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Raymond Chollet
University of Nebraska - Lincoln, rchollet1@unl.edu

Jean Vidal
Université de Paris-Sud

Marion H. O'Leary
University of Nebraska - Lincoln

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Raymond Chollet

Department of Biochemistry,
University of Nebraska–Lincoln,
Lincoln, NE 68588-0664, USA

Jean Vidal

UA CNRS D-1128, Institut de Biotechnologie des Plantes,
Université de Paris-Sud,
Orsay Cedex, 91405 France

Marion H. O'Leary

Department of Biochemistry,
University of Nebraska–Lincoln,
Lincoln, NE 68588-0664, USA

Abstract

Since plant phospho*enol*pyruvate carboxylase (PEPC) was last reviewed in the *Annual Review of Plant Physiology* over a decade ago (O'Leary 1982), significant advances have been made in our knowledge of this oligomeric, cytosolic enzyme. This review highlights this exciting progress in plant PEPC research by focusing on the three major areas of recent investigation: the enzymology of the protein; its posttranslational regulation by reversible protein phosphorylation and opposing metabolite effectors; and the structure, expression, and molecular evolution of the nuclear PEPC genes. It is hoped that the next ten years will be equally enlightening, especially with respect to the three-dimensional structure of the plant enzyme, the molecular analysis of its highly regulated protein-Ser/Thr kinase, and the elucidation of its associated signal-transduction pathways in various plant cell types.

Keywords: PEP carboxylase (PEPC), catalytic reaction mechanism, regulatory protein phosphorylation, gene structure, expression, and evolution

Contents

Introduction

Phospho*enol*pyruvate carboxylase (PEPC; EC 4.1.1.31) is a ubiquitous cytosolic enzyme in higher plants and is also widely distributed in bacteria, cyanobacteria, and green algae (68, 114). It catalyzes the irreversible β-carboxylation of Phospho*enol*pyruvate (PEP) in the presence of HCO_3^- and Me^{2+} to yield oxaloacetate (OAA) and Pi and thus is involved intimately in C_4-dicarboxylic acid metabolism in plants. Besides its cardinal roles in the initial fixation of atmospheric CO_2 during C_4 photosynthesis and Crassulacean acid metabolism (CAM), PEPC functions anaplerotically in a variety of nonphotosynthetic systems such as C/N partitioning in C_3 leaves, seed formation and germination, and fruit ripening (66, 68). Nonphotosynthetic isoforms of PEPC also play specialized roles in guard-cell C metabolism during stomatal opening (90) and plant host–cell C_4-acid formation in N_2-fixing legume root nodules (19, 115).

Since 1982, when PEPC was reviewed last in the *Annual Review of Plant Physiology* (87), many new and significant findings about this oligomeric enzyme have been made. In addition to the further elucidation of its catalytic reaction mechanism and the initiation of structure-function analyses by site-directed mutagenesis, there has been an explosion in research related to the posttranslational regulation of the enzyme's activity and allosteric properties by reversible protein phosphorylation and to PEPC gene (*Ppc*) structure, expression, and molecular evolution. These exciting new advances in plant PEPC research are the primary focus of this review, while only limited refer-

ence will be made to the microbial enzyme. The interested reader should consult earlier reviews on PEPC for additional breadth and detail (3, 19, 35, 54, 68, 84, 87, 90, 96, 104, 114, 117).

Enzymology of PEP Carboxylase

Comments on Isolation of PEPC

It is now amply documented that native leaf and recombinant forms of PEPC are highly susceptible to limited proteolysis near the N-terminus during extraction and subsequent purification (6, 9, 23, 77, 82, 121). While such modification has no major influence on the enzyme's electrophoretic mobility, V_{max}, and carbon-isotope effects, removal of this plant-invariant N-terminal domain markedly decreases the in vitro phosphorylatability and sensitivity of PEPC to its negative allosteric effector L-malate. Thus, it is our view that many earlier kinetic analyses of purified or commercial plant PEPC have probably been compromised by this N-terminal truncation (see comments in 23, 54, 68). More recent studies have preserved the enzyme's integrity during isolation by the inclusion of glycerol, L-malate, and proteinase inhibitors (especially chymostatin) and by the use of rapid purification protocols that exploit fast-protein liquid chromatography, HPLC, or immunochromatography (4, 7, 9, 23, 58, 77, 82, 119, 121, 136). With such strategies, preparations of intact, N-blocked leaf (C_4, CAM, C_3), nodule, and recombinant PEPC are readily obtained.

Carboxylation and Hydrolysis of PEP Analogs

A variety of PEP analogs have been examined as substrates for C_4 PEPC (see 35 and Table 1). Although a number of compounds are processed by the en-

Table 1. Activity of PEP analogs with PEPC[a]

Compound	Vmax (rel)[b]	% Carboxylation	% Hydrolysis	References
PEP	100	97	3	6
(E)-3-fluoro-PEP	5	86	14	32, 50
(Z)-3-fluoro-PEP	5	3	97	32, 50
(Z)-3-chloro-PEP	25	25	75	71
Alleno-PEP	90	0	100	126
Thio-PEP	9	0	100	103
(Z)-3-methyl-PEP	4	0	100	31, 33, 34, 86
(Z)-3-bromo-PEP	25	0	100	21
3,3-dimethyl-PEP	2	0	100	31

[a] Values given are for the maize leaf enzyme in the presence of Mg2+.
[b] Carboxylation plus hydrolysis.

zyme, most are not carboxylated but instead are hydrolyzed to pyruvate derivatives (Equation 1) by a mechanism that shares several steps with catalysis (see section on Catalytic Mechanism of PEPC). This phosphatase activity is probably not related to the much slower bicarbonate-independent hydrolysis of phosphoglycolate and phospholactate that is also catalyzed by the enzyme (48, 50).

$$R-CH=\overset{\overset{\displaystyle O-PO_3{}^{2-}}{\displaystyle |}}{C}-CO_2{}^- + HCO_3{}^- \rightarrow {}^-O_2C-CHR-\overset{\overset{\displaystyle O}{\displaystyle \|}}{C}-CO_2{}^- + R-CH_2-\overset{\overset{\displaystyle O}{\displaystyle \|}}{C}-CO_2{}^- + Pi \quad (1)$$

PEP itself also undergoes a few percent of an $HCO_3{}^-$-dependent pyruvate formation. This hydrolysis is a minor component of the overall reaction flux with Mg^{2+} under in vivo conditions (<5%), but it increases with other metal ions and constitutes over 50% of the total reaction flux when Ni^{2+} is used (6). Interestingly, the PEP analog in which the phosphate has been replaced by a sulfate is not a substrate for the enzyme and, in fact, this compound does not bind to the active site [but it is a substrate for pyruvate kinase (93)].

Functional analogs for CO_2 and $HCO_3{}^-$ are rare in enzymatic reactions. In the case of PEPC, $HCO_2{}^-$ can replace $HCO_3{}^-$, forming formyl-P and pyruvate at a rate that is about 1% of that for PEP carboxylation (48).

Kinetic and Isotopic Studies

Early thinking about the catalytic mechanism was dominated by the seminal observation of Maruyama et al (72) [recently confirmed by O'Leary & Hermes (88)] that ^{18}O-labeled $HCO_3{}^-$ gives products containing one equivalent of ^{18}O in Pi and two in the γ-carboxyl of OAA. This isotope transfer persists with a number of other substrates, including those that undergo hydrolysis rather than carboxylation. (Z)-3-methyl-PEP gives more than one equivalent of ^{18}O in Pi and also gives ^{18}O incorporation into reisolated starting material after partial reaction (29, 86). A similar phenomenon is observed with 3-fluoro-PEP; exchange is eight times faster than substrate consumption (50). These observations indicate that the initial steps in the carboxylation mechanism are reversible (see section on Catalytic Mechanism of PEPC).

PEPC has been subjected to a variety of kinetic studies over the years, but these have generally been qualitative in nature because investigators failed to rigorously control $HCO_3{}^-$ concentrations and to account for the presence of PEP-metal complexes. Recent studies of initial velocity patterns varying the two substrates and Me^{2+} indicate that there is a high level of synergism in the binding of substrates (49). Mg^{2+} binds first, and this binding is at equilibrium; PEP binds second; $HCO_3{}^-$ binds third; and all three have to be present before the reaction begins.

The small carbon-isotope effect ($k^{12}/k^{13} = 1.003$) that accompanies the carboxylation of PEP by PEPC has been of interest in connection with studies of isotope fractionation in plants (28). The carbon-isotope fractionation by PEPC is independent of the phosphorylation state of the enzyme and the presence or absence of the N-terminal phosphorylation domain, and nearly independent of pH (50, 89, 124; P Paneth & S Madhavan, unpublished data). This fractionation is small compared to what would be expected if C-C bond formation were simply rate determining. Instead, some step prior to C-C bond formation must be rate limiting.

The oxygen-isotope effect for the bridging oxygen of PEP is large (k^{16}/k^{18} = 1.0056) when the HCO_3^- concentration is low, but the value decreases to 0.994 at high [HCO_3^-], consistent with the ordered stepwise mechanism given below (30). Deuterium-isotope effects for PEP-3,3-d$_2$ are 0.94 on *V* and 0.95 on *V/K*, also consistent with the stepwise mechanism (D Arnelle & MH O'Leary, unpublished data).

Carbon-isotope effects on the (*E*) and (*Z*) isomers of 3-fluoro-PEP provide an interesting contrast (50). The (*E*) isomer has a small carbon-isotope effect (1.009), consistent with rate-determination phosphate transfer. However, the (*Z*) isomer [which mostly gives hydrolysis rather than carboxylation (Table 1)] shows a large isotope effect (1.049), which is apparently associated with the loss of CO_2 from the complex during catalysis.

Several stereochemical probes have been used to define PEPC catalysis. Early work by Rose et al (97) demonstrated that carboxylation of PEP occurs on the si face of the substrate, and carboxylation of the two isomers of 3-fluoro-PEP occurs on the same face (50). When (*Z*)-3-methyl-PEP is hydrolyzed by PEPC in D$_2$O, the 3-D-α-ketobutyrate that is produced is racemic, which indicates that protonation of the enolate occurs in solution rather than on the surface of the enzyme (33). The stereochemistry of substitution at phosphorus can be determined by using PEP containing S, ^{16}O, and ^{17}O in nonbridging positions of the phosphate ester. Carboxylation in H$_2$^{18}O produces a chiral thiophosphate with inversion of configuration at phosphorus (39). Thus, substitution at phosphorus occurs by an in-line mechanism.

Active-Site Structure

Mn-EPR studies of PEPC with PEP and various substrate analogs suggest that PEP itself is bidentate coordinated to the metal. Metal coordination in the enolate intermediate is to the enolate oxygen, the carboxyl oxygen, and a phosphate oxygen (5).

Results of chemical modification studies on various plant PEPCs with group-selective reagents have suggested that Cys, His, Arg, and Lys are essential for activity (3, 96, 104). To date, only one such residue has been identified in the plant primary structure—Lys-606 in maize PEPC (57). Further-

more, the complete absence of Cys in PEPC from *Thermus* sp., a thermophilic bacterium, excludes the direct involvement of these residues in catalysis (79A). Site-directed mutagenesis studies of the active-site domain of PEPC have thus far been performed only with the enzyme from *Escherichia coli*. His-138 (*E. coli* numbering) is required for carboxylation, but the mutant H138N is able to catalyze PEP hydrolysis to pyruvate in the presence of HCO_3^- (109, 112). His-579 is not obligatory for catalysis, in spite of the fact that it is species- invariant (111). Replacement of conserved Arg-587 by Ser also gives an enzyme that catalyzes hydrolysis, but not carboxylation (112, 134). Figure 1 indicates these targeted, species-invariant Lys, His, and Arg residues in the deduced primary structure of *Sorghum* C_4 PEPC.

Along with site-directed mutagenesis, X-ray crystallography has become the sine qua non of enzymology. Alas, PEPC does not yet seem to have yielded to the efforts of crystallographers. The *E. coli* enzyme has been reported to give crystals that diffract X-rays (46). We are also aware of attempts in other laboratories to obtain diffraction-quality crystals of recombinant PEPC from various plant sources, but no substantial progress in this area has been reported.

Catalytic Mechanism of PEPC

The information cited above permits presentation of a relatively convincing mechanism for action of PEPC (Figure 2). Substrates and Me^{2+} bind in the preferred order metal, PEP, HCO_3^-. The first chemical step is phosphate transfer to form carboxyphosphate and the enolate of pyruvate, as perhaps first suggested by Walsh (118). Stereochemical studies require that the transition state for this step is linear at phosphorus; thus, the carbonyl carbon in the intermediate carboxyphosphate following transfer is quite far from carbon-3 of the enolate, and a conformational change is required to place the two carbons near each other. The most parsimonious way to accomplish this is to have an enzyme base deprotonate the carboxyl group of carboxyphosphate, after which carboxyphosphate decomposes to form enzyme-bound CO_2 and Pi. Earlier mechanisms (87) did not recognize this aspect. This step brings CO_2 above the plane of the enolate and within bonding distance of its carbon-3. CO_2 in this intermediate is sequestered so that under optimum catalytic conditions it seldom escapes [3% (6)], but under other circumstances CO_2 is lost easily, as when the metal ion is changed in such a way as to lower the reactivity of the enolate. In the case of a variety of PEP analogs, loss of CO_2 competes effectively with carboxylation (cf Table 1). In some cases, the formation of enzyme-bound CO_2 must be reversible. Isotope exchange studies on (Z)-3-methyl-PEP (29, 86) and 3-fluoro-PEP (50) require that CO_2 is formed reversibly and can scramble isotopes and return to starting material. It is not clear whether CO_2 formation is reversible in the case of PEP. Isotope-effect results suggest that it is not.

1   *
MASERHHSIDAQLRALAPGKVSEELIQYDALLVDRFLDILQDLHGPSLREFVQECYEVSADYEGKKDTSKLGELG

76
AKLTGLAPADAILVASSILHMLNLANLAEEVELAHRRRNSKLKHGDFSDEGSATTESDIEETLKRLVSLGKTPAE

151   •
VFEALKNQSVDLVFTAHPTQSARRSLLQKNARIRNCLTQLSAKDVTVEDKKELDEALHREIQAAFRTDEIRRAQP

226   **1**
TPQDEMRYGMSYIHETVWNGVPKFLRRVDTALKNIGINERLPYDVPLIKFCSWMGGDRDGNPRVTPEVTRDVCLL

301   **2**
SRMMAANLYINQVEDLMFELSMWRCNDELRARAEEVQSTPASKKVTKYYIEFWKQIPPNEPYRVILGAVRDKLYN

376   **3**   **4 5**
TRERARHLLATGFSEISEDAVFTKIEEFLEPLELCYKSLCECGDKAIADGSLLDLLRQVFTFGLSLVKLDIRQES

451
ERQTDVIDAITTHLGIGSYRSWPEDKRMEWLVSELKGKRPLLPPDLPMTEEIADVIGAMRVLAELPIDSFGPYII

526   •
SMCTAPSDVLAVELLQREMWHSPAVPVVPLFERLADLQAAPASVEKLFSTDWYINHINGKQQVMVGYSDSGKDAG

601   •   •
RLSAAWQLYVAQEEMAKVAKKYGVKLTLFHGRGGTVGRGGGPTHLAILSQPPDTINGSIRVTVQGEVIEFMFGEE

676
NLCFQSLQRFTAATLEHGMHPPVSPKPEWRKLMEEMAVVATEEYRSVVVKEPRFVEYFRSATPETEYGKMNIGSR

751
PAKRRPGGGITTLRAIPWIFSWTQTRFHLPVWLGVGAAFKWAIDKDIKNFQKLKEMYNEWPFFRVTLDLLEMVFA

826
KGDPGIAGLYDELLVAEELKPFGKQLRDKYVETQQLLLQIAGHKDILEGDPYLKQGLRLRNPYITTLNVFQAYTL

901   **960**
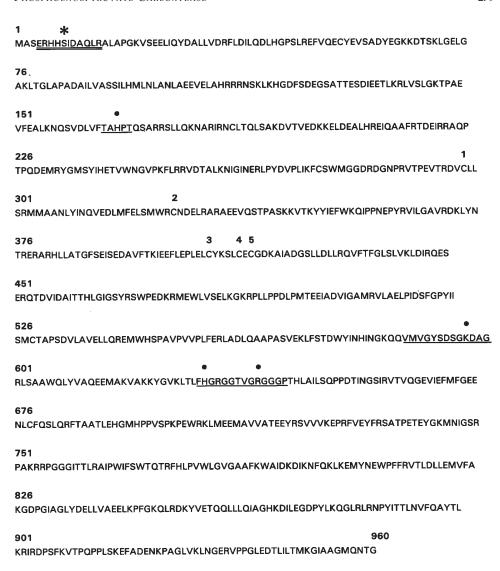KRIRDPSFKVTPQPPLSKEFADENKPAGLVKLNGERVPPGLEDTLILTMKGIAAGMQNTG

Figure 1. Deduced amino-acid sequence of the C_4-PEPC isoform from *Sorghum* (67). The plant-invariant phosphorylation domain, with its target Ser (*), is underlined twice, whereas the species-invariant functional regions identified to date are underlined singly. The specific His, Lys, and Arg residues targeted by site-directed mutagenesis or chemical modification (see text) are indicated within these three domains (•). C (1–5), plant-invariant Cys residues.

In the final chemical step of the overall reaction, CO_2 combines with the metal-stabilized enolate. It is interesting to note that there is no evidence that this step is reversible, even though the reverse reaction (decarboxylation of metal-chelated OAA) is well known in other systems. Finally, Pi and OAA are released.

$$HCO_3^- + \ ^=O_3P\text{-}O \quad\quad\quad HO_2C\text{-}O\text{-}PO_3^= \quad + \ O^-$$

$$| \quad\quad\quad\quad\quad\quad\quad\quad\quad\quad |$$

$$CH_2{=}C\text{-}CO_2^- \ \rightleftharpoons \quad\quad\quad\quad CH_2{=}C\text{-}CO_2^- \ \rightleftharpoons$$

PEP   **Carboxy-P**   **Enolate**

$$HPO_4^= + CO_2 + \quad\quad O^- \quad\quad\quad\quad\quad\quad O$$

$$| \quad\quad\quad\quad\quad\quad\quad ||$$

$$CH_2{=}C\text{-}CO_2^- \ \rightarrow \ ^-O_2C\text{-}CH_2\text{-}C\text{-}CO_2^- + \ HPO_4^=$$

OAA

$$\downarrow$$

$$\quad\quad\quad\quad\quad\quad\quad\quad O$$

$$\quad\quad\quad\quad\quad\quad\quad\quad ||$$

$$HPO_4^= + CO_2 + CH_3\text{-}C\text{-}CO_2^-$$

PYR

Figure 2. Mechanism of carboxylation and hydrolysis of PEP by PEPC. PYR, pyruvate.

Posttranslational Regulation of PEP-Carboxylase Activity

It is well documented that the activity of the various isoforms of plant PEPC are subject to allosteric control by a variety of positive [e.g. glucose 6-P (G6P), triose-P] and negative (e.g. L-malate, Asp) metabolite effectors, especially when assayed at suboptimal pH values that approximate that of the cytosol (e.g. 3, 23, 65, 66, 68, 90, 100, 101, 104). For example, the K_i(L-malate) of the intact recombinant C_4 enzyme from *Sorghum* is decreased about 25-fold at pH 7.3 compared with pH 8.0 (23). Although changes in the cytosolic levels of these opposing allosteric effectors and H^+ likely contribute to the overall regulation of PEPC activity in vivo (22, 26, 54, 66, 90), research over the past decade has focused primarily on the reversible phosphorylation of the enzyme. In fact, cytosolic PEPC and sucrose-P synthase presently represent the two best-defined examples of in vivo regulatory enzyme phosphorylation in plants (40A, 41).

Regulatory Phosphorylation of Photosynthetic PEPC

The regulatory phosphorylation of photosynthetic PEPC has been intensively studied and recently reviewed (41, 54, 68, 84, 96, 117) since the initial observations were published about ten years ago on the CAM and C_4 isoforms (10, 38, 51, 80, 81, 82). As an important prelude to these protein-phosphorylation studies, several reports had appeared that indicated that both photosynthetic PEPC isoforms were subject to a striking diel regulation in vivo that altered the enzyme's activity and/or sensitivity to L-malate under near-physiological assay conditions, without accompanying changes in V_{max} or PEPC amount (e.g. 42, 60, 81, 125). It thus became evident that the CAM enzyme was upregulated at night and downregulated during the day, thereby paralleling the classical changes in CAM physiology (e.g. leaf atmospheric CO_2 fixation and titratable acidity) (66). Related investigations of several CAM plants under *continuous* night or day conditions indicated that CAM physiology, as well as the L-malate sensitivity of PEPC, was controlled by an endogenous circadian rhythm rather than by light or dark signals per se (83, 125). In marked contrast, C_4 PEPC was shown to be reversibly light activated in vivo by a mechanism that was dependent, either directly or indirectly, on photosynthesis and modulated by the incident photosynthetic photon flux density above a minimum threshold of about 300 µmol m^{-2} s^{-1} (7, 36, 55, 60, 78, 98).

It is now established unequivocally by a wealth of in vivo and in vitro data that this striking diel regulation is caused by changes in the phosphorylation state of a single serine residue near the ~110-kDa subunit's N-terminus (e.g. Ser-8 and Ser-15 in the *Sorghum* and maizee C_4 enzymes, respectively, and Ser-11 in PEPC from the facultative CAM plant *Mesembryanthemum crystallinum*) (9, 23, 53, 58, 110, 121). Upregulation/phosphorylation of the target enzyme is catalyzed by a highly regulated (see below) protein kinase and downregulation/dephosphorylation by a typical mammalian-type protein phosphatase 2A (7, 11, 12, 12A, 27, 52, 53, 55, 56, 78). It is notable that this target Ser resides in a plant-invariant motif [E/DR/Kxx-SIDAQL/MR (see Figure 1)] that is absent in the bacterial and cyanobacterial primary structures deduced to date (67, 68, 79A, 96A, 114). Moreover, in vitro studies with the intact, recombinant *Sorghum* C_4 enzyme have established that phosphorylation of this N-terminal domain not only renders PEPC considerably less sensitive to inhibition by L-malate under near-physiological assay conditions (~sevenfold increase in K_i) but, conversely, both more active and more sensitive to activation by G6P (~fivefold decrease in K_a) (23, 26). Thus, this reversible means of fine tuning the activity and allosteric properties of PEPC is unique to the plant enzyme.

The molecular mechanism by which protein phosphorylation regulates C_4 PEPC has recently been addressed by site-directed mutagenesis and

chemical modification. The introduction of a monoanionic residue at position 8 in the recombinant *Sorghum* enzyme by directed mutagenesis (S8D) or sequential mutagenesis (S8C) and *S*-carboxymethylation functionally mimics the specific effects of regulatory phosphorylation on the target enzyme. In contrast, various neutral substitutions (S8T, S8Y, S8C, *S*-carboxamidomethylated S8C) are without major influence (23, 25, 121; GB Maralihalli, V Pacquit, B Li, JA Jiao, G Sarath, et al, unpublished data). Consequently, addition of negative charge to this N-terminal domain by reversible phosphorylation appears crucial to this regulatory mechanism, but the exact details must await the high-resolution crystal structures of the dephospho and phospho (or S8D) enzyme-forms.

Recent research on the phosphorylation of C_4 and CAM PEPC has focused on the physiologically relevant protein kinase and its requisite signal-transduction chain. This work took on special significance with the near-simultaneous discoveries that the C_4 and CAM PEPC kinases were both activated reversibly in vivo by some mechanism involving cytosolic protein turnover, thereby resulting in the upregulation of the kinase and, thus, its target enzyme in the light (C_4) or at night (CAM) (7, 12, 27, 55, 56, 78). Not only is the CAM kinase activated at night under the control of a circadian rhythm, but it is also coinduced with its protein-substrate during C_3 to CAM switching in the facultative CAM species *M. crystallinum* (12, 12A, 70). In contrast, the activity state of the type 2A PEPC-phosphatase catalytic subunit appears to be relatively constant during light-dark (C_4) or day-night (CAM) transitions (12, 56, 78), further underscoring the critical role of the kinase in the PEPC-phosphorylation cycle.

Following the initial report by Jiao & Chollet (52), the extremely low-abundance PEPC kinase has been partially purified about 4000-fold. It is likely to be a monomer of ~37/30-kDa (C_4) or ~39/32-kDa (CAM) polypeptides (69, 70, 120). As isolated, this protein kinase catalyzes neither autophosphorylation nor the phosphorylation of heterologous substrates (e.g. casein, histone III-S, BSA, leaf sucrose-P synthase). Similarly, position-8 *Sorghum* C_4-PEPC mutants (e.g. S8Y, S8D, S8C) are not phosphorylated except for the Thr substitution (70, 120, 121; GB Maralihalli, V Pacquit, B Li, JA Jiao, G Sarath, et al, unpublished data). In contrast, all plant PEPC isoforms examined to date (C_4, CAM, C_3-leaf, root nodule) serve as substrates in vitro (70, 119, 120), but with a distinct preference for the corresponding PEPC kinase (B Li, XQ Zhang & R Chollet, unpublished data). Considerable effort has been expended to (re)investigate the Ca^{2+}-dependency of this protein kinase. It is our view that although a variety of other protein-Ser/Thr kinases, including C_4-leaf calmodulin-like domain protein kinase (CDPK) and mammalian protein kinase A, specifically phosphorylate the single target Ser in plant PEPC in vitro (7, 53, 69, 85, 110), *only* the Ca^{2+}-independent, 30- to 39-kDa PEPC kinase has been shown to be light-dark (C_4) or day-night (CAM)

regulated in vivo (69, 70). Notably, these differential activity states of the kinase are maintained throughout chromatography on various matrices and even following SDS-PAGE and subsequent renaturation (69; B Li & R Chollet, unpublished data). Thus, PEPC kinase is likely up/downregulated in vivo by some mechanism that modulates its amount (7, 12, 56, 69) or else by covalent modification rather than by some noncovalent means (e.g. regulatory subunit, tight-binding effector). Repeated attempts to demonstrate an effect of in vitro dephosphorylation by alkaline phosphatase on the activity states of the light (active) and dark (inactive) C_4 kinase and its component ~37/30-kDa polypeptides have proven unsuccessful (B Li & R Chollet, unpublished data).

The signal-transduction chains that impinge upon the highly regulated PEPC kinases are also a focus of current research. Initial studies using a chemical inhibitor-based approach with detached leaves (7, 12, 55, 56, 69, 78) have been supplanted by in situ analyses with isolated C_4 mesophyll cells and protoplasts and cell biology techniques (24A, 30A, 94, 117). It is now established that the light-induced C_4 transduction cascade is initiated in the illuminated chloroplast by photosynthesis and likely involves some "signal" from the light-activated Calvin cycle in the neighboring bundle sheath, possibly 3-P-glycerate (Figure 3). In addition, there is mounting in situ evidence for the involvement of increases in mesophyll-cytosol pH and $[Ca^{2+}]$, the latter perhaps modulating an upstream protein kinase (24A, 30A, 94, 117), together with the inhibitor-based data that implicate a key role for a cytosolic protein-synthesis event (7, 8, 30A, 56, 69, 94). In contrast, not much is known about the CAM PEPC kinase signal-transduction pathway other than its light independency and the involvement of a circadian rhythm and cytosolic protein turnover (Figure 3) (12, 12A, 83, 84). Clearly, this area would benefit from detailed in situ analyses of intact mesophyll protoplasts isolated from night and day leaves performing CAM.

Finally, the results from leaf CO_2-exchange studies have underscored the impact of the PEPC regulatory-phosphorylation cycle on C_4 photosynthesis and dark CO_2 fixation during CAM (8, 12, 12A). For example, when the activity states of PEPC kinase and, thus, its target enzyme were downregulated in vivo by short-term pretreatment with cytosolic protein-synthesis inhibitors in the light (C_4) or prior to the night period (CAM), net leaf CO_2 uptake was diminished markedly. In contrast, no effects were observed on the activation states of other nuclear-encoded, photosynthesis-related enzymes, stomatal conductance, or CO_2 uptake by a C_3 leaf (7, 8, 56). Thus, the phosphorylation of photosynthetic PEPC is a cardinal regulatory event that influences atmospheric CO_2 fixation; this mechanism enables this primary carboxylase to function in the leaf cytosol even in the presence of the millimolar levels of C_4 acids (e.g. L-malate) required for C_4 photosynthesis and CAM.
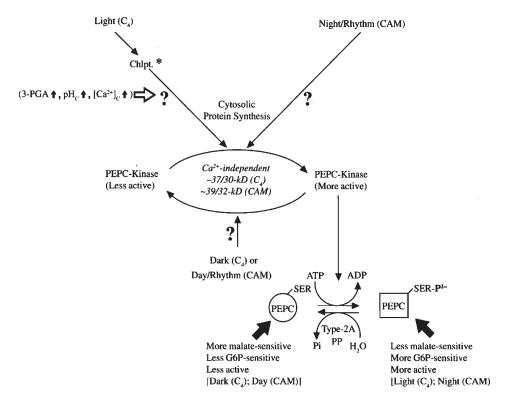
Figure 3. Proposed molecular mechanism for the light-dark (C_4) or night-day (CAM) regulation of the effector sensitivity [L-malate (negative), G6P (positive)] and activity of photosynthetic PEPC in the leaf mesophyll cell by reversible phosphorylation of a single target serine near the subunit's N-terminus [e.g. Ser-8 in *Sorghum* (see Figure 1)]. Chlpt. *, illuminated chloroplast; pH_c and $[Ca^{2+}]_c$, mesophyll cytosolic pH and $[Ca^{2+}]$, respectively; 3-PGA, 3-P-glycerate; PP, protein phosphatase. [Updated from Jiao & Chollet (54).]

Regulatory Phosphorylation of Nonphotosynthetic PEPC Isoforms

There is now convincing evidence that the reversible phosphorylation of the N-terminal domain of plant PEPC is widespread, if not ubiquitous. In vivo studies with [32]Pi have demonstrated the reversible phosphorylation of nonphotosynthetic PEPC in soybean root nodules (136) and in wheat leaves excised from N-deficient seedlings (24, 116). Complementary measurements of in vivo changes in PEPC activity and/or malate sensitivity under near-physiological assay conditions (i.e. low pH, low [PEP] relative to K_m) have underscored the regulatory nature of this covalent modification in nodules (136), illuminated C_3 leaves (24, 116; B Li, XQ Zhang & R Chollet, unpublished data), and *Vicia faba* guard cells microdissected from opening stomata

(135). Furthermore, related in vitro studies have established that PEPC kinase activity is present in soybean and alfalfa root nodules (102, 115), wheat and tobacco leaves (24, 119), and *Sorghum* roots (91), and have demonstrated this kinase's similarity to the C_4 and CAM enzymes with respect to its Ca^{2+} independency, chromatographic properties, and catalytic subunit(s) (24, 91, 119). The activity state of this PEPC kinase is modulated reversibly in vivo by a complex interaction between photosynthesis and N (C_3 leaves) or photosynthate supply to N_2-fixing root nodules (24; B Li, XQ Zhang & R Chollet, unpublished data). Thus, the phosphorylation of cytosolic PEPC by a highly regulated protein-Ser/Thr kinase is likely the major posttranslational mechanism for altering the allosteric properties and activity of this "multifaceted" plant enzyme in vivo.

Other Proposed Regulatory Mechanisms

Two other mechanisms have been proposed for the diel regulation of C_4 and CAM PEPC activity and/or sensitivity to L-malate based wholly on in vitro observations.

Dimer-Tetramer Interconversion — The Wedding laboratory found that CAM PEPC purified from day- and night-adapted *Crassula argentea* leaves exists as kinetically distinct but interconvertible oligomers (128). The day enzyme was mainly a malate-sensitive homodimer (α_2) and the night form a malate-"insensitive" homotetramer (α_4), with about a twofold higher K_i. PEP, G6P, Mg^{2+}, or a higher [PEPC] favors conversion of α_2 to α_4, whereas L-malate or a lower [PEPC] shifts the equilibrium toward the dimeric form (79, 128, 129). Similar in vitro association/dissociation properties have been reported for the active C_4 homotetramer from maize (123, 127). There is no evidence, however, to support the involvement of these aggregation-state changes in the diel regulation of the CAM and C_4 isoforms in vivo. On the contrary, several reports document that the phospho and dephospho C_4 and CAM enzyme forms are isolated in the same aggregation state while retaining the characteristic differential sensitivity to L-malate (4, 63, 77, 82, 122). Thus, it is our opinion that there is not a significant regulation of photosynthetic PEPC in vivo by changes in its aggregation state.

Redox Regulation — Even more speculative in our view is the proposal that the regulation of cytosolic C_4 PEPC may be primarily under the control of the redox state of certain critical cysteines (13, 45). While there are, indeed, five plant-invariant Cys residues in the various PEPC isoforms that are absent in the microbial enzymes (Figure 1) (67, 68, 79A, 114), none of them have been shown specifically to be involved in regulation of activity or L-malate sensitivity. On the contrary, related observations with the dephospho maize

enzyme indicate no effect of reduced cytosolic thioredoxin *h* on the properties of C_4 PEPC in vitro (52).

PEPC Gene Structure, Expression, and Molecular Evolution

Multigene Families

PEPC isoforms have been characterized in both photosynthetic and nonphotosynthetic tissues of various plants (reviewed in 68, 114). Consistent with the enzyme's functional diversity, small multigene families have been found. For example, three PEPC nuclear genes—*SvC3*, *SvC3RI*, and *SvC4*—have been characterized in *Sorghum* that encode the C_3-like housekeeping and root forms and the C_4-photosynthetic isoform, respectively (67). The maize family possesses at least five genes (37) that can be classified into three distinct groups (99). The C_4-PEPC gene is unique and is located near the centromere of chromosome 9. Three other genes have been mapped to different loci on chromosomes 4L, 5, and 7 (37, 47, 61). Both C_3 and C_4 species in the dicot genus *Flaveria* contain very similar families of distinct *Ppc* subgroups (40, 95). The C_4 isoform in *Flaveria trinervia* is encoded by the *PpcA* subgroup of the family. Homologous *PpcA* genes are found in the C_3 species *Flaveria pringlei*; however, they are weakly expressed, and their transcripts do not show the strict leaf-specific accumulation pattern found in the related C_4 species (40). In the facultative CAM plant *M. crystallinum*, two isogenes (*Ppc1*, *Ppc2*) have been described, and another distinct member might exist (17, 18); the transcriptional activity of *Ppc1* is strongly and selectively enhanced during C_3 to CAM switching induced by salt stress (18). The *Brassica napus* genome contains more than four highly similar PEPC genes, but some of them lack specific introns (133). PEPC gene families have also been found or suggested to exist in sugarcane, *Amaranthus*, tobacco, alfalfa, rice, wheat (reviewed in 68), and *Arabidopsis* (79B).

Ppc and PEPC Sequence Comparisons

The plant PEPC genes contain nine introns (with the exception reported in 133) of variable length but identical location with respect to the coding regions. Consensus intron/exon splice sites (a*GGT*aag—tgc*AG*g) are conserved. Generally, a classical gene organization is observed, although in some C_4- and C_3-type *Ppc* genes there is no typical TATA box, and multiple polyadenylation sites are found in the 3'-untranslated region (15, 43, 74, 132).

In alignments of all the deduced PEPC amino acid sequences reported, several highly conserved residues and motifs are found, and these likely contribute to the domains involved in the active site and/or regulation of the enzyme (see sections on Active-Site Structure and Regulatory Phosphorylation)

(67, 68, 79A, 96A, 114). Figure 1 exemplifies these structural features in the deduced sequence of the C_4-isoform from *Sorghum* (SvC4). The phosphorylation motif near the N-terminus (E/DR/KxxSIDAQL/MR), including the target Ser, and five cysteine residues, some of which have been proposed to be involved in redox regulation and/or stabilization of the tetrameric structure of the holoenzyme (13, 45), are specific to plant PEPC (68, 79A, 114). In addition, there are several species-invariant motifs in all PEPCs examined to date (TA**H**PT, VMxGYSDSx**K**DxG, F**H**GRGxxxx**R**GxxP) that contain specific His, Lys, and Arg residues implicated in the active-site domain (see section on Active-Site Structure; 3, 57, 79A, 96, 96A, 112). In general, the C-terminal half of the ~110-kDa PEPC polypeptide contains most of these presumed active-site determinants, whereas the N-terminal half appears to include the motifs that are regulatory in nature (53, 57, 110, 114). Further insight into the structure/function relationships of PEPC must await continued mutagenesis of these and other (114) highly conserved domains and, most importantly, high-resolution crystallographic analysis of the plant and microbial proteins.

Ppc Promoter Analysis and Transcription

The C_4-PEPC gene is expressed in photosynthetic tissues during greening via a phytochrome-mediated response (113). Expression of this gene is not necessarily coupled to the development of Kranz leaf anatomy because, in maize, it also occurs in such tissues as the inner leaf sheaths and tassels (43). In addition to light, cytokinins upregulate the transcriptional activity of the C_4-PEPC gene in maize leaves recovering from N deficiency (106), whereas in *Sorghum* abscisic acid (ABA) stimulates specific *Ppc* mRNA accumulation (2). In *M. crystallinum*, CAM-PEPC gene expression is induced by salt stress and/or ABA during C_3 to CAM switching, and these effects are moderated by light (76). In the CAM plant *Kalanchoë blossfeldiana*, changes in photoperiod and ABA are also involved in the induction of the photosynthetic PEPC gene (108). Lastly, C_3-type PEPC mRNAs are accumulated during the development of alfalfa root nodules (92, 115) and in recovering roots of N-deficient *Sorghum* (P Gadal, L Lepiniec & S Santi, unpublished data).

Light-responsive elements corresponding to those in the nuclear genes encoding the small subunit of Rubisco are lacking in the C_4-*Ppc* promoters of maize and *Sorghum*. Other conserved, direct repeated sequences (TTAC-CACTAGCTA), or the light-responsive element (CCTTATCCT) characterized previously in the promoter of light-inducible phytochrome genes, could play such a role, at least in part (15, 68, 74). The maize nuclear factor (MNF) (see below; 131) and *SV40* Sp1 (15, 68, 74) binding sites—(AAGG) and (CCGCCC), respectively—are also found in C_4-*Ppc* promoters. In addition, the presence of CpG islands (68) is consistent with the possible regulation of specific sites in

the promoters of both C_4 and C_3 PEPC genes by changes in DNA methylation status (64). In the *Sorghum* SvC3 and SvC3RI *Ppc* promoters, sequences homologous to the light-responsive element AT-1 (AATATTTTATA) and nod- (TCTACGTAGA) and G-boxes (CCACGTGG) are found (68). Both C_4 and C_3 species of *Flaveria* have orthologous C_4 genes (*PpcA* subgroup), the 5'-flanking regions of which are essentially homologous and share CCAAT, AT-1, and GT-1 III/IIIα boxes and an octameric motif known to confer cell-type specificity (40). It has been suggested that certain specific features of the C_4-PEPC gene promoter in *F. trinervia* could account for the much higher expression level in this C_4 species, including a light-responsive box II element, the microheterogeneity of the sequence around the TATA box, and the presence of a putative scaffold attachment region near the promoter that is often associated with highly expressed genes (40). Recent experiments using transgenic tobacco plants have shown that the sequences responsible for the enhanced, leaf-specific expression of C_4 *Ppc* in *F. trinervia* are located between positions −2118 and −500 relative to the transcription start site in the *PpcA* promoter (105); whether these sequences involve the above-mentioned proximal elements is not known.

Three leaf-specific DNA-binding proteins (MNF1, MNF2a, MNF2b) have been shown to interact specifically with the promoter of the maize C_4-PEPC gene (130, 131). Among these nuclear factors, MNF2a is presumed to act as a negative transcriptional effector (130). Two cDNA clones (MNB1a, MNB1b) encoding proteins that bind to an AAGG motif at the MNF1 site have been identified (131). Two other clones (designated 281, 282) may encode PEP1, a light-dependent factor interacting with the promoter of the maize C_4-PEPC gene (59). In *M. crystallinum*, salt stress causes three protein factors (PCAT-1, -2, and -3) to differentially recognize two AT-rich regions in the *Ppc1* promoter (16). Recently, several salt-responsive enhancer regions and one silencer region have been identified in this promoter (98A).

From the above, it is clear that data on nuclear *trans*-acting factors and the corresponding regulatory *cis*-acting DNA sequences of the *Ppc* promoters are still relatively scarce. Thus, no clear picture has emerged concerning the regulatory mechanisms that control the transcription rate of the different classes of PEPC genes in plants.

Transgenic Plants

In transgenic tobacco transformed with maize C_4-*Ppc1* genes containing the upstream regulatory region (about 2 kb), a low level of PEPC transcripts was produced; although their size was aberrantly large, accumulation still required light (44). These transformants possessed a twofold increase in PEPC activity that was correlated with the appearance of a high-K_m(PEP) C_4 form of the enzyme and an elevated level of leaf malate. However, these biochemical

changes did not result in any detectable physiological effects with respect to the rate of leaf net photosynthesis in air and to the CO_2 compensation concentration. In a related study, the maize C_4-PEPC gene was placed under the control of a CaMV 35S promoter (62). Although the transgenic tobacco plants contained *Ppc* transcripts of the correct size and about twice as much PEPC protein, their growth rate was retarded relative to that of the nontransformed plants. Transgenic tobacco plants transformed with either the C_4-PEPC gene from *Sorghum* or chimeric constructs containing the promoter of the C_4 gene from maize fused to the *gus*A reporter gene showed a high expression of transcripts as well as leaf mesophyll-cell specificity (75, 107). Similar results have been reported recently in transgenic rice using the same experimental strategy (73). Transgenic tobacco plants also expressed constructs containing various parts of the 5′-flanking region of the *PpcA1* (C_4-type) genes from both C_4 and C_3 species of *Flaveria* (105). In this heterologous system, only the C_4-*Ppc* promoter from the C_4 species conferred a high level of reporter gene expression, thus showing that it contains regulatory *cis*-elements responsible for abundant expression. In addition, a leaf palisade mesophyll-cell specificity was partially maintained in these transgenic tobacco plants. Hence, it appears that most of the regulatory elements that control the light-inducible expression of *Ppc* in C_4 leaves are also present in C_3 plants. On the other hand, although the CAM-specific *Ppc* promoter from the *M. crystallinum* gene is highly active in transgenic tobacco, it directs transcript synthesis in most cell types and lacks the salt inducibility found in its natural cellular environment (17). Finally, in homologous transient-expression systems using leaf-, stem-, and root-derived protoplasts from maize, a cell-specific expression pattern is largely dependent on the specific *Ppc* promoter used (99). In this system, transcript accumulation is not immediate but rather is related to light-dependent developmental changes, in contrast with other photosynthetic genes. This latter observation has led to the suggestion that distinct transduction pathways operate for the coordination of light-dependent genes encoding photosynthetic enzymes (99).

Molecular Evolution

Phylogenetic trees have been constructed using unambiguously aligned sites from the available PEPC amino acid sequences as well as on the basis of parsimony or distance analyses (1, 47, 61, 67, 68, 114). The cyanobacterial and bacterial PEPCs consistently group with prokaryotic phylogenetic relationships (68). As for the plant enzymes, phylogenetic relationships have been studied with particular emphasis on the molecular mechanisms that have shaped the expression characteristics and kinetic properties of PEPC during the evolutionary transition from C_3 into CAM and C_4 plants. The acquisition of these new photosynthetic strategies by a wide variety of plant species indicates that they have originated independently and on many

separate occasions during the evolution of flowering plants, with CAM being the antecedent of C_4 (47, 61, 67). Thus, an obvious question is how to account for the polyphyletic evolution of C_4 plants. From the various independently derived trees it can be inferred that all plant PEPC sequences diverged from a single common ancestral gene. On the other hand, the presence of different genes could have preceded angiosperm diversification and perhaps also that of higher plants. C_4-PEPC genes could have arisen from a duplication event long before the monocot-dicot divergence and thus prior
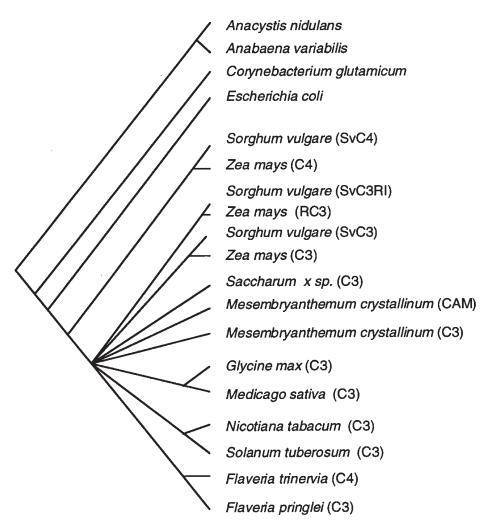

Figure 4   Consensus phylogenetic tree of 19 microbial and plant PEPCs. Branch lengths have no significance (see 67, 68 for details). SvC4, SvC3RI, and SvC3 are the photosynthetic, root, and housekeeping isoforms of *Sorghum vulgare* PEPC, respectively; *Zea mays* C4, RC3, and C3 are the corresponding isoforms in maize. (Redrawn from Lepiniec et al (68), with permission from Elsevier Science Ireland Ltd.)

to the appearance of C_4 plants. In this manner, the PEPC gene for C_4 photosynthesis could have evolved in a limited number of species while disappearing in others (47, 61, 67). In the consensus tree depicted in Figure 4, C_4-PEPCs from the monocots *Sorghum* and maize are clearly distinguishable from the various C_3 and CAM isoforms and also from their indigenous C_3 counterparts (e.g. SvC3RI, SvC3). In contrast, the photosynthetic enzyme in the C_4-dicot *F. trinervia* is more closely related to the various isoforms in C_3 and CAM dicots (40, 95) than to the two monocot C_4-PEPCs (68). Furthermore, because the promoters of the C_4-PEPC gene in *F. trinervia* and the orthologous gene in *F. pringlei* (C_3) are very similar, it has been suggested that a C_3 promoter could have been "tuned" to meet the special demands of C_4 photosynthesis (40). The possibility that an alternative evolution has led to the formation of C_4 enzymes in the various genera containing C_4 species could account for the observed divergence between monocots and dicots (40). Finally, it is not clear why a homologous form of C_4-PEPC is not found in dicots because, as mentioned above, a primordial PEPC form could have arisen before the divergence of monocots and dicots (68). Further investigations involving PEPC sequences from different genera are required to refine the phylogenetic relationships of the microbial and plant enzymes, including sequence analysis of green algal PEPCs and additional gymnosperm species (*Picea abies*) (96A).

Conclusions and Future Prospects

While the past decade has seen a number of truly impressive revelations concerning PEPC, future research awaits the results of three-dimensional structure studies that will provide another important chapter in PEPC mechanism, regulation, allosteric effects, and other areas. In addition, the emerging pictures of the highly regulated PEPC kinase, together with its requisite signal-transduction cascades, must be completed. Related work on the heteromeric intracellular form of the type 2A protein phosphatase that dephosphorylates plant PEPC in the cytosol will also be important (cf 122A). With the recent generation of the first C_4 PEPC-deficient mutant in the dicot *Amaranthus edulis* (20) and the development of an efficient, *Agrobacterium*-mediated transformation system for C_4 dicots (14), the stage is finally set for the genetic manipulation of C_4 photosynthesis in vivo by engineering the regulatory properties and amount of PEPC in the leaf cytosol. We anticipate that these and other fertile avenues for future research on PEPC will continue to deepen our understanding of this "multifaceted" enzyme in plants. Finally, we hope that this survey has reminded the reader that there is, indeed, another CO_2-fixing enzyme in plants besides Rubisco that is worthy of detailed study.

Acknowledgments

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