Chapter 1

Introduction and Review of Literature
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C₄ photosynthesis (C₄ pathway) is one of the three types of photosynthetic carbon metabolism operating in higher plants, the other two being Calvin cycle (C₃ pathway) and Crassulacean Acid Metabolism (CAM) (Edwards and Walker, 1983; Leegood, 1993; Raghavendra and Das, 1993). C₄ pathway requires a co-ordinated functioning of mesophyll and bundle sheath cells in leaves (Hatch, 1987; Leegood and Osmond, 1990). The operation of C₄ pathway of carbon fixation in mesophyll cells leads to a marked increase in the concentration of CO₂ in the bundle sheath cells (Furbank and Hatch, 1987; Furbank and Foyer, 1988), thereby optimizing the function of the ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). Readers interested in further information on C₄ photosynthesis may consult the articles of Edwards and Huber (1981), Hatch (1987, 1992), Furbank and Foyer (1988), Leegood (1993) and Raghavendra and Das (1993).

The key enzyme involved during primary carboxylation in C₄ photosynthesis as well as CAM pathway is phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31). The existence of PEPC in C₄ plants helps to build up a pool of dicarboxylic acids, which on decarboxylation raise the CO₂ concentration in bundle sheath cells and minimize the process of photorespiration. As a result, C₄ plants can achieve high growth rates under conditions of high temperatures, strong illumination and atmospheric oxygen levels, which are not optimal for C₃ plants (Edwards et al., 1985). Due to the importance of PEPC in not only C₄ and CAM but also C₃ plants (during anaplerotic carbon fixation), studies on PEPC were always of interest. The literature on PEPC has been reviewed frequently by several authors. Some of the recent reviews on PEPC are by Jiao and Chollet (1991), Lepiniec et al. (1994), Rajagopalan et al. (1994) and Toh et al. (1994). Earlier reviews on

**Occurrence and Importance**

PEPC occurs in all plants and is believed to be absent in animal tissues (Utter and Kolenbrander, 1972), yeast or fungi (O’Leary, 1982; Lepiniec et al., 1994). Green algae also possess PEPC activity, while its presence in Chromophytes and Rhodophytes is uncertain (Lepiniec et al., 1994).

The activities of PEPC levels in leaves of C₄ plants are about twenty fold higher on a chlorophyll basis than those in C₃ plants (Edwards and Walker, 1983). The ratio of PEPC : Rubisco is about 2 in leaves of C₄ plants, compared to the ratio of 0.1 in those of C₃ plants (Williams and Kennedy, 1978). 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). There are reports that PEPC may be present in the chloroplasts of C₃ plants (Perrot-Rechenmann et al., 1982; Latzko and Kelly, 1983), but this is to be confirmed further.

**Physiological Role**

PEPC is a principal enzyme in C₄ and CAM plants, mediating the primary carbon assimilation (O’Leary, 1982). It catalyses β-carboxylation of oxalacetate to yield PEP and Pi. The reaction is irreversible and exergonic.

One of the characteristic features of C₄ pathway is the occurrence of Kranz-like anatomy, which results in division of labour and spatial separation of biochemical reactions. Most of C₄-pathway enzymes, involved in fixation of atmospheric CO₂ and C₄-acid formation, including PEPC and pyruvate Pi-dikinase (PPDK) are located in mesophyll cells, while bundle sheath cells lodge the enzymes of Calvin cycle, along with those of C₄-acid decarboxylation.
system. In CAM plants, the function of PEPC is similar to \( \text{C}_4 \) plants. Primary carbon fixation by PEPC occurs during the night, followed by decarboxylation of \( \text{C}_4 \) acids and refixation of \( \text{CO}_2 \) by rubisco during day (Kluge, 1983). The operation of CAM minimizes the loss of water in these plants, as the stomata open in the night but are kept closed during most of the day.

PEPC plays an anaplerotic role in \( \text{C}_3 \) plants, while producing \( \text{C}_4 \) acids (i.e., oxalacetate and malate), to provide carbon skeletons for amino acid biosynthesis, nitrogen assimilation, and replenishment of tricarboxylic acid (TCA) cycle intermediates (Gadal, 1983; Melzer and O’Leary, 1987). Further, PEPC plays an important role in generation of NADPH for fatty acid synthesis in developing seeds, fruit maturation (Latko and Kelly, 1983) and maintenance of cytoplasmic pH and electroneutrality (Davis, 1979). PEPC is also known to be involved in regulating stomatal movement (Willmer, 1983; Outlaw, 1990).

**Form and Structure**

Four isoforms of PEPC have been reported in higher plants: \( \text{C}_4 \) photosynthetic form, \( \text{C}_3 \) photosynthetic form, CAM-form and dark or non-autotrophic PEPC (O’Leary, 1982; Andreo et al., 1987; Rajagopalan et al., 1994). These forms can be distinguished by chromatographic, immunological and kinetic properties.

Thomas et al. (1987) reported two isoforms of PEPC in sorghum leaves (E-PEPC and G-PEPC). The E-form occurred in etiolated leaves and exhibited \( \text{C}_3 \) characteristics, while the G-form was present in green leaves and had characteristics of \( \text{C}_4 \) photosynthetic form. Vidal and Gadal (1983) have reported that etiolated sorghum leaves contain only one form (\( \text{C}_3 \) form) of the enzyme and a new isoform of enzyme appears on illumination upon greening (\( \text{C}_4 \) form). The expression of PEPC-gene encoding the \( \text{C}_4 \) isozyme was not leaf specific, since high accumulation of its transcripts was found in also other
parts of maize plant i.e., inner leaf sheaths, tassels and husks (Hudspeth and Grula, 1989). On the other hand, Schaffner and Sheen (1992) reported that the expression of $\text{C}_4$-specific PEPC gene occurred only in illuminated (greening) leaves of maize. No signal of $\text{C}_4$-specific PEPC genome was detected in roots or stems or etiolated leaves of maize. The major form in maize leaves is the $\text{C}_4$-type and is the most abundant protein in mesophyll cells. However, 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).

There is a lot of variation in the number of PEPC isoforms reported from the leaves of $\text{C}_3$ plants and CAM species. Four major isoforms of PEPC are reported in leaves of a $\text{C}_3$ plant Flaveria conquistii, $\text{C}_3$-$\text{C}_4$ intermediate Flaveria floridana and a $\text{C}_3$ performing Mesembryanthemum crystallinum (Adams et al., 1986; Slocombe et al., 1993). Three isoforms in leaves of Gossypium hirsutum, a $\text{C}_3$ species (Mukerji and Ting, 1971) and also two in $\text{C}_3$ performing Kalanchoë blossfeldiana (Brulfert et al., 1979). The four isoforms of PEPC are encoded by different genes in $\text{C}_4$ 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 $\text{C}_4$ plants, a mechanism has developed for preferential expression of $\text{C}_4$ specific PEPC gene (Lepiniec et al., 1994; Stockhaus et al., 1994).

PEPC is a homotetramer (Andreo et al., 1987). Although it is suggested that the enzyme may exist in different oligomeric forms in vivo (Wu and Wedding, 1985; Walker et al., 1986) there are no convincing evidences. The quaternary structure of PEPC in vitro, depends on protein and effector concentrations (Jiao and Chollet, 1991). In Crassula argentea, the enzyme purified during night existed as tetramer while the day form existed in dimer (Wu and Wedding, 1985). In $\text{C}_4$ plants, dissociation of PEPC occurs on incubation with NaCl in a time- and protein-concentration dependent manner.
The phenomenon of oligomerization is further reviewed in the following pages, under the section “Post-translational Modification”.

Chemical modifications of the enzyme have shown that cysteine, arginine and lysine residues are essential for the catalytic activity of PEPC from corn and sorghum leaves (Andreo et al., 1987; Wagner et al., 1988; Willeford et al., 1990; Terada et al., 1991). Histidine and cysteine residues of enzyme may be involved in activation of PEPC by glucose-6-phosphate (G-6-P) (Manetas and Gavalas, 1982; Wedding et al., 1989).

The primary structure of PEPC from several C₄ plants, besides lower micro-organisms like, *Escherichia coli* or *Anabaena variabilis*, has been deduced through sequence analysis of cDNA (Lepiniec et al., 1994; Rajagopalan et al., 1994; Toh et al., 1994). A comparison on the amino acid sequences of PEPC from variety of these organisms shows that the enzyme molecule contains a conserved C-terminal half and a variable N-terminal one (Ishijima et al., 1985; Izui et al., 1986). C₄-PEPC from maize has 970 amino acids (Izui et al., 1986; Hudspeth and Grula, 1989) compared to 952 of sorghum (Cretin et al., 1990), 966 in *F. trinervia* (Poetsch et al., 1991) and 883 of *E. coli* (Fujita et al., 1984; Ishijima et al., 1985). There is 88% sequence homology of sorghum PEPC with that of maize but only 40-50% with *E. coli* and *Anacystis nidulans* (Ishijima et al., 1985; Izui et al., 1986; Cretin et al., 1990).

Phylogenetic trees, constructed from amino acid sequences of as many as 26 different forms of PEPC reveal that enzyme from C₄ dicot (*F. trinervia*) is closer to the C₃ (tobacco and soybean) and C3/CAM isoform than to monocotyledonous C₄ PEPC (Koizumi et al., 1991; Poetsch et al., 1991; Kawamura et al., 1992; Sugimoto et al., 1992; Lepiniec et al., 1993). This observation suggests that monocot and dicotyledonous C₄ plants have evolved separately during the course of evolution.
Purification

One of the first attempts to purify PEPC from plant tissues was by Bandurski and Grciner (1953), who attempted to purify partially the enzyme from spinach leaves. Partial purifications have been made from leaves of cotton, *Pennisetum purpureum*, sorghum, maize, lupin root nodules, soybean nodules, maize root tips, guard cells of *Vicia faba* and epidermis of *Commelina communis* (O'Leary, 1982).

Purification of PEPC to homogeneity has been done using peanut cotyledons (Maruyama and Lane, 1962), spinach (Miziorko et al., 1974), maize (Uedan and Sugiyama, 1976), *Bryophyllum fedtschenkoi* (Jones et al., 1978), *Amaranthus viridis* (Iglesias et al., 1986), sugarcane (Iglesias and Andreo, 1989), sorghum leaves (Arrio-Dupont et al., 1992) and soybean nodules (Schuller and Werner, 1993).

The components of purification-protocol in most of these cases include ammonium sulphate fractionation of crude leaf extracts, followed by dialysis, and gel-filtration through DEAE-cellulose, hydroxylapitite (HAP) and Sephadex column (Uedan and Sugiyama, 1976; Iglesias et al., 1986; Iglesias and Andreo, 1989; Schuller and Werner, 1993). However, in recent reports, FPLC (Jiao and Chollet, 1988, 1989; Jiao et al., 1991) or HPLC (Cretin et al., 1984) have also been used for purification of PEPC. During purification by gel filtration, the enzyme is eluted by high concentration of salt, often chloride (Uedan and Sugiyama, 1976; Mukerji, 1977; Hatch and Heldt, 1985; Iglesias et al., 1986) and occasionally phosphate (Hague and Sims, 1980) or acetate (Smith and Woolhouse, 1984). Chloride, however, can affect the activity of the enzyme (Manetas et al., 1986; Wagner et al., 1987), presumably by inducing dissociation of the tetrameric form into dimers or monomers (Wagner et al., 1987; Manetas, 1990). On the other hand, phosphate is considered to be a stabilizer of the enzyme (Yancey et al., 1982). Instead of ammonium sulphate, polyethylene glycol (PEG) had been used to precipitate the enzyme (Selinioti et
al., 1987; Angelopoulos and Gavalas, 1991), since the yield of PEPC by using PEG was reportedly much better than that with ammonium sulphate. Techniques are now available for rapid purification of enzyme from leaves by immunoadsorbant columns (Vidal et al., 1980; Arrio-Dupont et al., 1992).

During purification, the enzyme is highly susceptible to proteolysis at the N-terminal end. The loss of N-terminal is reflected in the decrease in the sensitivity of enzyme to malate. As a result, the enzyme becomes less prone for phosphorylation and not so sensitive to malate. Therefore, addition of proteolytic inhibitors like phenylmethylsulphonylfluoride (PMSF) or chymostatin is essential during purification (McNaughton et al., 1989).

The specific activity of purified PEPC, reported in literature, varied from a very low average value of 4-10 U mg⁻¹ protein (Coombs et al., 1973; Hayakawa et al., 1981; O'Leary et al., 1981; Sugiyama et al., 1984; Wedding and Black, 1986) and to as high values as 180-220 U mg⁻¹ protein (Mukerji, 1977; Reibach and Benedict, 1977). The latter high values are presumably in error. In case of CAM plants, the best specific activity reported in Kalanchoë daigremontiana is around 35 U mg⁻¹ protein. However, the specific activity from other CAM plants varied from 17-300 U mg⁻¹ protein (Nott and Osmond, 1982). Reviewing the available literature, O'Leary (1982) commented that the specific activity of purified PEPC could be around 25 U mg⁻¹ protein. Similar range of specific activities have been obtained by several authors (Hatch and Heldt, 1985; Iglesias et al., 1986; Wedding et al., 1988; McNaughton et al., 1989). Recently a specific activity of 7.68 U mg⁻¹ protein was reported for PI:PC purified from developing seeds of Brassica campestris (Mehta et al., 1995).

**Stability of PEP carboxylase**

Cytosolic enzymes such as PEPC are present in vivo at far higher concentrations than that are used during in vitro assays. The enzyme is
unstable and may dissociate into inactive di- or monomer on dilution (Wu et al., 1990). The addition of solutes (such as glycerol or PEG) during extraction and storage helps to maintain the tetrameric state of several enzymes (Rhodes and Hanson, 1993). Natural solutes, like betaine and proline can protect enzymes against heat denaturation (Paleg et al., 1981; Nash et al., 1982). Similarly, synthetic polymers like PEG are used often for protein stabilization (Reinhart, 1980).

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 (Pollard and Wyn Jones, 1979; Manetas et al., 1986; Manetas, 1990) and improvement of catalytic efficiency (Stamatakis et al., 1988; Podesta and Andreo, 1989). Karabourniotis et al. (1983) and Medina et al. (1985) have used glycerol and other solutes as stabilizers of activity and regulatory properties of PEPC during and after extraction. The presence of glycerol stabilized the maize PEPC activity by promoting the tetrameric form and enhancing the $V_{\max}$ 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 (Podesta and Andreo, 1989).

*Regulation of PEP carboxylase*

PEPC is an allosteric enzyme which is regulated by several internal and external factors (e.g. metabolic regulation by effectors, light, temperature and pH). The influence of these factors varies depending on the enzyme source and other interacting factors.

*Metabolites (Inhibitors/Activators):*

PEPC is highly regulated by feed-back inhibition by dicarboxylic acids oxaloacetate and allosteric activation by metabolites, particularly phosphate-compounds (Raghavendra and Das, 1976; Gonzalez et al., 1984; Andreo et al.,
1987). Malate and aspartate (besides oxalacetate) are among the typical feedback inhibitors. G-6-P is a powerful activator of PEPC, particularly in C₄ plants.

**L-malate,** which is a product of C₄-pathway, is a competitive inhibitor of the PEPC (Huber and Edwards, 1975). Malate is a known inhibitor not only of C₄ PEPC, but with different effectiveness also in C₃ and CAM forms (Kluge et al., 1988; Echevarria et al., 1990; Jiao and Chollet, 1990). Aspartate also inhibits the enzyme, quite strongly in some C₄ plants (Huber and Edwards, 1975; Iglesias et al., 1986). Aspartate may protect the enzyme against thermal inactivation (Rathnam, 1978; Mares and Leblova, 1980). Organic acids and analogues of PEP/pyruvate are powerful inhibitors of the C₄ enzyme (Rajagopalan et al., 1994). Based on this property, several analogues of PEP are employed to study the reaction mechanism of the enzyme (Gonzalez and Andreo 1989; Janc et al., 1992 a, b). The extent of malate inhibition depends on various factors like assay pH, phosphorylation status of enzyme, proteolytic loss of N-terminal region and presence of activators, e.g. G-6-P (McNaughton et al., 1989, 1991; Jiao and Chollet, 1991; Ausenhus and O’Leary, 1992; Wang et al., 1992). Willeford et al. (1990) postulated that malate causes changes in the oligomeric structure of the enzyme, enhancing the formation of less active dimer. In contrast, 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 dephosphorylated form of PEPC is extremely sensitive to malate, while the phosphorylated form is not so sensitive.

Besides G-6-P, a typical allosteric activator of the C₄ enzyme (Andreo et al., 1987), PEPC is activated by many phosphate-esters (Podesta et al., 1990). Walker et al. (1988) suggested that phosphatase activity of PEPC may be related to the activation process by phosphate compounds. However, in
maize, the activation of PEPC occurred without dephosphorylation of the activator (Bandarian et al., 1992).

The activation of PEPC by glycine is reported only in C₄ monocots such as maize (Nishikido and Takanashi, 1973; Doncaster and Leegood, 1987; Bandarian et al., 1992). Glycine exerted no effect on PEPC from dicotyledonous C₄ plants or from C₃ plants (Nishikido and Takanashi, 1973). This observation correlates well with the structural information, now available, on PEPC which suggests 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).

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

**Light:**

Illumination enhances, by 2-3 fold, the activity of PEPC, particularly in leaves of C₄ plants. Light-activation is a feature of key photosynthetic enzymes in C₃ plants (Buchanan, 1992). A two-fold activation of PEPC was first observed in *Amaranthus palmeri* leaves by Slack (1968). After a preliminary confirmation of light activation in *Atriplex tatarica* (Gavalas et al., 1981), light activation of PEPC has been reported in leaves of several C₄ species (Karabourniotis et al., 1983). 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). Sims and Hague (1981) have reported an increase in level of mesophyll cell mRNA and PEPC-protein synthesis during leaf development and greening of etiolated maize leaves.
Besides the change in activity, the kinetic and regulatory properties of PEPC are markedly modulated by light/dark (L/D) transitions in vivo (Andreo et al., 1987; Jiao and Chollet, 1991). The enzyme exhibits two or three fold more activity on illumination 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 and exhibits a marked stimulation by G-6-P. On the other hand, the dark-form is quite sensitive to malate and is less activated by G-6-P. The response of PEPC in C₃-leaves to light is much less than in C₄ plants. The increase in PEPC on exposure to light is marginal (about 10-15%) in C₃ species (Rajagopalan et al., 1993). Further, the addition of an allosteric positive effector (G-6-P) provided much greater protection against malate inhibition of the enzyme from C₄ species than that from C₃ species (Gupta et al., 1994).

Marginal increase in light activation was reported in mesophyll protoplasts of maize (Devi and Raghavendra, 1992). Pierre et al. (1992) have demonstrated light induced phosphorylation of PEPC in mesophyll protoplasts in sorghum and its dependence on calcium and pH. Light induced phosphorylation also was observed in guard cell protoplasts of Vicia faba 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). Using an artificial photosensitive dye in a reconstituted system, marked photo-activation of purified PEPC was demonstrated by Maheswari and Bharadwaj (1991), but this phenomenon needs to be confirmed.

The phenomenon of light activation of photosynthetic enzymes can be due to changes in the thiol groups (Iglesias and Andreo, 1984) or by phosphorylation-dephosphorylation of amino acid residues like serine (Jiao and Chollet, 1991). Buchanan (1991, 1992) have reported the involvement of thiol groups in modulation of several C₃ enzymes, but this phenomenon may not be well applicable to cytosolic enzymes like C₄-PEPC (Jiao and Chollet, 1991).
On the other hand, pH may be an important factor during light activation of PEPC, located in cytosol of mesophyll cells. Light induces alkalization of cytosol and can lead to activation of PEPC and PEPC-protein kinase (PEPC-PK) or both. This has been shown in "cytosol enriched" cell sap of *Alternanthera pungens*, 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 case of C₄ plants (Selinioti et al., 1986). Attempts have been made to correlate the poor rate of C₄ photosynthesis at low temperature with cold lability of PPDK (Shirahashi et al., 1978) and thermal response of PEPC (Selinioti et al., 1986). However, there is no clear correlation in response of PEPC in cold-exposed leaves with behaviour of the enzyme *in vitro* (Petropoulou et al., 1990; Krall and Edwards, 1993).

Yet, the activity of PEPC in C₄ as well as CAM plants is known to be regulated by temperature. At higher temperature, there is an increase in $V_{\text{max}}$ and a decrease in apparent $K_m$ (PEP) of the C₄ isoform. Cold inactivation of PEPC was observed at higher pH in *Cynodon dactylon*, *Atriplex halimus* and *Zea mays* (Angelopoulos et al., 1990). The oligomeric status of the PEPC enzyme may vary depending on the temperature. Above 25 °C, there is an aggregation of PEPC in case of C₄ or dissociation in case of CAM (Wu and Wedding, 1987). A change may occur on exposure to cold/chilling in form of PEPC: from active tetrameric to less-active dimers or monomers (Shi et al., 1981; Walker et al., 1986).

Krall and Edwards (1993) reported that the PEPC enzyme was very stable at even low temperatures in *Panicum miliaceum*, while the enzyme from *Panicum maximum*, lost 50% of its activity on incubation for 60 min at 0 °C. A
temperature dependent increase in PEPC activity, in presence of solutes, was reported in *Cynodon dactylon* (Drilias et al., 1994). The effect was more pronounced at temperature above 30 °C. PEPC is protected against cold inactivation by addition of compatible solutes like proline or betaine (Krall and Edwards, 1993). Temperature can affect **oligomeric/aggregation** status of enzymes. However, this is not well corroborated in case of C₄-PEPC (Shi et al., 1981; Walker et al., 1986).

**pH:**

PEPC is highly regulated by cytosolic pH (Davis, 1973, 1979; Andreo et al., 1987; Rajagopalan et al., 1993). The enzyme is active at pH 8.0 and becomes inactive at acidic pH, thereby slowing down carboxylation. The enzyme shows competitive inhibition at pH 8.0 but non-competition at pH 7.0 with malate (Gonzalez et al., 1984).

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, 1973, 1979). Recent reports by Raghavendra et al. (1993), Yin et al. (1993) using pH-dependent fluorescent dyes, have demonstrated the marked changes in the intracellular pH on illumination. Light induces an alkalization of cytosol and acidification of vacuole within the leaves. The extent of cytosolic alkalization in mesophyll cells of C₄ plants was much greater than that in C₃ leaves. The changes in pH could modulate the intracellular calcium, as in sorghum mesophyll protoplasts (Pierre et al., 1992). The response to pH may affect the catalytic activity of PEPC or PEPC-PK or both (Rajagopalan et al., 1993).

**Salt/Water stress:**

Water stress, increases the activity of PEPC in leaves of *Salsola soda*, similiar to the effects of warm-temperatures on PEPC in C₄ plants. In contrast, PEPC in *Cynodon dactylon* is activated by betaine, while proline is a competitive inhibitor of PEPC (Manetas et al., 1986). However, the effect of salt stress on
PEPC is not manifested in C₄ plants as strongly as in CAM plants. 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 (Mc Elwain et al., 1992). Photoperiodism or water stress can shift young leaves of *Kalanchoe blossfeldiana* from C₃-type photosynthesis to CAM. It has been shown that endogenous levels of ABA preceded PEPC increase, independent of CAM induction in isolated leaves of *Kalanchoe blossfeldiana* (Taybi et al., 1995). Dai et al. (1994) provided evidence that in *Mesembryanthemum crystallinum*, the increase in PEPC activity upon ABA treatment was due to increased levels of CAM-specific isoform of the enzyme.

**Effect of salts/inorganic ions**

Stimulation of dark CO₂ fixation by ammonium is a well known phenomenon (e.g. Hammel et al., 1979; Miyachi and Miyachi, 1985). Ammonium ions enhance assimilation of carbon into C₄ acids in higher plants, algal cells and cyanobacteria (Ohmori et al., 1986; Müller et al., 1990; Vanlerberghe et al., 1990). Such stimulation was assumed to be due to the increase in the activity of PEPC in ammonia-treated cells. The rates of ammonia assimilation in vivo were well 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 (Miller, 1970; Kanazawa et al., 1972; Peterson and Evans, 1978). Although the increase in PEPC activity has been correlated with elevated rates of dark carbon fixation, the direct effects of ammonium salts on PEPC activity in vitro were not assessed. Gayathri and Raghavendra (1994) have recently reported that ammonium ion can stimulate PEPC in vitro. 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 $\text{NH}_4^+$. Ammonia diffusion into cells may cause alkalization of cytosol and the rise in pH could in turn stimulate PEPC.

Biosynthesis of PEPC in maize leaves was affected by the extent and form of nitrogen available to the plant. For e.g., the levels of PEPC are increased on feeding maize leaves with nitrate or ammonium (Sughiharto et al., 1990; Sughiharto and Sugiyama, 1992). Ammonium salt was two-fold more effective inducer of PEPC biosynthesis than that of nitrate (Sughiharto and Sugiyama, 1992). These effects can be termed as long-term ones.

Van Quy et al. (1991a, b) have reported in wheat leaves the light activation of PEPC is further enhanced in presence of nitrate. Van Quy and Champigny (1992) and Duff and Chollet (1995) suggested that presence of 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 are of two kinds: long-term enhancement of PEPC biosynthesis and short-term: increase in the activity of PEPC/PEPC-PK.

Another important ion which is involved in regulation of PEPC is calcium. PEPC is one of the few plant proteins, photoregulated and controlled by a complex cascade of events, possibly involving calcium. Light dependent phosphorylation of PEPC in mesophyll protoplasts of sorghum was promoted by weak bases such as ammonium chloride and methylamine (Pierre et al., 1992). These ions were expected to increase the cytosolic pH, raise the levels of calcium and activate PEPC or PEPC-PK or both. However, the evidences on the involvement and regulation by calcium of PEPC-PK are contradictory. These are further discussed in the following pages. Further studies are needed to resolve the role of pH and calcium in regulating the activity of PEPC and its phosphorylation by PEPC-PK in C_4 and CAM plants.
**Post-translational modification of PEP carboxylase**

The post-translational modification of PEPC in plants involves two types of phenomena: Phosphorylation and oligomerization.

**Phosphorylation-Dephosphorylation:**

Regulation of enzyme activity in plants by reversible phosphorylation has been reviewed (Budde and Chollet, 1988; Huber et al., 1994). Regulatory phosphorylation can result in inactivation or activation and/or changes in the allosteric properties of the target enzyme. However, the extent of these changes can vary greatly among different target enzymes. For example, in case of PPDK, the difference in the catalytic activity between the phosphorylated and dephosphorylated forms can be so large, up to ten-fold, between the light- and dark-forms (Huber et al., 1994). On the other hand, alteration in phosphorylation status of some enzymes, may result only in two-three fold changes in the activity, as in case of PEPC. The phosphorylation of PEPC occurs at one or more seryl residues in C₄ and CAM plants (Nimmo, 1993). Regulatory phosphorylation of PEPC by a PEPC protein-serine kinase is established, both in vitro and in vivo (Jiao and Chollet. 1991). The phosphorylation of PEPC occurs on ser in sorghum (Jiao et al., 1991; Wang et al., 1992), ser¹⁵ in maize (Jiao and Chollet, 1990; Jiao et al., 1991), ser¹” in tobacco (Wang and Chollet, 1993) ser¹” in soybean root nodule (Zhang et al., 1995). The phosphorylation occurs on ser (day) in *Mesembryanthemum crystallinum* (Jiao and Chollet, 1991; Baur et al., 1992; Nimmo, 1993).

Comparison of amino acid sequences around N-terminal region reveals that the important structural amino acid motif of “Lys’Arg-X-X-Ser”, which appears to interact with PEPC-PK, is present in all C₃, C₃·C₄, and C₄ forms of PEPC but not in those of prokaryotes: *E. coli* ox *A. nidulans* (Cretin et al., 1991; Poetsch et al., 1991; Pathirana et al., 1992; Schaffner and Sheen, 1992). This observation suggests that PEPC of only higher plants possesses marked regulatory properties. It is now known that PEPC of also C₃ plants undergoes
reversible phosphorylation and changes its **allosteric** properties, like that of $C_4$ and CAM-PEPC (Gupta et al., 1994).

Limited information is available on **post-translational** regulation of the non photosynthetic $C_3$ enzyme, although it too is a subject of **allosteric** control. Phosphorylation of PEPC has been reported with purified PEPC of maize and tobacco (Wang and Chollet, 1993), and also in leaves of $C_3$ plants (Van Quy et al., 1991 a, b; Duff and Chollet, 1995), **stomatal** guard cells (Schnabl et al., 1992; Zhang et al., 1994), in legume root nodule extracts **in vitro** (Vance and Gantt, 1992; Schuller and Werner, 1993) and **in vivo** (Zhang et al., 1995). Phosphorylation of alfalfa and soybean root nodules in crude extracts by endogenous protein kinase **in vitro** has also been reported (Pathirana et al., 1992). The phosphorylation site is accessible to both homologous and heterologous protein kinases, e.g. mammalian protein-kinase A (Jiao and Chollet, 1990; Terada et al., 1990).

The post-translational modification by phosphorylation of PEPC is promoted further in light by virtue of **reversibly** 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 **de novo** synthesis of the enzyme. McNaughton et al. (1991) speculated that a signal generated in chloroplast may initiate a sequence of events that leads to a significant increase in activity of PEPC-PK and phosphorylation of PEPC in the cytosol. On feeding with photosynthetic inhibitors like DCMU or $DL$-glyceraldehyde, phosphorylation of PEPC decreased indicating the co-ordination of both mesophyll and bundle sheath cells (Jiao and Chollet, 1992). However, Pierre et al. (1992) have shown that maize mesophyll protoplasts possess the intrinsic ability, similar to whole leaf, in performing PEPC phosphorylation.

While phosphorylation of PEPC is catalyzed by PEPC-PK, the dephosphorylation is brought about by type 2A protein phosphatase(s) (Carter
et al., 1991; Mcnaughton et al., 1991). Although PEPC has been purified from leaves of several C₄ plants (maize and sorghum), CAM species (Bryophyllum fedtschenkoi) and C₃ species (tobacco), attempts to purify PEPC-PK from C₄ or CAM plants are still very limited.

Calcium-dependent and calcium-independent protein kinases were purified from sorghum leaves (Bakrim et al., 1992). Ogawa and Izui (1992) also have shown that phosphorylation of PEPC is by a calcium-dependent protein kinase in maize leaves. PEPC from sorghum was phosphorylated in a calcium-calmodulin dependent manner (Echevarria et al., 1988). However, Jiao and Chollet (1988, 1990, 1991) have reported that phosphorylation of PEPC in maize occurs in a calcium independent manner. Such discrepancy in the reports on the role of calcium during phosphorylation of PEPC could be due to presence of multiple kinases in cytosol (Ogawa and Izui, 1992).

The information about PEPC-phosphatase(s) from C₄ plants is even scarce. On treating with alkaline phosphatase, the malate inhibition of PEPC increases and enzyme functions in a manner similar to that of dark-form (Jiao and Chollet, 1988; Arrio-Dupont et al., 1992). The coordination of both PEPC-PK and PEPC-phosphatase(s) may determine the net phosphorylation and its sensitivity to malate during L/D transitions.

**Oligomerization:**

PEPC is a homotetramer (Andreo et al., 1987) and exists as dimer or monomer, depending on several factors: pH, ionic strength (Walker et al., 1986; Wagner et al., 1987), temperature (Wu and Wedding, 1987) and PEPC concentration (Willeford and Wedding, 1992). Presence of NaCl causes dissociation of enzyme into dimer at pH 7.0, and into dimers/monomers at pH 8.0. Presence of PEP, magnesium or G-6-P prevented dissociation of the enzyme (Wagner et al., 1987). Effectors such as G-6-P and malate, or presence of solutes (PEG, glycerol) can effect the aggregation of the enzyme (Podesta and Andreo, 1989; Manetas, 1990; Wedding et al., 1994). G-6-P increases the aggregation of the
enzyme (Wu and Wedding, 1994). Wang et al. (1992) have reported that phosphorylation has no effect on G-6-P and the activation of the enzyme by G-6-P occurs by a more complex mechanism than the activation by PEP or inhibition by malate.

Glycerol and high PEPC concentration shifts the enzyme to active tetrameric form (Podesta and Andreo, 1989). However, Weigend and Hincha (1992) have reported that there is no relation between the malate sensitivity and the oligomeric status of the enzyme. Dilution of the enzyme in vitro can change the oligomeric status of the enzyme (Wu et al., 1990). 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 yet to be investigated critically and its relevance to phosphorylation would be of great interest.

A lot of work has been done on the physiology, biochemistry and molecular biology of PEPC. But there is still a large scope for further work, on PEPC particularly from C₄ plants and C₃-C₄ intermediates. For example, PEPC has been purified to homogeneity from leaves of several C₃- and C₄ plant species like spinach, maize, sorghum (Rajagopalan et al., 1994), and even from Amaranthus viridis (Iglesias et al., 1986). Nevertheless, a method of rapid purification, along with long-term storage, is extremely useful for detailed studies. We have therefore, attempted to purify PEPC from Amaranthus hypochondriacus and evolve an acceptable method of storage.

Addition of glycerol stabilizes PEPC (Karabourniotis et al., 1983), since otherwise the enzyme is unstable, particularly on dilution (Selinioti et al., 1987). Inclusion of solutes, like PEG, has been conventionally used for stabilization of proteins (Reinhart, 1980). The effects of different solutes like PEG on stabilization and properties of PEPC from C₄ plants, is not yet studied in detail.
Although the existence of at least four isozymic forms of PEPC have been described in a number of \( \text{C}_3 \), \( \text{C}_3\text{-C}_4 \) intermediate and \( \text{C}_4 \) plants (Ting and Osmond, 1973a, b; Peterson and Evans, 1979; Vidal and Gadal, 1983), there is ambiguity about their distinction. Kinetic and characterization of PEPC from *Amaranthus hypochondriacus*, a \( \text{C}_4 \) plant in comparison to that of other \( \text{C}_3 \) or \( \text{C}_4 \) dicots/monocots is naturally a topic of interest.

PEPC has become a classic example of enzyme regulation by post-translational modification by phosphorylation-dephosphorylation cascade (Jiao and Chollet, 1991; Huber et al., 1994). However there is a lot of debate about the nature of PEPC-PK regulation of PEPC by calcium. There are conflicting reports that PEPC-PK is \( \text{Ca}^{2+} \) dependent or \( \text{Ca}^{2+} \) independent.

In the present investigation, PEPC was purified from the leaves of *Amaranthus hypochondriacus*, a NAD-malic enzyme type \( \text{C}_4 \) plant and used to answer some of those questions. The approach and objectives are further elaborated in the next chapter.