Purification and characterization of high- and low-molecular-mass isoforms of phosphoenolpyruvate carboxylase from *Chlamydomonas reinhardtii*

Kinetic, structural and immunological evidence that the green algal enzyme is distinct from the prokaryotic and higher plant enzymes

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Phosphoenolpyruvate carboxylase (PEPC) is a key enzyme in the supply of carbon skeletons for the assimilation of nitrogen by green algae. Two PEPC isoforms with respective native molecular masses of 400 (PEPC1) and 650 (PEPC2) kDa have been purified from *Chlamydomonas reinhardtii* CW-15 cc1883 (Chlorophyceae). SDS/PAGE, immunoblot and CNBr peptide-mapping analyses indicate the presence of the same 100 kDa PEPC catalytic subunit in both isoforms. PEPC1 is a homotetramer, whereas PEPC2 seems to be a complex between the PEPC catalytic subunit and other immunologically unrelated polypeptides of 50–70 kDa. Kinetic analyses indicate that these PEPC isoforms are (1) differentially regulated by pH, (2) activated by glutamine and dihydroxyacetone phosphate and (3) inhibited by glutamate, aspartate, 2-oxoglutarate and malate. These results are consistent with the current model for the regulation of anaplerotic carbon fixation in green algae, and demonstrate that

green algal PEPCs are uniquely regulated by glutamine. Several techniques were used to assess the structural relationships between *C. reinhardtii* PEPC and the higher plant or prokaryotic enzyme. Immunoblot studies using anti-(green algal or higher plant PEPC) IgGs suggested that green algal (*C. reinhardtii*, *Selenastrum minutum*), higher plant (maize, banana fruit, tobacco) and prokaryotic (*Synechococcus leopoliensis*, *Escherichia coli*) PEPCs have little or no immunological relatedness. Moreover, the N-terminal amino acid sequence of the *C. reinhardtii* PEPC subunit did not have significant similarity to the highly conserved corresponding region in enzymes from higher plants, and CNBr cleavage patterns of green algal PEPCs were distinct from those of higher plant and cyanobacterial PEPCs. These results point to significant evolutionary divergence between green algal, higher plant and prokaryotic PEPCs.

INTRODUCTION

Phosphoenolpyruvate carboxylase [PEPC, orthophosphate: oxaloacetate carboxy-lyase (phosphorylating), EC 4.1.1.31] catalyses the irreversible β -carboxylation of phosphoenolpyruvate (PEP) to form oxaloacetate and P_i . PEPC is a ubiquitous cytosolic enzyme in plants [1]. It is also found in a wide range of prokaryotes [2] including the archaeobacteria [3]. The C_4 and crassulacean acid metabolism (CAM) PEPCs are primarily responsible for the fixation of atmospheric CO_2 [1] and have been the subject of extensive research [1,2]. These PEPCs are homotetramers subject to allosteric regulation, mainly by glucose 6-phosphate and malate. They are also regulated by a Ca^{2+} -independent protein serine/threonine kinase that reversibly phosphorylates a Ser residue in the highly conserved N-terminal domain [1,2]. Phosphorylation decreases the sensitivity of the enzyme to inhibition by malate. PEPCs from C_3 leaves and non-photosynthetic plant tissues are implicated in a number of physiological processes. Because of this situation, the regulation of C_3 PEPCs has been studied in a variety of model systems (nearly all of which, however, are higher plants). Existing data suggest that regulatory mechanisms *in vivo* for PEPC in C_3 higher plants are similar to those found in C_4 and CAM organisms [1,4–6]. Compared with eukaryotic PEPCs, the prokaryotic enzyme has received only a modest amount of attention. It has nevertheless been established that, in prokaryotes, PEPC serves an anaplerotic function [7] and is allosterically regulated [7,8]. In some cases it is also involved in autotrophic assimilation of CO_2

[9]. In prokaryotes, however, PEPC is not regulated by reversible phosphorylation and the N-terminal domain of the prokaryotic enzymes does not contain the phosphorylation motif found in higher plants [2].

The steady progress in research on higher plant PEPCs contrasts with the poor understanding of the enzymes in lower plants, in particular in green algae. Even physiological data on the role of PEPC are limited primarily to a few Chlorophyceae [10], an algal family containing the widely used model organism, *Chlamydomonas reinhardtii*. These data show that Chlorophyceae are C_3 -type organisms in which PEPC has a cardinal anaplerotic function by replenishing tricarboxylic acid cycle carbon skeletons utilized during nitrogen assimilation [10,11]. Physiological data demonstrate that in nitrogen-limited cells, there is a rapid (within a few seconds) activation of PEPC when NH_4^+ is resupplied [11]. We have recently reported detailed physical and kinetic characterization of purified PEPC from *Selenastrum minutum* (Chlorophyceae) [12]. This study provided evidence that four isoforms of PEPC, falling into two structurally and kinetically distinct classes, are present in this organism. In addition to a 'classical' homotetrameric form, PEPC was also associated with high-molecular-mass complexes. All isoforms shared a similar 102 kDa catalytic subunit, but the association of PEPC with other polypeptides in the high-molecular-mass complexes was correlated with differences in the physical and kinetic properties of the enzyme [12]. In *C. reinhardtii*, an earlier study also reported the presence of two PEPC isoforms with different sedimentation coefficients when analysed by analytical ultracentrifugation [13]. Thus, these

Abbreviations used: CAM, crassulacean acid metabolism; DTT, dithiothreitol; PEP, phosphoenolpyruvate; PEPC, phosphoenolpyruvate carboxylase; PEG, poly(ethylene glycol).

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reports provide an indication that an interesting complexity in PEPC biochemistry might exist in lower plants.

The availability of various PEPC sequences has facilitated the construction of a PEPC phylogenetic tree [1,2,14]. By using parsimony or distance analyses, it was shown that PEPCs from higher plants are derived from a single common ancestral gene. The C_3 and CAM PEPCs group together, whereas the origin of the C_4 genes is not yet clear: in some species they are closely related to the C_3 and CAM forms [1,14,15] but not in others [1,2,14]. The relations between prokaryotic and higher plant PEPCs follow established phylogenetic trees. Prokaryotic sequences are only distantly related to those of higher plants, and cyanobacterial and bacterial sequences do not group together [1,2,14]. Owing to the lack of molecular data, nothing is known about the phylogenetic and molecular relationships between green algal and higher plant or prokaryotic PEPCs. The report of the existence of PEPC isoforms in *S. minutum* and that novel mechanisms might be implicated in their regulation [12] suggests that green algal PEPCs might not be closely related to the higher plant enzyme.

The present work was undertaken for several reasons. The primary goal was to evaluate the relationship between green algal, higher plant and prokaryotic PEPCs. Indeed, this information is needed for a better, more complete understanding of the evolution of this important enzyme [1]. As a first step towards the manipulation of PEPC in green algae, there is also a need to study this enzyme in a genetically well characterized organism, amenable to genetic transformation. We also wanted to discover whether the existence of different PEPC isoforms in *S. minutum* (taxon Chlorellales) [12] could be considered broadly applicable to green algae and to a widely used model system. We have found two PEPC isoforms in *C. reinhardtii* (taxon Volvocales) that apparently share a common catalytic subunit. Microsequencing of the N-terminus, peptide mapping and immunological data suggest that the *C. reinhardtii* PEPC catalytic subunit is not related to the prokaryotic enzyme and is only distantly related to higher plant C_4 and C_3 PEPCs. This suggests that green algae contain a novel type of PEPC.

EXPERIMENTAL

Biological material

C. reinhardtii CW-15 cc1883 was grown in chemostat cultures at 20 °C on NO_3^- -sufficient medium as described previously [16] except that the concentration of K_2HPO_4 was 1 mM. Cells were harvested from the chemostat outflow every 3 days by centrifugation at 1000 *g* followed by gentle resuspension in growth medium (1 ml/litre of culture). Harvested cells were frozen in liquid N_2 and stored at -80 °C. *Synechococcus leopoliensis* (UTEX 625) cells were grown at 25 °C in 15 litre batch cultures at a light intensity of approximately $50 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$. Cells were grown in an aerated modified Allen's medium [17] from which Na_2SiO_3 was omitted and to which 50 M Hepes, pH 8.5, and 10 mM NaHCO_3 were added. Cells were harvested when the D_{665} of the culture reached 1.5. The cells were initially concentrated over eight 0.45 μm ultrafiltration plates with a Minitan device (Millipore). The cells were then centrifuged for 10 min at 8000 *g* and the pellets were resuspended in 2 vol. of buffer G (see below), frozen in liquid nitrogen and stored at -80 °C. Purified banana and maize PEPCs were prepared as described previously [6]. For the purification of recombinant tobacco PEPC, we used the *Escherichia coli* mutant PCR1, a PEPC-deficient strain [18], harbouring the plasmid pTE6 (containing a tobacco PEPC cDNA under the control of the *LacZ* promoter) [19]. Cells were grown at 37 °C in batches of 1 litre in Luria-Bertani broth

medium to a D_{600} of 0.3. Isopropyl β -D-thiogalactoside was then added to the culture to a final concentration of 40 μM . The culture was then transferred to 25 °C for a further 6 h. The cells were then harvested by centrifugation for 10 min at 10000 *g* and the pellets were frozen in liquid nitrogen.

Chemicals

All chemicals were of A.R. grade from Sigma Chemical Co. (St. Louis, MO, U.S.A.) or BDH Chemicals (Toronto, ON, Canada) except isopropyl β -D-thiogalactoside (Chemical Diagnostics, Charlottetown, PEI, Canada), dithiothreitol (DTT) and malate dehydrogenase (Boehringer Mannheim, Montréal, PQ, Canada). Cell culture reagents were from Gibco (Burlington, ON, Canada). Butyl Sepharose-4 Fast Flow, Superose 6 HR and Mono Q HR columns were from Pharmacia (Baie d'Urfé, PQ, Canada). Bio-Gel HT hydroxyapatite was from Bio-Rad (Mississauga, ON, Canada).

Enzyme and protein assays

Unless otherwise stated, PEPC was assayed by using a coupled assay with malate dehydrogenase by the method of [12], except that the concentration of PEP was varied as follows: for *C. reinhardtii* PEPCs, 5 mM; for *S. leopoliensis* PEPC, 2 mM; for recombinant tobacco PEPC, 0.5 mM. One unit of enzyme activity is defined as the amount of PEPC catalysing the production of 1 μmol of oxaloacetate/min at 25 °C. Determination of protein concentration was by the dye-binding method of Bradford [20], using the Bio-Rad protein assay reagent (Bio-Rad Laboratories) and bovine γ -globulin as a standard.

Buffers used in PEPC purification

Buffer A contained 50 mM Hepes/KOH, pH 7.5, 40 % (v/v) glycerol, 2 mM EDTA, 2 mM EGTA, 20 mM MgCl_2 , 5 mM malate, 0.2 % (v/v) Triton X-100, 50 mM NaF, 4 mM PMSF, 4 mM DTT and 10 $\mu\text{g/ml}$ chymostatin. Buffer B contained 10 mM Hepes/KOH, pH 7.5, 20 % (v/v) glycerol, 1 mM EDTA, 5 mM MgCl_2 , 5 mM malate and 2 mM DTT. Buffer C contained 10 mM Hepes/KOH, pH 7.5, 20 %-satd. $(\text{NH}_4)_2\text{SO}_4$, 10 % (v/v) glycerol, 1 mM EDTA, 5 mM MgCl_2 , 5 mM malate and 1 mM DTT. Buffer D contained 10 mM Hepes/KOH, pH 7.5, 30 % (v/v) ethylene glycol, 1 mM EDTA, 5 mM MgCl_2 , 5 mM malate and 1 mM DTT. Buffer E contained 5 mM potassium phosphate, pH 6.8, 20 % (v/v) glycerol, 5 mM malate and 1 mM DTT. Buffer F contained 25 mM Hepes/KOH, pH 7.5, 20 % (v/v) glycerol, 50 mM KCl, 1 mM EDTA, 5 mM MgCl_2 , 5 mM malate, 0.02 % NaN_3 and 2 mM DTT. Buffer G contained 50 mM imidazole/HCl, pH 7.2, 1 mM EDTA, 1 mM EGTA, 20 mM NaF, 10 mM thiourea, 15 % (v/v) glycerol, 0.1 % (v/v) Triton X-100 and 25 mM KCl. Buffer H contained 50 mM potassium phosphate, pH 7.1, 5 mM MgCl_2 , 1 mM EDTA, 15 %-satd. $(\text{NH}_4)_2\text{SO}_4$ and 1 mM DTT. Buffer I contained 50 mM potassium phosphate, pH 7.1, 5 mM MgCl_2 , 1 mM EDTA, 10 % (v/v) ethylene glycol and 1 mM DTT.

Purification of *C. reinhardtii* PEPC isoforms

All procedures were conducted at 4 °C and all chromatographic steps were performed with an FPLC system (Pharmacia). Cells (100 g) were thawed in 250 ml of buffer A. The lysed cells were vigorously stirred for 20 min and then centrifuged at 24000 *g* for 20 min. The clarified homogenate was brought to 20 % (w/v) poly(ethylene glycol) (PEG) with a 50 % (w/v) PEG-8000

Figure 1 Final chromatographic steps in the isolation of two PEPC isoforms from *C. reinhardtii*

(A) Tandem Superose 6 FPLC of the hydroxyapatite-purified fraction. The pooled fractions obtained after the hydroxyapatite step were concentrated to less than 150 μ l and subjected to gel filtration on two Superose 6 HR 10/30 columns arranged in tandem. PEPC activity ($\circ$) was resolved as two peaks, PEPC1 and PEPC2. V_0 designates the void volume. (B,C) Mono Q FPLC profiles of *C. reinhardtii* PEPC1 (B) and PEPC2 (C). Pooled fractions corresponding to the respective Superose 6 PEPC peaks shown in (A) were applied separately to a Mono Q HR 5/5 column. PEPC activity ($\circ$) was eluted by a 0–500 mM KCl gradient. Abbreviation: U, unit.

solution containing 25 mM Hepes/KOH, pH 7.5, and 2 mM EDTA. The solution was stirred for 30 min and the precipitate was collected by centrifugation as above. The pellet was resuspended in 50 ml of buffer B containing 25 mM NaF, then brought to 20% satn. with $(\text{NH}_4)_2\text{SO}_4$ with a saturated solution of $(\text{NH}_4)_2\text{SO}_4$ containing 25 mM Hepes/KOH, pH 7.5, and 2 mM EDTA. This solution was stirred for 30 min then clarified by centrifugation at 37000 g for 20 min. The resulting supernatant was designated the 20% $(\text{NH}_4)_2\text{SO}_4$ cut. This was adsorbed batchwise for 30 min on 30 ml of Butyl Sepharose 4 Fast Flow previously equilibrated in a solution composed of 95% (v/v) buffer C and 5% (v/v) buffer D. The slurry was poured into a column (1.6 cm $\times$ 15 cm) and washed at 2 ml/min with 90 ml of fresh equilibration buffer. PEPC activity was eluted by a linear gradient of 95–0% buffer C simultaneously with

5–100% buffer D. Fractions of 6 ml were collected. PEPC activity was eluted as a single peak at approx. 80% buffer D. The pooled fractions (56 ml) were brought to 1 mM EGTA, 2 mM PMSF and 10 μ g/ml chymostatin and subjected to diafiltration on an Amicon YM 30 membrane against buffer B containing the protease inhibitors mentioned above. The process was stopped when the conductivity of the solution fell below 400 μ S. This solution was brought to 30 ml and loaded at 1 ml/min on a Fractogel EMD DEAE-650 (S) column (1.1 cm $\times$ 6 cm) previously equilibrated in buffer B. The column was washed with 18 ml of buffer B. PEPC activity was then eluted with a 60 ml linear 0–500 mM KCl gradient in buffer B. Fractions of 1 ml were collected. PEPC was eluted as a single peak (typically over 10 fractions) at approx. 300 mM KCl. Pooled peak fractions were dialysed twice (14 h then 2 h) against 1 litre of buffer E. The protein sample (adjusted to 12 ml with buffer E) was loaded at 0.25 ml/min on a hydroxyapatite column (1 cm $\times$ 5 cm) previously equilibrated with buffer E. The column was washed with 8 ml of buffer E and PEPC was eluted by a 5–125 mM potassium phosphate gradient in buffer E. Fractions of 1 ml were collected. PEPC activity was eluted as a broad peak (10 ml) centred on 75 mM potassium phosphate. The pooled fractions were concentrated to 200 μ l with Amicon YM30 centricon concentrators and applied at 0.2 ml/min to two Superose 6 HR 10/30 arranged in tandem and equilibrated in buffer F. Fractions of 0.4 ml were collected. PEPC activity was eluted as two discrete peaks designated PEPC1 and PEPC2 in inverse order of elution (Figure 1A). Estimation of native molecular masses was done with Superose 6 FPLC as previously described [12]. Each of the PEPC peaks obtained after Superose 6 FPLC was loaded at 0.25 ml/min on a Mono Q HR 5/5 column pre-equilibrated with buffer B. The columns were washed with 5 ml of buffer B and PEPC activity was eluted with a 15 ml linear gradient of 0–500 mM KCl in buffer B. Fractions of 0.5 ml were collected. Each PEPC isoform was eluted as a single symmetrical peak coinciding with a protein peak (Figures 1B and 1C). Pooled fractions were desalted against buffer B lacking malate, concentrated with Amicon YM30 centricon concentrators, brought to 50% (v/v) glycerol and stored at -20°C .

Purification of *S. minutum* PEPC1 and PEPC2

S. minutum PEPC1 and PEPC2 were purified from 50 g of cells by the method of [12].

Partial purification of *S. leopoliensis* PEPC

S. leopoliensis PEPC was partly purified from 50 g of cells. The cells, stored in buffer G, were thawed, brought to 2 mM PMSF and 1 mM DTT and then disrupted by passage through a French press at 18000 lb/in² (124 MPa). The lysate was clarified by a centrifugation for 15 min at 16000 g. The resulting supernatant was brought to 5% (w/v) PEG, stirred for 30 min and the solution was centrifuged as above. The resulting supernatant was brought to 15% (w/v) PEG. The solution was stirred and centrifuged as above. The resulting pellet was resuspended in 85 ml of buffer H, stirred for 10 min and clarified by centrifugation at 35000 g for 15 min. The supernatant was loaded at 2 ml/min on a 19 ml Butyl Sepharose 4 Fast Flow column (2.2 cm $\times$ 5 cm) previously equilibrated in buffer H. The column was washed with buffer H until the A_{280} returned to baseline level. PEPC was eluted with a 200 ml linear gradient (0–100% buffer I simultaneously with 100–0% buffer H). Fractions of 6.5 ml were collected. PEPC activity was eluted at 0% buffer H. Pooled PEPC fractions (32.5 ml) were brought to 1 mM EGTA,

2 mM PMSF and 10 $\mu\text{g/ml}$ chymostatin, then desalted by diafiltration on an Amicon YM 30 membrane against buffer B containing the above additions as described for the purification of *C. reinhardtii* PEPC. *S. leopoliensis* PEPC was further purified by chromatography on Fractogel EMD DEAE-650 (S) as described above except that elution was achieved with a 0–600 mM KCl gradient. The pooled PEPC fractions were concentrated to less than 150 μl and subjected to Superose 6 FPLC, as described above, with buffer F. The PEPC activity peak was eluted with an approximate molecular mass of 400 kDa. Pooled fractions (2.8 ml) were loaded at 0.25 ml/min on a Mono Q HR 5/5 column and eluted with a 15 ml linear gradient of 0–400 mM KCl. The final preparation had a specific activity of 2.1 units/mg of protein and was more than 50% pure, as judged from SDS/PAGE analysis followed by staining with Coomassie Blue.

Purification of recombinant tobacco PEPC

Cells (10 g) of *E. coli* PCR1 mutant overexpressing recombinant tobacco PEPC were thawed in 100 mM Hepes/KOH (pH 7.5)/10 mM MgCl_2 /5 mM DTT and lysed by a passage through a French press cell at 18000 lb/in² (124 MPa). The extract was then subjected to $(\text{NH}_4)_2\text{SO}_4$ fractionation, desalting on Sephadex G-25 and chromatography on hydroxyapatite as described previously [19] except that 1 mM DTT was used as a reducing agent in all buffers. The pooled hydroxyapatite fractions were then dialysed twice (14 h then 2 h) against 11 of buffer B and subjected to ion exchange chromatography on Fractogel EMD DEAE-650 (S) and Mono Q HR 5/5 as described above for *C. reinhardtii* PEPC. The final preparation had a specific activity of 25.7 units/mg of protein and was more than 95% pure on the basis of SDS/PAGE analysis followed by staining with Coomassie Blue.

Native PAGE, SDS/PAGE and immunoblot analyses

All PAGE analysis and activity detection procedures were performed as described previously [12]. Crude sera raised against banana PEPC and *S. minutum* PEPC1 were obtained as described previously [12,21]. For immunoblot analysis, proteins were resolved by SDS/PAGE and transferred to PVDF as described previously [12]. In the experiment described in Figure 7, purified PEPCs from various origins, with the exception of *E. coli*, were used. In this latter case, crude extracts of the wild-type K12 strain and the PEPC-deficient mutant PCR1 were analysed. Anti-(banana fruit PEPC) IgG was affinity purified with 20 μg of recombinant tobacco PEPC and anti-(*S. minutum* PEPC1) IgG (serum 1 in a previous study, [12]) was affinity-purified with 25 μg of *S. minutum* PEPC1 as described previously [22].

Kinetic analysis

For the kinetic analysis, PEPC fractions of the highest purity were used. Kinetics were done on a Dynatech microplate reader [23]. Assays were initiated by the addition of PEP and were linear with respect to time and protein concentration. Kinetic parameters were determined with a non-linear least-squares regression computer program [24]. All the results are the means of at least three independent determinations on two distinct enzyme preparations and were reproducible to within $\pm 15\%$ S.E.M.

Protein extraction under denaturing conditions

Extraction of *C. reinhardtii* under denaturing conditions was done with a modification of a published method [25]. An aliquot (approx. 50 mg) of frozen *C. reinhardtii* cells were homogenized in 900 μl of a mixture of 50% (v/v) phenol and 50% (v/v) buffer

A. The extract was vortex-mixed vigorously for 1 min then centrifuged for 5 min at 16000 *g*. The lower phase, containing the phenol-denatured proteins, was extracted three times with 900 μl of ether. The final aqueous phase was dried and resuspended in SDS sample buffer before immunoblot analysis.

Peptide mapping by CNBr fragmentation

The PEPC catalytic subunits from the various purified PEPCs were excised from a preparative SDS/PAGE gel and fragmented *in situ* with CNBr [26]. SDS/PAGE analysis of the degradation products was done as previously described [26].

N-terminus microsequencing and amino acid composition of *C. reinhardtii* PEPC catalytic subunit

For N-terminus sequencing and the determination of the amino acid composition of the PEPC catalytic subunit, 10 μg of PEPC polypeptide from purified PEPC2 was subjected to SDS/PAGE on a 7.5% (w/v) polyacrylamide gel and transferred to PVDF (Bio-Rad). The protein band was excised from the membrane after a 10 s staining with freshly prepared Ponceau S Red [0.1% (w/v) in 2% (v/v) acetic acid]. The membrane strip was washed seven times (5 min each) with 1.8 ml of MilliQ water and dried overnight. Amino acid composition was determined on a 1 μg aliquot with an ABI 420A automatic amino acid analyser at Harvard Microchem facility. N-terminal sequencing was done with Edman degradation on the remaining 9 μg . Similarity searches were conducted with the BLAST program (version 2.0.2) available on the National Center for Biotechnology Information World Wide Web site [27] and the GenBank database.

RESULTS

Purification of two PEPC isoforms from *C. reinhardtii*

The purification of PEPC from *C. reinhardtii* is summarized in Table 1. The PEPC1 and PEPC2 isoforms were purified approx. 2500-fold and 2000-fold respectively. Resolution of the two isoforms was achieved by using two Superose 6 columns arranged in tandem (Figure 1A). Calibration of the gel-filtration column with molecular mass standards yielded respective native molecular masses of 400 ± 17 and 650 ± 32 kDa ($n = 3$) for PEPC1 and PEPC2. Further purification of Superose 6-purified PEPCs on Mono Q FPLC led to chromatographically homogenous preparations of PEPC1 (Figure 1B) and PEPC2 (Figure 1C). On the Mono Q column, PEPC2 was eluted slightly after PEPC1, indicating that it carries more negative charges than PEPC1 at pH 7.5.

Electrophoretic and immunoblot analysis of PEPC1 and PEPC2

Each purified PEPC isoform was analysed by native PAGE followed by transfer to PVDF (Figure 2). The native gel was first stained for PEPC activity (Figure 2A). In both PEPC1 and PEPC2, a single but distinct band of activity was detected. The migration position of these activity bands coincided exactly with the position of the only protein bands detected after staining of the native gels with Coomassie Blue (Figure 2B). The results of native PAGE analysis were also consistent with the determination of molecular masses by gel filtration, with PEPC1 being smaller and migrating further than PEPC2. These results further document the presence of distinct PEPC isoforms in *C. reinhardtii* and demonstrate the electrophoretic homogeneity of each of the two isoforms. The native gel was blotted on PVDF and probed with antibodies raised against the *S. minutum* PEPC1 catalytic

Table 1 Purification of PEPC isoforms from *C. reinhardtii*

Step	Protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Clarified homogenate	14714	139	0.009	1	100
Butyl Sepharose 4 Fast Flow	159	37.6	0.236	26.2	27
Fractogel EMD DEAE-650 (S)	24	25.4	1.06	117.6	18.3
Bio-Gel HT hydroxyapatite	9.3	19.8	2.13	236.6	14.2
Tandem Superose 6 PEPC1	1.62	1.85	1.14	126.6	1.3
PEPC2	0.70	7.60	10.86	1207.1	5.5
Mono Q PEPC1	0.053	1.20	22.25	2472	0.8
PEPC2	0.261	4.73	18.11	2012	3.4

Figure 2 Native PAGE and immunoblot analysis of purified *C. reinhardtii* PEPC isoforms

Purified PEPC (2 μ g) (lanes 1, PEPC1; lanes 2, PEPC2) were separated by native PAGE [7% (w/v) separating gel] as described in the Experimental section. (A) The gel was stained for PEPC activity. (B) The gel was stained with Coomassie Blue R-250. (C) The gel was transferred to PVDF and subjected to immunodetection with a serum raised against the *S. minutum* PEPC catalytic subunit. The positions of the anode (+), the cathode (–) and the direction of migration (arrow) are indicated at the left.

subunit (Figure 2C). The results show that the two isoforms are immunologically related to each other and to *S. minutum* PEPC. To examine further the relationship between PEPC1 and PEPC2, the purified isoforms were analysed by SDS/PAGE followed by immunoblotting (Figure 3). PEPC1 was resolved as a single polypeptide with a molecular mass of 100 kDa, whereas PEPC2 contained a major polypeptide with a molecular mass of 100 kDa as well as several polypeptides in the range 50–70 kDa (Figure 3A). Immunoblot analysis was performed with affinity-purified anti-(*S. minutum* PEPC1) IgG. The 100 kDa polypeptides present in PEPC1 and PEPC2 both cross-reacted with the IgG, indicating that they are immunologically related and that this polypeptide is probably a PEPC catalytic subunit (Figure 3B). Moreover, the other polypeptides present in PEPC2 were not recognized by the IgG, indicating that they are not degradation products but distinct proteins associated with the catalytic subunit present in PEPC2. In a control experiment, *C. reinhardtii* cells were extracted under denaturing conditions in the presence of phenol, thereby eliminating the potential action of proteases during extraction and purification. Immunoblot analysis of this extract revealed the presence of a 100 kDa polypeptide identical in size with purified PEPC (results not shown). This result indicates that no proteolytic degradation of PEPC occurred during purification.

Figure 3 SDS/PAGE and immunoblot analysis of purified *C. reinhardtii* PEPC isoforms

Purified PEPC1 (lanes 1) and PEPC2 (lanes 2) were separated by SDS/PAGE [7.5% (w/v) separating gel]. (A) PEPC1 (2 μ g) and PEPC2 (4 μ g) were analysed and the gel was stained for proteins with Coomassie Blue R-250. (B) Immunoblot analysis was performed with affinity-purified anti-(*S. minutum* PEPC) IgG as described in the Experimental section (250 ng of protein per lane). The positions of various molecular mass standards are indicated at the right.

Kinetic analysis of PEPC1 and PEPC2

Effect of pH

The effect of pH on PEPC1 and PEPC2 $V_{\max}$ was examined in the pH range 7–9 (Figure 4A). The purified isoforms exhibited different pH optima, with that of PEPC1 being more basic (pH 8.8) than that of PEPC2 (pH 8.1). In the physiologically relevant pH range (7–8) the $V_{\max}$ of PEPC2 increased more rapidly than that of PEPC1. In this same pH range, there was a decrease in K_m (PEP) for both isoforms (Figure 4B). For PEPC1, K_m (PEP) decreased 4-fold between pH 7.1 and pH 8.2, whereas that of PEPC2 decreased more than 7-fold. The catalytic efficiency [$V_{\max}/K_m$ (PEP)] of both isoforms was also examined in the physiological pH range (Figure 4C). At pH values close to neutrality, the catalytic efficiencies of PEPC1 and PEPC2 were almost identical. However, with increasing pH, the catalytic efficiency of PEPC2 rose faster than that of PEPC1 to reach twice that of PEPC1 at pH 8.2. The observed differences in $V_{\max}/K_m$ (PEP) between the two isoforms were the result of

Figure 4 Effect of assay pH on V_{max} , K_m and on catalytic efficiency of *C. reinhardtii* PEPC isoforms

(A) Activity of PEPC1 and PEPC2 as a function of pH. Activity was assayed as described in the Experimental section except that the assay pH was varied. Assays were buffered with 50 mM Bistris/propane/HCl. (B) Effect of pH on the K_m (PEP) of PEPC1 and PEPC2. Assay pH was varied as above. (C) Effect of pH on the catalytic efficiency of PEPC1 and PEPC2. Assay pH was varied as above. Units were units/mg of protein for V_{max} , and mM for K_m .

differences in binding of PEP rather than V_{max} . These results also demonstrate that PEPC1 and PEPC2 differ in their sensitivity to pH, with PEPC2 being more efficient than PEPC1 at basic pH values.

Regulation by metabolites

The apparent affinity for PEP of the two isoforms was also examined at physiological (7.4) and near-optimal (8.4) pH values in the absence or presence of physiologically relevant concentrations of known effectors for green algal PEPCs (Table 2). Under all conditions tested, PEPC2 had a higher affinity for PEP than PEPC1. Glutamine and dihydroxyacetone phosphate activated the enzyme, whereas glutamate, aspartate, 2-oxoglutarate and malate were inhibitors. In the presence of glutamate and malate, sigmoidal kinetics were observed with PEPC1. The effect of these various metabolites was largely pH-dependent. For the

Table 2 Effect of pH and various metabolites on the apparent K_m (PEP) or $S_{0.5}$ (PEP) of *C. reinhardtii* PEPC isoforms

Assays were conducted at pH 7.4 or 8.4 in the absence and/or presence of the listed effectors. When significantly different from 1, Hill coefficients are given in parentheses.

		K_m or $S_{0.5}$ (mM)			
		PEPC1		PEPC2	
Condition	pH ...	7.4	8.4	7.4	8.4
Control		0.64	0.24	0.37	0.10
+ 2 mM glutamine		0.28	0.11	0.15	0.09
+ 2 mM dihydroxyacetone phosphate		0.31	0.21	0.23	0.11
+ 2 mM glutamate		1.21	0.38 (1.4)	0.58	0.27
+ 2 mM 2-oxoglutarate		0.76	0.53	0.68	0.27
+ 2 mM aspartate		0.88	0.38	0.43	0.36
+ 2 mM malate		0.92 (1.3)	0.80 (1.5)	0.62	0.23

Figure 5 Electrophoretic patterns of CNBr-cleavage fragments generated by PEPC polypeptides from various origins

CNBr-cleavage fragments were prepared from gel slices containing 4 μ g of the various PEPC polypeptides and analysed by SDS/PAGE. The gel was silver-stained by the method of Wray et al. [34]. The position of molecular mass standards are indicated at the right. Lane 1, *C. reinhardtii* PEPC1; lane 2, *C. reinhardtii* PEPC2; lane 3, *S. minutum* PEPC1; lane 4, *S. minutum* PEPC2; lane 5, *S. leopoliensis* PEPC; lane 6, recombinant tobacco PEPC; lane 7, maize leaf PEPC; lane 8, banana fruit 100 kDa PEPC subunit.

activator dihydroxyacetone phosphate, the effect was more pronounced at pH 7.4 than at pH 8.4 for both isoforms. Conversely, 2-oxoglutarate, aspartate and malate were more potent inhibitors at pH 8.4 for PEPC1 and PEPC2. The pH dependences of the effect of glutamine and glutamate were different for PEPC1 and PEPC2. The sensitivity of PEPC1 to glutamine was not affected by pH, whereas PEPC2 was more sensitive to glutamine at pH 7.4. For PEPC1, glutamate was more potent at neutral pH, whereas for PEPC2 the effect was stronger at pH 8.4.

Structural relationships between green algal, prokaryotic and higher plant PEPCs

Peptide mapping

Peptide mapping is a powerful method for evaluating structural relationships between polypeptides. We therefore analysed the CNBr cleavage fragments generated by the PEPC subunits from various origins (Figure 5). The cleavage patterns of *C. reinhardtii*

Table 3 Amino acid compositions of *C. reinhardtii*, *Zea mays*, *Nicotiana tabacum* and *E. coli* PEPC catalytic subunits

Values indicate the molar fraction of each amino acid. The number of amino acid residues per subunit is given in parentheses. Results for *C. reinhardtii* were obtained from amino acid analysis of the purified PEPC2 subunit. The other results are derived from the translation of cDNA sequences available in the literature: *Z. mays* [35]; *N. tabacum* [39]; *E. coli* [42]. Abbreviation: n.d., not determined.

Amino acid	Amino acid composition			
	<i>C. reinhardtii</i>	<i>Z. mays</i>	<i>N. tabacum</i>	<i>E. coli</i>
Asx	8.4 (77)	8.5 (83)	9.2 (89)	8.7 (77)
Glx	8.8 (82)	12.6 (122)	12.6 (121)	13.2 (117)
Ser	6.8 (63)	5.5 (53)	5.8 (56)	5.8 (51)
Gly	7.8 (72)	6.8 (66)	5.6 (54)	6.3 (56)
His	2.2 (20)	2.1 (20)	2.4 (23)	1.5 (13)
Arg	8.1 (75)	6.9 (67)	7.7 (74)	6.6 (58)
Thr	5.5 (50)	5.2 (51)	5.2 (50)	4.6 (41)
Ala	9.2 (85)	7.4 (72)	7.4 (71)	9.1 (80)
Pro	5.2 (48)	5.7 (55)	4.8 (46)	5.1 (45)
Tyr	2.7 (25)	2.8 (27)	3.4 (33)	2.6 (23)
Val	6.9 (64)	6.5 (63)	5.2 (50)	5.7 (50)
Met	4.0 (37)	2.2 (21)	2.2 (21)	2.7 (24)
Ile	3.5 (33)	4.9 (48)	5.4 (52)	4.9 (43)
Leu	13.6 (126)	11.2 (109)	12.1 (117)	13.4 (118)
Phe	3.5 (32)	3.9 (38)	3.6 (35)	2.0 (18)
Lys	3.8 (36)	5.4 (52)	5.1 (49)	4.8 (42)
Trp	n.d.	1.3 (13)	1.3 (13)	2.2 (19)
Cys	n.d.	1.0 (10)	1.0 (10)	0.9 (8)

HIGHER PLANTS:	1	↓	50
<i>C. Z. mays</i>	MAST-KAPGPG SEKHS IDAQLRQLVPGKVSDDKLIYDALIVDRFLNQLDHLGPSL		
<i>S. vulgare</i>	-----R:Q:-----L:A:-----V:-----D:-----H:		
<i>F. trinerva</i>	--NRNV-----LA:-----L:-----V:-----L:K:D:-----ED:		
CAM <i>M. crystallinum</i>	--V:L-----DRLT:-----L:A:K:-----L:-----D:N:--EDI		
<i>C. N. tabacum</i>	--RSL-----LA:-----A:-----V:-----L:-----D:-----ED:		
<i>S. tuberosum</i>	--T:RNL-----LA:-----A:-----V:-----L:-----D:-----ED:		
<i>M. crystallinum</i>	--RNI-----MA:-----L:-----A:K:-----V:-----L:-----D:-----EDI		
PROKARYOTES:			
<i>E. coli</i>	MNSEQYSAIRSNVSMGLKVLGETIKDALGEHILERVETIRKL-SKSSRA-GNDANRQE		
<i>C. glutamicum</i>	:TDF-----DDIRF:QI::V:AEQE:QEVY:L:QA:-:T:FDI-K:-:EM:-		
<i>A. variabilis</i>	-----MVD:L:QL:-:D:C:PE:-:-:Q:-TKDQ		
GREEN ALGAE:			
<i>C. reinhardtii</i>	ODLRGTGFANFLSDDL		

Figure 6 Comparison of N-terminal amino acid sequences of *C. reinhardtii* PEPC2 with those of PEPC from higher plants and prokaryotes

The *C. reinhardtii* N-terminal sequence was determined by Edman sequencing of the PEPC2 100 kDa subunit. The other sequences are derived from the translation of the corresponding nucleotide sequences. The higher plant sequences are aligned on the maize PEPC sequence. The prokaryotic sequences are aligned on the *E. coli* PEPC sequence. The numbering is based on the maize PEPC sequence. The arrow indicates the position of the regulatory serine phosphorylation site; the bold characters indicate the consensus target site for higher plant PEPC protein kinase. Gaps (—) have been introduced to maximize alignment. A colon indicates an identical amino acid residue. Literature references for the sequences are as follows: *Z. mays* [35], *S. vulgare* [36], *Flaveria trinerva* [37], *Mesembryanthemum crystallinum* [38], *N. tabacum* [39], *S. tuberosum* [40], *M. crystallinum* (*C*₃) [41], *E. coli* [42], *Corynebacterium glutamicum* [43], *Anabaena variabilis* [44].

PEPC1 and PEPC2 were identical, as they were for *S. minutum* PEPC isoforms (Figure 5) [12]. These results demonstrate that in *C. reinhardtii* the 100 kDa catalytic subunit in PEPC1 and PEPC2 is the same polypeptide. The CNBr cleavage patterns obtained with *C. reinhardtii* were different from those observed with *S. minutum*, the higher plant and cyanobacterial enzymes.

Figure 7 Immunoblot analysis of PEPCs from green algae, higher plants and prokaryotes

(A) Detection of PEPC polypeptides with anti-(*S. minutum* PEPC1) IgG. (B) Detection of PEPC polypeptides with anti-(banana fruit PEPC) IgG. Samples were subjected to immunoblot analysis as described in the Experimental section. Each lane was loaded with 250 ng of purified PEPC or, in lanes 9 and 10, 20 µg of crude *E. coli* extracts. Lane 1, *C. reinhardtii* PEPC1; lane 2, *C. reinhardtii* PEPC2; lane 3, *S. minutum* PEPC1; lane 4, *S. minutum* PEPC2; lane 5, recombinant tobacco PEPC; lane 6, maize leaf PEPC; lane 7, banana fruit PEPC; lane 8, *S. leopoliensis* PEPC; lane 9, *E. coli* K12 extract; lane 10, *E. coli* PCR1 mutant extract. The positions of various molecular mass standards are indicated at the right.

The higher plant PEPCs, however, shared some common CNBr fragments. CNBr fragmentation patterns depend on the position and number of Met residues in the protein [26]. These results therefore demonstrate that the primary structure of the green algal PEPC is different from the cyanobacterial and higher plant *C*₄ and *C*₃ forms of the enzyme.

Amino acid composition

The 100 kDa PEPC2 polypeptide was subjected to amino acid analysis. The results are compared with the amino acid composition of PEPC from maize (*C*₄ form), tobacco (*C*₃ form) and *E. coli* (Table 3). Overall, *C. reinhardtii* PEPC had an amino acid composition comparable to those of PEPCs from other sources but was somewhat closer to those of higher plant PEPCs, particularly with respect to His and Phe residue content. However, the green algal PEPC had some particular features that distinguished it from other PEPCs: a lower content in Glx, Ile and Lys residues and a much higher content of Met residues.

N-terminal sequence comparison

The N-terminal 15-residue sequence of the PEPC2 100 kDa catalytic subunit was determined (Figure 6). No protein having

a statistically significant sequence similarity to *C. reinhardtii* PEPC was found in the computer search of the proteins in GenBank. Also, as shown in Figure 6, the *C. reinhardtii* N-terminal sequence did not align with the corresponding region in higher plant or prokaryotic PEPCs. This feature is important because the N-terminal region is highly conserved in higher plant PEPCs and contains the regulatory serine phosphorylation site of the enzyme.

Immunological characterization

We performed an immunological comparison of PEPCs from green algal, higher plant and prokaryotic origins. We purified PEPC from green algae (*C. reinhardtii* and *S. minutum*), from higher plants [maize leaves (C_4 form), banana fruit (C_3 form) and tobacco (recombinant C_3 form)] as well as the cyanobacterium *S. leopoliensis*. We also analysed crude extracts of *E. coli* K12 and the *E. coli* mutant PCR1, which lacks PEPC [18]. The immunoblot analysis was done with anti-(*S. minutum* PEPC1) and anti-(banana fruit PEPC) IgGs (Figures 7A and 7B respectively). The anti-(*S. minutum*) IgG recognized only green algal PEPCs. The anti-(banana fruit PEPC) IgG, in contrast, strongly cross-reacted with PEPCs from higher plants but only weakly with the green algal PEPCs. Both IgGs failed to recognize *E. coli* and *S. leopoliensis* PEPCs. These results indicate that green algal PEPCs are immunologically distant from higher plant PEPCs and distinct from the prokaryotic enzymes. They do, however, have few common epitopes with the higher plant enzyme, because a weak cross-reactivity was observed between the anti-(banana fruit PEPC) and the green algal polypeptides.

DISCUSSION

Purification and characterization of *C. reinhardtii* PEPC isoforms

This work was undertaken with the goals of characterizing PEPC from a genetically well characterized green alga and to compare it structurally and kinetically with other green algal, higher plant and prokaryotic PEPCs. Two PEPC isoforms were purified over 2000-fold from *C. reinhardtii* (Table 1). These results are consistent with a previous report of the presence of two PEPC isoforms in *C. reinhardtii* [13]. We determined that PEPC1 and PEPC2 had respective specific activities of 22 and 18 units/mg of protein. These specific activities are lower than, but comparable to, those reported for purified *S. minutum* PEPC isoforms, higher plant and prokaryotic PEPCs [12,21]. The other PEPCs so far reported are all tetrameric enzymes except *S. minutum* high-molecular-mass PEPCs. These results therefore suggest that the existence of multiple PEPCs isoforms, some of which are due to the formation of protein complexes, might indeed be a feature common to Chlorophyceae because it is found in two different taxa of this green algal family. Further studies are needed to determine whether or not the existence of high-molecular-mass PEPC isoforms is widespread among other groups of lower plants.

The existence of multiple PEPC isoforms raises the question of their function. It is not yet possible to answer this question. However, there are clear differences in the kinetic properties of PEPC1 and PEPC2 that suggest that they could be engaged in anaplerotic carbon fixation under different physiological conditions. More specifically, PEPC2 seems to be more responsive to variation in assay pH than is PEPC1. PEPC2 also has a greater affinity for PEP (Figure 4 and Table 2). There are also differences in the sensitivity to the various effectors (Table 2). These properties are reminiscent of those of the two classes of PEPC found in the green alga *S. minutum* [12]. The clarification of

the function of PEPC1 and PEPC2 awaits the identification of the polypeptides that seem to be associated with PEPC2 and the generation of transgenic organisms with altered contents in PEPC1 and PEPC2.

Unique kinetic characteristics of green algal PEPCs

The examination of the kinetic properties of *C. reinhardtii* PEPC reveals that green algal PEPCs share several common effectors with higher plant C_3 -PEPCs. For example, dihydroxyacetone phosphate and 2-oxoglutarate have been reported as effectors of soybean nodule PEPC [28]; malate, aspartate and glutamate are potent inhibitors of several PEPCs [1,10,21,29].

Our results also expose some features that distinguish green algal PEPCs from the higher plant and prokaryotic counterparts. Such a distinctive feature is sensitivity to regulation by glutamine. C_3 PEPCs are generally considered as anaplerotic enzymes with a key role in providing carbon skeletons for nitrogen assimilation [2,12,21]. Measurements of changes in the intracellular concentrations of various amino acids have been made in nitrogen-deficient green algal cultures that were resupplied with NH_4^+ [30] and in cultures growing at different NH_4^+ regimes [31]. These studies have shown that the intracellular glutamine-to-glutamate ratio changes markedly in response to NH_4^+ supply. Indeed, it has been shown that, in *S. minutum*, the steady-state NH_4^+ assimilation rate *in vivo* was proportional to the intracellular glutamine-to-glutamate ratio [31]. The green algal PEPCs are the only ones so far known to be allosterically regulated by both glutamine and glutamate (Table 2) [11,12]. This property supports their role in the provision of carbon skeletons for the assimilation of nitrogen. Although some higher plant PEPCs are highly sensitive to inhibition by glutamate [6,29], allosteric regulation by glutamine has been reported only in green algal PEPCs. It has been shown, however, that glutamine induces PEPC gene expression in maize leaf [32]. Therefore intracellular glutamine could act as an early 'nitrogen status' signal in both higher plants [2] and green algae, although through different mechanisms.

Structural relationships of *C. reinhardtii* PEPC with PEPCs from higher plants and prokaryotes

We performed several experiments aimed at evaluating the structural relationship between green algal, higher plant and prokaryotic PEPCs. Several lines of evidence show that green algal and higher plant PEPCs are only distantly related. First, the N-terminal sequence of *C. reinhardtii* 100 kDa PEPC subunit does not have any significant similarities to higher plant or prokaryotic PEPCs (Figure 6). This is a particularly important result because the N-terminal domain contains the regulatory phosphorylation site of higher plant PEPCs. This result could indicate that the *C. reinhardtii* PEPC purified here is not regulated by phosphorylation. However, these results do not rule out the possibility of a phosphorylation site being a little further along the sequence. In this case, there would be significant sequence differences between the conserved N-terminus of PEPC from higher plants and the corresponding region in the *C. reinhardtii* enzyme. Secondly, CNBr fragmentation patterns of purified green algal, higher plant and prokaryotic PEPC subunits were obtained. The results show that the cleavage patterns of *C. reinhardtii* and *S. minutum* PEPCs are different from those of purified *S. leopoliensis*, recombinant tobacco, maize leaf and banana fruit PEPCs (Figure 5). Again, the results of this experiment are consistent with the idea that the green algal enzymes are divergent from both higher plants and prokaryotic enzymes. Thirdly, an immunological comparison of PEPCs from various origins was performed with affinity-purified IgG against

higher plant PEPC and green algal PEPC. On immunoblots, a strong cross-reaction was obtained between the anti-(banana fruit PEPC) IgG and the other higher plant PEPCs (Figure 7). This IgG also gave a weak cross-reaction with the green algal PEPCs. However, anti-(*S. minutum* PEPC) IgG did not cross-react at all with PEPCs from higher plants, bacteria (*E. coli*) or cyanobacteria (*S. leopoliensis*). Thus there seems to be the conservation of only a few common epitopes between higher plants and green algal PEPCs.

Within the subkingdom Chlorobionta (green plants), the green algae constitute a quite diverse group of organisms. On the basis of ultrastructural, morphological and molecular studies, several green algal orders (e.g. Charales and Coleochaetales) are even considered close relatives to land plants [33] and are classified within the same group (Streptophyta). Ultrastructural and molecular data suggest that other green algae (Chlorophyceae, Trebouxiophyceae and Ulvophyceae) represent a lineage distinct from Streptophyta [33]. The Chlorophyceae therefore represent a group of organisms that is phylogenetically only distantly related to higher plants. The finding that *C. reinhardtii* PEPC is structurally different from that of higher plants, but is weakly immunologically related to them, is consistent with the current phylogenetic classification. The fact that *C. reinhardtii* PEPC is immunologically closer to the higher plant enzymes than it is to the prokaryotic ones is also consistent with established classifications.

Taken as a whole, our results lead to the conclusion that green algal PEPC high- and low-molecular-mass isoforms such as those purified from *C. reinhardtii* (Chlorophyceae) constitute a novel class of PEPCs. The green algal PEPCs can be distinguished from higher plant and prokaryotic PEPCs on the basis of structure (formation of isoforms, N-terminal sequence and CNBr fragmentation patterns), kinetic properties (unique allosteric regulation by glutamine, and apparent regulation by protein-protein interactions [12]) and immunological properties. These last results, however, suggest that *C. reinhardtii* PEPC is more closely related to higher plant PEPCs than it is to cyanobacterial or bacterial PEPCs.

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