Biochem. J. (1989) 261, 349–355 (Printed in Great Britain)

Purification, oligomerization state and malate sensitivity of maize leaf phosphoenolpyruvate carboxylase

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A method was developed for the purification of phosphoenolpyruvate carboxylase from darkened maize leaves so that the enzyme retained its sensitivity to inhibition by malate. The procedure depended on the prevention of proteolysis by the inclusion of chymostatin in the buffers used during the purification. The purified enzyme was indistinguishable from that in crude extracts as judged by native polyacrylamide-gel electrophoresis, SDS/polyacrylamide-gel electrophoresis followed by immunoblotting, and Superose 6 gel filtration. Gel-filtration studies showed that the purified enzyme and the enzyme in extracts of darkened or illuminated leaves showed a concentration-dependent dissociation of tetrameric into dimeric forms. Purified phosphoenolpyruvate carboxylase and enzyme in crude extracts from darkened leaves were equally sensitive to inhibition by malate (K_i approx. 0.30 mM) under conditions where it existed in the tetrameric or dimeric forms, but the enzyme in crude extracts from illuminated leaves was less sensitive to malate inhibition (K_i approx. 0.95 mM) whether it was present as a tetramer or as a dimer. It is concluded that changes in the oligomerization state of phosphoenolpyruvate carboxylase are not directly involved in its regulation by light.

INTRODUCTION

Phosphoenolpyruvate carboxylase (EC 4.1.1.31) is a key enzyme in the fixation of atmospheric CO_2 in C_4 and crassulacean acid metabolism (CAM) plants. It catalyses the conversion of phosphoenolpyruvate and HCO_3^- to oxaloacetate and inorganic phosphate, the first committed step in the fixation of external CO_2 in these plants. The enzyme from leaves of C_4 plants such as maize has been shown to be regulated by light. The enzyme is less sensitive to inhibition by malate when it is extracted from illuminated leaves than when it is extracted from darkened leaves [1–4]. In addition, phosphoenolpyruvate carboxylase from illuminated leaves has been shown to be much more phosphorylated than enzyme from darkened leaves [2,4]. This finding has led to the proposal that phosphorylation of maize phosphoenolpyruvate carboxylase in the light is responsible for its reduced sensitivity to malate [2,4].

It is generally thought that active purified phosphoenolpyruvate carboxylase is a tetramer ($M_r \sim 400000$) of four identical subunits [5–7]. There have, however, been many reports that the enzyme can exist in different oligomeric forms [7–19]. These studies have been carried out on phosphoenolpyruvate carboxylase from a number of C_4 and CAM species using several different techniques such as gel filtration [7–17], non-denaturing polyacrylamide-gel electrophoresis [11,18] and ultracentrifugation [19]. However, the question of whether changes in oligomerization state are involved in the regulation of phosphoenolpyruvate carboxylase activity by light remains unanswered. In this study, a systematic investigation was therefore made of conditions which might cause alterations in the aggregation state of purified maize-leaf phosphoenolpyruvate carboxylase. We first developed a procedure for purifying the enzyme in an undegraded form that maintained the same sensitivity to malate as was observed in leaf extracts. We then identified conditions where the purified enzyme behaves as a tetramer and as a dimer, and went on to study the effects of illumination on the aggregation state and malate sensitivity of phosphoenolpyruvate carboxylase in extracts.

EXPERIMENTAL

Materials

Chymostatin, M_r marker proteins for SDS/polyacrylamide-gel electrophoresis, Hepes and D-isoascorbic acid were obtained from Sigma Chemical Co., Poole, Dorset, U.K. Ferritin was from Pharmacia, Milton Keynes, Bucks., U.K. Enzymes were from Boehringer Corp. (London) Ltd., Lewes, Sussex, U.K. The sources of other materials were as given previously [2,8,20–22].

Plants

Maize (*Zea mays* L. var. Bastille) plants were grown from seed in a greenhouse for 3–5 weeks before being transferred to growth rooms (12 h photoperiod, 27 °C day/18 °C night, 21 $W \cdot m^{-2} \cdot s^{-1}$ radiant fluence rate at plant height from fluorescent and tungsten lamps) for 1–3 weeks before use.

Detached fully expanded leaves were either darkened for at least 2 h or illuminated at a total radiant fluence rate of 32 $W \cdot m^{-2} \cdot s^{-1}$ for 1 h at 27 °C as described in [2].

Radiant fluence rates were measured using a Kipp and Zonen thermopile.

Purification of phosphoenolpyruvate carboxylase

All steps were carried out at 4 °C except Mono Q chromatography which was done at room temperature. The pH of all buffers was adjusted at room temperature.

Abbreviation used: CAM, crassulacean acid metabolism.

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Extraction

Maize leaves (10 g) were taken from plants which had been maintained in darkness for at least 2 h in the growth rooms. The leaves were chopped up with scissors and homogenized in 40 ml of 50 mM-Hepes/KOH, pH 7.3, containing 5 mM-$MgCl_2$, 5 mM-dithiothreitol, 1 mM-EDTA, 10 μg of chymostatin/ml, 2% (w/v) insoluble polyvinylpyrrolidone and 0.5% (w/v) isoascorbate (Buffer A) in a Waring blender for 30 s at low speed. The extract was filtered through two layers of muslin and centrifuged for 15 min at 10000 ***g***.

Ammonium sulphate fractionation

The supernatant was brought to 30% saturation with ammonium sulphate and centrifuged for 15 min at 15000 ***g***. The supernatant from this centrifugation was then taken to 50% ammonium sulphate saturation, recentrifuged and the supernatant discarded. The pellet from the 30–50% ammonium sulphate fraction, which contained the phosphoenolpyruvate carboxylase activity, was dissolved in 2 ml of 50 mM-Hepes/KOH, pH 7.1, containing 5 mM-$MgCl_2$, 5 mM-dithiothreitol, 1 mM-EDTA and 10 μg of chymostatin/ml. This buffer (Buffer B) was used in all subsequent purification steps.

Sephadex G-25M chromatography

The dissolved pellet from the 30–50% ammonium sulphate fraction was desalted into Buffer B on a 1 cm × 12 cm (10 ml) column of Sephadex G-25M. The flow rate was 4 ml/min and 0.5 ml fractions were collected.

Hydroxyapatite chromatography

Active fractions from the Sephadex column were pooled and loaded on to a 3.3 cm × 1.2 cm (10 ml) column of hydroxyapatite Bio-Gel HTP equilibrated in Buffer B. After washing the column in this buffer until the A_{280} fell to zero, phosphoenolpyruvate carboxylase was eluted by running a 0–150 mM-potassium phosphate gradient in 200 ml of Buffer B. The flow rate was 2 ml/min and 2 min fractions were collected. Enzyme was eluted from 80–120 mM-potassium phosphate and fractions containing enzyme activity were pooled and desalted on a 2.2 cm × 45 cm (175 ml) Sephadex G-25 M column into Buffer B as described above.

Mono Q chromatography

The active fractions from the Sephadex column were pooled and chromatographed on a Mono Q column (0.5 cm × 5 cm) connected to a Pharmacia f.p.l.c. system. A linear 25 ml gradient of 50–400 mM-KCl in the same buffer was used to elute the phosphoenolpyruvate carboxylase from the column. The flow rate was 1 ml/min and 0.5 min fractions were collected. Enzyme activity was eluted at 210–240 mM-KCl. Purified enzyme from the Mono Q column was mixed with an equal volume of glycerol and stored at −20 °C.

Assay procedures

The standard spectrophotometric assay mixture [8] for the measurement of phosphoenolpyruvate carboxylase activity contained, in 1 ml, 50 mM-Tris/HCl, pH 7.8, 5 mM-$MgCl_2$, 2 mM-phosphoenolpyruvate, 0.2 mM-NADH, 10 mM-$NaHCO_3$, 5 units of malate dehydrogenase and the enzyme sample (approx. 2–20 munits unless otherwise stated). For the determination of the malate sensitivity of the enzyme, the assay mixture consisted of 50 mM-Hepes/KOH, pH 7.0, 10 mM-$MgCl_2$, 10 mM-$NaHCO_3$, 0.2 mM-NADH, 5 mM-glucose 6-phosphate, 0.5 mM-phosphoenolpyruvate, 5 units of malate dehydrogenase, malate (0–1.8 mM) and the enzyme sample (concentration as described in the text) in 1 ml as in [2]. This reaction mixture is based on that of Huber & Sugiyama [1], and was used because it shows the maximum difference in malate sensitivity between phosphoenolpyruvate carboxylases extracted from illuminated and darkened leaves. One unit of enzyme activity is the amount required to catalyse the production of 1 μmol of oxaloacetate/min at 25 °C.

Pyruvate, phosphate dikinase was assayed as in [23].

Protein was measured by the method of Bradford [24], using bovine serum albumin as a standard.

Polyacrylamide-gel electrophoresis

SDS/polyacrylamide slab gels (8%) were run as in [25]. The proteins used as M_r markers were: myosin (205000), β-galactosidase (116000), phosphorylase *b* (97400), bovine serum albumin (66000), ovalbumin (45000) and carbonic anhydrase (29000).

Non-denaturing polyacrylamide (7%) tube gels were run as in [26]. Activity staining for phosphoenolpyruvate carboxylase was carried out at 30 °C as in [27] using 50 mM-Tris/HCl, pH 8.0, 5 mM-$MgCl_2$, 10 mM-$NaHCO_3$, 2 mM-phosphoenolpyruvate, 0.1% (v/v) 2-mercaptoethanol and 10 mM-$CaCl_2$ as a staining mixture.

Gel-filtration of phosphoenolpyruvate carboxylase

This was carried out at room temperature on a Superose 6 column linked to a Pharmacia f.p.l.c. system. The column buffer contained 50 mM-Hepes/KOH, pH 7.1, 5 mM-$MgCl_2$, 5 mM-dithiothreitol and 1 mM-EDTA as well as the relevant additions shown in Table 2. In one set of experiments the effect of magnesium was investigated by omitting it from the column buffer. Enzyme activity (0.01–1 unit) was loaded on to the column in a volume of 100 μl. This represented 0.5–50 μg of purified phosphoenolpyruvate carboxylase or about 5–500 μg of crude extract protein. Where additions were made to the buffer, the enzyme was preincubated with the addition at room temperature for 30 min before it was loaded on the column. The flow rate was 0.4 ml/min and 0.5 min fractions were collected. The column was calibrated using the following M_r markers: thyroglobulin (669000), ferritin (440000), aldolase (157000), lactate dehydrogenase (144000), hexokinase (100000) and ovalbumin (45000).

Crude extracts for gel-filtration and kinetic studies were made by extracting, in liquid N_2, 0.5 g leaf portions in 2 ml of Buffer A as in [2].

RESULTS

Purification of phosphoenolpyruvate carboxylase

The purification of phosphoenolpyruvate carboxylase from maize leaves as described in the Experimental section is summarized in Table 1. The purified phosphoenolpyruvate carboxylase was essentially homogeneous as judged by electrophoresis on denaturing and non-denaturing polyacrylamide gels (Figs. 1 and 2) and retained the malate sensitivity of the enzyme found in the crude extract (Table 1). The final specific activity varied

Table 1. Purification of phosphoenolpyruvate carboxylase

Step	Volume (ml)	Total protein (mg)	Total enzyme activity (units)	Specific activity (units/mg)	Yield (%)	Purification (fold)	K_i for malate (mM)
Homogenate	40	92	189	2.0	100	1.0	0.35
10000 *g* supernatant	39	62	201	3.2	106	1.6	0.30
Desalted 30–50% ammonium sulphate fraction	4	44	174	4.0	92	2.0	0.35
Desalted hydroxyapatite pool	50	30	140	4.7	74	2.3	0.30
Mono Q pool	3	4.6	96	20.8	51	10.4	0.30

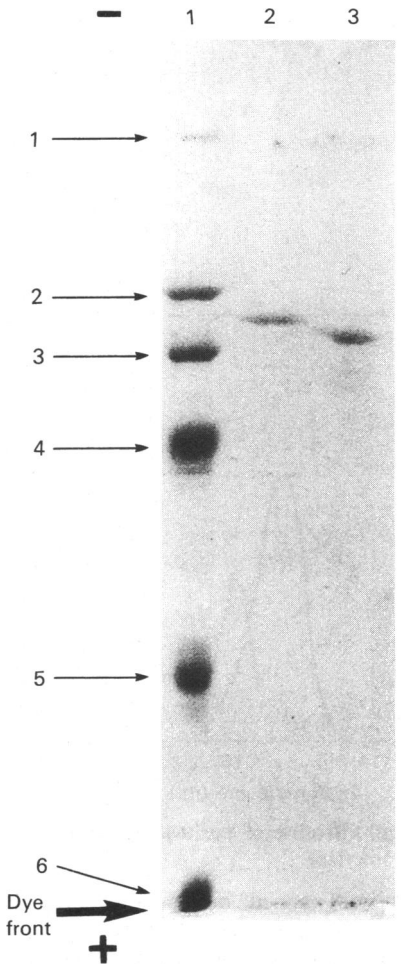

Fig. 1. SDS/polyacrylamide (8%, w/v)-gel electrophoresis of purified phosphoenolpyruvate carboxylase

Track 1, M_r marker proteins. As indicated by the arrows these are: 1, myosin; 2, β-galactosidase; 3, phosphorylase *b*; 4, bovine serum albumin; 5, ovalbumin and 6, carbonic anhydrase. Track 2, phosphoenolpyruvate carboxylase purified in the presence of chymostatin (0.25 μg). Track 3, phosphoenolpyruvate carboxylase purified in the absence of chymostatin (0.25 μg).

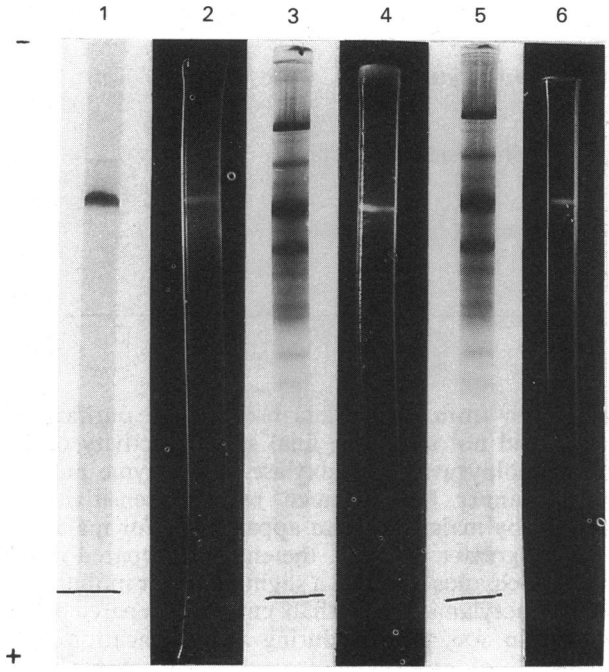

Fig. 2. Non-denaturing polyacrylamide-gel electrophoresis of phosphoenolpyruvate carboxylase

Gels 1 and 2, purified phosphoenolpyruvate carboxylase (2.5 μg); gels 3 and 4, crude extract of darkened leaf (from 0.01 g of leaf); gels 5 and 6, crude extract of illuminated leaf (from 0.01 g of leaf). Gels 1, 3 and 5 were stained for protein with Coomassie Brilliant Blue. Gels 2, 4 and 6 were stained for phosphoenolpyruvate carboxylase activity (see Experimental section). The position of the tracking dye was marked with wire.

from 17 to 22 units/mg of protein, and the apparent K_i values for malate from 0.2 to 0.4 mM, amongst different enzyme preparations. Phosphoenolpyruvate carboxylase purified by this method was free from contamination by pyruvatephosphate dikinase activity, another enzyme of the C_4 pathway which has a similar M_r to phosphoenolpyruvate carboxylase and has previously been reported to be a contaminant in purified phosphoenolpyruvate carboxylase [18].

The presence of the protease inhibitor chymostatin throughout the enzyme preparation was found to be essential for the maintenance of the malate sensitivity of phosphoenolpyruvate carboxylase during the purification. The inclusion of malate, which has previously been used to stabilize the C_4 and CAM enzymes [4,11,19,20,28,29], or other protease inhibitors such as benzamidine or phenylmethanesulphonyl fluoride in the buffers used in the preparation, did not prevent the enzyme from losing its sensitivity to malate. Omission of

Table 2. Apparent M_r values for purified phosphoenolpyruvate carboxylase under various conditions

M_r values and recoveries obtained from Superose 6 gel-filtration are expressed as means ± S.D. for the number of experiments shown in parentheses in the final column. Levels of significance are a comparison of M_r values with the M_r value obtained when 50 μg of protein was loaded without any addition: n.s., not significantly different at the 0.1 level; * significantly different at the 0.01 level; ** significantly different at the 0.001 level.

Addition to column buffer	Protein loading (μg)	10^{-3} × Apparent M_r	Level of significance	Recovery of activity from column (%)
None	50	415 ± 40	–	93 ± 10 (8)
	25	405 ± 35	n.s.	87 ± 13 (14)
	10	410 ± 50	n.s.	78 ± 14 (22)
	0.5	260 ± 50	**	55 ± 16 (25)
200 mM-NaCl	25	380 ± 65	n.s.	98 ± 15 (8)
	10	255 ± 60	**	80 ± 15 (8)
	0.5	200 ± 50	**	46 ± 12 (8)
5 mM-malate	25	330 ± 40	*	101 ± 12 (8)
	10	270 ± 35	**	83 ± 16 (8)
	0.5	270 ± 35	**	74 ± 24 (8)
5 mM-phosphoenolpyruvate	25	405 ± 20	n.s.	110 ± 12 (5)
	10	395 ± 15	n.s.	90 ± 5 (5)
	0.5	365 ± 40	n.s.	74 ± 20 (8)
None, but buffer pH 8.0	25	420 ± 20	n.s.	29 ± 9 (4)
	10	410 ± 30	n.s.	18 ± 7 (5)
	0.5	260 ± 25	**	10 ± 5 (4)

chymostatin from the buffers used in the purification protocol did not affect the final specific activity of the phosphoenolpyruvate carboxylase, but enzyme purified in this manner had a much reduced sensitivity to inhibition by malate with an apparent K_i for malate of 1.8 mM or greater. Further, the enzyme prepared in the absence of chymostatin had a slightly greater mobility on SDS/polyacrylamide gels than enzyme prepared when chymostatin was present during the purification. The subunit M_r of phosphoenolpyruvate carboxylase was about 109000 for enzyme prepared in the presence of chymostatin, while enzyme prepared in its absence had a subunit M_r of about 105000 (Fig. 1). The two species could be resolved by gel electrophoresis only if protein loading on the gel was low (0.25 μg). The difference in M_r values was confirmed by immunoblotting (results not shown). These results suggest that chymostatin protects the enzyme against the cleavage of a peptide bond close to one end of the phosphoenolpyruvate carboxylase subunit and thus prevents the loss of the enzyme's malate sensitivity. The malate sensitivity and specific activity were stable for several weeks when the purified enzyme was stored in 50% (v/v) glycerol at −20 °C.

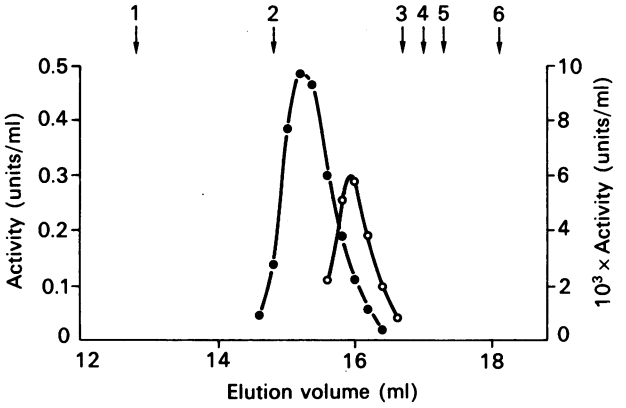

Fig. 3. Superose 6 gel-filtration of purified maize phosphoenolpyruvate carboxylase

This experiment was carried out in the absence of effectors (see Experimental section). Phosphoenolpyruvate carboxylase activity is indicated on the left-hand ordinate for a 25 μg loading (●) and for a 0.5 μg loading (○) on the right-hand ordinate. The arrows indicate the following M_r marker proteins: 1, thyroglobulin; 2, ferritin; 3, aldolase; 4, lactate dehydrogenase; 5, hexokinase and 6, ovalbumin.

Analysis of the oligomerization state of phosphoenolpyruvate carboxylase by gel filtration

Table 2 summarizes the effects on the apparent M_r of purified phosphoenolpyruvate carboxylase of changing the protein concentration under various conditions. At high protein loadings (10–50 μg of purified enzyme) phosphoenolpyruvate carboxylase activity was eluted from the Superose 6 column as a single, slightly asymmetrical peak (Fig. 3) with an apparent M_r which corresponds to a tetrameric aggregation state. At low protein loadings (0.5 μg of purified enzyme), enzyme activity was eluted from the column, again as a single slightly asymmetrical peak, with a lower apparent M_r equivalent to that which might be expected for a dimer of phosphoenolpyruvate carboxylase (Fig. 3).

The inclusion of 200 mM-NaCl in the buffer appeared to promote the dissociation of phosphoenolpyruvate carboxylase (Table 2). This is most clearly seen at a loading of 10 μg, where the apparent M_r is significantly lower than that observed in the absence of NaCl. At a loading of 25 μg, the enzyme behaved as a tetramer whereas at loadings of 10 μg and 0.5 μg the elution

volume was close to that expected for a dimer. A similar effect was observed if 5 mM-malate was present (Table 2). Phosphoenolpyruvate carboxylase was eluted with a lower apparent M_r than when malate was omitted from the buffers. This was the case for 25 μg loadings as well as for lower loadings, although the M_r was slightly larger than might have been expected for a dimer in the former case. The apparent M_r values obtained in the presence of malate were significantly different from the apparent M_r values in the absence of malate for 10 and 25 μg but not for 0.5 μg loadings of protein. The differences in the apparent M_r values amongst the loadings in the presence of malate were not significant, indicating that malate probably induces dissociation of phosphoenolpyruvate carboxylase to dimers over the entire concentration range of enzyme tested. The inclusion of 5 mM-phosphoenolpyruvate in the buffer resulted in the elution of phosphoenolpyruvate carboxylase at an apparent M_r corresponding to the tetramer even at a loading of 0.5 μg (Table 2). Increasing the pH of the column buffer from pH 7.1 to pH 8.0 did not affect the oligomeric state of phosphoenolpyruvate carboxylase although the recovery of enzyme activity from the column was much lower at pH 8.0 (Table 2).

In the absence of magnesium from the buffer, phosphoenolpyruvate carboxylase was eluted from the column at about 13.2 ml, a much greater apparent M_r than in the presence of magnesium. The aggregation was so great that the M_r could not be estimated accurately by Superose 6 chromatography. Further, there was no concentration-dependent dissociation of this aggregation state.

Extracts from illuminated or darkened leaves were subjected to gel-filtration in the absence of additions to the buffer. There were no detectable differences in the behaviour of phosphoenolpyruvate carboxylase between the two extracts (Table 3). In both cases the enzyme showed a dissociation to dimers occurring at low enzyme concentrations, similar to that found with the purified enzyme under the same conditions.

Non-denaturing polyacrylamide-gel electrophoresis

Analysis by non-denaturing polyacrylamide-gel electrophoresis of purified phosphoenolpyruvate carboxylase showed a single protein band with the same mobility at either high (10 μg) or low (0.5 μg) loadings. This band was coincident with a single band of enzyme activity (Fig. 2). Crude extracts from both darkened and illuminated leaves gave a single band of phosphoenolpyruvate carboxylase activity with the same mobility when electrophoresed under the same conditions as the purified enzyme (Fig. 2). Only one band of activity was observed when darkened and illuminated crude extracts were electrophoresed together on one gel (results not shown). These findings with the crude extracts are in contrast to the results of Budde & Chollet [18], who showed the existence of dissociated forms of phosphoenolpyruvate carboxylase in leaf extracts from illuminated plants using a similar procedure.

Table 3. Apparent M_r values for phosphoenolpyruvate carboxylase in extracts prepared from darkened and illuminated leaves

M_r values and recoveries of activities from Superose 6 gel-filtration are expressed as means ± S.D. for four experiments.

	Enzyme loading* (units)	$10^{-3}\times$ Apparent M_r	Recovery of activity from column (%)
Darkened leaf extract	0.2	420 ± 25	104 ± 18
	0.01	260 ± 35	46 ± 15
Illuminated leaf extract	0.2	410 ± 35	86 ± 16
	0.01	285 ± 50	40 ± 16

* Loadings were equivalent in enzyme activity to 10 μg (0.2 units) and 0.5 μg (0.01 units) of purified phosphoenolpyruvate carboxylase (see Table 2).

Concentration dependence of malate sensitivity of purified phosphoenolpyruvate carboxylase and enzyme in extracts of illuminated and darkened leaves

These experiments were designed to investigate the effects of enzyme concentration and oligomerization state on the malate sensitivity of phosphoenolpyruvate carboxylase. The apparent K_i for malate was measured by diluting enzyme samples to concentrations analogous to those at which it was eluted from the Superose 6 column as a tetramer (approx. 10 μg/ml of eluate) or a dimer (approx. 0.5 μg/ml of eluate). The enzyme was diluted 30 min before assay in order to mimic the time taken for the enzyme to pass down the column. It was then assayed at final concentrations in the cuvette (2 or 0.2 munits/ml respectively) which were equivalent to those used when assaying samples from a column loaded with 100 μl volumes containing 10 μg (tetramer) or 0.5 μg (dimer) of protein.

At both concentrations of phosphoenolpyruvate carboxylase, non-linear progress curves were seen which became more curved as the concentration of malate present in the assay was increased. In experiments involving purified phosphoenolpyruvate carboxylase, when the reaction was initiated by adding phosphoenolpyruvate, a slight acceleration period in the rate occurred before a steady-state rate was reached, as would be expected for any coupled assay system. When the enzyme was added last, the rate slowed for the first few minutes before becoming linear. In experiments using crude extracts the rate accelerated during a short lag period before linearity was reached, irrespective of whether assays were initiated with phosphoenolpyruvate carboxylase or phosphoenolpyruvate. Desalted extracts showed the same kinetic behaviour as crude extracts. In all cases the final linear rate was measured; it was the same irrespective of the order of addition of enzyme and phosphoenolpyruvate, and was proportional to enzyme concentration. The discrepancy in kinetic behaviour between purified phosphoenolpyruvate carboxylase and enzyme in crude extracts observed when assays were started by addition of enzyme is difficult to explain. It might imply that there is some difference between purified phosphoenolpyruvate carboxylase and the enzyme in crude extracts that is not manifested on polyacrylamide-gel electrophoresis or gel-filtration.

Dilution of purified phosphoenolpyruvate carboxylase to 10 or 0.5 μg/ml (2 or 0.2 munits/ml in the reaction mixture), concentrations at which it behaves as a tetramer or as a dimer respectively, had no effect on its sensitivity to malate (Table 4). The malate sensitivity remained

Table 4. Malate sensitivity of phosphoenolpyruvate carboxylase at high and low concentrations of the enzyme in the reaction mixture

Purified phosphoenolpyruvate carboxylase was diluted to 10 or 0.5 μg/ml and then assayed as described in the text. Crude extracts were diluted to 0.2 or 0.01 units/ml and then assayed. The concentrations were chosen to be equivalent to the concentrations at which the enzyme was eluted from the Superose 6 column loaded with 10 μg or 0.5 μg of protein respectively. Values are expressed as means ± S.D. for four experiments.

	Phosphoenolpyruvate carboxylase concentration in the reaction mixture (munits/ml)	Apparent K_i for malate (mM)
Purified enzyme	2.0	0.26 ± 0.04
	0.2	0.21 ± 0.06
Enzyme in an extract from a darkened leaf	2.0	0.32 ± 0.04
	0.2	0.32 ± 0.04
Enzyme in an extract from an illuminated leaf	2.0	0.95 ± 0.16
	0.2	0.97 ± 0.18

constant over an even wider range of concentrations of enzyme, up to 0.2 units/ml in the reaction mixture. At 10 μg/ml, phosphoenolpyruvate carboxylase activity was stable. At 0.5 μg/ml, the specific activity of the enzyme declined slowly (a 50% decrease in 90–120 min) while the malate sensitivity was not affected. Crude extracts were diluted to give phosphoenolpyruvate carboxylase activities of 0.2 and 0.01 units/ml (2 and 0.2 munits/ml in the reaction mixture), concentrations at which the enzyme behaves as a tetramer or a dimer (Table 3). This did not affect the malate sensitivity of the enzyme. The characteristic difference in sensitivity between extracts from darkened or illuminated leaves was observed at both concentrations (Table 4). Similar results were obtained at higher enzyme concentrations (up to 0.2 units/ml in the reaction mixture). As found for the purified enzyme, the specific activity of the crude enzyme declined with time at the lower concentration. This instability of the dimer could explain the varied recovery of enzyme activity from gel-filtration, depending on the conditions used.

DISCUSSION

The method described in this paper affords a rapid purification of phosphoenolpyruvate carboxylase from darkened maize leaves to apparent homogeneity. The final specific activity of 17 to 22 units/mg of protein is comparable with most other reported maximum specific activities of purified maize phosphoenolpyruvate carboxylase [6,30,31]. Although several other groups have isolated phosphoenolpyruvate carboxylase from maize, in some cases the enzyme has apparently been partially proteolysed during the purification (e.g. [32,33]) and in others there was little or no attempt to show that the purified enzyme had retained its regulatory properties which are present *in vivo*. It was therefore necessary to assess whether the enzyme had been isolated in its native state by the present procedure. In our hands, the inclusion of chymostatin in the buffers during purification was necessary to prevent proteolysis of the enzyme and to preserve its sensitivity to malate. The purified phosphoenolpyruvate carboxylase was indistinguishable from enzyme from crude extracts as judged by non-denaturing polyacrylamide-gel electrophoresis (Fig. 2), Superose 6 gel-filtration, malate sensitivity (Table 4) and SDS/polyacrylamide-gel electrophoresis and immunoblotting (results not shown). Darkened leaves contain a dephosphorylated form of phosphoenolpyruvate carboxylase [2,4] and an important finding is that phosphoenolpyruvate carboxylase purified by this new method can by phosphorylated *in vitro*, while preliminary experiments suggest that the proteolysed form of the enzyme, of subunit M_r 105000, cannot (G. A. L. McNaughton, unpublished work).

Using gel-filtration, a concentration-dependent dissociation of tetramers to a less stable form with a lower apparent M_r was detected. Dissociation of the enzyme was not detected using non-denaturing polyacrylamide-gel electrophoresis, possibly because of the higher protein concentration used with this technique. Similar effects were observed with purified phosphoenolpyruvate carboxylase and with enzyme in extracts of darkened and illuminated leaves. We believe that the dissociated enzyme form observed on gel-filtration is a dimer, even though the elution volume is slightly lower than would be expected. It is unlikely that cross-linking experiments could resolve this point because the enzyme loses activity rapidly under the slightly alkaline conditions (pH 8.0) normally used for such experiments. Jones *et al.* [8] found a similar concentration-dependent dissociation of phosphoenolpyruvate carboxylase purified from the CAM plant *Bryophyllum fedtschenkoi*. The maize dimer appears to be unstable whereas the dimer of *Bryophyllum* phosphoenolpyruvate carboxylase was stable but had only about 50% of the activity of the tetramer. Nevertheless, it appears that phosphoenolpyruvate carboxylases from C_4 and CAM plants undergo similar concentration-dependent changes in oligomerization state. The results of the kinetic experiments on purified maize phosphoenolpyruvate carboxylase show that the tetrameric and dimeric forms of phosphoenolpyruvate carboxylase are equally sensitive to inhibition by malate (Table 4). Wu & Wedding [16] found that gel-filtration of partially purified phosphoenolpyruvate carboxylase from the CAM plant *Crassula argentea* yielded a mixture of dimers and tetramers, with the tetramer being much less sensitive to inhibition by malate that the dimer. This does not seem to be the case for purified maize phosphoenolpyruvate carboxylase.

The apparent M_r of phosphoenolpyruvate carboxylase was also affected by the composition of the buffer used for gel-filtration. The presence of 200 mM-NaCl promoted the dissociation of the enzyme into dimers at concentrations above those at which dilution alone caused this effect (Table 2). A fast dissociation into dimers induced by high ionic strength was also observed by Wagner *et al.* [15] using h.p.l.c. gel-filtration and similar conditions to those employed in this investigation. In the presence of malate the enzyme exhibited a lower apparent M_r than the tetramer, which was only slightly affected by changes in the protein concentration. These

results could indicate that in the presence of malate the dissociation does not go to completion. Alternatively, the binding of malate to dimers may induce a conformational change which results in increased asymmetry and a higher apparent M_r. Huber *et al.* [28] found that in the presence of 2 mM-malate during gel-filtration, partially purified phosphoenolpyruvate carboxylases from illuminated and darkened maize leaves had slightly different elution profiles which both corresponded to tetrameric conformations of the enzyme. If malate was omitted from the buffers, no such subtle difference was seen. The enzyme concentrations that they used were much higher than those used in the present study. Presumably the enzyme does not dissociate to dimers at such high concentrations even in the presence of malate. The presence of 5 mM-phosphoenolpyruvate in the buffers maintained the tetrameric state of the enzyme on dilution, with no dissociation to dimers being observed at low enzyme concentrations (Table 2). Similar results have been observed by others [10,15,34]. Wu & Wedding reported that the phosphoenolpyruvate carboxylase from *C. argentea* dissociated from tetramers to dimers in the absence of a bivalent metal ion [10], whereas our results indicate that the absence of magnesium causes aggregation of the maize enzyme. Wu & Wedding [10] suggested that magnesium might play some role in the light regulation of the CAM enzyme. However studies on the purified enzyme in the presence of single effectors are of limited relevance in this context, since it is probably the relative concentrations of several effectors in the cytosol that is of importance.

Conditions have been identified which affect the oligomerization state of maize phosphoenolpyruvate carboxylase. This has allowed the comparison of the association/dissociation behaviour of phosphoenolpyruvate carboxylase from illuminated and darkened extracts. Both forms of the enzyme dissociated at low enzyme concentrations, as did the purified enzyme. This dissociation did not affect the characteristic difference in K_i for malate between the enzyme from illuminated and darkened leaves. These results therefore suggest that changes in the oligomerization state of phosphoenolpyruvate carboxylase as reported here are not directly involved in its regulation by light.

This work was supported by grant no. PG 17/186 from the Agricultural and Food Research Council.

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Received 28 December 1988/3 February 1989; accepted 20 February 1989