Regulation of NADP- Malic Enzyme and Phosphoenolpyruvate Carboxylase in Leaves of C_4 Plants

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DECLARATION

I hereby declare that the work presented in this thesis entitled "**Regulation of NADP- malic enzyme and phosphoenolpyruvate carboxylase in leaves of C_4 plants**" has been carried out by me under the supervision of Professor A. S. Raghavendra in the Department of Plant Sciences, School of Life Sciences, University of Hyderabad and this work has not been submitted for any degree or diploma of any other University or Institute.

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CERTIFICATE

This is to certify that ***Mr. Jhadeswar Murmu*** *has carried out the research work embodied in the present thesis entitled "****Regulation of NADP- malic enzyme and Phosphoenolpyruvate carboxylase in Leaves of C₄ Plants****" for the degree of Doctor of Philosophy under my supervision in the Department of Plant Sciences, School of Life Sciences, University of Hyderabad.*

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ABBREVIATIONS

CAM	=	crassulacean acid metabolism
CD	=	circular dichroism
CDPK	=	calmodulin-like-domain protein kinase or Ca^{2+}-dependent protein kinase
EGTA	=	ethyleneglycol-bis-(β-aminoethyl ether) N,N,N',N'-tetra acetic acid
FBPase	=	fructose 1,6-bisphosphate
FPLC	=	fast-protein liquid chromatography
Glc-6-P	=	glucose-6-phosphate
HAP	=	hydroxylapatite
MDH	=	NAD malic dehydrogenase
OAA	=	oxaloacetate
PEG	=	polyethylene glycol
PEP	=	phosphoenolpyruvate
PEPC	=	PEP carboxylase
PEPC-PK	=	PEPC-protein kinase
Pi	=	inorganic phosphate
PMSF	=	phenylmethylsulphonyl fluoride
PPDK	=	pyruvate Pi dikinase
PVP	=	polyvinylpyrrolidone

All the remaining abbreviations are all standard ones, and as indicated in *Plant Physiology* issue, 2004, Instructions for contributors, website: http://www.aspb.org

Contents

Chapter 1

Introduction and Review of Literature

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The NADP malic enzyme (NADP-ME; EC 1.1.1.40) and phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) are among the key enzymes of C_4 photosynthesis. NADP-ME catalyzes the oxidative decarboxylation of L-malate to yield pyruvate, CO_2 and NADPH (Edwards and Andreo, 1992). NADP-ME is localized in the chloroplasts of bundle sheath cells for decarboxylation of malate in C_4 species (Maurino et al., 1997). Being an important enzyme, the regulation of NADP-ME has been studied in detail, particularly in C_4 plants (Drincovich et al., 2001).

Phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) is involved in the primary carbon fixation in leaves of C_4 and Crassulacean acid metabolism (CAM) plants (Andreo et al., 1987). PEPC yields oxaloacetate from phosphoenolpyruvate (PEP) and bicarbonate (HCO_3), liberating Pi in the presence of Mg^{2+}. The enzyme plays an important role also in leaves of C_3 plants, and tissues other than leaves (e.g. root nodules, fruit pods, seeds and cotton fibers), since PEPC is responsible for channeling of carbon into keto-acids required for amino acid synthesis and ensuing protein synthesis.

Enormous progress has been made in our knowledge of biochemistry and molecular biology of PEPC in not only C_4 plants, but also C_3 species and legume root nodules. For the first time the three-dimensional (3D) structures were obtained for PEPCs from *Escherichia coli* (Kai et al., 1999) and *Zea mays* (Matsumura et al., 2002). This structural information, together with information obtained by site-directed mutagenesis, have all helped to throw light on the molecular mechanisms of catalytic and allosteric regulation of PEPC. Another

important development is that PEPC protein kinase (PEPC-PK) was purified to homogeneity (Saze et al., 2001), and cDNAs for the kinase were cloned, initially from CAM plants (Hartwell et al., 1999; Taybi et al., 2000) and C_3 plants (Fontaine et al., 2003; Nakagawa et al., 2003; Xu et al., 2003), and then from C_4 plants (Tsuchida et al., 2001). Several authors have periodically reviewed the literature on the functions, properties and regulation of C_4 PEPC, PEPC kinase and its use in metabolic engineering (O'Leary 1982; Lepiniec et al., 1994; Rajagopalan et al., 1994; Toh et al., 1994; Chollet et al., 1996; Vidal and Chollet, 1997; Nimmo 2000; 2003; Matsuoka et al., 2001; Chinthapalli et al., 2002; Vidal et al., 2002; Miyao 2003; Svensson et al., 2003; Kai et al., 2003; Izui et al., 2004).

Physiological role of NADP-ME

The information on NADP-ME in C_4 plants is much less than that on PEPC. The primary role of NADP-ME is decarboxylation of malate in bundle sheath cells and to provide CO_2 for Rubisco in Calvin cycle (Edwards and Andreo, 1992). The cytoslic isoform of NADP-ME is involved in plant defenses, possibly by providing NADPH for the biosynthesis of lignin and flavonoids (Casati et al., 1999; Drincovich et al., 2001). NADP-ME in fruit tissues of tomato and grape berries was implicated in respiration during ripening, providing pyruvate and/or NADPH as substrate for respiration, maintenance of intracellular pH (Edwards and Andreo, 1992; Famiani et al., 2000). In submerged aquatic macrophytes such as *Hydrilla verticillata* and *Egeria densa* where availability of CO_2 is low, NADP-ME facilitates maintenance of high rates of decarboxylation of malate and delivery of CO_2 to Rubisco in the chloroplasts (Lara et al., 2002). NADP-ME activity is also associated with the

plastids from developing embryos of oil seeds and fatty acid biosynthesis in castor bean endosperm (Shearer et a., 2004).

The levels of NADP-ME increase during the greening of etiolated seedlings of C_4 plants (Maurino et al., 1996). The marked fold increase in activity of NADP-ME during greening is mainly due to light stimulated synthesis of protein. To our knowledge, there has been only one attempt to examine the short-term modulation by light of NADP-ME in C_4 plants. Drincovich and Andreo (1994) demonstrated that the activity of NADP-ME in crude extracts increased by 50%, when the extracts were illuminated for a long time of 10 h. This observation is rather intriguing.

NADP-ME isoforms

Seven isoforms of NADP-ME are found in photosynthetic plants depending on their physiological roles and localization. The photosynthetic isoform is $C_{4(1)}$-NADP-ME and is specifically located in bundle sheath chloroplasts of C_4 plants such as maize, sugar cane, sorghum, *Haloxylon persicum and* C_3-C_4 *Flaveria floridana* or C_4 *Flaveria* species (Drincovich et al., 2001). The best studied $C_{4(1)}$-NADP-ME is from maize leaves. The enzyme has been purified and its kinetic parameters, molecular properties and amino acid residues essential for catalysis have been determined (Maurino et al., 1996). The corresponding cDNA of this enzyme was the first to be determined among plant NADP-ME, and the N-terminal sequence of the mature protein was also determined.

Transgenic rice expressing high levels of maize NADP-ME under the control of the rice light-harvesting chlorophyll a, b binding protein promoter was recently obtained (Hausler et al., 2002). Interestingly the chloroplasts in the

transgenic plants were agranal without stacking of thylakoids. As bundle sheath chloroplasts of NADP-ME type C_4 plants are unique in having reduced grana formation, the authors postulated that high levels of chloroplastic NADP-ME activity in transgenic C_3 plants could strongly affect the development of this organelle by generating excessive reducing power (Tsuchida et al., 2001).

$C_{4(2)}$-NADP-ME is non-photosynthetic isoform found in plastids of C_4 plants such as maize and *F. bidentis*. In maize the enzyme was purified from both etiolated leaves (Maurino et al., 1997) and roots (Maurino et al., 2001). The cDNA clone and expression pattern of root form of enzyme was studied in detail by Maurino et al., 2001. This enzyme is under transcriptional or posttranscriptional regulation by effectors related to plant defense responses, as in the case for C_3 plants. Marshall et al. (1996) characterized the genomic clone of the enzyme from *F. bidentitis*. $C_{4(3)}$-NADP-ME is cytosolic isoform found in C_4 plants maize. The gene coding for this enzyme has been cloned and the gene is apparently expressed in embryo root epidermis from maize but particular function of this enzyme is not yet known.

$CAM_{(1)}$-NADP-ME is a photosynthetic isoform of NADP-ME found in cytosol of some CAM plants (Cushman 1992; Honda et al., 2000). $C_{3(1)}$-NADP-ME is a non-photosynthetic isoform of NADP-ME found in the cytosol of some C_3 plants such as bean, poplar, grape berries, tomato and *Apium graveolens* (Dricovich et al., 2001). $C_{3(2)}$-NADP-ME is a non-photosynthetic isoform of NADP-ME present in plastids of some C_3 plants such as rice, wheat, *Flaveria pringlei*, tomato, *Arabidopsis*, *Ricinus communis*, *Glycine max*, *Hydrilla verticillata, Egeria densa* (Drincovich et al., 2001).

Evolution of NADP-ME

Most of the evolutionary studies on C_4 enzymes was carried out in *Flaveria*, as this genus contains C_3, C_3-C_4 and C_4 photosynthetic sub-types (Borsch and Westhoff, 1990). Full length cDNA of NADP-ME, isolated from *F. trinervia* (C_4) and *F. pringlei* (C_3) encodes mature proteins of about 62 kD (Drincovich et al., 1998). They suggested that among *Flaveria* spp. there is a 72-kD constitutive form, a 64-kD form that may have appeared during evolution of C_4 metabolism, and a 62-kD form that is necessary for the complete functioning of C_4 photosynthesis. Lipka et al. (1994) reported that the gene encoding the C_4 NADP-ME isoform descended from a common ancestral gene already present in C_3 species. Marshall et al. (1996) isolated three genomic clones of NADP-ME from the C_4 species *F. bidentis*. Through southern blot analysis and sequence similarity, they reported that possibly 4 isoforms are present in *Flaveria* species. Drincovich et al. (1998) reported three isoforms of NADP-ME in *Flaveria* species and suggested that a differential expression of existing NADP-ME gene encoding the chloroplastic forms in a tissue and cell-specific manner was involved in the evolution of C_4 photosynthesis in the genus *Flaveria*.

The phylogenetic tree constructed with the performed alignment of different plant NADP-MEs clearly shows three groups. Group I includes cytosolic dicot NADP-MEs; group II includes the plastidic dicot NADP-MEs and group III includes the monocot NADP-MEs suggests that they may have originated from two different ancestral genes. It is possible that one of the genes may become silent during the evolution of some species or that all the dicot plants have both the cytosolic and plastidic enzyme such as tomato and

grape. It is also clear that both cytosolic and plastidic ancestral genes have the potential to originate photosynthetic NADP-MEs independently, as CAM and C_4 NADP-ME are found in different groups. On the other hand, both plastidic and cytosolic monocot NADP-MEs are found in within group III, except for the $C_{4\,(3)}$-NADP-MEs of maize, which is found in group I. Presence of common amino acid residues in NADP-MEs of group I, represents typical dicot NADP-MEs. Thus, an ancestral gene has been maintained in the monocot during evolution. The phylogenetic tree shows that C_4 and CAM photosynthetic isoforms appeared several times in different branches of the tree, suggesting the polyphyletic origin of the C_4 and CAM photosynthesis. However, the divergence between dicots and monocots occurred earlier that the appearance of both C_4 and CAM photosynthesis, as the specific forms of NADP-ME associated with those pathways appear in well different branches of the tree.

Drincovich et al. (2001) proposed a classification of plant NADP-MEs based on their physiological function and localization with recent advances in the characterization of each isoform. Based on the alignment of amino acid sequences of plant NADP-MEs, putative binding sites for the substrates were analyzed and the phylogenetic origin of each isoform, revealing several features of the molecular evolution of this ubiquitous enzyme. Recently, Tausta et al. (2002) proposed a scheme for the origin of the C_4-specific NADP-ME gene, based on developmental stage of the leaf, light conditions, and tissue type and on sequence comparison of ME families in other species.

UV-B induction of NADP-ME

Light is essential for plant growth and development not only as a source of energy but also as a signal that regulates developmental and metabolic

processes. Plant responses to light as a signal are perceived by three major classes of photoreceptors the phytochrome family ultraviolet-A (UV-A) blue light (cytochrome) receptors and UV-B receptors. Light regulated genes respond to one or more of these photo receptors via different signal transduction pathways and the responses may be tempered by the other environmental factors or developmental stages of the plants. NADP-ME in etiolated maize seedlings are increased with UV-B and UV-A radiation. NADP-ME from bean and wheat could be associated with lignin biosynthesis by providing NADPH for the two NADP-dependent reductive steps in monolignol biosynthesis (Casati et al., 1997). Drincovich et al. (1998) reported increase in content and activity, as well as in amount of steady state RNA of photosynthetic isoform of NADP-ME, after treatment with environmentally relevant levels of UV-B radiation in the presence of low PAR, in a manner similar to the induction of NADP-ME in high PAR in etiolated and green maize seedlings. NADP-ME was induced by UV-B radiation to almost same extent as by high levels of white light. The increase in the steady state level of NADP-ME RNA may be attributed to a high rate of transcription or higher stability of the synthesized RNA, or both.

Genetic engineering of NADP-ME

Tsuchida et al. (2001) over expressed the full length cDNA encoding NADP-ME from rice and maize under the control of the rice *Cab* promoter. Transformant of rice cDNA for NADP-ME resulted very low amount of NADP-ME in rice compared with that of maize cDNA. But in maize leaves the transformant of maize cDNA resulted about 60% increase in NADP-ME activity. Two sets of transgenic rice plants overproducing the maize C_4-specific isoform (Takeuchi et al., 2000; Tsuchida et al., 2001) and the rice C_3- specific isoform

of NADP-ME (Tsuchida et al., 2001) have been reported. The transformants overproducing the rice enzyme with some fold increase in activity did not show any detectable differences in their growth, while those overproducing the maize enzyme at the same activity level showed serious stunting and bleaching of leaf colour, due to enhanced photoinhibition of photosynthesis under natural light conditions. It is proposed that the action of the maize NADP-ME in the chloroplasts increases the NADPH/NADP ratio and suppresses photorespiration, rendering photosynthesis more susceptible to photoinhibition (Takeuchi et al., 2000; Tsuchida et al., 2001). The C_4- specific NADP-ME has a higher V_{max} value, lower K_m values for substrates, and higher optimum pH, as compared with the C_3-specific isoform (Casati et al., 1997). Such features are suitable for strict regulation of the enzyme activity in the bundle sheath cell chloroplasts of C_4 plants, but they allow the enzyme to continue operating in the leaves of C_3 plants even when serious damage occurs.

Occurrence of PEPC

PEPC is ubiquitous and is distributed widely in photosynthetic and non-photosynthetic tissues of higher plants, green algae, bacteria, protozoa and legume root nodules and apparently absent in animal tissues, yeast or fungi (Andreo et al., 1987; Vance and Gantt, 1992; Lepiniec et al., 1994; Kai et al., 2003; Izui et al., 2004). The activities of PEPC levels in leaves of C_3 plants are about 2 to 5% of that found in C_4 plants (Edwards and Walker, 1983; Latzko and Kelly, 1983).

PEPC constitutes about 15% of total soluble protein in maize (Hague and Sims, 1980). The enzyme is confined to the cytoplasm of mesophyll cells in C_4 and CAM plants. In C_3 plants, PEPC may be localized in both cytosol and

chloroplasts of the leaves (Perrot-Rechenmann et al., 1982; Latzko and Kelly, 1983). Thus, PEPC is considered as a typical marker enzyme for cytosol and for C_4 mesophyll cells. PEPC has many faceted physiological roles in plants (Latzko and Kelly, 1983), which are distributed among several isoforms with different catalytic and regulatory properties including, important roles in the photosynthesis of C_4 and CAM plants, supplying carbon to N_2 fixing legume root nodules, and maintaining cellular pH.

There is a significant evolutionary divergence between green algal, higher plant and prokaryotic PEPCs. Studies on immunoblot analysis using anti-PEPC (green algal or higher plant) antibodies 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, the N-terminal amino acid sequences and CNBr cleavage patterns suggest that prokaryotic or green algal PEPC is distinct from higher plant PEPC (Rivoal et al., 1998).

PEPC isoforms and molecular evolution

In higher plants, four types of PEPC isoforms have been reported so far, namely, C_4-, C_3-, CAM- and dark/non-autotrophic forms. 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 (C_3 form) of the enzyme and a new isoform of enzyme appears on illumination upon greening (C_4 form) (Vidal and Gadal, 1983). C_4 specific gene expression occurs only in illuminated (greening) leaves (Shäffner and Sheen, 1992). The expression of PEPC-gene encoding the C_4 isozyme was not leaf specific, since high accumulation of its

transcripts was observed in also 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, C_4-type is the major form in maize leaves and is the most abundant protein in mesophyll cells. In maize, two form of C_3 PEPC were found, one is C_4 form and other is root form. The amino acid residue similarity between these two forms of PEPC was 77%, and the K_m values of recombinant C_4-PEPc for PEP, Mg^{2+} and HCO_3- at pH 7.3 were about 30-, 10-, 2-fold higher than that of root form PEPC, respectively (Dong et al., 1998).

There is a lot of variation in the number of PEPC isoforms reported from the leaves of C_3 plants and CAM species. Four major isoforms of PEPC were reported in leaves of a C_3 plant *Flaveria conquistii*, C_3-C_4 intermediate *F. floridana* and a C_3 performing *Mesembryanthemum crystallinum* (Adams et al., 1986; Slocombe et al., 1993). Three isoforms were noticed in leaves of two C_3 species: *Gossypium hirsutum* and *Vicia faba* (Mukerji and Ting, 1971; Schulz et al., 1992). Two kinetically and immunologically distinct isoforms of PEPC were identified in developing castor oilseeds (Blonde and Plaxton, 2003).

The four isoforms of PEPC are encoded by different genes in C_4 plants (Hermans and Westhoff, 1990). Genomic, cDNA sequences and Southern hybridizations showed that both *F. trinervia* (C_4) and *F. pringlei* (C_3) contain four distinct classes of *ppc* genes, which are named *ppc*A to *ppc*D. The classes of *ppc* genes identified in *F. trinervia* and *F. pringlei* relate to one another by

gene-to-gene relationship (Hermans and Westhoff, 1990). Recently, Bläsing et al. (2002), has shown the existence of four PEPC gene class in grasses. The hypothesis that the grass C_4 PEPC gene could have derived from root pre-existing PEPC gene was further substantiated by analyzing the amino acid sequence of PEPC from different plant families (Bläsing et al., 2002).

C_4 plants have evolved several times independently from ancestral C_3 plants due to selective environmental conditions during the evolution of higher plants (Monson 1999) and gained distinct kinetic and regulatory properties compared to C_3 isoforms. According to Sanchez and Cejuda (2003) that both plant-type and bacterial-type PEPCs diverged early during the evolution of plants from a common ancestor, probably the PEPC from gamma-proteobacteria. The dicotyledonous genus *Flaveria*, comprises not only C_3 and C_4 species but also a large number of C_3-C_4 intermediates which makes *Flaveria* a good model system for the studying evolution of PEPC in which C_4 photosynthetic traits are expressed (Westhoff et al., 1997; Bläsing et al., 2000; Svensson et al., 2003). The kinetic properties of various isoforms of PEPC from genus Flaveria are similar to that of maize isoforms, though there was more (95%) similarity between amino acid residue (Blässing et al., 2002). Amino acid swapping and site-directed mutagenesis revealed that Ser at position 774 (780 in maize) is a C_4-determining residue and Ala at this position is determinant for C_3 and CAM PEPCs. Along with this residue another segment of 296-437 (301-442 in maize) was found important for the C_4 characteristics (Engelmann et al., 2002). Comparative and functional analysis of the PEPC promoter from *F. trinervia* and *F. Pringlei* make it possible to identify the cis-regulatory sequences for mesophyll specific gene expression

and to search for the corresponding trans-regulatory factors (Gowik et al., 2004; Westhoff and Gowik, 2004).

Purification of PEPC

For the first time, Bandurski and Greiner (1953) had partially purified PEPC from spinach leaves. Since then PEPC has been purified from a wide variety of sources such as cotton, *Pennisetum*, *Sorghum* and maize (all from leaves), lupin and soybean root nodules, maize root tips, *Vicia faba* guard cells and epidermis of *Commelina communis* (O'Leary 1982; Rajagopalan et al., 1994). Among the recent reports are the purification of PEPC from *Amaranthus hypochondriacus* (Gayathri et al., 2000), *Brassica napus* (Moraes and Plaxton, 2000), *Selenastrum minutum* (Rivoal et al., 2002) and developing castor oilseed (Blonde and Plaxton, 2003).

The native structure 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 (Chollet et al., 1996). 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 using FPLC, HPLC or immunoaffinity chromatography (Wang and Chollet, 1993a; Duff et al., 1995; Zhang et al., 1995). With proper precautions and suitable protocols of rapid purification, the preparation of PEPC with an intact N-terminal region is possible from leaves (C_4, CAM and C_3) and root nodules.

The cloning and expression of recombinant PEPC in *E. coli* has been successfully employed with the enzyme from *Sorghum* (Crétin et al., 1991) or maize (Yanagisawa and Izui, 1990) or *Flaveria* (Westhoff et al., 1997). The

recombinant DNA technology made it possible to produce large amounts of C_4-type or C_3-type PEPC in *E. coli* (Pacquit et al., 1993; Svensson et al., 1997; Bläsing et al., 2000).

Structure of PEPC protein

PEPC at a native state is a homotetrameric enzyme of about 400-kD with four identical subunits, each with a molecular mass of 95 to 110-kD (Andreo et al., 1987). Most of PEPCs are regulated by allosteric effectors depending on the species. However, its quaternary structure depends on protein concentration, presence of effectors and the protein environment.

The primary structure of PEPC was first deduced from *ppc* gene cloned from *E. coli* in 1984 (Fugita et al., 1984). Since then, till date more than 75 molecular species of PEPC have been established from their primary structure, including the enzymes from plant such as maize (Matsumura et al., 2002; Kai et al., 2003), and bacteria such as *Anacystis nidulans*, a cyanobacterium (Katagiri et al., 1985), and *Thermus* sp., an extreme thermophilic bacterium (Nakamura et al., 1995; Chen et al., 2002). About 500 partial sequences reside within GenBank, generated primarily for phylogenetic reconstruction (Gehrig et al., 2001; Besnard et al., 2002). A phylogenetic tree showed that these PEPCs had evolved from the same ancestral origin (Chollet et al., 1996; Svensson et al., 2003). The size of PEPC polypeptide varies significantly depending on the kind of organism: the approximate number of amino acid residues is 870 (100 kDa), 970 (110 kDa), 1010 (116 kDa), and 1150 (134 kDa) for PEPCs from bacteria, vascular plants, cyanobacteria, and protozoa (e.g., malaria pathogen), respectively. No sequence data are available for PEPC from archaea, though it is known that their subunit sizes are extremely small (about 60 kDa) and no

common allosteric regulator is known (Sako et al., 1996; 1997). The sequence data of algal PEPCs are also sought because one of the purified isoforms from *Selenastrum minutum* is composed of three different kinds of subunits (Rivoal et al., 2001). The alignment of all the deduced amino acid sequences and the construction of related phylogenetic trees showed that several PEPCs had evolved from the same ancestral origin. The amino acid similarities between various pairs of enzyme forms were more than 50% (Lepiniec et al., 1994; Toh et al., 1994; Kai et al., 2003; Svensson et al., 2003). Another new entry to higher plant PEPC was identified in the genome sequence of *Arabidopsis thaliana* as the fourth isoform (Sanchez and Cejudo, 2003). The deduced enzyme is similar to cyanobacterial PEPC in molecular size and is missing the N terminus phosphorylation site, a hallmark of plant PEPC. Such unexpected variety and wide distribution in the plant and microbial kingdoms make PEPC one of the most interesting targets of phylogenetic analysis. Alignment of PEPC sequences shows that about 100 residues are invariably conserved (identical) and another 100 residues are conserved with similar amino acid residues. For pairs of land plant enzymes the identities were more than 71%. Because the C terminus is highly conserved, any difference in length seems to be due to either addition or insertion of extra sequences to the N terminus or to the internal region (at about 10 loci). The number of recombinant PEPCs prepared and characterized is still limited with not more than 15 molecular species identified, necessitating further work. The structural basis of acquiring a variety of regulatory properties during evolution is a very intriguing problem that needs exploration.

Chemical modification of amino acid residues have shown that Arg, His, and Lys residues are essential for the catalytic activity of PEPC (Podestá et al., 1986; Wagner et al., 1988). Some of these conserved residues or regions are expected to be associated with substrate binding and catalytic function. The conserved positively charged amino acid residues (11 arginine, 2 histidine and 2 lysine) may be particularly important, since both the substrates, PEP and HCO_3^-, are anions at physiological pH (Toh et al., 1994; Dong et al., 1999). Using site-directed mutagenesis and PEPC of *Flaveria trinervia,* Gao and Woo (1996) observed that Arg^{450} and Arg^{767} were essential for PEPC function.

The structural elements that give the C_4 PEPC its specific kinetic and regulatory properties were examined by following the strategy of domain swapping and making C_3-C_4 chimerical enzymes (Westhoff et al., 1997; Bläsing et al., 2000; Svensson et al., 2003). The C_3 enzyme (FP966) was progressively interchanged with corresponding parts of the C_4 enzyme (FT966) starting from the amino terminus, while the reciprocal strategy was applied, i.e. regions of the C_4 enzyme were swapped with corresponding segments of the C_3 PEPC. Detailed studies on these chimeric C_3/C_4 enzymes indicated that region 2 (position 296 to 437) and region 5 (position 645 to 966) contains the major determinants for C_4 specific kinetic and regulatory properties. Region 2 was essential for the allosteric regulation by glucose-6-phosphate (Glc-6-P), where as region 5 was the key factor for K_m (PEP; phosphoenolpyruvate) of the nonactivated enzymes. The central determinant in this region was amino acid position 774, which held a serine in all C_4 enzymes but an alanine in C_3 or CAM enzyme (Bläsing et al., 2000; Svensson et al., 2003).

Further, studies on molecular structure of PEPC can provide clues for the development of innovative strategies for the augmentation of productivity of photosynthetic organisms.

Reaction mechanism and properties of PEPC

PEPC, a cytosolic enzyme located in mesophyll cells of C_4 and CAM plants, catalyzes the carboxylation of PEP to yield oxaloacetate (OAA) and inorganic phosphate (Pi). β-Carboxylation of PEP by PEPC occurs in a two step mechanism (O'Leary 1982; Andreo et al., 1987). On the basis of 3D structures of the *E. coli* PEPC and maize C_4-PEPC, a detailed reaction mechanism for PEPC was proposed (Matsumura et al., 2002; Kai et al., 2003).

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 oxaloacetate and Pi (Andreo et al., 1987; Rajagopalan et al., 1994; Chollet et al., 1996; Vidal and Chollet, 1997). First Mg^{2+} binds to PEPC, and when this Mg-Enzyme complex is at equilibrium; PEP binds onto it followed by HCO_3^- binding. Mg^{2+}, PEP and HCO_3^- have to be present on PEPC before the enzyme reaction begins (Chollet et al., 1996).

It is assumed that structures of the Mn^{2+}-bound and Mn^{2+}-DCDP bound *E. coli* PEPC closely mimic the situations in the first and second steps of catalysis. Chlorine atoms of DCDP occupy a hydrophobic pocket consisting of W248, L504, and M538 (Matsumura et al., 1999a; b), and H177 is a critically important catalytic base (Terada and Izui, 1991) located in the hydrophobic pocket. H177 is supposed to play a role in stabilizing the carboxyphosphate intermediate and abstracting a proton from its carboxyl group. The side chain

of R647 on Loop I is located 3 °A away from the phosphate group of PEP and R647 could partially neutralize the negative charge of the phosphate group. Because the structure of the Mn^{2+}-bound *E. coli* PEPC was not so different from that bound with Mn^{2+}-DCDP as to prevent PEP access, the proposal that the true substrate of PEPC is solely a Mg^{2+}-PEP complex (Tovar-Mendez et al., 1998) is questioned.

A serious problem with many of these studies on PEPC is the uncertain state of protein phosphorylation and concomitant malate sensitivity. Frank et al. (2001) have studied the reaction of PEPC by stopped flow fluorometry and suggested that the binding of PEP to PEPC is biphasic. Tovar-Méndez et al. (1998) reported the effects of PEP and Mg^{2+} on the activity of the non-phosphorylated and phosphorylated forms of PEPC from *Zea mays* leaves. At pH 7.3, Mg-PEP binds to the active site and the free PEP to an activating allosteric site.

The optimal pH for the activity of PEPC is around 8.0. The activity of PEPC therefore depends on the cytosolic pH (Andreo et al., 1987; Rajagopalan et al., 1993). 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).

Regulation of PEPC

PEPC is regulated by external factors (e.g. light, temperature, and photoperiod) as well as internal factors (metabolites, inorganic phosphate, and cytosolic pH). Nutrition, particularly nitrogen, can mediate long-term regulation of PEPC. Nitrate, ammonium ions, glutamine and amino acids (i.e. glycine and

alanine) promote the biosynthesis of PEPC protein, and lead to an increase in PEPC activity.

Light and temperature

Light modulates markedly the activity of PEPC in leaves of C_4 plants. On illumination, the activity of PEPC in leaves of C_4 plants is enhanced by 2-3 fold along with a marked decrease in the malate sensitivity of the enzyme (Rajagoplan et al., 1993; Parvathi et al., 2000b). These changes during the light activation are due to the phosphorylation of the enzyme by a PEPC-protein kinase (PEPC-PK (Chollet et al., 1996; Vidal and Chollet, 1997, Parvathi et al., 2000b). See the section on 'Phosphorylation' for further details. The temperature optimum of the C_4 PEPC is around 40-45 ^{0}C and its activity sharply drops below the optimal temperature. At low temperature, the sensitivity of PEPC to malate was very high in maize (Wu and Wedding, 1987) and *A. hypochondriacus* (Chinthapalli et al., 2003). The temperature dependent regulation of PEPC was shown to be independent of phosphorylation, particularly at warm temperature (Chinthapalli et al., 2003).

Cytosolic pH

The activity as well as the pattern of malate sensitivity of PEPC is dependent on pH. A change in cytosolic pH would dramatically affect the activity of PEPC and PEPC-PK (Wang and Chollet, 1993b; Echevarría et al., 1994). Illumination causes a marked alkalization of cytosolic pH especially in C_4 plants (Raghavendra et al., 1993; Yin et al., 1993) and such alkalization can modulate the PEPC phosphorylation and thus the PEPC activity (Giglioli-Guivarc'h et al., 1996; Rajagopalan et al., 1998). The changes in cytosolic pH may modulate properties of PEPC either directly or indirectly through regulation

of PEPC-PK or protein phosphatase (Rajagopalan et al., 1998). There could be a cross talk between the RPP pathway in bundle sheath and pH changes in mesophyll cells of C_4 plant (Giglioli-Guivarc'h et al., 1996). However, this concept has been questioned (Smith et al., 1998). The changes in cytosolic pH might affect induction of the PEPC-PK in CAM plants too (Paterson and Nimmo, 2000).

Calcium

Changes in cytosolic concentration of free calcium regulate various cellular processes through Ca^{2+}-modulated proteins and their targets (Bush 1995). The marked change on illumination in the level of cytosolic calcium implies that calcium could be a part of light-transduction mechanism in plants. There are strong evidences of the importance of Ca^{2+} during phosphorylation of PEPC *in situ* (Duff et al., 1996; Giglioli-Guivarc'h et al., 1996; Parvathi et al., 2000a). Calcium ionophore (A23187) and EGTA inhibited the phosphorylation of PEPC and Ca^{2+} can protect against such inhibition (Chollet et al., 1996; Duff et al., 1996). Thus, cytosolic Ca^{2+} can be a dynamic secondary messenger during light activation of PEPC.

Bicarbonate

Compared to the extensive literature on the kinetic and regulatory properties of PEPC, the studies on modulation of PEPC by HCO_3^- are quite limited. There is a lot of discrepancy for the K_m for HCO_3^- of PEPC in C_4 plants, with the reported values ranging from 20 to 100 μM (Dong et al., 1997; Parvathi et al., 1998). The level of HCO_3^- in the cytosol of C_4 mesophyll is expected to be 80 μM (Jenkins et al., 1989). Dong et al. (1997) reported that HCO_3^- plays a major role in the allosteric regulation of PEPC. The activity of PEPC increased

by 2-fold as the concentration of HCO_3^- was raised from 0.05 mM to 10 mM (Parvathi et al., 1998). Only marginal effect on K_m for PEP was observed but the affinity of PEPC to Mg^{2+} increased by >2-fold. Bicarbonate-induced changes in PEPC were independent of phosphorylation, and possibly involve conformational changes of the protein (Parvathi et al., 1998).

Metabolites: Malate/Glc-6-P/Gln

PEPC is modulated by metabolites such as oxalacetate, malate, aspartate, glycine and Glc-6-P (Andreo et al., 1987). L-malate, a product of carboxylation, is a competitive inhibitor of PEPC, not only C_4 but also the C_3 and CAM forms and also its phosphorylation *in vitro* (Bakrim et al., 2001). Glc-6-P and related phosphorylated metabolites activate PEPC, by decreasing the K_m for PEP (Andreo et al., 1987). Glc-6-P can also protect the enzyme from malate inhibition and induce aggregation of PEPC into the tetrameric form (Willeford and Wedding, 1992; Wu and Wedding, 1994). Although structurally quite different from phosphorylated activators of the enzyme, alanine and glutamine also reduce the K_m of the maize PEPC for PEP (Doncaster and Leegood, 1987). Glycine (Gln) activates the PEPC of C_4 monocots (Tovar-Mendez et al., 1998), but has no effect on PEPC from C_4 dicots. The reason for such specific response of gln on PEPC from monocots is not known.

Nitrogen nutrition

The biosynthesis of PEPC in leaves of C_3- and C_4 plants is highly regulated by the availability and source of nitrogen (Murchie et al., 2000). The rise in level of PEPC-mRNA and PEPC protein in maize plants was more pronounced supplemented with NH_4^+ or glutamine than those with NO_3^- (Sugiyama and Sakakibara, 2002). NH_4^+ also stimulate PEPC activity *in vitro*

mainly due to allosteric activation of enzyme (Gayathri and Raghavendra, 1994). The extent of light activation of PEPC is increased in presence of NH_4^+, due to the modulation of PEPC-PK activity (Giglioli-Guivarc'h et al., 1996; Murchie et al., 2000). Recent studies have demonstrated that roots may sense nitrogen signals, by producing cytokinins, which are transported to leaves to activate gene expression in C_4 plants (Sugiyama and Sakakibara, 2002).

Hormones

Among the plant hormones, known to modulate PEPC in different plant systems are: abscisic acid (ABA), cytokinin, gibberellic acid (GA) and fusicoccin (FC). Extensive work has been made on the induction of PEPC in CAM plants, while similar work with C_4 plants, is limited. ABA induces Kranz anatomy, a new form of C_4-PEPC and expression of other C_4 genes in *Eleocharis vivipara*, a C_3-C_4 intermediate (Uchino et al., 1998). ABA also promotes PEPC biosynthesis and induces CAM in succulent plants (Taybi et al., 1995). Incubation of guard cell protoplasts with FC led to the rapid activation of PEPC and reduced its sensitivity to malate, due to enhanced PEPC phosphorylation (Du et al., 1997; Meinhard and Schnabl, 2001). Treatment of GCPs with ABA decreased the phosphorylation of PEPC (Du et al., 1997) and suppressed the activation of PEPC. Induction of PEPC was observed by exogenous treatment of GA in *Mesembryanthemum crystallinum* (Guralnick et al., 2001). Treatment of wheat seedlings with cytokinin increased the PEPC activity and protected against dehydration induced decrease in enzyme activity (Cherniad'ev and Monakhova, 2001).

Induction of PEPC

Submerged aquatic macrophytes employ a CO_2 concentrating mechanisms so as to use effectively dissolved HCO_3^- (Raven 1970; Bowes and Salvucci, 1989). An appreciable shift from C_3 photosynthesis to a Kranz-less C_4 acid metabolism has been observed in at least three members of the Hydrocharitaceae, *H. verticillata*, *Elodea canadensis* and *Egeria densa*, when plants were grown with air levels CO_2, high temperature, and long photoperiods (Bowes and Salvucci, 1989; Reiskind et al., 1997; Casati et al., 2000; Rao et al., 2002). Two isoforms of PEPC were highly expressed under high temperature and high light. Under these conditions, an increase in total PEPC activity was due to the expression of lower molecular weight isoform that was strongly phosphorylated in the light. The changes in kinetic and regulatory properties of PEPC were correlated with changes in the phosphorylation state of enzyme (Lara et al., 2001).

In another instance, the amphibious leafless sedge *E. vivipara* developed Kranz anatomy and shifted to C_4 photosynthesis (including high activity of PEPC) under terrestrial conditions, but retained C_3-like traits and operated C_3 photosynthesis when submerged in water (Ueno, 1996, 1998). The transition from water to land could signal a water-deficient condition, therefore anatomical development and new gene expression could represent an adaptational response to water stress. Abscisic acid (ABA), induced Kranz anatomy, a new form of C_4-PEPC and expression of other C_4 genes in *E. vivipara* (Uchino et al., 1998). This observed transition is unique because normally ABA represses the expression of genes involved in C_4 photosynthesis (Sheen 1999).

ABA also promotes the induction of PEPC, along with CAM in succulent plants (Dai et al., 1994; Taybi et al., 1995). The patterns of accumulation of these photosynthetic enzymes in ABA induced plants were similar to those after temperature induction (Casati et al., 2000). Another pattern of induction was noticed in *Portulaca oleracea*, a succulent C_4 plant during the exposure to short photoperiods or water-stress, which induce CAM (Lara et al., 2004). During such induction, a new form of CAM-form of PEPC was synthesized, besides the original C_4-form of PEPC. These two forms of PEPC (C_4 and CAM) were quite distinct in their kinetic and regulatory properties (Mazen 2000). Recently, Wakayama et al. (2003) reported in *Arundinella hirta*, C_4 grass, has not only mesophyll cells (MCs) and bundle sheath cells (BSCs, usual Kranz cells) but also another type of Kranz cells (distinctive cells; DCs) that are not associated with vascular bundles. Immunogold electron microscopy revealed that PEPC and Rubisco were detected in the MC cytosol and in the BSC and DC chloroplasts, respectively. It is demonstrated that, although the DCs are not associated with veins, they behaved like BSCs with respect to enzyme induction and cellular differentiation.

Single cell C_4 photosynthesis

The C_4 photosynthesis makes use of both the mesophyll and the bundle sheath cells arranged in a wreath-like manner which is referred to as 'Kranz anatomy". Single-cell C_4 photosynthesis was first reported by Elena Voznesenskaya and her colleagues in the semi-succulent halophyte *Borszozowia aralocaspica* of the family Chenopodiaceae, which has the ability to perform C_4 photosynthesis in absence of Kranz anatomy (Voznesenskaya et

al., 2001; 2002). These observations disproved the requirement of Kranz anatomy for C_4 photosynthesis (Edwards et al., 2004).

More recently Voznesenskaya et al. (2004) reported light induced formation of dimorphic chloroplasts from the single plastid pool, synthesis of C_4 enzyme, and biochemical and structural polarization leading to the single cell syndrome in *B. aralocaspica*. It was found that the enzyme PEPC is not localized differentially but rather distributed throughout the cytoplasm, indicating that photosynthetic carbon assimilation (PCA) and photosynthetic carbon reduction (PCR) are carried out by the differential localization of Rubisco, the decarboxylase enzymes and PPDK (pyruvate phosphate dikinase). *B. cycloptera*, another halophytic species of the same tribe, exhibits the isotopic signature characteristic of a C_4 plant and the cytoplasm of *B. cycloptera* is divided into a region at the cell periphery containing the PCA region and a central area containing the PCR region. The vacuole plays an important role in minimizing CO_2 efflux while cytoplasmic strands serve as the channels for metabolic change. Some aquatic angiosperms such as *H. verticillata* and *E. densa* and some diatoms such as *Thalassiosira weissflogii* carry out C_4 photosynthesis in individual photosynthetic cells (Sage 2002).

Posttranslational modification

The characteristics of PEPC are modulated by posttranslational modification of the enzyme. One of the strongest modes is the reversible phosphorylation at single Ser residue near the N terminus resulting in a striking up- or down-regulation of the enzyme's allosteric properties (Chollet et al., 1996; Vidal and Chollet, 1997; Nimmo 2000; 2003). The second type of posttranslational modification is the change in oligomeric state of the enzyme,

studied mostly *in vitro* (Jiao and Chollet, 1991; Rajagopalan et al., 1994; Chollet et al., 1996). A third possibility is the regulation by modulation of redox states of the enzyme, and reduction in cysteine residues. However, the significance of the latter two *in vivo* is not yet clear.

Phosphorylation

PEPC is phosphorylated in light by a PEPC-protein kinase (PEPC-PK) and dephosphorylated in dark by a type 2A PEPC-protein phosphatase (Vidal and Chollet, 1997; Nimmo 2003). The regulatory phosphorylation occurs on serine residue of the enzyme, for e.g. Ser15 in maize and Ser8 in *Sorghum*. In PEPC of *A. hypochondriacus* the serine residue located at 11th position (Rydzik and Berry, 1996). The phosphorylation state of PEPC is largely determined by the action of a Ca^{2+}-independent protein kinase (Vidal and Chollet, 1997; Tsuchida et al., 2001; Garcia-Maurino et al., 2003), although regulation by other Ca^{2+}-dependent protein kinase has also been demonstrated (Zhang and Chollet, 1997; Ogawa et al., 1998; Parvathi et al., 2000a; Osuna et al., 2004).

PEPC-PK seems to be synthesized *de novo* on illumination, as pretreatment of leaves with cycloheximide suppressed the synthesis of PEPC-PK and prevented light activation of PEPC (Parvathi et al., 2000b). The modulation by light of PEPC-PK is further mediated through changes in the level of metabolites of photosynthesis and/or energy charge. For example, 3-PGA formed in the bundle sheath cells, during C_4 photosynthesis could activate PEPC-PK and PEPC (Chollet et al., 1996; Giglioli-Guivarc'h et al., 1996). Illumination induced the cytosolic alkalization in mesophyll cells of C_4 plants (Raghavendra et al., 1993; Rajagopalan et al., 1998) and such increase

in cytosolic pH could raise cytosolic calcium and increase the activity of PEPC-PK or an upstream protein kinase, which modulates PEPC-PK. Light presumably provides ATP and/or NADPH via photosynthesis for some step(s) in the transduction pathway, particularly for protein synthesis related to PEPC protein kinase (Vidal and Chollet, 1997). Parvathi et al. (2000a) reported that the regulation by Ca^{2+} or CAM could be at an upstream level of regulation of PEPC-PK. For example, a CDPK-like protein kinase may modulate the Ca^{2+}-independent PEPC-PK. In a recent study, a Ca^{2+}-dependent phosphoinositide-specific phospholipase C (PI-PLC) has been found to participate in the light dependent cascade leading to C_4-PEPC phosphorylation in mesophyll cell protoplasts of *Digitaria sanguinalis* (Coursol et al., 2000). A simplified schematic representation during transduction of light signal leading to PEPC phosphorylation is elucidated (Fig 1.1A).

The transcription of the PEPC kinase gene and the abundance of PEPC kinase mRNA responds to photosynthesis in C_3 and C_4 plants (Hartwell et al., 1996) and metabolic triggers in maize (Hartwell et al., 1999). Intense efforts were made to isolate, purify and clone the C_4-PEPC-PK from maize, *Sorghum*, *Flaveria* and two CAM plants: *Kalanchoe fedtschenkoi* and *M. crystallinum* (Tsuchida et al., 2001; Vidal et al., 2002; Nimmo 2003). Ogawa et al. (1998) 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 Ca^{2+}-dependent and Ca^{2+}-independent) are involved in the regulation of PEPC phosphorylation (Bakrim et al., 1992; Giglioli-Guivarc'h et al., 1996; Nhiri et al., 1998).

The molecular masses of PEPC-protein kinases from maize, *Sorghum*, *M. crystallinum* were in the range of 30 to 39-kD (Saze et al., 2001; Vidal et al., 2002; Garcia-Maurino et al., 2003) and these PEPC-PK were largely calcium-independent. In contrast, Ca^{2+}-dependent PEPC-PK also was detected in maize with a molecular weight of 50 to 60-kD. These PEPC-PK were inhibited by the calmodulin antagonist W7 and KT5926 (Izui et al., 1995). The light activation of PEPC was insensitive to type 2A protein phosphatase inhibitor, okadaic acid and microcystin-LR, which suggested that the phosphorylation of PEPC is modulated by PEPC-PK more effectively than by the phosphatase (Bakrim et al., 1992). Compared to the extensive literature in PEPC-PK, the studies on PEPC-protein phosphatase are very few (Dong et al., 2001).

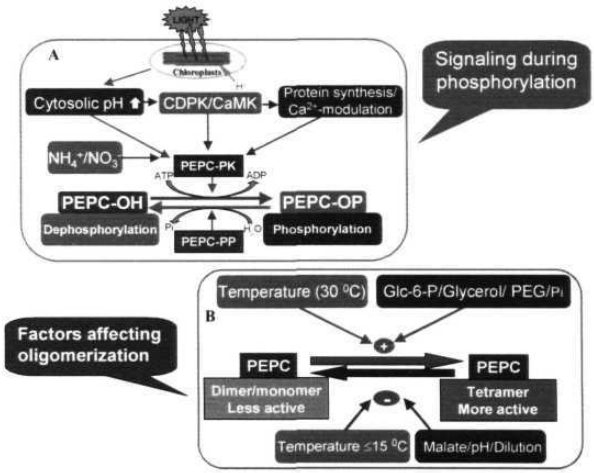

Figure 1.1: (A) Schematic model of the multimodal regulation of PEPC in the mesophyll cells. Many of these factors act by converging at the crucial step of reversible phosphorylation of PEPC at a single serine residue near N-terminus. The light signal is transduced through a series of secondary messengers, cytosolic pH, cytosolic Ca^{2+}, CDPK/CaMK, PEPC-PK, leading to phosphorylation. Besides light, nutritional factors modulate the expression of PEPC gene. PI-PLC, phosphoinositide specificphospholipase C; Ins(1,4,5)P3, Inositol-1,4,5-triphosphate. (B) The significance and modulation of the oligomeric status of the PEPC protein by temperature, compatible solutes and other metabolites. PEPC becomes oligomerized at optimal temperature and in presence of Pi or compatible solutes, such as glycerol or PEG (indicated by '+'). PEPC is dissociated at warm temperature, low pH and in presence of malate or on dilution of enzyme (indicated by '-' symbol).

Oligomerization

PEPC is a homotetrameric enzyme having four identical subunits. PEPC is very active when it is in tetrameric shape, while the activity and malate sensitivity decreases when the enzyme dissociated into monomer or dimer (Walker et al., 1986; Willeford et al., 1990). But the enzyme can exist as a dimer or monomer depending on several factors: pH, ionic strength (Wagner et al., 1987), temperature (Wu and Wedding, 1987; Chinthapalli et al., 2003) and concentration of PEPC (Willeford and Wedding, 1992). The oligomerization /dissociation of PEPC has been shown to regulate PEPC *in vitro,* but so far, experimental evidence *in vivo* is lacking. The presence of PEP, Mg^{2+}, Glc-6-P, malate or compatible solutes like PEG-6000 or glycerol promoted the aggregation of the PEPC (Podestá and Andreo, 1989; Manetas 1990; Wedding et al., 1994). Activity of PEPC is also dependent on dilution factor; activity being very high at concentrated protein (Selenioti et al., 1986) and this is due to the formation of active tetramer (Wu et al., 1990; Meyer et al., 1991; Willeford and Wedding, 1992; Drillius et al., 1997). It is suggested that Pi can shift the dimer/tetramer equilibrium towards tetramer (Salahas and Gavalas, 1997). A schematic representation of various factors that causes oligomerization/ dissociation is represented in Fig 1.1B.

Regulatory phosphorylation may not always be involved during the increase in PEPC activity and subsequent decrease in malate sensitivity of the enzyme. The temperature dependent changes in PEPC activity and malate sensitivity are independent of phosphorylation, and possibly due to changes in aggregation status of PEPC (Wu and Wedding, 1987; Chinthapalli et al., 2003).

The reversible dissociation/association PEPC is not the reason for the diurnal variation of PEPC activity (Weigend and Hindcha, 1992).

Reduction of -SH Groups

The regulation of cytosolic C_4 PEPC may be under the control of the redox state of certain critical cysteine residues (Iglesias and Andreo, 1984; Chardot and Wedding, 1992). Five to seven cysteine residues are present in plant PEPC that are absent in microbial enzymes (Vidal and Chollet, 1997). It is not known which of these residues are involved in regulation of activity or malate sensitivity. In contrast, reduced cytosolic thioredoxin had no effect on the properties of C_4 PEPC *in vitro,* when the dephosphorylated maize enzyme was used (Jiao and Chollet, 1989). Cysteine residues may be involved also in the maintenance of enzyme quaternary structure.

Conformational Changes

The activity of enzymes depends on conformational status of the protein, which can be monitored, by diverse techniques such as intrinsic/extrinsic fluorescence, circular dichroism spectra, limited proteolysis (Maralihalli and Bhagwat, 2001; Nakamura et al., 2002). However, studies on conformational changes in PEPC are very few. Change in temperature or presence of allosteric effectors or compatible solutes can cause conformational changes in the protein and regulate the PEPC activity. Recently, Alvarez et al. (2003) reported that the native C_4 PEPC could be in two different conformational states, as indicated by the binding of antibodies raised against peptide C19.

Characterization and Regulation of PEPC Kinase gene

For the first time Nimmo's group had cloned the cDNA for PEPC-PK (*PPCK*) from a CAM plant, *K. fedtschenkoi*, using an elegant method (Hartwell

et al., 1999). Although PEPC-K was purified to homogeneity from cell extracts of maize leaves by106-fold, the amount obtained was too small to get the partial sequence information useful for cDNA cloning (Saze et al., 2001). Subsequently, *PPCK* cDNAs were found in the DNA database or actually cloned from various sources including C_3 and C_4 plants. Till date, 14 cDNA sequences of *PPCK* are available on the DNA databank listed by Nimmo (2003). PEPCK proteins are comprised of 274–307 amino acid residues with molecular weight of 31-33 kDa. PPCK is the smallest protein kinase yet reported, consisting of only a core kinase domain. Alignment of PPCK sequences revealed that the sequences are rather diverse from one another, and that the amino acid identity and similarity are approximately 25% and 19%, respectively.

A phylogenetic tree constructed with a core domain of protein kinase showed that calcium-dependent protein kinase with a calmodulin domain (CDPK) is the nearest neighbor, PPCK is thought to have evolved from a common ancestor with the CDPK group (Nimmo 2003). From the expression patterns, the physiological role of each PPCK isoform can be inferred. PPCKs, which are supposed to be involved in C_4 and CAM photosynthesis, and carbon metabolism in nodules of the legume plant, were produced successfully in *E. coli* and characterized (Nakagawa et al., 2003; Taybi et al., 2000; Tsuchida et al., 2001; Xu et al., 2003). The enzymatic properties of PPCKs from a CAM plant, *M. crystallinum*, and a C_4 plant, *F. trinervia*, were essentially the same as those of native PEPC-K purified from maize leaves (Saze et al., 2001): e.g., the strict substrate specificity, Ca^{2+} independency, pH profile, and K_m values for ligands (Tsuchida et al., 2001; Ermolova et al., 2003). Because a synthetic

peptide with a sequence of N-terminus of C_4-PEPC was a poor substrate for maize PEPC-K, the other site of PEPC is required for recognition by PEPC-K (Li et al., 1997). Recently, a 19 amino acid peptide sequence of the C terminus of PEPC was identified to be inhibitory for PEPC-K (Alvarez et al., 2003).

Previously PEPC-PK activity was thought to be regulated by *de novo* synthesis in view of its rapid degradation (Chollet et al., 1996). Nimmo et al. (2001) identified a 55-kDa inhibitor protein in *K. fedtschenkoi* and maize leaf cell extracts, and proposed that its function is to mask the basal level of the kinase activity. Two forms of PPCK (PEPC-PK gene) have been identified recently in tomato (Marsh et al., 2003), these PPCK are regulated after alternative splicing to form an active PEPC-PK and conserved among species. Saze et al. (2001) reported possible redox regulation of purified maize PEPC-K. The PEPC-K could be readily inactivated under mild oxidative conditions and reactivated efficiently by thioredoxin-mediated reduction. Similar phenomena were observed with PPCK from *F. trinervia*, and intramolecular disulfide formation was shown under oxidative conditions (Tsuchida et al., 2001). Further studies are necessary to establish whether these two regulatory mechanisms operate *in vivo*.

Crystallization and three-dimensional structure

Kai et al. (1999) have successfully crystallized the PEPC from *E. coli* at a 2.8 °A resolution after the first preliminary crystallization (Inoue et al., 1989). The X-ray diffraction study revealed "dimer-of-dimer" form with respect to subunit contact. Despite the continious efforts, the progress in crystallization of C_4 plant PEPC has been slow (Matsumura et al., 1999b; 2002). However, recently maize PEPC has been crystallized and characterized at 3 °A resolution

(Kai et al., 2003; Izui et al., 2004). Dynamic movements were observed for several loops due to the binding of an allosteric inhibitor, a metal cofactor, and a PEP analogue or a sulfate anion, indicating the functional significance of these loops in carboxylation and regulation. Detailed comparison of *E. coli* PEPC with maize PEPC implicates an allosteric transition. Based on these studies, models are proposed for the reaction mechanism and allosteric regulation of PEPC (Kai et al., 2003). The details of structure and location of functional sites in crystallization are described recently by Izui et al. (2004).

Future prospects for research on NADP-ME and PEPC

Compared to extensive studies on the characterization of NADP-ME isoforms and their evolution, the reports on NADP-ME expression in *E. coli*, crystallization and 3-D structure are very limited. Further work is required on the catalytic and regulatory mechanisms of NADP-ME. The molecular basis to control the chloroplast aberrant upon over expression of NADP-ME is to be studied in detail. The use of recombinant enzymes for mutagenesis and crystal structure studies will help to understand the C_4 specific properties of NADP-ME (Detarsio et al., 2003). All the C_4 enzymes appear to be developmentally regulated and the expression of each is greatly enhanced by light. In the case of NADP-ME, the fact that the promoter of this enzyme is also active in mesophyll protoplasts, the details of this mechanism need to be studied. The potential effect on photosynthetic capacity of increased levels of NADP-ME and possibly other photosynthetic enzymes, induced by UV-B radiations need to be evaluated.

In the past decades, a significant progress has been made on the biochemistry and molecular biology of PEPC. However, there is still scope for

further studies on C_4-PEPC, particularly on the structural biology. From the crystal structure the details of reaction mechanism as well as the regulatory binding site with respect to allosteric effector can be derived. Comparison of various and novel isoforms of PEPC from different type of photosynthetic plants or algae will be a novel approach.

In tropical climates there is a huge fluctuation of light and temperature along with changes in nutrient availability. It is therefore important to study these interactions between light, temperature and nitrogen nutrition on PEPC. For e.g. the nitrogen requirements may differ among C_4 monocots and dicots particularly in relation to the sub-classification of C_4 plants, namely NADP-ME, NAD-ME and PEP-CK. Further, the effect of temperature on PEPC seems to be quite different in C_4 and CAM.

The extent of carbon fixation is very high during the day. It is, however, not clear if the activity of PEPC follows a strong diurnal rhythm. It is possible that the levels of PEPC change (protein and mRNA) during day/night cycles. In a related study, Hartwell et al. (1996) found that the mRNA levels of PEPC-protein kinase start increasing well before the sunrise in leaves of maize. Further, studies are needed to determine mRNA and protein levels of PEPC as well as the post-translational modification of PEPC, if any. Besides the PEPC-PK, there are factors affecting the regulatory phosphorylation, for e.g. protein synthesis, cytosolic pH, calcium and phosphoinositide, which should be taken up for further studies. The nature and importance of PEPC-PK is to be reassessed, as the reports on the Ca^{2+}-dependence or Ca^{2+}-independence are contradictory. The recent success (Nakagawa et al., 2003) in cloning and expression of PEPC-PK would be highly helpful in this regard.

Mutants and transgenics offer a great potential for further studies on PEPC. Bundle sheath defective (*bsd*) mutants in maize, that shows specific disruption in bundle sheath cells have been identified and analyzed for studies on PEPC (Hall et al., 1998). Mutants from *A. edulis* with reduced activity of PEPC and other C_4 enzymes are generated and are being studies. PEPC-deficient mutants would be quite attractive systems for studies on regulation of PEPC. Similarly, after the initial limited success, recent efforts have been quite successful in overexpressing C_4 PEPC in C_3 crop plants (Miyao and Fukayama, 2003). These attempts with tobacco, potato and rice resulted in a wide range of increase (5- to >100-fold) in PEPC activity (Leegood 2002). Overexpression of *Sorghum bicolor* PEPC gene in transgenic *Zea mays* was also made. These plants can be further analyzed.

There are still several aspects about NADP-ME and PEPC in C_4 plants, which need to be studied further. The objectives and scope of present work are outlined in the next chapter.

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Chapter 2

Approaches and Objectives

Chapter 2

Approach and Objectives

The NADP malic enzyme (NADP-ME; EC 1.1.1.40) and phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) are among the key enzymes of C_4 photosynthesis NADP-ME catalyzes the oxidative decarboxylation of L-malate to yield pyruvate, CO_2 and NADPH (Edwards and Andreo, 1992). NADP-ME, which is localized in the chloroplasts of bundle sheath, cells catalyze the decarboxylation of malate in C_4 species (Maurino et al., 1997). Being an important enzyme, the regulation of NADP-ME has been studied in detail, particularly in C_4 plants.

The levels of NADP-ME increase during the greening of etiolated seedlings of C_4 plants (Maurino et al., 1996). The marked increase in the activity of NADP-ME during greening is mainly due to the light stimulated synthesis of protein. To our knowledge, there has been only one attempt to examine the short-term modulation by light of NADP-ME in C_4 plants. Drincovich and Andreo (1994) demonstrated that the activity of NADP-ME in crude extracts increased by 50%, when the extracts were illuminated for a long time of 10 h. This observation is rather intriguing.

The first part of the present work is an effort to re-examine the pattern and characteristics of light activation of NADP-ME in leaf discs of *Zea mays*. Being located in chloroplasts of bundle sheath, it is quite likely that light may regulate the activity/properties of NADP-ME in C_4 plants. A comparative study was made of kinetic and regulatory properties of NADP-ME in extracts made from dark-adapted or illuminated leaf discs. Since the modulation by light of

NADP-ME was not as pronounced as we expected, the subsequent work was focused on PEPC from *A. hypochondriacus*.

A. hypochondriacus, a NAD-ME type of C_4 plant, is an important grain crop and leafy vegetable, grown in semi-arid, sub-tropical and tropical regions. Our laboratory has been using *A. hypochondriacus* as a model system to study the properties and regulation of C_4-PEPC (Rajagopalan et al., 1994; Parvathi et al., 2000a, b; Gayathri et al., 2000; Chinthapalli et al., 2003; Murmu et al., 2003). The activity and properties of PEPC are highly regulated by the environmental factors such as light and temperature. Phosphorylation of PEPC is regulated by light/dark transitions *in vivo* (Rajagopalan et al., 1994; Chollet et al., 1996; Vidal and Chollet, 1997; Parvathi et al., 2000b). On illumination, PEPC is phosphorylated by PEPC protein kinase (PEPC-PK) on a single serine residue and dephosphorylated in dark by type 2A protein phosphatases. *De novo* synthesis of PEPC-PK is an important component during PEPC phosphorylation.

Extensive work on the regulation of PEPC by light has been done in C_4 plants and CAM plants. Gavalas and Manetas (1980) speculated that C_4 photosynthesis and mineral nutrition are closely interconnected. The present work examines three essential components of nutrition, namely calcium, nitrogen and Pi on the properties and regulation of PEPC. We have used both leaf discs, and purified PEPC from *A. hypochondriacus*.

Calcium plays a key role as a secondary messenger in a variety of physiological responses and activates signaling cascades involving Ca^{2+}-modulated protein-kinases and protein phosphatases (Bush 1995). The reports on the modulation of PEPC by Ca^{2+} are contradictory. There are reports

describing Ca^{2+}-independent PEPC-protein kinase (PEPC-PK) in regulation of PEPC (Vidal et al., 2002). On the other hand, some authors reported that PEPC phosphorylation was mediated by Ca^{2+}-dependent PEPC-PK (Vidal et al., 2002).

Among the inorganic nutrients, nitrogen has a profound influence on PEPC in C_3, C_4 as well as CAM plants, at the level of both *de novo* synthesis of enzyme and the phosphorylation of protein (Murchie et al., 2000). Reports on the effects of feeding NO_3^- on PEPC in leaves of C_3 and C_4 plants, are contradictory. Therefore, it is quite interesting to study the short-term, *in vivo* effects of NO_3^- on the activity and light-activation of PEPC from plant species, grown under normal field conditions. NH_4^+ ions promote the biosynthesis of PEPC *in vivo* (Sugiyama and Sakakibara, 2002) and stimulate the PEPC activity *in vitro* (Gayathri and Raghavendra, 1994).

The cytosolic Pi concentration can rise up to 30 mM (Mimura et al., 1998). Being an end product of PEPC reaction, the possible role of Pi is not well corroborated. Pi plays an important role in plant metabolism: not only as a substrate for oxidative and photophosphorylation but also as a key component of several metabolic processes (Theodoron and Plaxton, 1993). The effect of Pi on PEPC is interesting, as Pi is an end product of PEPC reaction. However, studies on the direct effects of Pi on PEPC are limited and also contradictory. Further, more the material used in these experiments also varied from extracts from etiolated or illuminated leaves to purified PEPC, making it difficult for a comparison.

The modulation of Ca^{2+}, NH_4^+, NO_3^- and Pi on PEPC from a typical C_4 plant, *A. hypochondriacus* was assessed. Attempts were made to modulate

the level of PEPC *in vivo* by feeding leaves with those factors. Properties of PEPC were checked from dark-adapted and illuminated leaves for comparison. Experiments were extended to study the pattern of PEPC phosphorylation in the presence of Ca^{2+}, NH_4^+, NO_3^- and Pi. Studies were extended with purified PEPC of *A. hypochondriacus* (C_4) to assess the changes induced up on Pi treatment. Purification of PEPC was done from leaves of *A. hypochondriacus* by conventional techniques, according to Gayathri et al., 2000. Polyclonal antibodies were raised in rabbits against the purified PEPC from *A. hypochondriacus* leaves using the procedure of Nimmo et al. (1986), and the anti-PEPC antiserum was used for immunoprecipitation of protein involving PEPC phosphorylation.

Conformational changes in PEPC protein were studied using fluorescence spectra and circular dichroism (CD) spectra. Comparative effect of Pi was cross-checked with the effect of PEG-6000 (compatible solute and protein stabilizer) and urea (protein denaturant) on PEPC protein.

The following were the specific objectives:

1. Investigate if NADP-ME is light activated in maize, a C_4 plant? If so, changes in the properties of enzyme and possible mechanism of regulation.
2. Reevaluate the modulation of PEPC by Ca^{2+} *in vitro* and *in vivo* in leaves of *A. hypochondriacus*.
3. Examine the modulation of PEPC *in vivo* by NH_4^+ and NO_3^- in leaves of *A. hypochondriacus*.
4. Assess the effect of phosphate *in vitro* as well as *in vivo* on PEPC of *A. hypochondriacus*, a C_4 plant.
5. Study the effect of Pi on the structural properties of purified PEPC protein.

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Chapter 3

Materials and Methods

Chapter 3

Materials and Methods

Plant material

Plants of *Zea mays* L. (cv. VMH 404) or *Amaranthus hypochondriacus* L. (cv. AG-67) were raised from seeds in earthen pots filled with soil, supplemented with farmyard manure (in a ratio of 5:1). Plants were grown outdoors in the field under a natural photoperiod of approximately 12 h and average temperatures of 30-40 °C day/25-30 °C night. The upper fully expanded leaves of 2 week-old maize plants (Fig 3.1A) or 3-4 week-old plants of *A. hypochondriacus* (Fig 3.1B) were harvested, about 2-3 h after sunrise and were used for experiments.

(A) *Zea mays*

(B) *Amaranthus hypochondriacus*

Figure 3.1: (A) View of 2- to 3-weeks old plant of *Zea mays* VMH 404 and (B) 3- to 4-weeks old plant of *Amaranthus hypochondriacus* AG-67, grown in the field (outdoors)

Preparation of leaf discs

Discs of *ca.* 0.2 cm^2 were punched from leaves, under water, with the help of a sharp paper punch. Leaf discs were kept in a Petri dish (5-cm diameter) containing 10 mL distilled water and left in darkness for 2 h.

Light activation of NADP-ME

Leaf discs (each of ca. 0.2 cm^2 and total weight of 250 mg) were floated in 10 ml of 2 mM $NaHCO_3$ (unless and otherwise specified) in a 5 cm diameter Petri dish. They were illuminated with white light (Philips Comptalux R95 flood bulbs) at an intensity of 1000 $\mu E\ m^{-2}\ s^{-1}$. The light was passed through a circulatory water filter of 10 cm thickness to prevent heating. After 30 min of illumination (or dark incubation for comparison), the leaf discs were extracted quickly for assaying the NADP-ME.

Extraction of NADP-ME

The leaf discs (illuminated or dark-adapted) were extracted in 1 ml of chilled extraction buffer using a pre-chilled mortar and pestle. The extraction buffer contained: 100 mM Tris-HCl (pH 7.3), 10 mM $MgCl_2$, 2 mM K_2HPO_4, 1 mM EDTA, 1 mM PMSF, 20% (v/v) glycerol and 10 mM β-mercaptoethanol (modified from the method of Iglesias and Andreo 1989). The extract was centrifuged at 9000 g for 10 min in a Hermle Z 320 K centrifuge. The clear extract was used for NADP-ME assay.

NADP-ME assay

The assay of NADP-ME was based on the principles described by Iglesias and Andreo (1989). Enzyme activity at 30 °C was determined spectrophoto- metrically by monitoring NADPH production at 340 nm in a Shimadzu 160 A UV-visible spectrophotometer. The assay medium, in a

volume of 1 ml, contained 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 0.5 mM NADP and leaf extract equivalent to 1μg chlorophyll. The enzyme activity was initiated by the addition of malate. The enzyme was assayed at either 0.01 mM (limiting) or 4 mM malate (saturating substrate concentration).

The ranges of concentration used in case of different compounds were: K_m (malate) - (0.01 to 10 mM), K_a (citrate) - (0.1 to 4 mM), K_a (succinate) - (0.1 to 4 mM), K_i (pyruvate) - (1 to 10 mM), K_i (oxalate) - (0.1 to 1 mM).

Extraction of PEPC

Leaf discs (22 each of *ca.* 0.2 cm^2 and total weight of 125 mg) were quickly extracted using a prechilled mortar and pestle with 500 μl of extraction medium containing 100 mM Hepes-KOH (pH 7.3), 5 mM $MgCl_2$, 2 mM KH_2PO_4, 1 mM EDTA, 10% (v/v) glycerol, 10 mM β-mercaptoethanol, 10 mM NaF, 2 mM PMSF, 5 mM DTT, 2% (w/v) PVP. The homogenate was centrifuged at 15,000 g for 5 min. The supernatant was desalted through a small Sephadex G-25 column and was used for enzyme assays. A small aliquot was kept aside, prior to centrifugation, for chlorophyll estimation.

Assay of PEPC activity

The activity of PEPC was assayed coupling to NAD malic dehydrogenase (MDH) and monitoring NADH oxidation at 340 nm in a Shimadzu UV-Vis Spectrophotometer (Parvathi et al., 2000a). The assay mixture (1 mL) contained 50 mM Hepes-KOH (pH 7.3), 5 mM $MgCl_2$, 0.2 mM NADH, 2 Units of MDH, 2.5 mM PEP, 10 mM $NaHCO_3$ and leaf extract (equivalent to 1 μg of Chlorophyll). The sensitivity of PEPC to malate was checked using 0.5 mM malate in a separate assay. One unit of enzyme is

defined as the amount of enzyme which carboxylates 1 μmol of PEP min^{-1} under standard assay conditions.

The ranges of concentration used for various parameters were as follows: K_m (PEP) - 0.1 to 5 mM, K_i (malate) - 0.1 to 2 mM, K_a (Glc-6-P) - 0.5 to 5 mM.

Light activation of PEPC

Leaf discs (30 discs each of ca. 0.2 cm^2 and total weight of 125 mg) were floated on 10 ml distilled water (unless and otherwise specified) in a 5 cm diameter Petri dishes. They were illuminated under white light (Philips Comptalux R95 flood bulbs) at an intensity of 1000 μE m^{-2} s^{-1}. The light was passed through a water filter of 10 cm thickness to prevent heating. After 30 min of illumination (or dark incubation for comparison), the leaf discs were extracted quickly for PEPC assay.

Estimation of chlorophyll and protein

Chlorophyll was estimated by extraction with 80% (v/v) acetone as per (Arnon 1949). An aliquot of 12.5 μL of crude leaf extract was added to 5 mL of 80% (v/v) acetone and the absorbance of solution was measured at 652 nm (for chlorophyll) and at 710 nm (for assessing turbidity). Total chlorophyll content was estimated by using the following formula:

$$Chl\ (mg\ mL^{-1}) = \Delta A_{(652\text{-}710)} \times 11.1$$

and protein was estimated by method of Lowry et al. (1951), with Bovine serum albumin as the standard.

Feeding of leaves: $CaCl_2$, EGTA-buffered or EGTA, NH_4^+ or NO_3^-

The required concentrations of $CaCl_2$, Ca^{2+}-EGTA buffer or EGTA or NH_4Cl or KNO_3 were fed to leaves through the petioles at 200 $\mu E\ m^{-2}\ s^{-1}$ for 3 h. Leaves fed with water served as control sets, for comparison.

Evaluation of the effects of Pi or mannose

Whenever needed, Pi was added in the form of K_2HPO_4 (pH adjusted to 7.3 with HCl). The leaves were fed through petiole, with either 30 mM Pi or 10 mM mannose, under low light (200 $\mu E\ m^{-2}\ s^{-1}$). After feeding for required time, leaf discs were prepared and kept in either darkness or illuminated (1000 $\mu E\ m^{-2}\ s^{-1}$) for 30 min. Then the discs were homogenized and their extract was used for assaying PEPC.

***In vivo* labelling of PEPC with ^{32}Pi**

Labelling of PEPC with ^{32}Pi *in* vivo was done using the procedure described by Bakrim et al. (1992) and modified slightly by Parvathi et al. (2000b). Excised leaves were fed through petiole with 100 µL (60 µCi) of $KH_2{}^{32}PO_4$ (Specific activity of 10 mCi/mmol) under moderate illumination (200 $\mu E\ m^{-2}\ s^{-1}$) and then with $CaCl_2$, Ca^{2+}-EGTA, and EGTA (Ca^{2+} chelator), NH_4^+, NO_3^-, Pi, mannose (Pi sequester) for 3 h. Then the leaves were left in darkness for 3 h to ensure that the PEPC is dephosphorylated. A set of leaves was used for preparing leaf discs, which were to be either illuminated (1000 $\mu E\ m^{-2}\ s^{-1}$) or kept in darkness for 60 min (Parvathi et al., 2000a). These leaf discs were then extracted with 0.5 mL of extraction buffer as described above. Another set of leaves fed with only distilled water served as control. The leaf extracts were examined for the protein levels and the phosphorylation status of PEPC as described (Parvathi et al., 2000b).

Kinetic and Regulatory Properties of PEPC

The maximum velocity of the enzyme (V_{max}) and K_m for PEP were determined by using varying concentrations of PEP (0.5 to 5 mM). V_{max} and K_m values were calculated from Lineweaver-Burk plots. The K_i for malate was determined by using computer program developed by Brooks (1992). The activation of PEPC by allosteric activator, glucose-6-phosphate (Glc-6-P) was also studied in a manner similar to that described above, except that different concentrations of Glc-6-P (0.05 to 5 mM) were added instead of malate in the assay medium and PEPC was assayed at pH 7.3 and 2.5 mM PEP. K_a (Glc-6-P) values were calculated from double reciprocal plots.

Treatment of PEPC with PEG or urea

The effects of PEG-6000 (a compatible solute and protein stabilizer) and urea (protein denaturant) were checked, so as to compare the effects with those of Pi. Purified PEPC (50 μg mL^{-1}, unless otherwise specified) was incubated with 1.25% (w/v) PEG-6000 or 3M urea at room temperature for 2 h. An aliquot of PEPC protein was taken out and used for study.

Purification of PEPC

Extraction and Ammonium Sulfate Fractionation

Leaves (40 g) of *Amaranthus hypochondriacus* were picked from the field-grown plants (exposed to sunlight for 2-3 h), washed, chopped into small pieces and suspended in 160 mL of buffer containing 100 mM phosphate buffer (pH 7.3), 25% (v/v) glycerol, 5 mM DTT, 10 mM $MgCl_2$, 1 mM EDTA, 2 mM PMSF, 50 μg mL^{-1} chymostatin, 10 mM NaF and 2% (w/v) solid insoluble PVP. The leaves were then homogenized using a mixie [1.5 min; maximum speed, Remi Equipments (Ato-mix Blender)]. The homogenate was filtered through

four layers of cheese-cloth and the filtrate was centrifuged at 40,000*g* for 10 min.

The supernatant (160 mL) was brought to 40% saturation with saturated ammonium sulfate solution. The suspension was stirred slowly for 30 min and then centrifuged at 40,000*g* for 40 min. The precipitate was discarded, the supernatant was brought to 60% saturation by further addition of saturated ammonium sulfate solution and the precipitate was collected by centrifugation at 40,000*g* for 30 min. The extraction and $(NH_4)_2SO_4$ precipitation were performed at 4°C and the subsequent steps were carried out in an air-conditioned room with a temperature of 15-20°C.

DEAE-Sepharose Chromatography

The precipitate from 60% ammonium sulfate treatment was dissolved in 15-20 mL of 200 mM potassium phosphate buffer (pH 7.3) plus 10% (v/v) glycerol and was dialyzed against 20 mM potassium phosphate buffer (pH 7.3) and 10% (v/v) glycerol. The dialyzed solution was loaded onto a DEAE-Sepharose CL-6B column (1 x 12 cm), equilibrated with 20 mM potassium phosphate buffer [pH 7.3] and 10% (v/v) glycerol. The column was washed with same buffer at a flow rate of 0.5 mL min^{-1} until A_{280} returned to baseline. A linear gradient of 40 to 200 mM phosphate buffer (pH 7.3) containing 10% (v/v) glycerol was used to elute PEPC. The active fractions containing maximum PEPC activity were pooled and the enzyme was precipitated with 60% (v/v) saturated ammonium sulfate solution.

Hydroxylapatite (HAP) Chromatography

The precipitate from the above step, after ammonium sulfate precipitation, was dissolved in 200 mM phosphate buffer (pH 7.3) containing

10% (v/v) glycerol, and dialyzed against 20 mM phosphate buffer (pH 7.3) containing 10% (v/v) glycerol. The dialyzed sample was applied onto a 1 x 12 cm HAP column.

HAP column was prepared as described by Oishi (1971). 25 mL each of 0.5 M $CaCl_2$ and 0.5 M Na_2HPO_4 from separate burettes were mixed drop wise in a beaker containing 2.5 mL of 1 M NaCl. A flow rate of 4 mL min^{-1} was maintained from each burette. The brushite formed was allowed to settle and the supernatant was decanted. The precipitate was washed twice and boiled with simultaneous stirring for 1 h with 500 mL of double distilled water containing 1.25 mL of 1 M NaOH solution. The precipitate was allowed to settle completely. The supernatant was decanted; the precipitate was washed twice with distilled water and was allowed to settle. The precipitate was taken out and added to 10 mM sodium phosphate buffer (pH 6.8) and allowed to reach boiling point (avoid boiling). The gel (HAP) was washed with 20 mM phosphate buffer (pH 7.3) and stored at room temperature until required. Later it was transferred onto a column of 1 x 12 cm and equilibrated with 20 mM phosphate buffer (pH 7.3) containing 10% (v/v) glycerol.

The dialyzed eluate was applied slowly on the column and the eluate, which passes out of the column, was again recycled (5 to 6 times) into the column. This ensures complete binding of the enzyme to the column and removal of non-specific proteins from the column. PEPC was eluted with a linear gradient of 40-200 mM phosphate buffer (pH 7.3) plus 10% (v/v) glycerol. The active fractions were pooled.

Concentration and Storage

The pooled active fractions were transferred into a dialysis bag (2.1 x 5 cm) and concentrated by covering with solid PEG 20,000 (Sigma Chemical Co., USA). The concentrated and pure PEPC was stored in multiple aliquots with 50% (v/v) glycerol in -70 ^{0}C.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Mini-gels (8 x 8 cm) of 10% SDS-polyacrylamide were used and electrophoresis was performed, as per the principles of Laemmli (1970). The stacking gel (2 x 8 cm) contained 125 mM Tris-HCl (pH 6.7), 4% (w/v) of acrylamide, 0.1% (w/v) of SDS. The resolving gel (6 x 8 cm) was polymerized using 375 mM Tris-HCl (pH 8.8), 10% (w/v) of acrylamide and 0.1% (w/v) of SDS.

The electrode buffer contained 25 mM Tris-HCl, 192 mM glycine, (pH 8.3) and 0.1% (w/v) SDS. Proteins were dissolved in sample buffer [250 mM Tris-HCl (pH 6.8), 8% (w/v) SDS, 50% (v/v) glycerol, 10 mM β mercaptoethanol, 0.04% (w/v) bromophenol blue] and boiled at 100°C for 2 min and loaded onto 10% SDS-PAGE. Electrophoresis was performed at 60 V until the dye front migrated into the resolving gel and later the voltage was raised to 120 V. Power was supplied through Atto Digi-Power (SJ-1081) for about 2 h. After the electrophoresis, the gels were fixed for 1 h with fixative solution containing 40% (v/v) methanol and 7% (v/v) glacial acetic acid.

The gels were then stained with Coomassie Blue-staining solution [0.25% (w/v) Coomassie Brilliant Blue R-250 in 50% (v/v) methanol and 12.5% (v/v) acetic acid] and destained with a destaining solution containing 50% (v/v) methanol and 12.5% (v/v) acetic acid.

In some cases, the gels were visualized by silver staining, as per the procedure of Blum et al. (1987). After electrophoresis, the gel was fixed in fixative-solution containing 50% (v/v) methanol, 12.5% (v/v) acetic acid and 0.5 mL of commercial [37% (v/v)] formaldehyde/liter for 1 h. Later the gel was washed thrice with 50% (v/v) ethanol for 20 min each. The gel was pretreated with 0.02% (w/v) sodium thiosulfate solution for 1 min and rinsed with water for 1 min (3 washes, each for 20 s). The gel was impregnated with 0.2% (w/v) silver nitrate and 0.75 mL of formaldehyde/liter for 20 min. The gel was again washed with water for 1 min (three washes, each for 20 s) and developed with 6% (w/v) sodium carbonate and 0.5 mL of formaldehyde/liter for 10 min. The reaction was stopped with a mixture containing 50% (v/v) methanol and 12.5% (v/v) acetic acid for 10 min and then the gel was washed thoroughly with water for 4 min. Finally, the gel was washed well with 50% (v/v) methanol (for more than 20 min).

Pre-stained molecular weight markers (29 to 116-kD, from Sigma) were used as standards, for assessing molecular weight of proteins on SDS gels.

Preparation of Anti-PEPC Antiserum

Anti-PEPC antiserum was raised in 6 month-old white rabbits, as per the principles of Nimmo et al. (1986), and described by Gayathri et al. (2001).

Pre-immune serum was collected from ear-vein of the rabbit. Subsequently 0.5 mg of purified PEPC in 500 μL emulsified in equal volume (500 μL) of 50% Freund's complete adjuvant and was injected subcutaneously at about 10 sites. Four weeks later, the animal was given (through subcutaneous injections) a booster dose of 0.25 mg in 250 μl of purified enzyme, emulsified with equal volume (250 μl) of 50% Freund's incomplete

adjuvant. After 2 weeks, blood was collected from the ear vein. The blood was allowed to coagulate and the antiserum was collected by centrifugation at 10,000*g* for 30 min. The antiserum was split into several small aliquots and stored at -20°C.

The animal was again inoculated with a further 0.25 mg in 250 μL of enzyme emulsified with equal volume (250 μL) of 50% Freund's incomplete adjuvant. Blood was collected after a further period of 6 to 8 days. Anti-PEPC antiserum was collected as described above by centrifugation and stored in multiple aliquots.

SDS-PAGE and Autoradiography

The soluble proteins were separated by 10% SDS-PAGE, as already described in the previous pages. The gels were dried and examined by autoradiography to assess the incorporation of ^{32}P label into PEPC-protein.

Gels were stained with Coomassie Brilliant Blue R-250 and destained thoroughly by using destaining solution with constant shaking. These gels were dried under vacuum with a gel dryer (Bio-Rad Laboratories, USA). The X-ray film was cut to the gel-size and was placed on top of the gel. The gel and X-ray film were placed between two intensifiers, inside an X-ray cassette. The gel position was marked by cutting the corner of the X-ray film. The cassette was left in a Deep-Freezer (-80°C). After 4 to 5 days, the X-ray film was developed using X-ray film-developer and fixed with X-ray film-fixer. The developed and fixed X-ray film was washed thoroughly with water and was allowed to dry.

Non-denaturing PAGE (Native PAGE)

Native gel was performed according to Law and Plaxton (1995). A 7% (w/v) polyacrylamide gel (8 x 8 cm) was polymerized without SDS, using 200 mM Tris (pH unadjusted), 100 mM glycine (pH unadjusted), 20 % (v/v) glycerol, 10 % (v/v) ethylene glycol, 0.15 % APS, 0.05 % TEMED as resolving gel and the gel was allowed to polymerize for 30 min at room temperature. For preparation of stacking gel, 4% (w/v) acrylamide, 20 % (v/v) glycerol, 10 % (v/v) ethylene glycol, 70 mM Tris-HCl (pH 6.7), 4 mM EDTA, 0.25 % APS, 0.05 % TEMED was used and allowed to polymerize for 30 min. The polymerized gel was precooled at 4°C before loading the protein.

The electrode buffer contained 100 mM Tris and 150 mM glycine (pH unadjusted). 20 μg of purified PEPC was loaded into each of the wells with or without Pi. Electrophoresis was performed at 4°C continuously at 200 volts for 3 hours through Atto Digi-Power (SJ-1081).

Intrinsic and Extrinsic Fluorescence of PEPC

The intrinsic fluorescence of PEPC protein was studied by monitoring the fluorescence pattern, typical of tryptophan amino acid residues. The samples were prepared by taking purified PEPC (10 μg mL^{-1}) into 50 mM Tris-HCl (pH 7 .3) and 10% (v/v) glycerol and in presence of required concentration of Pi or 1.25 % (w/v) PEG-6000 or 3 M urea. The fluorescence emission spectrum of the sample was recorded at room temperature with a spectrofluorimeter (Spec Fluoromax 3). The samples were excited at 280 nm and the emission spectrum was measured between 300 to 400 nm, using a light path of 1 cm. Further details are indicated in the results.

The extrinsic fluorescence was monitored using 1-anilinonaphthalene-8-sulfonic acid (ANS), a fluorescent probe that binds to the hydrophobic region of protein. ANS was dissolved in 50 mM Tris-HCl (pH 7 .3) and 10% (v/v) glycerol. 1 μM ANS was dissolved with PEPC sample (10 μg mL^{-1}) in 50 mM Tris-HCl (pH 7 .3) and 10% (v/v) glycerol. Varying concentrations of Pi or 1.25 % (w/v) PEG-6000 or 3 M urea was incubated with PEPC. After incubating the samples for 2 h at room temperature, the fluorescence emission of the sample was recorded at room temperature. The excitation was at 350 nm and emission spectra were measured between 400 to 600 nm. The spectra of necessary controls (protein without ANS and buffer with or without ANS) were collected and subtracted from the spectra of enzyme with buffer.

CD spectra

CD measurements were recorded with a spectropolarimeter (Jasco J-810) using 1.0 cm path length cell and an average of 3 repetitive scans between 250 and 200 nm. The spectra were recorded with a scan speed of 20 nm min^{-1} and with a response time of 1 s. The purified PEPC (50 μg mL^{-1}) protein taken in a buffer (Tris-HCl, pH 7.3 and 10% (v/v) glycerol) was incubated with varying concentration of Pi or 1.25 % (w/v) PEG-6000 or 3 M urea. The spectra of buffer were subtracted from the respective spectra of enzyme with buffer, which gives the spectra of the enzyme. The CD results were expressed as mean residue ellipticity (θ), obtained by the equation,

$$[\theta] = \frac{\theta * M_{MRW}}{10*d*c} \qquad \text{(Eq. 1)}$$

in which M_{MRW} (the mean amino acid residue weight), d is the cell path in cm,

and c is the concentration of the protein in mg mL^{-1} (Sievers 1978). These values were M_{MRW} = 414.93, d = 0.1, and c = 0.05.

Estimation of α-helicities percentages were made using the method suggested by Greenfield and Fasman (1969) and modified as given below. Using the ellipticity at 222 nm, the fractional helicities were estimated as per the equation,

$$f_\eta = ([\theta] - [\theta]^0) / ([\theta]^{100} - [\theta]^0) \qquad \text{(Eq. 2)}$$

where [θ] represents the experimentally observed mean residue ellipticity. Values for $[\theta]^0$ and $[\theta]^{100}$ corresponding to 0 and 100% helical content at 222 nm were estimated to be 6000 and 40,000 degrees cm^2/dmol, respectively.

Replication and Statistical Analysis

The data presented are the average values (± SE) of results from three to four experiments conducted on different days. Statistical analysis of the data was done using a computerized program written in a basic language.

Chemicals/Materials

Most of the biochemicals, were from either Sigma (Sigma Aldrich, St Louis, USA) or Boehringer, Germany) or Qiagen. DEAE-Sepharose CL-6B and Sephadex G-25, from Pharmacia, Sweden. [^{32}P]-labelled KH_2PO_4 was procured from Board of Radiation and Isotope Technology, Bombay. The X-ray film was from Konica Photofilms Mfg. Co. Ltd., X-ray Developer and Fixer from Allied Photographers India Limited.

All other chemicals were of analytical grade from Sisco Research Laboratories, E-Merck (India), Spectrochem, Loba Chemie, Himedia Laboratories all from Bombay or Ranbaxy Laboratories New Delhi, India.

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Chapter 4

Light Activation of NADP Malic Enzyme in Leaves of Maize: Marginal Increase in Activity but Marked Change in Regulatory Properties of Enzyme

Chapter 4

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The NADP malic enzyme (NADP-ME; L-malate: NADP oxidoreductase [oxaloacetate decarboxylating] (EC 1.1.1.40) catalyzes the oxidative decarboxylation of L-malate to yield pyruvate, CO_2 and NADPH (Edwards and Andreo, 1992; Drincovich et al., 2001). The enzyme plays an important role in different metabolic pathways in plants. NADP-ME, localized in either the chloroplasts of bundle sheath cells of C_4 species (Maurino et al., 1997) or cytosol of CAM plants, mediates the decarboxylation of malate and releases CO_2 required for carbon fixation by Rubisco (Edwards and Huber, 1981; Ting 1985; Cushman and Bohnert 1997). NADP-ME along with phosphoenolpyruvate carboxylase can regulate the intracellular pH (Davies 1986). Besides the housekeeping function of malate metabolism, NADP-ME serves also to meet the demand for reductive power, providing building blocks of carbon and energy for biosynthesis of defense compounds (Casati et al., 1999).

Being an important enzyme, the regulation of NADP-ME has been studied in detail, particularly in C_4 plants. The C_4 isoform of NADP-ME maintains a substantial rate of catalysis under the high pH (8.0) and high Mg^{2+} concentration, common in illuminated chloroplasts (Asami et al., 1979; Holaday and Lowder, 1989), while the C_3 plant enzyme appears to have an optimum activity around pH 6.4 to 7.3 (Dhillon et al., 1985; Tomaszewska et al., 1982). The activity of decarboxylation by NADP-ME in C_4 plants is much higher (40 to

50 fold) than that in C_3 plants. The deduced primary sequences of maize leaf malic enzyme have been reported (Rothermel and Nelson, 1989). The chemical modifications of amino acid residues have implicated His, Arg and cysteine residues essential for activity (Drincovich and Andreo, 1992; Jawali and Bhagwat, 1987).

Several enzymes localized in chloroplasts are activated on illumination. This phenomenon of light activation has been reported in case of several enzymes of Calvin cycle such as Rubisco, glyceraldehyde 3-phosphate dehydrogenase, ribulose 5-phosphate kinase, fructose-1,6-bisphosphatase (Buchanan 1980; Faske et al., 1995; Sage and Seemann, 1993; Scheibe 1987; Vivekanandan and Saralabai, 1997). Further, some of the enzymes localized in mesophyll chloroplasts of C_4 plants, also are light activated, e.g PPDK or NADP-MDH (Buchanan 1980; Edwards et al., 1985; Vivekanandan and Saralabai, 1997).

The levels of NADP-ME increase during the greening of etiolated seedlings of C_4 plants (Pupillo and Bossi, 1979; Collins and Hauge, 1983; Scagliarini et al., 1988; Maurino et al., 1996). The several fold increase in activity of NADP-ME during greening is mainly due to light stimulated synthesis of protein. The long-term effect of light in promoting the *de novo* synthesis of NADP-ME during the seedling growth is well established. To our knowledge, there has been only one attempt to examine the short-term modulation by light of NADP-ME in C_4 plants. Drincovich and Andreo (1994) demonstrated that the activity of NADP-ME in crude extracts increased by 50%, when the extracts were from illuminated at 400 $\mu E\ m^{-2}\ s^{-1}$ for 10 h. This is rather intriguing, as the illumination needs to be done preferable of intact leaf discs.

Being located in chloroplasts of bundle sheath cell, it is quite likely that light may regulate the activity/properties of NADP-ME in C_4 plants. The present work is an effort to reexamine the pattern and characteristics of light activation of NADP-ME in leaf discs of *Zea mays*. A comparative study was made of kinetic and regulatory properties of NADP-ME in extracts made from dark-adapted or illuminated leaf discs. On illumination, there was a small but consistent increase (about 30% over dark-control) in the activity of NADP-ME. Besides the marginal increase in the activity, the illuminated form of NADP-ME exhibited marked changes in its regulatory properties.

Results

The leaf discs of *Zea mays* were illuminated in the presence of 2 mM bicarbonate (unless otherwise specified). The properties of NADP-ME were determined using the extracts prepared from either illuminated or dark-adapted leaf discs. The assay was performed at pH 8, with either saturating (4 mM) or limiting (0.01 mM) concentration of malate.

Light activation of NADP-ME

The activity of NADP-ME increased when the leaf discs were illuminated at an intensity of 1000 $\mu E\ m^{-2}\ s^{-1}$. The optimal period of illumination for increase in NADP-ME activity was 30 min, irrespective of malate concentration during the assay, 4 mM (Fig 4.1A) or 0.01 mM malate (Fig 4.1B). The activation of NADP-ME was higher in presence of bicarbonate than that without bicarbonate. Maximal activation occurred at 2 mM or higher $NaHCO_3$ (Fig 4.2). The stimulation of light activation of NADP-ME by bicarbonate could be noticed when the enzyme was assayed at either 4 mM malate (Fig 4.2A) or 0.01 mM malate (Fig 4.2B).

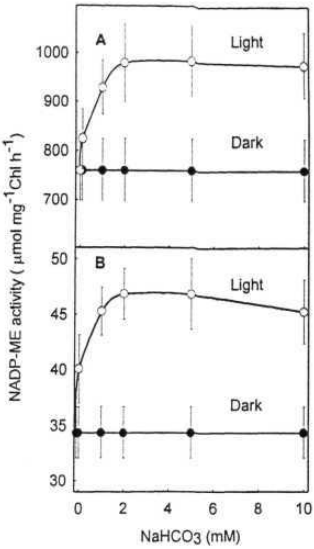

Figure 4.1: The activity of NADP-ME in leaf discs of maize, after different periods of illumination (at an intensity of 1000 µE m^{-2} s^{-1}). The medium during illumination contained 2 mM $NaHCO_3$. The activity was assayed at either 4 mM malate (A) or at 0.01 mM malate (B). The experiments were done on at least three different days and the average values are ± SE represented.

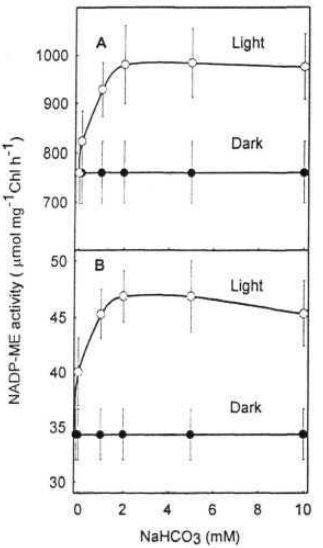

Figure 4.2: The response of NADP-ME in leaf discs of maize to illumination, in relation to varying concentration of $NaHCO_3$ in the medium after 30 min of illumination. The illumination was at an intensity of 1000 µE m^{-2} s^{-1}. The activity of NADP-ME was assayed at either 4 mM malate (A) or 0.01 mM malate (B). The experiments were done on at least three different days and the average values are ± SE represented.

Changes in the regulatory properties of NADP-ME in response to dark and illumination

The kinetic and regulatory properties of NADP-ME were studied with extracts prepared from illuminated or dark-adapted leaf discs. To check the regulatory properties of NADP-ME, we have chosen two activators (citrate and succinate) and two inhibitors (pyruvate and oxalate). Comparative study of NADP-ME to effectors at either limiting (0.01 mM) or saturating (4 mM) malate showed that the effect of activators was more pronounced at limiting concentration than that at saturating concentration of malate. But the pattern of inhibition was similar at either concentration of malate (Table 4.1). Upon illumination, the sensitivity of NADP-ME towards the inhibitors as well as activators decreased quite significantly.

Table 4.1: The activity of NADP-ME in the absence or presence of effectors. The enzyme was extracted from maize leaf discs, which were either illuminated or dark-adapted. The illumination was done for 30 min at 1000 $\mu E\ m^{-2}\ s^{-1}$ and in presence of 2 mM bicarbonate. The enzyme was assayed at either limiting (0.01 mM) or saturating (4 mM) malate. Figures in parenthesis represent the activity in presence of effectors, as % of control (without effectors). The experiments were done on at least three different days and the average values are ± SE represented. The changes due to light activation were all statistically significant, as indicated.

Effector (concentration)	Assay at					
	0.01 mM Malate			4 mM Malate		
	Dark	Light	L/D	Dark	Light	L/D
	Activity ($\mu mol\ mg^{-1}$ Chl h^{-1})					
None (control)	36.1 (100)* ± 2.7	46.9 (100)* ± 3.6	1.30	833 (100)* ± 74	1080 (100)* ± 93	1.30
Citrate (2 mM)	56.3 (156)** ± 4.8	64.7 (138)** ± 5.9	1.15	757 (91)* ± 68	1061 (98)* ± 89	1.40
Succinate (2 mM)	55.4 (153)** ± 4.3	59.4 (126)** ± 4.6	1.07	1000 (120)** ± 82	1061 (98)** ± 87	1.06
Pyruvate (10 mM)	6.4 (18)* ± 0.8	14.4 (31)* ±1.2	2.25	337 (41)** ± 27	448 (42)** ± 37	1.33
Oxalate (0.5 mM)	12.7 (35)* ± 1.4	20.8 (44)* ± 2.1	1.64	357 (43)* ± 33	408 (38)* ± 34	1.14

* Significant at $P < 0.01$.
** Significant at $P < 0.05$.

On illumination of NADP-ME, the V_{max} increased by about 30% at either saturating or optimal malate concentration (Table 4.2). There was not much change in K_m for malate. The K_a for citrate and succinate increased by 36% and 32%, respectively (Table 4.2). K_i for pyruvate and oxalate increased by 100% and 67% respectively, on illumination. Thus, NADP-ME becomes less sensitive to both inhibitors and activators, on illumination than that in darkness.

Table 4.2: Changes induced in illumination in the kinetic and regulatory properties of NADP-ME leaf discs of maize. Effectors were checked at 0.01 mM malate. The illumination was done for 30 min at 1000 $\mu E\ m^{-2}\ s^{-1}$ and in presence of 2 mM bicarbonate. The experiments were done on at least three different days and the average values are ± SE represented.

Parameters	Dark form	Light form
V_{max} ($\mu mol\ mg^{-1}Chl\ h^{-1}$)	679 ± 68*	990 ± 82*
K_m (malate) mM	0.28 ± 0.02**	0.26 ± 0.02**
K_a (Citrate) mM	0.14 ± 0.01*	0.19 ± 0.01*
K_a (Succinate) mM	0.19 ± 0.01*	0.25 ± 0.02*
K_i (Pyruvate) mM	1.3 ± 0.1*	2.60 ± 0.2*
K_i (Oxalate) mM	0.18 ± 0.01*	0.30 ± 0.02*

* Significant at $P < 0.01$.
** Significant at $P < 0.05$.

Damping of light induced changes by DTE

The light activation of several chloroplastic enzymes is modulated by the reduction of dithiols on the enzyme. This possibility is checked by incubating the extracts from illuminated or dark-adapted leaf discs with 10 mM DTE and assaying the enzyme at either 0.01 mM or 4 mM malate. Incubation with DTE enhanced the activity of enzyme in extracts from dark-adapted leaf discs and

reduced the extent of light activation (Table 4.3). As a result, the L/D ratio of enzyme activity was less in the presence of DTE than that in the absence.

Table 4.3: The effect of preincubation with 10 mM DTE on the activity of NADP-ME in the extracts prepared from illuminated or dark-adapted leaf discs of maize. The illumination was done for 30 min at 1000 $\mu E\ m^{-2}\ s^{-1}$ and in presence of 2 mM bicarbonate. The experiments were done on at least three different days and the average values are ± SE represented.

Activity assayed at	Preincubation					
	No DTE			+10 mM DTE		
	Dark	Light	L/D	Dark	Light	L/D
	Activity ($\mu mol\ mg^{-1}\ Chl\ h^{-1}$)					
0.01 mM malate	21.7 ± 1.8	28.6 ± 2.6	1.32	24.1 ± 2.1	28.4 ± 2.3	1.18
4 mM malate	482 ± 43	621 ± 58	1.29	636 ± 56	559 ± 48	0.88

Discussion

The present article is the first detailed report on the characteristics of light activation of NADP-ME in maize. There was limited but significant activation of NADP-ME (Fig 4.1), along with marked changes in the regulatory properties of enzyme, when compared to dark-form (Table 4.2). The light induced increase in the NADP-ME activity was greater when the illumination medium contained 2 mM (or higher) bicarbonate than that in the absence (Fig 4.2). This observation is physiologically relevant. During C_4 photosynthesis, the C_4 acids formed in mesophyll move into the bundle sheath and are decarboxylated leading to a high rise in CO_2 of bundle sheath cells. The high concentration of CO_2 in the bundle sheath can be a contributing factor to light activation of NADP-ME in leaves of C_4 plants *in situ*.

The reason for limited photoactivation is not clear. However, the decrease in the sensitivity of NADP-ME to pyruvate appears quite logical. On illumination, the concentration of pyruvate in bundle sheath cells is likely to increase and reach levels as high as 9 mM (Leegood 1999). Also, the level of CO_2 in bundle sheath cells rises at least ten times above the atmospheric levels (Furbank and Hatch, 1987; Jenkins et al., 1989; Dai et al., 1993). Thus, the dissolved bicarbonate concentration in bundle sheath cells could be >1mM. It is therefore logical that NADP-ME is modified to tolerate and function at high levels of CO_2 and pyruvate. Figure 4.2 demonstrate that NADP-ME responds positively to external bicarbonate.

The light-activated form of NADP-ME becomes less sensitive to feedback inhibition by pyruvate, as indicated by the increase in K_i pyruvate (Table 4.2). The process of desensitization of NADP-ME to inhibitors strikes a strong similarity to the phenomena with other enzymes in leaves of C_3 and C_4 plants. A decrease in the sensitivity to feedback inhibition and/or regulation, after illumination of leaves has been recorded in case of PEPC (Rajagopalan et al., 1994; Vidal and Chollet, 1997), nitrate reductase (NR, Campbell 1999) and sucrose phosphate synthase (SPS, Huber and Huber, 1996). Thus, the decrease in the extent of feedback inhibition by suitable posttranslational modification may be a common feature of key enzymes of photosynthetic carbon metabolism.

The changes in kinetic and regulatory properties of enzymes are often and easily mediated by posttranslational modification of the protein. Among the possibilities for such modification of enzymes are: (i) Phosphorylation-dephosphorylation of protein; (ii) Oligomerization-dissociation of the enzyme;

(iii) Reduction-oxidation of vicinal dithiols. The light induced changes in properties of PEPC, NR and SPS are mediated due to the phosphorylation-dephosphorylation of the enzyme. In contrast, the changes in NADP-ME appear to be due to the reduction of dithiols, as the incubation with DTE dampened the light-activation, by enhancing the activity of enzyme even in dark (Table 4.3). Thus, NADP-ME is similar to enzymes, such as NADP-MDH or fructose 1, 6-bisphosphatase, which are light activated principally through the reduction of dithiols and subsequent oligomerization of enzyme (Buchanan 1980; Edwards et al., 1985; Vivekanandan and Saralabai, 1997). There are at least seven cysteinyl residues on each monomer of maize NADP-ME and there is evidence that these are oxidized-reduced through ferredoxin/thioredoxin system (Drincovich and Andreo, 1994).

Our results establish that although there was only a marginal increase in the activity of enzyme on illumination of leaf discs, the changes in regulatory properties of NADP-ME were marked. Further experiments are needed to study the mechanism of light-induced changes in the regulatory properties of NADP-ME from C_4 plants.

Major conclusions from the results presented in this chapter are:

1. The extent of light activation was much more in the presence of bicarbonate than that in the absence. Upon illumination, the V_{max} of NADP-ME increased by about 30%. Although small, the increase was consistent and significant.
2. The extent of light activation was similar when substrate (malate) concentration was either 4 mM (saturating) or 0.01 mM (limiting).

3. The light-induced change seems partially due to the reduction of dithiols, as incubation of leaf extracts with DTE dampened the extent of light activation of NADP-ME.
4. We conclude that the properties of NADP-ME do change on illumination. Although there was only a marginal increase in the activity, the changes in regulatory properties of NADP-ME were marked.

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Chapter 5

Modulation *in vivo* by Ca^{2+} of Phosphoenolpyruvate Carboxylase in *Amaranthus hypochondriacus*: Possible Involvement of Ca^{2+} in Up-regulation of PEPC Protein Kinase *in vivo*

Modulation *in vivo* by Ca^{2+} of Phosphoenolpyruvate Carboxylase in *Amaranthus hypochondriacus*: Possible Involvement of Ca^{2+} in Up-regulation of PEPC Protein Kinase *in vivo*

Phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) is a ubiquitous enzyme occurring in cytosol of photosynthetic and non-photosynthetic tissues of C_3, C_4 and CAM plants (Andreo et al., 1987; Rajagopalan et al., 1994; Vidal and Chollet, 1997). The enzyme is regulated strongly by external factors, which include light, temperature and inorganic nutrients. On illumination, the activity of PEPC in leaves of C_4 plants is enhanced by 2 to 3- fold along with a marked decrease in the malate sensitivity of the enzyme (Huber and Sugiyama, 1986; Doncaster and Leegood, 1987; Parvathi et al., 2000a). These changes during the light activation are due to the phosphorylation of the enzyme by a PEPC-protein kinase (PEPC-PK) (Vidal and Chollet, 1997, Parvathi et al., 2000a). PEPC is also highly regulated by temperature in C_4 plants (Phillips and McWilliam, 1971; Wu and Wedding, 1987; Chinthapalli et al., 2003). The regulation of PEPC by temperature or Pi was recently shown to be not through phosphorylation but rather by conformational changes in protein (Chinthapalli et al., 2003; Murmu et al., 2003).

Calcium plays a key role as a secondary messenger in a variety of physiological responses, through Ca^{2+}-modulated proteins and activation of protein-kinases or protein phosphatases (Bush 1995). The reports on the modulation of PEPC by Ca^{2+} are contradictory. There are reports describing Ca^{2+}-independent PEPC-protein kinase (PEPC-PK) in regulation of PEPC and thus a non-involvement of Ca^{2+} (Carter et al., 1991; Jiao and Chollet, 1991;

Bakrim et al., 1992; Wang and Chollet, 1993a). On the other hand, some authors reported that PEPC phosphorylation was mediated by Ca^{2+}-dependent PEPC-protein kinase (Echevarria et al., 1988; Bakrim et al., 1992; Ogawa and Izui, 1992; Ogawa et al., 1992; Pierre et al., 1992). The marked changes on illumination in the levels of cytosolic Ca^{2+} suggested that Ca^{2+} could be a part of light-transduction mechanism in plants. Pretreatment of mesophyll cell protoplasts in *Digitaria* with calcium ionophore A23187 (calcimycin) and EGTA inhibited the phosphorylation of PEPC and Ca^{2+} can recover such inhibition (Chollet et al., 1996; Duff et al., 1996). It is possible that Ca^{2+} exerts a marked effect *in vivo* and such effect is not discernible *in vitro*.

The present study is an attempt to reevaluate the role of Ca^{2+} on PEPC in leaves of *Amaranthus hypochondriacus*, a NAD-malic enzyme type C_4 plant. Attempts were made to modulate PEPC *in vivo* by feeding leaves with either Ca^{2+} (EGTA-buffered) or EGTA (Ca^{2+} chelator). Properties of PEPC were checked from dark-adapted or illuminated leaves for comparison. Experiments were extended to study the pattern of PEPC phosphorylation in leaves with or without Ca^{2+} pretreatment of leaves *in vivo*.

Results

Effect of Ca^{2+} or EGTA on activity and malate sensitivity in vitro

Inclusion of Ca^{2+} during enzyme assay (at μM ranges) increased the PEPC activity in concentration dependent manner, peak at 10 μM Ca^{2+}. PEPC activity increased in dark-adapted leaf extracts up to 30% but in illuminated extracts it was marginal (Fig 5.1A). In parallel, the inhibition by malate decreased by 26% and 8% respectively, in extracts from dark-adapted and

illuminated leaves (Fig 5.1B). EGTA, a Ca^{2+} chelator at mM range has no remarkable effect on PEPC activity during enzyme assay (data not shown).

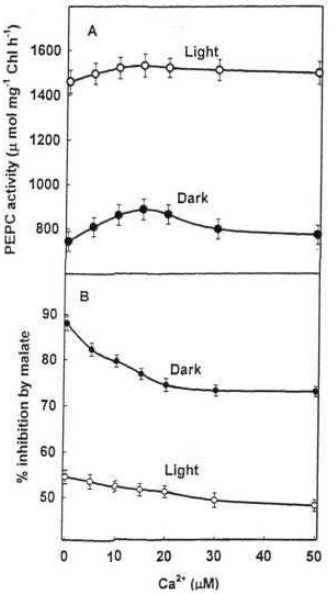

Figure 5.1: The effect of addition of Ca^{2+} during the enzyme assay on the activity (A) or the extent of inhibition by 0.5 mM malate (B) of PEPC in extracts from dark-adapted leaves of *A. hypochondriacus*. The changes caused by Ca^{2+} were all statistically significant ($P < 0.01$).

Effect of Ca^{2+} or EGTA on activity and malate sensitivity in vivo

To mimic the *in vivo* situation, the leaves were fed with 10 μM Ca^{2+} (EGTA-buffered) and an optimal time for feeding was 3 h (Fig 5.2). Feeding of Ca^{2+}-EGTA resulted in a 40% and 80% increase in PEPC activity in dark-adapted and illuminated leaves, respectively (Fig 5.3A). The malate sensitivity decreased by 15 to 20% (Fig 5.3B).

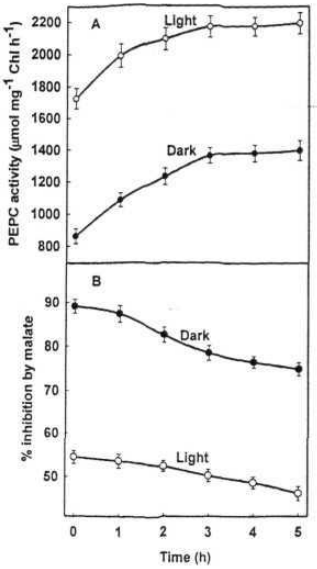

Figure 5.2: The effect of feeding 10 μM Ca^{2+}-EGTA through petioles on the activity (A) or inhibition by 0.5 mM malate (B) of PEPC in extracts from illuminated or dark-adapted leaves of *A. hypochondriacus*. The changes caused by Ca^{2+} were all statistically significant ($P < 0.01$).

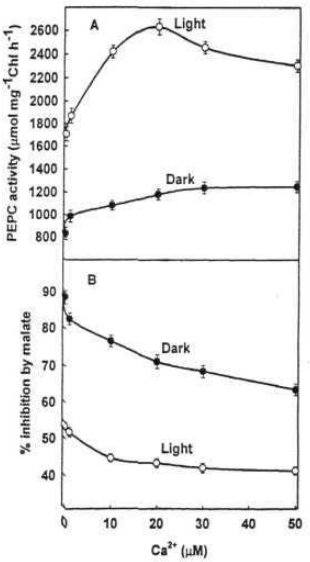

Figure 5.3: The effect of feeding Ca^{2+}-EGTA through petioles on the activity (A) or inhibition by 0.5 mM malate (B) of PEPC, extracted from illuminated or dark-adapted leaves of *A. hypochondriacus*. The changes caused by Ca^{2+} were all statistically significant ($P < 0.01$).

Feeding of EGTA through petiole to leaves resulted in a 30% increase in PEPC activity in dark-adapted leaves but the activity fell significantly in illuminated leaves (Fig 5.4A). However, malate inhibition decreased by 15% in dark-adapted leaves but had only marginal effect upon illumination (Fig 5.4B). Feeding Ca^{2+}-EGTA not only increased the PEPC activity in dark-adapted leaves but also promotes the light activation, thus the L/D ratio increased at optimal concentration of Ca^{2+}-EGTA (Fig 5.5A). But with EGTA feeding, the L/D ratio fell drastically (Fig 5.5B).

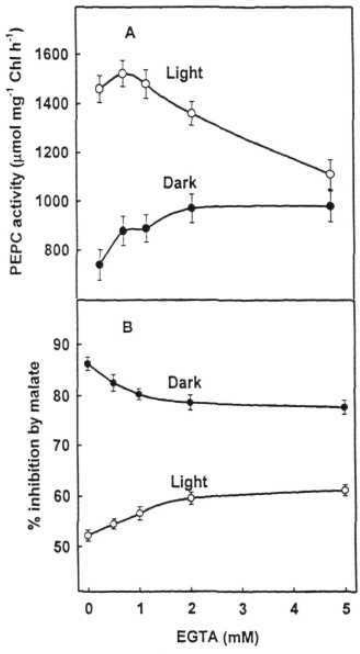

Figure 5.4: Effect of feeding EGTA to leaves of *A. hypochondriacus*, on the activity (A) or the inhibition by malate (B) of PEPC in extracts from dark-adapted or illuminated leaves. The changes caused by Ca^{2+} were all statistically significant ($P < 0.01$).

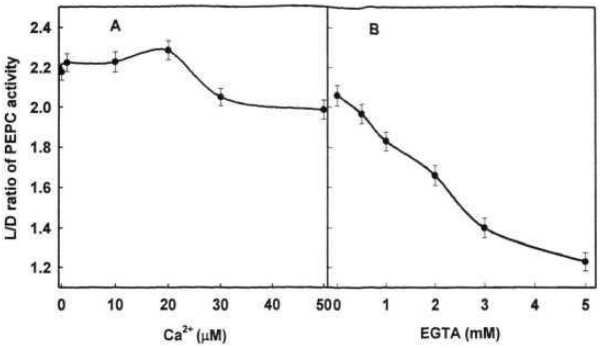

Figure 7.5: The extent of light activation of PEPC in leaf extracts, after feeding either $CaCl_2$ or EGTA to leaves. The above changes caused were all statistically significant ($P < 0.01$).

Phosphorylation status of PEPC with respect to modulation of Ca^{2+}

The level of PEPC phosphorylation was much higher in Ca^{2+} or Ca^{2+}-EGTA fed leaves compared with control (no Ca^{2+}). On the other hand, the light activated phosphorylation was suppressed in EGTA fed leaves (Fig 5.6). No significant phosphorylation was detected in dark, either in the control or treated leaf.

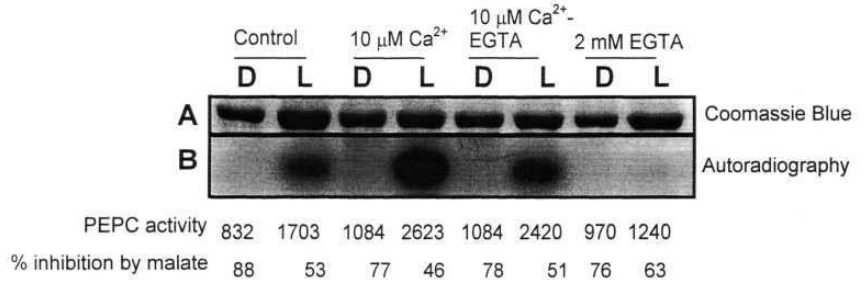

Figure 5.6: The pattern of protein levels and the phosphorylation of PEPC in leaves of *A. hypochondriacus* exposed to either dark (D), light (L) treatment or fed with either $CaCl_2$, Ca^{2+}-EGTA or EGTA. The leaves labelled with $K_2H^{32}PO_4$ were extracted after the required treatment. The PEPC protein was immunoprecipitated from leaf extracts, separated on 10% SDS-PAGE and examined for either protein (A) by using Coomassie Blue or radioactivity (B) by autoradiography. The amount of protein in each lane was 15 μg. The activity (μmol mg^{-1} Chl h^{-1}) and sensitivity to malate (0.5 mM) of PEPC are also shown for ready comparison.

Discussion

The light activation of PEPC is achieved by posttranslational modification of the enzyme, by phosphorylation of a serine residue near the N-terminus of PEPC and dephosphorylated in dark by a type 2A PEPC-protein phosphatase. (Rajagopalan et al., 1994; Chollet et al., 1996; Vidal and Chollet, 1997). The regulatory phosphorylation occurs on serine residue of the enzyme, for e.g. Ser15 in maize, Ser8 in sorghum (Vidal and Chollet, 1997) and a putative Ser11 in *Flaveria trinervia* (Peotsch et al., 1991) and *A. hypochondriacus* (Rydzik and Berry, 1996). The phosphorylation of PEPC has a strong regulatory impact on not only PEPC but also on the overall functioning of C_4 photosynthesis (Chollet et al., 1996; Vidal and Chollet, 1997).

While the mechanism and significance of phosphorylation in regulation of PEPC is well established, there are contradictory reports on the Ca^{2+}-dependence of PEPC-PK. Some reports suggest the existence of a Ca^{2+}-independent protein kinase and others by a Ca^{2+}-dependent protein kinase (Vidal et al., 2002; Nimmo 2003). Presence of both Ca^{2+}-dependent and independent PEPC-PK was observed in maize (Li and Chollet, 1993; Nihiri et al., 1998; Ogawa et al., 1998), in sorghum (Bakrim et al., 1992). The molecular masses of PEPC-PK from maize, Sorghum or *Mesembryanthemum* were in the range of 30-39 kDa (Saze et al., 2001; Vidal et al., 2002) and these PEPC-PK were Ca^{2+}-independent. In contrast, Ca^{2+}-dependent PEPC-PK also was detected in maize with a molecular weight of 50-60 kDa, and sensitive to calmodulin antagonist W7 and KT5926 (Izui et al., 1995). There are evidences of the importance of Ca^{2+} during phosphorylation of PEPC *in situ* (Duff et al., 1996; Giglioli-Guivarc'h et al.,

1996; Parvathi et al., 2000a). Calcium ionophore (A23187) and EGTA inhibited the phosphorylation of PEPC and Ca^{2+} can protect against such inhibition (Chollet et al., 1996; Duff et al., 1996). Thus, cytosolic Ca^{2+} can be a dynamic secondary messenger during light activation of PEPC.

The present study points out the marked modulation of light activation of PEPC upon Ca^{2+} feeding to leaves. The increase in PEPC activity and that of decrease in the sensitivity of enzyme to L-malate (Fig 5.3) and finally pronounced phosphorylation of PEPC protein in Ca^{2+} fed leaves, all suggest that the Ca^{2+}-dependent protein kinase is involved during light mediated phosphorylation of PEPC in leaves of *A. hypochondriacus*. Obviously the effect of Ca^{2+} is pronounced *in vivo* and is not discernible in experiments done *in vitro*.

Illumination induces the cytosolic alkalization in mesophyll cells of C_4 plants (Raghavendra et al., 1993) and such increase in cytosolic alkalization can raise cytosolic Ca^{2+}, leading to a rise in the activity of PEPC-PK and PEPC. Parvathi et al. (2000a) reported that the regulation by Ca^{2+} or calmodulin could be at an upstream level of regulation of PEPC-PK. A Ca^{2+}-dependent phosphoinositide-specific phospholipase C (PI-PLC) has been found to participate in the light dependent cascade leading to C_4-PEPC phosphorylation in mesophyll cell protoplasts of *Digitaria sanguinalis* (Coursol et al., 2000).

The role of Ca^{2+} was further confirmed with our observation on the effect of EGTA. The marked decrease in the light activation (Fig 7.5) and phosphorylation of PEPC, after feeding of EGTA to *A. hypochondriacus* leaves (Fig 7.6) demonstrates the significant involvement of Ca^{2+}-dependent protein kinase during light activation of PEPC in leaves of *A. hypochondriacus*.

We conclude that optimal levels of Ca^{2+} are required for PEPC activity. Further, Ca^{2+} is essential for the light activation of PEPC, through a significant modulation of PEPC phosphorylation, obviously through a Ca^{2+}-dependent protein kinase. Such Ca^{2+}-dependent protein kinase may well be operating at up stream of PEPC-protein kinase, as suggested by earlier reports (Parvathi et al., 2000a; Giglioli-Guivarc'h et al., 1996).

Major conclusions from the results presented in this chapter are:

1. Ca^{2+} during assay increased the PEPC activity by 30% in dark-adapted leaf extracts but marginally inhibited in illuminated leaf-extracts.
2. Up on feeding 10 μM Ca^{2+}, light activation of PEPC increased by 3-fold in leaves with 24% decrease in malate sensitivity.
3. EGTA had no effect during enzyme assay but up on feeding dampened the light activation of PEPC.
4. Kinetic and regulatory properties of PEPC do changes up on modulation of Ca^{2+} *in vivo*.
5. The phosphorylation level of PEPC was higher in Ca^{2+} fed leaves than that in control. But EGTA decreased the light modulated phosphorylation.

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Chapter 6

Marked Modulation by NH_4^+, But Limited Effects of NO_3^- On Phosphoenolpyruvate Carboxylase in Leaves of *Amaranthus hypochondriacus*

Chapter 6

Marked Modulation by NH_4^+, But Limited Effects of NO_3^- on Phosphoenolpyruvate Carboxylase in Leaves of *Amaranthus hypochondriacus*

Phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) is a ubiquitous enzyme occurring in cytosol of photosynthetic and non-photosynthetic tissues of C_3, C_4 and CAM plants (Andreo et al., 1987; Rajagopalan et al., 1994; Chollet et al., 1996; Vidal and Chollet, 1997; Nimmo 2000). The enzyme is regulated strongly by external factors, which include light, temperature and inorganic nutrients. On illumination, the activity of PEPC in leaves of C_4 plants is enhanced by 2-3 fold along with a marked decrease in the malate sensitivity of the enzyme (Huber and Sugiyama, 1986; Doncaster and Leegood, 1987; Parvathi et al., 2000a). These changes during the light activation are due to the phosphorylation of the enzyme by a PEPC-protein kinase (PEPC-PK) (Chollet et al., 1996, Vidal and Chollet, 1997, Parvathi et al., 2000a). PEPC is also highly regulated by temperature in C_4 plants (Phillips and McWilliam, 1971; Wu and Wedding, 1987; Chinthapalli et al., 2003). The regulation of PEPC by temperature is possibly not through phosphorylation rather than conformational changes in protein (Chinthapalli et al., 2003).

PEPC is regulated markedly by metabolites. L-malate, a product of carboxylation, is a competitive inhibitor of PEPC and is a key player of feed back regulation in not only C_4 but also C_3 and CAM plants (Vidal and Chollet, 1997). Glucose-6-P (Glc-6-P) is an allosteric activator of PEPC increases the V_{max} of enzyme and decreases K_m for PEP (Andreo et al., 1987; Vidal and

Chollet, 1997). The K_i (malate) and K_a (Glc-6-P) values would reflect the modulation of regulatory properties of PEPC.

Among the inorganic nutrients, nitrogen has profound influence on PEPC in C_3, C_4 as well as CAM plants, at the level of both *de novo* synthesis of enzyme and the phosphorylation of protein (Murchie et al., 2000). NH_4^+ ions promote the biosynthesis of PEPC *in vivo* (Sugiyama and Sakakibara, 2002) and stimulate the PEPC activity *in vitro* (Gayathri and Raghavendra, 1994). Reports on the effects of feeding NO_3^- on PEPC in leaves of C_3 and C_4 plants, are contradictory. *In vivo* feeding of NO_3^- starved leaves with NO_3^- salts enhanced the light-activation of PEPC from the C_3 and C_4 species, e.g., wheat (Van Quy et al., 1991; Van Quy and Champigny, 1992; Duff and Chollet, 1995; Li et al., 1996) and maize (Gupta et al., 1994). However, Gupta et al. (1994) reported that feeding of NO_3^- to NO_3^- starved plants had no effect on light-activation of PEPC in wheat. Therefore, it is quite interesting to study the short-term *in vivo* effects of NO_3^- on the activity and light-activation of PEPC from plant species, grown under normal field conditions. In contrasts to the reports with NO_3^-, the reports on the effect of NH_4^+ are very few.

The present study is an attempt to reevaluate *in vitro* as well as *in vivo*, the role of N nutrition particularly NH_4^+ and NO_3^- on PEPC from *Amaranthus hypochondriacus*, a NAD-malic enzyme type C_4 plant. Effect of NH_4^+ or NO_3^- was assessed *in vitro* during enzyme assay. Attempts were made to modulate the level of PEPC *in vivo* by feeding leaves with NH_4Cl or KNO_3. Properties of PEPC were checked from dark-adapted or illuminated leaves for comparison. Experiments were extended to study the pattern of PEPC phosphorylation in the presence of NH_4^+ or NO_3^-.

Results

Effect of NH_4^+ on activity and malate sensitivity of PEPC in vitro and in vivo

Inclusion of varying concentration of NH_4^+ during the enzyme assay increased the activity of PEPC in dark-adapted leaf extracts with the maximum response at 40 μM NH_4^+. However, NH_4^+ inhibited the enzyme activity marginally in illuminated leaf extracts (Fig 6.1A). On the other hand, malate sensitivity in dark-adapted leaf extracts decreased by 20% on exposure to NH_4^+ but in illuminated extracts the effect of NH_4Cl was marginal (Fig 6.1B). In an extended study, high concentrations of NH_4^+ (mM ranges) were used, but the effect was quite similar: activation of PEPC at lower concentrations and inhibition at high concentration (Fig 6.2).

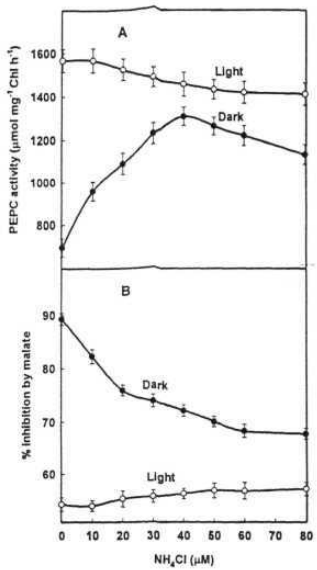


Figure 6.1: The effect of NH_4Cl on activity (A) or inhibition by 0.5 mM malate (B) of PEPC in extracts from dark-adapted or illuminated leaves of *A. hypochondriacus*. The average values of 3 to 5 experiments are represented as ± SE. The changes caused by NH_4Cl were statistically significant ($P<0.01$) in most of the points.

When fed to leaves (optimum period of 3 h), NH_4^+ increased the PEPC activity up to 50% in dark-adapted leaves but dramatically increased the light activation of the enzyme up to 2.7-fold (Fig 6.3A). The increase in PEPC activity by NH_4^+ feeding was correlated with the decrease in malate sensitivity (Fig 6.3B). When these values were recalculated, the extent of light activation increased significantly from 2.1-fold (No NH_4Cl) to 2.8-fold, with 5 mM NH_4Cl (Fig 6.4).

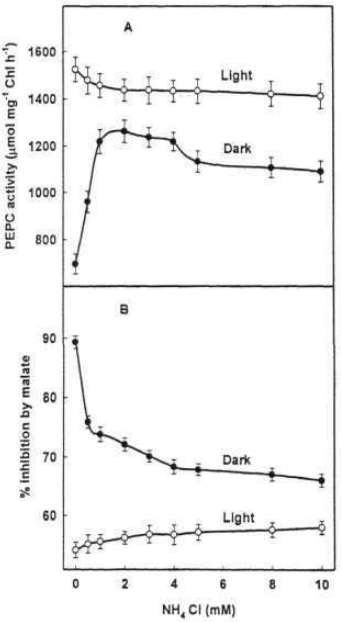


Figure 6.2: The effect of NH_4Cl on activity (A) or inhibition by 0.5 mM malate (B) of PEPC in extracts from dark-adapted or illuminated leaves of *A. hypochondriacus*. The average values of 3 to 5 experiments are represented as ± SE.

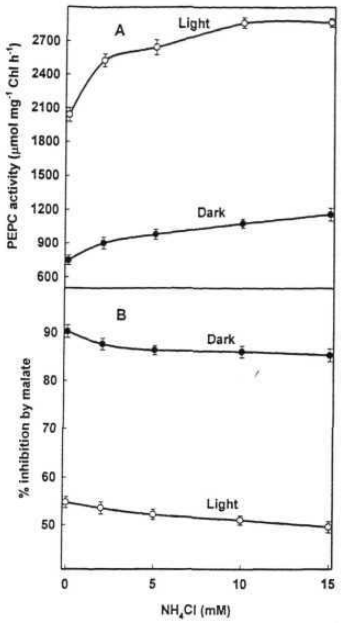


Figure 6.3: The effect of feeding varying concentrations of NH_4Cl on the activity (A) and the inhibition by 0.5 mM malate (B) of PEPC in extracts from illuminated or dark-adapted leaves of *A. hypochondriacus*. The average values of 3 to 5 experiments ± SE are represented. The changes caused by NH_4Cl were all statistically significant ($P<0.01$).

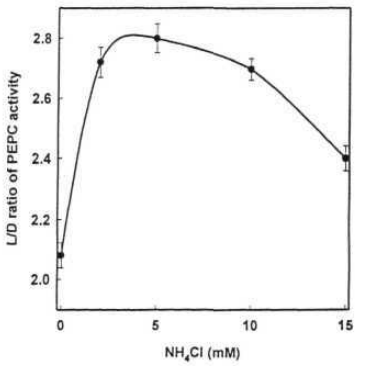


Figure 6.4: The effect of feeding varying concentration of NH_4Cl feeding on the extent of light activation of PEPC in leaves of *A. hypochondriacus*. The average values of 3 to 5 experiments are represented as ± SE.

Effect of NO_3^- on activity and malate sensitivity of PEPC in vitro and in vivo

In leaf extracts, when added in μM range, NO_3^- decreased marginally PEPC activity and increased malate sensitivity of the enzyme. This effect was quite similar in the extracts of illuminated or dark-adapted leaves (Fig 6.5). At higher concentrations (mM range) too, NO_3^- had only marginal effect on the PEPC activity (Fig 6.6A) and malate sensitivity (Fig 6.6B) of PEPC. After feeding NO_3^- to leaves *in vivo*, increased the activity of PEPC in leaf extracts by >50% in case of both dark-adapted or illuminated leaves (Fig 6.7). As the stimulatory effect of NO_3^- was more in dark-adapted leaves, the extent of light activation decreased marginally, as indicated by L/D ratio (Fig 6.8).

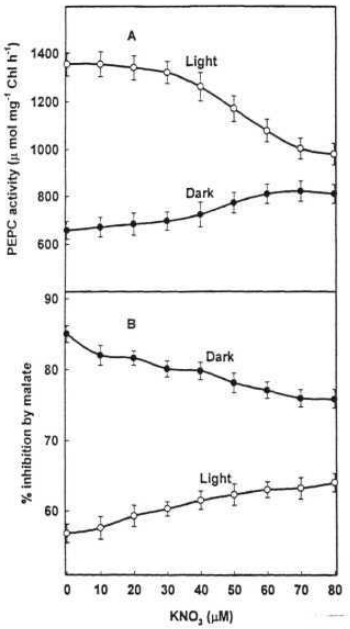


Figure 6.5. The effect of KNO_3 addition during the enzyme assay on the activity (A) or the extent of inhibition by 0.5 mM malate (B) of PEPC in extracts from illuminated or dark-adapted leaves of *A. hypochondriacus*. The average values of 3 to 5 experiments ± SE are represented. The changes caused by KNO_3 were all statistically significant ($P<0.01$).

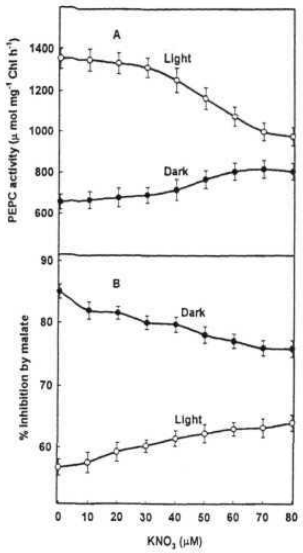


Figure 6.6: The effect of KNO_3 addition during the enzyme assay on the activity (A) or the extent of inhibition by 0.5 mM malate (B) of PEPC in extracts from illuminated or dark-adapted leaves of *A. hypochondriacus*. The average values of 3 to 5 experiments ± SE are represented. The changes caused by KNO_3 were all statistically significant ($P<0.01$).

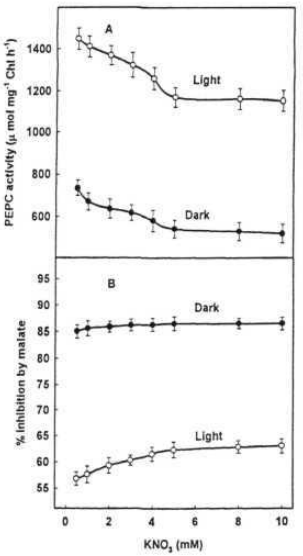


Figure 6.7: The effect of feeding varying concentration of KNO_3 on the activity (A) or the extent of inhibition by 0.5 mM malate (B) of PEPC in extracts from illuminated or dark-adapted leaves of *A. hypochondriacus*. The average values of 3 to 5 experiments ± SE are represented. The changes caused by KNO_3 were all statistically significant ($P<0.01$).

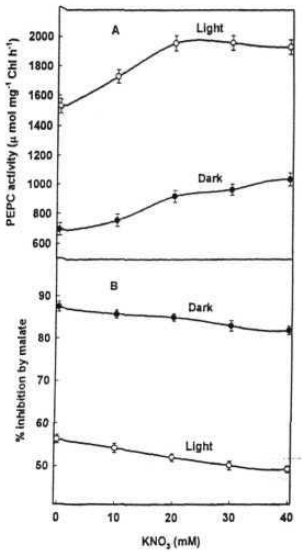


Figure 6.8: The effect of feeding varying concentration of KNO_3 on the extent of light activation of PEPC in leaves of *A. hypochondriacus*. The average values of 3 to 5 experiments are represented as ± SE.

Changes in kinetic and regulatory properties of PEPC in leaves of Amaranthus hypochondriacus by NH_4^+

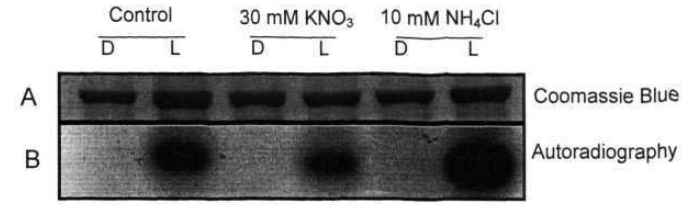
Table 6.1 summarizes the changes in kinetic and regulatory properties of PEPC with respect to NH_4^+ feeding to leaves. The V_{max} of PEPC increased by almost 2.7-fold compared with that of unfed leaves as 2.1-fold. There was >50% increase in K_i (malate) up on NH_4^+ feeding in dark-adapted leaves and further up on illumination it increased almost 4.8-fold. There was an only marginal change in K_m (PEP) up on NH_4^+ feeding in dark-adapted leaves but up on illumination it increased further by 40%. The similar pattern of changes was observed in K_a (Glc-6-P) up on illumination of NH_4^+ fed leaves with 3.5-fold increase in K_a (Glc-6-P).

Table 6.1: Changes induced in the kinetic and regulatory properties of PEPC in extracts of illuminated or dark-adapted leaves of *A. hypochondriacus* in response to 10 mM NH_4^+ feeding to leaves. NH_4^+ was fed to leaves at 200 μE m^{-2} s^{-1} for 3 hours for control, taken as dark and illuminated for another 30 min at 1000 μE m^{-2} s^{-1} for light activation of the enzyme. Another set of leaves without NH_4^+ was taken as control. The experiments were done on at least three different days and the average values are ± SE represented. The changes caused by NH_4^+ were all significant at P <0.01.

Parameters	No NH_4^+		Δ change (-fold)	+ NH_4^+		Δ change (-fold)
	Dark	illuminated		Dark	illuminated	
V_{max} (μmol mg^{-1} Chl h^{-1})	773 ± 54	1624 ± 63	2.1	1106 ± 65	2877 ± 86	2.6
K_m (PEP) mM	0.29 ± 0.02	0.18 ± 0.02	0.6	0.26 ± 0.01	0.17 ±0.01	0.7
K_i (Malate) mM	0.11 ± 0.01	0.41 ± 0.02	3.7	0.17 ± 0.01	0.53 ± 0.02	3.1
K_a (Glc-6-P) mM	0.32 ± 0.02	1.03 ± 0.13	3.2	0.33 ± 0.04	1.12 ± 0.14	3.4

Phosphorylation status of PEPC with respect to nitrogen source

The increase in PEPC activity and decrease in malate sensitivity is a strong indication of phosphorylation of PEPC protein. The phosphorylation levels of PEPC with respect to N-source were therefore assessed after NH_4^+ or NO_3^- feeding to leaves. The phosphorylation level of PEPC in NH_4^+ fed leaves was much higher than that of control but in NO_3^- fed leaves effect was marginal (Fig 6.9). No phosphorylation was detected in dark, either in the control or treated leaf. The increase in PEPC activity and decrease in malate inhibition were all correlated with the level of phosphorylation.



PEPC activity	748	1647	1070	2633	1081	2865
% inhibition by malate	90	55	81	52	83	51

Figure 6.9: The pattern of protein levels and the phosphorylation of PEPC in leaves of *A. hypochondriacus* exposed to either dark (D), light (L) treatment or fed with either 30 mM KNO_3, 10 mM NH_4Cl. The leaves of *A. hypochondriacus* labelled with $K_2H^{32}PO_4$ were extracted after the required treatment. The PEPC protein was immunoprecipitated from leaf extracts, separated on 10% SDS-PAGE and examined for either protein levels by using Coomassie Blue (A) or radioactivity, by using autoradiography (B). The amount of protein in each lane was 15 μg. The activity and sensitivity to malate (0.5 mM) of PEPC are also shown for ready comparison.

Discussion

There is a high variability in the reports on the effects NO_3^- on PEPC in leaves of C_3 and C_4 plants. *In vivo* feeding of NO_3^- starved leaves with NO_3^- salts enhanced the light-activation of PEPC from the C_3 and C_4 species, e.g., wheat (Van Quy et al., 1991; Van Quy and Champigny, 1992; Manh et al., 1993; Duff and Chollet, 1995; Li et al., 1996) and maize (Gupta et al., 1994; Murchie et al., 2000). However, Gupta et al. (1994) reported that feeding of NO_3^- to NO_3^- starved plants had no effect on light-activation of PEPC in wheat. The extent of stimulation by KNO_3 in leaves ranged from 30% to 3.8-fold (Table 2).

Table 6.2: *Comparative changes in the kinetic and regulatory properties of PEPC in C_3 and C_4 plants in response to light (L) and NO_3^-.*

Experimental plant and type	Changes in PEPC in response to NO_3^-		Reference
	Increase in activity	Change in malate sensitivity	
Maize (C_4)	2.3-fold	No change	Foyer et al., 1994
Maize (C_4)	1.62-fold	4.1-fold increase in K_i (malate)	Gupta et al., 1994
Maize (C_4)	>40%	No significant change	Murchie et al., 2000
Wheat (C_3)	1.5-fold	No change	Van Quy et al., 1991
Wheat (C_3)	3.8-fold	2.5-fold increase in K_i (malate)	Van Quy et al., 1991
Wheat (C_3)	70%	No change	Mahn et al., 1993
Wheat (C_3)	>50%	No significant change	Duff and Chollet, 1995
Tobacco (C_3)	30%	2-fold decrease	Murchie et al., 2000

Similarly, the change in malate sensitivity was also quite variable. The effect of NO_3^-feeding on PEPC activity and malate sensitivity was more pronounced in C_4 plants than C_3 (Murchie et al., 2000). In the present study, we observed that NO_3^- had no effect of PEPC *in vitro* during enzyme assay and had significant effect *in vivo* up on feeding (Fig 6.5 and 6.7). There was 2.5-fold increase in light activation and increase in phosphorylation of PEPC in *A. hypochondriacus*.

We need to also point out that the experimental conditions of several of the reports, vary. In several cases, the studies on PEPC were made during a transfer of plants from low to the high NO_3^- leaves (Van Quy et al., 1991; Champigny and Foyer, 1992; Sugiharto and Sugiyama, 1992; Duff and Chollet,

1995). The transition from low to NO_3^- triggers an increase in the activity of PEPC, high activation of PEPC, leading to an increase in flow of carbon into amino acid synthesis (Van Quy et al., 1991; Sugiharto and Sugiyama, 1992; Foyer et al., 1994). This occurs in both C_3 and C_4 leaves.

Gayathri and Raghavendra (1994) reported an allosteric activation by NH_4^+ of PEPC *in vitro*. In the present study, we observed that NH_4^+ during enzyme assay increased the PEPC activity and decreased the sensitivity (Fig 6.1). The above effect could be due to allosteric activation of PEPC by NH_4^+. Giglioli-Guivarc'h et al. (1996) reported an increase in phosphorylation status of PEPC up on treatment with 10 mM NH_4Cl of *Digitaria sanguinalis* protoplasts and these authors correlated the effect with increased in cytosolic pH. In the present study, treatment of leaves *in vivo* with NH_4^+ increased markedly the extent of light activation up on feeding to leaves and this was partly due to increased phosphorylation *in vivo* (Fig 6.9). Our results suggest that the effects of NH_4^+ on PEPC can be of two types. The activation of PEPC *in vitro* (i.e. when added during enzyme assay) appears to be allosteric and *in vivo* (i.e. feeding leaves) the increase in PEPC activity is due to the phosphorylation of enzyme.

The marked increase in light activation of PEPC was observed when leaves were fed with 10 mM NH_4^+, was observed in C_3 plants, such as wheat (Manh et al., 1993) and C_4 plants, such as maize or Digitaria (Duff and Chollet, 1995; Giglioli-Guivarc'h et al., 1996; Murchie et al., 2000). Even in non-photosynthetic tissues of wheat roots, PEPC activity increased in response to NH_4^+ and was due to *de novo* synthesis of PEPC protein (Koga and Ikeda, 2000).

In present study too, we could observe a 3-fold increase in light activation of PEPC up on feeding 10 mM NH_4^+ to *A. hypochondriacus* leaves (Fig 6.3) and this was possibly due to the enhanced phosphorylation status of PEPC (Fig 6.9).

Our results demonstrate the marked modulation of PEPC by N-source *in vitro* as well as *in vivo*. The modulation by NH_4^+ was strong both *in vitro* as well as *in vivo*, while the effect of NO_3^- was discernible only *in vivo*. Thus, a close relationship appears to exist between PEPC and NO_3^- reduction and ammonia assimilation. This is not surprising as the chloroplasts, mitochondria, and peroxisomes and cytosol work together and keep up photosynthesis along with N metabolism and respiration (Raghavendra and Padmasree, 2003).

Our results also suggest that NO_3^- or NH_4^+ regulates PEPC of *A. hypochondriacus*, in two ways. In short-term incubation, i.e., during enzyme assay NH_4^+ causes allosteric activation of PEPC. In a long term effect, i.e., feeding, there is a marked increase in phosphorylation level, due to the biosynthesis of PEPC and/or PEPC-PK.

Major conclusions from the results presented in this chapter are:

1. Lower conc. of NH_4^+ during assay increased the PEPC activity in dark-adapted leaf extracts and but marginally inhibited in illuminated extracts, but NO_3^- had only marginal effect.
2. NH_4^+ or NO_3^- feeding to leaves dramatically enhanced the light activation of PEPC with decrease in malate sensitivity.
3. The phosphorylation level of PEPC was much higher in NH_4^+ fed leaves than that with NO_3^-.

4. In short-term incubation, i.e., during *enzyme assay*, NH_4^+ causes allosteric activation of PEPC. In a long term effect, i.e., feeding, there is a marked increase in phosphorylation level, due to the biosynthesis of PEPC and/or PEPC-PK.

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Chapter 7

Marked Modulation by Phosphate of Phosphoenolpyruvate Carboxylase in Leaves of *Amaranthus hypochondriacus*, a NAD-ME type C_4 Plant: Decrease in Malate Sensitivity but no Change in the Phosphorylation status

Marked Modulation by Phosphate of Phosphoenolpyruvate Carboxylase in Leaves of *Amaranthus hypochondriacus*, a NAD-ME type C_4 Plant: Decrease in Malate Sensitivity but no Change in the Phosphorylation Status

Phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) is a key enzyme involved in primary carbon fixation of C_4 plants (Andreo et al., 1987; Rajagopalan et al., 1994; Chollet et al., 1996; Vidal and Chollet, 1997; Nimmo 2000). The enzyme is regulated strongly by factors, such as light, temperature or inorganic nutrients. On illumination, the activity of PEPC in leaves of C_4 plants is enhanced by 2-3 fold along with a marked decrease in the malate sensitivity of the enzyme (Huber and Sugiyama, 1986; Doncaster and Leegood, 1987; Parvathi et al., 2000a). These changes during the light activation are due to mostly the phosphorylation of the enzyme (Chollet et al., 1996; Vidal and Chollet, 1997; Parvathi et al., 2000a).

The C_4 plants are tolerant to heat but quite sensitive to cold temperature (Phillips and McWilliam, 1971). At low temperature, activity of PEPC decreased but its malate sensitivity increased in C_4 plants, while at high temperature the activity of enzyme increased and malate sensitivity decreased. The changes in PEPC induced by temperature appear to be due to conformational changes in the protein (Wu and Wedding, 1987; Chinthapalli et al., 2003).

Among the inorganic nutrients, nitrogen has profound influence on PEPC, at both the levels of *de novo* synthesis of enzyme and the phosphorylation of PEPC in C_3, C_4 as well as CAM plants. NH_4^+ promotes the

biosynthesis of PEPC (Sugiyama and Sakakibara, 2002) and stimulate the PEPC activity *in vitro* (Gayathri and Raghavendra, 1994). The extent of light activation of PEPC was increased in presence of NO_3^- or NH_4^+ (Vidal et al., 2002). The stimulation by NO_3^- or NH_4^+ appears to be through the enhancement of PEPC-PK activity and an increase the phosphorylation status of PEPC (Sugiyama and Sakakibara, 2002; Vidal et al., 2002).

Pi also plays an important role in plant metabolism: not only as the substrate for oxidative and photophosphorylation but also as a key component of several metabolic processes (Theodoron and Plaxton, 1993). Pi modulates the activity of several enzymes such as, fructose and sedoheptulose bisphosphatases, ribulose-1,5-bisphosphate carboxylase and sucrose phosphate synthase (Iglesias et al., 1993; Rao 1997). The role of Pi in C_3 photosynthesis is studied extensively (Cséke and Buchanan, 1986). But the role of Pi in C_4 photosynthesis is yet to be examined in detail.

The effect of Pi on PEPC is interesting, as Pi is an end product of PEPC reaction. However, studies on the direct effects of Pi on PEPC are quite limited (Wong and Davies, 1973; Podesta et al., 1990; Meyer et al., 1989; Salahas and Gavalas, 1997). There are contradictory reports in the literature on the effects of Pi on the activity of C_4 PEPC: inhibition of enzyme activity (O' Leary 1982; Doncaster and Leegood, 1987), marginal or marked activation (Podesta et al., 1990; González et al., 1987) or no effect (Wong and Davies, 1973). Usuda and Shimogawara (1992) reported decrease in PEPC activity in maize when plants were grown Pi deprived condition. Further, the material used in these experiments also varied from extracts from etiolated or illuminated leaves to purified PEPC, making it difficult for a comparison.

PEPC is regulated also by metabolites. L-malate, a product of carboxylation, is a competitive inhibitor of PEPC and a key player of feed- back regulation not only in C_4 but also in C_3 and CAM plants (Vidal and Chollet, 1997). Glc-6-P is an allosteric activator of PEPC increases the V_{max} of enzyme and decreases K_m for PEP (Andreo et al., 1987; Vidal and Chollet, 1997). The K_i (malate) and K_a (Glc-6-P) values would reflect the modulation of regulatory properties of PEPC.

The present study is an attempt to reevaluate critically the effect of Pi on the properties of PEPC from *A. hypochondriacus,* a C_4 plant. Experiments were conducted at different levels; Pi effects were analyzed *in vitro* by its addition during assay, while using leaf extracts or purified PEPC, and *in vivo* by measuring the effect of Pi fed to intact leaves. Initially, the effect of Pi on the kinetic and regulatory properties of PEPC was studied in leaf extracts and with purified protein. The Pi status of leaves was modulated by feeding with either Pi or mannose, a Pi sequester (Loughman et al., 1989). The phosphorylation status of PEPC was evaluated after feeding the leaves with Pi. The presence of Pi increased the activity of the enzyme (particularly the dark-form) and decreased the malate sensitivity. The changes caused by Pi appear to be independent of phosphorylation of PEPC.

Results

Effect of Pi during assay on PEPC in leaf extracts

The activity of PEPC was enhanced in presence of Pi in leaf extracts (Fig 7.1A). The activity of enzyme increased by >2.5-fold at 15 or 20 mM Pi, while the sensitivity of PEPC to malate decreased (Fig 7.1B) from about 90% (no Pi) to about 70% (in presence of 30 mM Pi). Due to marked stimulation of

PEPC activity by Pi even in extracts from dark-adapted leaves, the extent of light activation got masked as the Pi concentration was increased in the assay medium. As a result, the L/D ratio of PEPC in leaf extracts decreased (Fig 7.2).

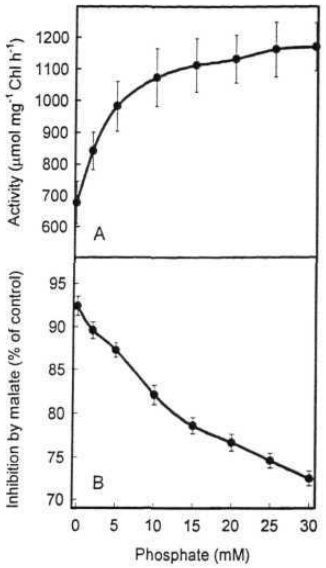

Figure 7.1: The effect of addition of Pi during the enzyme assay on the activity (A) or the extent of inhibition by 0.5 mM malate (B) of PEPC in extracts from dark-adapted leaves of *A. hypochondriacus*. The average values of 3 to 5 experiments are represented as ± SE. The changes caused by Pi were all statistically significant ($P < 0.01$).

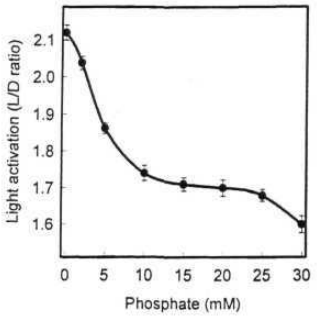

Figure 7.2: The extent of light activation of PEPC in leaf extracts, in relation to different concentration of Pi in the assay medium. The average values of 3 to 5 experiments are represented as ± SE. The changes caused by Pi were all statistically significant ($P < 0.01$).

Purification of PEPC

Purification of PEPC from leaves of *A. hypochondriacus* was done, as already described (Gayathri et al., 2000). PEPC was purified to homogeneity from *A. hypochondriacus* leaves by using the conventional steps of extraction: 40-60% ammonium sulfate precipitation, followed by successive chromatography through columns of DEAE-Sepharose and Hydoxylapatite (HAP). PEPC was eluted from the DEAE-Sepharose column as a broad peak at around 120-150 mM Pi with specific activity of 21.7 U mg^{-1} protein (Fig 7.3). When eluted from the hydrophobic interaction chromatography using DEAE-Sepharose substantially enriched the PEPC. But the use of HAP helped to improve the purity of PEPC further. The enzyme was eluted as a single peak at 70-90 mM Pi with an activity of 31 U mg^{-1} protein from HAP column (Fig 7.4). The most notable property observed in the course of purification procedure was that the enzyme strongly bound on hydrophobic columns. Finally, the enzyme was concentrated by using solid PEG-20,000.

After the above three steps, PEPC was purified by 81.5-fold, with a final specific activity of 31 U mg^{-1} protein, and an overall recovery of about 61% (Table 7.1). The purity of the enzyme was confirmed by the appearance of a single band of about 100-kD on 10% SDS-PAGE (Fig 7.5) and 400 kD on 7% native-PAGE (Fig 7.6)

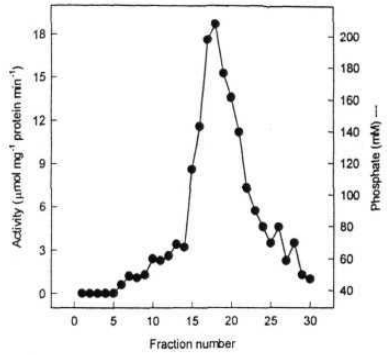

Figure 7.3: The pattern of PEPC activity during elution from a DEAE-Sepharose column. The enzyme from 40-60% ammonium sulfate fractions of leaf extracts was loaded after dialysis, onto a DEAE-Sepharose column (1x12 cm), preequilibrated with 20 mM phosphate buffer (pH 7.2) containing 10% (v/v) glycerol. The column was washed with the same buffer and was eluted with a linear gradient from 40-200 mM phosphate buffer (pH 7.2) containing 10% glycerol. The activity of PEPC was assayed at pH 7.3 with 2.5 mM PEP.

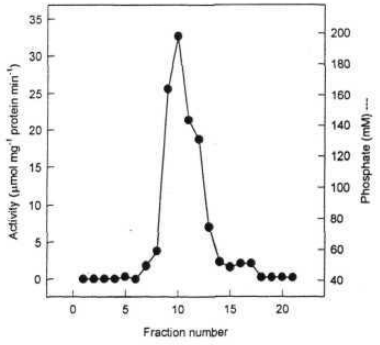

Figure 7.4: The pattern of PEPC activity during elution from a hydroxylapatite (HAP) column. The active fractions obtained from DEAE-Sepharose column were pooled and precipitated by 60% ammonium sulfate. The precipitate was dissolved in 200 mM phosphate buffer (pH 7.2) containing 10% (v/v) glycerol and dialyzed against 20 mM phosphate buffer (pH 7.2) containing 10% glycerol. The dilacerate was applied onto HAP column (1x 12 cm), equilibrated with the same buffer. PEPC was eluted with a linear gradient of 40-200 mM phosphate buffer (pH 7.2) containing 10% glycerol. The activity of PEPC was assayed at pH 7.3 with 2.5 mM PEP.

Table 7.1: The purification pattern of PEPC from leaves of *A. hypochondriacus* by conventional methods involving three steps.

Step	Total activity (μmol min^{-1})	Total protein (mg)	Specific activity (μmol mg^{-1} protein min^{-1})	Purification (Fold)	Yield (%) of initial)
Crude extracts	820	2160	0.38	1.0	100
40-60% $(NH_4)_2SO_4$	604	336	1.8	4.7	73.6
DEAE-Sepharose	648	30.9	21.7	57.1	79
Hydroxylapatite	505.3	16.3	31.0	81.5	61

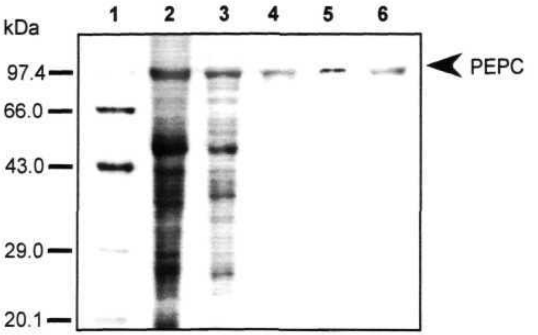

Figure 7.5: Purification of PEPC from *A. hypochondriacus* leaves by conventional method involving three steps. Samples from various steps were analyzed by SDS-PAGE and the gel was stained with silver nitrate. Lane 1: Protein molecular weight marker, Lane 2: Crude extracts, Lane 3: 40-60% ammonium sulfate precipitate, Lane 4: Pool of active fractions from DEAE-Sepharose column, Lane 5: Active pool from HAP column, Lane 6: Purified PEPC, after concentrating with PEG 20,000 and storage in presence of 50% (v/v) glycerol. The molecular weight markers are indicated on the left, while the location of PEPC is shown with an arrow on the right. Lane 2 and 3 contained 8 μg protein, while lane 4 contained 6 μg protein and lane 5 contained 2 μg protein and lane 6 contained 5 μg protein.

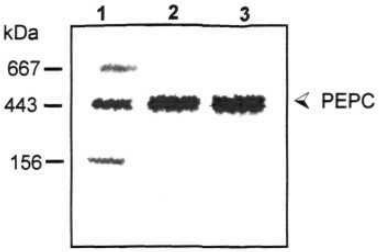

Figure 7.6: Native-PAGE of purified PEPC from *A. hypochondriacus* leaves by conventional method. The molecular weight markers are indicated on the left, while the location of PEPC is shown with an arrow on the right. Lane 2 and 3 contained 20 μg protein.

Effect of Pi during the assay on the properties of purified PEPC

Studies were extended to purified form of PEPC and its response to 30 mM Pi during enzyme assay. The V_{max} of the purified PEPC increased upon addition of Pi (Fig 7.7A) and there was drastic decrease in its malate sensitivity (Fig 7.7B).

Table 7.2 summarizes the response to Pi of catalytic and regulatory properties of purified PEPC from dark-adapted and light-adapted leaves. The presence of Pi increased V_{max} by >1.8-fold and 1.6 fold whereas K_i (malate) increased by >2.3-fold and >1.9-fold in dark-adapted and light adapted leaves respectively. The K_m (PEP) increased by about >1.5-fold and 1.3-fold respectively. K_a (Glc-6-P) increased by about 1.7-fold and >1.6-fold in dark-adapted and light adapted leaves respectively. Thus, the effects of Pi were much more pronounced in purified PEPC from dark-adapted leaves than that of light-adapted ones.

Effect of feeding Pi or mannose to leaves

Feeding leaves with Pi through transpiration stream increased PEPC activity and decreased the extent of inhibition by malate (Fig 7.8A). The effect of Pi was dependent on the duration of Pi feeding. By about 3 h of feeding, there was >2-fold increase in PEPC activity, while there was decrease in malate sensitivity, from 92 to 76% in dark-adapted leaves (Fig 7.8B), all in extracts from dark-adapted leaves. Again, the effects of Pi were low on PEPC in extracts from illuminated leaves.

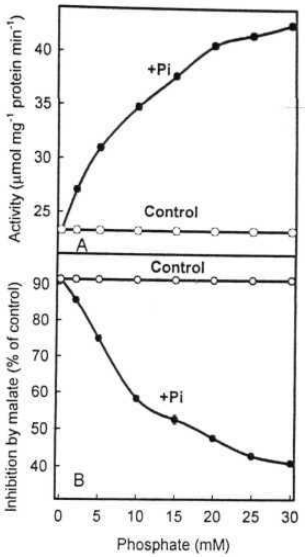

Figure 7.7: The effect of added Pi on the activity (A) or the extent of inhibition by 0.5 mM malate (B) of purified PEPC from dark-adapted leaves of *A. hypochondriacus*. The average values of 3 to 5 experiments are represented as ± SE. Some errors are within the symbols. The changes caused by Pi were all statistically significant (P <0.01).

Table 7.2: Changes in the kinetic and regulatory properties of purified PEPC from dark-adapted and light adapted leaves of *A. hypochondriacus*, respectively in response to 30 mM Pi during enzyme assay. The average values of 3 to 5 experiments are represented as ± SE. The changes caused by Pi were all statistically significant (P <0.01).

Parameters	PEPC from dark-adapted leaves.		Δ Change (- fold)	PEPC from light-adapted leaves.		Δ Change (- fold)
	Control	+ 30 mM Pi		Control	+ 30 mM Pi	
V_{max} (μmol mg^{-1} protein min^{-1})	22.6 ± 0.63	41.4 ± 0.52	1.8	29.7 ± 0.47	49.9 ± 0.62	1.6
K_m (PEP) mM	0.33 ± 0.01	0.49 ± 0.01	1.5	0.14 ± 0.01	0.18 ± 0.02	1.3
K_i (Malate) mM	0.13 ± 0.01	0.34 ± 0.02	2.6	0.21 ± 0.02	0.40 ± 0.08	1.9
K_a (Glc-6-P) mM	0.21 ± 0.01	0.38 ± 0.01	1.8	0.29 ± 0.02	0.47 ± 0.02	1.6

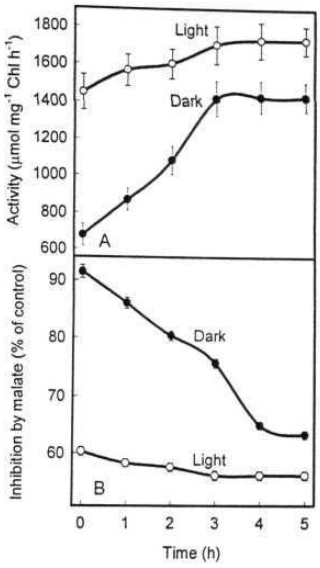

Figure 7.8: The effect on the activity (A) or the extent of inhibition by 0.5 mM malate (B) of PEPC, extracted from illuminated or dark-adapted leaves of *A. hypochondriacus*, after varying periods of feeding 20 mM Pi through petioles. The average values of 3 to 5 experiments are represented as ± SE. Some errors are within the symbols. The changes caused by Pi were all statistically significant ($P < 0.01$).

Mannose, a Pi sequestering agent was fed to the leaves for 90 min and the properties of PEPC extracted from these leaves (after illumination or dark-adaptation) were examined. The effect of mannose was concentration dependent and peaked at 10 mM mannose. Feeding of leaves with 10 mM mannose decreased the activity of PEPC; the decrease being more pronounced in illuminated leaves than in the dark-adapted ones (Fig 7.9A). There was small effect on the extent of malate inhibition, either in illuminated or dark-adapted leaves (Fig 7.9B).

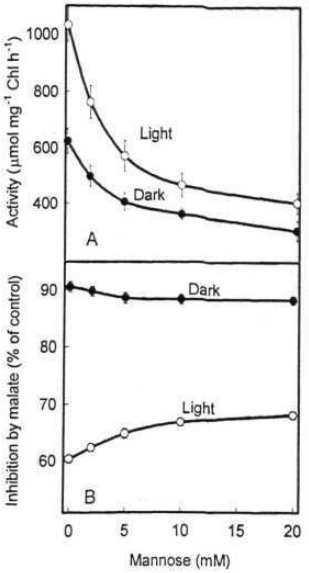

Figure 7.9: Effect of feeding mannose to leaves of *A. hypochondriacus* on the activity (A) or the malate sensitivity by 0.5 mM malate (B) of PEPC extracted from dark-adapted or illuminated leaves. The average values of 3 to 5 experiments are represented as ± SE. Some errors are within the symbols.

Effect of Pi on the phosphorylation status of PEPC

The phosphorylation status of PEPC was examined, with or without pretreatment with Pi and after illumination of leaves (Fig 7.10). There was a marked phosphorylation of PEPC in extracts from illuminated leaves. However, there was no change in PEPC phosphorylation due to presence of Pi. The phosphorylation of PEPC was suppressed in the presence of mannose, particularly in illuminated leaves. No phosphorylation was detected in dark, either in the control or Pi treated leaf (Fig 7.10).

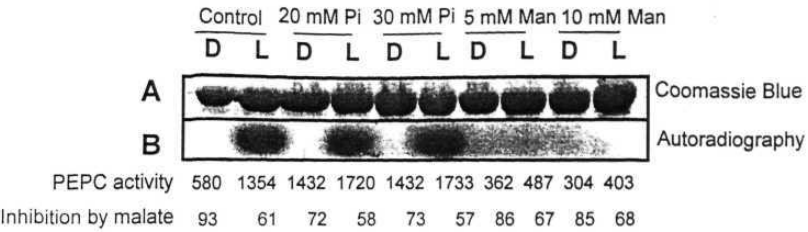

Figure 7.10: The pattern of protein levels and the phosphorylation of PEPC in leaves of *A. hypochondriacus* exposed to either dark (D), light (L) treatment or fed with either Pi (20 and 30 mM) or mannose (5 and 10 mM). The leaves of *A. hypochondriacus* labeled with ^{32}Pi were extracted after the required treatment. The PEPC protein was immunoprecipitated from leaf extracts, separated on 10% SDS-PAGE and examined for either protein levels by using Coomassie Blue (A) or radioactivity, by using autoradiography (B). The amount of protein in each lane was 15 µg. The activity and sensitivity to malate (0.5 mM) of PEPC are shown for a ready comparison.

Discussion

Our results demonstrate the marked regulation of PEPC by Pi *in vivo* (leaves) as well as *in vitro* (purified protein). The presence of Pi not only increased PEPC activity but also decreased the malate sensitivity of the enzyme from *A. hypochondriacus* (Fig 7.1 and Fig 7.3). Our study endorses the earlier suggestions that Pi can stabilize the PEPC (Podesta et al., 1990; Salahas and Gavalas, 1997; Salahas et al., 1997b). High levels of Pi (~100 mM) can protect PEPC against heat denaturation (Salahas et al., 1997a; Jensen et al., 1995) as well as cold temperature (Salahas et al., 1997a). It is therefore important that PEPC is extracted in Pi buffer for maximum activity and stability.

The reports on the effect of Pi on PEPC have been quite contradictory. Doncaster and Leegood (1987) reported >40% inhibition of enzyme activity, whereas Podesta et al. (1990) reported an increase of 150% in PEPC activity. The activity of Pi was unaffected, as per Wong and Davies (1973). Our results,

therefore, clear the ambiguities and confirm that the PEPC from *A. hypochondriacus* is stimulated by Pi. The concentration of Pi in mesophyll cells of plants is expected to be around 20 mM (Mimura et al., 1998) and can be an important factor.

The internal levels of Pi in leaves increase after feeding with external Pi (Mimura et al., 1990). When the leaves of *A. hypochondriacus* were fed with Pi, the activity of PEPC increased, while the extent of malate inhibition decreased (Fig 7.8). These results suggest that the changes in Pi levels in the leaves would lead to marked modulation *in vivo* of PEPC activity and its malate sensitivity. Marked changes in PEPC, during growth in the presence or absence of Pi were noticed in maize seedlings (Usuda and Shimogawara, 1992) and suspension cell cultures of *Brassica napus* (Moraes et al., 2000).

Rapid utilization of cytoplasmic Pi occurs upon the addition of sequestering agents like mannose (Loughman et al., 1989). Mannose is phosphorylated in cytoplasm and hence, decreases not only the cytoplasmic Pi concentration (Lee and Ratcliffe, 1993) but also the levels of ATP (Van Quy and Champigny, 1992). We have, therefore, attempted to decrease the level of Pi by feeding mannose to leaf. Mannose feeding in dark has not only decreased the PEPC activity but has also markedly suppressed the light activation of PEPC (Fig 7.9). In a similar study Van Quy and Champigny (1992) found that mannose feeding had inhibited kinase activity and restricted light activation of PEPC in wheat leaves. This could be the reason of inhibition of phosphorylation in mannose fed leaves even upon illumination (Fig 7.10).

Mannose at high concentration may affect various metabolites by sequestering Pi into mannose-6-phosphate, and may cause an imbalance in

metabolism (Herold and Lewis, 1977; Brouquisse et al., 2001). The properties of PEPC may, therefore, be affected due to the altered level of the metabolites. However, this possibility would reaffirm the importance of Pi *in vivo*, as these effects of mannose are consequences of lowered Pi.

It is interesting note that the leaves treated with mannose still exhibit limited light activation of PEPC and a decrease in malate inhibition of enzyme. Obviously, these changes must be due to a process other than phosphorylation. It would be interesting to examine light-activation of PEPC in mannose-treated leaves. For e g. the cytosolic alkalization of mesophyll cells of C_4 leaves (Raghavendra et al., 1993) could mediate some of these changes in PEPC.

The phenomenon of the increase in PEPC activity along with decrease in malate sensitivity due to Pi is quite similar to the effects of light on PEPC in C_4 leaves. The light activation of PEPC is achieved by posttranslational modification of the enzyme, by phosphorylation of a serine residue near the N-terminus of PEPC (Rajagopalan et al., 1994; Chollet et al., 1996; Vidal and Chollet, 1997). It is, therefore, possible that there is a change in the phosphorylation status of PEPC in presence of Pi. The extent of PEPC phosphorylation was much higher in illuminated discs than that in dark-adapted leaves. However, there was no change in phosphorylation status of PEPC due to Pi treatment (Fig 7.10). Feeding mannose or Pi could affect the specific radioactivity of leaf ATP. Thus, our results may be termed as qualitative indications. However, the high level of PEPC phosphorylation under light, even after feeding Pi, indicated that the process of PEPC phosphorylation was stable

and similar. We, therefore, conclude that the changes in PEPC induced by Pi are not dependent on phosphorylation.

Our results suggest that Pi induced decrease in malate sensitivity is not related to the phosphorylation status of PEPC. Similar situation has recently been reported, during temperature modulated decrease in malate sensitivity of PEPC (Chinthapalli et al., 2003). Although the suggestion is speculative at this stage, the presence of Pi could either stabilize the tetrameric shape of PEPC or change the conformational status of protein or both. Further experiments on fluorescence and circular dichroism spectra of PEPC are necessary to assess this possibility.

Table 7.2: Relative changes in kinetic and regulatory properties of PEPC to various regulatory factors from leaves of *A. hypochondriacus*. The illumination was done for 30 min at 1000 μE m^{-2} s^{-1} and PEPC was incubated for 45 min at 45 ^{0}C. During enzyme assay 1.25% (w/v) PEG or 30 mM Pi was added. The values represented are as compared with control (100%). Dark, 15 ^{0}C, zero PEG and zero Pi are taken as controls.

Regulatory factor	Parameters				Reference
Transition	V_{max}	K_m (PEP)	K_i (Malate)	K_a (Glc-6-P)	
Dark ⟶ Light	234	70	430	315	Parvathi 1998; Parvathi et al., 2000
Temperature (15 ^{0}C ⟶ 45 ^{0}C)	165	22	155	52	Chinthapalli et al., 2003
PEG (0 ⟶ 1.25%)	169	60	151	140	Parvathi 1998
Pi (0 ⟶ 30 mM)	183	130	233	160	Present work

Table 7.2 summarizes the changes in properties of PEPC from *A. hypochondriacus* due to light or temperature and compares with the effects of PEG-6000 or Pi. The increases in V_{max}, K_i (malate) and K_a (Glc-6-P) on illumination are all due to the phosphorylation of enzyme (Parvathi 1998;

Parvathi et al., 2000a). But the changes in V_{max} or K_i (malate) induced by warm temperature were due to a process other than phosphorylation, possibly changes in the oligomerization or conformation of the enzyme (Chinthapalli et al., 2003). The effects of Pi as recorded in present work are quite interesting as the presence of Pi caused an increase V_{max} or K_i (malate) but had no significant effect on K_m (PEP) or K_a (Glc-6-P). We speculate that the effects of Pi on the kinetic properties and stability of PEPC are due to a shifting of the dimer/tetramer equilibrium towards the tetramer. Further experiments are necessary to test this hypothesis.

Our results establish that cytoplasmic Pi is an important factor in regulating PEPC of C_4 plants such as, *A. hypochondriacus*. Further, Pi could be used as a tool to analyze the properties of C_4 PEPC.

Major conclusions from the results presented in this chapter are:

1. Pi caused marked increase in PEPC activity and decrease in malate sensitivity, during enzyme assay or feeding to leaves in a conc. dependent manner.
2. PEPC was purified from leaves of *A. hypochondriacus* with a specific activity of 31 units mg^{-1} protein.
3. Pi not only modulated the PEPC activity in leaf extracts but also in purified protein.
4. Pi is important for PEPC *in vivo* as sequestering by mannose decreased the PEPC activity as well as phosphorylation status.
5. Marked changes in kinetic and regulatory properties of PEPC by Pi are independent of phosphorylation.

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Chapter 8

Marked Conformational Changes in Purified PEPC by Phosphate: Studies on Fluorescence and CD sepectra

Chapter 8

Marked Conformational Changes in Purified PEPC by Pi: Studies on Fluorescence and CD sepectra

Phosphoenolpyruvate carboxylase ((PEPC, EC 4.1.1.31), a key enzyme involved in primary carbon fixation of C_4 plants (Vidal and Chollet, 1997), is regulated strongly by factors, such as light, temperature or inorganic nutrients. The activity of PEPC in leaves of C_4 plants is enhanced by light and such light activation is mostly due to the phosphorylation of the enzyme (Vidal and Chollet, 1997; Parvathi et al., 2000a). In contrast, the changes in PEPC induced by temperature and Pi appears to be due to conformational changes in the protein (Chinthapalli et al., 2003; Murmu et al., 2003).

PEPC has an average molecular mass of 400-kD and is a homotetramer (Andreo et al., 1987; Chollet et al., 1996). The enzyme exists in monomer, dimer and tetramer depending on protein concentration or regulating factor. The stability of PEPC is strongly associated with the maintenance of tetrameric structure. The monomeric or dimeric structures tend to lose enzyme activity and are susceptible to inhibition by malate (Willeford et al., 1990; Willeford and Wedding, 1992; Jensen et al., 1995). The presence of PEP, Mg^{2+}, Glc-6-P, malate or compatible solutes like PEG-6000 or glycerol can promote the aggregation of the PEPC (Podestá and Andreo, 1989; Manetas 1990; Wedding et al., 1994; Chinthapalli et al., 2003).

Compared to the extensive literature on posttranslational modification of enzyme in C_4 PEPC, studies on conformational changes other than phosphorylation and oligomerization of PEPC are limited (Wu and Wedding,

1987; Chinthapalli et al., 2003). Conformational changes of the protein can be studied using different approaches like monitoring intrinsic, extrinsic fluorescence or circular dichroism (CD) spectra. Changes in the intrinsic fluorescence as indication of conformational changes have been demonstrated in PEPC from *Rhothermus obamensis* (Takai et al., 1997), invertase of yeast (Cavaille and Combes, 1995), thermostable D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from the *Thermotoga maritime* (Wrba et al., 1990) and in soyabean peroxidase (Kamal and Behere, 2002). Conformational changes can be studied by also studying the extrinsic fluorescence of dyes, which bind to the proteins. For e.g., fluorescent probes such as ANS (1-anilinonaphthalene-8-sulfonic acid), bis-ANS [1,1'-Bis (4-anilino-5 naphthalenesulfonic acid)] or Nile Red (Greenspan et al., 1985; Shi et al., 1994), are widely used to probe the hydrophobic surfaces of proteins.

CD spectroscopy measures the difference in absorption of left- and right-circularly polarized light as it passes through optically active or chiral samples. Spectra in the far UV-CD wavelength range (about 190 nm to 250 nm) can provide information on the polypeptide conformation of protein (Wallace and Janes, 2001).

Our previous study showed that modulation of PEPC by Pi is independent of phosphorylation (Murmu et al., 2003). The present study is an attempt to characterize the conformational changes, if any, of PEPC from C_4 plant, *A. hypochondriacus* with varying Pi. The conformational changes were monitored by recording either intrinsic fluorescence or extrinsic fluorescence with ANS binding. Further, the conformational changes of PEPC protein of *A. hypochondriacus*, visualized through CD-spectra. A comparative study of Pi

effects with PEG (aggregating agent/stabilizer) and urea (disaggregant /denaturant) were made.

Results

Intrinsic fluorescence

The intrinsic fluorescence of PEPC exhibited a peak at 335 nm. The intrinsic fluorescence of PEPC decreased significantly with increased Pi concentration (Fig 8.1A). Table1 represents the quantitative increase in intrinsic emission fluorescence of PEPC with Pi treatment. PEG-6000 a well known protein stabilizer and promote oligomerization of PEPC (Huber and Sugiyama, 1986; Chinthapalli et al., 2003) and urea is a well known protein denaturant and dissociates proteins (Encinas et al., 2002). Attempts were made, therefore to study the fluorescence in presence of PEG-6000 and urea. The presence of PEG-6000 increased the intrinsic fluorescence of PEPC and in contrast, the intrinsic fluorescence emission of PEPC was markedly reduced up on treatment with urea (Fig 8.1B). The quantitative increase in intrinsic emission fluorescence of PEPC with respect to varying Pi is represented in table 8.1.

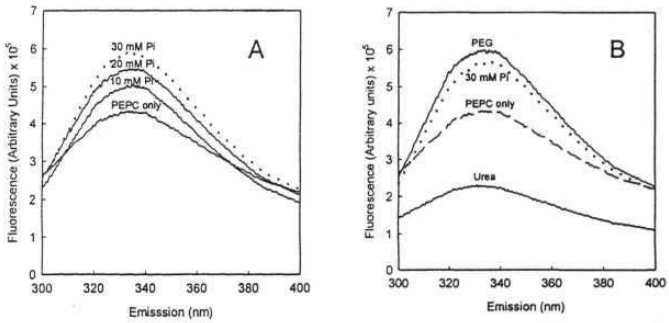

Figure 8.1: Effect of Pi on the intrinsic fluorescence (A) or the comparative effect of with PEG-6000 or urea (B) of PEPC purified from *A. hypochondriacus* leaves. PEPC protein (10 µg/ml) was incubated with varying concentration of Pi for 10 min or PEG-6000 (1.25%) and 3 M urea for 3 h. Excitation of protein was done at 280 nm.

Table 8.1: Effect of Pi on the intrinsic fluorescence of purified PEPC from *A. hypochondriacus* leaves. PEPC protein (10 µg/ml) was incubated with varying concentration of Pi for 10 min, PEG or urea for 3 h. Excitation of protein was done at 280 nm and the intrinsic fluorescence was measured between 300-400 nm. The values in parenthesis are % of control.

Effectors	Intrinsic fluorescence at 354 nm (Arbitrary units)
None (Control)	4.31 (100)
10 mM Pi	4.95 (115)
20 mM Pi	5.43 (126)
30 mM Pi	5.85 (136)
1.25% PEG-6000	5.92 (137)
3 M urea	2.3 (53)

Extrinsic fluorescence

ANS (1-anilinonaphthalene-8-sulfonic acid) is a dye, particularly binds to the hydrophobic regions of the proteins and increases their fluorescence emission (Takai et al., 1997). In the absence of any protein, ANS exhibits a weak fluorescence with the maximum emission at 524 nm. When the dye binds to a protein, the fluorescence of ANS increases, and the peak of emission shifting to 462 nm. Treatment of varying concentration of Pi with PEPC increased the ANS emission peak and a slight shift in the peak of emission from 454 nm to 458 nm has been observed (Fig 8.2A). The effects of Pi were compared with the effect of PEG-6000 and urea. Inclusion of 1.25% PEG-6000 decreased the extrinsic fluorescence of PEPC, while urea increased the extrinsic fluorescence (Fig 8.2B). The quantitative increase in extrinsic emission fluorescence of PEPC with respect to varying Pi is represented in table 8.2.

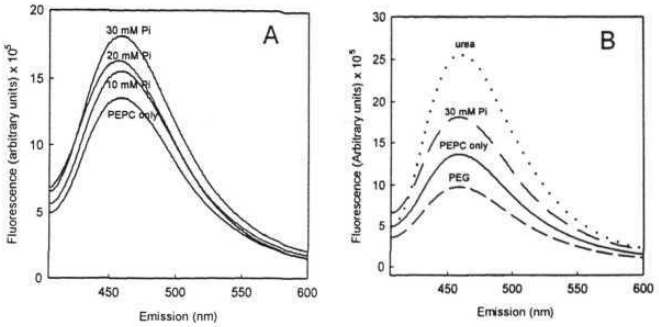

Figure 8.2: Effect of Pi on the extrinsic fluorescence (A) or the comparative with PEG-6000 and urea (B) of PEPC purified from *A. hypochondriacus* leaves. PEPC protein (10 μg/ml) was incubated with varying concentration of Pi for 10 min or 1.25% PEG-6000 or 3M urea for 3 h in presence of 1 mM ANS. Excitation of protein was done at 350 nm.

Table 8.2: Effect of Pi on the extrinsic fluorescence of purified PEPC from *A. hypochondriacus* leaves. PEPC protein (10 μg/ml) was incubated with varying concentration of Pi for 10 min, PEG or urea for 3 h. Excitation of protein was done at 340 nm and the extrinsic fluorescence was measured between 400-600 nm. The values in parenthesis are % of control.

Effectors	Extrinsic fluorescence at 562 nm (Arbitrary units)
None (Control)	13.62 (100)
10 mM Pi	15.57 (114)
20 mM Pi	16.36 (120)
30 mM Pi	18.13 (133)
1.25% PEG-6000	9.78 (72)
3 M urea	25.58 (188)

CD spectra

The far UV-CD spectrum of PEPC showed a broad negative band from about 210 to 220 nm, which is characteristic of a protein having α-helices. The mean residue negative ellipticity of PEPC decreased markedly as the varying concentration of Pi was increased during incubation (Fig 8.3A). The effect was significant by 30 mM Pi treatment. There was increase in % of α-helices

content with varying Pi while, decrease in % of β-sheet and random coil was observed (Table 8.3).

Comparative effects of PEG-6000 or urea

As PEG-6000 is well-known protein stabilizer and promotes oligomerization of PEPC (Huber and Sugiyama, 1986; Chinthapalli et al., 2003). We have compared the effect of Pi with PEG-6000 or urea (protein denaturant, that dissociates proteins) (Encinas et al., 2002). There was increase in the negative ellipticity of PEPC protein at 222 nm with 1.25% (w/v) PEG-6000. On the other hand, the mean residue negative ellipticity of PEPC decreased markedly in presence of 3 M urea, with a maximum loss of secondary structure of PEPC protein (Fig 8.3B). There was increase in % of α-helix with PEG-6000 and decrease in % of random coil. However, treatment with urea decreased % of α-helix with decrease in % of β-sheet or random coil.

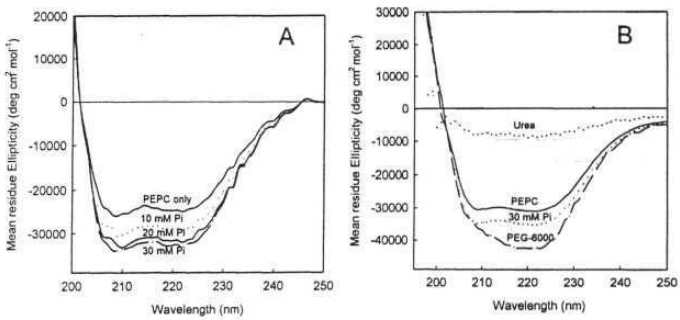

Figure 8.3: CD spectra of PEPC (50μg/ml) purified from *A. hypochondriacus*, with respect to varying concentration of Pi (A) or the comparative effect of 30 mM Pi, 1.25% (w/v) PEG-6000 and 3 M urea (B).

Table 8.3: Secondary structure content of PEPC protein purified from *A. hypochondriacus* with respect to inclusion of varying concentration of Pi, PEG-6000 or urea. The estimated % of α-helix, β-sheet and random coil from CD curves are represented. The details of measurement and calculations are described in "Materials and Methods".

Effectors	α-helix	β-sheet	Random coil
	Percentage %		
None	64	2.7	33.4
10 mM Pi	71.7	2.1	26.2
20 mM Pi	78.4	2.0	19.6
30 mM Pi	84.7	2.3	13.0
1.25 % PEG-6000	85.6	8.4	6.0
3% urea	14.6	52.8	32.6

Discussion

Fluorescence is a useful tool for monitoring the conformational changes in protein molecules. Proteins exhibit significant intrinsic fluorescence, due to presence of aromatic amino acids: tryptophan, tyrosine and phenylalanine. The fluorescence of tryptophan is influenced by several factors such as pH, temperature and the solvent. Tryptophan in non-polar solvent exhibits maximum emission at 320 nm and in aqueous environment at 355 nm. The fluorescence intensity decreases on protonation of tryptophan and is also quenched by neighboring acidic groups as well as high temperature (Lakowicz 1983).

The PEPC protein from *A. hypochondriacus* exhibited high intrinsic fluorescence, peak at 335 nm suggests that most of the protein's intrinsic fluorescence is possibly due to tryptophan residues. Up on Pi treatment there was significant increase in the intrinsic as well as the extrinsic fluorescence of PEPC protein. These changes in fluorescence suggest that possibly the Pi is

stabilizing protein in to active form. PEG-6000 is known to promote the aggregation of several enzymes, including PEPC, pyruvate kinase and fructose-1, 6-bisphosphatase (Huber and Sugiyama, 1986; Podestá and Plaxton, 1993; Hodgson and Plaxton, 1995; Chinthapalli et al., 2003). While urea, at concentration of 3 M or higher dissociates the proteins effectively (Encinas et al., 1998; 2002). Thus, the increase in intrinsic fluorescence by PEG can be taken as an indication of aggregation of the protein. In contrast, the decrease in intrinsic fluorescence in presence of urea suggests the dissociation/denaturation of the enzyme.

In presence of ANS, the fluorescence peaked at 462 nm, indicating binding of the probe to PEPC protein. Increase in extrinsic fluorescence up on Pi treatment suggests an increase in the binding of dye due to exposure of hydrophobic regions to the surface due to aggregation of protein structure. Possibly Pi stabilizes PEPC in partially or completely unfolded protein. Gupta et al. (2003) demonstrated that 2,2,2-trifluoroethanol (which increases α-helical conformation in protein) caused an increase in extrinsic ANS fluorescence with stem bromelain. PEG promotes the aggregation of protein and decreases the extrinsic fluorescence of castor oil seed fructose-1, 6-bisphosphatase (Hodgson and Plaxton, 1995). In contrast, denaturation of PEPC with 3 M urea caused the increase in fluorescence emission intensity. Similar changes were obtained in presence of urea-induced unfolding of tetrameric PEP carboxykinase from *E. coli* and *S. cerevisiae* (Encinas et al., 1998; 2002).

Far UV circular dichroism reflects the secondary structure of a protein and is hence used frequently as a tool to monitor protein conformation. It was

therefore undertaken to study the CD-spectra of PEPC with respect to Pi treatment. The CD-spectra of C_4 PEPC protein from *A. hypochondriacus* shows characteristic dual peak at 208 and 222 nm (Fig 8.1) suggesting that the major part of protein is in α-helical form.

Quantitative analysis of the CD spectra revealed that the PEPC of *A. hypochondriacus* too contained significant proportion of α-helices, while the β-sheets were <10% (Table 8.3). The use of CD-spectra has revealed interesting changes in several proteins with other factors such as temperature (Nölting et al., 1997; Kamal and Behere, 2002; Nakamura et al., 2002). Up on increasing the Pi concentration there was marked increase in negative ellipticity of CD spectrum of PEPC from *A. hypochondriacus* indicating the decrease of α-helicity and stabilization of PEPC protein with Pi.

PEG-6000 and urea are known to induce aggregation and dissociation of proteins: for e.g., barstar and yeast PEP carboxykinase (Nölting et al., 1997; Encinas et al., 2002). The CD-spectra of PEPC showed an increase in negative ellipticity and dual peak nature, in presence of PEG-6000 suggesting that PEG induced the protein to attain a high α-helical conformation. In contrast, 3 M urea caused a marked decrease in the α-helicity and increased % of random coil of PEPC, indicating a complete denaturation of secondary structure of the protein.

The present study emphasizes that the PEPC protein undergoes marked stabilization/conformational change in its secondary structure in presence of Pi. A comparison of the effects of Pi with those of PEG-6000 or urea, suggests that Pi stabilizes PEPC protein by promoting the aggregation of protein, along with an increase in α-helicity.

Major conclusions from the results presented in this chapter are:

1. The increase in intrinsic fluorescence of PEPC protein with Pi treatment provides conformational changes in protein.
2. Comparative effect of PEG-6000 (protein stabilizer) or urea (protein denaturant) with that of Pi, on intrinsic fluorescence of PEPC indicates stabilization of purified PEPC.
3. The increase in extrinsic fluorescence of PEPC protein with Pi treatment suggests increase in the binding of ANS dye to protein, possibly due to formation of stable secondary structure in protein.
4. With increasing concentration of Pi, the negative ellipticity of PEPC protein increased, suggesting stabilization of PEPC protein with Pi.
5. PEG-6000 (protein aggregant/stabilizer) increased the % of α-helicity, however 3 M urea decreased the % of α-helicity, suggesting stabilization of PEPC protein by PEG and disruption of secondary structure of the protein by urea.
6. In a similar way, Pi increased the % of α-helicity in PEPC protein suggesting stabilization/conformational changes in PEPC protein.

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Chapter 9

General Discussion

Chapter 9

General Discussion

Several photosynthetic enzymes are activated on illumination. This phenomenon of light activation has been reported in case of enzymes related to not only Calvin cycle such as Rubisco, glyceraldehyde 3-phosphate dehydrogenase, ribulose 5-phosphate kinase, fructose-1,6-bisphosphatase (Vivekanandan and Saralabai, 1997), but also chloroplasts of C_4 plants e.g. PPDK or NADP-MDH (Edwards et al., 1985; Vivekanandan and Saralabai, 1997).

Being located in chloroplasts of bundle sheath cell, it is quite likely that light may modulate the activity/properties of NADP-ME in C_4 plants. The present work is an effort to reexamine the pattern and characteristics of light activation of NADP-ME in leaf discs of *Zea mays*. A comparative study was made of kinetic and regulatory properties of NADP-ME in extracts made from dark-adapted or illuminated leaf discs. On illumination, there was a small but consistent increase (about 30% over dark-control) in the activity of NADP-ME (Fig 4.1 and 4.2). Besides the marginal increase in the activity, the illuminated form of NADP-ME exhibited marked changes in its regulatory properties (Table 4.2). The light induced increase in the NADP-ME activity was greater when the illumination medium contained 2 mM (or higher) bicarbonate than that in the absence. In contrast, the changes in NADP-ME appear to be due to the reduction of dithiols, as the incubation with DTE dampened the light-activation, by enhancing the activity of enzyme even in dark. The present results suggested that NADP-ME do get light activated to limited extent with marked changes in kinetic and regulatory properties.

Since the modulation of NADP-ME up on illumination was only marginal, subsequent work was focused on the regulation of phosphoenolpyruvate carboxylase (PEPC) from *A. hypochondriacus*, particular emphasis to nutritional factors. Among the nutrients nitrogen source, calcium and phosphate are essential for plant as well as PEPC enzyme.

There are contradictory reports on the modulation of PEPC by Ca^{2+}. Some reports described Ca^{2+}-independent PEPC-protein kinase (PEPC-PK) and thus a non-involvement of Ca^{2+} in regulation of PEPC (Carter et al., 1991; Jiao and Chollet, 1991; Bakrim et al., 1992; Wang and Chollet, 1993). On the other hand, some authors reported that PEPC phosphorylation was mediated by Ca^{2+}-dependent PEPC-protein kinase (Echevarria et al., 1988; Bakrim et al., 1992; Ogawa and Izui, 1992; Ogawa et al., 1992; Pierre et al., 1992).

In the present study, the role of Ca^{2+} on PEPC in leaves of *A. hypochondriacus*, a C_4 plant, was reevaluated. Attempts were made to modulate PEPC *in vivo* by feeding leaves with either Ca^{2+} (EGTA-buffered) or EGTA (Ca^{2+} chelator). Properties of PEPC were checked from dark-adapted or illuminated leaves for comparison. Experiments were extended to study the pattern of PEPC phosphorylation in leaves with or without Ca^{2+} pretreatment of leaves *in vivo*.

During enzyme assay lower concentration of Ca^{2+} increased PEPC activity (>30%) and decreased inhibition by malate in dark adapted leaves rather up on illumination (Fig 5.1). There was almost 3-fold modulation of light activation of PEPC upon Ca^{2+} feeding to leaves (Fig 5.2 and 5.3). Further, EGTA feeding to leaves decreased the light activation of PEPC as well as phosphorylation of PEPC. The increase in PEPC activity and that of decrease

in the sensitivity of enzyme to L-malate and finally pronounced phosphorylation of PEPC protein in Ca^{2+} fed leaves, all suggest that the Ca^{2+}-dependent protein kinase is involved during light mediated phosphorylation of PEPC in leaves of *A. hypochondriacus*. Obviously the effect of Ca^{2+} is pronounced *in vivo* and is not discernible in experiments done *in vitro*.

Our result suggests Ca^{2+} is essential for the light activation of PEPC, through a significant modulation of PEPC phosphorylation, obviously through a Ca^{2+}-dependent protein kinase. Such Ca^{2+}-dependent protein kinase may well be operating at up stream of PEPC-protein kinase, as suggested by earlier reports (Parvathi et al., 2000a; Giglioli-Guivarc'h et al., 1996).

Nitrogen source has profound influence on *de novo* synthesis of PEPC and the phosphorylation of protein (Murchie et al., 2000). NH_4^+ stimulates PEPC activity both *in vitro* and *in vivo*: by promoting the biosynthesis of PEPC protein *in vivo* (Sugiyama and Sakakibara, 2002) and stimulating the enzyme activity *in vitro* (Gayathri and Raghavendra, 1994). Reports on the effects of NO_3^- on PEPC in leaves of C_3 and C_4 plants, are contradictory. *In vivo* feeding of NO_3^- to N-starved leaves enhanced the light-activation of PEPC from the C_3 and C_4 species, e.g., wheat (Van Quy et al., 1991; Van Quy and Champigny, 1992; Duff and Chollet, 1995; Li et al., 1996) and maize (Gupta et al., 1994). However, Gupta et al. (1994) reported that feeding of NO_3^- to NO_3^- starved plants had no effect on light-activation of PEPC in wheat. Therefore, it is necessary to reevaluate the short-term *in vivo* effects of NO_3^- on the activity and light-activation of PEPC in leaves. In contrast to several reports on NO_3^- effect on PEPC, studies on the effect of NH_4^+ are very few.

The present study is undertaken to examine the role of nitrogen, particularly NH_4^+ and NO_3^- on PEPC from *A. hypochondriacus in vitro* as well as *in vivo*. Effect of NH_4^+ or NO_3^- was assessed *in vitro* during enzyme assay and *in vivo* by feeding leaves with NH_4Cl or KNO_3. Properties of PEPC were checked from dark-adapted or illuminated leaves for comparison. Experiments were extended to study the pattern of PEPC phosphorylation in the presence of NH_4^+ or NO_3^-.

Nitrate had no effect of PEPC *in vitro* during enzyme assay and had significant effect *in vivo* up on feeding (Fig 6.5 and 6.6). There was almost 2.5-fold increase in light activation of PEPC up on NO_3^- feeding. NH_4^+ during enzyme assay at lower concentration increased the PEPC activity and decreased the sensitivity of enzyme to malate. Up on feeding to leaves there was 3-fold increase in light activation of PEPC (Fig 6.8) and this was possibly due to the enhanced phosphorylation status of PEPC (Fig 6.9). Our results demonstrate the marked modulation of PEPC by N-source *in vitro* as well as *in vivo*. The modulation by NH_4^+ was strong both *in vitro* as well as *in vivo*, while the effect of NO_3^- was discernible only *in vivo*. Our results also suggest that NO_3^- or NH_4^+ regulates PEPC of *A. hypochondriacus*, in two ways. In short-term incubation, i.e., during enzyme assay NH_4^+ causes allosteric activation of PEPC. In a long term effect, i.e., feeding, there is a marked increase in phosphorylation level, due to the biosynthesis of PEPC and/or PEPC-PK.

Phosphate also plays an important role in plant metabolism: not only as the substrate for oxidative and photophosphorylation but also as a key component of several metabolic processes (Theodoron and Plaxton, 1993).

Pi modulates the activity of several enzymes such as, fructose and sedoheptulose bisphosphatases, ribulose-1,5-bisphosphate carboxylase and sucrose phosphate synthase (Iglesias et al., 1993; Rao 1997). The role of Pi in C_3 photosynthesis is studied often (Cséke and Buchanan, 1986), but studies on the role of Pi in C_4 photosynthesis are very few.

The concentration of Pi in mesophyll cells of plants is expected to rise up to 20 mM (Mimura et al., 1998) and can be important stabilizing factor of PEPC. The effect of Pi on PEPC is interesting and need attention, as Pi is an end product of PEPC reaction. Further, there have been contradictory reports in the literature on the effects of Pi on the activity of C_4 PEPC: inhibition of enzyme activity (O' Leary 1982; Doncaster and Leegood, 1987), marginal or marked activation (Podesta et al., 1990; González et al., 1987) or no effect (Wong and Davies, 1973).

The present work aims at studying in detail the effect of Pi on the properties of PEPC from *A. hypochondriacus*. Experiments were conducted at different levels. Pi effects were analyzed *in vitro* by its addition during assay, using leaf extracts or purified PEPC, and *in vivo* by measuring the effect of feeding Pi to intact leaves. Initially, the effect of Pi on the kinetic and regulatory properties of PEPC was studied in leaf extracts and with purified protein.

Inclusion of Pi during enzyme assay not only increased PEPC activity but also decreased the malate sensitivity of the enzyme from *A. hypochondriacus* (Fig 7.1). When the leaves were fed with Pi, the activity of PEPC increased, while the extent of malate inhibition decreased (Fig 7.8). These results suggest that the changes in Pi levels in the leaves would lead to marked modulation *in vivo* of PEPC activity and its malate sensitivity.

Attempts were made to decrease the level of Pi by feeding mannose (Pi sequester) to leaves. Mannose feeding in dark has not only decreased the PEPC activity but has also markedly suppressed the light activation of PEPC (Fig 7.9). In a similar study, Van Quy and Champigny (1992) found that mannose feeding had inhibited kinase activity and restricted light activation of PEPC in wheat leaves. The phenomenon of the increase in PEPC activity along with decrease in malate sensitivity due to Pi is quite similar to the effects of light on PEPC in C_4 leaves.

The phosphorylation status of PEPC was examined, with or without pretreatment with Pi and after illumination of leaves (Fig 7.10). There was no significant increase in phosphorylation of PEPC due to presence of Pi. However, the phosphorylation of PEPC was suppressed in the presence of mannose, particularly in illuminated leaves. As the above results suggest that modulation of Pi on PEPC is independent of phosphorylation status of PEPC, in a pattern very similar to the modulation of PEPC by temperature (Chinthapalli et al., 2003). Thus, the next part of work was undertaken to see the conformational changes if any in purified PEPC protein.

PEPC has an average molecular mass of 400-kD and is a homotetramer (Andreo et al., 1987; Chollet et al., 1996). The enzyme exists in monomer, dimer and tetramer depending on protein concentration or regulating factor. The stability of PEPC is strongly associated with the maintenance of tetrameric structure. The monomeric or dimeric structures tend to lose enzyme activity and are susceptible to inhibition by malate (Willeford et al., 1990; Willeford and Wedding, 1992; Jensen et al., 1995). The presence of PEP, Mg^{2+}, Glc-6-P, malate or compatible solutes like PEG-6000 or glycerol can promote the

aggregation of the PEPC (Podestá and Andreo, 1989; Manetas 1990; Wedding et al., 1994; Chinthapalli et al., 2003).

Our previous study showed that modulation of PEPC by Pi is independent of phosphorylation (Murmu et al., 2003). The conformational changes of purified PEPC from C_4 plant, *A. hypochondriacus* were monitored in presence of varying Pi. The conformational changes were monitored by recording either intrinsic fluorescence or extrinsic fluorescence with ANS binding. A comparative study of Pi effects with PEG (aggregating agent/stabilizer) and urea (disaggregant/denaturant) were made.

The PEPC protein from *A. hypochondriacus* exhibited high intrinsic fluorescence, peak at 335 nm suggests that most of the protein's intrinsic fluorescence is possibly due to tryptophan residues. Up on Pi treatment there was significant increase in the intrinsic as well as the extrinsic fluorescence of PEPC protein. These changes in fluorescence suggest that possibly the Pi is stabilizing protein in to active form. PEG-6000 is known to promote the aggregation of several enzymes, including PEPC, pyruvate kinase and fructose-1, 6-bisphosphatase (Huber and Sugiyama, 1986; Podestá and Plaxton, 1993; Hodgson and Plaxton, 1995; Chinthapalli et al., 2003). While urea, at concentration of 3 M or higher dissociates the proteins effectively (Encinas et al., 1998; 2002). Thus, the increase in intrinsic fluorescence by PEG can be taken as an indication of aggregation of the protein. In contrast, the decrease in intrinsic fluorescence in presence of urea suggests the dissociation/denaturation of the enzyme.

PEG promotes the aggregation of protein and decreases the extrinsic fluorescence of castor oil seed fructose-1, 6-bisphosphatase (Hodgson and

Plaxton, 1995). In contrast, denaturation of PEPC with 3 M urea caused the increase in fluorescence emission intensity. Similar changes were obtained in presence of urea-induced unfolding of tetrameric PEP carboxykinase from *E. coli* and *S. cerevisiae* (Encinas et al., 1998; 2002).

Far UV circular dichroism reflects the secondary structure of a protein and is hence used frequently as a tool to monitor protein conformation. It was therefore undertaken to study the CD-spectra of PEPC with respect to Pi treatment. The CD-spectra of C_4 PEPC protein from *A. hypochondriacus* shows characteristic dual peak at 208 and 222 nm (Fig 8.3) suggesting that the major part of protein is in α-helical form. Quantitative analysis of the CD spectra revealed that the PEPC of *A. hypochondriacus* too contained significant proportion of α-helices, while the β-sheets were <10% (Table 8.3). The use of CD-spectra has revealed interesting changes in several proteins with other factors such as temperature (Nölting et al., 1997; Kamal and Behere, 2002; Nakamura et al., 2002). Up on increasing the Pi concentration there was marked increase in negative ellipticity of CD spectrum of PEPC from *A. hypochondriacus* indicating the decrease of α-helicity and stabilization of PEPC protein with Pi.

PEG-6000 and urea are known to induce aggregation and dissociation of proteins: for e.g., barstar and yeast PEP carboxykinase (Nölting et al., 1997; Encinas et al., 2002). The CD-spectra of PEPC exhibited an increase in negative ellipticity and dual peak nature, in presence of PEG-6000 suggesting that PEG induced the protein to attain a high α-helical conformation. In contrast, 3 M urea caused a marked decrease in the α-helicity and increased

% of random coil of PEPC, indicating a complete denaturation of secondary structure of the protein.

The present study establishes that the PEPC protein undergoes marked stabilization/conformational change in its secondary structure in presence of Pi. A comparison of the effects of Pi with those of PEG-6000 or urea, suggests that Pi stabilizes PEPC protein by promoting the aggregation of protein, along with an increase in α-helicity. The objectives and major conclusions of the preset study are summarized in the next chapter.

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Chapter 10

Summary and Conclusions

Chapter 10

Summary and Conclusions

The NADP malic enzyme (NADP-ME; EC 1.1.1.40) and phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) are among the key enzymes of C_4 photosynthesis. NADP-ME catalyzes the oxidative decarboxylation of L-malate to yield pyruvate, CO_2 and NADPH (Edwards and Andreo, 1992). NADP-ME is localized in the chloroplasts of bundle sheath cells for decarboxylation of malate in C_4 species (Maurino et al., 1997). Being an important enzyme, the regulation of NADP-ME has been studied in detail, particularly in C_4 plants.

The levels of NADP-ME increase during the greening of etiolated seedlings of C_4 plants (Maurino et al., 1996). The marked fold increase in activity of NADP-ME during greening is mainly due to light stimulated synthesis of protein. To our knowledge, there has been only one attempt to examine the short-term modulation by light of NADP-ME in C_4 plants. Drincovich and Andreo (1994) demonstrated that the activity of NADP-ME in crude extracts increased by 50%, when the extracts were illuminated for a long time of 10 h. This observation is rather intriguing. Being located in chloroplasts of bundle sheath, it is quite likely that light may regulate the activity/properties of NADP-ME in C_4 plants. A comparative study was made of kinetic and regulatory properties of NADP-ME in extracts made from dark-adapted or illuminated leaf discs.

Upon illumination, the V_{max} of NADP-ME increased by about 30% in presence of optimal concentration of bicarbonate in medium. The extent of light activation was similar either at 4 mM (saturating) or 0.01 mM (limiting)

substrate concentration. The light-induced change seems to be due to partially the reduction of dithiols, as incubation of leaf extracts with DTE dampened the extent of light activation of NADP-ME. Our results suggest that properties of NADP-ME do change on illumination. Although there was only a marginal increase in the activity of enzyme on illumination of leaf discs, the changes in regulatory properties of NADP-ME were marked. Since the modulation by light of NADP-ME was not as pronounced as we expected, the subsequent work was focused on PEPC from *A. hypochondriacus*.

The enzyme PEPC is involved in primary carbon fixation of C_4 plants (Vidal and Chollet, 1997). The enzyme is regulated strongly by factors, such as light, temperature or inorganic nutrients. On illumination, the activity of PEPC in leaves of C_4 plants is enhanced by 2-3 fold along with a marked decrease in the malate sensitivity of the enzyme (Parvathi et al., 2000). These changes during the light activation are mostly due to the phosphorylation of the enzyme (Vidal and Chollet, 1997). In contrast, the changes in PEPC induced by temperature appear to be due to conformational changes in the protein (Chinthapalli et al., 2003). There has been tremendous progress in understanding the biochemistry and molecular biology of C_4 PEPC, for e.g cloning and expression of native and recombinant PEPC in *E. coli*; characterization of PEPC phosphorylation and PEPC-PK; evolutionary tendency of PEPC based on amino acid sequence and nucleotide sequence alignment, crystallization and three dimensional studies of PEPC (Vidal et al., 1997; Nimmo 2003; Westhoff and Gowik, 2004; Izui et al., 2004).

Most of the work on regulation of PEPC deals with mostly light and temperature and to a limited extent with nitrogen. The present work attempts to study the regulation of PEPC by selected nutritional factors. Experiments were designed to modulate of PEPC by Ca^{2+}, NH_4^+, NO_3^- and Pi and kinetic and regulatory properties of PEPC were studied. Further, the modulations of PEPC by these factors were studied in vitro as well as *in vivo*. Studies were than extended to see the phosphorylation levels or conformational changes in purified PEPC in case of Pi.

Calcium plays a key role as a secondary messenger in a variety of physiological responses and activates signaling cascades involving Ca^{2+}-modulated protein-kinases and protein phosphatases (Bush 1995). The reports on the modulation of PEPC by Ca^{2+} are contradictory. There are reports describing Ca^{2+}-independent PEPC-protein kinase (PEPC-PK) in regulation of PEPC (Vidal et al., 2002). On the other hand, some authors reported that PEPC phosphorylation was mediated by Ca^{2+}-dependent PEPC-PK (Vidal et al., 2002).

C_4 plants maintain soluble Ca^{2+} at low levels in their leaves and therefore appear to be calciphobes (Gavalas and Manrtas, 1980). The effects of Ca^{2+} on the activity of PEPC in leaves were assessed. Further, the Ca^{2+} levels in leaves were modulated by feeding $CaCl_2$ or Ca^{2+}-EGTA through petiole to leaves. During enzyme assay, Ca^{2+} (at μM ranges) increased the PEPC activity in dark adapted leaf extracts and decreased the inhibition by malate marginally. Upon feeding low concentration of Ca^{2+} to leaves, light activation of PEPC increased dramatically and up on feeding EGTA (a Ca^{2+} chelator), there was restriction of light activation. The level of PEPC

phosphorylation was much higher in Ca^{2+} fed leaves compared with control but EGTA decreased the light modulated phosphorylation. These results suggest that significant involvement of Ca^{2+}-dependent protein kinase during light activation of PEPC in leaves of *A. hypochondriacus*.

PEPC is located in cytosol of mesophyll cell in leaves of C_4 plants. The reduction of NO_3^- to NH_4^+ also occurs in C_4 mesophyll cells (Hatch 1987). Among the inorganic nutrients, nitrogen has profound influence on PEPC in C_3, C_4 as well as CAM plants, at the level of both *de novo* synthesis of enzyme and the phosphorylation of protein (Murchie et al., 2000). Reports on the effects of feeding NO_3^- on PEPC in leaves of C_3 and C_4 plants, are contradictory. Therefore, it is quite interesting to study the short-term *in vivo* effects of NO_3^- on the activity and light-activation of PEPC from plant species, grown under normal field conditions. NH_4^+ ions promote the biosynthesis of PEPC *in vivo* (Sugiyama and Sakakibara, 2002) and stimulate the PEPC activity *in vitro* (Gayathri and Raghavendra, 1994). Allosteric activation by NH_4^+ of PEPC fits well with intercellular enzymatic distribution. An increase in the availability of NH_4^+ can stimulate PEPC and thus promote carbon and amino acid metabolism.

Inclusion of varying concentration of NH_4^+ during the enzyme assay increased the activity of PEPC in dark-adapted leaf extracts with the maximum response at 40 μM NH_4^+. However, NH_4^+ inhibited the enzyme activity marginally in illuminated leaf extracts. On the other hand, malate sensitivity in dark-adapted leaf extracts decreased by 20% on exposure to NH_4^+ but in illuminated extracts the effect of NH_4Cl was marginal. In an extended study, high concentrations of NH_4^+ (mM ranges) were used, but the

effect was quite similar: activation of PEPC at lower concentrations and inhibition at high concentration.

When fed to leaves (optimum period of 3 h), NH_4^+ increased the PEPC activity upto 50% in dark-adapted leaves but dramatically increased the light activation of the enzyme up to 2.7-fold. The increase in PEPC activity by NH_4^+ feeding was correlated with the decrease in malate sensitivity. When these values were recalculated, the extent of light activation increased significantly from 2.1-fold (No NH_4Cl) to 2.8-fold (with 5 mM NH_4Cl).

In leaf extracts, when added in μM range, NO_3^- decreased marginally PEPC activity and increased malate sensitivity of the enzyme. This effect was quite similar in the extracts of illuminated or dark-adapted leaves. At higher concentrations (mM range) too, NO_3^- had only marginal effect on the PEPC activity and malate sensitivity of PEPC. After feeding NO_3^- to leaves *in vivo*, increased the activity of PEPC in leaf extracts by >50% in case of both dark-adapted or illuminated leaves. As the stimulatory effect of NO_3^- was more in dark-adapted leaves, the extent of light activation decreased marginally, as indicated by L/D ratio.

The kinetic and regulatory properties of PEPC do change with respect to NH_4^+ feeding to leaves. The phosphorylation level of PEPC in NH_4^+ fed leaves was much higher than that of control but in NO_3^- fed leaves effect was marginal. No phosphorylation was detected in dark, either in the control or treated leaf.

Our results suggest that NH_4^+ regulates PEPC of *A. hypochondriacus*, in two ways. In short-term incubation, i.e., during enzyme assay, NH_4^+ causes allosteric activation of PEPC. In a long term effect, i.e., feeding, there is a

marked increase in phosphorylation level, due to the biosynthesis of PEPC and/or PEPC-PK.

Pi plays an important role in plant metabolism: not only as the substrate for oxidative and photophosphorylation but also as a key component of several metabolic processes (Theodoron and Plaxton, 1993). The concentration of Pi in mesophyll cells of plants can rise up to 20 mM (Mimura et al., 1998) and can be important factor. The effect of Pi on PEPC is interesting, as Pi is an end product of PEPC reaction. However, studies on the direct effects of Pi on PEPC are limited and also contradictory. Further, the material used in these experiments also varied from extracts from etiolated or illuminated leaves to purified PEPC, making it difficult for a comparison.

The activity of enzyme increased by >2.5-fold at 15 or 20 mM Pi, while the sensitivity of PEPC to malate decreased from about 90% to about 70% (in presence of 30 mM Pi). Due to marked stimulation of PEPC activity by Pi even in extracts from dark-adapted leaves, the extent of light activation got masked as the Pi concentration was increased in the assay medium. As a result, the L/D ratio of PEPC in leaf extracts decreased. The V_{max} of the purified PEPC increased upon addition of Pi and there was drastic decrease in its malate sensitivity.

The presence of Pi increased V_{max} by >1.8-fold and 1.6 fold whereas K_i (malate) increased by >2.3-fold and >1.9-fold in dark-adapted and light adapted leaves respectively. The K_m (PEP) increased by about >1.5-fold and 1.3-fold respectively. K_a (Glc-6-P) increased by about 1.7-fold and >1.6-fold in dark-adapted and light adapted leaves respectively.

By about 3 h of Pi feeding to leaves, there was >2-fold increase in PEPC activity with decrease in malate sensitivity in dark-adapted leaves while, the effects of Pi were low on PEPC in extracts from illuminated leaves. Feeding of leaves with 10 mM mannose (Pi sequester) decreased the activity of PEPC; the decrease being more pronounced in illuminated leaves than in the dark-adapted ones. There was small effect on the extent of malate inhibition, either in illuminated or dark-adapted leaves.

There was no significant increase in phosphorylation level of PEPC in leaves of *A. hypochondriacus* due to the feeding of 30 mM Pi. However, feeding with mannose decreased light enhanced phosphorylation of PEPC. Thus, the marked modulation of PEPC by Pi was independent of the phosphorylation of PEPC. The extent of PEPC phosphorylation was much higher in illuminated discs of *A. hypochondriacus* than that in dark adapted leaves. However, the Pi modulation of PEPC was independent of phosphorylation. But Pi is seems to be important in vivo as sequestering of Pi by mannose decreased the light induced phosphorylation of PEPC.

Since there was no change in phosphorylation status of PEPC with Pi, we have analysed the conformational changes of purified PEPC. PEPC exists predominately as a tetramer along with dimer or monomeric forms (Walker et al., 1986). PEPC is very active when it is in a tetrameric shape, while its activity and malate sensitivity decreases when the enzyme dissociates into a monomer or dimer (Walker et al., 1986). As cytosolic Pi concentration can be expected to rise 30 mM. Pi would modulate the oligomeric status of the enzyme PEPC. Aggregation state of PEPC is known to influence the stability and properties of PEPC from CAM as well as C_4

plants (Willeford and Wedding, 1992). PEPC, a homotetramer, dissociates in to dimer and or monomer, on dilution of protein solution or on exposure to effector such as malate (Meyer et al., 1991). On the other hand, high concentration of protein, presence of PEG, glycerol, PEP or Glc-6-P can induce aggregation of PEPC (Vidal et al., 2002). PEG is shown to promote the self association and/or activation a number of regulatory enzymes in dilute solutions (Podestá and Plaxton, 1993). Compatible solutes like PEG-6000 can promote the oligomerization of PEPC increase the enzyme activity and decrease its malate sensitivity (Huber and Sugiyama, 1986). Thus, the effects of Pi were compared with the effect of PEG-6000 or urea, a well known denaturant and dissociates proteins (Encinas et al., 2002).

Changes in intrinsic and extrinsic fluorescence of purified PEPC demonstrate the qualitative indication of conformational changes in protein up on Pi treatment. When excited at 280 nm, the intrinsic fluorescence of PEPC protein from *A. hypochondriacus* (C_4) exhibited a maximum at 335 nm. The intrinsic fluorescence of PEPC increased markedly with the increase in concentration of Pi. These results suggest that Pi stabilizes PEPC protein. ANS is a polycyclic aromatic fluorescent probe that interacts with hydrophobic sites on the proteins (Takai et al., 1997). The extrinsic fluorescence of PEPC (due to ANS) increased significantly with incubation with Pi, although the results showed that effect of Pi could be different than that of PEG.

PEG-6000, which causes an aggregation of PEPC, increased the intensity of the fluorescence emission spectra but the Pi induced increase in fluorescence emission was slight lower than the PEG effect. These results established that conformational changes in PEPC, with a shift towards

hydrophobic environment, in presence of PEG-6000 (Lakowicz 1983) so with Pi in the present study. In contrast, denaturation of PEPC with 3 M urea decreased the intrinsic fluorescence emission of PEPC due to unfolding of the protein.

Studies were extended to check the conformational changes in the PEPC protein through fluorescence and circular dichroism (CD) spectra. The CD-spectra of C_4-PEPC of *A. hypochondriacus* at room temperature showed broad negative band, with dual peaks at around 208 nm and 222 nm, suggesting that the major part of the protein was in α-helical conformation. The CD-spectra of PEPC increased with incubation of Pi and % of α-helicity increased. Further, the CD spectra of Pi were compared with PEG-6000 or urea. PEG-6000 further increased the CD spectra of PEPC compared with Pi but urea decreased the negative ellipticity of PEPC. The calculated % of secondary contents of PEPC increased % of α-helicity with PEG but urea decreased the % of α-helicity while, % of β-sheet or random coil increased. Suggesting that Pi induced conformational changes in PEPC.

The following are the conclusions, with references to the five objectives undertaken in present work.

1. Upon illumination, the V_{max} of NADP-ME increased by about 30%. Although small, the increase was consistent and significant. The extent of light activation was similar when substrate (malate) concentration was either 4 mM (saturating) or 0.01 mM (limiting). The light-induced change seems partially due to the reduction of dithiols, as incubation of leaf extracts with DTE dampened the extent of light activation of NADP-ME. We conclude that the properties of NADP-ME do change on illumination.

Although there was only a marginal increase in the activity, the changes in regulatory properties of NADP-ME were marked.2. Inclusion of Ca^{2+} during assay increased the PEPC activity *in vitro* by 30% in dark-adapted leaf extracts but marginally inhibited in illuminated leaf-extracts. Upon feeding 10 μM $CaCl_2$, light activation of PEPC increased by 3-fold in leaves with 24% decrease in malate sensitivity. EGTA (Ca^{2+} chelator) had no effect during enzyme assay but up on feeding to leaves dampened the light activation of PEPC. The phosphorylation level of PEPC was greater in Ca^{2+} fed leaves than that in control. But EGTA decreased the light induced phosphorylation of PEPC.

3. Lower conc. of NH_4^+ during assay increased the PEPC activity in dark-adapted leaf extracts and but marginally inhibited in illuminated extracts. NO_3^- had only a marginal effect on PEPC *in vitro*. NH_4^+ or NO_3^- feeding to leaves dramatically enhanced the light activation of PEPC with decrease in malate sensitivity. The phosphorylation level of PEPC was much higher in NH_4^+ fed leaves than that with NO_3^-. Our results suggest that in short-term incubation, i.e., during enzyme assay, NH_4^+ causes allosteric activation of PEPC. In a long term effect, i.e., feeding, there is a marked increase in phosphorylation level, due to the biosynthesis of PEPC and/or PEPC-PK
4. Presence of Pi caused marked increase in PEPC activity and decrease in malate sensitivity, during enzyme assay or feeding to leaves in a conc. dependent manner. Pi modulated the PEPC activity not only in leaf extracts but also in purified protein. Pi is important for PEPC *in vivo* as sequestering by mannose decreased the PEPC activity as well as

phosphorylation status. Marked changes in kinetic and regulatory properties of PEPC by Pi were independent of phosphorylation.

5. Changes in intrinsic and extrinsic fluorescence of purified PEPC demonstrated the conformational changes in purified PEPC upon Pi treatment. Comparative studies of the effect of Pi with that of PEG (protein stabilizer) or urea (protein denaturant) suggested the stabilization of PEPC with Pi. The increase in α-helicity % of PEPC with Pi treatment from CD spectra suggested the stabilization of PEPC with Pi. The marked increased in negative ellipticity of PEPC with increasing concentration of Pi, indicated significant changes in the secondary structure of protein. The effect of PEG-6000 or urea on the CD spectra of PEPC confirmed that the presence of Pi stabilized PEPC protein.

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Chapter 11

Literature Cited

Literature Cited

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Appendix: Research Papers Published

(First page of the article is attached)

List of Publications in Referred Science Journals

1. **Jhadeswar Murmu,** Bhaskarrao Chinthapalli and Agepati S. Raghavendra (2003) Phosphoenolpyruvate carboxylase from leaves of C_4 plants: Biochemistry and molecular biology of regulation. ***Indian Journal of Plant Physiology*** (*special issue*): 164-173.

2. **Jhadeswar Murmu** and Agepati S. Raghavendra (2003) Biochemistry of Photosynthesis: Recent trends. ***Journal of Plant Biology*** 30:241-252.

3. **Jhadeswar Murmu**, Bhaskarrao Chinthapalli and Agepati S. Raghavendra (2003). Marked modulation by phosphate of phosphoenolpyruvate carboxylase in leaves of *Amaranthus hypochondriacus,* a NAD-ME type C_4 plant: Decrease in malate sensitivity but no change in the phosphorylation status. ***Journal of Experimental Botany*** 54: 2661-2668.

4. Bhaskarrao Chinthapalli, **Jhadeswar Murmu** and Agepati S. Raghavendra (2003) Dramatic difference in the response of phosphoenolpyruvate carboxylase to temperature in leaves of C_3 and C_4 plants ***Journal of Experimental Botany*** 54: 707-714.

5. **Jhadeswar Murmu**, Bhaskarrao Chinthapalli and Agepati S. Raghavendra (2003) Light activation of NADP malic enzyme in leaves of maize: Marginal increase in activity but marked change in regulatory properties of enzyme. ***Journal of Plant Physiology*** 160: 51-56

6. Bhaskarrao Chinthapalli, Nasser Syed, **Jhadeswar Murmu**, and A. S. Raghavendra (2001) Cytosolic pH as a secondary messenger during light activation of phosphoenolpyruvate carboxylase in mesophyll cells of C_4 plants. In: ***Signal Transduction in Plants: Current Advances***. (Eds. S.K. Sopory, R. Oëlmuller and S.C. Maheswari). Kluwer Acdemic Publishers, New York, Pp 39-48

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Paper No. 178

PHOSPHOENOLPYRUVATE CARBOXYLASE FROM LEAVES OF C_4 PLANTS: BIOCHEMISTRY AND MOLECULAR BIOLOGY OF REGULATION

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SUMMARY

Phosphoenolpyruvate carboxylase (PEPC) is the primary carboxylating enzyme in C_4 and crassulacean acid metabolism (CAM) plants. PEPC is regulated by reversible phosphorylation and/or oligimerization, depending on the causal factor. On illumination, in C_4 leaves, the enzyme is phosphorylated by a PEPC-protein kinase (PEPC-PK). It is a matter of debate if the PEPC-PK is Ca^{2+}-dependent or independent. PEPC-PK has been purified and characterized from a range of C_4 and CAM plants and has recently been cloned in *E. coli.* Oligomerization or conformational changes could be important mechanisms of regulation, during modulation by temperature, Pi or certain metabolites. Long-term regulation by nutrition, particularly nitrogen source is quite important and involves increased biosynthesis of PEPC protein and PEPC-PK. Further studies are needed to understand the nature of conformational changes in PEPC and their possible implication in phosphorylation of PEPC.

Key words: **C_4 plant, oligomerization, PEPC, PEPC-protein kinase, phosphorylation, posttranslational regulation**

INTRODUCTION

The primary carbon fixation in C_4 and CAM plants is catalyzed by phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31). Being a key enzyme of C_4 photosynthesis or CAM, the regulation of PEPC has been of considerable interest. Extensive studies have been carried out on C_4 PEPC particularly in the past two decades. The properties and regulation of PEPC are summarized in several reviews (Andreo *et al.* 1987, Rajagopalan *et al.* 1994, Chollet *et al.* 1996, Vidal and Chollet 1997, Nimmo 2000, Chinthapalli *et al.* 2002, Vidal *et al.* 2002, Nimmo 2003). The present article focuses on the regulation of PEPC mainly in C_4 plants, although occasional references are made to CAM or C_3 plants.

PROPERTIES OF PEPC

PEPC, a cytosolic enzyme located in mesophyll cells of C_4 and CAM plants, catalyzes the carboxylation of phosphoenolpyruvate (PEP) to yield oxaloacetate (OAA) and inorganic phosphate (Pi). In the initial phase of reaction, Mg^{2+} binds to PEPC, and when Mg-enzyme complex is at equilibrium, PEP binds onto it followed by HCO_3^- binding. Mg^{2+}, PEP and HCO_3^- have to be present on PEPC before the enzyme reaction begins (Chollet *et al.* 1996). The enzyme acts as both carboxylase and phosphatase. The b-carboxylation of PEP by HCO_3^- is catalyzed by PEPC in a two-step reaction (Andreo *et al.* 1987). *In vitro,* Mn^{2+} can replace Mg^{2+} as a cofactor. The first step involves the reversible, rate limiting formation of carboxyphosphate and the enolate of pyruvate. The second step involves the carboxylation of the enolate with the formation of oxalacetate (OAA) and the release of Pi (Andreo *et al.* 1987).

PEPC is a homotetramer, each subunit having an approximate mass of 110 kDa (Chollet *et al.* 1996). The optimal pH for the activity of PEPC is around 8.0. The activity of PEPC therefore depends on the cytosolic pH (Rajagopalan *et al.* 1993). A serious problem with many of these studies on PEPC is the uncertain state of protein

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REVIEW ARTICLE

Biochemistry of Photosynthesis: Recent Trends

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The process of photosynthesis begins with photochemical reactions, which derive assimilatory power (ATP and NADPH) from solar/radiant energy. The assimilatory power is then used for a series of reactions involving the assimilation of either carbon or nitrogen or sulfur. This review attempts to summarize the important trends, particularly in the last five years, in the photosynthetic carbon metabolism through the C_3 or C_4 or C_2 (photorespiration) or CAM pathways. Dramatic progress has been achieved in genetic manipulation of several C_4 enzymes. Attention is drawn to the marked interaction of photosynthesis with mitochondrial respiration and N-metabolism. Significant work in the area of physiology and biochemistry of photosynthesis has been accomplished in India.

Keywords: Calvin cycle, crassulacean and metabolism, carbon assimilation, mitochondrial respiration, nitrogen metabolism, phosphoenolpyruvate carboxylase, photorespiration, Rubisco.

Introduction

Photosynthesis, the primary source of energy for our world, can be distinguished into two phases. The first phase of photochemical reactions involves the conversion of radiant/solar energy into chemical forms like ATP and NADPH (or reduced ferredoxin) with concomitant evolution of oxygen. In the second biochemical phase, the ATP and NADPH (or reduced ferredoxins) are utilized to reduce carbon dioxide (or NO_2, NH_4 or SO_4) into energy-rich carbon (or nitrogen or sulfur) compounds.

This article is an attempt to highlight the recent trends in the area of photosynthetic carbon assimilation in higher plants. In view of the vast literature and limited space, references are made mostly to reviews and selected literature of the past few years. Readers interested in the subject may consult several books, which deal in detail with different biochemical aspects of photosynthesis (Heldt, 1997; Pessarakli, 1997; Wild and Ball, 1997; Raghavendra, 1998; Hall and Rao, 1999; Yunus *et al.*, 2000; Lawlor, 2001; Blankenship, 2002). These books are all published within the last six years. There is also an excellent series of volumes on photosynthesis, being published by Kluwer Academic Publishers, Dordrecht, The Netherlands. Some of these volumes deal with carbon metabolism in extensive detail (Leegood *et al.*, 2000; Aro and Andersson, 2001; Foyer and Noctor, 2002).

Carbon assimilation by plants and other photosynthetic organisms is a very important event in the global carbon cycle. Plants fix carbon primarily into 3-phosphoglycerate (PGA, a 3-carbon compound) and hence the process is named as C_3 photosynthesis or C_3 pathway or Calvin cycle. The other two variants of photosynthetic carbon assimilation are C_4 photosynthesis (or C_4 pathway) and Crassulacean acid metabolism (CAM). However, the carbon from C_4 acids formed initially during these two pathways has to be refixed ultimately through the C_3- or Calvin cycle. Thus, the C_3 photosynthesis is the basic route of carbon-assimilation while C_4 pathway and CAM function as carbon-concentrating mechanisms and form adjuncts of Calvin cycle. Plants possessing only the Calvin cycle are called C_3 plants, while the other two categories are named C_4 plants and CAM plants.

C_3 photosynthesis is the principal route of carbon fixation in most of the plants, including the lower groups and appears to have evolved much earlier to the CAM or C_4 pathway. Among the ca. 300,000 higher plants on the earth, almost 90% are C_3 plants, while the CAM and C_4 species constitute about 10% and 3% respectively. Most of the crops (particularly cereals, legumes and oilseed crops) are of the C_3 type. Therefore, C_3 plants have attracted the attention of several scientists.

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Journal of Experimental Botany

RESEARCH PAPER

Marked modulation by phosphate of phosphoenolpyruvate carboxylase in leaves of *Amaranthus hypochondriacus*, a NAD-ME type C_4 plant: decrease in malate sensitivity but no change in the phosphorylation status

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Abstract

The effect of Pi on the properties of phosphoenolpyruvate carboxylase (PEPC) from *Amaranthus hypochondriacus*, a NAD-ME type C_4 plant, was studied in leaf extracts as well as with purified protein. Efforts were also made to modulate the Pi status of the leaf by feeding leaves with either Pi or mannose. Inclusion of 30 mM Pi during the assay enhanced the enzyme activity in leaf extracts or of purified protein by >2-fold. The effect of Pi on the enzyme purified from dark-adapted leaves was more pronounced than that from light-adapted ones. The K_i for malate increased >2.3-fold and >1.9-fold by Pi in the enzyme purified from dark-adapted leaves and light-adapted leaves, respectively. Pi also induced an almost 50–60% increase in K_m for PEP or K_a for glucose-6-phosphate. Feeding the leaves with Pi also increased the activity of PEPC in leaf extracts, while decreasing the malate sensitivity of the enzyme. On the other hand, Pi sequestering by mannose marginally decreased the activity, while markedly suppressing the light activation, of PEPC. There was no change in phosphorylation of PEPC in leaves of *A. hypochondriacus* due to the feeding of 30 mM Pi. However, feeding with mannose decreased the light-enhanced phosphorylation of PEPC. The marked decrease in malate sensitivity of PEPC with no change in phosphorylation state indicates that the changes induced by Pi are independent of the phosphorylation of PEPC. It is suggested here that Pi is an important factor in regulating PEPC *in vivo* and could also be used as a tool to analyse the properties of PEPC.

Key words: *Amaranthus hypochondriacus*, glucose-6-phosphate, light activation, malate sensitivity, mannose, PEPC, phosphate, phosphorylation.

Introduction

Phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) is a key enzyme involved in primary carbon fixation of C_4 plants (Andreo *et al.*, 1987; Rajagopalan *et al.*, 1994; Chollet *et al.*, 1996; Vidal and Chollet, 1997; Nimmo, 2000). The enzyme is regulated by factors, such as light, temperature or inorganic nutrients. On illumination, the activity of PEPC in leaves of C_4 plants is enhanced by 2–3-fold along with a marked decrease in the malate sensitivity of the enzyme (Huber and Sugiyama, 1986; Doncaster and Leegood, 1987; Parvathi *et al.*, 2000*a*). These changes during light activation are mostly due to the phosphorylation of the enzyme (Chollet *et al.*, 1996; Vidal and Chollet, 1997; Parvathi *et al.*, 2000*a*).

The C_4 plants are tolerant to heat but quite sensitive to cold temperatures (Phillips and McWilliam, 1971). At low temperature, the activity of PEPC decreased but its malate sensitivity increased in C_4 plants, while at high temperature the activity of the enzyme increased and malate sensitivity decreased. The changes in PEPC induced by temperature appear to be due to conformational changes in the protein (Wu and Wedding, 1987; Chinthapalli *et al.*, 2003).

Among the inorganic nutrients, nitrogen has profound influence on PEPC, at both the levels of *de novo* synthesis of enzyme and the phosphorylation of PEPC in C_3, C_4 as well as in CAM plants. Ammonium ions promote the

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Abbreviations: G-6-P, glucose-6-phosphate; PEPC, phosphoenolpyruvate carboxylase; Pi, phosphate; NAD-MDH, NAD-malate dehydrogenase.

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Journal of Experimental Botany

RESEARCH PAPER

Dramatic difference in the responses of phospho*enol*pyruvate carboxylase to temperature in leaves of C_3 and C_4 plants

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Abstract

Temperature caused phenomenal modulation of phospho*enol*pyruvate carboxylase (PEPC, EC 4.1.1.31) in leaf discs of *Amaranthus hypochondriacus* (NAD-ME type C_4 species), compared to the pattern in *Pisum sativum* (a C_3 plant). The optimal incubation temperature for PEPC in *A. hypochondriacus* (C_4) was 45 °C compared to 30 °C in *P. sativum* (C_3). *A. hypochondriacus* (C_4) lost nearly 70% of PEPC activity on exposure to a low temperature of 15 °C, compared to only about a 35% loss in the case of *P. sativum* (C_3). Thus, the C_4 enzyme was less sensitive to supra-optimal temperature and more sensitive to sub-optimal temperature than that of the C_3 species. As the temperature was raised from 15 °C to 50 °C, there was a sharp decrease in malate sensitivity of PEPC. The extent of such a decrease in C_4 plants (45%) was more than that in C_3 species (30%). The maintenance of high enzyme activity at warm temperatures, together with a sharp decrease in the malate sensitivity of PEPC was also noticed in other C_4 plants. The temperature-induced changes in PEPC of both *A. hypochondriacus* (C_4) and *P. sativum* (C_3) were reversible to a large extent. There was no difference in the extent of phosphorylation of PEPC in leaves of *A. hypochondriacus* on exposure to varying temperatures, unlike the marked increase in the phosphorylation of enzyme on illumination of the leaves. These results demonstrate that (i) there are marked differences in the temperature sensitivity of PEPC in C_3 and C_4 plants, (ii) the temperature induced changes are reversible, and (iii) these changes are not related to the phosphorylation state of the enzyme. The inclusion of PEG-6000, during the assay, dampened the modulation by temperature of malate sensitivity of PEPC in *A. hypochondriacus*. It is suggested that the variation in temperature may cause significant conformational changes in C_4-PEPC.

Key words: Cold sensitivity, conformational change, malate sensitivity, PEPC, PEG-6000, temperature.

Introduction

Phospho*enol*pyruvate carboxylase (EC 4.1.1.31) is a ubiquitous enzyme occurring in the cytosol of photosynthetic and non-photosynthetic tissues of C_3, C_4 and CAM plants (Andreo *et al.*, 1987; Rajagopalan *et al.*, 1994; Chollet *et al.*, 1996; Vidal and Chollet, 1997; Nimmo, 2000). C_4 plants differ from C_3 plants in several features, including their light and temperature responses (Berry and Björkman, 1980; Sugiyama *et al.*, 1979). The temperature optima for photosynthesis and growth in C_4 plants are usually higher than those for C_3 plants (Berry and Björkman, 1980). The C_4 plants, in general, are tolerant to heat, but are quite sensitive to cold temperatures (Du *et al.*, 1999*a*). The cold sensitivity of the C_4 pathway has been suggested to be related to the cold sensivitity of key C_4 enzymes, such as pyruvate phosphate dikinase (PPDK) or PEPC (Potvin and Simon, 1990; Burnell, 1990; Du *et al.*, 1999*a*).

The cold sensitivity of PPDK in C_4 plants is well established and the mechanism of cold inactivation of PPDK is studied in detail (Krall *et al.*, 1989; Burnell, 1990; Du *et al.*, 1999*b*). By contrast, the reports on cold sensitivity of PEPC have been conflicting. There are

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Abbreviations: MDH, NAD-malate dehydrogenase; NAD-ME, NAD-malic enzyme; PEG-6000, polyethylene glycol 6000; PEPC, phospho*enol*pyruvate carboxylase; PMSF, phenylmethylsulphonyl fluoride; PPDK, pyruvate phosphate dikinase.

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JOURNAL OF PLANT PHYSIOLOGY

Light activation of NADP malic enzyme in leaves of maize: Marginal increase in activity, but marked change in regulatory properties of enzyme

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Summary

This article reports the characteristics of light activation of NADP-malic enzyme (NADP-ME, EC 1.1.1.40) in leaf discs of maize (*Zea mays* cv. VMH 404) for the first time. The leaf discs were illuminated in the presence of 2 mmol/L bicarbonate, as light activation increases in the presence of bicarbonate. Upon illumination, the V_{max} of NADP-ME increased by about 30 %. Although small, the increase was consistent and significant. The changes in regulatory properties of NADP-ME were quite pronounced. The extent of light activation was similar when substrate (malate) concentration was either 4 mmol/L (saturating) or 0.01 mmol/L (limiting). There was only a marginal change in the K_m for malate, but there was marked change in the response of NADP-ME to activators or inhibitors. The K_i for pyruvate and oxalate increased by 100 and 67 % respectively, while the K_a for the citrate and succinate increased by 36 and 32 % respectively. These results suggest that the NADP-ME becomes less sensitive to feedback inhibition on illumination. The light-induced change seems to be due, at least partially, to the reduction of dithiols, as incubation of leaf extracts with DTE dampened light activation of NADP-ME. We conclude that the properties of NADP-ME do change on illumination. Although there was only a marginal increase in the activity of the enzyme on illumination of leaf discs, the changes in regulatory properties of NADP-ME were marked.

Key words: Bundle sheath – C_4 plants – enzyme activity – light activation — maize – NADP-malic enzyme

Abbreviations: NADP-MDH = NADP-malate dehydrogenase. – NADP-ME = NADP malic enzyme. – NR = nitrate reductase. – PEPC = phospho*enol*pyruvate carboxylase. – PPDK = pyruvate phosphate dikinase. – SPS = sucrose phosphate synthase. – PMSF = phenylmethylsulfonyl fluoride. – PVPP = polyvinylpolypyrrolidone

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Cytosolic pH as a Secondary Messenger During Light Activation of Phospho*enol*pyruvate Carboxylase in Mesophyll Cells of C_4 Plants

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1. INTRODUCTION

The primary carbon fixation in C_4 and CAM plants is catalyzed by phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31). With a pH optimum of about 8.0, the activity of PEPC is modulated markedly by pH. The enzyme, PEPC, is localized in cytosol of mesophyll cells higher plants. Obviously that cytosolic pH would be a very important factor in determining the activity of PEPC.

Being a key enzyme of C_4 photosynthesis or CAM, the regulation of PEPC has been of considerable interest. PEPC is feed-back inhibited by L-malate and is activated by glucose-6-phosphate (Glc-6-P) (Andreo *et al* 1987, Rajagopalan *et al* 1994, Chollet *et al* 1996, Vidal and Chollet 1997). The sensitivity of PEPC to malate is further influenced by various factors like light or pH. Malate inhibition is competitive at pH 7.0, and non competitive at pH 8.0 and Glc-6-P protects the enzyme from malate inhibition. On illumination, the concentration of malate in mesophyll cells can rise up to 30 mM and can lead to a strong feedback inhibition of PEPC. However, when leaves are illuminated, there is a marked decrease in sensitivity of PEPC to malate besides an increase in activity. The light activation of PEPC is therefore considered to be an adaptive feature to sustain enzyme activity in presence of high malate concentrations.

The present article attempts to review the observations on the light induced increase in the activity of PEPC and relate them to changes in pH of cytosol in mesophyll cells. We propose that the cytosolic pH could be a secondary messenger during such light activation of PEPC, particularly in leaves of C_4 plants.

Readers interested in the properties and regulation of PEPC in leaves of C_4 and CAM plants are referred to several recent reviews (Andreo *et al*