Phosphoenolpyruvate carboxylase (PEPC) is believed to play an important role in producing malate as a substrate for fatty acid synthesis by leucoplasts of the developing castor oilseed (COS) endosperm. Two kinetically distinct isoforms of COS PEPC were resolved by gel filtration chromatography and purified. PEPC1 is a typical 410-kDa homotetramer composed of 107-kDa subunits (p107). In contrast, PEPC2 exists as an unusual 681-kDa hetero-octamer composed of the same p107 found in PEPC1 and an associated 64-kDa polypeptide (p64) that is structurally and immunologically unrelated to p107. Relative to PEPC1, PEPC2 demonstrated significantly enhanced thermal stability and a much lower sensitivity to allosteric effectors (Glc-6-P, Glc-1-P, Fru-6-P, glycerol-3-P) and inhibitors (Asp, Glu, malate) and pH changes within the physiological range. Nondenaturing PAGE of clarified extracts followed by in-gel PEPC activity staining indicated that the ratio of PEPC1:PEPC2 increases during COS development such that only PEPC1 is detected in mature COS. Dissimilar developmental profiles and kinetic properties support the hypotheses that (i) PEPC1 functions to replenish dicarboxylic acids consumed through transamination reactions required for storage protein synthesis, whereas (ii) PEPC2 facilitates PEP flux to malate in support of fatty acid synthesis. Interestingly, the respective physical and kinetic properties of COS PEPC1 and PEPC2 are remarkably comparable with those of the homotetrameric low Mr, Class 1 and heteromeric high Mr, Class 2 PEPC isoforms of unicellular green algae.

Phosphoenolpyruvate carboxylase (PEPC) is a ubiquitous cytosolic enzyme in vascular plants that is also widely distributed in green algae and bacteria (1). It catalyzes the irreversible β-carboxylation of PEP in the presence of Mg2+ and HCO3− to yield oxaloacetate and P1; PEPC is abundant in C4 and crassulacean acid metabolism (CAM) leaves where it participates in photosynthesis by catalyzing the initial fixation of atmospheric CO2. Both allosteric mechanisms and covalent modification are involved in PEPC control in C4 and CAM leaves (1–3). Early work established that C4 and CAM PEPCs are controlled by a diurnal cycle that modulates their sensitivity to L-malate inhibition (1–3). This cycle is the result of phosphorylation of the PEPC homotramer by an endogenous Ca2+-independent PEPC protein kinase and dephosphorylation by a protein phosphatase type 2A at a highly conserved seryl residue localized near the N terminus of the 100–110-kDa PEPC subunit (1–3).

Relative to C4 and CAM PEPCs, the properties of the enzyme from non-green plant tissues are less well understood. Although proposed roles for nonphotosynthetic PEPCs are diverse, a crucial PEPC function is the anaplerotic replenishment of citric acid cycle intermediates consumed during biosynthesis and nitrogen assimilation (1). As with C4 and CAM PEPCs, the PEPC of C3 leaves and nonphotosynthetic tissues can be controlled by allosteric effectors and reversible phosphorylation (4–10). However, despite the probable central role of PEPCs in the metabolism of developing and germinating seeds (11–16), no seed PEPC has been fully purified and thoroughly characterized.

Storage lipids account for as much as 65% of the weight of mature castor oilseeds (COS). Triacylglyceride accumulation depends on the synthesis of long chain fatty acids, which in developing oilseeds occurs in specialized plastids termed leucoplasts. This process requires the transport of both sucrose-derived carbon skeletons and energetic intermediates across the plastid envelope (17). L-Malate supports significant rates of fatty acid synthesis by isolated leucoplasts (18). Malate imported from the cytosol into the leucoplast stroma is mediated by a malate/Pi translocator within the COS leucoplast envelope (19). Sangwan and co-workers (16) hypothesized that the large increase in PEPC activity and concentration that accompanies COS development facilitates malate production for fatty acid synthesis. The increased PEP to malate flux would also serve as an anaplerotic source of C-skeletons for transamination reactions associated with COS storage protein synthesis.

The aim of this study was to purify and characterize PEPC from developing COS. Here we present unexpected evidence for two PEPC isoforms from developing COS and examine their structural and kinetic properties. Although one isoform is a typical PEPC homotramer, the other represents a unique high Mr PEPC complex unprecedented in vascular plants but remarkably reminiscent of Class 2 PEPC isoforms recently described in unicellular green algae (20–23). We provide evidence that the association of a common 107-kDa PEPC catalytic subunit with an unrelated but PEPC-like 64-kDa polypeptide is responsible for the dramatic differences in the physical properties of PEPC in photosynthesis by catalyzing the initial fixation of atmospheric CO2. Both allosteric mechanisms and covalent modification are involved in PEPC control in C4 and CAM leaves (1–3). Early work established that C4 and CAM PEPCs are controlled by a diurnal cycle that modulates their sensitivity to L-malate inhibition (1–3). This cycle is the result of phosphorylation of the PEPC homotramer by an endogenous Ca2+-independent PEPC protein kinase and dephosphorylation by a protein phosphatase type 2A at a highly conserved seryl residue localized near the N terminus of the 100–110-kDa PEPC subunit (1–3).

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Plant Material—Castor plants (Ricinus communis L., cv Baker 206) were cultivated in a greenhouse at 24°C and 60% relative humidity under natural light supplemented with 16 h of artificial light. COS were harvested at stages of development previously described (24). Dissected endosperm (free of cotyledon) was frozen in liquid N2 and stored at −80°C.

Enzyme and Protein Assays and Kinetic Studies—PEPC activity was assayed at 25°C using a Molecular Devices microplate reader as previously described (8). Standard assay conditions were: 100 mM Hepes-KOH (pH 8), 10% (v/v) glycerol, 2.5 mM PEP, 5 mM KHCO3, 5 mM MgCl2, 2 mM dithiothreitol, 0.15 mM NADH, and 5 units/ml desalted malate dehydrogenase. All assays were corrected for background NADH oxidation and were linear with respect to time and the concentration of enzyme assayed. One unit of PEPC activity is defined as the amount of enzyme resulting in the production of 1 mol of oxaloacetate min−1.

Protein concentration was determined by the Coomassie Blue G-250 (25) or bicinchoninic acid (26) colorimetric methods using bovine γ-globulin as the protein standard.

Apparent Vmax, (Vmax,app), Kcat, and kcat/n values (concentrations of inhibitors or activators producing 50% inhibition or activation of PEPC activity, respectively) were calculated using Brooks' computer program (27). All kinetic parameters represent means of at least three separate determinations and are reproducible to within ±10% (S.E.) of the mean value. Stock solutions of metabolites were made equimolar with MgCl2 and adjusted to pH 7.5.

PEPC Purification—All procedures were carried out at 0–4°C, and 10 μg/ml chymostatin, 0.5 μg/ml leupeptin, and 50 μg/ml microcystin-LR were added to all resuspended pellets and pooled fractions. Malate and 2,2'-dipyridyl disulfide were omitted during the purification of proteolyzed PEPC. All buffers contained 1 mM dithiothreitol, 5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 20 mM NaF, and 5 mM malate in addition to the following. Buffer A contained 50 mM Hepes-KOH (pH 7.5), 0.1% (v/v) Triton X-100, 50% (v/v) glycerol, 4% (w/v) PEG 8000, 1% (w/v) insoluble polyvinylpolypyrrolidone, 5 mM thiose, 2 mM 2,2′-dipyrindil disulfide, 2 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml leupeptin, 10 μg/ml chymostatin, and 50 mM microcystin-LR. Buffer B contained 50 mM Hepes-KOH (pH 7.1) and 20% (saturation) (NH4)2SO4. Buffer C was buffer B lacking (NH4)2SO4 but containing 10% (v/v) glycerol. Buffer D contained 50 mM Hepes-KOH (pH 8) and 15% (v/v) glycerol. Buffer E contained 50 mM Hepes-KOH (pH 7.5), 15% (v/v) glycerol, 100 mM KCl, and 0.02% (w/v) Na3VO4.

Stage VII developing COS endosperm (100 g) was homogenized (1:2, w/v) in buffer A using a Polytron. After centrifugation, the supernatant was filtered through two layers of Miracloth and recentrifuged. PEG 8000 (50% (w/v) in 50 mM Hepes-KOH, pH 7.5) was added to the supernatant to a final concentration of 20% (w/v) and stirred for 30 min. After centrifugation, solid (NH4)2SO4 was added to the supernatant to 20% (w/v) and centrifuged and subsequently purified (1). With these buffer additions, partial degradation of p107 was prevented (Fig. 2, A and B, lane 1). BLAST analysis of the N-terminal amino acid sequence of each polypeptide indicated that p98 probably arose via the action of a COS endopeptidase that hydrolyzed an approximate 120-amino acid polypeptide from the N terminus of p107 (Fig. 3). Thus, we modified the purification protocol by adding 2 mM 2,2′-dipyridyl disulfide to the extraction buffer and 5 mM malate to all purification buffers. 2,2′-Dipyridyl disulfide is an active site-directed covalent affinity label of papain (29) that also suppresses the activity of COS cysteiny1 endopeptidase(s) (30). Malate helps to preserve the integrity of the N-terminal phosphorylation domain of vascular plant PEPCs during extraction and subsequent purification (1). With these buffer additions, partial degradation of p107 was prevented (Fig. 2, A and B). Moreover, two distinct peaks of PEPC activity were resolved during Superdex 200 FPLC (Fig. 1B).

Additional gel filtration via Superose 6 FPLC resulted in an approximately 200-fold purification of PEPC1 and PEPC2 to a final specific activity of approximately 10 units/mg (Table 1). Although Superose 6 FPLC did not increase the specific activity of PEPC1 or PEPC2 beyond that achieved at the Superdex 200 step, it was included to ensure a clean separation of PEPC1 from PEPC2. With the bichemonic acid protein assay (26) the specific activity of the final PEPC1 and PEPC2 preparations was increased to 24.2 and 29.2 units/mg, respectively. Calibration of the Superdex 200 column with molecular mass standards yielded respective native molecular masses of 410 ± 5 kDa for PEPC1 and 681 ± 9 kDa for PEPC2 (n = 3).

PAGE and Immunoblot Analysis—A Coomassie Blue- and PEPC activity-staining polypeptide that cross-reacted with anti-(B. napus PEPC) IgG was observed after the nondenaturing PAGE of PEPC1 and PEPC2 (Fig. 4, A–C, lanes 1 and 2). This analysis was consistent with the respective native Mr estimates.
resolved a protein-staining p107 that cross-reacted with anti-PEPC IgG (Fig. 4, lanes 2 and 3). However, PEPC2 contained two additional protein-staining polypeptides of approximately 98-kDa (p98) and 57-kDa (Fig. 2, lane 3). When the protein- and PEPC activity-staining band obtained after nondenaturing PAGE of PEPC2 was excised, equilibrated with SDS, and subjected to SDS-PAGE, p107 and p64 were resolved (Fig. 2A, lane 4), indicating that the p64 was complexed with p107 in the native PEPC2. This result was corroborated by SDS-PAGE of fractions collected during analytical Mono-Q FPLC of PEPC2 in which both the p107 and p64 co-eluted with PEPC activity and a symmetrical A280 peak (Fig. 1C). The p64 was not recognized by the anti-(B. napus PEPC) IgG (Fig. 3A). Densitometric analysis of Sypro Red-stained SDS gels of Mono-Q-purified PEPC2 allowed us to estimate a p107:p64 molar ratio of 1:1. The native PEPC2 therefore appears to exist as an unusual hetero-octomeric complex composed of four p107 and four p64 subunits with a combined theoretical molecular mass of 684 kDa. This value closely agrees with the molecular mass of 681 kDa estimated for native PEPC2 during Superdex 200 FPLC. PEPC1, by contrast, is a typical PEPC homotetramer of p107 subunits, which likely correspond to the same p107 found in PEPC2.

Nondenaturing PAGE of clarified extracts (prepared in buffer A) followed by in-gel PEPC activity staining indicated that the ratio of PEPC1:PEPC2 progressively increases during COS development such that only PEPC1 is detected in stage IX endosperm (Fig. 4, lane 1). By contrast, PEPC2 was much less heat labile, losing 0, 20, and 100% of its original activity when incubated for 3 min at 45, 50, and 55 °C, respectively. PEPC2 was also much less heat labile, losing 0, 19, 25, 40, 82, and 100% of its original activity when incubated for 3 min at 45, 50, and 55 °C, respectively.

N-terminal Sequencing and Mass Spectrometry—The 20 N-terminal amino acids of the p107 and p98 of proteolyzed PEPC were sequenced by Edman degradation (Fig. 3). BLAST analysis revealed significant matches with the corresponding region of various plant PEPCs and included the conserved regulatory seryl phosphorylation site found in all plant PEPCs.
examined to date (Fig. 3). The sequences of 12 amino acid residues of the N termini of the p107 of PEPC1 and PEPC2 were determined and found to be identical to that of the p107 of proteolyzed PEPC preparation (see Fig. 2, A and B, lane 1). Other PEPC sequences were derived by translation of the corresponding genes. Sequence numbering represents amino acid position relative to the N terminus. Hyphens denote amino acid residues that are identical to those of the respective COS PEPC sequences. An asterisk indicates the conserved regulatory seryl phosphorylation site, and underlined letters indicate the consensus target sequence for plant PEPC protein kinase (1).

Kinetic Properties

Effect of pH and PEP Saturation Kinetics—Similar to other plant PEPCs (1), PEPC1 and PEPC2 activity increased with pH in the range of 6.5–8.0. However, PEPC1 exhibited optimal activity at pH 8.5, whereas PEPC2 displayed optimal activity at pH 8.0 (Fig. 6). Moreover, PEPC1 displayed a significantly greater sensitivity to pH changes within the physiological range. Between pH 8 and 6.5, PEPC1 activity decreased by more than 30-fold, whereas PEPC2 activity decreased by less than 3-fold (Fig. 6).

Pep saturation kinetics and response to metabolite effectors

Table I

| Step               | Activity | Protein | Specific activity | Purification | Yield |
|--------------------|----------|---------|------------------|--------------|-------|
| Clarified extract  | 270      | 5.472a  | 0.049            | 1            | 100   |
| PEG fractionation  | 234      | 2.848a  | 0.082            | 1.9          | 87    |
| Butyl-Sepharose    | 173      | 538a    | 0.32             | 6.5          | 64    |
| DEAE Fractogel     | 162      | 24.8a   | 6.5              | 133          | 60    |
| Superdex 200       |          |         |                  |              |       |
| PEPC1              | 32.4     | 3.4a    | 9.5              | 194          | 12    |
| PEPC2              | 43.0     | 4.1a    | 10.5             | 214          | 16    |
| Superose 6         |          |         |                  |              |       |
| PEPC1              | 11.0     | 1.1a    | 10.0             | 204          | 4.1   |
| PEPC2              | 9.5      | 0.92a   | 10.3             | 210          | 3.5   |

a Determined with Coomassie Blue G-250 (25).

b Determined with bicinchoninic acid (26).
Fig. 4. Nondenaturing PAGE (5% gel) analysis of COS PEPC isoforms. A, staining was performed with Coomassie Blue R-250. Lanes 1 and 2, respectively, contain 1 μg each of purified PEPC1 and PEPC2. B, immunoblot analysis was performed using affinity-purified rabbit anti-(B. napus PEPC) IgG (8). Lanes 1 and 2, respectively, contain 50 ng of purified PEPC1 and PEPC2. C, in-gel PEPC activity staining was performed as described (9). Lanes 1 and 2, respectively, contain 1 μg each of purified PEPC1 and PEPC2. The remaining lanes contain clarified extracts from various stages of COS development, as described in the legend for Fig. 2. Inset, corresponding PEPC activity stained in COS endosperm (means ± S.E., n = 3).

Fig. 5. Q-TOF MS/MS analysis of p64 tryptic peptides. A, Q-TOF data were submitted to the Mascot search engine and used to match the nonredundant NCBI database. All significant matches (MOWSE score > 34) are shown. B, predicted primary sequence of a putative PEPC from rice (O. sativa cv. Japonica) (GenBankTM GI number 13486658). Peptides found in the tryptic digest of p64 are underlined. Boldface sequences represent conserved functional domains (1).

were determined at pH 8 and 7.3. Hyperbolic PEPC saturation kinetics was always observed. At pH 8.0 and 7.3 the respective V_max,app values of PEPC1 and PEPC2, which were both approximately 0.06 and 0.12 mU at pH 8 and 7.3, respectively.

**Metabolite Effects**—A wide variety of compounds were tested as possible effectors of PEPC1 and PEPC2 at pH 7.3 and pH 8 with subsaturating PEP (0.2 mM). The following compounds exerted little or no influence on the activity of either isoform (± 20% of the control rate): 2-P-glycerate, dihydroxyacetone-P, Fru-1,6-P_2, NAD^+^, Gly, Glu, Arg, Ala, Leu, Asn, Phe, pyruvate, and AMP (2 mM each); CoA, malonyl-CoA, acetyl-CoA, and oleoyl-CoA (50 μM each). Table II lists those compounds that significantly influenced PEPC activity. Similar to other plant PEPCs (1), PEPC1 and PEPC2 displayed pH-dependent modulation by several metabolites that were more effective at pH 7.3 than at pH 8. PEPC1, however, was much more sensitive to the various metabolite effectors than PEPC2 (Table II). PEPC1 was potently activated at pH 7.3 by the hexose-mono-Ps and by glycerol-3-P, whereas PEPC2 was only weakly activated by these compounds. Similarly, PEPC1 was far more sensitive to inhibition by malate, Asp, Glu, and ATP, relative to PEPC2. Fig. 7 demonstrates the marked differential response of PEPC1 and PEPC2 activity to increasing concentrations of the most widely recognized allosteric effectors of plant PEPC (1), namely malate and Glc-6-P.

**DISCUSSION**

When partial *in vitro* proteolysis of p107 was prevented, two COS PEPC isoforms that significantly differed in their physical and kinetic properties were resolved by Superdex 200 FPLC and highly purified. Tissue- and/or developmentally specific PEPC isoforms are known to occur in vascular plants (1) and widely recognized allosteric effectors of plant PEPC (1), namely malate and Glc-6-P.
Influence of various metabolites on the activity of COS PEPC1 and PEPC2

| Addition    | PEPC1 pH 7.3 | PEPC2 pH 7.3 | PEPC1 pH 8 | PEPC2 pH 8 |
|-------------|--------------|--------------|------------|------------|
| Glc-6-P     | 200 (0.17)   | 121          | 118        | 103        |
| Glc-1-P     | 164          | 118          | 107        | 101        |
| Fru-6-P     | 200 (0.87)   | 121          | 127        | 108        |
| Glycerol-3-P| 193 (1.2)    | 122          | 116        | 107        |
| Malate      | 4 (0.07)     | 41 (1.6)     | 78         | 83         |
| Glu         | 26 (2.0)     | 73 (11.0)    | 97         | 95         |
| Asp         | 5 (0.4)      | 51 (7.0)     | 95         | 101        |
| ATP         | 35           | 87           | 83         | 98         |

**TABLE II**

![Graph showing the influence of various metabolites on the activity of COS PEPC1 and PEPC2.](image)

**Fig. 7.** Influence of malate and Glc-6-P on the activity of PEPC1 and PEPC2. Assays were conducted at pH 7.3 with substratating PEP (0.2 mM) in the presence of various concentrations of malate or Glc-6-P. All values represent the means (±S.E.) of three separate determinations.

transamination reactions required to support storage protein synthesis. The inhibition of PEPC1 by Asp and Glu provides a tight feedback control that could closely balance PEPC1 activity with the production of C-skeletons (i.e. oxaloacetate, 2-oxoglutarate) required for NH₃ assimilation or transamination reactions. The “effector-insensitive” PEPC2, by contrast, may facilitate PEP flux to malate in support of leucoplast fatty acid synthesis despite the significant malate levels present in developing COS (18). Nonenaturing PAGE of clarified COS extracts followed by in-gel PEPC activity staining revealed that PEPC2 increases during COS development, peaking at stage VII, and then rapidly disappears during COS maturation (Fig. 4C). This pattern parallels triglyceride accumulation in this tissue, which also peaks at stage VII (34). The developmental profile for PEPC1 (Fig. 4C), by contrast, parallels that of storage protein accumulation (24), with both becoming maximal during the maturation phase of COS development. Further studies using transgenic plants and/or pharmacological inhibitors will help to fully evaluate the metabolic functions of COS PEPC1 and PEPC2.

It remains to be determined whether and how COS PEPC1 and PEPC2 interconvert. However, protein-kinase-mediated phosphorylation of p102 appears to be involved in the control and structural organization of green algal (S. minutum) Class 2 PEPCs (23). COS p107 contains the N-terminal regulatory seryl phosphorylation site characteristic of most plant PEPCs (Fig. 3A). It will be of interest to determine whether COS PEPC1 and PEPC2 are interconverted via a phosphorylation-dephosphorylation mechanism involving p107 and/or p64.

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