Phosphoenolpyruvate Carboxylase (PEPC)

Phosphoenolpyruvate Carboxylase (PEPC, EC 4.1.1.31) is a key enzyme involved in primary carbon fixation in C4 and CAM plants (Edwards and Walker, 1983). PEPC is localized in mesophyll cytosol of plant cells. PEPC in C4 plants primarily fixes CO₂ into C₄ dicarboxylic acids, which on decarboxylation raise the CO₂ concentration in bundle sheath cells and minimize the process of photorespiration. Thus, C₄ plants can achieve high growth rates under conditions of high temperature, strong illumination and atmospheric oxygen levels, which are not optimal for C₃ plants (Edwards et al., 1985). PEPC plays an anaplerotic role in C₃ plants. Due to the importance of PEPC in C₄-, C₃- as well as CAM plants, studies on PEPC are always of great interest.

Stupendous progress has been made in our knowledge of biochemistry and molecular biology of PEPC in not only C₄ plants, but also C₃ species and legume root nodules. The literature on the properties, regulation and functions of PEPC has been reviewed by several authors during the present decade (Gonzalez and Andreo, 1989; Jiao and Chollet, 1991; Vance and Gantt, 1992; Nimmo, 1993; Lepiniec et al., 1994; Rajagopalan et al., 1994; Toh et al., 1994; Chollet et al., 1996; Vidal and Chollet, 1997). There are also several reviews which appeared before 1988 (Utter and Kolenbrander, 1972; O'Leary, 1982, 1983; Latzko and Kelly, 1983; Gadal, 1983; Guern et al., 1983; Kluge, 1983; Andreo et al., 1987; Deroche and Carrayol, 1988; Stiborova, 1988).
PEPC is almost ubiquitous and is distributed widely in photosynthetic and non-photosynthetic tissues of higher plants, green algae, bacteria and legume root nodules and is absent in animal tissues, yeast or fungi (Andreo et al., 1987; Deroche and Carrayol, 1988; Vance and Gantt, 1992; Lepiniec et al. 1994; Toh et al., 1994; Rajagopalan et al., 1994; Chollet et al., 1996). It is an important enzyme involved in primary CO₂ fixation in C₄ and CAM plants (O'Leary, 1982). The activities of PEPC levels in leaves of C₃ plants are about 2 to 5% of that found in C₄ plants (Edwards and Walker, 1983; Kluge, 1983; Latzko and Kelly, 1983).

PEPC constitutes about 15% of total soluble protein in maize (Hague and Sims, 1980; Hayakawa et al., 1981). The enzyme is confined to the cytoplasm of mesophyll cells in C₄ and CAM plants (Perrot-Rechenmann et al., 1982). In C₃ plants, PEPC may be localized in both cytosol and chloroplasts of the leaves (Perrot-Rechenmann et al., 1982. Latzko and Kelly, 1983). The activity of PEPC in bundle sheath preparations was only about 3 to 7% of that in mesophyll cells (Meister et al., 1996). Thus, PEPC is considered as a typical marker enzyme for C₄ mesophyll cells.

There is a significant evolutionary divergence between green algal, higher plant and prokaryotic PEPCs. The studies on immunoblot analysis using anti-(green algal or higher plant PEPC) IgGs suggested that PEPC from different sources of green algae (Chlamydomonas, Selenastrum), higher plants (maize, banana fruit, tobacco) and prokaryotes (Synechococcus leopoliensis, E. coli) has very little or no immunological relatedness. Further, N-terminal amino acid sequence and CNBr cleavage patterns suggest that prokaryotic or green algal PEPC is distinct from higher plant PEPC (Rivoal et al., 1998).
**Physiological role**

The occurrence of 'Kranz-like anatomy' in C4 plants results in the division of labor and spatial separation of biochemical reactions. In C4 plants, the initial carbon fixation through PEPC occurs in mesophyll cells, while the subsequent decarboxylation reactions take place in the bundle sheath cells, where the Calvin cycle enzyme, Rubisco refixes the released CO₂. The function of PEPC in CAM plants is similar to C4 plants. Primary carbon fixation by PEPC occurs during the night, followed by decarboxylation of C4 acids and refixation of CO₂ by Rubisco during day (Kluge, 1983). PEPC is a key enzyme involved in such primary CO₂ fixation in C4 and CAM plants (O’Leary, 1982). It catalyzes the irreversible β-carboxylation of PEP in the presence of HCO₃⁻ and Me²⁺ to yield OAA and Pi.

PEPC plays more than one metabolic role, its precise function depending on the organ and plant in which it is found. For e.g. PEPC is involved in the 'anaplerotic function' which involves mainly the replenishment of TCA cycle intermediates (i.e. oxalacetate and malate), thus providing the carbon skeletons necessary for nitrogen assimilation and amino acid biosynthesis (Melzer and O’Leary, 1987). PEPC plays an important role during fruit maturation (Blanke and Lenz, 1989), seed formation and germination (Watson and Duffus, 1988; Khayat et al., 1991; Macnicol and Jacobsen, 1992; Sangwan et al., 1992; Sugimoto et al., 1992), metabolic interactions between the style and elongation of pollen tube (Jansen et al., 1992), plant cell division and organogenesis (Coudret and Ducher, 1993), regulation of stomatal movement (Parvathi and Raghavendra, 1997) and maintenance of cytosolic pH and electroneutrality (Davis, 1979).

**Types of isoforms**

In higher plants, four types of PEPC isoforms have been reported so far, namely, C4 photosynthetic form, C3 photosynthetic form, CAM form and dark or
non-autotrophic PEPC. Chromatographic, immunological and kinetic properties of PEPC can be used to distinguish these isoforms (O’Leary, 1982; Andreo et al., 1987; Rajagopalan et al., 1994).

Etiolated *Sorghum* leaves contain only one form (C3 form) of the enzyme and a new isoform of enzyme appears on illumination upon greening (C4 form) (Vidal and Gadal, 1983). C4 specific gene expression occurs only in illuminated (greening) leaves (Schäffer and Sheen, 1992). On the other hand, the expression of PEPC-gene encoding the C4 isozyme was not leaf specific, since high accumulation of its transcripts was also found in other parts of maize plant, i.e., inner leaf sheaths, tassels and husks (Hudspeth and Grula, 1989). There is a possibility that a small amount of etiolated form of the enzyme may exist also in green tissue, which could explain the detection of the two major isozymes of PEPC in leaves of maize (Ting and Osmond, 1973a, b; Mukerji, 1977). However, C4-type is the major form in maize leaves and is the most abundant protein in mesophyll cells.

There is a lot of variation in the number of PEPC isoforms reported from the leaves of C3 plants and CAM species. Four major isoforms of PEPC are reported in leaves of a C3 plant *Flaveria conquistii*, C3-C4 intermediate *Flaveria floridana* and a C3 performing *Mesembryanthemum crystallinum* (Adams et al., 1986; Slocombe et al., 1993). Whereas, three isoforms are noticed in leaves of *Gossypium hirsutum*, a C3 species (Mukerji and Ting, 1971) and also two in C3 performing *Kalanchoë blossfeldiana* (Brulfert et al., 1979). Three distinct isoforms were identified in *Vicia faba*, by immunological determination. Those are mesophyll-PEPC, epidermal-PEPC and guard cell-PEPC and they represent an isoform specific physiological function in a given cell type (Schulz et al., 1992). The four isoforms of PEPC are encoded by different genes in C4 plants (Hudspeth et al., 1986; Hudspeth and Grula, 1989; Hermans and Westhoff, 1990). The occurrence of multiple forms of PEPC suggests that during the evolution of C4 plants, a mechanism has developed
for preferential expression of C4 specific PEPC gene (Nelson and Langdale, 1992; Lepiniec et al., 1994; Stockhaus et al., 1994; Ku et al., 1996).

**PEPC structure**

PEPC is a homotetrameric enzyme of about 400 kD (Andreo et al., 1987). However, its quaternary structure (i.e. tetramer/dimer) depends on protein and effector concentrations and it has been proposed that this specific property is involved in the regulation of PEPC in vitro, but so far, in vivo experimental evidence is lacking (Jiao and Chollet, 1991).

C4-PEPC from *Amaranthus hypochondriacus* is composed of 964 amino acid residues (Rydzik and Berry, 1996) compared to 970 of maize (Hudspeth and Grula, 1989), 952 of *Sorghum* (Cretin et al., 1990), 966 of *Flaveria trinervia* (Poetsch et al., 1991), 956 of potato, 967 of soybean, 964 of tobacco, 976 of *Flaveria pringeli*, 1025 of *Anacystis variabilis*, 1016 of *Anacystis nidulans*, 919 of *Corynebacterium glutamicale* and 883 of *Escherichia coli* (Lepiniec et al., 1993, 1994; Rajagopalan et al., 1994, Chollet et al., 1996).

A number of putatively important domains are identified in the primary sequence of PEPC based on biochemical experiments (Andreo et al., 1987; Jiao and Chollet, 1991), amino-acid alignments (Lepiniec et al., 1993) and site-directed mutagenesis (Terada et al., 1992; Wang et al., 1992). Four possible sites are responsible for the regulation of the activity of plant enzyme by direct interactions with PEP, glucose 6-phosphate (Glc-6-P) and L-malate, as well as protein phosphorylation (Lepiniec et al., 1994). The histidine residue of the amino acid motif ‘VfTAHPT’ (residues with capital letters being conserved) is essential for the carboxylation activity of the enzyme (Lepiniec et al., 1993). The highly conserved sequence ‘QqVMvGYDSgKDαG’ contains the species-invariant lysine residue implicated in the active site (Jiao et al., 1990). The glycine-rich motif
'FHGRGGtGvGRGGGgP' has been proposed to be part of the substrate binding site (Jiao et al., 1990). Seven cysteine residues are conserved in all plant PEPC sequences (Lepiniec et al., 1993) and these may be involved in the proposed redox regulation of PEPC activity (Chardot and Wedding, 1992) or subunit-interactions to maintain the tetrameric structure (Andreo et al., 1987).

A serine residue in the N-terminal domain E/D/K/R xx S1DAQLR (Ser^8, Ser^15 and Ser^11 in Sorghum, maize and Flaveria, respectively) is involved in the phosphorylation of PEPC. Such post-translational modification of PEPC increases several-fold the enzyme's apparent K_i for malate and its catalytic activity at suboptimal levels of PEP and pH, without affecting markedly K_m (PEP) or V_max at optimal pH (Jiao and Chollet, 1991; Wang et al., 1992). Reversible phosphorylation of PEPC is a cardinal event in the regulation by light or darkness of the C4 and CAM enzymes (Carter et al., 1991; Jiao and Chollet, 1991; Bakrim et al., 1993; Nimmo, 1993; Rajagopalan et al., 1994).

**Evolutionary aspects**

It has been widely accepted that C3 photosynthesis predates both CAM and C4 metabolism that have originated independently and on many occasions (Ehleringer et al., 1991). It has been hypothesized that C4 photosynthesis evolved in response to a reduction in atmospheric CO2 level (Ehleringer et al., 1991).

A number of C4 traits, including 'Kranz' leaf anatomy, intercellular compartmentation and specific activities of key C4 enzymes such as PEPC, are inherited independently. The genetic origins of these modifications are not yet fully understood. These characteristics might have evolved separately in one or several species in response to different selective pressures like water stress, salinity, low CO2 pressure or high temperature. In this regard, three biochemical sub-types or modes of C4 carbon metabolism are known (Leegood and Osmond, 1990; Hatch,
which could reflect different possible adaptive combinations (Lepiniec et al., 1994).

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 (Albert et al., 1992; Hermans and Westhoff, 1992; Izui et al., 1992; Kawamura et al., 1992; Lepiniec et al., 1993, 1994; Toh et al., 1994). For the plant PEPC enzyme, phylogenetic relationships have been studied with particular emphasis on the molecular mechanisms. PEPC appears to have originated independently and on many separate occasions during the evolution of flowering plants, with CAM being the antecedent of C4 (Izui et al., 1992; Kawamura et al., 1992; Lepiniec et al., 1993). Polyphyletic evolution of C4 plants is accounted from the various independently derived trees that all plant PEPC sequences diverged from a single common ancestral gene. C4-PEPC genes could have arisen from a duplication event long before the monocot-dicot divergence and thus prior to the appearance of C4 plants. In this manner, the PEPC gene for C4 photosynthesis could have evolved in a limited number of species while disappearing in others (Izui et al., 1992; Kawamura et al., 1992; Lepiniec et al., 1993). The photosynthetic enzyme in the C4 dicot *Flaveria trinervia* is more closely related to the various isoforms in C3 and CAM dicots (Poetsch et al., 1991; Hermans and Westhoff, 1992) than to the two monocot C4-PEPCS (Lepiniec et al., 1994).

Based on the PEPC gene sequences, the divergence of monocots/dicots has been estimated (Lepiniec et al., 1994). Therefore, PEPC may eventually help to clarify the present view of plant evolution, but first, additional sequences are required (e.g. algal PEPC).

Another view is that plant PEPC might have arisen from an endosymbiotic origin. Indeed, PEPC distribution in prokaryotes indicates that the enzyme was already present in the ancestor of the Chloroplast and is still present in their
'descendants' (i.e., cyanobacteria) (Katagiri et al., 1985; Luinenburg and Coleman, 1992). Moreover, it is difficult to explain how a loss of PEPC could have occurred recently (after plant appearance) and concomitantly in all eukaryotic taxa, with the exception of plants and *Euglena* (O'Leary, 1982; Lepiniec et al., 1994) which are not related phylogenetically (Martin et al., 1992). Nevertheless, uncertainties arise from the facts that the phylogenetic relationships between plants, fungi and animals are still a matter of debate and the *Euglena* is thought to have attained a secondary endosymbiosis with a eukaryotic chlorophyte (Martin et al., 1992). In addition, no PEPC gene has been found in the chloroplastic genomes sequenced to date (Lepiniec et al., 1994).

**Purification**

Bandurski and Greiner (1953) partially purified PEPC from spinach leaves for the first time. PEPC has been purified from a wide variety of sources: cotton, *Pennisetum purpureum*, *Sorghum*, maize leaves, lupin root nodules, soybean nodules, maize root tips, guard cells of *Vicia faba* and epidermis of *Commelina communis* (O'Leary, 1982; Rajagopalan et al., 1994).

The native leaf and recombinant forms of PEPC are highly susceptible to limited proteolysis and as a result their N-terminus is frequently lost during extraction and purification (Nimmo et al., 1986; McNaughton et al., 1989; Ausenhus and O'Leary, 1992; Baur et al., 1992; Wang et al., 1992; Duff et al., 1995). The integrity of the enzyme can be maintained during isolation of PEPC by the inclusion of glycerol, L-malate and protease inhibitors (especially chymostatin) and by the use of rapid purification protocols that exploit FPLC, HPLC or immunoaffinity chromatography (Nimmo et al., 1986; McNaughton et al., 1989; Jiao et al., 1991a; Arrio-Dupont et al., 1992; Bakrim et al., 1992; Baur et al., 1992; Wang et al., 1992; Wang and Chollet, 1993; Duff et al., 1995; Zhang et al., 1995). With proper precautions and suitable protocols of rapid purification, the
preparations of PEPC with an intact N-terminal region is possible from leaves (C4, CAM and C3) and root nodules.

The cDNA encoding the C4- or C3-type PEPC, (ppc) gene was cloned and expressed in bacteria. Cloning of PEPC gene in E. coli is a widely used phenomenon (Chollet et al., 1996; Vidal and Chollet, 1997). The successful cloning of ppc paved the way for the studies on site-directed mutagenesis, and a powerful tool to elucidate the importance and function of individual amino acid residues in the catalytic and regulator)’ properties of any enzyme (Rajagopalan et al., 1994, Dong et al., 1997b; Grisvard et al., 1998).

PEPC-deficient mutants of E. coli have been used to complement with a plasmid bearing a full-length cDNA encoding the C4-type form of Sorghum ppc (Crétin et al., 1991). The transformed bacteria produced a functional and full-sized enzyme, as determined by activity, immunochemical behavior and SDS-PAGE analysis. In addition, the recombinant PEPC could be phosphorylated in vitro by the Sorghum PEPC-protein kinase (PEPC-PK) or mammalian protein kinase A (Crétin et al., 1991; Pacquit et al., 1993).

The recombinant protein technology was successfully applied in previous work with Sorghum (Crétin et al., 1991) or maize (Yanagisawa and Izui, 1990), and was used to raise large amounts of C4-PEPC. As C3-type enzyme forms are present in relatively little amount in Sorghum and therefore difficult to purify extensively, they have received much less attention and consequently little is known about their regulation and phosphorylation. This recombinant technology made it possible to produce large amounts of C3-type PEPC form Sorghum leaves, which are used for biochemical studies (Pacquit et al., 1993).

Thus, genetically engineered E. coli cells could produce a genuine, phosphate-free, higher plant PEPC. This system is a versatile tool to prepare large quantities of pure protein for biochemical studies.
Stability of PEPC in vitro

C4 plant leaves contain PEPC as 15% of the soluble protein (Hague and Sims, 1980). Its concentrations in situ can therefore be expected to be extremely high. The enzyme is unstable on dilution and dissociates into inactive di- or monomer (Wu et al., 1990). However, the inclusion of solutes (such as glycerol or PEG) during extraction and storage helps to maintain the tetrameric state of several enzymes (Rhodes and Hanson, 1993).

The integrity of PEPC is affected by compatible solutes in several ways: stabilization of enzyme during storage (Selinioti et al., 1987), protection of the enzyme against NaCl inhibition (Manetas et al., 1986; Manetas, 1990) and improvement of catalytic efficiency (Stamatakis et al., 1988; Podestá and Andreo, 1989). Betaine and proline, which are known to accumulate during stress conditions, can protect enzymes against heat denaturation (Paleg et al., 1981; Nash et al., 1982). Similarly, synthetic polymers like PEG are often used for protein stabilization (Reinhart, 1980). Glycerol and other solutes have also been used as stabilizers to maintain the activity and regulatory properties of PEPC during and after extraction (Karabourniotis et al., 1983; Medina et al., 1985). The presence of glycerol stabilized the maize PEPC activity by promoting the tetrameric form and enhancing the $V_{\text{max}}$ of the enzyme (Uedan and Sugiyama, 1976). However, glycerol was unable to prevent the dissociation of PEPC or shift the equilibrium of enzyme to active tetrameric form at pH 8.0 (Podestá and Andreo, 1989).

Kinetic properties of PEPC

The kinetic mechanism of the enzyme is central to the understanding of any enzyme. The exergonic $\beta$-carboxylation of PEP by HCO$_3^-$ ($\Delta G = -7 \text{ kcal mol}^{-1}$) is catalyzed by PEPC in the presence of a divalent cation, particularly Mg$^{2+}$. As PEPC plays an important role in C4 and CAM carbon fixation, it is essential to
understand the kinetic properties of the enzyme, and the mechanism of regulation of the enzyme by substrates, pH and other metabolites (O’Leary, 1982).

β-Carboxylation of PEP by PEPC occurs in a two step mechanism (O’Leary, 1982; Andreo et al., 1987). The first step involves the reversible, rate-limiting formation of carboxyphosphate and the enolate of pyruvate from the substrates. The second step would be the carboxylation of the enolate with the formation of products (Andreo et al., 1987; Rajagopalan et al., 1994; Chollet et al., 1996; Vidal and Chollet, 1997).

Magnesium ion is required for PEPC activity (Utter and Kolenbrander, 1972; O’Leary, 1982). In vitro, Mn$^{2+}$ can replace Mg$^{2+}$ as a cofactor (O’Leary et al., 1981). The $K_m$ values for Mg$^{2+}$ vary from about 0.1 mM to 1 mM (O’Leary, 1982). An enzyme-metal-PEP bridge complex is formed at the active site of the Carboxylase. However, from kinetic studies it has been deduced that the active substrate is the free form of PEP rather than the metal-PEP complex. Chemical modification studies confirmed that Mg$^{2+}$ is not essential for the binding of PEP to the Carboxylase, but increases the affinity of the substrate to enzyme, probably by inducing conformation changes in the enzyme (O’Leary, 1982; Andreo et al., 1987).

$K_m$ for PEP values are about five fold lower for C3 enzyme than that from C4, considerably varies within each group. The maize enzyme shows hyperbolic kinetics at pH 8 with a $K_m$ near 1 mM, whereas, at pH 7, the kinetics are sigmoid with a $K_m$ that is several fold higher than at pH 8 (O’Leary, 1982).

In spite of the extensive research that has been focused on the regulation of PEPC, the studies on kinetic characteristics of the enzyme are rather limited. There are no studies on the mechanism of binding of bicarbonate (HCO$_3$-). This may be due to the technical problem of complete elimination of exogenous HCO$_3$- during such studies.
PEPC uses HCO$_3^-$ for the carboxylation of PEP. The $K_m$(HCO$_3^-$) of PEPC is lower than for other enzymes. The relative low value indicates that PEPC, which is able to catalyze the rapid carboxylation at low HCO$_3^-$ concentrations, can serve as an effective HCO$_3^-$ trap (Maruyama et al., 1966). Carbon atom of HCO$_3^-$ is ordinarily unreactive towards the nucleophilic attack and the way by which the C-C bond formation is achieved by PEPC is not completely understood (Iglesias and Andreo, 1983). Besides carboxylation, PEPC catalyzes a HCO$_3^-$-dependent hydrolysis of PEP to pyruvate and Pi (phosphatase activity). Only 5% of the total reaction is diverted to hydrolysis under optimal conditions. The extent of phosphatase activity of PEPC is increased up to 50% in presence of Ni$^{2+}$ as a metal ion (Chollet et al., 1996).

Regulation

PEPC is an allosteric enzyme, and is highly regulated by several internal and external factors, such as metabolites, light, temperature, pH, water stress and nutrition. The effect of these factors varies depending on the source of the enzyme and other interacting factors, as described below.

Metabolites (Inhibitors/Activators)

The properties of PEPC are regulated markedly by metabolites through either inhibition (by dicarboxylic acids, such as oxalacetate, L-malate and aspartate) or activation particularly by phosphate-compounds (Raghavendra and Das, 1976; González et al., 1984; Andreo et al., 1987).

L-Malate, which is a product of carboxylation, is a competitive inhibitor of PEPC (Huber and Edwards, 1975). Malate not only inhibits C4-PEPC, but also the C3 and CAM forms (Kluge et al., 1988; Echevarria et al., 1990; Jiao and Chollet, 1990). Aspartate also inhibits the enzyme (Huber and Edwards, 1975; Iglesias et al., 1986). The enzyme is protected against thermal inactivation by aspartate (Rathnam,
Several other analogues of PEP/pyruvate are powerful inhibitors of the C4 enzyme and are employed to study the reaction mechanism of the enzyme (González and Andreo, 1989; Janc et al., 1992 a, b; Rajagopalan et al., 1994).

The extent of inhibition of PEPC by malate depends on various factors like assay pH, phosphorylation status of enzyme, proteolytic cleavage of N-terminal region and presence of activators, e.g. Glc-6-P (McNaughton et al., 1989, 1991; Jiao and Chollet, 1991; Ausenhus and O'Leary, 1992; Wang et al., 1992). Malate is known to dissociate the enzyme into inactive or less active dimers (Willeford et al., 1990). Perhaps, as an adaptive feature, PEPC from C4 and CAM plants can change their sensitivity to malate inhibition, by modification of enzyme-protein. Such change in malate sensitivity is achieved through a regulatory seryl-phosphorylation (Nimmo et al., 1984; Kluge et al., 1988; Jiao and Chollet, 1990). The phosphorylated form of PEPC is less sensitive to malate, while the dephosphorylated form is extremely sensitive (Vidal and Chollet, 1997).

Glc-6-P activates PEPC from C4 plants (O'Leary, 1982; Andreo et al., 1987). The major effect of Glc-6-P is an increase in $V_{\text{max}}$ and decrease in $K_m$ for PEP (Coombs et al., 1973; Walker et al., 1986a). Glc-6-P protects the enzyme against malate inhibition from C4 species than that from C3 species (Gupta et al., 1994). PEPC is also activated by many phosphate-esters (Podesta et al., 1990). Some of these phosphate-esters are dephosphorylated by the phosphatase reaction of the enzyme, which may be related to the activation process (Walker et al., 1988).

Glc-6-P is also an efficient stabilizer of the activity of the enzyme in storage (Wedding et al., 1989) as well as during the assay. Glc-6-P induces aggregation of PEPC into the tetrameric form (Wedding et al., 1989; Willeford and Wedding, 1992; Wu and Wedding, 1994). Further, Glc-6-P protects PEPC against inactivation by the modification of essential cysteine residues (Manetas and Gavalas, 1982) and
against inactivation by urea (Wedding et al., 1992). Wang et al. (1992) reported that phosphorylation of PEPC has no effect on its response to Glc-6-P and suggested that the activation by Glc-6-P may occur by a more complex path than activation by PEP and inhibition by malate.

The activation of PEPC by glycine has so far been reported only in C4 monocots such as maize (Nishikido and Takanashi, 1973; Doncaster and Leegood, 1987; Bandarian et al., 1992; Gillinta and Grover, 1995; Gao and Woo, 1996a). This observation correlates well with the structural information, now available, on PEPC which suggests that the C4 enzyme of the monocots (e.g. maize and Sorghum) may have evolved separately from other C4 dicots, CAM and C3 plants (Lepiniec et al., 1993, 1994; Chollet et al., 1996).

Apart from the above, other known activators of PEPC that could be physiologically important, are: fructose 2,6-bisphosphate (Doncaster and Leegood, 1987), Pi (Podestá et al., 1990), dihydroxyacetone phosphate, fructose 6-phosphate (Doncaster and Leegood, 1987), AMP (Rustin et al., 1988), carbamyl phosphate (González et al., 1987) and ribulose 1,5-bisphosphate (Leblova et al., 1991).

Light

The kinetic and regulatory properties of C4-PEPC in leaves are modulated markedly by light/dark transitions in vivo (Andreo et al., 1987; Jiao and Chollet, 1991; Rajagopalan et al., 1994; Chollet et al., 1996; Vidal and Chollet, 1997). The activity of PEPC, particularly in leaves of C4 plants is enhanced by 2 to 3 fold upon illumination. Light-activation is a feature of key photosynthetic enzymes in C3 plants (Buchanan, 1992). The light activation of PEPC is distinct from light-induced synthesis of PEPC-protein, which is observed typically during greening of Sorghum or maize leaves (Sims and Hague, 1981; Vidal and Gadal, 1983).
The enzyme exhibits two or three fold more activity on illumination, when assayed at sub-optimal but physiological levels of PEP and pH (Jiao and Chollet, 1988). The light-form is less sensitive to feedback inhibition by malate than that of dark-form (Vidal and Chollet, 1997). The response of PEPC in C3 leaves to light is much less than that in C4 plants. The activation of PEPC on exposure to light is marginal (about 10-15%) in C3 species (Rajagopalan et al., 1993). A marginal increase in PEPC activity on exposure to light was reported in mesophyll protoplasts of maize (Devi and Raghavendra, 1992).

Light induces an increase in cytosolic calcium and pH in mesophyll protoplasts of *Sorghum*, which can result in the phosphorylation of PEPC (Pierre et al., 1992). Light induced phosphorylation also was observed in guard cell protoplasts of *Vicia faha* L. (Schnabl et al., 1992), although no light activation of PEPC could be detected in guard cell protoplasts of *Commelina communis* L. (Willmer et al., 1990).

Cytosolic pH in mesophyll cells may be an important factor during light activation of PEPC. Illumination induces the marked cytosolic alkalization in mesophyll cells of C4 plants (Raghavendra et al., 1993). The increase in cytosolic pH can rise cytosolic calcium and lead to an increase in the activity of PEPC and PEPC-PK or both. This has been shown in "cytosol enriched" cell sap of *Alternantherapungens*, a NAD-ME type plant (Rajagopalan et al., 1993). Light is also known to induce marked alkalization of cytosol in mesophyll cells of C4 plants, as documented by the use of pH-dependent fluorescent probes (Raghavendra et al., 1993; Yin et al., 1993).

**Temperature**

The effects of temperature on growth are often correlated to corresponding changes in activity of several enzymes, including PEPC in C4 plants (Selinioti et al., 1986).
High temperatures increase activity and the apparent allostERICity towards PEP. The day-form of the enzyme has a slightly higher affinity for PEP and the temperature for optimum affinity to the substrate is around 35°C (Karabourniotis et al., 1985). Attempts have been made to correlate the poor rate of C4 photosynthesis at low temperature with cold liability of PPDK (Shirahashi et al., 1978) and thermal response of PEPC (Selinioti et al., 1986). Cold inactivation of PEPC was observed at higher pH in *Cynodon dactylon*, *Atriplex halinus* and *Zea mays* (Angelopoulos et al., 1990).

Temperature can affect the oligomeric status of PEPC. An aggregation of PEPC in case of C4 or dissociation in case of CAM was observed above 25°C (Wu and Wedding, 1987). Cold/chilling temperature may cause the enzyme dissociation from active tetramer to less-active dimers or monomers. However, this is not well corroborated in case of C4-PEPC (Walker et al., 1986a).

C4-PEPC shows a temperature optimum of 37°C (Huber and Edwards, 1975). McWilliam and Ferrar (1974) have suggested that the high temperature tolerance of C4 plants is not due to the greater thermostability of their PEPC, but to a more heat-resistant protein synthesizing machinery, which can replace the primary Carboxylase denatured by high temperatures. 1-Malate and aspartate, the photosynthetic intermediates of the C4 pathway protect PEPC against heat inactivation (Rathnam, 1978). Since the C4 plants can withstand temperatures up to 50 to 55°C, it is possible that the enzyme may be forming a complex, *in vitro* with aspartate that protects it against heat inactivation (Rathnam, 1978). Proline and betaine protect the enzyme against heat inactivation by inducing the conformational change in the protein important for enzyme activity (Krall and Edwards, 1993; Rajagopalan et al., 1994).
**pH**

PEPC is active at pH 8.0 and becomes inactive at acidic pH and can therefore expected to be regulated by cytosolic pH (Andreo et al., 1987; Rajagopalan et al., 1993). Besides the regulation by pH, PEPC has been proposed to be involved in the regulation of intracellular pH, and thus forms an important part of the biochemical pH-stat, particularly in plant cells (Davis, 1979).

Cytosolic pH is shown to be an important factor during light activation of PEPC in mesophyll protoplasts of maize (Devi and Raghavendra, 1992) and *Sorghum* (Pierre et al., 1992). Illumination increases the cytosolic pH in C4 mesophyll cells and such changes in cytosolic pH may modulate the catalytic activity of PEPC either directly or indirectly through regulation of PEPC-PK or PEPC-protein phosphatase or both (Rajagopalan et al., 1993).

**Salt/Water stress**

Salt or water stress plays an interesting role in modulating the expression and regulation of PEPC in higher plants. The activity of PEPC in leaves of *Salsola soda* increased by water stress, which is similar to the effects of high-temperatures on PEPC in C4 plants. The affinity of PEPC to PEP increased upon the exposure to salt stress in *Cynodon dactylon* and *Sporobolus pungens* (Manetas et al., 1986). Proline and betaine, osmoregulants, are known to accumulate under water stress in many plants and these compounds can stimulate the activity of PEPC by protecting the enzyme from degradation (Manetas et al., 1986).

The effect of salt stress on PEPC is manifested much more strongly in CAM plants than that in C3 or C4 species. For e.g. in an inducible-CAM plant, *Mesembryanthemum crystallinum*, PEPC-activity rises remarkably on exposure to salt or water stress, due to enhanced transcription of the *ppc* gene (McElwain et al., 1992). Photoperiodism or water stress can shift young leaves of *Kalanchoë*
blossfeldiana from C3-type photosynthesis to CAM. A rise in endogenous levels of ABA preceded PEPC-increase, independent of CAM induction in isolated leaves of Kalanchoë blossfeldiana (Taybi et al., 1995). In Mesembryanthemum crystallinum, the increase in PEPC activity upon ABA treatment was due to increased levels of CAM-specific isoform of the enzyme (Dai et al., 1994).

**Nutrition**

The biosynthesis of PEPC in leaves of C3-, C4- and CAM plants is highly regulated by availability and source of nitrogen (Champigny and Foyer, 1992; Foyer et al., 1994; Gadal et al., 1995; Lara et al., 1995). The rise in the level of PEPC-mRNA and PEPC-protein (particularly the C4 isoform) was more pronounced in maize plants supplemented with NH4⁺ or glutamine than those with NO3⁻. The biosynthesis of PEPC in maize leaves increased on feeding the leaves with nitrate or ammonium. Ammonium salt induces two-fold more PEPC biosynthesis than that of nitrate (Sugiharto et al., 1990, 1992; Sugiharto and Sugiyama, 1992).

Ammonium ions are known to stimulate the dark CO₂ fixation into C4 acids in higher plants, algal cells and cyanobacteria (Miyachi and Miyachi, 1985; Ohmori et al., 1986; Müller et al., 1990; Vanlerberghe et al., 1990). Such stimulation could be due to the increase in the activity of PEPC in ammonia-treated cells. The rates of ammonia assimilation *in vivo* were correlated with PEPC activity in a green alga, Selanastrum minutum (Vanlerberghe et al., 1990).

Ammonia is a toxic metabolite and inhibits several enzymes of plant and animal metabolism (WHO, 1986). Nevertheless, a few enzymes such as pyruvate kinase are stimulated by ammonium (Peterson and Evans, 1978). Ammonium ions can stimulate PEPC *in vitro* (Gayathri and Raghavendra, 1994). The effect of ammonium on PEPC was at the regulatory allosteric site on the enzyme. Another possibility is the modulation of intracellular pH by externally added NH4⁺.
Ammonia diffusion into cells may cause alkalization of cytosol and the rise in pH could in turn stimulate PEPC.

The light activation of PEPC is enhanced in presence of nitrate in wheat leaves (Van Quy et al., 1991a, b; Van Quy and Champigny, 1992; Duff and Chollet, 1995). The reason is that nitrate enhances the PEPC-PK activity which phosphorylates PEPC in wheat leaves in the light, leading to greater light activation. Thus, the presence of nitrate/ammonia can lead to the long-term enhancement of PEPC biosynthesis and short-term increase in the activity of PEPC/PEPC-PK.

Post-translational modification of PEPC

Post-translational modification of the enzyme regulates the activity and kinetic characteristics of PEPC. Two types of post-translational modification of PEPC are known. One is regulatory phosphorylation mediated by protein kinases, while the other is a change in the oligomeric state of the enzyme induced in vitro (Jiao and Chollet, 1991; Rajagopalan et al., 1994; Chollet et al., 1996). Both processes are reversible. A third possibility is the regulation by modulation of redox state of the enzyme, but this phenomenon did not draw much attention (Chollet et al., 1996).

Phosphorylation/dephosphorylation

Phosphorylation of PEPC has been studied extensively (Jiao and Chollet, 1991; Nimmo, 1993; Huber et al., 1994; Lepiniec et al., 1994; Rajagopalan et al., 1994; Chollet et al., 1996; Vidal and Chollet, 1997). C4-PEPC is phosphorylated in light by a PEPC-PK and dephosphorylated in dark by a type 2A protein phosphatase. In vitro phosphorylation of PEPC in Sorghum leaves causes only a modest effect on the $K_m$ for PEP, but an approximately two fold increase in the $V_{max}$, a 7-fold increase in the $K_i$ for malate and a 4.5-fold decrease in the $K_a$ for
Glc-6-P, when assayed at suboptimal pH and PEP concentrations (Duff et al., 1995; Vidal and Chollet, 1997). These changes are reversed by dephosphorylation.

Phosphorylation of PEPC occurs on a serine residue near N-terminus by an ATP-dependent and soluble protein kinase. Ser$^8$, Ser$^{11}$, Ser$^{15}$ in *Sorghum*, *Flaveria trinervia* and maize are phosphorylation sites in C4 enzymes (Jiao and Chollet, 1991; Rajagopalan et al., 1994; Vidal and Chollet, 1997). The tryptic phosphopeptide, (Asp/Glu)-(Lys/Arg)-X-X-Ser(P)-Ile-Asp-Ala-Gln-(Leu/Met)-Arg was suitable for seryl phosphorylation in C4 and CAM plants (Toh et al., 1994; Chollet et al., 1996; Relle and Wild, 1996; Vidal and Chollet, 1997). This sequence is highly conserved in all plant PEPCs, and is absent in bacterial and cyanobacterial enzymes, analyzed so far (Cretin et al., 1991; Poetsch et al., 1991; Pathirana et al., 1992; Schäffner and Sheen, 1992; Lepiniec et al., 1994; Toh et al., 1994; Chollet et al., 1996; Vidal and Chollet, 1997).

The regulatory phosphorylation of PEPC was mimicked by substitution of Ser-8 to Asp in *Sorghum* (Wang et al., 1992; Duff et al., 1993) or by S-carboxymethylation of Cys introduced in place of Ser-8 (Duff et al., 1995). Mutant enzymes in which the serine residue at position 8 was replaced by either aspartate (S8D) or cysteine (S8C) clearly showed that phosphorylation can be functionally mimicked by the introduction of negative charge (S8D) to the N-terminal domain of the protein (Wang et al., 1992; Duff et al., 1993, 1995; Lepiniec et al., 1994; Chollet et al., 1996).

The phosphorylation site is only two amino acid residues removed from a basic amino acid group (Lys-12 in the case of maize PEPC) in the primary sequence, a feature similar to that of various protein serine/threonine kinase substrates (Jiao and Chollet, 1991; Fallon and Trewavas, 1993; Macintosh and Macintosh, 1993; Hunter, 1995). Thus, a specific phosphorylation site is accessible
to both homologous and heterologous protein kinases, for e.g., mammalian protein kinase A (Jiao and Chollet, 1990; Terada et al., 1990; Rajagopalan et al., 1994).

An increase in the catalytic activity and decrease in malate sensitivity of C4-PEPC are directly correlated with the changes in the status of seryl phosphorylation \textit{in vitro}. This is proved by the experiments performed with an homologous reconstituted phosphorylation system comprised of purified, dark-form maize PEPC, a partially purified protein kinases from light-adapted leaves and ATP.Mg^{2+}. An ATP-dependent, soluble leaf protein kinase is a key component in the post-translational regulation of C4-PEPC activity by light/dark transitions \textit{in vivo} (Vidal et al., 1990).

The post-translational modification by phosphorylation of PEPC is promoted in light by virtue of light-activated nature of PEPC-PK (Echevarria et al., 1990; Jiao and Chollet, 1991; McNaughton et al., 1991; Bakrim et al., 1992; Jiao and Chollet, 1992). The activity of PEPC-PK is increased, possibly due to \textit{de novo} synthesis of either PEPC-PK or a regulatory protein.

\textit{Physiological role of phosphorylation}

On illumination, PEPC phosphorylation reached its maximum by about an hour and was associated with corresponding marked changes in the enzyme's properties: increase in catalytic activity and apparent affinity for Glc-6-P and decrease in inhibition by malate (as determined at suboptimal but near-physiological conditions of pH and PEP concentration) (Bakrim et al., 1992; Duff et al., 1995). Such modulation of PEPC is believed to enable the enzyme to cope with the high concentration of L-malate that occurs during CO$_2$ fixation in mesophyll cells of an illuminated C4 leaf (10-20 mM) (Stitt and Heldt, 1985; Doncaster and Leegood, 1987). The fully phosphorylated form of C4-PEPC had a markedly reduced sensitivity to L-malate (IC$_{50} = 15$ mM) compared to
dephosphorylated enzyme form (Kj (malate) about 0.2 mM) (Echevarria et al., 1994).

Phosphorylation of PEPC and ultimate CO₂ fixation was correlated by inhibition of PEPC-PK (Bakrim et al., 1992, 1993; Li and Chollet, 1993). These observations suggest that PEPC phosphorylation has a critical regulatory impact on the overall functioning of C4 photosynthesis (Bakrim et al., 1993; Echevarría et al., 1994). Phosphorylation not only protects C4-PEPC against 1-malate, but also adjusts its catalytic activity to meet the demand of the Calvin cycle for an acid-derived supply of CO₂. The light transduction pathway is present in mesophyll cell and a cross-talk between the mesophyll and bundle sheath cells is implied in the form of the photosynthetic metabolite, 3-PGA (Giglioli-Guivarc'h et al., 1996; Vidal and Chollet, 1997).

The phosphorylation-induced changes in the regulatory properties of PEPC are relatively slow and a hysteretic behavior of the enzyme is expected (Vidal and Chollet, 1997). A temporary imbalance between the light-dependent formation of PEP (mesophyll-chloroplast PPDK is rapidly activated in the light) and its utilization by PEPC would cause a pool of this metabolite to build up during the induction phase of C4 photosynthesis. Accumulation of millimolar concentrations of this substrate (0.3 mM in dark, 3 mM in light) (Doncaster and Leegood, 1987) in illuminated mesophyll cells is needed for PEPC to achieve maximum catalytic activity during C4 photosynthesis (Vidal and Chollet, 1997).

**Oligomerization**

The active form of PEPC is a tetramer. But the enzyme can exist as a dimer or monomer depending on several factors: pH, ionic strength (Walker et al., 1986a; Wagner et al., 1987), temperature (Wu and Wedding, 1987) and concentration of PEPC (Willeford and Wedding, 1992). NaCl causes dissociation of enzyme into a
dimer at pH 7.0 and into dimers/monomers at pH 8.0. The presence of PEP, Mg\(^{2+}\) or Glc-6-P prevented dissociation of the enzyme (Wagner et al., 1987; Wu and Wedding, 1994). Effectors such as Glc-6-P and malate or presence of solutes (PEG and/or glycerol) can also effect the aggregation of the enzyme (Podestá and Andreo, 1989; Manetas, 1990; Wedding et al., 1994).

Dilution of the enzyme in vitro can change the oligomeric status of the enzyme and thereby affect the activity of PEPC (Wu et al., 1990). The activity of PEPC is more at high concentrations of the enzymic protein (Selinioti et al., 1987) that favors the formation of the most active tetramer (Wu et al., 1990; Meyer et al., 1991; Willeford and Wedding, 1992). High concentration of PEPC-protein or the addition of glycerol or PEG can shift the enzyme to active tetrameric form (Podestá and Andreo, 1989). Enzyme dilution can be minimized by using compatible solutes (Krall and Edwards, 1993). The inclusion of an appropriate cosolute in the assay medium promotes the self-association of the enzymic-protein and, therefore, mimics the intracellular situation, where the enzyme is much concentrated (Stamatakis et al., 1988). The significance of the oligomeric transition of PEPC during light activation/dark deactivation is debatable since oligomerization/deoligomerization have mostly been observed in vitro (Wu and Wedding, 1987, 1992). The reversible oligomeric conversion of PEPC may not be a mechanism that regulates the light/dark transition of PEPC in C4-, C3- and CAM species (McNaughton et al., 1989; Weigend and Hincha, 1992).

Recently, Drilias et al. (1997) reported in Cynodon dactylon PEPC that the enzyme is more active at high concentration and the amino acids of the active site are more accessible, when the active form (tetramer) predominates. At high concentration the essential cysteine residues are protected against their specific modifiers, whereas arginine, histidine and lysine residues become more vulnerable. PEP acts not only as a substrate, but also favors the formation of the most active tetramer (Wu and Wedding, 1987; Wu et al., 1990; Meyer et al., 1991; Willeford
and Wedding, 1992). Glc-6-P also favors the formation of the most active tetramer of the enzyme (Wagner et al., 1987; Meyer et al., 1991; Willeford and Wedding, 1992).

Most of these experiments on oligomerization of PEPC have been done in vitro and not much information is available on the form of PEPC under in vivo conditions. The physiological condition of the oligomerization of the enzyme in vivo is not studied critically and its relevance to phosphorylation would be of great interest. Protein phosphorylation can induce oligomeric interconversion of enzymes (Frieden, 1989). It is therefore possible that phosphorylation and oligomerization of PEPC interact with each other, but this possible phenomenon is yet to be evaluated.

Redox regulation

The regulation of cytosolic C4-PEPC may be under the control of the redox state of certain critical cysteines (Iglesias and Andreo. 1984; Chardot and Wedding, 1992). Five to seven Cys residues are present in plants in the various PEPC isoforms that are absent in the microbial enzymes (Lepiniec et al., 1993, 1994; Toh et al., 1994; Nakamura et al., 1995; Vidal and Chollet. 1997). It is not known which of these Cys residues are involved in regulation of activity or L-malate sensitivity. In contrast, reduced cytosolic thioredoxin h had no effect on the properties of C4-PEPC in vitro, when the dephospho maize enzyme was used (Jiao and Chollet 1989). Cysteine residues may be involved also in the maintenance of enzyme quaternary structure.

Transduction of light-signal during PEPC-activation

Although the phenomenon of marked activation by light of PEPC in C4 leaves, is well known, the mechanism of light activation is not completely understood. Nevertheless, a few factors have been identified to be involved as the messengers. Light-signal transduction can be mediated through changes in the level
of a metabolite of photosynthesis and/or energy charge (Doncaster and Leegood, 1987). A transcellular message (3-PGA) formed in the bundle sheath during C4 photosynthesis would be delivered to the adjacent mesophyll cells where the physiological responses with respect to PEP carboxylase-kinase and PEPC were observed (Chollet et al., 1996; Giglioli-Guivarc'h et al., 1996). This suggested that the signal transduction chain involves intracellular cross talk between these neighboring photosynthetic tissues. McNaughton et al. (1991) speculated that a signal generated in Chloroplast may initiate a sequence of events that lead to a significant increase in activity of PEPC-PK and phosphorylation of PEPC in the cytosol. On feeding the leaves with photosynthetic inhibitors like DCMU or D, 1-glyceraldehyde, decreased phosphorylation of PEPC indicating the coordination of both mesophyll and bundle sheath cells (Jiao and Chollet, 1992).

Illumination induces the marked cytosolic alkalization in mesophyll cells of C4 plants (Raghavendra et al., 1993). The increase in cytosolic pH can rise cytosolic calcium and lead to an increase in the activity of PEPC and PEPC-PK. Light provides the energy required for photosynthetic carbon assimilation in bundle sheath cells, which, in turn, produces the intercellular metabolite message 3-PGA and an upward shift in the cytosolic pH of adjoining mesophyll cells. Light presumably provides ATP and/or NADPH via photosynthesis for some step(s) in the transduction pathway, particularly if a protein synthesis event related to the photoregulation of PEPC kinase is involved. It could also act as a thioredoxin pathway (Buchanan et al., 1994).

The light transduction pathway in an illuminated C4 leaf involves, sequentially: 3-PGA, as an intercellular message indicating photosynthesis, an increase in cytosolic pH in mesophyll, IP3, Ca$^{2+}$ channels on tonoplast, cytosolic Ca$^{2+}$, a Ca$^{2+}$-dependent protein kinases and finally the upregulation of PEPC-PK via stimulation of cytosolic protein synthesis event. Recently, Vidal and Chollet
(1997) proposed a model showing the likely events during the transduction of light signal to control C4-PEPC-PK and, thus, PEPC phosphorylation.

Secondary messengers likely to be involved during light activation of PEPC

Calcium

Many of the responses of plants to growth regulators, light, environmental stress and pathogen attack are mediated by changes in the cytosolic concentration of free calcium. Calcium-modulated proteins such as calmodulin and calmodulin-like domain protein kinases (CDPKs) are capable of both sensing the increases in the cytosolic concentration of free calcium and effecting changes in cellular metabolism, and have been proposed to play important roles as secondary messengers (Roberts and Harmon, 1992).

Calcium is a dynamic secondary messenger in a variety of physiological responses, particularly those related to hormones and/or light (Bush, 1993, 1995; Poovaiah and Reddy, 1993). The marked changes on illumination in the levels of cytosolic calcium suggested that calcium could be a part of light-transduction mechanism in plants. Pretreatment of MCP in Digitaria with the calcium ionophore A23187 (calcimycin) and EGTA inhibited the phosphorylation of PEPC. A recovery from such inhibition was possible, only when Ca\(^{2+}\) was provided (Chollet et al., 1996; Duffet et al., 1996).

Cytosolic pH

Felle (1989) was among the first to suggest that cytosolic pH is an intracellular messenger in plants. In mesophyll cells of an illuminated C4 leaf, cytosolic pH plays an important role in modulating the PEPC phosphorylation and as a result the activity of PEPC (Giglioli-Guivarc'h et al., 1996). NH\(_4\)Cl and methylamine, weak bases trigger the phosphorylation of PEPC. Both compounds
are known to induce cytosolic pH. Intracellular calcium levels could be modulated by the changes in pH in *Sorghum* mesophyll protoplasts (Pierre et al., 1992). The phosphorylation state of PEPC or a putative activation protein factor of PEPC, depends on the amplitude of the cytosolic pH jump in mesophyll cells, reaching a maximum at a pH value close to 7.3. While, a change in cytosolic pH would dramatically affect both PEPC kinase and PEPC activities because these two enzymes strongly respond *in vitro* to H\(^+\) concentrations in the range of pH 7 to 8 (Wang and Chollet, 1993; Echevarria et al., 1994).

Alkalization of the cytosolic pH in C4 mesophyll cells in the light might result from the uptake of bundle sheath cell-generated 3-PGA, in its partially protonated form, into the Chloroplast stroma (Yin et al., 1990). According to Yin et al. (1993), the cytosolic (H\(^+\)) decrease has two major components: protonated PGA transferred into chloroplasts and pumping of protons into the vacuole. This can raise the cytosolic pH, which in turn leads to phosphorylation of PEPC. When 3-PGA is used as a weak base in the mesophyll protoplast suspension medium, this metabolite elicited the predicted *in situ* changes in cytosolic pH and resulted in the upregulation of PEPC kinase and the phosphorylation state of PEPC (Giglioli-Guivarc'h et al., 1996).

A change in cytosolic pH is expected to trigger calcium efflux from the mesophyll cell vacuole in *Digitaria sanguinalis* protoplasts (Giglioli-Guivarc'h et al., 1996). Calcium channels on tonoplast membranes are sensitive to pH and are subject to long term regulation (Sanders et al., 1992; Brosnan and Sanders, 1993; Bush, 1993). Thus, the intracellular alkalization in mesophyll-cell protoplasts was implicated as an early signaling element in the C4-PEPC phosphorylation circuitry. Therefore, both light and alkalization of cytosolic pH favor the PEPC phosphorylation or PEPC kinase activity, and enhance the activity of PEPC.
Characterization of PEPC-protein kinase

PEPC is phosphorylated by a PEPC-protein serine kinase. However, there are conflicting reports on the exact type of this PEPC-PK, particularly in relation to the involvement of Ca\(^{2+}\). Calcium-dependent and calcium-independent PEPC-PKs were purified from *Sorghum* leaves (Bakrim et al., 1992) and were shown to be capable of phosphorylating PEPC.

The first demonstration of the regulatory phosphorylation of C4-PEPC *in vitro* was obtained using a soluble protein fraction partially purified from illuminated maize leaves (Jiao and Chollet, 1989). A low abundant protein kinase was partially purified about 4000-fold from the leaves of maize and *Mesembryanthemum crystallinum*. The molecular masses of these protein kinases were in the range of 30-39 kD (Wang and Chollet, 1993; Li and Chollet, 1994). On contrary, the Ca\(^{2+}\)-dependent PEPC-PK from maize was found to have molecular weight of 50-60 kD and to be inhibited by the calmodulin antagonist W7 and potent inhibitor of myosin light chain kinase, KT5926 (Izui et al., 1995). This PEPC-PK was reminiscent of plant calmodulin-like domain (or Ca\(^{2+}\)-dependent) protein kinase (CDPK), a protein Ser/Thr kinase not found in animals. This latter kinase possesses an intrinsic calcium-binding regulatory domain, with four typical EF-hand motifs, linked to an N-terminal catalytic domain by an intervening junction domain (Roberts and Harmon, 1992).

It is proposed that PEPC-PK undergoes reversible light activation *in vivo* (Bakrim et al., 1992; Chollet et al., 1996). Pretreatment with cycloheximide (CHX), a cytosolic protein synthesis inhibitor, efficiently blocked both C4-PEPC-PK upregulation and PEPC phosphorylation during illumination (Bakrim et al., 1992; Chollet et al., 1996). Phosphorylation of PEPC was markedly blocked by CHX, but not by a-amanitin and actinomycin D (inhibitors of RNA polymerase II), thereby suggesting that protein translation is the regulatory step (Vidal and Chollet, 1997).
Feeding of CHX to an excised *Sorghum* or maize leaf performing photosynthesis at steady state caused a significant decrease in both its CO₂ assimilation rate and PEPC phosphorylation state (Bakrim et al., 1992). These results suggested that the light induced phosphorylation of PEPC was regulated by the synthesis of PEPC-PK or an unknown protein required for the activation of PEPC-PK. Further, it was recently reported that PEPC-PK from C4-, CAM and C3 species is regulated at the level of translatable mRNA in response to light (C4, C3), or circadian rhythm (CAM) (Hartwell et al., 1996).

Reconstituted assays containing the C4 leaf protein kinase or mammalian protein kinase A revealed that the phosphorylation rate of purified C4-PEPC was markedly inhibited by l-malate (Wang and Chollet, 1993; Echevarría et al., 1994). This inhibition was relieved by Glc-6-P. This indirect means of regulating phosphorylation of PEPC might allow for an individual, target-dependent control of a multisubstrate protein kinase. However, to date, available evidence is in favor of the argument that this highly regulated protein kinase is specific for plant PEPC (Chollet et al., 1996).

Possible involvement of other types of kinases

Ogawa et al. (1992) suggested that at least four types of protein kinases could be detected in their PEPC-PK preparation, of which two of them were calcium-dependent. It has been suggested that multiple forms of PEPC-PK (both Ca²⁺-dependent and Ca²⁺-independent) are involved in the regulation of PEPC phosphorylation (Bakrim et al., 1992; Giglioli-Guivarc’h et al., 1996). There are reports suggesting that the Ca²⁺-dependent (Vidal et al., 1990; Ogawa and Izui, 1992; Ogawa et al., 1992) and Ca²⁺-independent (Chollet et al., 1990; Echevarria et al., 1990; McNaughton et al., 1991) protein kinases are involved in PEPC phosphorylation. Immunopurified C4-PEPC from *Sorghum* and maize could be phosphorylated *in vitro* by various kinases, including calcium- or magnesium-
dependent protein kinase, both in crude extract and a reconstituted system (Bakrim et al., 1992) or catalytic-subunit of cAMP-dependent protein kinase from bovine heart (Terada et al., 1990; Bakrim et al., 1992).

**Protein kinases in plants**

Protein phosphorylation cascades make up an intricate circuitry within the plant cell, the complexity of which may be comparable to a computer CPU (Verslues et al., 1996). More than 70 genes coding for protein kinases have been identified in plants. In eukaryotes, 1 to 3% of functional genes encode protein kinases, suggesting that they are involved in many aspects of cellular regulation and metabolism in plants. However, the functional roles of specific protein kinases and phosphatases during plant growth and development are not completely understood (Stone and Walker, 1995).

Eukaryotic protein kinases have been subdivided into those that phosphorylate Ser and/or Thr and those that phosphorylate Tyr. However, a major classification of the superfamily of protein kinases is into five groups, based on a phylogenetic analysis of the alignment of protein kinase catalytic domains (Stone and Walker, 1995).

**AGC group**

The AGC group consists the cyclic nucleotide-dependent family (PKA and PKG), the PKC family, and the ribosomal S6 kinase family. These kinases are regulated by secondary messengers (i.e. cAMP, cGMP, diacylglycerol and Ca^{2+}) and are not common in plants. The involvement of cAMP in plants has been disputed (Spiteri et al., 1989), although cGMP may function in phytochrome responses (Bowler et al., 1994).
**CaMK group**

CaMK group consists calcium/calmodulin-dependent kinase and the SNF1/AMP-activated protein kinase families. These protein kinases are regulated by secondary messengers like Ca\(^{2+}\)/calmodulin. There are very few reports about the involvement of CaMK in plants (Pandey and Sopory, 1998). The plant SNF1 kinase play some role in carbon metabolism (Stone and Walker, 1995).

Calcium-dependent, calmodulin-independent CDPKs are predominant calcium-dependent kinases in plants. These CDPKs do not depend on the requirement of calmodulin, phospholipid or diacylglycerol for their activities, and thus differ from both CaMK and PKC families, present in mammals (Roberts and Harmon, 1992).

CDPK has been purified from soybean cell suspension cultures (Putnam-Evans et al., 1990). CDPK is a monomeric enzyme, having a native molecular weight ranging from 40 to 90 kD (Roberts and Harmon, 1992). The primary sequence of CDPK from soybean contains both a protein kinase catalytic domain and a calcium-binding regulatory domain similar to calmodulin. This unique molecular structure explains the direct activation of this enzyme by calcium and clearly establishes CDPK as the prototype of a new class of protein kinases (Harper et al., 1991).

Even though the CDPKs are the predominant Ca\(^{2+}\)-dependent PKs found in plant cells, an understanding of their physiological role(s) remains elusive (Poovaiah and Reddy, 1993; Stone and Walker, 1995).

**CMGC group**

CMGC group contains the cyclin-dependent kinase (CDK), mitogen-activated protein kinase (MAPK), glycogen synthase kinase (GSK-3), and casein
kinase II (CKII) families in plants. CDKs are involved in cell cycle regulation in plants. Whereas, MAPKs are activated by dual phosphorylation on Thr and Tyr residues and they play a role in cell proliferation. GSK3 plays an important role in plant development. CKII serves a critical function in transcriptional regulation in plants (Collinge and Walker, 1994; Stone and Walker, 1995).

**PTK group**

Protein Tyr kinase (PTK) group kinases have not yet been reported in plants. However, conservation of regulatory sites in the plant protein kinases suggest that Tyr phosphorylation may play an important physiological role in higher plants (Stone and Walker, 1995).

**Other group**

The “other” group of protein kinases are those, which have been cloned from plant sources, and do not fall into any of the four families described above. Receptor-like kinases (RLKs) are similar to receptor Tyr kinases (RTKs), which are transmembrane proteins that recognize extracellular signal. CTR1 is a plant protein kinase belonging to Raf family. CTR1 is proposed to be involved in negative regulation of ethylene signal transduction (Kieber et al., 1993). Tsl encodes a novel Ser-Thr kinase with a little similarity to other kinases (Roe et al., 1993) and it is involved in plant morphogenesis.

Secondary messengers are important in signal transduction in plant cells. The changes in cytosolic Ca^{2+} levels in response to external stimuli are often accompanied by changes in protein phosphorylation. Ca^{2+}-dependent phosphorylation in plants is regulated by CDPKs rather than by PKC or CaMK. Since plant-CDPKs have calcium-binding, calmodulin-like domain on the same polypeptide as a protein kinase catalytic domain, the plants may have surpassed the need for CaMK or PKC (Stone and Walker, 1995).
One of the aims of present work has been to study the characteristics of PEPC phosphorylation in *Amaranthus hypochondriacus* leaves. There are conflicting results about the involvement of Ca$^{2+}$-dependent and -independent protein kinases during the regulatory phosphorylation of PEPC in response to light. It has been proposed that CDPK may be involved in the upregulation of PEPC-PK, i.e., Ca$^{2+}$-independent protein kinase (Chollet et al., 1996; Vidal and Chollet, 1997). A direct study of the characteristics of CDPK or CCaMK in *Amaranthus hypochondriacus* leaf extracts, would be helpful to resolve this issue.

**Some of the points to be resolved**

Despite the extensive literature on certain aspects of physiology, biochemistry and molecular biology of C4-PEPC, there are still some gaps in our knowledge of regulation of this key enzyme of C4 pathway, a topic of interest.

PEPC has been purified from leaves and several plant tissues of C3 and C4 plant species, particularly from spinach, maize, *Sorghum*, root nodules, *Amaranthus viridis* and even from banana fruit (Iglesias et al., 1986; Law and Plaxton, 1995; Chollet et al., 1996). Nevertheless, a method of rapid purification along with long-term storage, is extremely useful for detailed studies of the enzyme. We have therefore, attempted to purify PEPC from *Amaranthus hypochondriacus* by conventional method. Further antibodies were raised in rabbits against PEPC purified from *A. hypochondriacus*. These antibodies were used later to evolve a method to rapidly purify PEPC from *Amaranthus* leaves by using immunoadsorbent column. The use of immunoadsorbent chromatography allowed a rapid preparation of a high quality PEPC, but the yield was quite poor.

The extraction and particularly the assay of activity are invariably linked to dilution and in addition, deprive the enzymes of their natural physico-chemical environment. Such dilution of the medium (e.g. encountered during enzyme assays)
affects the quaternary structure of PEPC by inducing dissociation and loss of activity (Willeford and Wedding, 1992). The addition of compatible solutes like glycerol or PEG can stabilize the enzyme (Reinhart, 1980; Karabourniotis et al., 1983). The inclusion of compatible solutes can provide a favorable microenvironment against dilution. The effects of these solutes during extraction and assay were therefore studied in this work.

Light activation of photosynthetic enzymes is a cardinal event in the plant growth and development. As PEPC is a key enzyme involved in CO₂ fixation in C₄- and CAM plants, the regulation of PEPC by light is a topic of interest. It is obvious that the light signal has to be perceived by the Chloroplast and the effect is transmitted to the cytosol, the site of PEPC in C₄ mesophyll cells. Although, the kinetic and regulator) properties of PEPC are modulated by light/dark transitions in vivo (Chollet et al., 1996; Vidal and Chollet, 1997), there is very limited information on the mechanism and pattern of transduction of light signal. Illumination of plant cells is known to rise the cytosolic pH and possibly free Ca²⁺. An attempt was therefore made to study in detail the effects of Ca²⁺ on the activity of PEPC.

Phosphorylation/dephosphorylation cascade is one of the most important modes of post-translational modification of enzymes in both plants and animals. PEPC is one of the enzymes, that undergoes regulatory phosphorylation in the plant tissue. Thus, PEPC has become a model system to study the regulatory changes brought out by protein phosphorylation in plant cells (Chollet et al., 1996; Vidal and Chollet, 1997). However, there is a lot of debate about the nature of regulation of PEPC-PK by calcium.

Compared to the extensive literature on the modulation of malate-sensitivity of PEPC, the studies on changes in kinetic characteristics of the enzyme and in particular the affinity to bicarbonate are quite limited. Recently, it has been reported
that bicarbonate plays an important role in the allosteric regulation of PEPC (Dong et al., 1997a; Ogawa et al., 1997). Such, the modulation of PEPC by one of the substrates, bicarbonate is extremely interesting and warrants further studies.

**Present work**

In the present investigation, PEPC was rapidly purified to homogeneity from the leaves of *Amaranthus hypochondriacus*, a NAD-malic enzyme type C4 plant and used to answer some of the above questions. The approach and objectives are further elaborated in the next chapter.