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Identification and expression analysis of two inorganic C-and N-responsive genes encoding novel and distinct molecular forms of eukaryotic phosphoenolpyruvate carboxylase in the green microalga Chlamydomonas reinhardtii

Summary

Phosphoenolpyruvate carboxylase (PEPC [Ppc]) has been previously purified and characterized in biochemical and immunological terms from two green microalgae, Chlamydomonas reinhardtii and Selenastrum minutum. The findings indicate that these algae possess at least two distinct PEPC enzyme-forms, homotetrameric Class1 and heteromeric Class-2 that differ significantly from each other and their plant and prokaryotic counterparts. Surprisingly, however, green-algal PEPC has been unexplored to date in molecular terms. This study reports the molecular cloning of the two Ppc genes in C. reinhardtii (CrPpc1, CrPpc2), each of which is transcribed in vivo and encodes a fully active, recombinant PEPC that lacks the regulatory, N-terminal serylphosphorylation domain that typifies the vascular-plant enzyme. These distinct catalytic subunit-types differ with respect to their (i) predicted molecular mass (108.9 [CrPpc1] versus 131.2 kDa [CrPpc2]) and critical C-terminal tetrapeptide; and (ii) immunoreactivity with antisera against the p102 and p130 polypeptides of S. minutum PEPC1/PEPC2 and PEPC2, respectively. Only the Ppc1 transcript encodes the p102 catalytic subunits common to both Class-1 and Class-2 enzyme-forms in C. reinhardtii. The steady-state transcript levels of both CrPpc1/2 are coordinately up-/down-regulated by changes in [CO2] or [$_{NH+4}$] during growth, and generally mirror the response of cytoplasmic glutamine synthetase (Gs1) transcript abundance to changes in inorganic [N] at 5% CO2. These collective findings provide key molecular insight into the Ppc genes and corresponding PEPC catalytic subunits in the eukaryotic algae.

Keywords: PEP carboxylase (PEPC [Ppc]), Chlamydomonas reinhardtii, green microalgae, C/N-assimilation, anaplerosis.

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Introduction

Phosphoenolpyruvate carboxylase (PEPC [Ppc]; E.C. 4.1.1.31) is a ubiquitous cytoplasmic enzyme in vascular plants, and is also widely distributed among archaeal, (cyano)bacterial, and unicellular green-algal species (Chollet et al., 1996; Ettema et al., 2004; Izui et al., 2004). It catalyzes the irreversible b-carboxylation of phosphoenolpyruvate (PEP) in the presence of HCO_3 and Me^{2}þ to yield inorganic phosphate and oxaloacetate (OAA), and thus is involved intimately in C4-dicarboxylic acid metabolism in these organisms. While the enzyme is clearly best known for its cardinal roles in C4 photosynthesis and Crassulacean acid metabolism (CAM), green-plant PEPC has also been widely studied in a diverse array of non-photosynthetic contexts, ranging from C4-acid synthesis during leaf stomatal opening to symbiotic N2-fixation in legume root nodules (Vidal and Chollet, 1997). In addition, in most non-photosynthetic organs and C3 leaves, PEPC is a major anaplerotic enzyme, providing OAA and/or malate to replenish citric-acid-cycle intermediates consumed in other primary metabolic pathways, most notably during the assimilation of ammonia into amino acids and amino-acid amides by the Gln synthetase/ Glu synthase (GS/GOGAT) cycle. It is in this latter anaplerotic/non-photosynthetic role that PEPC has been studied at the physiological and biochemical levels in the green microalgae (Giordano et al., 2003; Huppe and Turpin, 1994; Norici et al., 2002; Reinfelder et al., 2004).

During the past 15 years an impressive list of advances in PEPC research has been generated (reviewed in Chollet et al., 1996; Izui et al., 2004). In marked contrast to this wealth of information on vascular-plant and prokaryotic PEPC, until recently there was little or no biochemical or molecular insight into the green-microalgal enzyme (Chen and Jones, 1970; Schuller et al., 1990). However, starting in 1996 a series of detailed biochemical studies of the PEPC enzyme-forms purified from two unicellular green algae, Selenastrum minutum and Chlamydomonas reinhardtii, were published (Rivoal et al., 1996, 1998, 2001, 2002). These collective findings reveal that the immunological and biochemical features of these green-algal PEPCs are quite distinct from those of the vascular-plant and prokaryotic homotetrameric enzymes. For example, in both S. minutum and C. reinhardtii two novel classes of native PEPC isoforms exist in vivo. Whereas the lesser abundant Class-1 PEPCs are homotetramers of 102-kDa catalytic subunits (p102), the dominant Class-2 enzyme-forms are heteromeric, high-Mr complexes of identical p102 subunits and immunologically unrelated polypeptides of 65, 73 and 130 kDa (S. minutum PEPC2; Rivoal et al., 2001) or 50–70 and likely 130 kDa (C. reinhardtii PEPC2; Rivoal et al., 1998; J. Rivoal, personal communication). To date, such unusual, heteromeric Class-2 PEPCs have only been described in the green microalgae and, more recently, in the endosperm of developing castor oilseeds (Blonde and Plaxton, 2003). However, in neither of these cases have the various component subunits of the Class-1 and Class-2 enzymes been identified in rigorous molecular terms except for the typical vascular-plant catalytic subunit (p107) common to the two Ricinus enzyme-forms (Blonde and Plaxton, 2003). In contrast, N-terminal microsequencing of the corresponding p102 catalytic subunit of Chlamydomonas PEPC2 revealed a pentadecameric amino-acid sequence bearing no resemblance to the N-terminal seryl-phosphorylation domain that typifies green-plant PEPC (Rivoal et al., 1998; Vidal and Chollet, 1997). Consistent with these latter findings are (i) our continued failure to identify a plant PEPC-kinase (PpcK) homologue in the publicly available nuclear genome of C. reinhardtii and this alga's vast expressed sequence tag (EST) database when queried with representative vascular-plant PpcK sequences (see Sullivan et al., 2004); and (ii) the availability of only circumstantial, in-vitro data to suggest that the green-algal PEPC

enzyme-forms are possibly phosphorylatable in vivo (Rivoal et al., 2002).

Given this complete lack of molecular insight into the various component subunits of the novel Class-1 and Class2 PEPC enzyme-forms in the green microalgae, we first set out to identify the Ppc gene(s) encoding the catalytic polypeptide(s) of these two enzyme-classes in C. reinhardtii, a model photosynthetic eukaryote (Ball, 2005), and to analyze its (their) expression as influenced by varying levels of inorganic-N and -C during photoautotrophic growth. As a result of these initial efforts we have cloned and characterized two novel and distinct Ppc genes in this unicellular green alga, each of which is transcribed in vivo, similarly responsive to changes in culture-medium levels of NH^+_4 and CO_2, and directs the production of highly active (>20 U mg^{-1} protein), recombinant PEPC in Escherichia coli. While both catalytic subunits lack the N-terminal seryl-phosphorylation domain that typifies the green-plant enzyme, they differ markedly with respect to their (i) predicted Mr and critical C-terminal tetrapeptide (see Izui et al., 2004); and (ii) immunoreactivity with antisera against the p102 and p130 polypeptides of S. minutum PEPC1/PEPC2 and PEPC2, respectively.

Results and discussion

Cloning and sequence analyses of two novel and distinct Ppc genes in C. reinhardtii

This molecular effort was initiated prior to the first public release of the draft nuclear genome of this green microalga, a model photosynthetic eukaryote. Thus, by using the deduced protein sequence of Zea mays C4-form PEPC (nucleotide accession no. X15238) to search this alga's EST database (http://www.kazusa.or.jp/en/plant/chlamy/EST/) we identified four different EST clones (accession nos. AV629602, AV390217, AV631506, AV622208) that encompassed highly conserved motifs and subdomains in PEPC. The corresponding cDNAs of these EST clones were obtained from the Kazusa DNA Research Institute (Chiba, Japan), and the entire nucleotide sequences were determined. These sequences revealed that the first three ESTs represented the same gene and encoded a deduced, partial PEPC polypeptide ending with the plant-like, C-terminal tetrapeptide QNTG (see Dong et al., 1999), hereafter named CrPpc1 or CrQNTG, whereas the fourth clone encoded a deduced, partial PEPC ending in the non-archaeal, prokaryote-like RNTG motif, hereafter called CrPpc2 or CrRNTG. From this initial, pre-genomic research, these results led us to conclude that C. reinhardtii possessed at least two distinct Ppc genes (CrPpc1 and CrPpc2). Another EST sequence (K. Miura, personal communication) formed a contig with clone AV629602 and a genomic sequence (see scaffold_800) in the Chlamydomonas genome database (version 1). After analyzing this sequence we found it to encode a deduced amino-acid sequence that matched in 13 positions (underlined) with the QDLRTGPANFLSDLL pentadecameric sequence of the 102-kDa catalytic polypeptide from C. reinhardtii PEPC2 determined directly by N-terminal micro sequencing (Rivoal et al., 1998). Therefore, in order to isolate the full-length cDNA corresponding to CrPpc1,a forward primer was designed from this partial genomic sequence and a reverse primer was designed on the basis of sequencing and analyzing the aforementioned EST clones, as described in Experimental procedures.

When the nucleotide sequence of EST clone AV622208 was used as a query to BLAST the public database, we retrieved a 168.8-kb clone, cr-40a20 (AC087726) that likely encoded CrPpc2 and represented the same gene as the original EST clone. In order to isolate the full-length cDNA corresponding to CrPpc2, primers were designed from this genomic sequence (see Experimental procedures) after prediction of exons. The corresponding CrPpc1/2 cDNAs were isolated, sequenced and cloned, and their sequences deposited in GenBank under nucleotide accession nos. AY517644/AY517643, respectively.

The nucleotide sequences of these two distinct C. reinhardtii Ppc transcripts share 46% identity within both the open reading frame (ORF) and the 3'-UTR. Similarly, their 5'-genomic sequences are

45% identical within the first 1400 bp upstream of the ATG start codon (sequences were obtained from scaffold_800 and clone cr-40a20 for CrPpc1 and CrPpc2, respectively). A selected alignment of the deduced, full-length amino-acid sequences of these two polypeptides, along with representative plant and prokaryotic PEPCs, is depicted in Figure 1. Of special note is that both CrPpc1 and CrPpc2 (i) lack the N-terminal seryl-phosphorylation domain that typifies the green-plant enzyme, and thus they resemble the archaeal, (cyano)bacterial (and predicted malarial parasite) PEPCs (Ettema et al., 2004; Vidal and Chollet, 1997), and the recently described 'bacterialtype' plant enzymes in Arabidopsis (AtPpc4), rice (OsPpc-b) and soybean (GmPpc17) (Sa´ nchez and Cejudo, 2003; Sullivan et al., 2004); (ii) harbor all the conserved subdomains that contribute essential residues to the active site (e.g. see boxes I–III in Figure 1; Izui et al., 2004); and (iii) contain the conserved, hydrophobic C-terminal domain that participates in both negative allosteric regulation and maximal catalysis by PEPC (Izui et al., 2004). In contrast, the deduced CrPpc1 and CrPpc2 polypeptides also differ significantly in a number of important respects. First, they share only a 30% overall amino-acid sequence identity. Whereas a number of green-plant PEPCs possess a relatively high amino-acid identity with CrQNTG (e.g. 47% for rice [OsPpc1] and Arabidopsis [AtPpc1]), the predicted primary structure of CrRNTG is most similar to the 'bacterial-type' PEPCs recently found in a few green plants, with AtPpc4, OsPpc-b and GmPpc17 at 43–45% identity. The %-identity of the deduced

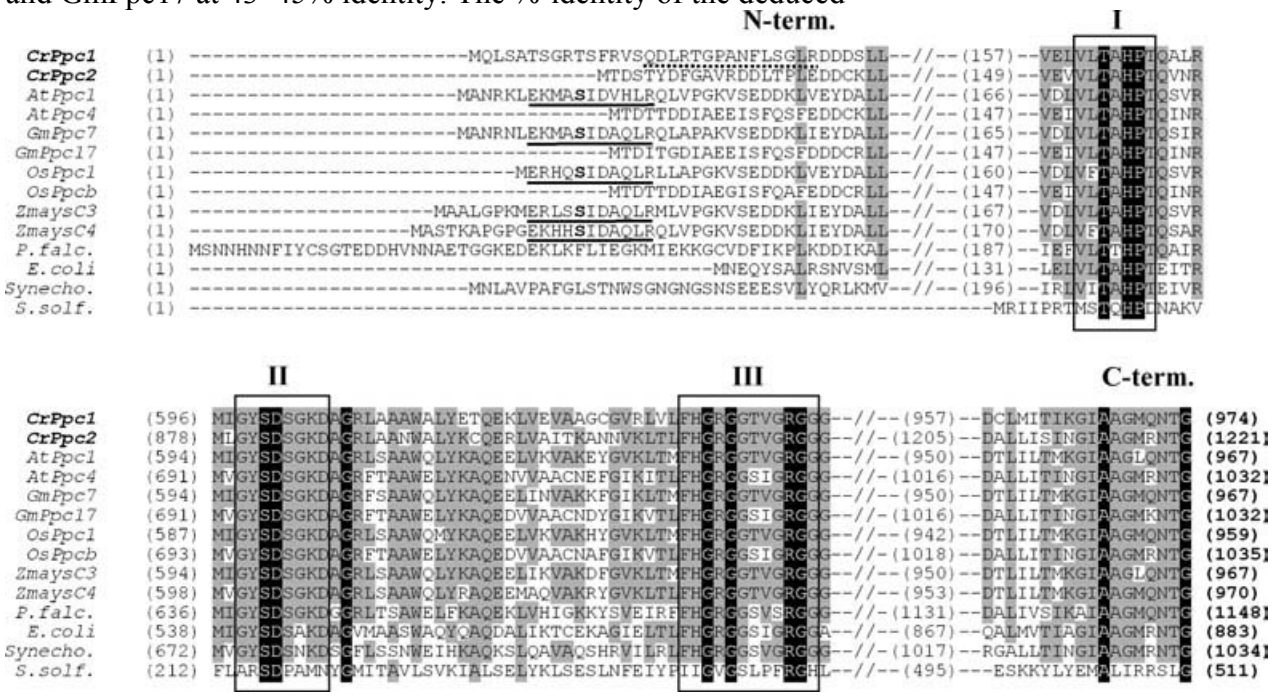

Figure 1. Selected amino-acid-sequence alignments of deduced regulatory and catalytic subdomains in PEPC from C. reinhardtii and representative vascular-plant and prokaryotic species. The abbreviated name of each aligned sequence is as follows: CrPpc1, C. reinhardtii Ppc1 (CrQNTG); CrPpc2, C. reinhardtii Ppc2 (CrRNTG); AtPpc1, Arabidopsis thaliana Ppc1; AtPpc4, A. thaliana Ppc4 (C-terminal RNTG ['bacterial-type']); GmPpc7, Glycine max Ppc7; GmPpc17, G. max Ppc17 (C-terminal KNTG ['bacterialtype']); OsPpc1, Oryza sativa Ppc1; OsPpcb, O. sativa Ppc-b (C-terminal RNTG ['bacterial-type']); ZmaysC3, Zea mays C3-form Ppc; ZmaysC4, Z. mays C4-form Ppc; P.falc., Plasmodium falciparum Ppc; E.coli, E. coli Ppc; Synecho., Synechocystis sp. PCC6803 Ppc; S.solf., Sulfolobus solfataricus Ppc (archaeal). Boxes I–III indicate conserved subdomains essential for catalysis by PEPC (see Izui et al., 2004). The deduced, N-terminal sequence in CrPpc1 underlined with dashes agrees at 13 positions with the 15 amino-acid sequence (QDLRTGPANFLSDLL) of the PEPC2 catalytic polypeptide (p102) of C. reinhardtii determined directly by N-terminal microsequencing (see Rivoal et al., 1998). The conserved, N-terminal seryl-phosphorylation domain typical of plant PEPCs is underscored with a solid line (target Ser is marked in bold).

amino-acid sequences of CrPpc1 and CrPpc2 with PEPC from E. coli is 38 and 32%, respectively, and the predicted primary structures of both forms share a low-level identity with PEPC from the

cyanobacterium Synechocystis sp. strain PCC6803 (27% with CrPpc1 and 25% with CrPpc2). Second, as noted above the deduced amino-acid sequence of CrPpc1 has a green-plant-like QNTG motif at its extreme C-terminus, whereas CrPpc2 has a non-archaeal, prokaryotic-like motif (RNTG) at its carboxy-terminus. Third, near the N-terminus only the deduced sequence of CrPpc1 agrees favorably [87% identity between Gln^{16}–Arg^{30} (Figure 1)] with the 15 amino-acid-residue sequence of the 102-kDa catalytic polypeptide of C. reinhardtii Class-2 PEPC determined directly by N-terminal microsequencing (Rivoal et al., 1998). This notable finding indicates that only this specific Ppc transcript encodes the p102 catalytic subunits common to both the Class-1 and Class-2 enzyme-forms in C. reinhardtii. Finally, the predicted molecular mass of the 974-residue CrPpc1 (CrQNTG) polypeptide is 108 887 Da, whereas that of CrPpc2 (CrRNTG) is 131 218 Da. Notably, the deduced molecular size of CrRNTG, encompassing 1221 amino acids, is the largest PEPC catalytic subunit reported to date (Figure 1). The insertions that account for the large size of CrPpc2, AtPpc4, GmPpc17, and OsPpc-b are flanked by two amino-acid motifs, LRFELS and KLDLRQE, that are conserved in most green-plant and bacterial PEPCs. Surprisingly, the deduced amino-acid sequence of this insert region in CrPpc2 has a number of repetitive Ala and Gly residues. Because of this unusual feature we confirmed this prediction by complementary PCR analyses using cDNA as template which was synthesized from purified mRNA prepared by two different protocols, and also by using two different C. reinhardtii cDNA libraries as template (see Experimental procedures). It should be noted that similarly unusual amino-acid sequences containing repetitive Ala, Gly and/or Gln residues have been reported for the deduced Mut11 (Zhang et al., 2002) and Cia5 (Xiang et al., 2001) proteins in C. reinhardtii.

Phylogenetic analysis of CrPpc1/2 and a number of other PEPCs revealed a clustering into three general groupings (Figure 2): (i) the typical vascular-plant enzymes, of which CrPpc1, with its 109-kDa Mr and C-terminal QNTG tetrapeptide, is a distant member; (ii) the recently described, 'bacterial-type' plant PEPCs reported, to date, in Arabidopsis (AtPpc4), rice (OsPpc-b) and soybean (GmPpc17), of which C. reinhardtii Ppc2, with its C-terminal RNTG tetrapeptide, is

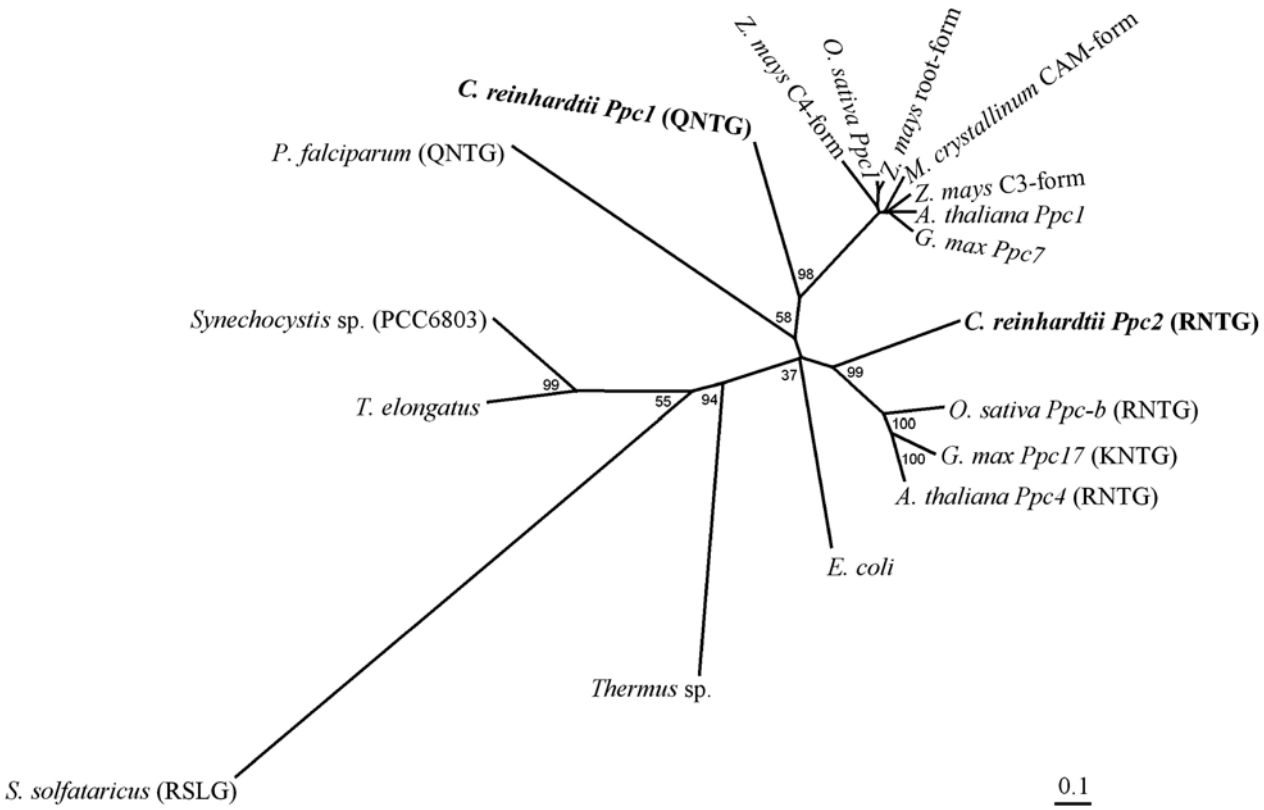

Figure 2. Phylogenetic relationships of PEPCs from C. reinhardtii and representative vascular plants and prokaryotes.

The phylogenetic tree was constructed with deduced, full-length PEPC amino-acid sequences, aligned using the ClustalX (version 1.81) program, as described in Experimental procedures. The bootstrap values, shown at selected nodes, are percentages for 1000 replications. The complete scientific names and GenBank accession numbers pertaining to this figure are found in Experimental procedures.

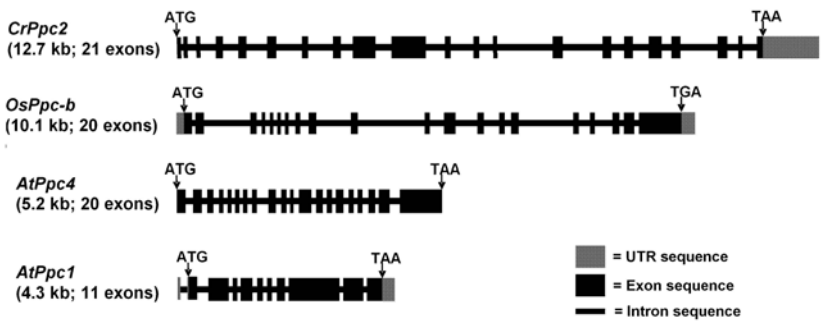

Figure 3. Gene structures of various eukaryotic Ppcs predicted from the Chlamydomonas, rice and Arabidopsis nuclear genomes. The intron and exon positions for A. thaliana Ppc1 and Ppc4 (bacterial-type), O. sativa Ppc-b (bacterial-type), and C. reinhardtii Ppc2 were deduced using SIM4 software (see Experimental procedures), and Vector NTI 7.0 software was used to create the image.

a distant member; and (iii) the archaeal and (cyano)bacterial PEPCs. The distant relationship of the CrQNTG catalytic subunit to typical green-plant PEPCs is consistent with previous findings that indicated a similarly remote relationship between the corresponding p102 polypeptides in the purified

Class-1 and Class-2 enzyme-forms of C. reinhardtii with representative C3 and C4 PEPCs based on CNBr-peptide mapping and immunoblotting (Rivoal et al., 1998).

At the level of overall gene structure, almost all vascular-plant Ppc genes have a highly conserved genomic structure composed of approximately 10 exons interrupted by introns, regardless of whether they are from C3 [e.g. AtPpc1 (Figure 3)], C4 or CAM plants (Chollet et al., 1996). However, the two known 'bacterial-type' Ppc genes in Arabidopsis and rice are predicted to have a very different and more complex structure: AtPpc4 is composed of 20 exons, and OsPpc-b is also predicted to contain 20 [Figure 3 (or 16; Sa´ nchez and Cejudo, 2003)]. The deduced gene structure of C. reinhardtii Ppc2, with its 21 exons, is thus most similar to these 'bacterial-type' plant Ppc genes (Figure 3). However, the longer introns found in CrPpc2 make it the largest Ppc gene (12.7 kb) identified to date. Unfortunately, full-length genomic sequence for

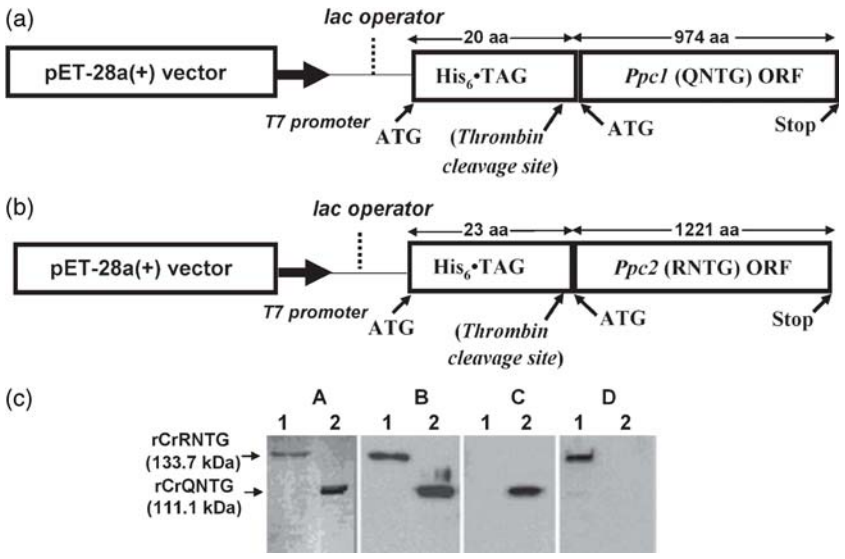

Figure 4. His-tagged recombinant CrPpc1 and CrPpc2 constructs, and subsequent SDS-PAGE and immunoblot analysis of the purified recombinant His6·CrQNTG and His6·CrRNTG fusion proteins. The pET-28a(+) expression vector was used to express the (a) CrPpc1 and (b) CrPpc2 ORFs in E. coli [strain BL21(DE3)] cells, ±IPTG induction, as a (cleavable) His6-tagged PEPC polypeptide of 994 and 1244 amino acids (aa) in length, respectively. (c) SDS-PAGE and immunoblot analysis of ~0.5 lgNi^{2+}-IMAC-purifiedrecombinantCrRNTG (lanes 1) and CrQNTG (lanes 2)His6·PEPCs. Protein staining with Coomassie Blue (A). Immunoblot analysis was performed using His-tag primary antibody (B), or affinity-purified S. minutum anti-p102 PEPC1/PEPC2 IgG (C) and anti-p130 PEPC2 IgG (D).

C. reinhardtii Ppc1 is not represented in the current version of the Chlamydomonas nuclear genome, and thus its exact gene structure remains unknown. Finally, the collective findings from query of this publicly available genomic database and the Chlamydomonas EST database, together with direct Southern analysis using two different Ppc probes (data not shown, and Experimental procedures), indicated that there are only these two Ppc genes in this green microalga. Consistent with PEPC's multi-faceted functional diversity in green plants, similarly small multigenic Ppc families have been reported previously (Chollet et al., 1996).

Heterologous expression, and activity-and immunoblotanalysis of the C. reinhardtii recombinant CrQNTG (Ppc1) and CrRNTG (Ppc2) proteins

In order to verify experimentally that CrPpc1/2 encode functional PEP-carboxylase enzymes, the corresponding ORFs were subcloned into the E. coli expression vector pET28a(þ) (Figure 4a,b) and transformed into expression strain BL21(DE3). The expressed recombinant proteins were found to be highly soluble and effectively purified from clarified cell extracts by Ni^{2}þ-immobilized metal affinity-chromatography (IMAC) as evidenced by SDS-PAGE analysis (Figure 4c, panel A). When assayed at optimal conditions of pH 8.4 and 5 mM PEP, the specific activities of purified recombinant CrQNTG and CrRNTG were 25 and 22 lmol $min^{)1}$ $mg^{)1}$ protein, respectively. These values are similar to the specific activities reported for the Class-1 and Class-2 PEPCs purified from C. reinhardtii cell extracts (Rivoal et al., 1998). These results indicate that the Chlamydomonas recombinant PEPCs produced in E. coli were fully active enzymes and that their modest, 20-to 23residue N-terminal extensions had no detrimental effect on activity. The high specific activity of rCrRNTG is especially notable in light of its unusual deduced primary structure and its closest phylogenetic relationship to the plant 'bacterialtype' PEPCs of undetermined functionality (see Figure 2, and Sa´ nchez and Cejudo, 2003; Sullivan et al., 2004). Acetyl-CoA, a potent allosteric activator of many bacterial PEPCs (Nakamura et al., 1996), but not of the vascular-plant, green-algal (S. minutum), cyanobacterial and archaeal enzymes (Chen et al., 2002; Ettema et al., 2004; Schuller et al., 1990), showed no effect on the activity of rCrQTNG and rCrRNTG at a final concentration of 0.5 mM. This is an important observation in that it further documents, together with the SDSPAGE analysis depicted in Figure 4c (panel A), that the IMAC-purified recombinant proteins are essentially free of contaminating host PEPC from the BL21(DE3) cells.

Figure 4c also summarizes the findings from SDS-PAGE and immunoblot analysis of the two C. reinhardtii recombinant PEPCs using different antibody preparations. As expected, a commercial monoclonal antibody against a His5-tag cross-reacted with the recombinant 111-and 134-kDa fusion proteins (panel B), indicating that the entire PEPC polypeptides were in-frame with the N-terminal His-tag and that they corresponded to the predicted mass of the intact, His6-tagged CrPpc gene products. In marked contrast, affinity-purified polyclonal antibodies raised against the p102 catalytic subunit common to both green-algal PEPC1 and PEPC2, and the interacting p130 polypeptide of PEPC2 from S. minutum (Rivoal et al., 1996, 2001) reacted immunospecifically with rCrQNTG or rCrRNTG, respectively (panels C and D). This is a notable finding in that it agrees with previous immunological data and mass spectroscopic and amino-acid sequence analyses which indicated that the p102 and p130 polypeptides in the Class-2 PEPC-complex from S. minutum are not closely related (Rivoal et al., 2001). In addition, the finding that the fully active, 134-kDa recombinant CrRNTG protein reacts immuno-specifically with anti-S. minutum p130 antibodies supports earlier claims, based largely on circumstantial biochemical evidence, that p130 is a novel, active PEP-carboxylase polypeptide in the green microalgae (Rivoal et al., 2001). As a result of these collective data, we conclude that there are likely two distinct PEPC catalytic subunit-types in the unusual but dominant Class-2 enzyme-complexes in the unicellular green algae, contributed by both p102 (e.g. CrPpc1) and the interacting p130 polypeptide (e.g. CrPpc2). Unfortunately, unlike the situation with C. reinhardtii p102 and CrPpc1 [see Figures 1 and 4c (panel C), and Rivoal et al., 1998] or S. minutum PEPC2 (Rivoal et al., 2001), there are no published biochemical findings on a p130 interacting protein in the PEPC2 complex purified from Chlamydomonas cells (Rivoal et al., 1998). This is likely an experimental artifact of the previously unrecognized, extreme sensitivity of this novel polypeptide to degradation in vitro by endogenous, green-algal protease activity (Rivoal et al., 2001; J. Rivoal, personal communication). Thus, at present it is not possible to directly compare our immunological and deduced amino-acid-sequence results with the active, 131-kDa CrRNTG protein (Figures 1 and 4c) with an authentic p130 polypeptide isolated from C. reinhardtii PEPC2.

Steady-state transcript analysis of CrPpc1, CrPpc2, and a known inorganic C-responsive gene (Cah1)in C. reinhardtii cells grown in high/low levels of CO2 and 10 mM NH_4^+

PEPC and Rubisco are the two major inorganic C (Ci)-fixing enzymes in green plants and unicellular green algae (Chollet et al., 1996; Huppe and Turpin, 1994). It was thus of interest to assess the effects of varying levels of Ci, initially supplied as CO2, on the expression of the two distinct Ppc genes in C. reinhardtii and the corresponding activity of total cellular PEPC. Likewise, given that the green microalgae possess a multi-component, low CO2-inducible Ci-concentrating mechanism (CCM) that services Rubisco with elevated levels of CO2 in order to ensure efficient photosynthesis under Ci limiting conditions (Miura et al., 2004, and references therein), analysis of the CrPpc1 and CrPpc2 transcripts in response to varying [CO2] assumed an even greater importance. Thus, the steady-state mRNA levels of these two distinct Ppc genes were determined in response to changes in CO2 concentration by northern hybridization using gene-specific probes designed from the respective 3'-UTRs. As shown in Figure 5, when C. reinhardtii cells grown at 5% CO2 were transferred to a low-CO2 condition (ambient air), the transcript levels of both Ppc genes increased transiently within 1 h, reached a maximum after 2 h, and then declined following 6 total hours of acclimation to these limiting-CO2 conditions. In addition, the levels of both Ppc transcripts were down-regulated in cells grown in air relative to high CO2-grown cells. However, when the air-grown cells were transferred to high-CO2, the transcript levels of both Ppc genes remained largely unchanged after 8 h of acclimation (Figure 5). These collective results indicate that both CrPpc1 and CrPpc2 are CO2-responsive genes, and their steady-state transcript levels are up-/down-regulated by varying levels of CO2 supplied to the growth medium. It is notable, however, that total cellular PEPC activity, measured in vitro at optimal

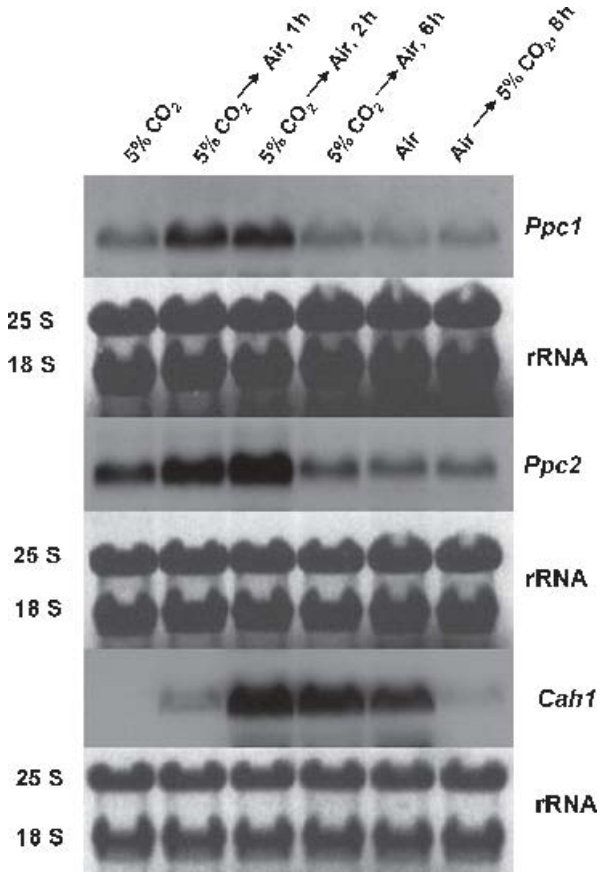

Figure 5. Northern blot analysis of Ppc1, Ppc2, and a Ci-responsive control transcript (Cah1)in C. reinhardtii cells grown in high/low levels of CO2. Cells were grown in HS medium (10 mM NH4Cl) and bubbled with air enriched with 5% CO2 or ordinary air alone. Where indicated, mid-log phase cultures grown in 5% CO_2 were switched to bubbling with air for 1, 2,

or 6 h, and air-grown cultures were switched to bubbling with 5% CO_2 for 8 h. Equal amounts of total RNA (8 lgfor Ppc1,5 lgfor Ppc2, and 3 lgfor Cah1) were separated, blotted, and hybridized to gene-specific probes as described in Experimental procedures. rRNA stained with methylene blue is shown below the corresponding hybridization as a loading control. Cah1 is a CCM-related, low CO_2-inducible control transcript (see text).

levels of PEP (5 mM) and pH (8.4), varied little (£18%) during these various perturbations in [Ci], when expressed on a $Umg^{)1}$ total soluble protein basis (data not shown).

Cah1, which encodes a periplasmic a-carbonic anhydrase [a-CA (Fukuzawa et al., 1990)], was used as a Ci-responsive reference transcript whose expression is related directly to the induction of the CCM in C. reinhardtii cells (Fukuzawa et al., 1990, 2001; Xiang et al., 2001). As shown in Figure 5, essentially no accumulation of the Cah1 transcript was detected under high-CO2 conditions, in contrast to Ppc1 and Ppc2. However, the level of Cah1 mRNA increased within 1 h of low-CO2 acclimation and attained its maximum after 2 h. Notably, during this transient period Ppc1 and Ppc2 responded in a similar manner. In contrast, there was a striking down-regulation of Cah1 expression when air-grown cells were transferred to 5% CO2 for 8 h, whereas Ppc1 and Ppc2 levels were essentially unchanged after this relatively brief period of acclimation to high CO2 (Figure 5). Thus, it is clear that neither of these two distinct Ppc genes responds to changes in [Ci] in a manner identical to Cah1 (Figure 5) and other transcripts whose expression patterns are correlated with the CCM in Chlamydomonas (e.g. Eriksson et al., 1998; Fukuzawa et al., 2001; Miura et al., 2004 and references therein). These differing trends in expression are not surprising given the non-photosynthetic, anaplerotic role of PEPC in the green microalgae (see introductory remarks, and Huppe and Turpin, 1994). However, it is notable that the response of CrPpc1 and $CrPpc_2$ to changes in [CO_2] during growth at high levels of NH^+_4 (Figure 5) differs significantly from the expression of the Mca1/2 genes in C. reinhardtii (Eriksson et al., 1998; Giordano et al., 2003). The protein-products of these latter two transcripts, which encode mitochondrial isoforms of b-CA (Eriksson et al., 1998), are hypothesized to be involved in the conversion of (photo)respiratory CO2 into bicarbonate for subsequent export in order to provide the specific carboxylation substrate for anaplerotic C-fixation in the cytoplasm catalyzed by PEPC (Giordano et al., 2003). Clearly, if these mitochondrial b-CAs are, indeed, anaplerotic enzymes like PEPC, the expression of these sets of 'anaplerotic' Mca and Ppc genes is not coordinately regulated in Chlamydomonas cells grown in these varying levels of Ci/NH^+_4.

Steady-state transcript analysis of CrPpc1, CrPpc2, and known inorganic N-responsive genes (Gs1 and Amt4) in C. reinhardtii cells grown in high/low levels of NH_4pat 5% CO2

As noted in the preceding section and the Introduction, PEPC is a major anaplerotic enzyme in the green microalgae, especially in the context of replenishing citric-acid-cycle intermediates, such as 2-oxoglutarate, consumed during the assimilation of ammonia by the GS/GOGAT cycle (Giordano et al., 2003; Huppe and Turpin, 1994; Norici et al., 2002). It was thus of considerable relevance to assess the effects of varying levels of inorganic N, initially supplied as NH4Cl, on the expression of the Ppc1 and Ppc2 genes in C. reinhardtii, and any concomitant changes in total cellular PEPC activity. As a complement to these Ppc northern analyses, the expression of two distinct, inorganic-N responsive reference transcripts was also monitored, namely Amt4 and Gs1. The former (AY542491) is one of at least four Amt genes in C. reinhardtii that is believed to encode a putative gas channel for the uncharged NH_3 species (Soupene et al., 2004). Notably, the steady-state transcript levels of Amt4 are increased specifically and dramatically under N-limiting conditions. Gs1 represents the sole gene in C. reinhardtii that encodes the cytoplasmic isoform of GS, an obvious component of the N-assimilating GS/GOGAT cycle (Chen and Silflow, 1996). In contrast to Gs2, which encodes the stromal enzyme

and whose expression is unaffected by growth in high [NH^+_4] versus [NO_3], the transcript levels of Gs1 are significantly down-regulated under conditions of excess $_{NH+4}$ (Chen and Silflow, 1996). Thus, the comparative expression responses of the Amt4 and Gs1 genes would likely provide additional insight into the effects of varying [NH^+_4] during growth on the levels of the CrPpc1/2 transcripts.

As shown in Figure 6, the transcripts for Ppc1, Ppc2, and Gs1 were all readily detected in cells grown in 10 mM NH^+_4 and air enriched with 5% CO2 (also see Figure 5). In contrast,

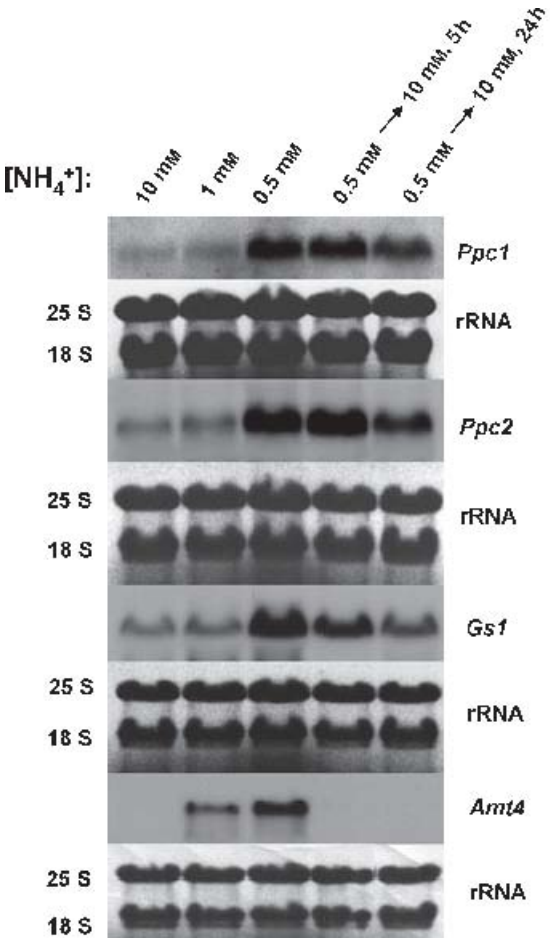

Figure 6. Northern blot analysis of Ppc1, Ppc2, and N-responsive control transcripts (Gs1 and Amt4)in C. reinhardtii cells grown in high/low levels of NH^+_4 . Cells were grown in HS medium bubbled with air enriched with 5% CO2. Inorganic N was supplied initially to the medium as 10, 1, or 0.5 mM NH4Cl, and cells originally grown in 0.5 mM NH^+_4 were also subjected to a subsequent 5-or 24-h growth-period in the presence of 10 mM NH_4Cl added directly to the low N-medium, as indicated in the figure. Equal amounts of total RNA (8 lg for Ppc1,5 lgfor Ppc2, and 4 lg, each, for Gs1 and Amt4) were separated, blotted, and hybridized to gene-specific probes. Gs1 is an NH^+_4 repressible control transcript, and Amt4 is induced specifically in limiting-N growth conditions (see text). rRNA stained with methylene blue is shown below each corresponding hybridization as a loading control.

the exquisitely N-sensitive Amt4 gene was not expressed under these conditions of N-sufficiency. With growth in decreasing levels of $_{NH+4}$, all four genes were progressively up-regulated, with the maximal increase in steady-state transcript abundance occurring at 0.5 mM. Clearly, Ppc1 and Ppc2 are N-responsive genes in C. reinhardtii, and their expression profiles in response to decreasing NH^+_4 levels from 10 to 0.5 mM at 5% CO2 mirrored that of Gs1. The latter finding is noteworthy in that the

presumably 'anaplerotic' Mca1/2 genes are not expressed at high CO2 and 0.1–10 mM $_{NH+4}$ (Giordano et al., 2003). Thus, as with the above [Ci] effects on Ppc expression summarized in Figure 5, the abundance of these two b-CA transcripts is clearly not coordinately regulated with Ppc1, Ppc2 and Gs1, whose protein-products are known components of the overall N-assimilation pathway in the green microalgae (Chen and Silflow, 1996; Huppe and Turpin, 1994; Norici et al., 2002).

When the low-N grown cells were re-supplied directly with 10 mM $_{NH+4}$, there was a modest but detectable down-regulation of Ppc1, Ppc2 and Gs1 expression within 24 h (Figure 6). In contrast, the exquisitely N-sensitive Amt4 gene was once again specifically and completely down-regulated within 5 h of growth under these N-sufficient conditions. It is noteworthy that while concomitant changes in total in vitro PEPC-specific activity (on a soluble protein basis) generally paralleled those for Ppc1 and Ppc2 transcript abundance with decreasing [$_{NH+4}$] from 10 to 0.5 mM, gradually increasing approximately threefold from about 0.04 to

0.12 U mg^{-1} protein, this was not the trend upon re-supply of $_{NH+4}$ to the low-N grown cells. In this case, total cellular PEPC activity reverted fully back to its original high-N value of 0.04 U $mg^{)1}$ within 5 h, whereas Ppc transcript levels were relatively unaffected during this brief period of acclimation (Figure 6). Whether these N-mediated changes in total PEPC specific activity are due to selective changes in the steady-state levels of the CrQNTG and CrRNTG catalytic polypeptides, and/or the Class-1 and Class-2 enzyme-forms, awaits future investigation. Finally, at the physiological level, related chemostat experiments with N-limited

S. minutum cells revealed an immediate and dramatic increase in the in vivo rates of non-photosynthetic, dark CO2-fixation by PEPC and concomitant N-assimilation within 10 min of resupply of mM-levels of $_{NH+4}$ to the N-deficient cells (Schuller et al., 1990; Vanlerberghe et al., 1990). By analogy with the above transcript and PEPC-activity findings in C. reinhardtii, such N-limited green-microalgal cells appear 'poised' in anticipation of such future N-sufficient conditions with elevated levels of the Ppc1, Ppc2 and Gs1 transcripts and potential PEPC activity.

Concluding remarks

In this paper, we provide seminal molecular insight into the two novel and distinct Ppc genes, and corresponding PEPC catalytic subunits, in the green alga C. reinhardtii. It is now evident that CrPpc1 is the specific gene whose protein-product (CrQNTG) corresponds to the p102 catalytic subunit common to both the homotetrameric Class-1 and heteromeric Class-2 PEPC-forms in C. reinhardtii (see Figure 1, and Rivoal et al., 1998). Similarly, the divergent but highly active,

131-kDa CrRNTG catalytic subunit is also void of the 'typical' N-terminal phosphorylation domain, and likely is equivalent to the interacting p130 polypeptide that is unique to the green-algal Class-2 PEPC complex (see Figure 4c, and Rivoal et al., 2001). Taken together, these and earlier biochemical findings (Rivoal et al., 2001) implicate the novel presence of two distinct PEPC catalytic subunit-types in the unusual but dominant Class-2 enzyme-complexes in the unicellular green algae. Current research in this laboratory is directed at the rigorous identification of all the in-vivo interacting protein-partners in the Chlamydomonas Class-2 PEPC heteromer.

At the level of expression, both of these 'anaplerotic' CrPpc1/2 genes are coordinately responsive to changes in inorganic-C and -N levels during growth, and they generally mirror the response of Gs1 transcript abundance to changes in [$_{NH+4}$] at high CO2. This correlation between Ppc1/2 and Gs1 expression in Chlamydomonas provides direct molecular evidence in support of previous physiological and biochemical studies that highlighted a key anaplerotic, non-photosynthetic role for PEPC in overall N-assimilation by the GS/GOGAT cycle in green microalgae (see the introductory remarks, and Huppe and Turpin, 1994). This important aspect of this ongoing Chlamydomonas Ppc/PEPC project will be

explored in greater detail by (i) complementary immunoblot analyses of the CrQNTG and CrRNTG catalytic subunits, and the Class-1 and Class-2 enzyme-forms, as a function of [$_{NH+4}$] during growth by exploiting isoform-specific peptide antibodies directed against the two divergent N-terminal sequences (see Figure 1); and (ii) functional genomics approaches to these two distinct CrPpc genes in relation to C/N-metabolism, including improved RNA interference strategies (Rohr et al., 2004).

Experimental procedures

Cells and growth conditions

Chlamydomonas reinhardtii cells (strains CC-125, CC-1883 and CC1021) were cultured at 25C in continuous light (~100 μmol m^{-2} sec^{-1}, 400–700 nm) in HS medium (Harris et al., 1989). Inorganic nitrogen was added as NH_4Cl at 0.5, 1 or 10 mM, unless otherwise stated. To achieve elevated CO_2 concentrations, the cultures were bubbled continuously with compressed air enriched with CO_2 to 5% by volume. For low-CO_2 conditions, cultures were bubbled with ordinary compressed air (0.036% CO_2) in the same medium. Cells were acclimated to each $_{NH+4}$ and CO_2 concentration for at least 10 days before the experiments were initiated, and all studies were performed with cells in the mid-exponential growth phase.

Cloning of the CrPpc1/2 genes

In order to isolate the cDNAs of the C. reinhardtii Ppc genes, total RNA was extracted from liquid nitrogen-frozen cells of strain CC-125 (grown in 10 mM $_{NH+4}$ and high CO2) and precipitated with 3 M LiCl. RNA samples (2 lg) were pre-treated with 2 units of DNase I for 1 h at 37C, after which the enzyme was inactivated at 65Cin the presence of 2 mM EDTA. This DNA-depleted RNA preparation was used for synthesis of the first-strand cDNA with oligo (dT)18 primer and an M-MLV Reverse Transcriptase-based RETROscript kit (Ambion, Inc., Austin, TX, USA) at 44C for 1 h. Samples of the first-strand cDNA were used in PCR reactions to amplify full-length Ppc encoding regions using the following gene-specific primer-pairs: CrPpc1 [5'-ATGCAGCTGTCTGCTACCAGCG-3' (forward) and 5'TTACCCCGTGTTCTGCATGCCCGCGG -3' (reverse)]; and CrPpc2 [5'ATGACTGCAAGCTTCTCGGTA-3' (forward) and 5'-TTAGCCGGTGTTGCGCATGCCGG-3' (reverse)]. PCR was performed with cloned Pfu DNA polymerase (Stratagene, La Jolla, CA, USA) with 35 cycles of 96C denaturation for 45 sec, a 55C annealing period of 45 sec and 72C extension period of 8 min, and final extension at 72Cfor 10 min, using cDNA, prepared as described above, as template. Both Ppc cDNA ORFs were cloned into pBluescript II()) phagemid vector (Stratagene) after digestion with EcoRV, sequenced at the Genomics Core Research Facility, University of Nebraska-Lincoln, and designated as CrPpc1 (AY517644) and CrPpc2 (AY517643).

In CrPpc2, the unusual insert sequence responsible for its overall large size (4.77 kb) was confirmed by PCR using cDNA templates which were synthesized from mRNA prepared with two different purification kits [PolyATtract (Promega, Madison, WI, USA), and Poly(A) Purist (Ambion)], and two different C. reinhardtii cDNA libraries (kindly provided by Dr Martin Spalding, Iowa State University, Ames, IA, USA) as a template.

Sequence-alignment, phylogenetic and gene-structure analyses

Deduced amino-acid-sequence alignments of C. reinhardtii Ppc1 and Ppc2, together with representative plant and prokaryotic PEPCs, were performed using VECTOR NTI 7.0 software. Likewise, a phylogenetic tree was constructed with predicted, full-length PEPC amino-acid sequences aligned using the ClustalX program (version 1.81) with manual adjustments (Thompson et al., 1997). A distance matrix for the alignment was calculated, and an unrooted tree was constructed using the Protdist (with JTT model) and Neighbor programs of the PHYLIP package (version 3.62), respectively (Felsenstein, 1996). Bootstrap analysis was performed with 1000 replications and the tree was visualized using TreeView 32 software. The GenBank nucleotide accession numbers of the various PEPCs used to construct the tree depicted in Figure 2 are as follows: E. coli (NC_002655); Sulfolobus solfataricus (NC_002754 [archaeal]); Thermus sp. (D42166); Thermosynechococcus elongatus (AP005375); Synechocystis sp. PCC6803 (NC_000911); Arabidosis thaliana Ppc1 (NM_104209); A. thaliana Ppc4 (AJ532903 [AtRNTG]); rice (Oryza sativa) OsPpc-b (AP002882 [OsRNTG]); rice OsPpc1 (AF271995); maize (Zea mays) C3-form (X61489); maize C4-form (X15238); maize root-form (AB012228); soybean (Glycine max) GmPpc7 (AB008540); soybean GmPpc17 (AY563043 [GmKNTG]); Mesembryanthemum crystallinum CAM-form (X14587 [Ppc1]); C. reinhardtii Ppc1 (AY517644 [CrQNTG]); C. reinhardtii Ppc2 (AY517643 [CrRNTG]); and Plasmodium falciparum (AE014820).

The intron and exon positions depicted in Figure 3 were predicted by comparing cDNA sequences of A. thaliana Ppc1 (NM_104209) and Ppc4 (AJ532903) with their corresponding genomic sequences available at the Arabidopsis database (http://www.Arabidopsis.org/),

O. sativa Ppc-b (AP002882) cDNA with its corresponding genomic sequence obtained from the rice genome database (http://www.tigr. org/tdb/e2k1/osa1/), and C. reinhardtii Ppc2 cDNA with its genomic sequence obtained from clone cr-40a20 (AC087726), using SIM4 software available at http://gamay.univ-perp.fr/analyse_seq/sim4/.

Construction and purification of recombinant, His6-tagged C. reinhardtii Ppc1 (rCrQNTG) and Ppc2 (rCrRNTG) proteins

In order to construct the recombinant protein expression plasmids pET-28a(+)-Ppc1(QNTG) and -Ppc2(RNTG), primer pairs were designed on the basis of the CrPpc1 and CrPpc2 sequences. PCR was performed as described above using pBluescript II(-))-Ppc1 or -Ppc2 as template. The respective sequences of the forward and reverse primers used to construct pET-28a(+)-Ppc1(QNTG) were 5'GATCACCATATGCAGCTGTCTGCTACCAGCGGCAG-3' and 5'-ACTGTCCTCGAGTTACCCCGTGTTCTGCATGCCCG-3'. The bold underlined nucleotide bases indicate restriction-enzyme digestion-sites for NdeI and XhoI, respectively. After amplification, the PCR product was digested by NdeI and XhoI for at least 12 h at 37C and then directionally ligated with pET-28a(+) vector (Novagen, Madison, WI, USA) that was previously digested with both enzymes. The ligated plasmid was transformed into competent cells of E. coli strain DH5a and then a positive clone was selected. Purified plasmid from DH5a was re-transformed into E. coli BL21(DE3) competent cells (Novagen) to express recombinant CrQNTG as a fusion protein (cf. Chen et al., 2002) with an extraneous, 20-residue N-terminal extension. The expression plasmids were re-isolated and re-sequenced in order to verify that the ligations and the His-tagged Ppc1 insert were correctly in-frame.

The sequences of the forward and reverse primers used to construct pET-28a(+)-Ppc2(RNTG) were 5'-GATCACGCTAGCATGACGGACTCCACATATGATTT-3' and 5'-ACTGTCCTCGAGTTAGCCGGTGTTGCGCATGCCGG-3', respectively. The bold underlined nucleotide bases were designed as restriction-enzyme sites for NheI and XhoI, respectively. After

amplification, the PCR product was digested and then directionally ligated with pET-28a(þ) vector as described above, using NheI and XhoI restriction enzymes. As a result, recombinant CrRNTG was expressed as a fusion protein with a 23-residue N-terminal extension.

Cultures of E. coli strain BL21(DE3), transformed with pET-28a(þ)Ppc1(QNTG) or -Ppc2(RNTG), were grown at 25C in 100 ml of LB medium containing 30 lgml^{-1} of kanamycin to an OD600 of 0.6. Recombinant proteins were induced with or without 1 mM isopropyl-b-D-thiogalactoside (IPTG) in order to express rCrQNTG and rCrRNTG, respectively, for 3 h at 25C. Subsequently, the cells were harvested at 4C by centrifugation at 2000 g for 15 min and resuspended in 4 ml of ice-cold 1X binding buffer (0.5 M NaCl, 20 mM Tris–HCl, 5 mM imidazole, pH 7.9) containing a Complete protease inhibitor tablet (Roche Applied Science, Indianapolis, IN, USA) dissolved at the recommended concentration. The re-suspended cells were lysed at 4C by sonicating eight times for 10 sec, each, and then centrifuged at 39 000 g for 20 min. The resulting supernatant fraction was desalted by passage through a PD-10 column (Amersham Biosciences, Piscataway, NJ, USA), and then applied to a 2-ml column of Ni-NTA HisÆBind resin (Novagen). Both columns were pre-equilibrated with 1X binding buffer containing 10% (v/v) glycerol and a Complete protease inhibitor tablet (Roche Applied Science) dissolved as recommended. The Ni^{2}þ-IMAC purification procedure was carried out at 4C according to the manufacturer's instructions, except for the addition of 10% (v/v) glycerol in all buffers for stabilizing PEPC activity. rCrQNTG and rCrRNTG were eluted at 1 and 0.1 M imidazole, pH 7.9, respectively. The fractions possessing high PEPC activity were combined, desalted and concentrated with a Centricon YM-30 centrifugal concentrator (Millipore Corp., Bedford, MA, USA) against 50 mM HEPES/KOH, pH 7.5, containing 20% (v/v) glycerol, 1 mM EDTA, 5 mM MgCl2,2 mM DTT, and a Complete protease inhibitor tablet (Roche Applied Science) dissolved at the recommended concentration, and stored at)20C until used.

Affinity-purification of S. minutum anti-p130 PEPC2 and anti-p102 PEPC1/2 IgGs, SDS-PAGE, and immunoblotting

Anti-S. minutum-p130 PEPC2 (Rivoal et al., 2001) and anti-p102 PEPC1/2 (serum 1 in Rivoal et al., 1996) IgGs were affinity-purified from the crude antisera (kindly provided by Dr Jean Rivoal, Universite´ de Montre´ al, Canada) against 20 or 30 lg of purified recombinant CrRNTG or CrQNTG, respectively, as described previously (Ermolova et al., 2003). For immunoblot analysis, SDS-PAGE was performed on 8 or 10% acrylamide gels, and the gels were subsequently blotted to polyvinylidene difluoride membranes using a 25 mM Tris base/150 mM Gly, pH 8.3, transfer buffer at 100 V for 1 (rCrQNTG) or 2 h (rCrRNTG). The membranes were first blocked with 5% (w/v) low-fat milk in TBS at room temperature for 1 h, and then probed with the antibody of interest [i.e. the affinity-purified, anti-p130 and anti-p102 antibodies described above or a His5ÆTag monoclonal antibody (Novagen)] in TBS containing 3% (w/v) low-fat milk for 1 h at room temperature. After three 10-min washes, the membranes were incubated with a horseradish peroxidase-labeled anti-IgG for 1 h at room temperature, followed by five 10-min washes with TBS, and finally chemiluminescent detection was performed with the ECL western-blotting reagents from Amersham Biosciences.

PEPC extraction and assay

Extraction of PEPC from liquid N2-frozen C. reinhardtii cells (strain CC-1883) was performed with 300–700 ll of extraction buffer, as described previously (Rivoal et al., 1998), but with the addition of 2mM 2,2'-dipyridyl, 10 lgml^{-1}, each, of pepstatin A, chymostatin and leupeptin, plus a Complete protease inhibitor tablet (Roche Applied Science) dissolved as recommended, followed by centrifugation for 20 min at 16 000 g. PEPC activity in these crude C. reinhardtii supernatant fractions,

or the IMAC-purified rCrQNTG and rCrRNTG preparations, was assayed spectrophotometrically at pH 8.4 and 340 nm by coupling to excess NADH-malate dehydrogenase (Sigma, St Louis, MO, USA; no. M-2634), as described previously (Rivoal et al., 1998), 5mM PEP. One unit (U) of PEP-dependent enzyme activity is defined as the amount of PEPC catalyzing the production of 1 μmol of OAA min^{-1} at 25C. Soluble protein concentration was determined using the Bio-Rad protein assay-reagent (Bio-Rad Laboratories, Hercules, CA, USA) and BSA as standard.

RNA and DNA hybridizations

For northern blot analysis, total RNA from cells of strain CC-1883 grown in various levels of NH_4Cl and CO_2 was isolated using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA), including on-column digestion with the RNase-free DNase Set (Qiagen), following the manufacturer's protocols. Equal amounts of total RNA (3–8 lg for each specific transcript of interest) were separated by electrophoresis in a formaldehyde-containing 0.8% (w/v) agarose gel and then blotted onto a Hybond-N+ membrane (Amersham Biosciences) by capillary transfer. After fixation by UV irradiation at 254 nm, the quality and loading of the transferred RNA were assessed by methylene blue staining of rRNA (Wilkinson et al., 1991). Destained blots were then hybridized overnight either at 50 (for CrPpc1)or 55C (for CrPpc2, CrCah1, CrGs1, and CrAmt4) in UL-TRAhyb buffer (Ambion) with gene-specific probes at a concentration of 10 pM. All probes were labeled with digoxiginen-11-dUTP by PCR as recommended in the manufacturer's kit (Roche Applied Science), using the following forward and reverse gene-specific primers, respectively: Ppc1 (5'-CCTGTGTGGAGCTGCACCAT-3' and 5'-CCTCCAGGTTCCAAACACTAAG-3'); Ppc2 (5'-GGAGGTGGTGCTGAAAATGT-3' and 5'-TCTGGGCTGCCTCAAATATC-3'); Cah1 (5'-AAGTCGACCGTACACGTCTCTGCCTC-3' and 5'-AAGCGGCCGCGATGGATGGTAGTGAG-3'); Gs1 (5'-CAGTGCCATACCATTTGTCG3' and 5'-CAAGTGCACAATCACCCAAC-3'); and Amt4 (5'-GTATTGCGTCCGATCTGC-3' and 5'-CGTGGAAATGCTGTAGGG-3').

For Southern blot analysis of CrPpc1 and CrPpc2, genomic DNA was isolated as described by Newman et al. (1990) from cells of strain CC-1021 grown in HS medium with 10 mM NH4Cl and 5% CO2, and 10-lg samples were digested with NdeI, BamHI, and XhoI restriction enzymes. The resulting fragments were separated on a 1.0% agarose gel and then transferred to a Hybond-Nþ membrane. Hybridization was performed as described above, at 50C, with probes designed from either a 180-bp cDNA corresponding to part of the coding region of CrPpc1, amplified using forward (5'AGAAGCTGGTGGAGGTGG-3') and reverse (5'-ACTGCTGCTCAATGATCTC-3') primers encompassing a highly conserved catalytic subdomain (box III in Figure 1), or the respective 3'-UTRs of CrPpc1 and CrPpc2 as described above.

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