 107000, but did not release the ^{32}P (results not shown).

Fig. 6. SDS/polyacrylamide (8%, w/v)-gel electrophoresis of purified ^{32}P -labelled phosphoenolpyruvate carboxylase

Track 1, phosphoenolpyruvate carboxylase ($6\ \mu\text{g}$) incubated for 1 h at 37°C in $100\ \mu\text{l}$ of 50 mM-Tris/HCl (pH 7.8)/1 mM- MgCl_2 . Track 2, as track 1 with the addition of 2 mM-phosphoenolpyruvate and 10 mM- NaHCO_3 . Track 3, phosphoenolpyruvate carboxylase ($6\ \mu\text{g}$) incubated for 2 h at 25°C in $100\ \mu\text{l}$ of 1,3-bis[tris(hydroxymethyl)methylaminolpropane]/HCl, pH 9.5, containing 1 mM- MgCl_2 , 0.1 mM- ZnSO_4 and 2.3 units of alkaline phosphatase. Track 4, as track 3, with the omission of alkaline phosphatase. The gel was dried and autoradiographed, then re-swollen and stained for protein. (a) Gel stained for protein with Coomassie Brilliant Blue. (b) Autoradiograph. The arrows on the left indicate the position of phosphoenolpyruvate carboxylase (subunit M_r 112000). Each track represents a separate incubation.

More heavily loaded gels showed that the 123000- M_r -subunit form of the enzyme also contained ^{32}P . A lower- M_r -subunit protein (~ 60000) containing ^{32}P can be seen in Fig. 6. This band was separated from phosphoenolpyruvate carboxylase by gel filtration on Superose 6.

DISCUSSION

The day and night forms of phosphoenolpyruvate carboxylase were highly purified, as judged by electrophoresis on denaturing and non-denaturing polyacrylamide gels (Figs. 1 and 2). Furthermore, when chromatographed on Mono Q and Superose 6 the fractions containing phosphoenolpyruvate carboxylase had constant specific activity. The two forms of the enzyme behaved identically with each other on electrophoresis (Figs. 1 and 2) and chromatography as described above, and were not distinguished by 'Cleveland mapping'. The purified enzyme was indistinguishable from the enzyme in fresh crude extracts, as judged by non-denaturing and denaturing polyacrylamide-gel electrophoresis (Figs. 1 and 4) and by immunoprecipitation. The specific activity of the purified enzyme forms (20–26 units/mg) (Table 1) was similar to the value of 26 units/mg obtained for an earlier preparation of the same enzyme [15]. However, unlike the earlier preparation, the present procedure retained the malate sensitivities observed in fresh crude extracts of leaves prepared during the day and at night (Table 1). The purified day and night forms had the same specific activity as each other under the standard assay conditions (Table 1). The native M_r value obtained from the gel-filtration experiments is less than 4 times the subunit M_r value obtained from SDS/polyacrylamide-gel electrophoresis. The cross-linking and gel-filtration experiments of Jones *et al.* [15] on the proteolysed form of phosphoenolpyruvate carboxylase showed it to be a tetramer that could dissociate to a dimer. The same could be true for the undegraded form of the enzyme studied in this paper.

Both purified forms of phosphoenolpyruvate carboxylase contained a minor protein of subunit M_r 123000 and a major protein of subunit M_r 112000 (Fig. 2). The two bands co-purified and were closely related in amino acid sequence, as judged by 'Cleveland mapping' (Fig. 3). Crude extracts, prepared under conditions which would prevent any but very rapid proteolysis, contained the two proteins in the same ratio (Fig. 4). The relationship of these two proteins *in vivo* is unclear.

Analysis of the night form of phosphoenolpyruvate carboxylase purified from ^{32}P -labelled leaves indicated that the conversion from the day form into the night form was accompanied by phosphorylation of one or more serine residues (Fig. 5). It will be necessary to isolate the kinase and carry out the conversion of the day form into the night form *in vitro* in order to discover whether the phosphorylation correlates directly with the decrease in the malate sensitivity of the enzyme and to determine the stoichiometry of phosphorylation. The covalently bound phosphate group was not a catalytic intermediate, and its stability to chemical and enzymic hydrolysis was consistent with the properties of a protein-bound phosphoserine and inconsistent with the properties of other known phosphorus-containing covalent modifications of proteins (Fig. 6). It seems likely that alkaline phosphatase mimics the action of the

putative *B. fedtschenkoi* phosphoenolpyruvate carboxylase phosphatase in removing the phosphate group and increasing the malate sensitivity of the enzyme.

A fairly rapid decline in the malate sensitivity of phosphoenolpyruvate carboxylase after extraction has been observed by several groups. The decline was slowed by the presence of malate and has been interpreted as conversion of the day form into the less-sensitive night form [8,12–14]. However, we observed that, although the malate sensitivity of phosphoenolpyruvate carboxylase in crude day extracts declined more rapidly than in night extracts, both preparations of enzyme declined to a form with a very high apparent K_i for malate (10–15 mM) in a few hours, although V_{max} was almost unaffected. The main factor in this irreversible decrease in malate sensitivity appeared to be the proteolysis of the enzyme to a species with a subunit M_r of 107000 (Fig. 2). It was not caused by phosphorylation. Although both phosphorylation and the binding of malate to phosphoenolpyruvate carboxylase partially protected the enzyme against proteolysis, they did not have the same effect as each other on the conformation of the enzyme, since only malate caused a decrease in the apparent M_r of the native enzyme. It is clearly important to distinguish between the reversible decrease in malate sensitivity that accompanies phosphorylation and the irreversible decrease that accompanies proteolysis.

An earlier preparation of purified phosphoenolpyruvate carboxylase from *B. fedtschenkoi* [15] had a much lower sensitivity to inhibition by malate than that observed for enzyme in fresh extracts [13], and was probably the proteolysed 107000- M_r -subunit species. This earlier enzyme preparation also differed from that reported in the present paper in being much more readily dissociated from tetramer to dimer on dilution or the addition of EDTA and phosphoenolpyruvate [15]. Preparations of purified enzyme from three Crassulacean plants (*B. pinnatum*, *Kalanchoë daigremontiana* and *Ananas comosus*) had malate sensitivities which were intermediate between those of the preparations discussed above, and the enzymes readily dissociated from tetramers to dimers on dilution [25]. More recently phosphoenolpyruvate carboxylase has been purified in the day and night forms from *C. argentea*, retaining the distinct malate sensitivities observed in crude day and night extracts [14]. The preparations from *C. argentea* differed in a number of respects from the preparations described here from *B. fedtschenkoi*. The day form from *C. argentea* was a dimer and was aggregated to form tetramers by concentration or the addition of Mg^{2+} ions or phosphoenolpyruvate, whereas the night form was a tetramer and was dissociated to dimers by dilution or the addition of malate or EDTA or by raising the pH [26]. Furthermore, the day and night forms were separated by non-denaturing polyacrylamide-gel electrophoresis [14]. In contrast, the day and night forms from *B. fedtschenkoi* both appeared to be tetramers and were not separated by polyacrylamide-gel electrophoresis (Fig. 1). They were not dissociated by dilution or the addition of EDTA or by raising the pH, although the apparent M_r was decreased by the addition of malate. This conformational change or dissociation may account for the hysteretic character of the malate inhibition which has been observed for many preparations of the enzyme [9,13–15,25,26]. The day form from *C. argentea* was about 10 times less sensitive to malate [14,26] than was the

day form from *B. fedtschenkoi*. The night form from *C. argentea* was only 25% inhibited by saturating malate concentrations [14,26], whereas the night form from *B. fedtschenkoi* was 100% inhibited. It is unclear at present whether the properties of phosphoenolpyruvate carboxylase differ greatly from one Crassulacean plant to another and whether phosphorylation and proteolysis of the enzyme occur in all these plants.

Phosphorylation of phosphoenolpyruvate carboxylase has been shown to occur *in vivo* in association with a diurnal rhythm in the malate sensitivity of the enzyme; the enzyme was phosphorylated at night [9]. Similarly phosphorylation has been shown to occur in association with circadian rhythms in the malate sensitivity of phosphoenolpyruvate carboxylase in plants maintained under constant environmental conditions (G. A. Nimmo, H. G. Nimmo, C. A. Fewson & M. B. Wilkins, unpublished work). The regulation of the kinase and phosphatase which presumably phosphorylate and dephosphorylate phosphoenolpyruvate carboxylase must be an important factor in the regulation of Crassulacean acid metabolism and of the diurnal and circadian rhythms of phosphoenolpyruvate carboxylase activity.

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