Purification, Properties and Regulation of Phosphoeno/pyruvate Carboxylase from Leaves of *Amaranthus hypochondriacus* in relation to Bicarbonate, Calcium and Light

Thesis submitted for the Degree of

DOCTOR OF PHILOSOPHY

By

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DECLARATION

I hereby declare that the work presented in this thesis entitled **"Purification, Properties and Regulation of Phosphoenol/pyruvate Carboxylase from Leaves of *Amaranthus hypochondriacus* in relation to Bicarbonate, Calcium and Light"** has been carried out by me under the supervision of Prof A.S. Raghavendra, Department of Plant Sciences, School of Life Sciences, University of Hyderabad, Hyderabad 500 046, and that this work has not been submitted for any degree or diploma of any other University.

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CERTIFICATE

This is to certify that the thesis entitled **"Purification, Properties and Regulation of Phosphoenolpyruvate Carboxylase from Leaves of *Amaranthus hypochondriacus* in relation to Bicarbonate, Calcium and Light"** is based on the results of the work done by **Ms K. Parvathi** for the degree of **Doctor of Philosophy** under my supervision. This work has not been submitted for any degree or diploma of any other University.

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My Parents

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ABBREVIATIONS

BAPTA	=	1,2-bis-(-2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid
BCIP	=	5-bromo-4-chloro-3-indolyl phosphate
CaM	=	calmodulin
CaMK or CCaMK	=	Ca^{2+}/CaM-dependent protein kinase
CDK	=	cyclin-dependent kinase
CDPK	=	CaM-like-domain protein kinase or Ca^{2+}-dependent protein kinase
CHX	=	cycloheximide
CKII	=	casein kinase II
EZ	=	ethoxyzolamide
FBPase	=	fructose 1,6-bisphosphatase
FPLC	=	fast-protein liquid chromatography
Glc-6-P	=	glucose 6-phosphate
GSK-3	=	glycogen synthase kinase
H_7	=	l-(5-isoquinolinylsulfonyl)-2-methylpiperazine
HAP	=	hydroxylapatite
IP_3	=	inositol 1,4,5-triphosphate
MAPK	=	mitogen-activated protein kinase
MDH	=	NAD malate dehydrogenase
ML7	=	1-(5-iodonaphthalene-1-sulfonyl)-1 H-hexahydro-1,4-diazepine
MLCK	=	myosin light chain kinase
NBT	=	nitro blue tetrazolium
OAA	=	oxalacetate
PEPC	=	PEP Carboxylase

PEPC-PK	=	PEPC-protein kinase
3-PGA	=	3-phosphoglyceric acid
PKA	=	cAMP-dependent protein kinase
PKC	=	Ca^{2+}/phospholipid-dependent protein kinase
PKG	=	cGMP-dependent protein kinase
PPDK	=	pyruvate Pi dikinase
PTK	=	protein Tyr kinase
RLK	=	receptor-like kinase
RTK	=	receptor Tyr kinase
S15D	=	mutant maize PEPC in which Ser-15 is replaced by Asp
S8D	=	mutant sorghum PEPC in which Ser-8 is replaced by Asp
SNF	=	Suc nonfermenting
TFP	=	tritluoperazine
W7	=	N-(6-aminohexyl)-5-chloro-l-naphthalenesulfonamide
WT	=	wild type

All the remaining abbreviations are all standard ones, and as per the list given in ***Plant Physiology***, January 1997, vol 113, No. 1. pp. xiii-xv or ***Plant*** *Physiology,* 1998, Instructions for contributors,web site: http://www.aspp.org.

CONTENTS

Chapter 1

Introduction and Review of Literature

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Phospho*enol*pyruvate Carboxylase (PEPC)

Phospho*enol*pyruvate Carboxylase (PEPC, EC 4.1.1.31) is a key enzyme involved in primary carbon fixation in C4 and CAM plants (Edwards and Walker, 1983). PEPC is localized in mesophyll cytosol of plant cells. PEPC in C4 plants primarily fixes CO_2 into C4 dicarboxylic acids, which on decarboxylation raise the CO_2 concentration in bundle sheath cells and minimize the process of photorespiration. Thus. C4 plants can achieve high growth rates under conditions of high temperature, strong illumination and atmospheric oxygen levels, which are not optimal for C3 plants (Edwards et al., 1985). PEPC plays an anaplerotic role in C_3 plants. Due to the importance of PEPC in C4-, C3- as well as CAM plants, studies on PEPC arc always of great interest.

Stupendous progress has been made in our knowledge of biochemistry and molecular biology of PEPC in not only C4 plants, but also C3 species and legume root nodules. The literature on the properties, regulation and functions of PEPC has been reviewed by several authors during the present decade (González and Andreo, 1989; Jiao and Chollet, 1991; Vance and Gantt, 1992; Nimmo, 1993; Lepiniec et al., 1994; Rajagopalan et al.. 1994; Toh et al., 1994; Chollet et al., 1996; Vidal and Chollet, 1997). There are also several reviews which appeared before 1988 (Utter and Kolenbrander, 1972; O'Leary, 1982, 1983; Latzko and Kelly, 1983; Gadal, 1983; Guern et al., 1983; Kluge, 1983; Andreo et al., 1987; Deroche and Carrayol, 1988; Stiborová, 1988).

Occurrence

PEPC is almost ubiquitous and is distributed widely in photosynthetic and non-photosynthetic tissues of higher plants, green algae, bacteria and legume root nodules and is absent in animal tissues, yeast or fungi (Andreo et al., 1987; Deroche and Carrayol, 1988; Vance and Gantt, 1992; Lepiniec et al.. 1994; Toh et al., 1994; Rajagopalan et al., 1994; Chollet et al., 1996). It is an important enzyme involved in primary CO_2 fixation in C4 and CAM plants (O'Leary, 1982). The activities of PEPC levels in leaves of C3 plants are about 2 to 5% of that found in C4 plants (Edwards and Walker, 1983; Kluge, 1983; Latzko and Kelly, 1983).

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

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

Physiological role

The occurrence of 'Kranz-like anatomy' in C4 plants results in the division of labor and spatial separation of biochemical reactions. In C4 plants, the initial carbon fixation through PEPC occurs in mesophyll cells, while the subsequent decarboxylation reactions take place in the bundle sheath cells, where the Calvin cycle enzyme, Rubisco refixes the released CO_2. The function of PEPC in CAM plants is similar to C4 plants. Primary carbon fixation by PEPC occurs during the night, followed by decarboxylation of C4 acids and refixation of CO_2 by Rubisco during day (Kluge, 1983). PEPC is a key enzyme involved in such primary CO_2 fixation in C4 and CAM plants (O'Leary, 1982). It catalyzes the irreversible β-carboxylation of PEP in the presence of HCO_3^- and Me^{2+} to yield OAA and Pi.

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

Types of isoforms

In higher plants, four types of PEPC isoforms have been reported so far, namely, C4 photosynthetic form, C3 photosynthetic form, CAM form and dark or

non-autotrophic PEPC. Chromatographic, immunological and kinetic properties of PEPC can be used to distinguish these isoforms (O'Leary, 1982; Andreo et al., 1987; Rajagopalan et al., 1994).

Etiolated *Sorghum* leaves contain only one form (C3 form) of the enzyme and a new isoform of enzyme appears on illumination upon greening (C4 form) (Vidal and Gadal, 1983). C4 specific gene expression occurs only in illuminated (greening) leaves (Schäfiner and Sheen, 1992). On the other hand, the expression of PEPC-gene encoding the C4 isozyme was not leaf specific, since high accumulation of its transcripts was also found in other parts of maize plant, i.e., inner leaf sheaths, tassels and husks (Hudspeth and Grula, 1989). There is a possibility that a small amount of etiolated form of the enzyme may exist also in green tissue, which could explain the detection of the two major isozymes of PEPC in leaves of maize (Ting and Osmond, 1973a, b; Mukerji, 1977). However, C_4-type is the major form in maize leaves and is the most abundant protein in mesophyll cells.

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

for preferential expression of C4 specific PEPC gene (Nelson and Langdale, 1992; Lepiniec et al., 1994; Stockhaus et al., 1994; Ku et al., 1996).

PEPC structure

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

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

A number of putatively important domains are identified in the primary sequence of PEPC based on biochemical experiments (Andreo et al., 1987; Jiao and Chollet, 1991), amino-acid alignments (Lepiniec et al., 1993) and site-directed mutagenesis (Terada et al., 1992; Wang et al., 1992). Four possible sites are responsible for the regulation of the activity of plant enzyme by direct interactions with PEP, glucose 6-phosphate (Glc-6-P) and L-malate, as well as protein phosphorylation (Lepiniec et al., 1994). The histidine residue of the amino acid motif 'VfTA*H*PT' (residues with capital letters being conserved) is essential for the carboxylation activity of the enzyme (Lepiniec et al., 1993). The highly conserved sequence 'QqVMvGYDSgKDaG' contains the species-invariant lysine residue implicated in the active site (Jiao et al., 1990). The glycine-rich motif

'FHGRGGtvGRGGgP' has been proposed to be part of the substrate binding site (Jiao et al., 1990). Seven cysteine residues are conserved in all plant PEPC sequences (Lepiniec et al., 1993) and these may be involved in the proposed redox regulation of PEPC activity (Chardot and Wedding, 1992) or subunit-interactions to maintain the tetrameric structure (Andreo et al., 1987).

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

Evolutionary aspects

It has been widely accepted that C3 photosynthesis predates both CAM and C4 metabolism that have originated independently and on many occasions (Ehleringer et al., 1991). It has been hypothesized that C4 photosynthesis evolved in response to a reduction in atmospheric CO_2 level (Ehleringer et al., 1991).

A number of C4 traits, including 'Kranz' leaf anatomy, intercellular compartmentation and specific activities of key C4 enzymes such as PEPC, are inherited independently. The genetic origins of these modifications are not yet fully understood. These characteristics might have evolved separately in one or several species in response to different selective pressures like water stress, salinity, low CO_2 pressure or high temperature. In this regard, three biochemical sub-types or modes of C4 carbon metabolism are known (Leegood and Osmond, 1990; Hatch,

1992), **which** could reflect different possible adaptive combinations (Lepiniec et **al., 1994).**

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

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

Another view is that plant PEPC might have arisen from an endosymbiotic origin. Indeed, PEPC distribution in prokaryotes indicates that the enzyme was already present in the ancestor of the Chloroplast and is still present in their

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

Purification

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

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

preparations of PEPC **with an intact N-terminal region** is possible **from** leaves (C4, **CAM and** C3) and root nodules.

The cDNA encoding the C4- or C_3-type PEPC, *(ppc)* gene was **cloned and** expressed in bacteria. Cloning of PEPC gene in *E. coli* is a widely used phenomenon (Chollet et al., 1996; Vidal and Chollet, 1997). The successful cloning of *ppc* paved the way for the studies on site-directed mutagenesis, and a powerful tool to elucidate the importance and function of individual amino acid residues in **the** catalytic and regulator}' properties of any enzyme (Rajagopalan et al., 1994, Dong et al., 1997b; Grisvard et al., 1998).

PEPC-deficient mutants of *E. coli* have been used to complement with a plasmid bearing a full-length cDNA encoding the C_4-type form of *Sorghum ppc* (Crétin et al., 1991). The transformed bacteria produced a functional and full-sized enzyme, as determined by activity, immunochemical behavior and SDS-PAGE analysis. In addition. the recombinant PEPC could be phosphorylated *in vitro* by the *Sorghum* PEPC-protein kinase (PEPC-PK) or mammalian protein kinase A (Crétin et al., 1991; Pacquit et al., 1993).

The recombinant protein technology was successfully applied in previous work with *Sorghum* (Crétin et al., 1991) or maize (Yanagisawa and Izui, 1990), and was used to raise large amounts of C4-PEPC. As C_3-type enzyme forms are present in relatively little amount in *Sorghum* and therefore difficult to purify extensively, they have received much less attention and consequently little is known about their regulation and phosphorylation. This recombinant technology made it possible to produce large amounts of C_3-type PEPC form *Sorghum* leaves, which are used **for** biochemical studies (Pacquit et al., 1993).

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

Stability of PEPC in vitro

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

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

Kinetic properties of PEPC

The kinetic mechanism of the enzyme is central to the understanding of any enzyme. The exergonic β-carboxylation of PEP by HCO_3^- (ΔG = -7 kcal mol^{-1}) is catalyzed by PEPC in the presence of a divalent cation, particularly Mg^{2+}. As PEPC plays an important role in C4 and CAM carbon fixation, it is essential to

understand the kinetic properties of **the** enzyme, and the mechanism of regulation of the enzyme by substrates, pH and other metabolites (O'Leary, 1982).

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

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

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

In spite of the extensive research that has been focused on the regulation of PEPC, the studies on kinetic characteristics of the enzyme are rather limited. There are no studies on the mechanism of binding of bicarbonate (HCO3-). This may be due to the technical problem of complete elimination of exogenous HCO3- during such studies.

PEPC uses HCO_3^- for the carboxylation of PEP. The K_m (HCO_3^-) of PEPC is lower than for other enzymes. The relative low value indicates that PEPC, which is able to catalyze the rapid carboxylation at low HCO3" concentrations, can serve as an effective HCO_3^- trap (Maruyama et al., 1966). Carbon atom of HCO3" is ordinarily unreactive towards the nucleophilic attack and the way by which the C-C bond formation is achieved by PEPC is not completely understood (Iglesias and Andreo, 1983). Besides carboxylation, PEPC catalyzes a HCO_3^--dependent hydrolysis of PEP to pyruvate and Pi (phosphatase activity). Only 5% of the total reaction is diverted to hydrolysis under optimal conditions. The extent of phosphatase activity of PEPC is increased up to 50% in presence of Ni^{2+} as a metal ion (Chollet et al., 1996).

Regulation

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

Metabolites (Inhibitors/Activators)

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

L-Malate, which is a product of carboxylation, is a competitive inhibitor of PEPC (Huber and Edwards, 1975). Malate not only inhibits C4-PEPC, but also the C3 and CAM forms (Kluge et al., 1988; Echevarria et al., 1990; Jiao and Chollet, 1990). Aspartate also inhibits the enzyme (Huber and Edwards, 1975; Iglesias et al., 1986). The enzyme is protected against thermal inactivation by aspartate (Rathnam,

1978; Mareš and Leblová, 1980). Several other analogues of PEP/pyruvate are powerful inhibitors of the C4 enzyme and are employed to study the reaction mechanism of the enzyme (González and Andreo, 1989; Janc et al., 1992 a, b; Rajagopalan et al., 1994).

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

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

Glc-6-P is also an efficient stabilizer of the activity of the enzyme in storage (Wedding et al., 1989) as well as during the assay. Glc-6-P induces aggregation of PEPC into the tetrameric form (Wedding et al., 1989; Willeford and Wedding, 1992; Wu and Wedding, 1994). Further, Glc-6-P protects PEPC against inactivation by the modification of essential cysteine residues (Manetas and Gavalas, 1982) and

against inactivation by urea (Wedding et al., 1992). Wang et al. (1992) reported that phosphorylation of PEPC has no effect on its response to Glc-6-P and suggested that the activation by Glc-6-P may occur by a more complex path than activation by PEP and inhibition by malate.

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

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

Light

The kinetic and regulatory properties of C4-PEPC in leaves are modulated markedly by light/dark transitions *in vivo* (Andreo et al., 1987; Jiao and Chollet, 1991; Rajagopalan et al., 1994; Chollet et al., 1996; Vidal and Chollet, 1997). The activity of PEPC, particularly in leaves of C4 plants is enhanced by 2 to 3 fold upon illumination. Light-activation is a feature of key photosynthetic enzymes in C3 plants (Buchanan, 1992). The light activation of PEPC is distinct from light-induced synthesis of PEPC-protein, which is observed typically during greening of *Sorghum* or maize leaves (Sims and Hague, 1981; Vidal and Gadal, 1983).

The enzyme exhibits two or three fold more activity on illumination, when assayed at sub-optimal but physiological levels of PEP and pH (Jiao and Chollet, 1988). The light-form is less sensitive to feedback inhibition by malate than that of dark-form (Vidal and Chollet, 1997). The response of PEPC in C3 leaves to light is much less than that in C4 plants. The activation of PEPC on exposure to light is marginal (about 10-15%) in C3 species (Rajagopalan et al., 1993). A marginal increase in PEPC activity on exposure to light was reported in mesophyll protoplasts of maize (Devi and Raghavendra, 1992).

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

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

Temperature

The effects of temperature on growth are often correlated to corresponding changes in activity of several enzymes, including PEPC in C4 plants (Selinioti et al, 1986).

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

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

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

pH

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

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

Salt/Water stress

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

The effect of salt stress on PEPC is manifested much more strongly in CAM plants than that in C3 or C4 species. For e.g. in an inducible-CAM plant, *Mesembryanthemum crystallinum,* PEPC-activity rises remarkably on exposure to salt or water stress, due to enhanced transcription of the *ppc* gene (McElwain et al., 1992). Photoperiodism or water stress can shift young leaves of *Kalanchoë*

blossfeldiana from C_3-type photosynthesis to CAM. A rise in endogenous levels of ABA preceded PEPC-increase, independent of CAM induction in isolated leaves of *Kalanchoë blossfeldiana* (Taybi et al., 1995). In *Mesembryanthemum crystallinum*, the increase in PEPC activity upon ABA treatment was due to increased levels of CAM-specific isoform of the enzyme (Dai et al., 1994).

Nutrition

The biosynthesis of PEPC in leaves of C3-, C4- and CAM plants is highly regulated by availability and source of nitrogen (Champigny and Foyer. 1992; Foyer et al., 1994; Gadal et al., 1995; Lara et al., 1995). The rise in the level of PEPC-mRNA and PEPC-protein (particularly the C4 isoform) was more pronounced in maize plants supplemented with NH_4^+ or glutamine than those with NO3". The biosynthesis of PEPC in maize leaves increased on feeding the leaves with nitrate or ammonium. Ammonium salt induces two-fold more PEPC biosynthesis than that of nitrate (Sugiharto et al., 1990, 1992; Sugiharto and Sugiyama, 1992).

Ammonium ions arc known to stimulate the dark CO_2 fixation into C4 acids in higher plants, algal cells and cyanobacteria (Miyachi and Miyachi, 1985; Ohmori et al.. 1986; Müller et al., 1990; Vanlerberghe et al., 1990). Such stimulation could be due to the increase in the activity of PEPC in ammonia-treated cells. The rates of ammonia assimilation *in vivo* were correlated with PEPC activity in a green alga, *Selanastrum minutum* (Vanlerberghe et al., 1990).

Ammonia is a toxic metabolite and inhibits several enzymes of plant and animal metabolism (WHO, 1986). Nevertheless, a few enzymes such as pyruvate kinase are stimulated by ammonium (Peterson and Evans, 1978). Ammonium ions can stimulate PEPC *in vitro* (Gayathri and Raghavendra, 1994). The effect of ammonium on PEPC was at the regulatory allosteric site on the enzyme. Another possibility' is the modulation of intracellular pH by externally added NH_4^+.

Ammonia diffusion into cells may **cause** alkalization **of cytosol and the rise in pH could in turn** stimulate PEPC.

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

Post-translational modification of PEPC

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

Phosphorylation/dephosphorylation

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

Glc-6-P, when assayed at suboptimal pH and PEP concentrations **(Duff** et **al.,** 1995; Vidal and Chollet, 1997). These changes are reversed by dephosphorylation.

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

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

The phosphorylation site is only two amino acid residues removed from a basic amino acid group (Lys-12 in the case of maize PEPC) in the primary sequence, a feature similar to that of various protein serine/threonine kinase substrates (Jiao and Chollet, 1991; Fallon and Trewavas, 1993; Macintosh and Macintosh, 1993; Hunter, 1995). Thus, a specific phosphorylation site is accessible

to both homologous and **heterologous protein** kinases, for e.g., mammalian protein kinase A (Jiao and Chollet, 1990; Terada et al., 1990; Rajagopalan et **al.,** 1994).

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

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

Physiological role of phosphorylation

On illumination, PEPC phosphorylation reached its maximum by about an hour and was associated with corresponding marked changes in the enzyme's properties: increase in catalytic activity and apparent affinity for Glc-6-P and decrease in inhibition by malate (as determined at suboptimal but near-physiological conditions of pH and PEP concentration) (Bakrim et al., 1992; Duff et al., 1995). Such modulation of PEPC is believed to enable the enzyme to cope with the high concentration of L-malate that occurs during CO_2 fixation in mesophyll cells of an illuminated C4 leaf (10-20 mM) (Stitt and Heldt, 1985; Doncaster and Leegood, 1987). The fully phosphorylated form of C4-PEPC had a markedly reduced sensitivity to L-malate (IC_{50} = 15 mM) compared to

dephosphorylated enzyme form (Kj (malate) about 0.2 mM) (Echevarria et al., 1994).

Phosphorylation of PEPC and ultimate CO_2 fixation was correlated by inhibition of PEPC-PK (Bakrim et al., 1992, 1993; Li and Chollet, 1993). These observations suggest that PEPC phosphorylation has a critical regulatory impact on the overall functioning of C4 photosynthesis (Bakrim et al., 1993; Echevarría et al., 1994). Phosphorylation not only protects C4-PEPC against L-malate, but also adjusts its catalytic activity to meet the demand of the Calvin cycle for a acid-derived supply of CO_2. The light transduction pathway is present in mesophyll cell and a cross-talk between the mesophyll and bundle sheath cells is implied in the form of the photosynthetic metabolite, 3-PGA (Giglioli-Guivarc'h et al., 1996; Vidal and Chollet, 1997).

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

Oligomerization

The active form of PEPC is a tetramer. But the enzyme can exist as a dimer or monomer depending on several factors: pH, ionic strength (Walker et al., 1986a; Wagner et al., 1987), temperature (Wu and Wedding, 1987) and concentration of PEPC (Willeford and Wedding, 1992). NaCl causes dissociation of enzyme into a

dimer at pH 7.0 and into dimers/monomers at pH 8.0. The presence of PEP, Mg^{2+} or Glc-6-P prevented dissociation of the enzyme (Wagner et al., 1987; Wu and Wedding, 1994). Effectors such as Glc-6-P and malate or presence of solutes (PEG and/or glycerol) can also effect the aggregation of the enzyme (Podestá and Andreo, 1989; Manetas, 1990; Wedding et al., 1994).

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

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

and Wedding, 1992). Glc-6-P also favors the formation of the most active tetramer of the enzyme (Wagner et al., 1987; Meyer et al., 1991; Willeford and Wedding, 1992).

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

Redox regulation

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

Transduction of light-signal during PEPC-activation

Although the phenomenon of marked activation by light of PEPC in C4 leaves, is well known, the mechanism of light activation is not completely understood. Nevertheless, a few factors have been identified to be involved as the messengers. Light-signal transduction can be mediated through changes in the level

of a metabolite of photosynthesis and/or energy charge (Doncaster and Leegood, 1987). A transcellular message (3-PGA) formed in the bundle sheath during C4 photosynthesis would be delivered to the adjacent mesophyll cells where the physiological responses with respect to PEP carboxylase-kinase and PEPC were observed (Chollet et al., 1996; Giglioli-Guivarc'h et al., 1996). This suggested that the signal transduction chain involves intracellular cross talk between these neighboring photosynthetic tissues. McNaughton et al. (1991) speculated that a signal generated in Chloroplast may initiate a sequence of events that lead to a significant increase in activity of PEPC-PK and phosphorylation of PEPC in the cytosol. On feeding the leaves with photosynthetic inhibitors like DCMU or D, L-glyceraldehyde, decreased phosphorylation of PEPC indicating the coordination of both mesophyll and bundle sheath cells (Jiao and Chollet, 1992).

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

The light transduction pathway in an illuminated C4 leaf involves, sequentially: 3-PGA, as an intercellular message indicating photosynthesis, an increase in cytosolic pH in mesophyll, IP3, Ca^{2+} channels on tonoplast, cytosolic Ca^{2+}, a Ca^{2+}-dependent protein kinases and finally the upregulation of PEPC-PK via stimulation of cytosolic protein synthesis event. Recently, Vidal and Chollet

(1997) proposed **a** model showing the likely events during the transduction of light signal to control C4-PEPC-PK and, thus, PEPC phosphorylation.

Secondary messengers likely to be involved during light activation of PEPC

Calcium

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

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

Cytosolic pH

Felle (1989) was among the first to suggest that cytosolic pH is an intracellular messenger in plants. In mesophyll cells of an illuminated C4 leaf, cytosolic pH plays an important role in modulating the PEPC phosphorylation and **as** a result the activity of PEPC (Giglioli-Guivarc'h et al., 1996). NH_4Cl and methylamine, weak bases trigger the phosphorylation of PEPC. Both compounds

are known to induce cytosolic pH. Intracellular calcium levels could be modulated by the changes in pH in *Sorghum* mesophyll protoplasts (Pierre et al., 1992). The phosphorylation state of PEPC or a putative activation protein factor of PEPC, depends on the amplitude of the cytosolic pH jump in mesophyll cells, reaching a maximum at a pH value close to 7.3. While, a change in cytosolic pH would dramatically affect both PEPC kinase and PEPC activities because these two enzymes strongly respond *in vitro* to H^+ concentrations in the range of pH 7 to 8 (Wang and Chollet, 1993; Echevarria et al., 1994).

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

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

Characterization of PEPC-protein kinase

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

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

It is proposed that PEPC-PK undergoes reversible light activation *in vivo* (Bakrim et al., 1992; Chollet et al., 1996). Pretreatment with cycloheximide (CHX), a cytosolic protein synthesis inhibitor, efficiently blocked both C4-PEPC-PK upregulation and PEPC phosphorylation during illumination (Bakrim et al., 1992; Chollet et al., 1996). Phosphorylation of PEPC was markedly blocked by CHX, but not by a-aminitin and actinomycin D (inhibitors of RNA polymerase II), thereby suggesting that protein translation is the regulatory step (Vidal and Chollet, 1997).

Feeding of CHX to an excised *Sorghum* or maize leaf performing photosynthesis at steady state caused a significant decrease in both its CO_2 assimilation rate and PEPC phosphorylation state (Bakrim et al., 1992). These results suggested that the light induced phosphorylation of PEPC was regulated by the synthesis of PEPC-PK or an unknown protein required for the activation of PEPC-PK. Further, it was recently reported that PEPC-PK from C4-, CAM and C3 species is regulated at the level of translatable mRNA in response to light (C4, C3), or circadian rhythm (CAM) (Hartwell et al., 1996).

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

Possible involvement of other types of kinases

Ogawa et al. (1992) suggested that at least four types of protein kinases could be detected in their PEPC-PK preparation, of which two of them were calcium-dependent. It has been suggested that multiple forms of PEPC-PK (both 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). There are reports suggesting that the Ca^{2+}-dependent (Vidal et al., 1990; Ogawa and Izui, 1992; Ogawa et al., 1992) and Ca^{2+}-independent (Chollet et al., 1990; Echevarria et al., 1990; McNaughton et al., 1991) protein kinases are involved in PEPC phosphorylation. Immunopurified C4-PEPC from *Sorghum* and maize could be phosphorylated *in vitro* by various kinases, including calcium- or magnesium-

dependent protein kinase, both in crude extract and a reconstituted system (Bakrim **et** al., 1992) or catalytic-subunit of cAMP-dependent protein kinase from bovine heart (Terada et al., 1990; Bakrim et al., 1992).

Protein kinases in plants

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

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

AGC group

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

CaMK group

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

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

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

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

CMGC group

CMGC group contains the cyclin-dependent kinase (CDK), mitogen-activated protein kinase (MAPK), glycogen synthase kinase (GSK-3), and casein

kinase **II** (CKII) families in plants. CDKs are involved in cell cycle regulation in plants. Whereas, MAPKs are activated by dual phosphorylation on Thr and Tyr residues and they play a role in cell proliferation. GSK3 plays an important role in plant development. CKII serves a critical function in transcriptional regulation in plants (Collinge and Walker, 1994; Stone and Walker, 1995)

PTK group

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

Other group

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

Secondary messengers are important in signal transduction in plant cells. The changes in cytosolic Ca^{2+} levels in response to external stimuli are often accompanied by changes in protein phosphorylation. Ca^{2+}-dependent phosphorylation in plants is regulated by CDPKs rather than by PKC or CaMK. Since plant-CDPKs have calcium-binding, calmodulin-like domain on the same polypeptide as a protein kinase catalytic domain, the plants may have surpassed the need for CaMK or PKC (Stone and Walker, 1995).

One of the aims of present work has been to study the characteristics of PEPC phosphorylation in *Amaranthus hypochondriacus* leaves. There are conflicting results about the involvement of Ca^{2+}-dependent and -independent protein kinases during the regulatory phosphorylation of PEPC in response to light. It has been proposed that CDPK may be involved in the upregulation of PEPC-PK, i.e., Ca^{2+}-independent protein kinase (Chollet et al., 1996; Vidal and Chollet, 1997). A direct study of the characteristics of CDPK or CCaMK in *Amaranthus hypochondriacus* leaf extracts, would be helpful to resolve this issue.

Some of the points to be resolved

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

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

The extraction and particularly the assay of activity are invariably linked to dilution and in addition, deprive the enzymes of their natural physico-chemical environment. Such dilution of the medium (e.g. encountered during enzyme assays)

affects the quaternary structure of PEPC by inducing dissociation and loss of activity (Willeford and Wedding, 1992). The addition of compatible solutes like glycerol or PEG can stabilize the enzyme (Reinhart, 1980; Karabourniotis et al., 1983). The inclusion of compatible solutes can provide a favorable microenvironment against dilution. The effects of these solutes during extraction and assay were therefore studied in this work.

Light activation of photosynthetic enzymes is a cardinal event in the plant growth and development. As PEPC is a key enzyme involved in CO_2 fixation in C4- and CAM plants, the regulation of PEPC by light is a topic of interest. It is obvious that the light signal has to be perceived by the Chloroplast and the effect is transmitted to the cytosol, the site of PEPC in C4 mesophyll cells. Although, the kinetic and regulator)' properties of PEPC are modulated by light/dark transitions *in vivo* (Chollet et al., 1996; Vidal and Chollet, 1997), there is very limited information on the mechanism and pattern of transduction of light signal. Illumination of plant cells is known to rise the cytosolic pH and possibly free Ca^{2+}. An attempt was therefore made to study in detail the effects of Ca^{2+} on the activity of PEPC.

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

Compared to the extensive literature on the modulation of malate-sensitivity of PEPC, the studies on changes in kinetic characteristics of the enzyme and in particular the affinity to bicarbonate are quite limited. Recently, it has been reported

that bicarbonate plays an important role in the allosteric regulation of PEPC (Dong **et al., 1997a;** Ogawa et al., 1997). Such, the modulation of PEPC by one of the substrates, bicarbonate is extremely interesting and warrants further studies.

Present work

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

Chapter 2

Approach and Objectives

Chapter 2

Approach and Objectives

Amaranthus hyochondriacus, a NAD-ME type of C4 plant, is an important grain crop/leafy vegetable, which is grown in semi-arid, sub-tropical and tropical regions. PEPC is localized in the cytosol of mesophyll cells of C4 plants, and constitutes about 15% of total soluble protein in maize (Hague and Sims, 1980; Hayakawa et al., 1981). Further, the leaf tissue of this C4 dicot is soft, easy to extract and contains very few interfering compounds. Therefore, we have used the leaves of *Amaranthus hypochondriacus* for studies on purification, characterization and regulation of PEPC.

PEPC was purified to homogeneity from several photosynthetic tissues. Purified PEPC is used extremely to study the kinetic and regulatory properties of the enzyme, so as to avoid interferences, if any of other enzymes, which would be present in crude extracts. Therefore, we have purified PEPC from *Amaranthus* leaves by conventional techniques of 40-60% ammonium Sulfate fractionation, followed by DEAE-Sepharose, phenyl-Sepharose and HAP column and the enzyme was concentrated with PEG 20,000.

Native leaf PEPC is highly susceptible to limited proteolysis near the N-terminal during extraction and subsequent purification steps (Chollet et al., 1996). The purified PEPC also is unstable, if proper precautions are not taken. The enzyme loses frequently its N-terminal end (during either extraction or storage) and becomes malate-insensitive (McNaughton et al., 1989; Chollet et al., 1996). Therefore, 50% glycerol was included along with PEPC and the mixture was stored in liquid nitrogen as small aliquots. The stability of purified PEPC was examined with or without glycerol.

Polyclonal antibodies were raised in rabbits against the purified PEPC from *Amaranthus hypochondriacus* leaves using the procedure of Nimmo et al. (1986). Ouchterlony double immunodiffusion method was employed to check the titer value of antiserum. Immunoprecipitation using the antibody confirmed that the PEPC was precipitated effectively in the crude extracts from leaves. Further, the cross reactivity of antiserum was checked and assessed by using Western blot analysis and Enzyme-Linked Immunosorbent Assay (ELISA) using alkaline phosphatase.

IgG were purified from antiserum by using DEAE-cellulose chromatography. These IgG were used to prepare the immunoadsorbent column and perform chromatography. The fast immunoaffinity-based purification would help to minimize the detrimental effects exerted by phosphatases and proteases encountered during the process of extraction. Immunoadsorbent chromatography is one of the fastest ways of obtaining the enzyme and detrimental effects are minimized.

The organic cosolutes, glycerol and PEG, have been shown to stabilize the activity and integrity of several enzymes, including PEPC, during adverse conditions of extraction (Selinioti et al, 1987; Rhodes and Hanson. 1993; Drilias et al., 1994; Podestá and Plaxton. 1994; Law and Plaxton, 1995). Although the reaction media containing organic cosolutes are not physiological, they resemble an environment closer to the conditions *in vivo,* than those which are associated with the extensive dilution that occurs during extraction and assays. The organic cosolutes promote self association of proteins and stabilize their structure by being preferentially excluded from contact with the protein surface (Timasheff, 1992). Under such a high protein concentration, the stability of oligomeric enzymes is enhanced. The interaction of compatible solutes on the stability and oligomeric status of the enzyme was studied in presence of PEG or glycerol or both by using gel filtration chromatography.

The uniqueness of C4-PEPC is that the enzyme in leaves is markedly regulated by light. The activity of PEPC in leaves of C4 plants is activated, by two to three fold by light, compared to that in dark-adapted ones. The kinetic and regulatory properties of PEPC are also modulated by light. We have therefore studied the kinetic and regulatory properties of PEPC in illuminated leaf samples and the interaction between the increased affinity for bicarbonate and light.

In spite of the extensive literature on the modulation of malate-sensitivity of the enzyme, the studies on changes in kinetic characteristics of PEPC, and in particular the affinity to HCO_3^- are quite limited (O'Leary, 1982; Chollet et al., 1996). One of the likely reasons is the practical difficulty in the complete removal of dissolved bicarbonate during PEPC assays. We have also examined the kinetic and regulatory properties of PEPC at two concentrations of bicarbonate: low or limiting (0.05 mM) and high or saturating (10 mM). Low bicarbonate (0.05 mM) reflects the physiological environment of the cell, while most of the literature on PEPC, used 10 mM bicarbonate.

Phosphorylation of PEPC is regulated by light/dark transitions *in vivo* (Rajagopalan et al.. 1994; Chollet et al., 1996; Vidal and Chollet, 1997). Phosphorylation of PEPC occurs on serine residue during illumination by protein kinase and dephosphorylation occurs during darkness by type 2A protein phosphatase. *De novo* synthesis of PEPC-PK is an important component during PEPC phosphorylation. Cytosolic pH and calcium are important factors during light activation and phosphorylation of PEPC (Pierre et al., 1992). However, the mechanism of action of calcium and its interaction with pH during PEPC phosphorylation is ambiguous.

The effect of calcium on PEPC and PEPC-PK activity were re-examined critically in the present work. As per the present results, Ca^{2+} inhibits PEPC at high concentration and such inhibition is due to competition with Mg^{2+} (Mukerji, 1977;

Gavalas and Manetas, 1980b; Gayathri and Raghavendra, 1994). Ca^{2+} also acts as a stabilizer at low concentration. Therefore, we have studied the mechanism of the effect of calcium on PEPC activity.

Experiments were conducted to examine the phosphorylation of PEPC using both direct and indirect methods. An indirect method is to incubate the crude extracts with ATP and $MgCl_2$ and determining the extent of activation of PEPC after incubation, whereas in direct method, the incorporation of ^{32}P into PEPC *in vitro* is monitored. Incubation of crude extracts prepared from illuminated leaf discs with ATP and $MgCl_2$ stimulated the activity and decreased the malate sensitivity. Indirect way was used first to check the effect of Ca^{2+} on the extent of phosphorylation of PEPC. In both direct and indirect methods phosphorylation of PEPC was observed with extracts prepared from only illuminated leaves but not from the dark-adapted ones. Further experiments were designed to evaluate the involvement of calcium and calmodulin as secondary messengers during the phosphorylation of PEPC.

It has been proposed that a CDPK or Ca^{2+}/CaM-dependent protein kinase (CCaMK) may be involved in an upstream-regulation of PEPC-PK (Vidal and Chollet, 1997). An attempt was therefore made to study the characteristics of CDPK or CCaMK in the leaf extracts *of Amaranthus hypochondriacus.* The kinase activity was monitored with different substrates related to CDPK or CCaMK. The cross reactivity of proteins in leaf extracts prepared from C4-, C3-C4 and C3 plants with anti-CCaMK antiserum raised against purified CCaMK from etiolated maize coleoptiles was examined.

The specific objectives of the present study are:

1. To evolve protocols for purification of PEPC from the leaves of *Amaranthus hypochondriacus* by conventional method as well as immunoadsorbent chromatography.

2. To study the effect of compatible solutes on PEPC and its interaction with stability and oligomeric status of the enzyme.

3. To observe the pattern and consequences of light activation of PEPC and to study the modulation by the affinity of PEPC to bicarbonate.

4. To assess the effect of bicarbonate on the catalytic and regulatory properties of PEPC.

5. To reevaluate the response of PEPC to calcium in crude and desalted extracts of PEPC.

6. To examine the effect of Ca^{2+} and Ca^{2+}-chelators on phosphorylation of PEPC.

7. To investigate the PEPC phosphorylation pattern in presence of typical inhibitors or activators of different types of protein kinases: BAPTA and EGTA (Ca^{2+} chelators); trifluoperazine and W7 (Calmodulin antagonists); Ca^{2+}, Phosphatidyl serine and diacylglycerol (Protein kinase C activators); H7 (Protein kinase C inhibitors); staurosporine (CaM-dependent kinase inhibitor); Ca^{2+} and calmodulin (CaMK and MLCK activators); and ML_7 (MLCK inhibitor).

8. To examine the properties of possible CDPK or CCaMK in leaf extracts of *Amaranthus hypochondriacus.*

Chapter 3

Materials and Methods

Chapter 3

Materials and Methods

Plant material

Plants of *Amaranthus hypochondriacus* L. (cultivar AG-67) were raised from seeds, supplied by National Botanical Research Institute, Lucknow (Courtesy: Dr M. Pal, Cytogenetics Division). The plants were grown in 25-cm earthen pots filled with soil supplemented with farm-yard manure (in a ratio of 5:1). They were grown outdoors in the field under a natural photoperiod of approximately 12 h and temperature of 30-40°C day/25-30°C night. The upper fully expanded leaves of 4- to 6-week-old plants (Plate 3.1) were harvested, about 2 - 3 h after sunrise.

In one experiment, the leaves of pea (*Pisum sativum* L., cv. Arkel) (from 8- to 10-day-old plants, raised from seeds), *Alternanthera sessilis* (L.) R. Br. *ex* DC (C3 species), *Alternanthera tenella* Colla (C3-C4 intermediate) and *Alternanthera pungens* H.B.K (C4 species) were used. Plants *of Alternanthera* species (3- to 4-week-old) were propagated by transplantation of cuttings. These plants also were grown in the field, as described in the above paragraph.

Preparation of leaf discs

Discs of *ca.* 0.2 cm^2 were cut from leaves under water with the help of a sharp paper punch. Thirty discs were kept in a Petri dish (5-cm diameter) containing 10 mL water and left in darkness for 2 h. In experiments involving light-activation the predarkened discs were illuminated as described in the following page.

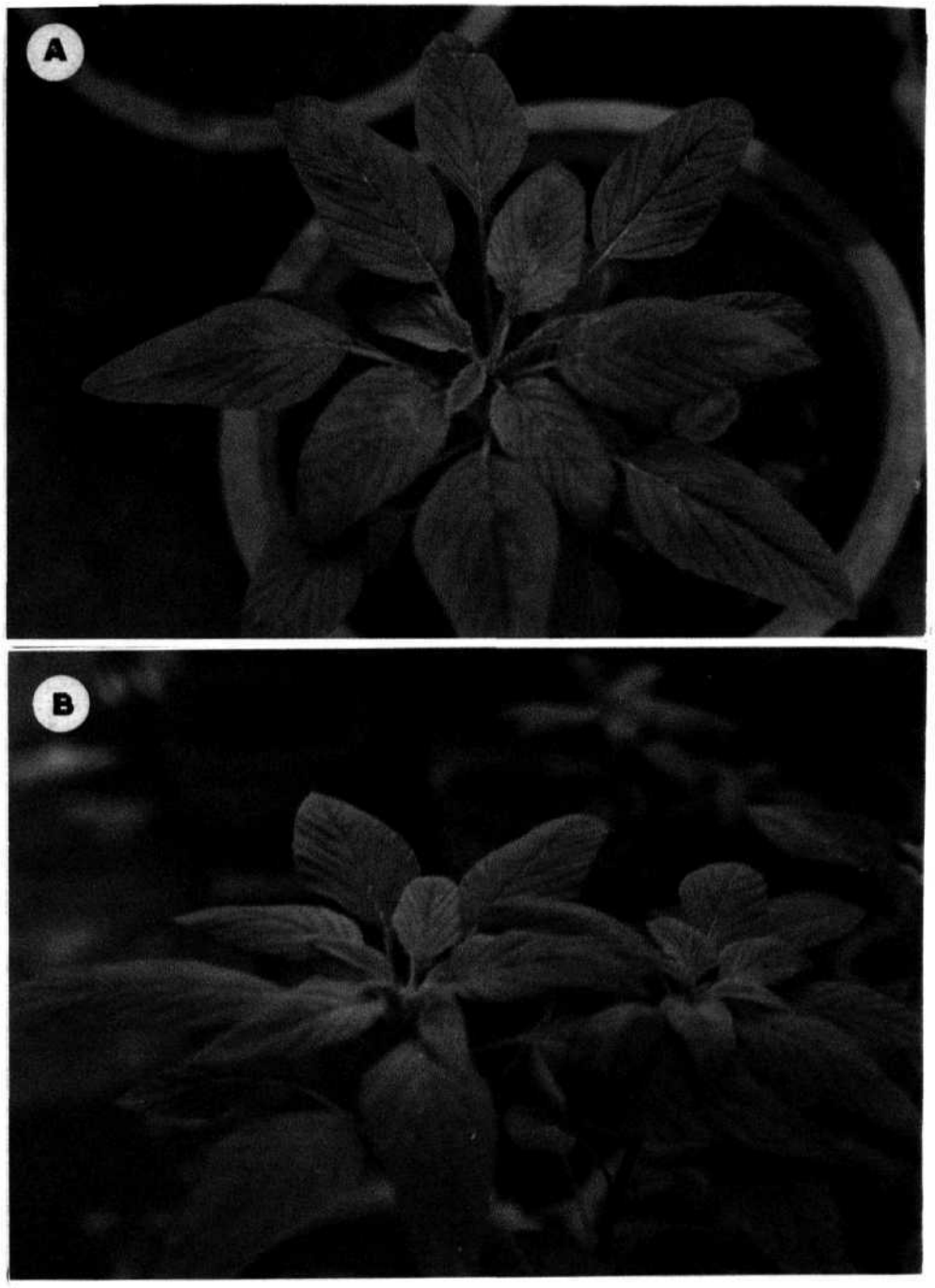


Plate 3.1. A view of 4- to 6-week-old plants of *Amaranthus hypochondriacus* (cultivar AG-67) grown in the field (outdoors). (A) top- and (B) side-views.

Extraction

Thirty leaf discs *(ca.* 120 mg) were extracted in a chilled mortar with **a** pestle using 1 mL of extraction medium containing 100 mM HEPES-KOH, pH 7.3, 10 mM MgCl2, 2 mM K2HPO4, 1 mM EDTA, 10% (v/v) glycerol, 10 mM β-mercaptoethanol, 10 mM NaF, 2 mM PMSF and 2% (w/v) insoluble PVP. The homogenate was centrifuged at 7000*g* for 5 min. The supernatant was used as "crude extract". In some of the experiments. 500 µL of crude cleared extract was rapidly desalted on a Sephadex G-25 column (0.5 x 2 cm) equilibrated with the above buffer.

A small aliquot was kept aside, prior to centrifugation for chlorophyll estimation.

Light activation of PEPC

Light activation of PEPC in leaf discs was carried out, as described already (Rajagopalan et al., 1993). Thirty leaf discs (each *of ca.* 0.2 cm^2) were floated on 10 mL of water in a 5-cm diameter Petri dishes under darkness for 2 h. After predarkening. the leaf discs were illuminated (white light; Philips Comptalux R95 flood bulbs) at an intensity of 1000 µmol m^{-2} s^{-1} (after passing through a water filter of 10-cm thickness) for 30 min. The 10-cm thick water filter helped to dissipate the heat and to maintain an optimal temperature, during illumination.

At the end of 30 min-illumination (or dark-incubation for comparison), the leaf discs were extracted (as described above) for studies on PEPC.

Assay of PEPC

The reaction of PEPC was coupled to NAD malic dehydrogenase and the enzyme activity was determined by monitoring NADH oxidation at 340 nm in a Shimadzu UV-Vis Spectrophotometer at 30°C.

The assay mixture (1 mL) contained 50 mM HEPES-KOH, pH 7.3 or pH 7.8, 5 mM $MgCl_2$, 0.2 mM NADH, 2 U MDH, 0.5 mM or 2.5 mM PEP, 10 mM $NaHCO_3$ (unless otherwise mentioned) and leaf extract (equivalent to 1 μg of chlorophyll or protein). The extract or the purified enzyme was incubated in the assay medium for 30 s and reaction was started by the addition of PEP.

Malate sensitivity of PEPC in crude/desalted extracts or purified preparation was routinely checked using 0.5 mM malate. After measuring the PEPC activity for 3 min, 0.5 mM L-malate was added to the cuvette and the activity monitored for further 3 min. Variations, if any, in the assay medium are described in the text.

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

Estimation of protein and chlorophyll

The total soluble protein was estimated by using either Bradford's reagent (Bradford, 1976) or Folin-Phenol reagent (Lowry et al., 1951), with bovine serum albumin as a standard.

Chlorophyll was estimated by extraction with 80% 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:

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

Cycloheximide (CHX) feeding

For some experiments, thirty leaf discs were floated on 5 μM CHX or water (control) in 5-cm Petri dishes and kept in darkness for 4 hours. These discs were

then **either left** in darkness for a further 30 min or exposed to light for 30 **min at 1000** μmol nr^2 s^{-1}. A stock solution of 10 mM CHX was prepared in absolute ethanol and diluted to 5 μM CHX with distilled water.

Kinetic characteristics of the enzyme

The maximum velocity of the enzyme (V_{max}) and K_m for PEP, Mg^{2+} and HCO3" were determined in the presence or absence of solutes/effectors. PEPC activity was measured at varying concentrations of PEP (0.5 to 5 mM). Similarly, K_m for Mg^{2+} and K_m for HCO3" were measured at different concentrations of $MgCl_2$ (0.5 to 10 mM) and NaHCC>3 (0 to 10 mM). The reaction was started by the addition of PEP. V_{max} and K_m values were calculated from Lineweaver-Burk or Eadie-Hofstee plots.

The response of PEPC to varying concentrations of malate (0.01 to 2.5 mM) was monitored at pH 7.3 and 2.5 mM PEP. The Kj (malate) was determined by using computer program developed by Brooks (1992).

The activation of PEPC by 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) was added instead of malate in the assay medium and PEPC was assayed at pH 7.3 and 0.5 mM PEP. K_a (Glc-6-P) values were calculated from double reciprocal plots.

Purification of PEPC by conventional method

Extraction and ammonium Sulfate fractionation

Leaves (40 g) of *Amaranthus hypochondriacus* picked from the field-grown plants (exposed to sunlight for 2-3 h) were washed, chapped and suspended in 160 mL of buffer containing 100 mM phosphate buffer pH 7.2, 25% (v/v) glycerol, 5 mM DTT, 10 mM $MgCl_2$, 1 mM EDTA, 2 mM PMSF, 50 μg/mL chymostatin,

5 μg/mL leupepetin, 10 mM NaF and 2% (w/v) solid insoluble PVP was added to the medium. The leaves were then homogenized using a blender (1.5 min; maximum speed, Remi). The homogenate was filtered through a 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% (v/v) saturation by further addition of saturated ammonium Sulfate solution and the precipitate was collected by centrifugation at 40,000*g* for 30 min.

The above procedure was 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% (v/v) ammonium Sulfate treatment was dissolved in 15-20 mL of 200 mM potassium phosphate buffer (pH 7.2) plus 10% (v/v) glycerol and was dialyzed against 20 mM potassium phosphate buffer (pH 7.2) 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 7.2) 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 7.2) containing 10% (v/v) glycerol was used to elute PEPC. The active fractions containing maximum PEPC activity were pooled (23 mL) and solid ammonium Sulfate was added to a final concentration of 0.5 M.

Phenyl-Sepharose column

The above enzyme suspension (in 0.5 M ammonium Sulfate) was loaded onto phenyl-Sepharose column (1 x 12 cm), equilibrated with 20 mM phosphate buffer (pH 7.2) containing 10% (v/v) glycerol and 0.5 M ammonium Sulfate. The column was washed with same buffer at a flow rate of 0.5 mL min^{-1} until A_{280} returned to baseline. A co-linear gradient of 0.5 M ammonium Sulfate and 0% ethylene glycol to 0 M ammonium Sulfate and 50% ethylene glycol in 20 mM phosphate buffer (pH 7.2) 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 7.2) containing 10% (v/v) glycerol, and dialyzed against 20 mM phosphate buffer (pH 7.2) 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 calcium chloride and 0.5 M disodium hydrogen phosphate from separate burettes were mixed dropwise in a beaker containing 2.5 mL of 1 M NaCl. A flow rate of 4 mL/min 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 just boil (avoid boiling). The gel (HAP) was washed with 20 mM phosphate buffer (pH 7.2) 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 7.2) 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 7.2) 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 liquid nitrogen.

Electrophoresis

SDS-Polyacrylamide *Gel Electrophoresis (SDS-PAGE)*

Mini-gels (8 cm long x 8 cm wide) of 10% SDS-polyacrylamide were used and electrophoresis was performed, as per the principles of Laemmli (1970). The stacking gel (2 cm long x 8 cm wide) contained 125 mM Tris-HCl (pH 6.7), 4% of acrylamide, 0.1% (w/v) of SDS. The resolving gel (6 cm long x 8 cm wide) was polymerized using 375 mM Tris-HCl buffer pH 8.8, 10% 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% (v/v) 2-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 a total period of about 2 h. The gels after the electrophoresis were fixed for 1 h with fixative solution containing 40% (v/v) methanol and 7% (v/v) glacial acetic acid.

The gels were 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.

Some of 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 prctreated 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.

Non-denaturing PA GE

Native gel was run as described by Davis (1964). A 10% polyacrylamide gel (8 cm long x 8 cm wide) was polymerized without SDS, using only 375 mM Tris-HCl buffer (pH 8.8). 4% acrylamide without SDS was used for stacking gel. The polymerized gel was cooled at 4°C before loading the protein.

The electrode buffer contained 25 mM Tris-HCl, 192 mM glycine, pH 8.3 and electrophoresis performed at 4°C. Electrophoresis was performed at 60 volts until the dye front migrates into the resolving gel (approximately for 1 h) and then power supply was raised to 120 volts through Atto Digi-Power (SJ-1081) for about 3 h. 20 µg of purified PEPC was loaded into each of the well.

Activity staining of PEPC on acrylamide gels

Activity staining for PEPC was carried out at 30°C as described by Nimmo and Nimmo (1982).

After the native gel was run, glycine (which may inhibit PEPC) was removed from the gels by presoaking them in 50 mM Tris-HCl (pH 8.0). 0.1% (v/v) 2-mercaptoethanol and 10 mM $CaCl_2$ for 10 min. The staining mixture contained 50 mM Tris-HCl (pH 8.0). 5 mM $MgCl_2$, 2 mM PEP, 10 mM $NaHCO_3$. 0.1% (v/v) 2-mercaptoethanol and 10 mM $CaCl_2$ at 30°C. The gel was incubated for about 30 min at room temperature with the staining mixture. On reaction with PEPC, white bands appeared on the gel.

The PEPC (active-form) present in the gel on reaction with PEP and $NaHCO_3$ catalyses the formation of oxalacetate and Pi. The released Pi reacts with $CaCl_2$ present in the medium and forms calcium phosphate which appears as white bands on the gel. Control sets were run without PEP in the staining mixture.

Gel filtration on Sephadex G-200

Sephadex G-200 was used to assess the molecular size of PEPC. Purified PEPC (approximately 100 µg protein) was loaded onto a Sephadex G-200 column (1 x 30 cm) with or without 1.25% PEG or 10% glycerol or both.

The standard buffer used for all gel filtration experiments was 50 mM HEPES-KOH (pH 7.3) with or without PEG and/or glycerol, as mentioned in text. The column was washed and equilibrated with 50 mM HEPES-KOH (pH 7.3) with additions as described. The flow rate was 10 mL/h at 30°C. Fractions of 1 mL were collected and the elution pattern of PEPC was checked by assaying the enzyme activity and/or A_{280} for protein.

The column was calibrated by using four protein-standards: thyroglobulin (669 kD), apoferritin (443 kD), alcohol dehydrogenase (150 kD), BSA (66 kD). The void volume V_O, was determined by using blue dextran (2,000 kD).

Immunological characteristics of PEPC

*Preparation of **anti-PEPC antiserum***

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

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, 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 uL 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.

Ouchterlony double-dimensional diffusion

The reactivity of anti-PEPC antiserum was checked by using 1% (w/v) agarose gels prepared on glass microscope slides. Five wells (0.5-cm diameter) were punched in agarose gels, with the help of a sharp gel-punch. One well was in the center and was surrounded by four wells located symmetrically in the outer ring. A graph sheet was placed below the glass slide, so as to achieve symmetry and precise distance between wells.

In the center-well, the purified PEPC or leaf extract from *A. hypochondriacus* was loaded. Different dilutions of crude anti-PEPC antiserum (1/20 to 1/150) were loaded into other four wells in the outer ring. The precipitin band was observed within 24 h of incubation at 4°C. The reaction was stopped by washing the gel several times with 0.9% (w/v) NaCl, to remove unbound proteins. Non-immunized serum showed no cross-reaction (i.e., no precipitation) with either purified PEPC or leaf extracts of *A. hypochondriacus.* The titer value of the enzyme was determined by using Ouchterlony double diffusion.

Immunoprecipitation of PEPC in leaf extracts

The efficacy of anti-PEPC antiserum was checked by performing immunoprecipitation, as described by Vidal et al. (1983).

The leaves of *Amaranthus hypochondriacus* were extracted as described above for PEPC assay. The leaf extracts were cleared by centrifugation for 5 min at 40,000*g* and the supernatants were assayed for PEPC. Extract containing 0.2 Units of PEPC-activity (approximately 300 μL) was mixed with different volumes of anti-PEPC antiserum (0 to 100 μL) solution and left overnight at 4°C. The mixture was later centrifuged for 5 min at 10,000*g*. The supernatant was assayed for PEPC activity, while the pellet was checked for PEPC-protein by SDS-PAGE. The control sets were run with the pre-immune serum from the same rabbits.

The pellet was washed twice with 0.5 M Tris-HCl, pH 8.0, 1.5 M NaCl and 1% (v/v) Triton X-100, and once with 0.1 M Tris-HCl, pH 8.0. The pellet was finally suspended in SDS sample buffer, boiled at 100°C for 2 min and were subjected to SDS-PAGE (Laemmli, 1970). These details are already described.

Western blotting of PEPC

The cross reactivity of PEPC to antibodies was checked by employing Western blots (Betz and Dietz, 1991), after transferring electrophoretically the proteins from the gel onto the polyvinylidene difluoridc (PVDF) membranes (Towbin et al., 1979).

Leaf extracts or purified PEPC were prepared and subjected to 10% SDS-PAGE, as described above. The proteins were transferred onto PVDF membranes (Immobilon-P, from Millipore, procured from Sigma Chemical Co., USA). The gel, PVDF membranes and Whatman No. 3 chromatography papers were soaked in transfer buffer containing 25 mM Tris-HCl/192 mM glycine (pH 8.3) and 20% (v/v) methanol for 30 min. The gel and membranes were sandwiched between the Whatman No. 3 filter papers (three on each side) saturated with the buffer and blotted using a semi-dry blotter (LKB 2117 Multiphor) for 2 h. A constant power of 90 volts was supplied (through Atto Digi-Power SJ-1081). The transfer of proteins

was confirmed by Ponceau's staining [0.2% (w/v) Ponceau's stain and 3% (w/v) TCA]. Ponceau's stain was removed by repeated washing with distilled water.

The membranes were blocked to saturate the non-specific binding sites with 5% (w/v) non-fat milk powder in Tris-buffered saline (TBS) containing 25 mM Tris-HCl (pH 7.5) and 150 mM NaCl. The blocking was allowed for 1 h at room temperature with constant shaking. The blocked membranes were treated with *A. hypochondriacus* anti-PEPC antiserum (diluted by 1:1000 in blocking solution), for 1 h. The blotted membranes were washed three times (15 min of each wash) with TBS and incubated with anti-IgG-alkaline phosphatase conjugate (1:7500) for one hour and washed for three times.

The washed blot was developed with 16.5 μL 5-bromo-4-chloro-3-indolylphosphate (BCIP) (50 mg mL^{-1} stock solution) and 30 μL of *p*-nitroblue-tetrazolium chloride (NBT) (50 mg mL^{-1} stock solution) in 10 mL of 16 mM Tris-HCl (pH 9.5), 4 mM NaCl and 0.2 mM $MgCl_2$.

Western blotting of protein kinase

For the detection of protein kinase in crude leaf extracts of C3-, C3-C4 and C_4 plants, the total protein was separated on 10% SDS-PAGE and transferred onto a PVDF membrane. The PVDF membranes were treated with 1:5000 dilution of *ant\-(Zea mays* CCaMK) IgG (provided by Prof S.K. Sopory, ICGEB, New Delhi). Further steps were followed as described above.

Western blotting of ***calmodulin-binding*** *proteins*

Calmodulin binding proteins in crude leaf extracts *of Amaranthus* was done according to Billingsley et al. (1985). The total protein in crude leaf extracts was separated on 10% SDS-PAGE and transferred onto a PVDF membrane.

Western blotting was done in the lab of Prof C. Subramanyam, Department of Biochemistry, Osmania University, Hyderbad. The membrane was blocked with buffer A (5% (w/v) non-fat milk powder in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl and 1 mM $CaCl_2$) for 1 h at room temperature and washed with TBS. Then the blot was incubated with 5 μg of biotinylated CaM in 2.5 mL of buffer A for 30 min. After the incubation, the blot was washed three times with buffer A for 30 min (10 min each wash). Then, the blot was treated with avidin-alkaline phosphatase in buffer A for 30 min. The blot was washed for three times with buffer A to remove unbound avidin. A mixture of BCIP (100 μL of 50 mg/mL in 50% dimethylformamide) and NBT (200 μL of 50 mg/mL in 50% dimethylformamide), both dissolved in 30 mL of 0.1 M Tris-HCl, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$, was used as the chromogen to develop the color.

***Enzyme-linked immunosorbent** assay (ELISA)*

Quantitative estimates of PEPC-protein *of A. hypochondriacus*, were made by the technique of ELISA, as per the procedure of Gillina and Greally (1993).

PEPC was diluted (ng level) in coating buffer (0.05 M carbonate buffer, pH 9.6). The ELISA plates (96 flat bottomed wells, Tarsons Products) were coated with 100 μL of the above PEPC in coating buffer in each well (in triplicates) and were left overnight at 4°C. Simultaneously triplicate wells were maintained as enzyme and substrate blanks. The unbound antigen from each well was discarded, after overnight incubation. All the wells in the plates were washed four times (250 μL per well per wash) with TBS. Then they were blocked by using 200 μL of blocking solution (containing 5% non-fat milk powder in TBS) and left for 1 h at room temperature with constant shaking. Later the wells were washed 5 times with TBS and were treated with 100 uL primary antibody (diluted by 1:3000) (anti-PEPC antiserum from *A. hypochondriacus)* in blocking buffer in each well for 2 h. The wells were washed again 5 to 6 times with coating buffer and incubated for 1 h

in secondary antibody conjugated to alkaline phosphatase (1:7500 in blocking buffer). The wells were thoroughly washed (5 or 6 times) with 200 μL of TBS containing Tween-20 and developed by adding 150 μL *p*-nitrophenylphosphate (1 mg/mL stock in coating buffer containing 5 mM $MgCl_2$) to each well. The plate was left at 37°C until the color developed and the reaction was stopped by the addition of 50 μL of 3 M NaOH.

The color was read at 405 nm using an ELISA-Plate Reader (Molecular Devices, USA, Model UVT-06050).

Purification of PEPC** by **immunoadsorbent column

Purification of IgG

Anti-PEPC antiserum (3.5 mL) was dialyzed for 2 days at 4°C against 5 mM sodium phosphate buffer, pH 7.0 (with four changes). The dialyzed antiserum was loaded onto DEAE-cellulose (1 x 12 cm) column, equilibrated with the above buffer. IgG were eluted with a linear gradient of 5 mM sodium phosphate buffer, pH 7.0 to 5 mM sodium phosphate buffer, pH 7.0 and 50 mM NaCl. IgG were eluted immediately after the void volume. Cross reactivity was checked with Ouchterlony double diffusion and Western blotting.

Preparation of Immunoadsorbent Column

The coupling of anti-PEPC IgG to CNBr-activated Sepharose was carried out by the method described in the practical guide.

CNBr-activated Sepharose (1 g) gel was washed with coupling buffer (0.1 M bicarbonate buffer, pH 8.4 containing 0.5 M NaCl). IgG (10 mg) was mixed with the swollen gel in a tube (capped) and mixture was rotated end over end at 4°C overnight. The coupled gel was gently centrifuged at 100*g* for 2 min and the supernatant was saved. The coupled gel was then treated with 10 mL coupling

buffer **to** remove the unbound material and again the supernatant was saved. The **gel was treated** with 1 mM ethanalamine (pH 8.0) at room temperature for 1 h by end over **end** rotation to remove remaining groups. The supernatant after this reaction was also saved.

Three washing cycles were used to remove non-covalently adsorbed protein to the gel each cycle consisting of an acid wash (15 mL of 0.1 mM acetate buffer, pH 4.0 plus 1 M NaCl) and an alkali wash (15 mL of 0.1 mM borate buffer, pH 8.0 containing 1 M NaCl). The supernatant obtained at each wash was separately saved. Finally the gel was suspended in 50 mM phosphate buffered saline (containing 0.15 M NaCl, pH 7.4) containing 0.05% sodium azide and stored in the refrigerator.

The amount of protein in the supernatants was determined by recording their A_{280}, against the corresponding blanks (buffer). The protein coupled to the gel was calculated by deducing the sum of the OD values of the supernatant from the total OD of the protein used for coupling. By this procedure about 90% of the protein was found to be coupled to the gel.

Immunoadsorption chromatography

Amaranthus leaves (7 g) were homogenized in extraction buffer. Extraction and 40-60% ammonium Sulfate precipitation were carried out as described above. The pellet was dissolved in 50 mM potassium phosphate buffer, pH 7.4 containing 0.15 M NaCl (1.6 mL). This suspension was spun at 40,000*g* for 30 min. 1.6 mL of cleared supernatant was loaded onto the immunoadsorbent column (1 x 4.5 cm), equilibrated with 50 mM potassium phosphate buffer, pH 7.4 containing 0.15 M NaCl.

PEPC was eluted with two step washing procedure with chilled 20% glycerol in distilled water. The first peak of activity was obtained by elution with **about** three column volumes (11 mL). The elution was arrested for 45 min before

restarted. PEPC was eluted as a sharp peak containing 42 U mg^{-1} protein. Active fractions were pooled and 100 mM HEPES-KOH (pH 7.3) buffer was added and concentrated with PEG 20,000. Purified PEPC was stored as small aliquots in liquid N_2. Purity was checked by 10% SDS-PAGE and stained with silver nitrate.

Interaction of PEPC with PEG and/or glycerol during assay and/or extraction

The effect of PEG or other compatible solutes on PEPC was checked by incubating purified PEPC (2 μg mL^{-1}, unless otherwise specified) with different concentrations of PEG-6000 and glycerol. The stock solutions (50%. w/v) of PEG were prepared in distilled water. The purified enzyme or the extract was left in 1 mL of the assay medium (± solutes) for 30 s and reaction was started by the addition of 50 μL of 50 mM PEP. The reaction was linear up to 5 min with purified enzyme.

In the another set of experiments, leaf discs were extracted without or with PEG or glycerol or both, as indicated. The details of the extraction of leaf discs are already described. PEPC activity was examined by including PEG-6000 during extraction alone, assay alone or during both extraction and assay.

Phosphorylation of PEPC and assay of PEPC-protein kinase **(PEPC-PK)**

Phosphorylation of PEPC in leaves was studied both indirectly and directly.

On incubation of leaf extracts (containing PEPC) with 1 mM ATP and 5 mM $MgCl_2$, the activity of PEPC increases while the inhibition of enzyme by malate decreases, both due to the phosphorylation of PEPC. Thus, the increase in PEPC activity/decrease in malate sensitivity after incubation with Mg-ATP reflects indirectly the extent of phosphorylation of PEPC.

The extent of PEPC phosphorylation (and thus PEPC-PK activity) can be assessed directly by using $AT^{32}P$. The leaf extracts containing PEPC are incubated

in vitro with $AT^{32}P$. The amount of radioactive label incorporated in the PEPC-protein is determined after stopping the reaction followed by separation of proteins on SDS-PAGE and autoradiography.

Indirect assay of PEPC-PK(ATP-dependent activation of PEPC)

Leaf discs after illumination (at a light intensity of 1000 μmol m^{-2} s^{-1} for 30 min; details described already) were extracted with a medium containing 100 mM HEPES-KOH, pH 7.8, 5 mM $MgCl_2$, 2 mM K_2HPO_4, 1 mM EDTA, 10% (v/v) glycerol, 10 mM β-mercaptoethanol and 2 mM PMSF. The homogenate was centrifuged at 7000*g* for 5 min. Phosphorylation was initiated by the addition of 1 mM ATP and 5 mM $MgCl_2$ to crude/desalted extract and the mixture was left at 30°C. Aliquots (5 μL) were withdrawn at the required time and assayed for PEPC activity.

The response to calcium of PEPC and PEPC-PK activity (indicated by the stimulation of PEPC by ATP-incubation) was assessed by adding various concentrations of calcium (0-20 μM) to the medium during the incubation with ATP + $MgCl_2$ (for 60 min, unless otherwise specified). Different concentrations of test compounds were added during incubation, as indicated.

In vitro *phosphorylation of purified PEPC by protein kinase A*

The reconstituted phosphorylation medium contained 100 mM HEPES-KOH (pH 7.3), 20% glycerol, 0.5 units of dark-form of purified PEPC from *Amaranthus* leaves, 10 units of the catalytic subunit of a cAMP-dependent protein kinase (PKA from Sigma), 5 mM $MgCl_2$ and 1 mM ATP in a total volume of 60 μL. The phosphorylation reaction was allowed to proceed for up to 120 min at 30°C. Besides the enzyme velocity, K_{m} (HCO_3^-) and Kj (malate) of the enzyme were determined at the specified times after the start of incubation.

In some of the experiments, two recombinant PEPC-forms from maize (wild type, WT; or mutant, S15D - Ser15 replaced by Asp) cloned and expressed in *E.coli* were used (Ueno et al., 1997).

In vitro *phosphorylation of PEPC with* $AT^{32}P$

In vitro phosphorylation of PEPC was performed for 60 min at 30°C according to Jiao and Chollet (1992).

The phosphorylation mixture (60 µL) contained 0.1 M Tris-HCl (pH 7.5), 20% (v/v) glycerol, 20 µL of leaf extract (60 fig of protein), 3 µg of purified dark-form PEPC, 10 mM $MgCl_2$, 4 mM phosphocreatine, 10 units creatine phosphokinase, 0.25 mM p^1,p^5-di (Adenosine 5') pentaphosphate (AP_5A), 5 mM DTT, 10 mM NaF, 2 mM PMSF and 100 µM of 15 µCi γ–$AT^{32}P$ (Specific activity of 3000 Ci/mmol). The mixtures were incubated at 30°C for 60 min. The reaction was stopped by addition of 30 µL of anti-PEPC antiserum of *Amaranthus* leaves and left at 4°C for overnight. The immunoprecipitates were processed as described already, were dissolved in 10 µL of SDS sample buffer [0.25 M Tris-HCl (pH 6.8), 8% (w/v) SDS, 50% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol and 0.04% (w/v) bromophenol blue] and were boiled for 2 min at 100°C.

Various concentrations of Ca^{2+}, EGTA or BAPTA or inhibitors were included in the reaction mixture, as required for the experiments.

SDS-PAGE and autoradiography

The soluble proteins were separated by 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 drygel jr (Hoefer Scientific Instruments, 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.

Quantification of autoradiographs

The spots on the autoradiographs were quantified by a computer program "Image Tools" developed by a team of Don Wilcox, Brent Dove, Doss McDavid, David Greer, The University of Texas Health Science Center in San Antonio. The autoradiographs were scanned by a HP scanner. The black areas corresponding to the radioactive spots on the scanned images were selected and the areas were measured. The areas of bands were taken as a measure of kinase activity.

Protein kinase assay

The assays of protein kinase were performed according to Pandey and Sopory (1998), with minor modifications. The standard assay medium (final volume 50 uL) contained 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 0.5 mM DTT, 1 mM PMSF, 50 µM cantharidin, free Ca^{2+} (as mentioned in the text), *Amaranthus* leaf crude extract (about 40 ug of protein), protein substrate [histone HIS (50 µg), dephosphorylated casein (50 µg), syntide-2 (2 ug), BSA (50 µg), MBP (50 µg) or PEPC (10 ug)] and 100 µM of 1 µCi (γ-^{32}P)ATP. Free Ca^{2+} concentrations in the phosphorylation media were maintained with CaCl2-EGTA buffers according to Veluthambi and Poovaiah (1986). In the phosphorylation assay medium, EGTA was maintained at 0.2 mM and total $CaCl_2$ concentrations were varied from 0 to 0.5 mM.

The reactions were initiated with 100 µM (y-32p)ATP and incubated at 30°C for 1 min. The reaction was stopped by spotting 10 µL of reaction mixture on P81 phosphocellulose paper squares (1.5 cm x 1.5 cm) (Whatman) and the cellulose paper was processed as described by Roskoski (1983). These P81 papers were washed thoroughly with 75 mM phosphoric acid for 30 min with frequent changes (about 6 to 7). The papers were dried and placed in scintillation vials containing 5 mL of Bray's scintillation fluid (60 g naphthalene, 4 g PPO, 200 mg POPOP, 100 mL (v/v) methanol, 20 mL (v/v) ethylene glycol in 1 L of 1-4 dioxane) and radioactivity was measured in liquid scintillation counter (Wallac).

Various protein kinase inhibitors were included in the phosphorylation assay medium, as indicated in the text.

Replication

The data presented are the average values (± SE) of results from three to four experiments conducted on different days.

Chemicals/Materials

Most of the biochemicals, unless otherwise specified, were procured from either Sigma (Sigma Chemical Company, St Louis, USA) or Boehringer (Boehringer Mannheim GmbH, Germany). PVDF membranes (Millipore) were obtained from Sigma. DEAE-Sepharose CL-6B, DEAE-cellulose, Phenyl-Sepharose and Sephadex G-200 were from Pharmacia, Sweden. 96-well ELISA plates (Flat bottom wells) were from Tarsons Products, Bombay.

Y[AT32p] was procured from Board of Radiation and Isotope Technology (Jonaki), CCMB campus, Hyderabad. Konica X-ray films from Konica Photofilms Co. X-ray Developer and Fixer from Allied Photographers India Limited.

Several compounds/antibodies were kind gifts from different sources: TFP, from SKF, Bombay; ML_7, from Prof K. Shimazaki, Department of Biology, Kyushu University, Japan; CaM kinase antibodies, from Prof Sopory, ICGEB, New Delhi; wild type and a mutant (S15D, Ser^{15} replaced by Asp) PEPCs (both from maize), from Prof K. Izui, Division of Applied Biosciences, Kyoto University, Japan; cantharidin, from Prof W.M. Kaiser, Julius-von-Sachs-Institut für Biowissenschaften der Universität, Würzburg, Germany; protein kinase A, from Dr A.S. Bhagwat, Molecular Biology and Agriculture Division, BARC, Mumbai; syntide-2, from Dr Aparna Datta Gupta, School of Life Sciences, University of Hyderabad, Hyderabad, and Prof S.K. Sopory, ICGEB, New Delhi; syntide-2, calmidazolium and biotinylated CaM, from Prof C. Subramanyam, Department of Biochemistry, Osmania Univerisity, Hyderabad; ethoxyzolamide and histone IIIS, from Dr K. Sarada Devi, Iowa State University, Iowa, USA.

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

Chapter 4

Purification and Immunology of PEPC-Protein

Chapter 4

Purification and Immunology of PEPC-Protein

Introduction

One of the early attempts of purifying PEPC used peanut cotyledons (Maruyama and Lane, 1962). PEPC has since been purified from leaves of different species like maize, cotton, *Amaranthus viridis*, sorghum, sugarcane, spinach and *Bryophyllum fedtschenkoi* (O'Leary, 1982; Iglesias et al., 1986; Iglesias and Andreo, 1989; Arrio-Dupont et al., 1992).

PEPC has also been purified from green algae, *Selenastrum minutum* and *Chlamydomonas reinhardtii* (Rivoal et al., 1996, 1998) and hyperthermophilic archaeon *Methanothermus sociabilis* (Sako et al., 1996), besides the non-photosynthetic tissues such as latex of *Hevea brasiliensis* (O'Leary, 1982), seeds of *Brassica campestris* (Mehta et al.. 1995), soybean root nodules (Schuller and Werner, 1993; Zhang et al., 1995), avocado fruit (Notion and Blanke, 1993) and banana fruit (Law and Plaxton, 1995).

The steps followed for purification-protocol in most of these cases include ammonium Sulfate fractionation of crude leaf extracts and passing through different column chromatography matrices, such as DEAE-cellulose, phenyl-Sepharose, hydroxylapatite (HAP) and Sephadex (Uedan and Sugiyama, 1976; Iglesias et al., 1986; Iglesias and Andreo, 1989; Schuller and Werner, 1993). However, in recent reports, FPLC (Jiao and Chollet, 1988, 1989; Jiao et al., 1991a) or HPLC (Crétin et al., 1984) have also been used for purification of PEPC.

During purification by gel filtration, the enzyme is eluted by high concentration of salt, often chloride (Hatch and Heldt, 1985; Iglesias et al., 1986) and occasionally phosphate (Hague and Sims, 1980) or acetate (Smith and

Woolhouse, 1984). Chloride, however, can affect the activity of the enzyme (Manetas et al., 1986; Wagner et al., 1987), presumably by inducing dissociation of the tetrameric form into dimers or monomers (Wagner et al., 1987; Manetas, 1990). Phosphate buffer for the extraction of PEPC offers several advantages by preventing the inactivation of the enzyme during storage or dilution (Salahas and Gavalas, 1997). On the other hand, phosphate is considered to be a stabilizer of the enzyme (Yancey et al., 1982). Instead of ammonium Sulfate, polyethylene glycol (PEG) had been used to precipitate the enzyme (Selinioti et al., 1987; Angelopoulos and Gavalas, 1991), since the yield of PEPC by using PEG was reportedly much better than that with ammonium Sulfate. Techniques are now available for rapid purification of enzyme from leaves by immunoadsorbent columns (Vidal et al., 1980; Arrio-Dupont et al., 1992).

The specific activity of purified PEPC, reported in literature, varied from low values of 4-10 U mg^{-1} protein (Hayakawa et al., 1981; O'Leary et al., 1981; Sugiyama et al., 1984; Wedding and Black, 1986) and to as high values as 180-220 U mg^{-1} protein (Mukerji, 1977). The latter high values are presumably in error (O'Leary, 1982). In case of CAM plants, the best specific activity reported in *Kalanchoë daigremontiana* is around 35 U mg^{-1} protein (O'Leary, 1982). Reviewing the available literature, O'Leary (1982) commented that the specific activity of purified PEPC could be around 25 U mg^{-1} protein. Similar range of specific activities have been obtained by several authors in later years (Hatch and Heldt, 1985; Iglesias et al., 1986; Wedding et al., 1988; McNaughton et al., 1989).

This chapter describes the attempts to achieve rapid and efficient purification of PEPC from leaves of *Amaranthus hypochondriacus*, a NAD-ME type of C4 plant. A method employing the conventional methods of ammonium Sulfate precipitation and chromatography on columns was evolved to prepare pure PEPC with one of the highest specific activities reported in literature. Having obtained the

pure enzyme, antibodies **were** raised against the PEPC-protein. These antibodies were employed for further immunological experiments.

Results

Purification of PEPC by conventional method

PEPC was purified to homogeneity from *Amaranthus hypochondriacus* leaves by using the conventional steps of extraction: 40-60% ammonium Sulfate precipitation, followed by successive chromatography through columns of DEAE-Sepharose, phenyl-Sepharose and finally HAP. PEPC eluted from the DEAE-Sepharose column as a broad peak at around 120-150 mM Pi with specific activity of 17.1 U rng-1 protein (Fig. 4.1). When eluted from phenyl-Sepharose column, with decreasing concentration of ammonium Sulfate and increasing concentration of ethylene glycol, enzyme had a specific activity of 24 U mg^{-1} protein (Fig. 4.2). The hydrophobic interaction chromatography using phenyl-Sepharose substantially enriched the PEPC. But the use of HAP helped to improve the purity further. The enzyme was eluted as a single peak at 70-90 mM Pi with an activity of 55 U mg^{-1} protein from HAP column (Fig. 4.3). 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 four steps, PEPC was purified by 138-fold, with a final specific activity of 55 U mg^{-1} protein, and an overall recovery of about 57% (Table 4.1). This is one of the highest activities reported for PEPC from C4 plants. The purity of the enzyme was confirmed by the appearance of a single band of about 100 kD on SDS-PAGE (Plate 4.1).

Another method was tried by avoiding phenyl-Sepharose column chromatography, with only three steps, to purify PEPC from *Amaranthus* leaves.

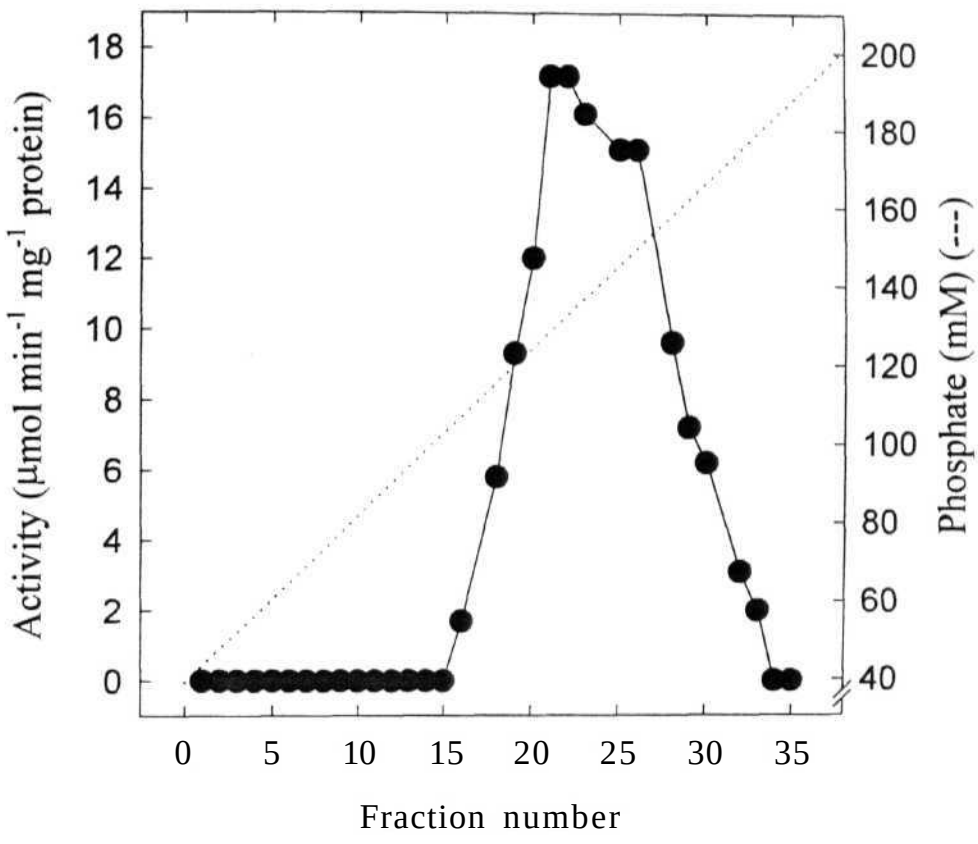


Figure 4.1. The pattern of PEPC activity during elution from a DEAE-Sepharosc column. The enzyme from 40-60% ammonium Sulfate fraction of leaf extract was loaded after dialysis, onto a DEAE-Sepharose (1 x 12 cm) column, preequilibrated with 20 mM potassium phosphate buffer (pH 7.2) containing 10% (v/v) 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. Further details are described in "Materials and Methods".

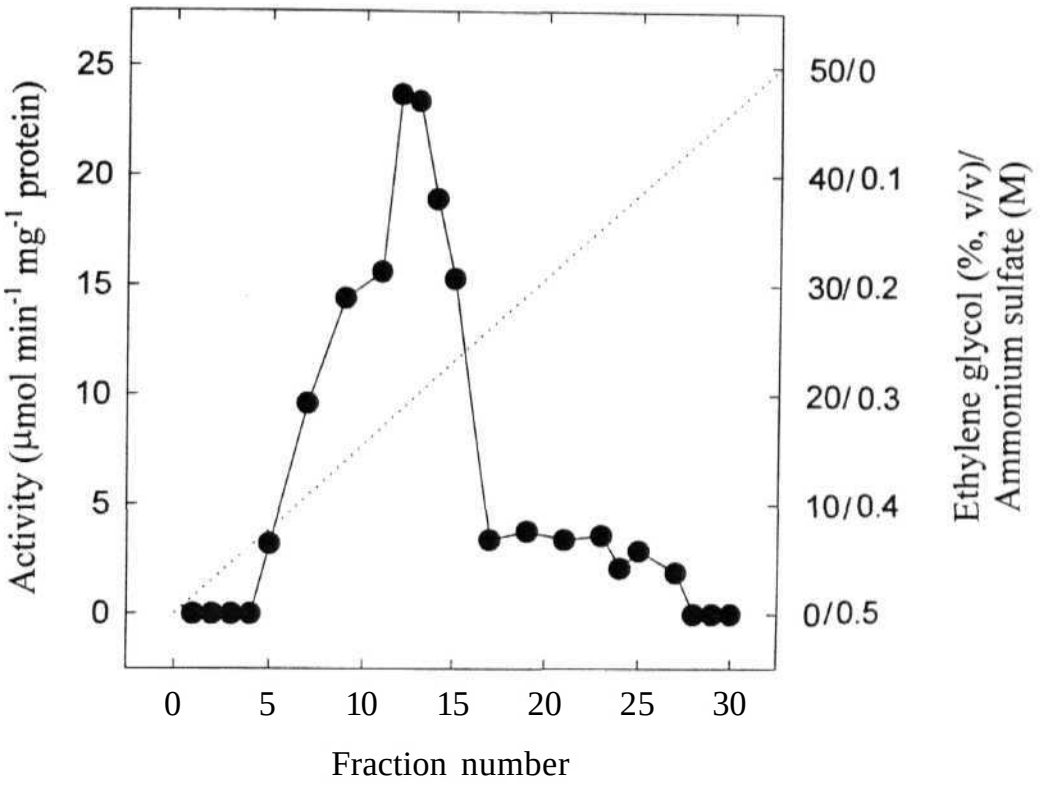


Figure 4.2. The elution profile of PEPC from phenyl-Sepharose column. The active fractions obtained through DEAE-Sepharose column were pooled and ammonium Sulfate was added to a final concentration of 0.5 M. This suspension was loaded onto phenyl-Sepharose column (1 x 12 cm), equilibrated with 20 mM phosphate buffer (pH 7.2) containing 10% glycerol and 0.5 M ammonium Sulfate. PEPC was eluted by employing a co-linear gradient of ammonium Sulfate (0.5-0 M) and ethylene glycol (0-50%) in 20 mM phosphate buffer (pH 7.2) containing 10% glycerol. Further details are described in "Materials and Methods".

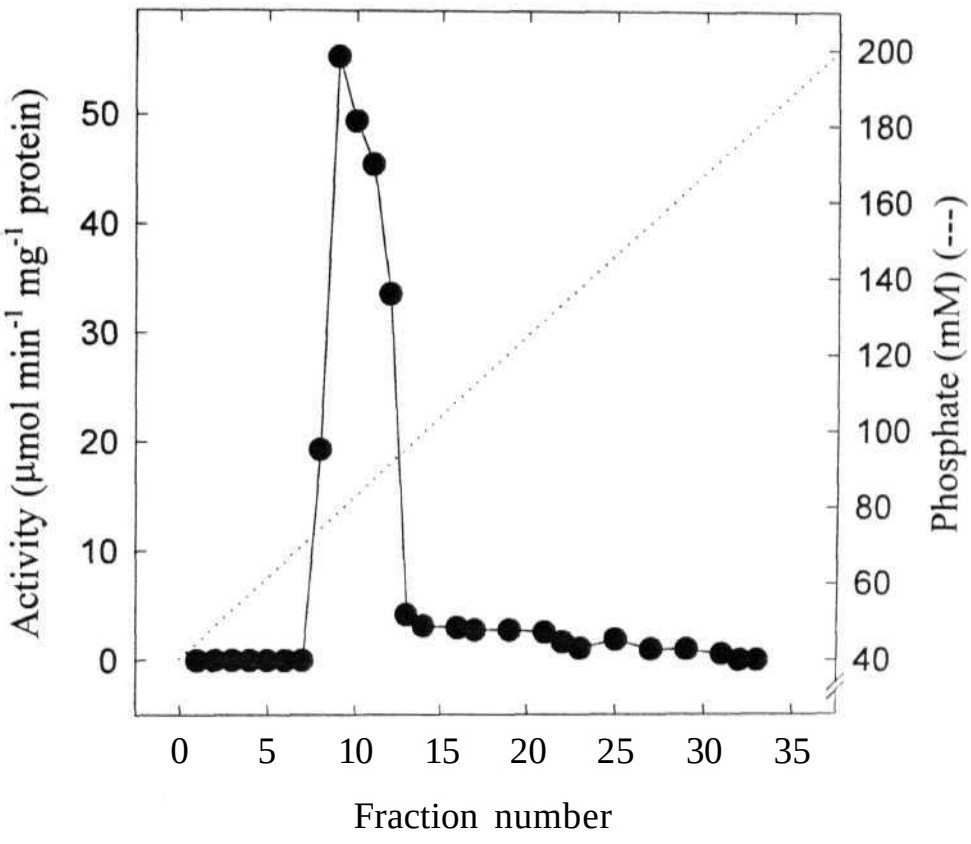


Figure 4.3. The pattern of elution profile of PEPC from hydroxylapatite column. The active fractions obtained from phenyl-Sepharose column were pooled and precipitated by 60% ammonium Sulfate. The precipitate was dissolved in 200 mM phosphate buffer (pH 7.2) containing 10% glycerol and dialyzed against 20 mM phosphate buffer (pH 7.2) containing 10% glycerol. The dialysate was applied onto HAP column (1 x 12 cm), equilibrated with the same buffer. PEPC was eluted with a linear gradient of 40-200 mM phosphate buffer plus 10% glycerol. Further details are described in "Materials and Methods".

Table 4.1. *Purification of PEPC from leaves of* Amaranthus hypochondriacus *by conventional method involving four steps.*

Step	Total Activity	Total Protein	Specific Activity	Purification	Yield
	(μmol min^{-1})	*(mg)*	*(μmol min^{-1} mg^{-1} protein)*	*(fold)*	*(%)*
Crude extract	300	750	0.4	1.0	100
40-60% $(NH_4)_2SO_4$	286	168	1.7	4.3	95
DEAE-Sepharose	258	15	17.2	43	86
Phenyl-Sepharose	213	9	23.7	59	71
Hydroxylapatite	171	3.1	55.3	138	57

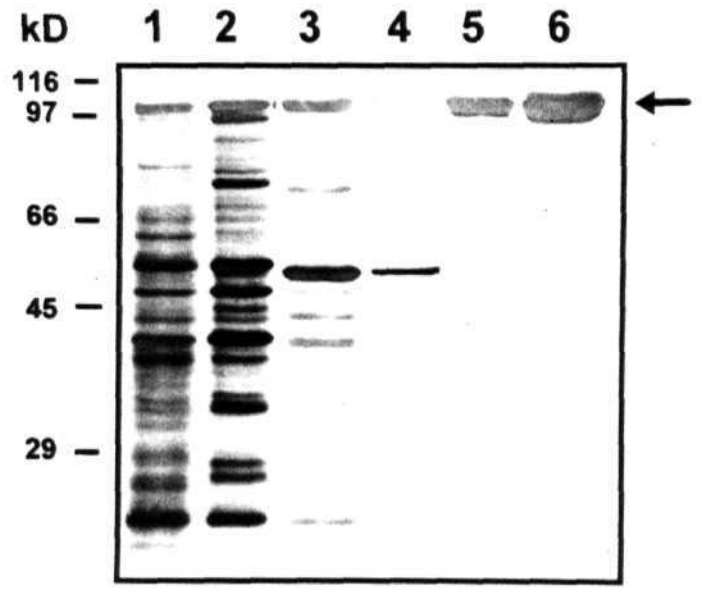


Plate 4.1. Purification of PEPC from *Amaranthus hypochondriacus* leaves by conventional method involving four steps. Samples from various steps were analyzed by SDS-PAGE and the gel was stained with silver nitrate. Lane 1: Crude extract, Lane 2: 40-60% ammonium Sulfate precipitate, Lane 3: Pool of active fractions from DEAE-Sepharose column, Lane 4: Active pool from phenyl-Sepharose column, Lane 5: Active pool from HAP column, Lane 6: Purified PEPC, after concentration with PEG 20,000 and storage in presence of 50% (v/v) glycerol. The positions of molecular weight markers are indicated on the left, while the location of PEPC is shown with a thick arrow on the right. Lanes 1 to 3 contained 7 μg protein, while lanes 4 to 5 contained 2 μg protein and lane 6 contained 3 μg protein. Further details are described in "Materials and Methods".

The purified enzyme had a specific activity of 35 U mg^{-1} protein (Table 4.2). Although the specific activity of the enzyme was less than that by the previous procedure (involving 4 steps), the PEPC preparation was quite pure, as indicated by single PEPC band on SDS-PAGE (Plate 4.2).

The preparation was examined for activity on non-denaturing gels. These experiments revealed the presence of two bands after the electrophoresis and both these bands stained for PEPC activity (Plate 4.3).

Immunological *characterization*

Polyclonal antibodies were raised in rabbits against purified PEPC from *Amaranthus* leaves. The anti-PEPC antiserum from rabbit showed a titer value of 1/100 against crude extract from *Amaranthus* leaves (Plate 4.4A).

The availability of antiserum facilitated its use for immunodiffusion and immunoelectrophoretic techniques. Antiserum was used to determine the amounts of protein-antigens by Western blot analysis. Purified PEPC was subjected to SDS-PAGE, followed by Western blotting analysis (Plate 4.5). Further, the anti-PEPC antiserum was used to quantitate the amount of protein-antigen in the given sample by ELISA. There was a linear response in the color (A405) with increase in concentration of PEPC-protein in the wells of ELISA plate (Fig. 4.4).

Since the purification techniques may affect the quality of antibody, the relative titer of the purified IgG was ascertained again (Plate 4.4B). The titer value of purified IgG was quite good at 1/200, against PEPC from leaf crude extracts.

Purification of PEPC by immunoadsorbent *column*

Attempts were made to develop a protocol for rapid purification of PEPC from *Amaranthus* leaves through immunoaffinity chromatography. Initially IgG were purified from anti-PEPC antiserum by DEAE-cellulose column. IgG were

Table 4.2. *Purification of PEPC from leaves of* Amaranthus hypochondriacus *by conventional method involving three steps.*

Step	Total Activity	Total Protein	Specific Activity	Purification	Yield
	(μmol min^{-1})	***(mg)***	*(fjmol min^{-1} mg^{-1} protein)*	*(fold)*	*(%)*
Crude extract	980	2450	0.4	1.0	100
40-60% $(NH_4)_2SO_4$	965	643	1.5	3.75	98.5
DEAE-Sepharose	949	73	13.0	32.5	96.8
Hydroxylapatite	385	11.2	35	87.5	39

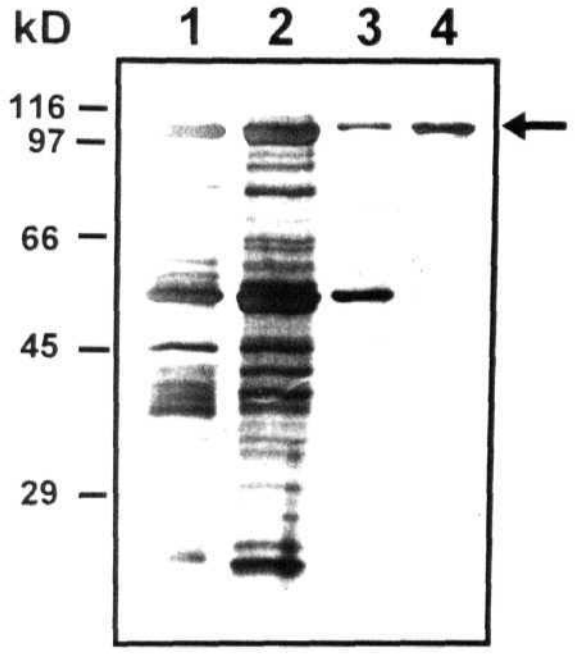


Plate 4.2. Purification of PEPC from *Amaranthus hypochondriacus* leaves by an alternative method involving only three steps. Samples from successive steps were analyzed by SDS-PAGE and the gel was stained with silver nitrate. Lane 1: Crude extract, Lane 2: 40-60% ammonium Sulfate precipitate, Lane 3: Pool of active fractions from DEAE-Sepharose column, Lane 4: Pool of active fractions from HAP column. Lanes 1 to 2 contained 7 μg protein, while lanes 3 and 4 contained 2 μg protein. Other details are as in Plate 4.1.

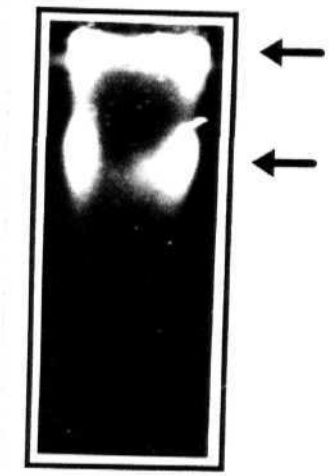


Plate 4.3. Activity staining of non-denaturing PAGE of purified PEPC, showing the presence of two isoforms (indicated by arrows). 20 μg of purified PEPC was loaded onto the lane. Further details are described in "Materials and Methods".

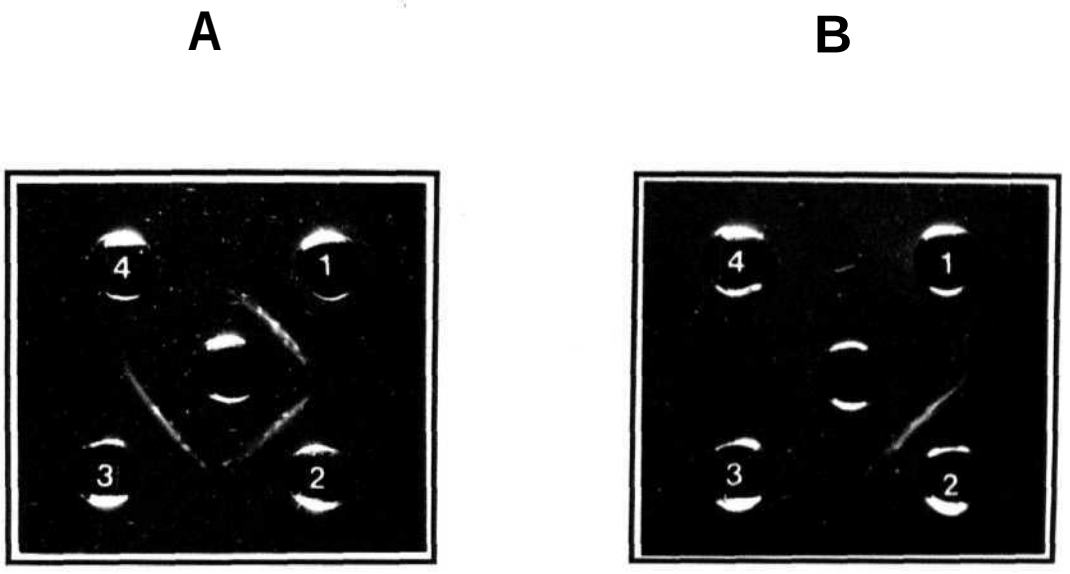


Plate 4.4. Ouchterlony double-difflision to determine the titer value of anti-PEPC antiserum. (A) The center well contained the crude extracts of *Amaranthus hypochondriacus.* Outer wells 1 to 4 contained the dilutions of anti-PEPC antiserum in the order of 1/20, 1/50, 1/100 and 1/150. (B) The center well contained the **crude** extract of *Amaranthus hypochondriacus.* Outer wells 1 and 2: 1/200 dilution of anti-PEPC antiserum and purified IgG from anti-PEPC antiserum, respectively. Outer wells 3 and 4: 1/500 dilution of purified IgG and anti-PEPC antiserum. Further details are described in "Materials **and** Methods".

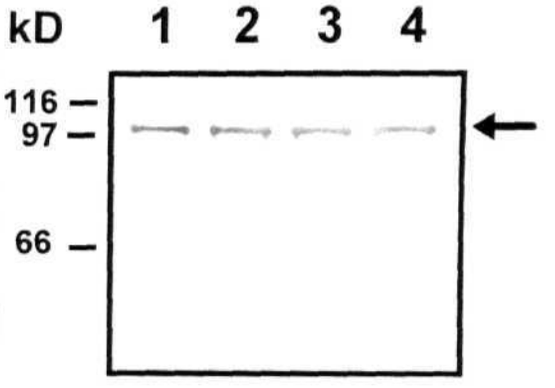


Plate 4.5. Western blot of PEPC purified from *Amaranthus hypochondriacus* leaves. Same amount of protein (0.5 μg protein) was used in all the four lanes to demonstrate the reproducibility. Proteins from SDS-PAGE was transferred onto PVDF membrane and probed with 1:1000 dilution of anti-PEPC antiserum and the complex was visualized with an alkaline phosphatase reaction. Further details are described in "Materials and Methods".

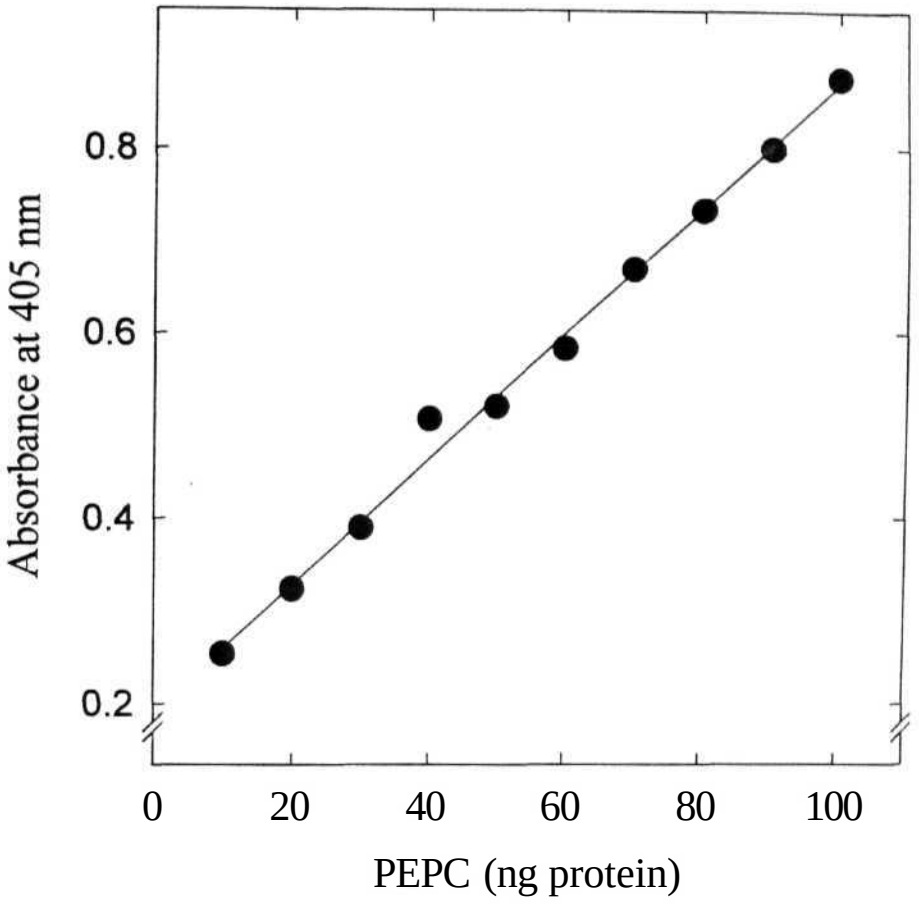


Figure 4.4. Application of ELISA to determine the quantity of PEPC purified from *Amaranthus hypochondriacus* leaves. The wells of an ELISA plate were loaded with varying amounts of PEPC-protein, as indicated. After ELISA, the color, based on assay with alkaline phosphatase was read at 405 nm. The absorbance at 405 nm was linear in **the** range of 10-100 ng PEPC protein in the wells. Further details **are** described in "Materials and Methods".

eluted as a sharp peak immediately after the void volume (Fig. 4.5). The SDS-PAGE analysis has confirmed the homogeneity of IgG preparation (Plate 4.6).

Immunoadsorbent column was prepared by coupling the anti-PEPC IgG with CNBr-activated Sepharose. PEPC was eluted from the column with chilled 20% glycerol in distilled water (Fig. 4.6). With this rapid purification procedure of two steps, the preparation of PEPC had a high specific activity (42.4 U mg^{-1} protein). The purification was by 120-fold with about 10% overall recover)' (Table 4.3). This enzyme showed a highly predominant protein band (>95%) on SDS-PAGE with PEPC activity (Plate 4.7).

Kinetic characteristics of purified PEPC

Table 4.4 summarizes the kinetic properties of the purified enzyme from conventional and immunoaffinity methods. K_m for PEP, Kj for malate and K_a (Glc-6-P) are within the range of reported values. However, there were slight differences in the kinetic properties of PEPC prepared by two different protocols. The affinity for PEP and malate of immunopurified PEPC were more than those of PEPC purified by conventional procedure (Table 4.4).

Discussion

PEPC purified in the present work had a very high activity of >55 U mg^{-1} protein. This is one of the highest specific activities reported for PEPC from C4 plants. Further, the yield also was as high as 57% (Table 4.1). Our method of purification of PEPC from leaves of *A. hypochondriacus* appears therefore to be one of the best. During purification, DEAE-Sepharose, phenyl-Sepharose and hydroxylapatite were quite effective in increasing purity.

The specific activity of the enzyme varies depending on the type of extraction, pH, ionic strength and salt used for elution. Podestá et al. (1990)

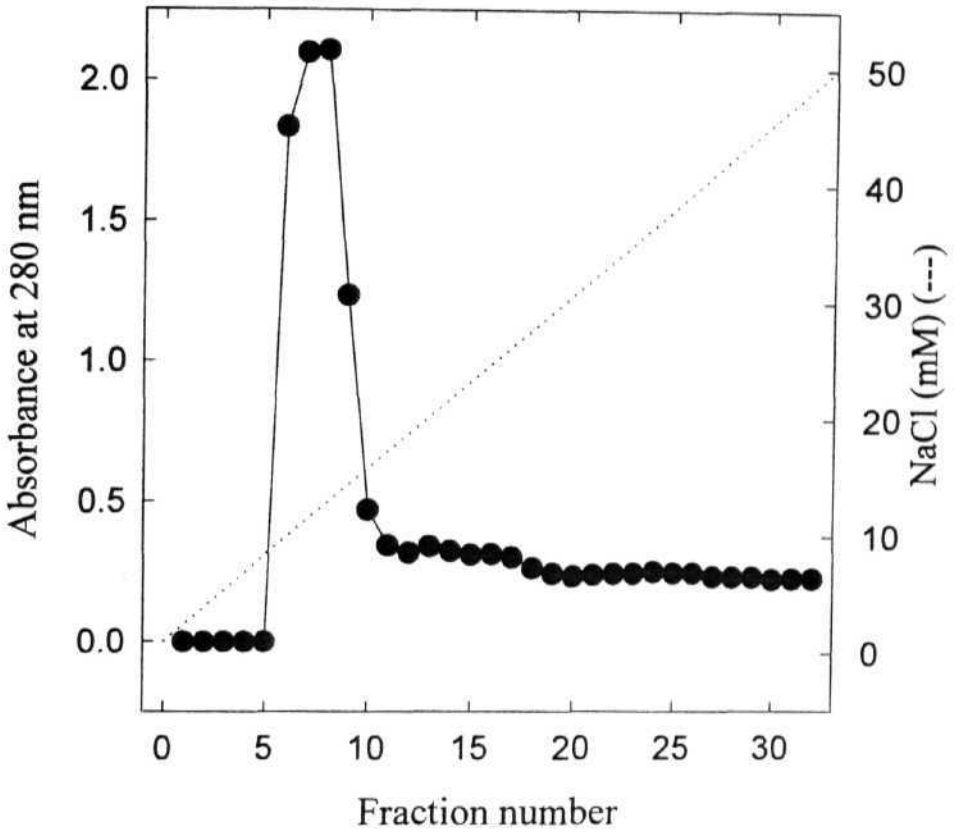


Figure 4.5. Elution profile of IgG purified from the anti-PEPC antiserum by chromatography on a DEAE-cellulose column. Anti-PEPC antiserum was dialyzed against 5 mM sodium phosphate buffer (pH 7.0). The dialysate was loaded onto a DEAE-cellulose column (1 x 12 cm), equilibrated with the same buffer. IgG were eluted with a linear gradient of 0-50 mM NaCl in 5 mM sodium phosphate buffer. Further details are described in "Materials and Methods".

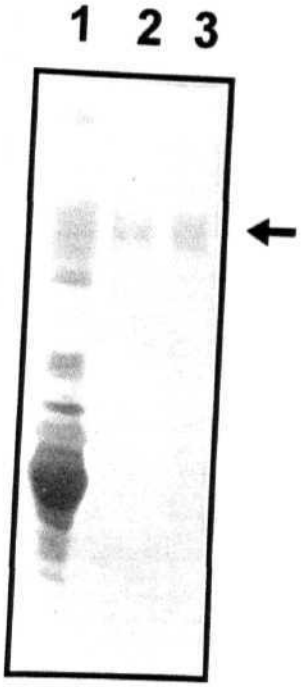


Plate 4.6. The purity of IgG as indicated by SDS-PAGE before and after chromatography on DEAE-cellulose. Lane 1: Crude antiserum (7 μg protein), Lane 2: Pooled fraction from DEAE-cellulose (3 μg protein), Lane 3: Purified IgG (3 μg protein). Further details are described in "Materials and Methods".

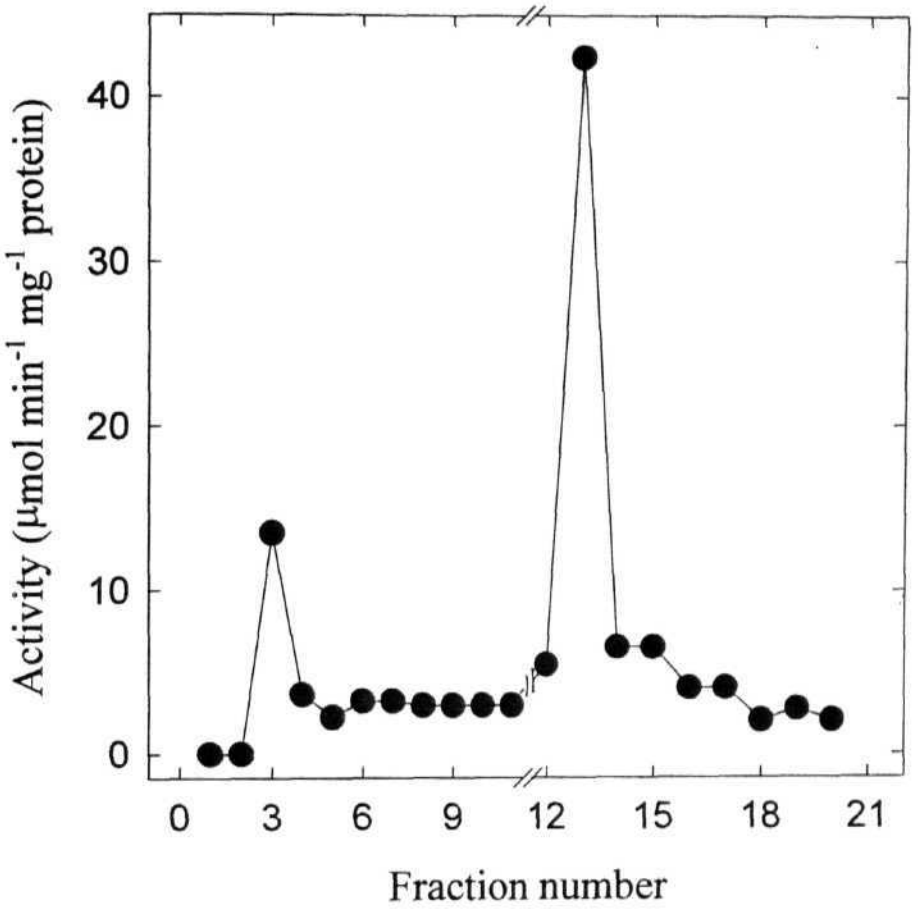


Figure 4.6. The pattern of PEPC elution from an immunoadsorbent column. The 40-60% ammonium Sulfate fraction was loaded onto the immunoadsorbent column (1 x 4.5 cm) and eluted with chilled 20% glycerol in water. After 11 ml of elution, the column was closed for 45 min and restarted. Further details are described in "Materials and Methods".

Table 4.3. *Purification of PEPC from leaves of* Amaranthus hypochondriacus *by immunoadsorbent column chromatography.*

Step	Total Activity	Total protein	Specific Activity	**Purification**	Yield
	(μmol min^{-1})	*(mg)*	*(μmol min^{-1} mg^{-1} protein)*	*(fold)*	*(%)*
Crude extract	42	120	**0.35**	1.0	**100**
40-60% $(NH_4)_2SO_4$	23	18	**1.3**	6.5	96
Immunoadsorbent column	4.2	0.1	**42.4**	120	**10**

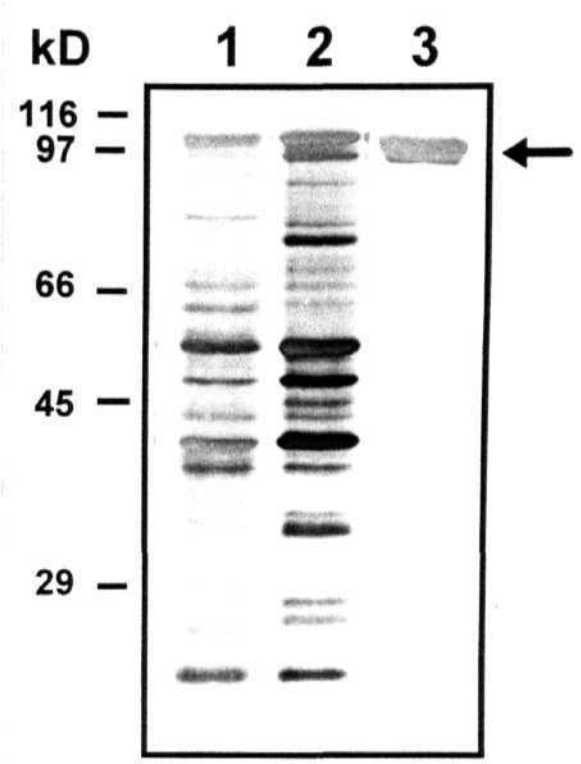


Plate 4.7. The status of purity of PEPC during purification from *Amaranthus hypochondriacus* leaves by immunoadsorbent chromatography. Samples from the various steps were analyzed by SDS-PAGE and the gel was stained with silver nitrate. Lane 1: Crude extract, Lane 2: 40-60% ammonium Sulfate precipitate, Lane 3: Pool of active fractions from immunoadsorbent column (purified PEPC). The positions of molecular weight markers are indicated on the left, while the location of PEPC is shown with a thick arrow on the right. Lanes 1 to 2 contained 7 μg protein and lane 3 contained 3 μg protein. Further details are described in "Materials and Methods".

Table 4.4. *A comparison of the kinetic characteristics of PEPC prepared by the conventional method of step wise column chromatography or a rapid purification by immunoaffinity chromatography.*

Purification procedure	Parameter			
	V_{max}	K_m (PEP)	K_i (malate)	K_a (Glc-6-P)
	(μmol min^{-1} mg^{-1} protein)	*(mM)*	*(mM)*	*(mM)*
Conventional method (3 columns/four steps)	55.3	0.44	0.55	0.6
Immunoaffinity	42.4	0.18	0.20	0.5

suggested that phosphate acts as a stabilizer during purification of PEPC from leaves of *Amaranthus viridis*. So far the highest activities reported in literature were obtained by using Pi buffer (Iglesias et al., 1986; Schuller and Werner, 1993; Zhang et al., 1995). Phosphate has also been used for elution of PEPC from soybean root nodules (Schuller and Werner, 1993; Zhang et al., 1995) and from leaves of maize (Hague and Sims, 1980). Iglesias et al. (1986) reported that PEPC from *A. viridis* eluted around 50-60 mM Pi. However, in sugarcane PEPC eluted at 120 mM Pi (Iglesias and Andreo, 1989). In our experiments, PEPC from leaves of *Amaranthus hypochondriacus* was eluted from HAP column, at a Pi concentration of about 70-90 mM (Fig. 4.3).

PEPC appeared as two distinct bands on non-denaturing gel electrophoresis and both these bands stained for PEPC activity (Plate 4.3), indicating that two bands are identical subunits of the holoenzyme. We suggest that these two bands may be isozymes of homotetramer, since dimer or monomer expected to be inactive and may not stain for PEPC activity. Further studies on amino acid sequence and corresponding mRNA patterns may be necessary to confirm the identity of these two bands on the native gel.

There are conflicting reports on the number of isoforms in higher plants. A few reports suggest that three PEPC isozymes exist in cotton leaf tissue (Mukerji and Ting, 1971), while others reported the presence of two isoenzymes in maize (Mukerji, 1977) and sugarcane (Goatly and Smith, 1974). Besides higher plant-PEPCs, two isozymes of PEPC were reported in an unicellular green alga, *Chlamydomonas reinhardtii* (Chen and Jones, 1970).

Earlier, Ting and Osmond (1973a,b) speculated that one of the PEPC-isozymes might have originated from mesophyll and other from adjacent bundle sheath cells, while purifying PEPC from maize. However, there is now overwhelming evidence that PEPC is present exclusively in mesophyll cells of C_4

plants (Perrot-Rechenmann et al., 1982). We therefore feel that these two bands of isozymes are present only in mesophyll cells. The function of these two isozymes of PEPC is not yet known. Coombs et al. (1973) and Wong and Davies (1973) suggested that they may have an anaplerotic function.

A single band of PEPC was observed when the SDS gels were loaded with either low or high amounts of pure PEPC-protein (Plate 4.1), indicating the subunit structure of PEPC was similar. Uedan and Sugiyama (1976) reported a single band of 100 kD protein after SDS-tube gel electrophoresis in maize leaves. In contrast, Hague and Sims (1980) have observed two bands of PEPC, again from maize leaves, on highly resolving SDS-PAGE slab gels. Budde and Chollet (1986) have reported that a doublet protein band is observed at 94 to 100 kD protein which may be PPDK and PEPC. However, these polypeptides get merged on the gel, particularly at loads of higher than 30 fig of protein.

PEPC purified from *Amaranthus hypochondriacus* (a C4 species) leaves exhibited a V_{max} of 55 U mg^{-1} protein and a K_m for PEP of 0.4 mM, at pH 7.3 (Table 4.4). The enzyme was inhibited by malate with a Kj of 0.55 mM and activated by Glc-6-P with a Ka of 0.6 mM. These values of our enzyme are similar to the kinetic parameters of C4-PEPC reported in the literature (Uedan and Sugiyama, 1976; Jiao and Chollet, 1988; Schuller and Werner, 1993; Duff et al., 1995; Mehta et al., 1995; Podestá et al., 1995; Svensson et al.. 1997) .

PEPC is highly unstable during extraction from leaves, due to proteolysis of N-terminal end. The cleavage of N-terminal from PEPC is a common problem during extraction and storage. The intactness of N-terminal of PEPC can be determined easily by checking the malate sensitivity of enzyme. The extent of malate inhibition of PEPC decreased as the enzyme lost its N-terminal portion (McNaughton et al., 1989). The inclusion of protease-inhibitors like PMSF, leupeptin or chymostatin during purification can protect the enzyme from

proteolysis. The addition of chymostatin is therefore necessary to prevent the proteolysis of PEPC and to retain the high malate sensitivity of the enzyme (McNaughton et al., 1989). We have also used the protease inhibitors such as PMSF, leupeptin and chymostatin, so as to avoid proteolysis and to maintain the stability of the enzyme during extraction and purification.

The inclusion of glycerol, apart from the need of protease inhibitors for stability, under *in vitro* conditions improves the stability of the enzyme during extraction (Uedan and Sugiyama, 1976; Manetas et al., 1986; Selinioti et al., 1987; Podestá and Andreo, 1989) as well as storage (Manetas, 1982; Karabourniotis et al., 1983). Therefore, we have used 10% glycerol through out our purification procedure and finally the purified enzyme was stored in 50% glycerol.

It is essential to store the purified enzyme under proper conditions to maintain the maximum activity and high malate sensitivity, since the purified enzyme is unstable. Several authors attempted PEPC-storage in different ways. The enzyme is usually stored with or without 5 to 50% (v/v) glycerol at either 4°C or below 0°C for several months (Vidal et al., 1980; Iglesias et al., 1986; Jiao and Chollet, 1988; Iglesias and Andreo, 1989; Bakrim et al.. 1992; Bandarian et al., 1992; Baur et al., 1992; Schuller and Werner, 1993; Mehta et al., 1995; Zhang et al., 1995). Thus, there is a lot of variation in published reports describing the storage of PEPC from different sources. Addition of 5-10 mM malate or Glc-6-P has frequently been recommended (Nimmo et al., 1986; Willeford et al., 1990; Zhang et al., 1995), besides the inclusion of protease inhibitors like PMSF or chymostatin (McNaughton et al., 1991; Arrio-Dupont et al., 1992).

We conclude that purified PEPC can be easily stored, along with 50% (v/v) glycerol, in liquid N_2 at least for 4 months. The addition of glycerol during storage is known to stabilize several other enzymes as well, for e.g. PPDK (Shirahashi et al., 1978) and cytosolic pyruvate kinase (Podestá and Plaxton, 1993). The exact

mechanism as how glycerol can **stabilize the** PEPC **during storage, still remains to be elucidated.**

Immunological characteristics

Polyclonal antibodies were raised in rabbits against PEPC purified from the leaves of *A. hypochondriacus.* The anti-PEPC antiserum could be used for several experiments: Western blots, ELISA and immunoadsorbent chromatography.

ELISA is highly useful technique to detect and quantitate the antigen-protein in given sample and can be employed in diagnostic kits. The standardization of ELISA technique with PEPC, has the potential of developing diagnostic kits for PEPC in leaf extracts of particularly C4 species. There arc very few reports on the use of ELISA with PEPC or other enzymes of C4 pathway. Saitou et al. (1994) used ELISA to evaluate the immunological difference between the NADP-malic enzymes extracted from C3- and CAM plants. ELISA has been used to identify and purify the multiple isoforms of stomatal PEPC after gel electrophoresis (Schnabl et al., 1993). The amount of PEPC-protein in crude extracts of *Amaranthus hypochondriacus* could be quantitatively determined by ELISA. The color development after ELISA was linear in the range of 0-100 ng ml^{-1} PEPC-protein purified from ,4. *hypochondriacus* leaves (Fig. 4.4). The antiserum against PEPC of *A. hypochondriacus* could therefore be used to detect at ng level of PEPC in extracts of *A. hypochondriacus.* Our experiments (Fig. 4.4) demonstrate that ELISA can be used as a diagnostic kit to detect and determine PEPC-protein in plants.

Immunoadsorption chromatography is one of the fastest ways of purifying the enzyme. The binding capacity of the immunoadsorbent column was approximately 80-90 units PEPC, i.e. most of the activity present in extracts. A typical elution diagram is shown in Figure 4.6. PEPC was eluted in two steps. **In the retarded peak, the** activity was about 3 times higher than the activity **eluted in the**

first **peak. In both** peaks, the recovery of active PEPC was usually 20-40%. Low pH citrate buffer was used to remove the remaining enzyme from the column, however it is irreversibly inactivated (Crétin et al., 1984). As judged by electrophoresis of denaturing PAGE and immunoprecipitation and/or Western blot analysis with **anti-**PEPC antiserum, the protein was of a high degree of purity. Furthermore, the sensitivity of the enzyme to malate inhibition was maintained. Although the yield of the immunoaffinity-based preparation is less than that of classical preparations, this disadvantage is largely compensated by the quality of the preparation and the short time required to obtain the purified enzyme.

Immunochemical techniques provide an efficient method for enzyme purification and localization. One of the possible draw-backs of conventional method is the loss of biological activity of protein/enzyme. A simple method based on the elution of an active, highly purified enzyme from an immunoadsorbent column has been developed by Vidal et al. (1980). PEPC could be rapidly purified by immunoadsorbent chromatography. The time taken for this procedure is very less compared to that of classical method (Arrio-Dupont et al., 1992).

As per the protocol employed in the present work 'immunoadsorbent column chromatography' could be used for rapid purification of PEPC from leaf extracts of *A. hypochondriacus*. It maintains the enzyme intact and with good catalytic and regulatory properties (Table 4.4). The disadvantages, such as enzyme degradation, which occurs during conventional method can thus be easily avoided by using this protocol. Further, it is also useful for rapid isolation of PEPC from leaves during light/dark transitions.

Major conclusions from the results presented in this chapter are:

1. PEPC was purified from leaves of *A. hypochondriacus* with a specific activity of 55 U mg^{-1} protein. Thus, this preparation had one of the highest specific activities of PEPC reported in the literature.

2. Polyclonal antibodies were raised in rabbits against purified PEPC from *A. hypochondriacus.* The anti-PEPC antiserum showed a titer value of 1:100.

3. The monospecifity of PEPC-antiserum was confirmed by Ouchterlony double diffusion.

4. Anti-PEPC antiserum could be used for Western blots, immunoprecipitation and ELISA.

5. A rapid purification protocol (immunoaffinity chromatography) for the isolation of highly active (and with intact N-terminal) PEPC was standardized.

6. An ELISA technique was evolved to detect and determine PEPC. It can be used as a diagnostic kit for the detection and quantification of PEPC-protein in plants.

Chapter 5

Interaction of PEPC with Compatible Solutes in relation to its Stability and Oligomeric Status

Chapter 5

Interaction of PEPC with Compatible Solutes in relation to its Stability and Oligomeric Status

Introduction

PEPC exists predominantly as a tetramer along with di- and monomeric forms (Walker et al., 1986a). The tetrameric form of PEPC is the most active form, while the dissociation of the enzyme results in a marked decrease in the catalytic activity of the enzyme (Walker et al., 1986a; Wagner et al., 1987; Podestá et al., 1990; Willeford et al., 1990; Wu et al., 1990). Oligomeric status of PEPC depends on factors like pH, ionic strength and temperature (Rajagopalan et al., 1994). The dilution of the enzyme *in vitro* leads to a marked instability of PEPC, due to the dissociation of PEPC into dimer and/or monomer (Andreo et al., 1987; Rajagopalan et al., 1994).

Glycerol protects the enzyme against dissociation induced by dilution (Uedan and Sugiyama 1976; Manetas, 1982; Karabourniotis et al., 1983; Manetas et al., 1986; Selinioti et al., 1987). Besides glycerol, other organic cosolutes (such as polyhydric and polyoxy compounds and some amino acids) arc known to promote self-association of proteins and stabilize their structure by a preferential exclusion (negative binding) from contact with protein surface (Gekko and Timasheff, 1981; Timasheff et al., 1982; Priev et al., 1996). The dissociation of active tetrameric form of PPDK, at low temperature, is prevented by glycerol, sucrose and sorbitol (Shirahashi et al., 1978). Besides PEPC, other cytosolic enzymes known to be activated by PEG are pyruvate kinase from germinating castor seed endosperm (Podestá and Plaxton, 1993) and fructose-1,6-bisphosphatase from endosperm of germinating castor seeds (Hodgson and Plaxton, 1995).

We also have noticed that PEPC becomes unstable when extracted, primarily due to dilution. Therefore studies were made with crude extracts to assess the stability and properties of PEPC in presence of compatible solutes, such as PEG or glycerol. These results were cross checked with similar experiments using purified PEPC-enzyme. Attempts were also made to assess the oligomeric status of the enzyme *in vitro* by gel filtration chromatography in the absence or presence of PEG and/or glycerol.

Results

The effect of PEG-6000 on PEPC was examined by including the PEG-6000 during either extraction or assay or both during extraction and assay. The activity of PEPC was assayed at suboptimal (0.5 mM) and optimal (2.5 mM) concentrations of PEP. The stimulation by PEG of PEPC activity was more when assayed at 0.5 mM PEP than that at 2.5 mM PEP (Fig. 5.1). The extent of stimulation by PEG was about 3-fold at 2.5 mM PEP, while being > 10-fold at 0.5 mM PEP. Maximum stimulation of PEPC occurred at 2.5% (w/v) PEG when PEPC was assayed with 2.5 mM PEP, while 5-10% (w/v) PEG was required to cause maximum stimulation, when assayed with 0.5 mM PEP. Maximum effect occurred when PEG-6000 was present during both extraction and assay.

The presence of PEG during assay stimulated the activity of even purified PEPC. Such stimulation of PEPC activity was more at 0.5 mM PEP (>2 fold) than that at 2.5 mM PEP (about 40%) (Fig. 5.2). The optimal concentration of PEG required for maximal stimulation of PEPC activity in purified preparation was 1.25%.

We have also studied the stability of purified PEPC in presence or absence of PEG and/or glycerol or both during incubation at room temperature. The activity of PEPC was significantly lost after 24 h in the absence of compatible solutes

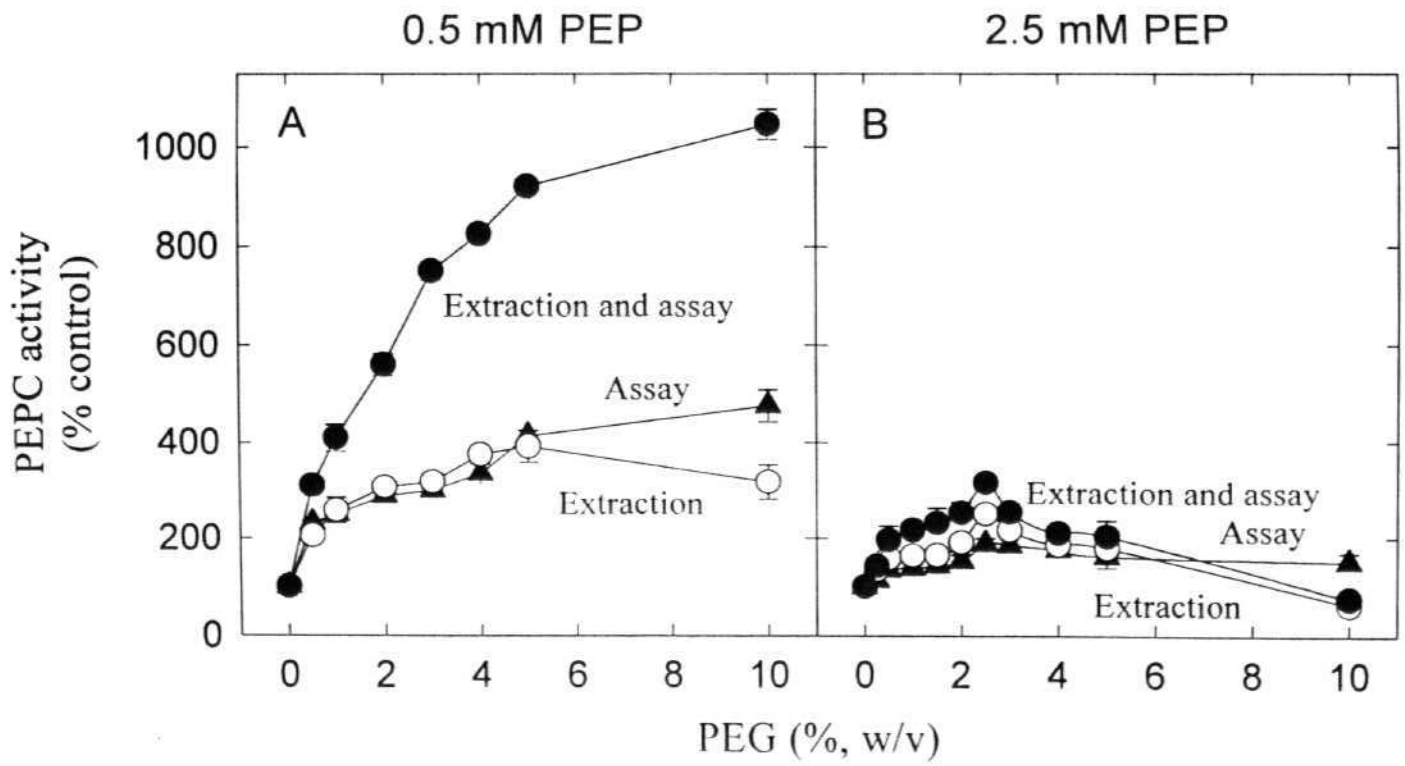


Figure 5.1. The effect of PEG-6000 on the activity of PEPC in crude extracts prepared from leaves of *Amaranthus hypochondriacus*. PEPC was assayed at pH 7.3 using either suboptimal (0.5 mM) PEP (A) or optimal (2.5 mM) PEP (B). PEG was included during either extraction (O) or assay (▲) or both (●). The activities of PEPC in the absence of PEG (control) were 174 ± 11 μmol h^{-1} mg^{-1} Chl and 419 ± 26 μmol h^{-1} mg^{-1} Chl, when assayed at 0.5 mM PEP and 2.5 mM PEP, respectively.

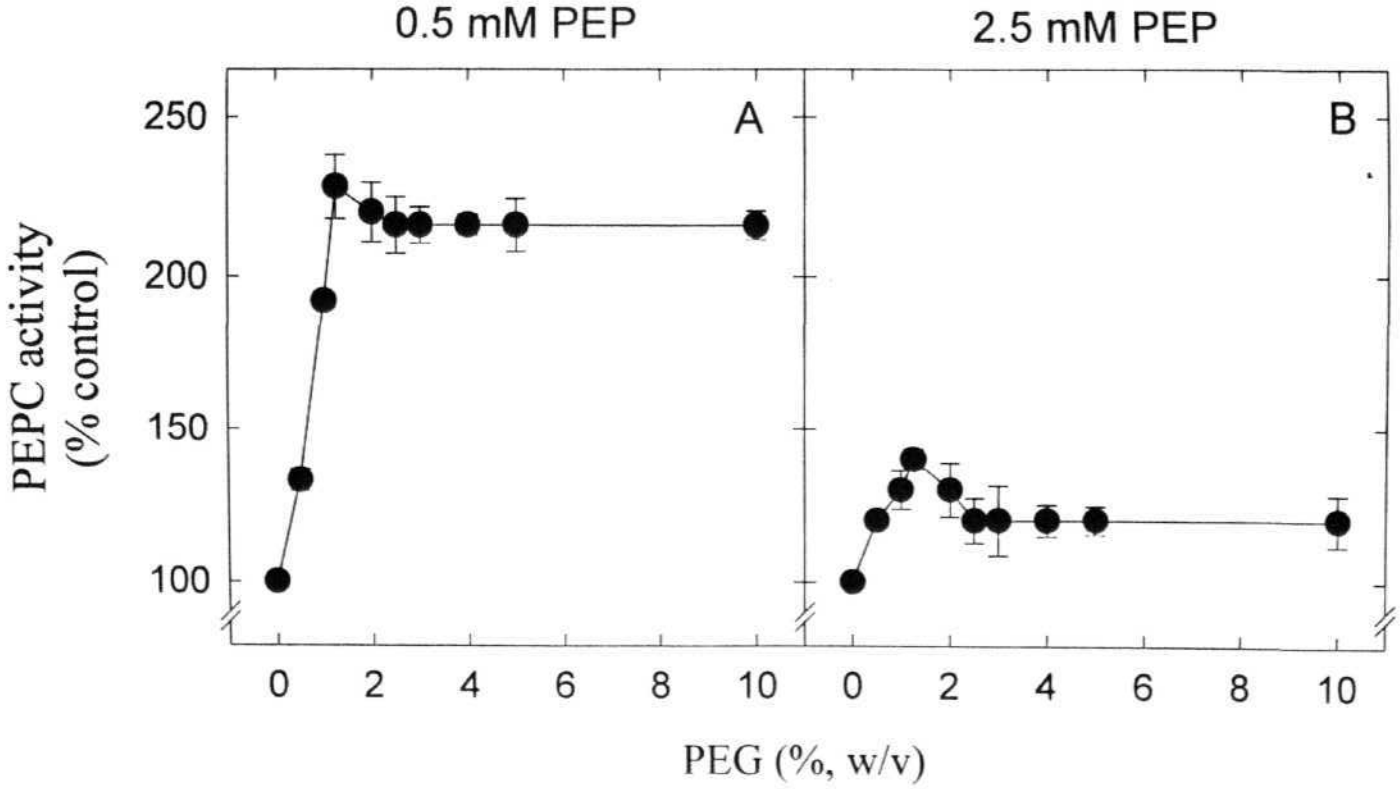


Figure 5.2. Activation by PEG of purified PEPC prepared from leaves of *Amaranthus hypochondriacus* when assayed at either suboptimal (0.5 mM) PEP (A) or optimal (2.5 mM) PEP (B) at pH 7.3. PEG was included during assay. The activities of PEPC in the absence of PEG (control) were 26 ± 5.1 μmol min^{-1} mg^{-1} protein and 38 ± 6.4 μmol min^{-1} mg^{-1} protein, when assayed at 0.5 mM PEP and 2.5 mM PEP, respectively.

(Fig. 5.3). In contrast, the enzyme was quite stable in presence of glycerol or PEG + glycerol, even after 24 h of incubation.

The kinetic characteristics of PEPC were studied in presence or absence of PEG and/or glycerol during both extraction and assay. Again maximal efTect was noticed in presence of both PEG and glycerol (Table 5.1). Although, the activation by PEG of PEPC activity was more at 0.5 mM PEP than that at 2.5 mM PEP, we preferred to use 2.5 mM in all subsequent experiments, as optimal concentration of PEP was needed for the determination of Kj (malate) and other kinetic characteristics of the enzyme. The maximal velocity (V_{max}) of the enzyme increased by almost 4-fold, in presence of PEG or glycerol or both. The affinity for PEP, was significantly enhanced while the Kj for malate increased (by >80%) when PEPC was extracted and assayed in presence of PEG or glycerol or both. In contrast, there was only a marginal decrease in the apparent K_m of the enzyme for HCO_3^- (Table 5.1).

All the above kinetic studies of the enzyme from crude extract were compared with the corresponding responses of purified PEPC preparation. The effects of PEG and/or glycerol on kinetic characteristics of purified PEPC were similar to those in crude extracts. On exposure to PEG and/or glycerol the V_{max} of PEPC increased, while K_m (PEP) decreased by 50-60%. In contrast, the sensitivity of the enzyme for malate drastically decreased (Table 5.2). However, the affinity of PEPC to HCO3- was not much affected.

Despite the marked increase in the velocity of enzyme, in presence of PEG and/or glycerol, the activity of PEPC in leaves was still enhanced by more than 2-fold on illumination. However, there was a marginal decrease in the extent of light activation, as indicated by the L/D ratio (Table 5.3).

It is possible that the presence of glycerol and/or PEG results in an oligomerization of PEPC leading to an increase in the V_{max} and changes in other

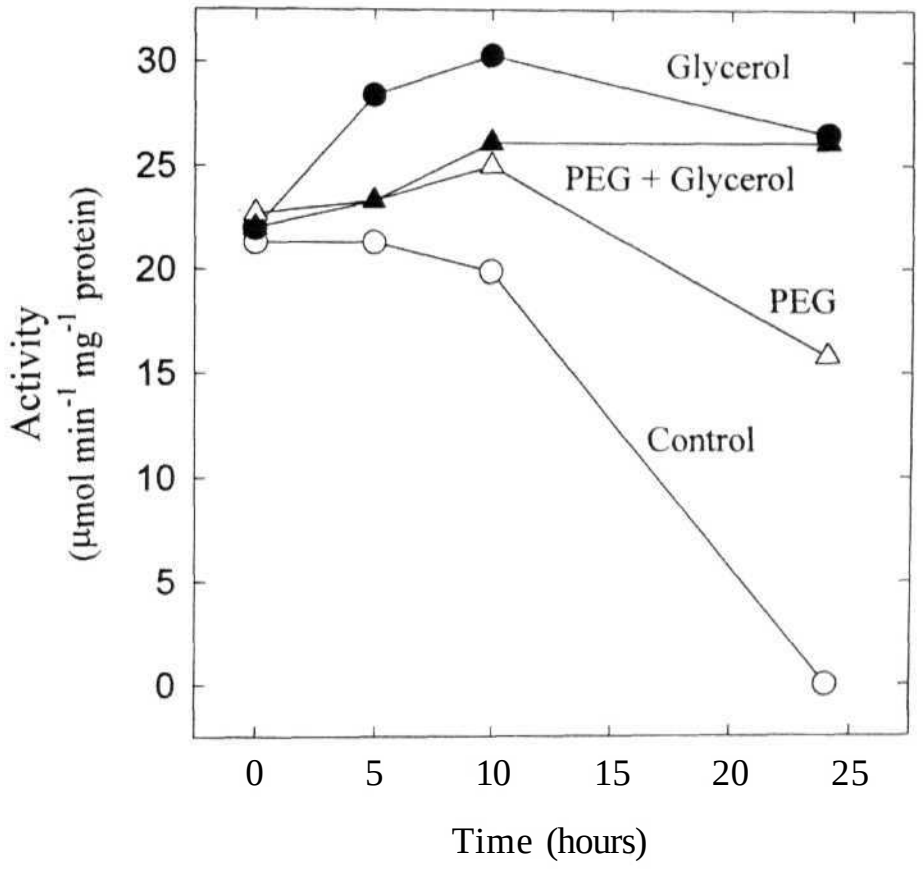


Figure 5.3. Stability of purified PEPC at room temperature in presence of glycerol or PEG or both. The purified enzyme was incubated with glycerol or PEG or both, during the incubation of purified PEPC. An aliquot was taken at specified times and assayed for PEPC activity.

Table 5.1. *Effect of 2.5% PEG and/or 10% glycerol during extraction and assay on the characteristics of PEPC in leaf extracts of* Amaranthus hypochondriacus.

PEPC was assayed at pH 7.3 and 2.5 mM PEP. Further details are described in "Materials and Methods".

Parameter	Control	Glycerol	PEG	PEG + Glycerol
V_{max} (μmol h^{-1} mg^{-1} Chl)	431 ±22	645 ± 31	1363 142	1531 ± 36
K_m [PEP] (mM)	0.19 ± 0.05	0.11 ± 0.01	0.1010.01	0.08 ± 0.01
K_m [HCO_3^-] (mM)	0.040 ± 0.006	0.047 ± 0.014	0.033 ± 0.001	0.026 ± 0.002
Kj [malate] (mM)	0.23 ± 0.04	0.31 ± 0.06	0.35 ± 0.01	0.42 1 0.02

Table 5.2. *Effect of 1.25% PEG and/or 10% glycerol during assay on the characteristics of purified PEPC from* Amaranthus hypochondriacus *leaves.*

PEPC was assayed at pH 7.3 and 2.5 mM PEP. Further details are described in "Materials and Methods".

Parameter	Control	Glycerol	PEG	PEG + Glycerol
V_{max} (μmol min^{-1} mg^{-1} protein)	35.1 ±0.7	55.6 ±0.3	59.6 ± 1.5	72.3 ± 1.2
K_m [PEP] (mM)	0.18 ± 0.014	0.12 ±0.004	0.09 ±0.01	0.08 ±0.01
K_m [HCO_3^-] (mM)	0.03 ± 0.002	0.03 ±0.001	0.04 ± 0.002	0.03 ±0.001
K_i [malate] (mM)	0.29 ±0.03	0.33 ±0.02	0.44 ± 0.05	0.63 ± 0.02

Table 5.3. *Effect of presence or absence of 10%(v/v) glycerol and 2.5% (w/v)* ***PEG*** *during extraction and assay on the extent of light activation of PEPC* ***in*** *leaf extracts.*

PEPC was assayed at pH 7.3 and 2.5 mM PEP. Further details are described in "Materials and Methods".

Addition during extraction and assay	Light	Dark	L/D
	$\mu molh^{-1} mg^{-1}$ Chl		*Ratio*
None (control)	898.8 ±10.8	359.5 ± 4.8	2.5
Glycerol	1158.4 ±13.4	609.7 ±9.7	1.9
PEG	1107.1 ±9.6	738.0 ±12.1	1.5
PEG + Glycerol	1668.3 ±12.3	758.3 ±15.3	2.2

properties. Experiments were therefore designed to assess the molecular size of PEPC in presence of these compatible solutes, by employing nondenaturing PAGE. Purified enzyme was incubated for 1 h with glycerol or PEG and PEG + glycerol and the nature of protein was examined on native PAGE. The protein on the gel was mostly in the form of monomer and very little in the form of dimer or tetramer, in the presence or absence of these two solutes (Plate 5.1 A). Similar pattern was noticed with also on Western blots (Plate 5.1B). It is possible that dilution occurred during the process of application of electrophoretic power or long duration of time taken to perform the nondenaturing PAGE (about 4 h) or developing Western blots. We have therefore re-examined the quaternary structure of PEPC by gel filtration on Sephadex G-200 column.

When the column was eluted with a medium containing neither glycerol nor PEG, PEPC eluted as three distinct peaks at fractions numbering 13, 20 and 27 (Fig 5.4A). The patterns of the elution profiles of PEPC were compared to the calibration of the column with protein standards of known molecular weights (Fig. 5.5). The protein peaks eluting at the fraction Nos. 13, 20 and 27 correspond to molecular weights of about 400 kD, 200 kD and 100 kD, respectively. These molecular weights correspond to tetramer, dimer and monomeric forms of PEPC. The PEPC peak corresponding to monomer appeared to be more predominant than the other two peaks in the absence of compatible solutes. When PEPC was incubated and column was eluted with 10% glycerol, two well defined peaks at fraction numbers 13 and 20 appeared with a small shoulder (Fig. 5.4B). In contrast, only the tetramer peak was obtained when the enzyme was incubated and the column was eluted with 1.25% (w/v) PEG (Fig. 5.4C) or a combination of PEG and glycerol (Fig. 5.4D).

The data in Figure 5.4 represent the profiles of elution of PEPC-protein only. It is necessary to ascertain the activity of the different forms of PEPC. The activity of PEPC in these fractions were therefore determined. The activity of PEPC was

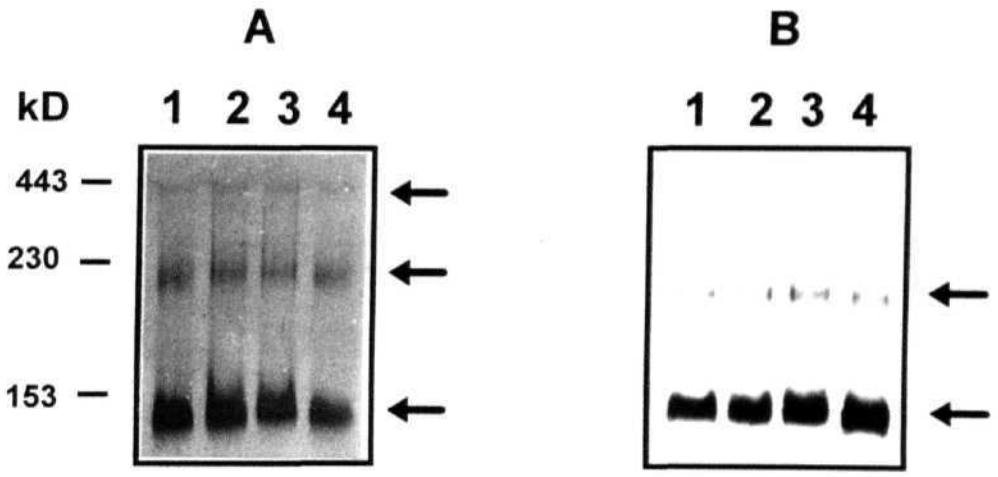


Plate 5.1. Effect of PEG or glycerol or both on the structure of PEPC during native PAGE. Lane 1: 50 mM HEPES-KOH, pH 7.3, control (none), lane 2: 10% glycerol, Lane 3: 1.25 % PEG, lane 4: 1.25% PEG plus 10% glycerol. PEPC was incubated with these compatible solutes for 1 h and subjected to nondenaturing PAGE. The gels were either stained with silver nitrate (A) or subjected to Western blotting (B), after transferring onto PVDF membrane. Further details are described in "Materials and Methods".

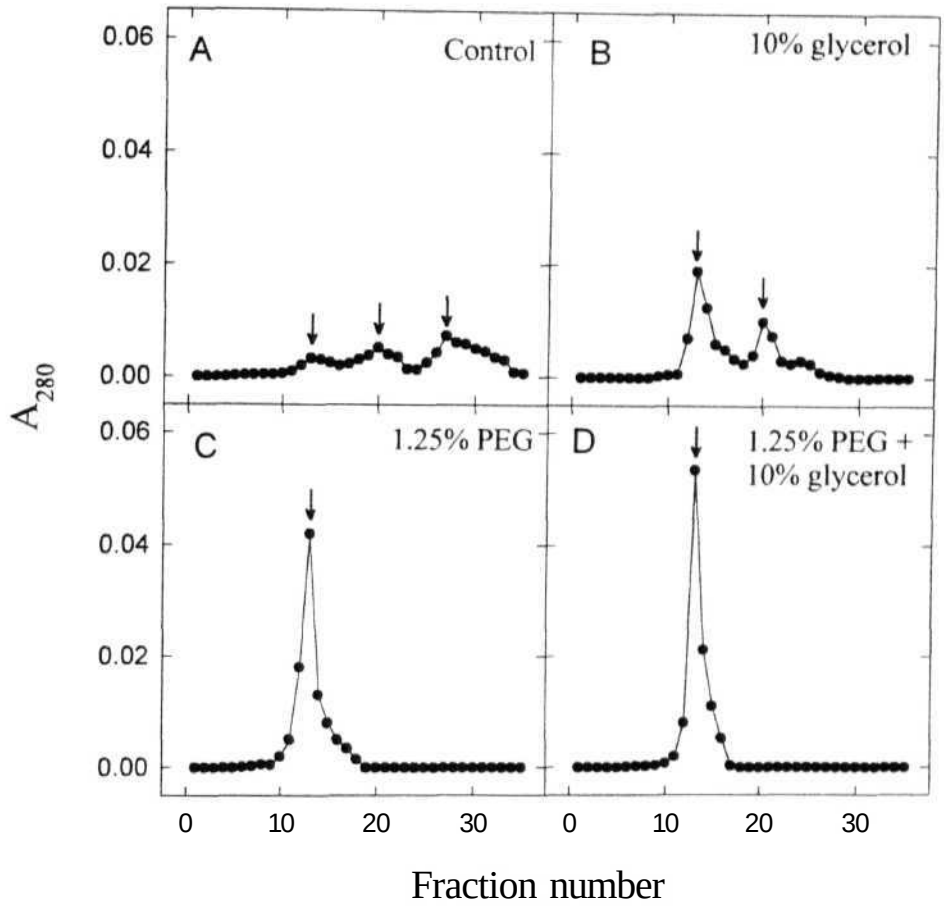


Figure 5.4. Elution profile of purified PEPC from Sephadex G-200 column (1 x 30 cm). 100 ug of PEPC protein was applied to the column and eluted with 50 mM HEPES-KOH, pH 7.3 without (A) or with 10% (v/v) glycerol (B) or 1.25% (w/v) PEG (C) or 1.25% PEG plus 10% glycerol (D). 1 ml fractions were collected and examined for the protein content by recording the absorbance at 280 nm. Further details are described in "Materials and Methods".

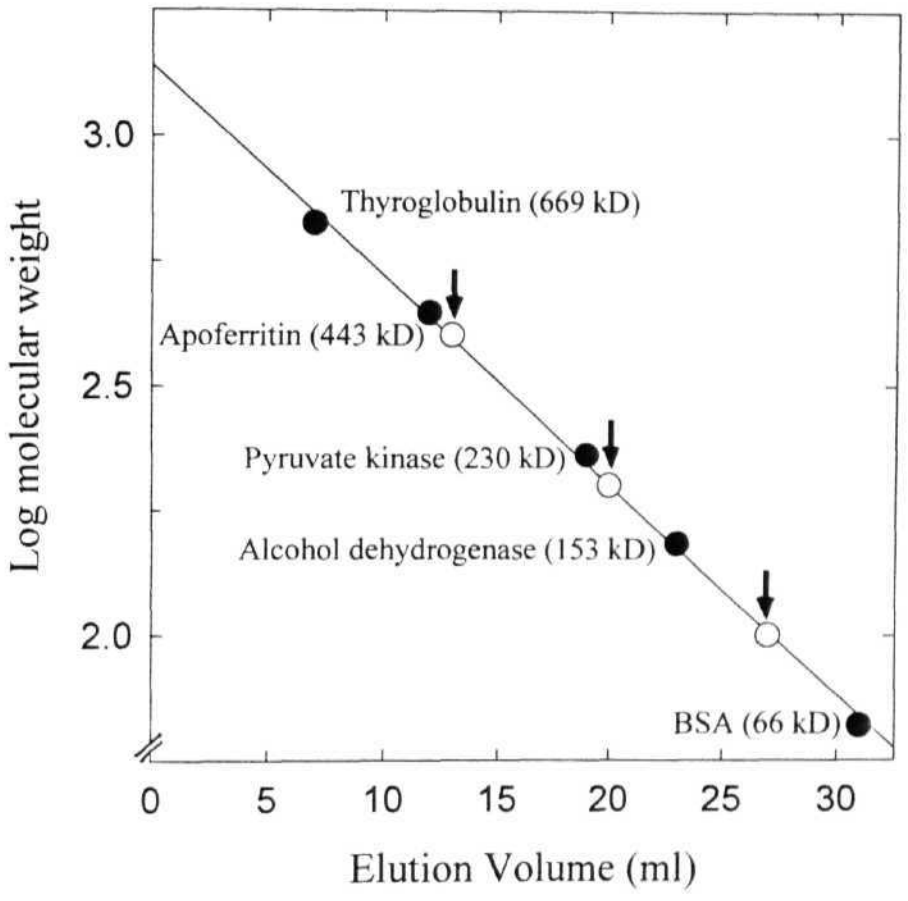


Figure 5.5. Calibration of the Sephadex G-200 column (1 x 30 cm) based on the elution profile of molecular standards. The buffer used for the elution, at a flow rate of 10 ml h^{-1}, contained 50 mM HEPES-KOH (pH 7.3). 100 µg each of protein was loaded. The location of mono-, di- and tetrameric forms of PEPC (O) are indicated with arrows. Further details are described in "Materials and Methods".

detectable only in fraction, with a peak at No. 13, **corresponding to tetramer** of PEPC. The location of peak was at the same No. of 13 in all treatments, irrespective of the presence of glycerol or PEG (Fig. 5.6, A-D). However, **the** height of peak, indicating the specific activity of PEPC was least in the absence of any solute (Fig. 5.6A) and maximal in presence of both PEG and glycerol (Fig. 5.6D).

Discussion

Compatible solutes protect the enzymes against a variety of adverse conditions *in vitro* by inducing the local concentration of protein (Timasheff et al., 1982; Rhodes and Hanson, 1993; Priev et al., 1996). PEG also acts by promoting self association of the enzyme in solution and therefore underlies its use for fractional precipitation of proteins (Miekka and Ingham, 1978). PEPC also is affected by compatible solutes in several ways: stabilization during storage (Selinioti et al., 1987), protection of the enzyme against salt inhibition (Manetas et al., 1986; Manetas, 1990) and improvement in catalytic efficiency of enzyme (Stamatakis et al., 1988; Podestá and Andreo, 1989).

Changes in the aggregation of the enzyme results in altered kinetic properties (Willeford et al., 1990) suggesting that the tetramer is the most active form (Wu et al., 1990). PEPC from maize can be dissociated from a tetra- to di- and monomer forms by high ionic strength, low pH and chemical modification (Walker et al., 1986a; Wagner et al., 1987). However, very little is known about the significance *in vivo* of C4 PEPC regulation by changes in its oligomeric state.

C4 plant leaves contain PEPC as 15% of the soluble protein (Hague and Sims, 1980) and as it is confined only in the cytosol of mesophyll cells (Perrot-Rechenmann et al., 1982), its concentration *in situ* should be extremely high. Inferences concerning the regulation of biochemical reactions *in vivo* are usually drawn from *in vitro* studies of the enzymes involved. The high concentration of the

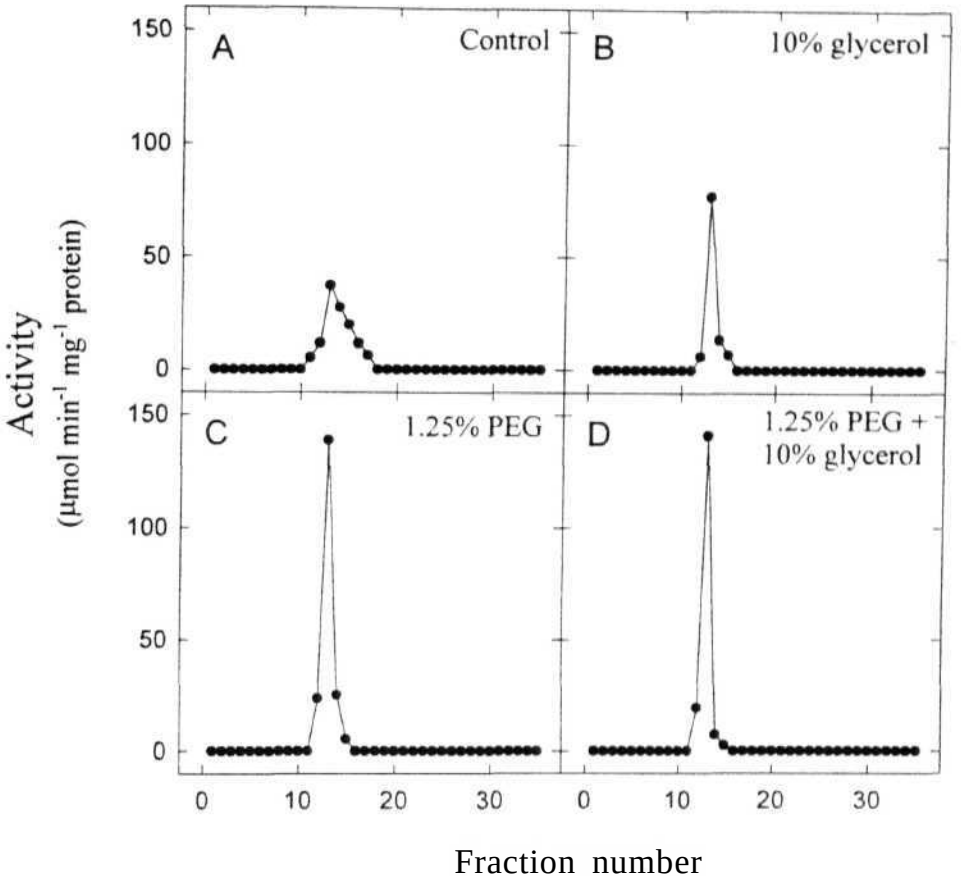


Figure 5.6. Elution profile of purified PEPC from Sephadex G-200 column. 100 µg of PEPC protein was applied at the top of the column and eluted with 50 mM HEPES-KOH, pH 7.3 without (A) or with 10% (v/v) glycerol (B) or 1.25% (w/v) PEG (C) or 1.25% PEG plus 10% glycerol (D). 1 ml fractions were collected and assayed for PEPC activity at pH 7.3 with 2.5 mM PEP. Further details are described in "Materials and Methods".

enzymic **protein** in the cytoplasm undoubtedly favors aggregation, **but** it is imaginable that even at high protein levels, variables such as pH, ionic strength or temperature might affect the equilibrium between oligomeric forms with diverse catalytic features (Stamatakis et al., 1988).

Dilution of the cellular contents, including enzymes, such as PEPC, is an unavoidable consequence during extraction and even the assay of the enzyme activity. Such dilution deprives the enzymes from their natural physico-chemical environment. In case of PEPC, the dilution of enzyme causes dissociation of PEPC from its original quaternary structure and subsequently loss of activity (Willeford and Wedding, 1992). These adverse effects can be counteracted either by high substrate (PEP) concentration (Wagner et al., 1987; Willeford and Wedding, 1992) or by the addition of compatible solutes (Selinioti et al., 1987; Stamatakis et al., 1988; Podestá and Andreo, 1989). Hence, we extended our studies on interaction of PEPC with PEG and glycerol in leaf crude extracts. This aspect serves two purposes: (i) to evaluate the effects of PEG and glycerol on PEPC activity in crude leaf extracts and (ii) to identify the best way of extraction/assay of enzyme.

PEG stimulated the activity of PEPC in leaf crude extracts. The extent of stimulation by PEG of PEPC activity was more when assayed at suboptimal PEP (0.5 mM) than that at optimal PEP (2.5 mM) (Fig. 5.1). Similar results were obtained with purified PEPC (Fig. 5.2). Since cosolutes do not promote the reaction rate at saturating PEP, it is obvious that the substrate exerts a stabilizing action on enzyme activity (Holaday and Black, 1981; Shi et al., 1981; Gavalas et al., 1982; Shomer-Ilan et al., 1985; Stamatakis et al., 1988).

Leaf extracts required higher concentration of PEG for maximal stimulation of PEPC activity than that of purified enzyme. It may be due to the dilution of the enzyme in crude preparation, which needs more concentration of PEG to aggregate the protein. However, further rise in concentration of PEG [6 to 40% (w/v)] can

cause precipitation of PEPC (Stamatakis et al., 1988; Angelopoulos and Gavalas, 1991).

The addition of glycerol in assay medium increased the enzyme activity when assayed at low PEP levels of 1 mM (Gavalas et al., 1982; Stamatakis et al., 1988; Podestá and Andreo, 1989). Ethylene glycol has been shown to stimulate dark- and light-form of purified PEPC from *A. viridis,* up to a concentration of 20% (w/v), while higher concentrations (>25% w/v) inhibit the enzyme (Podestá et al., 1995). PEP itself is capable of inducing aggregation, as shown by different experiments, including those involving light scattering (Willeford et al., 1990; Wu et al., 1990; Wedding et al., 1994).

PEG is known to promote the self-association and/or activation of number of regulatory enzymes in dilute solutions, for e.g. FBPase and pyruvate kinase (Podestá and Plaxton, 1993; Hodgson and Plaxton. 1995). Addition of glycerol, ethylene glycol and BSA increase pyruvate kinase activity, but to a lesser extent than PEG. The inclusion of PEG in the extraction medium stabilized the activity of enzymes (Chidwick, 1994).

Karabourniotis et al. (1983, 1985) reported that the inclusion of glycerol in the extraction medium is necessary for the detection of the differences between light and dark PEPC activities in several C4 plants. Prolinc and betaine completely protected the enzyme against heat inactivation *in vitro* and suggested that compatible solutes assist in maintaining the conformational characteristics and integrity of protein molecules (Paleg et al., 1981). Podestá et al. (1995) have shown that methanol or ethylene glycol stimulate the activity of PEPC, purified from *Amaranthus viridis.*

Concentration dependent dissociation of PEPC was reported in CAM plant *Bryophyllum* (Jones et al., 1978). PEPC from *Crassula* exists as both di- and tetramer, that during storage at -70°C the dimeric form showed a strong tendency to

aggregate to the tetramer and this aggregation was associated with less sensitivity to inhibition by malate. Further, malate itself induced dimerization and increased the malate inhibition in *Crassula argentea* (Wu and Wedding, 1987). Preincubation with PEP shifts the equilibrium towards the tetrameric form and reduces the maximum inhibition produced by 5 mM malate to <20%. However, none of the treatments used resulted in shifting the oligomerization equilibrium completely in either direction. Thus, the question of whether some covalent modification of the enzyme, such as phosphorylation, is required to permit complete changes in equilibrium remains open.

Most of the observations on changes in oligomeric status of PEPC were made *in vitro* (Wu and Wedding. 1985; Wagner et al., 1987; Podestá and Andreo, 1989; Willeford et al., 1990). Attempts to record such changes *in vivo* of oligomerization or dissociation of PEPC have so far not been successful (Wagner et al., 1987; Podestá and Andreo, 1989; Weigend and Hincha. 1992). As a result, the physiological significance of changes in oligomeric status of PEPC has been questioned (McNaughton et al., 1989; Wu et al., 1990).

Gel filtration technique has earlier been employed to assess the molecular size of PEPC. The oligomeric status of PEPC was found to change with dilution of enzyme suspension (McNaughton et al., 1989). PEPC was eluted as a tetramer on eluting with buffer containing glycerol (Podesta´ and Andreo, 1989). However, in the absence of glycerol in elution buffer the enzyme appeared as both tetramer and dimer at pH 7.0.

We have also used gel filtration technique to assess the changes in the quaternary structure of the enzyme in presence or absence of PEG and glycerol. We have observed three types of oligomers during the gel filtration. The absence of either PEG or glycerol in elution buffer resulted in mono-, di- and tetrameric forms of PEPC (Fig. 5.4A), with monomers being the predominant species. A prominent

peak of tetramers and another peak of dimers were obtained when 10% glycerol was used during preincubation and elution (Fig. 5.4B). Whereas the inclusion of PEG alone or PEG + glycerol resulted in tetramers (Fig. 5.4, C and D). These results suggest that the addition of compatible solutes protect the enzyme from dilution and promote the existence of tetrameric state. We therefore suggest that an increase in homologous protein-protein interaction, probably promoting subunit aggregation, is responsible for the PEG-mediated activation.

The information on the activity of monomer or dimer of PEPC is ambiguous. There are few reports suggesting that dimer is inactive or less active or inert (Stiborová and Leblová, 1985; Wu and Wedding. 1985; Walker et al., 1986 a, b; Selinioti et al., 1987; Wagner et al., 1987; McNaughton et al., 1989; Wu et al., 1990; Meyer et al.. 1991). Whereas, Willeford et al. (1990) have observed that the dimer of maize PEPC had considerable enzyme activity under standard assay conditions or is possibly converted to the tetrameric form in the assay. Maize dimer appears to be unstable, whereas the dimer of *Bryophyllum* PEPC was stable but had only about 50% of the activity of the tetramer (McNaughton et al., 1989). The activities of different oligomcrs during the gel filtration through Sephadex G-200 column were therefore of great interest (Fig. 5.6). Only the tetrameric form was found to be active (Fig. 5.6). Further, the activity of tetramer, was much greater in presence of glycerol and/or PEG than that in the absence of cosolutes (Fig. 5.6, A-D). It is obvious that the presence of PEG and glycerol are effective in maintaining the tetrameric structure of the enzyme but also keeping up high specific activity (Fig. 5.6, B and C).

The present work is the first attempt to assess in detail the status and properties of PEPC, on extraction from leaves in presence or absence of solutes in the medium. The kinetic properties of PEPC (in both leaf extracts and purified preparation) changed in response to the presence of PEG and/or glycerol. In presence of compatible solutes, the affinity for PEP of PEPC increased, while the

sensitivity to malate decreased as indicated by the increase in the values of their Kj (Tables 5.1 and 5.2). On the other hand, K_m (HCO3-) was not affected. These observations are similar to a earlier reports in the literature. Several organic cosolutes (glycerol, sorbitol, betaine, proline, PEG, PVP) or high concentration of protein in the assay medium increase the affinity of PEPC for PEP, whereas the maximum velocity remains unaffected (Stamatakis et al., 1988).

In case of pyruvate kinase, 5% PEG in the reaction medium increases 2.6-fold in maximal velocity and 12.5- to 2-fold reduction in K_{m} (PEP) and K_m (ADP), respectively (Podestá and Plaxton, 1993). Huber and Sugiyama (1986) have reported that PEG relieves malate inhibition in both crude extracts and purified enzyme. Even the presence of glycerol can help to overcome the inhibition of the enzyme by malate (Podestá and Andreo, 1989). We suggest that PEPC becomes less susceptible to malate inhibition in presence of PEG or glycerol, possibly because of the shift of enzyme towards active tetrameric form.

Our results demonstrate that PEG or glycerol help in keeping PEPC in its native active tetrameric form even in crude leaf extracts. These studies are quite relevant to the situation *in vivo*, and may be extended further to assess changes in oligomerization of PEPC, for e.g. on illumination. Glycerol is known to stabilize the activity of PEPC and is frequently included during extraction of enzyme from various plant tissues, including those of C4 plants (Manetas, 1982; Karabourniotis et al., 1983; Manetas et al., 1986; Stamatakis et al., 1988; Jawali, 1990; Manetas, 1990; Drilias et al., 1994). However, very few reports stressed the need for addition of glycerol during assays of PEPC activity. The use of compatible solutes during *in vitro* assay, appears to be highly relevant to *in vivo* situation, at least for oligomeric enzymes such as PEPC and PPDK (Salahas et al., 1990).

Our results demonstrate that the presence of PEG and/or glycerol during extraction and assay not only enhances the catalytic activity of PEPC but also

improves the stability of enzyme. The main reason for this effect appears to be the maintenance of oligomeric state of PEPC (Fig. 5.1), which is disturbed during dilution that occurs while extracting leaves. We therefore recommend that PEG and/or glycerol could be valuable additives to the medium to determine maximum catalytic efficiency of the enzyme. However, it should also be kept in mind that PEG and/or glycerol may change the kinetic and regulator)' properties of PEPC.

Our results suggest that the structural, kinetic and regulatory properties of PEPC are influenced by the microenvironment of the cell. The concentration of PEPC while being assayed *in vitro*, is far below to its expected concentration *in vivo*. To mimic the *in vivo* situation, precautions must therefore be taken. The observation from this chapter suggests that PEG and glycerol should be included during extraction of leaves as well as during the assay of enzyme, while studying the catalytic and regulatory properties of PEPC. A similar situation has been reported for another cytosolic enzyme, pyruvate kinase (Podestá and Plaxton, 1993). The quaternary structure of PEPC can be maintained only in presence of compatible solutes under *in vitro* conditions.

Major conclusions from the results presented in this chapter are:

1. The addition of PEG and glycerol during both extraction and assay stabilizes PEPC and helps to maintain high activities of the enzyme.

2. The presence of PEG and/or glycerol in the assay medium, stimulated the activity of even purified preparation of PEPC.

3. In the presence of PEG, the affinity for PEP of PEPC in leaf extracts or purified PEPC decreased, while the Kj for malate increased suggesting that the regulation of PEPC is dampened while promoting catalytic activity of the enzyme.

4. The extent of light activation of PEPC, observed in leaf extracts, was still high, but decreased marginally in presence of PEG and glycerol during extraction and assay.

5. Gel filtration of purified PEPC on Sephadex G-200 column showed the existence of three different forms with proportionally increasing molecular weight: monomer, dimer and tetramer. The absence of PEG or glycerol during elution resulted in a marked shift of the enzyme into di- and/or monomers, with only a small portion of tetramer. The presence of PEG and/or glycerol or both during elution of PEPC from Sephadex G-200 column, resulted in predominance of tetramer.

6. The present results strongly recommend the inclusion of 2.5% PEG and 10% glycerol during both extraction and assay, so as to stabilize the enzyme and maintain it in a tetrameric state.

Chapter 6

Modulation by Bicarbonate of Catalytic and Regulatory Properties of PEPC

Chapter 6

Modulation by Bicarbonate of Catalytic and Regulatory Properties of PEPC

Introduction

PEPC, a key enzyme mediating the primary carbon assimilation, is highly regulated in leaves of C4 plants. The activity of PEPC in C4 leaves is enhanced on illumination. The enzyme is also markedly modulated by metabolites, for e.g., PEPC is activated by Glc-6-P and feed-back inhibited by L-malate (Andreo et al., 1987; Rajagopalan et al, 1994; Chollet et al., 1996; Vidal and Chollet, 1997).

The sensitivity of PEPC to malate is influenced by various factors like light, pH and Glc-6-P. When leaves are illuminated, there is a marked decrease in sensitivity of PEPC to malate besides an increase in activity (Rajagopalan et al., 1993). Malate inhibition is competitive at pH 7.0, and non-competitive at pH 8.0 (González et al, 1984). Glc-6-P is an allosteric activator of PEPC, decreases K_m for PEP (Uedan and Sugiyama, 1976) and protects the enzyme from malate inhibition (Gupta et al, 1994).

Carboxylation of PEP is a bicarbonate-dependent reaction. As PEPC is an important enzyme mediating the primary carbon assimilation in C4 plants, the availability of HCO_3^- plays a major role in C4 photosynthesis. The effect of bicarbonate on the regulation of PEPC is obviously a topic of interest. We have therefore made an attempt to study the modulation of kinetic and regulatory characteristics of PEPC in relation to bicarbonate concentration.

Compared to the extensive literature on the modulation of malate-sensitivity of PEPC, the studies on changes in kinetic characteristics of the enzyme, **and** in particular the affinity to HCO_3^- are quite limited (O'Leary, 1982; Chollet et al.,

1996). This may be mainly due to the difficulty in the complete removal of dissolved bicarbonate during PEPC assays. There is also a lot of discrepancy for the K_m value for HCO_3^-, which ranges from 0.02 to 0.1 mM in C4 plants (at a pH range of 7 to 8) (Uedan and Sugiyama, 1976; Bauwe, 1986; Gao and Woo, 1995, 1996b; Dong et al., 1997a). Dong et al. (1997a) and Ogawa et al. (1997) have recently been reported that bicarbonate plays a major role in the allosteric regulation of PEPC. Such modulation of PEPC by one of the substrates, HCO_3^- is extremely interesting.

Most of the studies on PEPC have been made using 5 or 10 mM HCO3- (Doncaster and Leegood, 1987; Ausenhus and O'Leary, 1992; Bakrim et al., 1993; Rajagopalan et al., 1993; Gupta et al., 1994; Podestá et al., 1995; Gao and Woo, 1996b; Svensson et al., 1997) in the assay medium, while bicarbonate level in mesophyll cytosol (the site of PEPC) of C4 plants is expected to be 80 μM (Jenkins et al., 1989). We have therefore studied the kinetic and regulatory characteristics of PEPC at low or limiting (0.05 mM) and high or saturating (10 mM) concentrations of bicarbonate.

Results

Figure 6.1 A represents the typical response of PEPC from dark-adapted leaf discs to varying concentrations of HCO_3^-, in presence or absence of 0.5 mM malate. At a pH of 7.3, the inhibition of PEPC by malate (a feed-back inhibitor) was complete as indicated by no activity in presence of 0.5 mM malate. However, the inhibition by malate of PEPC was dependent on the concentrations of HCO3-. The inhibition by malate was markedly relieved by increasing concentration of bicarbonate. The relief by bicarbonate was more prominent at optimal concentration of PEP (2.5 mM) than that at suboptimal concentration (0.5 mM) (Fig. 6.1B). Similar results were obtained with PEPC purified from dark-adapted

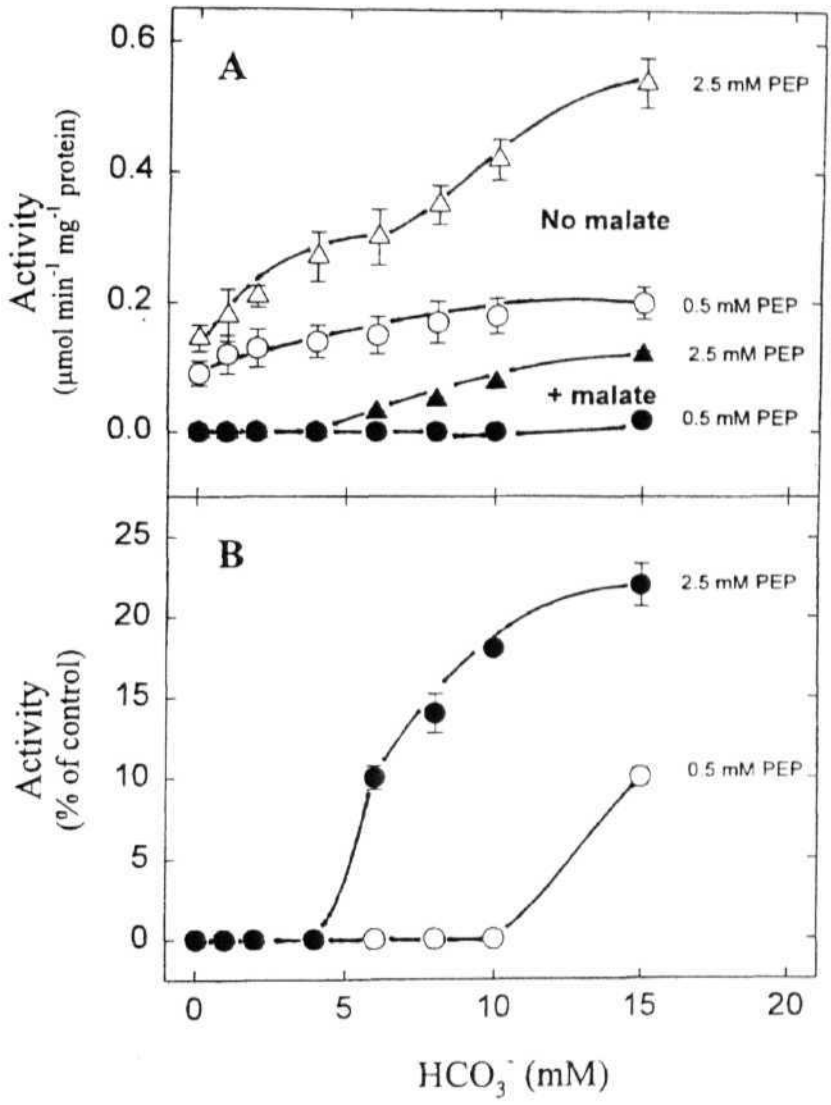


Figure 6.1. The sensitivity of PEPC from dark-adapted leaves of *Amaranthus hypochondriacus* to varying concentrations of HCO3". The enzyme was assayed at pH 7.3 in the presence or absence of 0.5 mM malate (A). The activities **are** represented as % of respective controls in Fig. B in the presence of 0.5 mM malate for effective comparison. Further details are described in "Materials and Methods".

leaves (Fig. 6.2). We have therefore examined the modulation of PEPC-properties by assaying the enzyme at 2.5 mM PEP, in all subsequent experiments.

The sensitivity of PEPC to malate or Glc-6-P was modulated significantly by HCO_3^-. The extent of malate inhibition was always more at 0.05 mM HCO3- than that at 10 mM HCO_3^- (Fig. 6.3)- Similarly, the extent of activation by Glc-6-P was much more at suboptimal bicarbonate (0.05 mM) than at saturating concentration (10 mM) of bicarbonate (Fig. 6.4).

Table 6.1 summarizes the catalytic as well as regulatory properties of PEPC at 0.05 mM and 10 mM bicarbonate. At high or saturating (10 mM) bicarbonate, the velocity of the enzyme increased by nearly 3 fold, while the K_m for PEP increased marginally. In contrast. K_m for Mg^{2+} was lowered by more than 2 fold in presence of 10 mM bicarbonate compared to that in the presence of 0.05 mM. Similarly, Kj for malate increased by about 3 fold and K_a of Glc-6-P rised by four fold as bicarbonate concentration during the enzyme assay was raised to 10 mM, from 0.05 mM.

The response of purified dark-form PEPC to bicarbonate was quite similar, as indicated by the decrease in sensitivity to malate or Glc-6-P (Table 6.1). We preferred to present the data with desalted extracts, so as to study the difference, if any, of the light- and dark-forms of PEPC in leaves.

It is known that the regulatory properties of light-form (phosphorylated) of PEPC are different from those of dark-form (non-phosphorylated). However, experiments using the extracts from illuminated leaves revealed that the typical responses of PEPC to high HCO_3^- [namely, the increase in Kj (malate) or Ka (Glc-6-P)] were occurred in the case of both light- and dark-forms of PEPC (Table 6.2).

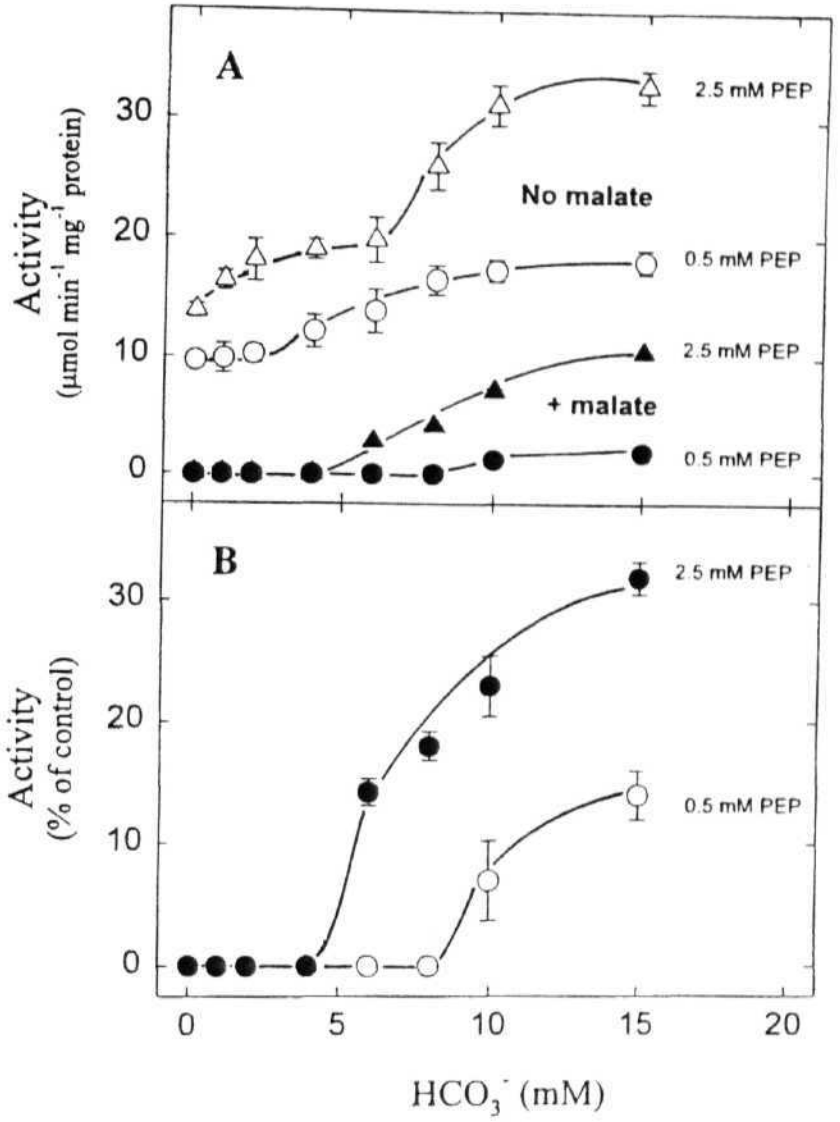


Figure 6.2. The sensitivity of PEPC, purified from dark-adapted leaves of *Amaranthus hypochondriacus* to varying concentrations of HCO3". The enzyme was assayed at pH 7.3 in the presence or absence of 0.5 mM malate (A). The activities are represented as % of respective controls in Fig. B in the presence of 0.5 mM malate for effective comparison. Further details are described in "Materials and Methods".

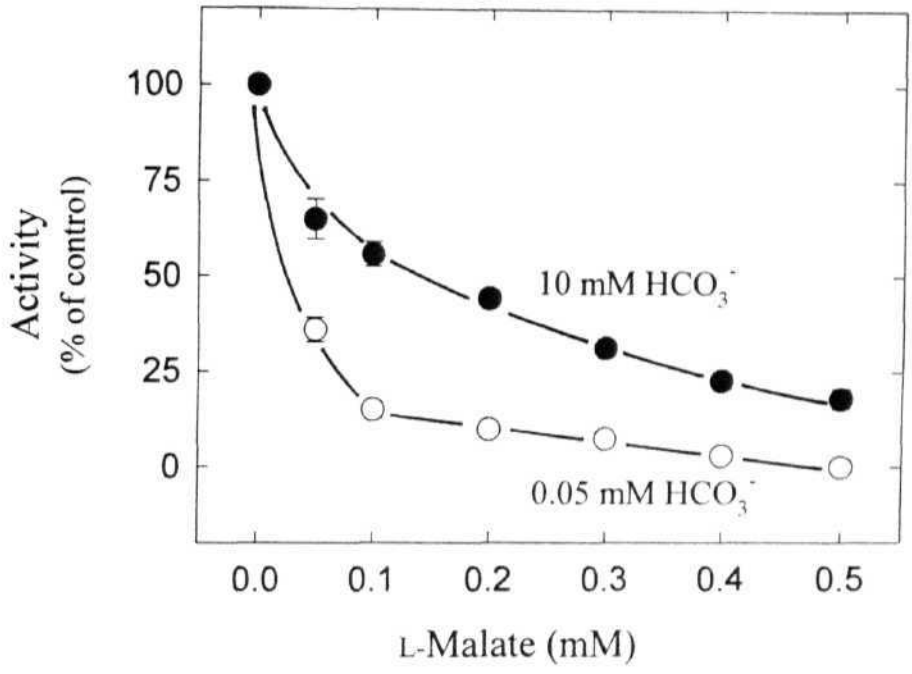


Figure 6.3. The sensitivity of PEPC from leaves of *Amaranthus hypochondriacus* to malate in presence of either 0.05 mM or 10 mM HCO_3^-. The activities of PEPC are represented as % of respective controls. The activity of PEPC, in the control sets (without malate) was 0.15 ± 0.02 μmol min^{-1} mg^{-1} protein and 0.42 ± 0.03 |imol min^{-1} mg^{-1} protein in presence of 0.05 and 10 mM HCO_3^-, respectively. Further details are as in Fig. 6.1.

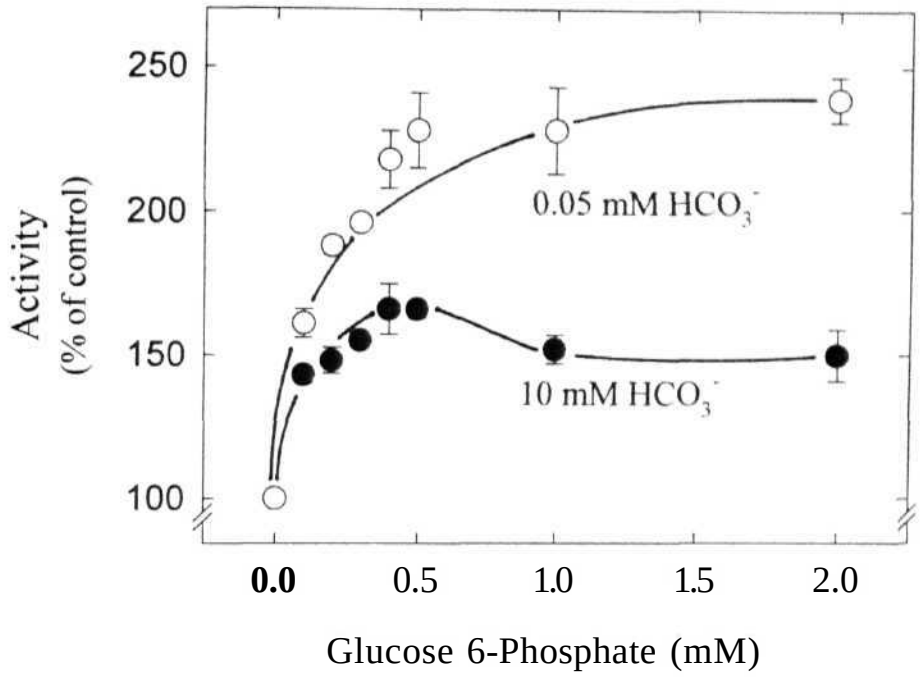


Figure 6.4. The sensitivity of PEPC from leaves of *Amaranthus hypochondriacus* to Glc-6-P in presence of either 0.05 mM or 10 mM HCO3". Further details are as in Fig. 6.3.

Table 6.1. *Kinetic and regulatory characteristics of PEPC from dark-adapted leaf extracts of* Amaranthus hypochondriacus.

The data obtained with purified dark-form PEPC are also presented, for a spontaneous comparison. PEPC activity was assayed at pH 7.3.

Parameter	0.05 mM HCO3-	10 mM HCO_3^-
Extract from dark-adapted leaves		
Kinetic characteristics		
Velocity (μmol min^{-1} mg^{-1} protein)	0.15 ±0.02	0.42 ± 0.03
K_m (PEP) (mM)	0.38 ±0.02	0.46 ± 0.02
K_m (Mg^{2+}) (mM)	4.7 ± 0.09	1.9 ±0.07
Regulator}' properties		
Kj (malate) (mM)	0.03 ±0.001	0.09 ± 0.007
K_a (Glc-6-P) (mM)	0.10 ±0.002	0.39 ± 0.003
Purified dark-form PEPC		
Kinetic characteristics		
Velocity (μmol min^{-1} mg^{-1} protein)	13.9 ±0.4	35 ±0.7
K_m (PEP) (mM)	0.16 ±0.02	**0.17** ±0.01
K_m (Mg^{2+}) (mM)	4.3 ±0.6	1.2 ±0.1
Regulatory properties		
K_i (malate) (mM)	0.05 ± 0.008	**0.16 ±0.002**
K_a (Glc-6-P) (mM)	0.10 ±0.001	**0.69 ± 0.003**

Table 6.2. *The properties of the enzyme from dark or light-adapted leaf extracts of* Amaranthus hypochondriacus.

PEPC activity was assayed at pH 7.3 and with 0.05 or 10 mM HCO3-. Further details are described in "Materials and Methods".

Form of PEPC/ Parameter	HCO_3^- A		change*
	0.05 mM	10 mM	-fold
Dark-form			
Velocity (μmol min^{-1} mg^1 protein)	0.22 ± 0.02	0.49 ± 0.03	2.2
K_i (malate) (mM)	0.03 ± 0.001	0.08 ± 0.002	2.7
K_a (Glc-6-P) (mM)	0.09 ± 0.002	0.32 ± 0.003	3.6
Light-form			
Velocity (μmol min^1 mg^{-1} protein)	0.74 ± 0.03	0.92 ± 0.04	1.2
K_i (malate) (mM)	0.09 ± 0.002	0.35 ± 0.01	3.9
K_a (Glc-6-P) (mM)	0.22 ±0.002	1.01 ±0.002	4.6

* Due to increase in bicarbonate.

Discussion

Our results reveal that bicarbonate modulates markedly both the kinetic and regulatory properties of PEPC. The major effect of bicarbonate is to desensitize PEPC to both malate, a feed-back inhibitor (Fig. 6.3) and Glc-6-P (allosteric activator) (Fig. 6.4). Besides the feed-back inhibition by malate, one of the products, the enzyme appears to be feed-forward regulated by bicarbonate, one of the substrates. This is the first detailed report on the marked desensitization of PEPC to malate and Glc-6-P, and sensitization to Mg^{2+}.

Being a NAD-malic enzyme type species, the leaf extracts of *Amaranthus hypochondriacus* arc likely to contain NAD-ME, which may interfere with measurements by leading to NADH production. This would cause an apparent reduction in PEPC activity. We have therefore conducted several checks with purified PEPC. However, a similar pattern was noticed with PEPC in either leaf extracts or in purified form. Figure 6.2 represents data with purified enzyme. The saturation curves of PEP were hyperbolic in the absence of malate and sigmoid in presence of malate indicating an active conformation of enzyme.

The pattern of PEPC response to HCO3" appears to be quite complex and unusual (Figs. 6.1 and 6.2). Double reciprocal plots as well as Eadie-Hofstee plots indicated that the response of PEPC to variable HCO3" concentration exhibits at least two phases. Such complex response may be one of the reasons for the wide variation in the K_m (HCO3") values of PEPC reported for different C4 species (Uedan and Sugiyama, 1976; Bauwe, 1986; Gao and Woo, 1995, 1996b; Dong et al., 1997a).

The required order of addition of reactants is Mg^{2+}, PEP and HCO_3^-, with a high level of synergism in the binding of substrates (Janc et al., 1992a). Svensson et al. (1997) reported that PEP and HCO3- binding sites are the same in C4 PEPC of *Flaveria trinervia.* We have therefore examined the response of PEPC to

bicarbonate by starting the reaction with either PEP or bicarbonate. But the decrease in the sensitivity to malate or Glc-6-P at high HCO_3^- occurred irrespective of the sequence of addition of the substrates. We therefore wonder if HCO3" has a separate binding site on PEPC, other than that of PEP.

The Kj (malate) values of purified enzyme were always more than that of the PEPC in leaf extracts (Table 6.1). A similar trend has been noticed in case of maize: Kj (malate), being 0.12 and 0.2 mM for crude and purified PEPC, respectively (Jiao and Chollet, 1988; Jiao and Chollet. 1992). While we do not know the exact reason, we feel that it could be due to the storage of enzyme with 50% glycerol.

It is known that C4 PEPC undergoes marked post-translational regulation by light. On illumination, PEPC is phosphorylated by a PEPC-protein kinase and dephosphorylated by a protein phosphatase when leaves are returned to darkness (Rajagopalan et a1, 1994; Chollet et al., 1996; Vidal and Chollet, 1997). However, our observations with PEPC from illuminated or dark-adapted leaves, confirmed that the marked decrease in the sensitivity of the enzyme to malate or Glc-6-P and increase in the affinity to Mg^{2+} occurred in both light-form (phosphorylated) and dark-form (nonphosphorylated) of PEPC. The present results therefore demonstrate marked modulation of PEPC by bicarbonate. by a mechanism independent of phosphorylation. Thus, HCO_3^- may modulate the conformation of the enzyme in such a way that enzyme loses its sensitivity to other metabolites, such as malate and Glc-6-P. The marked changes induced by bicarbonate in catalytic as well as regulatory properties of the enzyme (Table 6.1) suggest strongly that bicarbonate changes the conformation of the enzyme.

Since PEPC is extremely sensitive to pH and prefers alkaline range for optimal activity, any rise in the pH of the medium can result in enhancement of activity. Addition of HCO_3^- up to 20 mM in the reaction medium did not change

the pH of **the** medium. Thus, HCO_3^- induced catalytic or regulatory changes of PEPC are not due to any change in pH of the medium.

PEPC uses HCO3" during carboxylation of PEP. Therefore, the availability/ solubility of HCO_3^- plays a major role determining the rate of PEPC. The pH of **the** cytosol in mesophyll cells of C4 plants increases upon illumination (Raghavendra et al., 1993) and this can lead to increase the solubility of HCO3" in the cytosol of mesophyll. In fact, illumination increased the affinity of PEPC to bicarbonate (Chapter 7). The present results demonstrate that not only illumination of leaves but also presence of HCO_3^- desensitizes PEPC to malate-inhibition. We therefore suggest that the kinetic interactions between PEPC, bicarbonate and light are significant in moderating the inhibitor}' effects of malate.

Major conclusions from the results presented in this chapter are:

1. The sensitivity of PEPC to malate decreased markedly in presence of bicarbonate. The effect of bicarbonate was observed when PEPC was assayed either at 0.5 mM or 2.5 mM PEP.

2. The stimulation by Glc-6-P was more at low concentration of HCO_3^- (0.05 mM) than that at high concentration (10 mM).

3. Bicarbonate sensitizes the enzyme to Mg^{2+} and desensitizes to malate and Glc-6-P.

4. Bicarbonate modulates the regulator}' and kinetic properties of the enzyme not only in dark-form of PEPC but also in light-form of PEPC.

5. These results demonstrate that HCO3" acts as not only a substrate but also an allosteric regulator of enzyme at high concentrations.

Chapter 7

Illumination increases the Affinity of PEPC to Bicarbonate in Leaves

Chapter 7

Illumination increases the Affinity of PEPC to Bicarbonate in Leaves

Introduction

Photoregulation of enzymic activities appears to be a prevalent phenomenon in photosynthetic cells (Buchnan, 1980). This is clearly the case with also PEPC of C4 plants. However, the extent of photoactivation of PEPC ranges from 1.5 to 3.5 (depending on the extraction and assay conditions) and is not large enough to be considered of primary importance for the regulation of C_4 metabolism (Selinioti et al., 1986). Additional factors, such as pH, substrate and activator levels which may amplify the light effect (Karabourniotis et al., 1985), but the picture of PEPC regulation *in vivo* is far from complete (Selinioti et al., 1986).

Nevertheless, the catalytic and regulatory characteristics of C_4 PEPC are strongly regulated by light/dark transitions *in vivo* (Andreo et al., 1987; Jiao and Chollet, 1991; Rajagopalan et al., 1994; Chollet et al., 1996; Vidal and Chollet, 1997). Light reversibly induces a two to three fold increase in catalytic activity and decrease in malate sensitivity of the enzyme from a variety of C4 plants when assayed at suboptimal, but physiological levels of pH and PEP. Darkness reverses the effects of illumination on C_4-leaf PEPC activity. Moreover, light activation of PEPC in C4 plants is related, either directly or indirectly, to photosynthetic electron transport and/or photophosphorylation and is modulated by several photosynthesis-related environmental factors, including light intensity, CO_2 concentration, and temperature (Samaras et al., 1988).

Besides an increase in V_{max}, there is a marked decrease in the sensitivity to malate in the light-form of PEPC (Huber and Sugiyama, 1986; Doncaster and Leegood, 1987). A major mechanism of such modulation in enzyme activity is

phosphorylation-dephosphorylation cascade. PEPC is activated in light due to phosphorylation of enzyme by PEPC-protein kinase and dephosphorylated in dark by a type 2A protein phosphatase (Chollet et al., 1996; Vidal and Chollet, 1997). Phosphorylated form of PEPC is more active in catalysis and less sensitive to malate than the dephosphorylated form. The enzyme is also regulated by metabolites (e.g. malate, Glc-6-P. DHAP), whose levels change upon illumination (Doncaster and Leegood, 1987).

The pattern and mechanism of light activation of PEPC in leaves of C4 plants has been a topic of interest in our lab (Rajagopalan et al., 1993; Gayathri and Raghavendra, 1994). Surprisingly, most of the attention was focused on the regulatory properties of PEPC, i.e., the sensitivity of the enzyme to either malate or Glc-6-P. Factors such as pH, temperature or light exert much more marked effect on the sensitivity to malate, than that on the kinetic properties, i.e., affinity to PEP (Chollet et al., 1996; Vidal and Chollet, 1997). We have noticed that the affinity of PEPC to HCO3" in leaves of C4 plants markedly increased on illumination and examined this phenomenon in detail. The present study is the first report on marked modulation by light of K_{m} of PEPC to HCO3". one of the substrates of the enzyme.

PEPC is located in the cytosol of mesophyll cells. The level of dissolved HCO_3^- in the mesophyll cytosol (at a pH of 7.4 and in equilibrium with air) is expected to be 80 μM (Jenkins et al., 1989). However, the pH of the cytosol increases upon illumination (Pierre et al., 1992; Raghavendra et al., 1993; Yin et al., 1993) and this may lead to increase in the availability of HCO_3^- in the cytosol of mesophyll. This report on the increase in the affinity of PEPC to bicarbonate assumes a great significance as it accounts to sensitization of PEPC to physiological concentration in mesophyll cytosol on illumination of C4 leaves.

Results

The response of the activity of PEPC to varying concentrations of HCO_3^- was examined at both limiting (0.5 mM) as well as saturating PEP (2.5 mM) using the extracts prepared from dark-adapted or illuminated leaves (Fig. 7.1). **The** response of PEPC to HCO_3^- was higher at 2.5 mM PEP than that at 0.5 mM PEP. We have therefore employed 2.5 mM PEP for all further experiments.

The activity of PEPC from illuminated leaves was 2 to 5-fold more than that from the dark-adapted ones. Further, the activity of PEPC from illuminated leaves was very high even at low concentrations of HCO_3^- (Fig. 7.2). In spite of the precautions, it was impossible to remove completely the dissolved bicarbonate. There was a significant activity of PEPC, even if no bicarbonate was added to the assay medium obviously due to the endogenous bicarbonate, which is expected to be about 100 μM (Bauwe, 1986). The net-activities of PEPC at varying concentrations of HCO_3^- (after correcting by subtraction of enzyme activity without any added bicarbonate), in the extracts prepared from dark-adapted or illuminated leaves, are represented in the inset of Figure 7.2.

The double reciprocal plots illustrate the dramatic difference in the affinity of PEPC to HCO3-. The K_m (HCO3-) of PEPC in extracts from illuminated leaves was less than half of that from dark-adapted ones (Fig. 7.3) irrespective of the condition, that the endogenous (already dissolved) bicarbonate is considered while calculating the bicarbonate level during the assay of PEPC. Besides, there was an increase of at least 3-fold in V_{max} of PEPC from illuminated leaves.

As a result of this phenomenon, the stimulation of PEPC activity by light was pronounced at lower concentration of HCO_3^-. The increase in bicarbonate concentration during assay of PEPC activity, led to a marked decrease in the ratio of PEPC **activity** in light-exposed leaves to that in dark-adapted ones (L/D ratio).

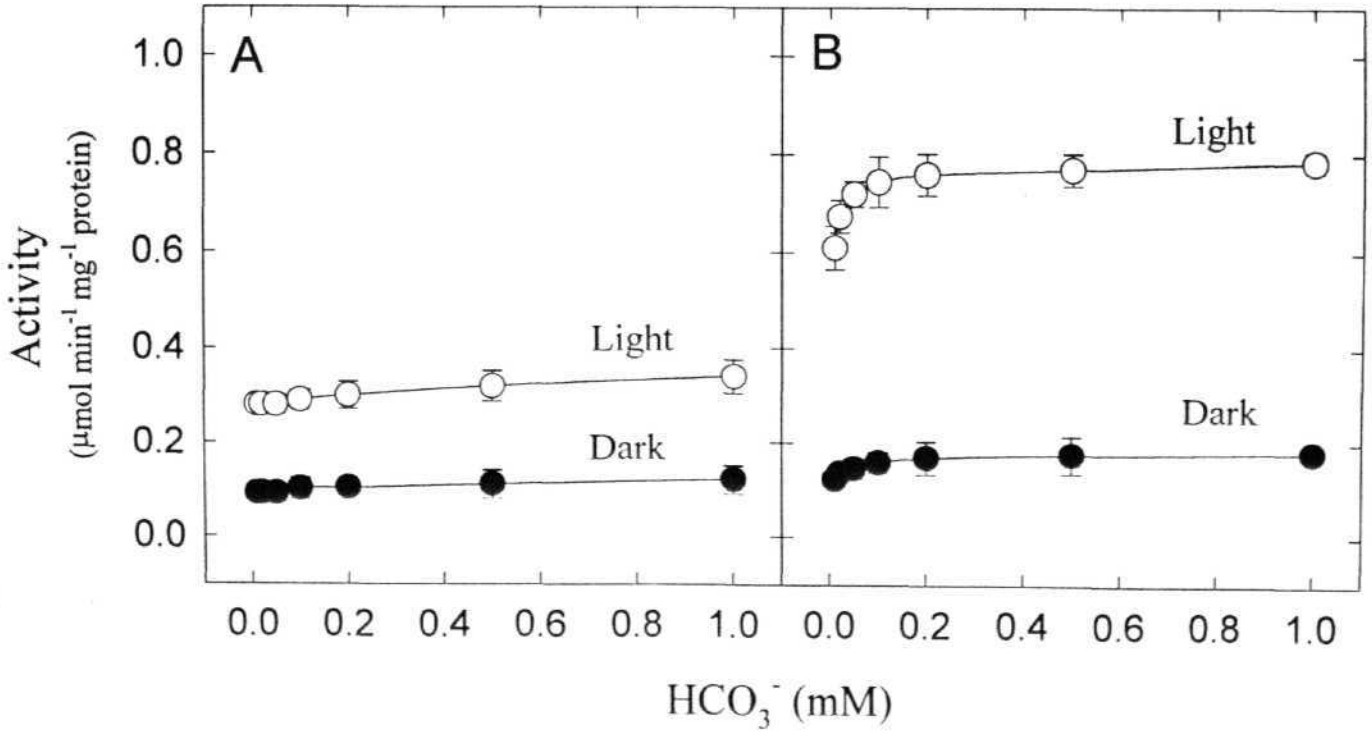
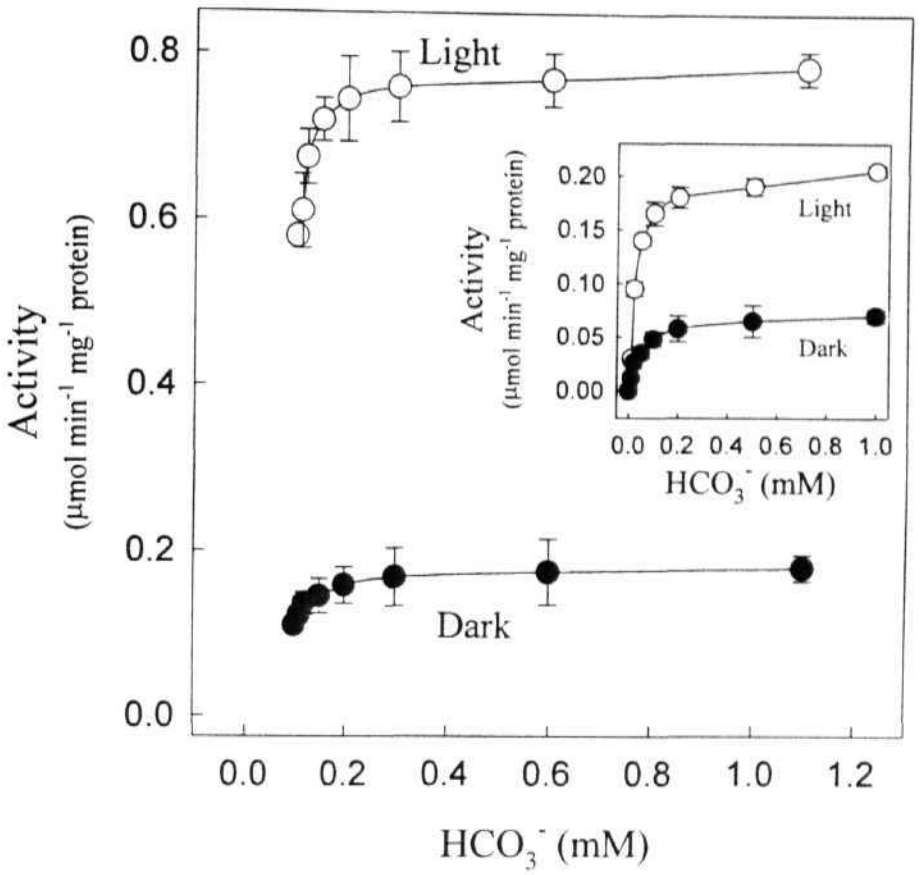


Figure 7.1. The response of PEPC to varying concentrations of HCO_3^- in desalted extracts prepared from illuminated or dark-adapted leaf discs. The enzyme was assayed at pH 7.3 and a PEP concentration of either 0.5 mM PEP (A) or 2.5 mM PEP (B). HCO_3^- was included in the assay medium at the concentration indicated. Further details are described in "Materials and Methods".



0.0 0.2 **0.4** 0.6 0.8 **1.0** **1.2**

HCO_3^- (mM)

Figure 7.2. The response of PEPC to HCO3" in desalted extracts prepared from illuminated or dark-adapted leaf discs. The enzyme was assayed at pH 7.3 and with 2.5 mM PEP. The dissolved endogenous bicarbonate is expected to be 0.1 mM at pH 7.3 (Bauwe, 1986). Extra HCO_3^- (0.01 to 1 mM) was added to the assay medium and the final concentrations of HCO3" are indicated in the Figure (0.11 to 1.1 mM). The activity of the enzyme without any added bicarbonate was 0.58 and 0.11 μmol min^{-1} mg^{-1} protein, in the extracts from illuminated and dark-adapted leaves, respectively. Inset: Corrected values derived by subtracting from **total** (gross) activity, the back ground activity of PEPC without any added bicarbonate. Further details are described in "Materials and Methods".

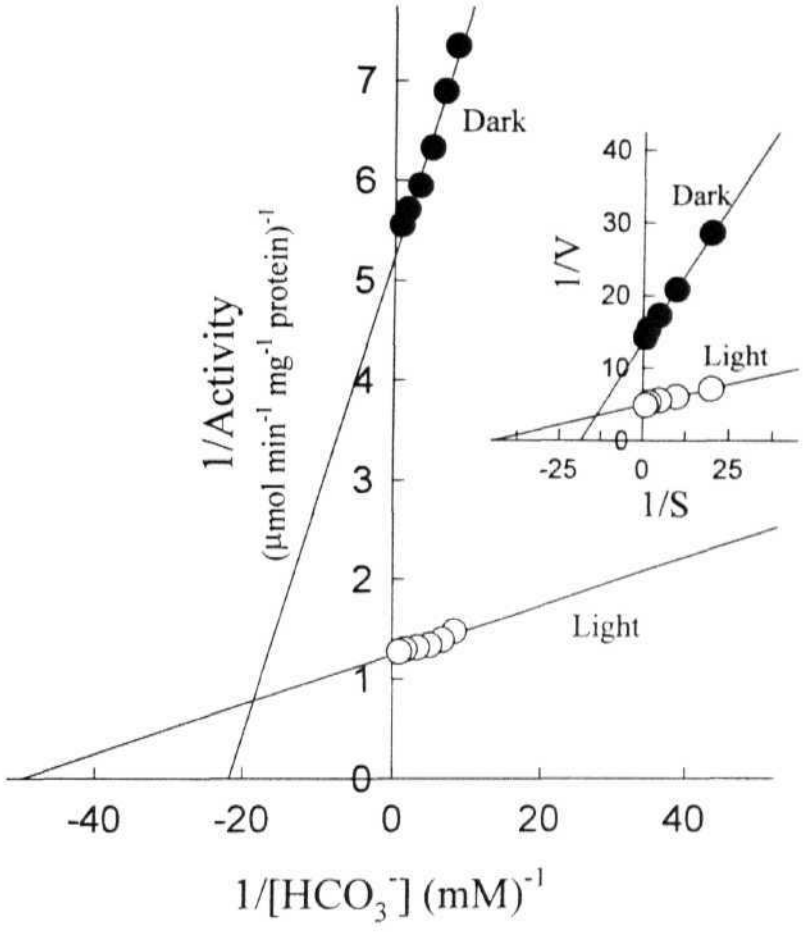


Figure 7.3. The double-reciprocal plots of PEPC activity (V) against bicarbonate concentration (S). The activity of PEPC is indicated in µmol min^{-1} mg^{-1} protein and the bicarbonate concentrations in mM. PEPC was assayed at pH 7.3 and 2.5 mM PEP. K_m (HCO_3^-) in extracts from illuminated leaves (o) was 0.020 mM and V_{max} was 0.81 µmol min^{-1} mg^{-1} protein while in dark-adapted extracts (•) K_m (HCO_3^-) was 0.046 mM and V_{max} was 0.19 µmol min^{-1} mg^{-1} protein. The inset shows the double reciprocal plots of data corrected for basal activity in the absence of externally added bicarbonate. The K_m (HCO_3^-) values of PEPC in illuminated and dark-adapted leaves were 0.023 mM and 0.054 mM respectively.

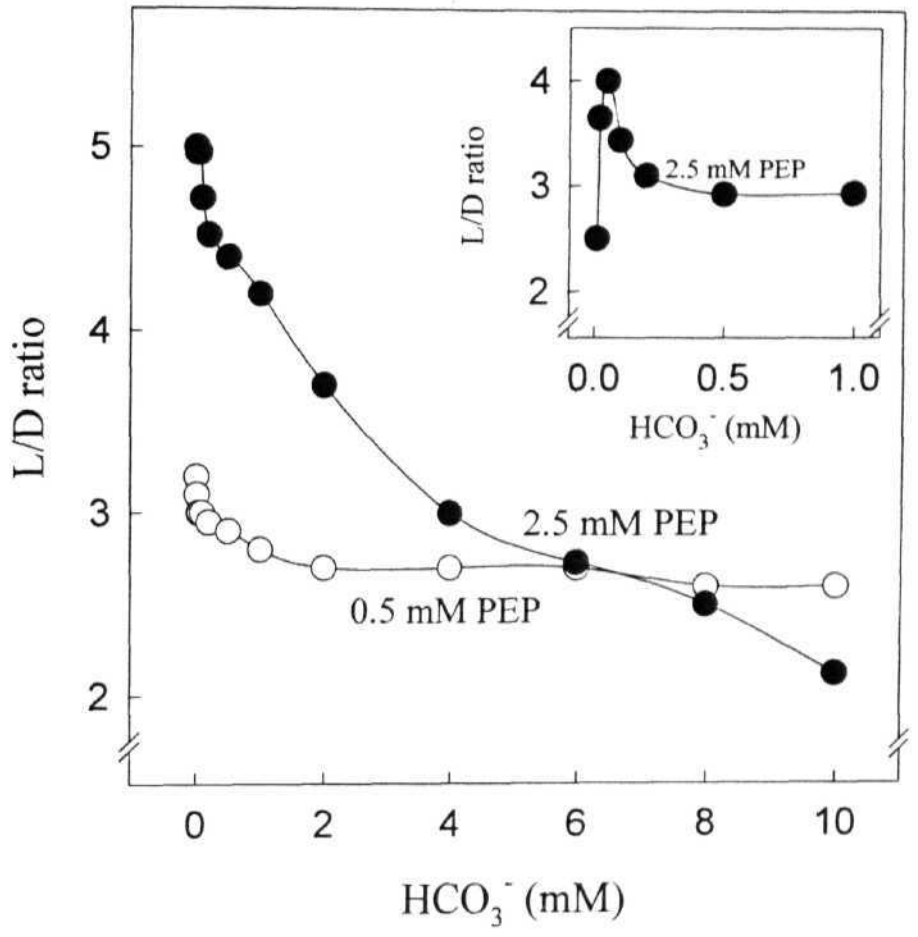
The decrease in L/D ratio was quite steep when PEPC was assayed at 2.5 mM PEP (Fig. 7.4).

Light stimulates the synthesis of PEPC-protein kinase and thus promotes PEPC phosphorylation (Hartwell et al., 1996). CHX, an inhibitor of cytosolic protein synthesis, blocks PEPC-protein kinase synthesis and light activation of PEPC (Carter et al., 1991; Jiao et al., 1991b; Bakrim et al., 1992, 1993). Feeding of 5 μM CHX to leaf discs restricted the light activation and light induced increase in the Kj (malate) (Table 7.1). The decrease in K_m (HCO_3^-) of PEPC in illuminated extracts, also was restricted by the CHX pretreatment of leaf discs.

The desalted extracts can be expected to contain carbonic anhydrase, which catalyses the interconversion of CO_2 and HCO_3^-. However, the presence of Ethoxyzolamide (EZ), an inhibitor of carbonic anhydrase during assay had no effect on either the extent of light activation or the decrease in K_m for HCO3" in light-activated PEPC (Table 7.1).

In view of the observed increase in the affinity of PEPC to bicarbonate of PEPC is enhanced on illumination, the kinetic and regulatory properties of light-activated and dark-adapted forms of PEPC were reexamined at either low (0.05 mM) or saturating (10 mM) levels of HCO_3^- (Table 7.2). The light-induced effects: enhancement of PEPC activity and the decrease in sensitivity to malate were greater at 0.05 mM bicarbonate than those at 10 mM bicarbonate and are reflected in high L/D ratios at 0.05 mM bicarbonate.

It is possible to phosphorylate C4 PEPC *in vitro* using protein kinase A. Figure 7.5 shows the changes in K_i (malate) and K_m (HCO3-) at different times of incubation of PEPC with ATP and PKA. The increase in phosphorylation status of PEPC is indicated by the increase in K_i (malate). On incubation with protein kinase A, there was more than two-fold increase in K_i (malate). In contrast, there was a more than two-fold decrease in K_m (HCO_3^-) of the enzyme (Fig. 7.5). No such



0 2 4 6 8 10
HCO3- (mM)

Figure 7.4. Effect of HCO_3^- on light/dark (L/D) ratio of gross-activities of PEPC. PEPC was assayed in presence of suboptimal (0.5 mM) and optimal (2.5 mM) concentrations of PEP at pH 7.3. The inset shows the response of HCO3- on L/D ratios of PEPC activity when the ratios were calculated from the corrected values of bicarbonate. Further details are as in Fig. 7.2.

Table 7.1. *Effect of cycloheximide (protein synthesis-inhibitor) and ethoxyzolamide (inhibits carbonic anhydrase) on the gross-activity and kinetic characteristics of PEPC in extracts prepared from leaves of* Amaranthus hypochondriacus, *kept in darkness or exposed to light.*

PEPC activity was assayed at pH 7.3 and 2.5 mM PEP. Further details are described in "Materials and Methods".

Exposure/Test compound	Parameter		
	V_{max}	$K_{i\ (malate)}$	$K_{m(HCCV)}$
	µmol min^{-1} mg^{-1} protein	mM	mM
Dark			
None (control)	0.24 ± 0.019	0.07 ± 0.01	0.05 ± 0.002
Light			
None (control)	0.83 ±0.035	0.22 ±0.02	0.02 ± 0.002
+ 5 µM Cycloheximide*	0.48 ± 0.015	0.06 ± 0.01	0.03 ± 0.001
+ 500 µM Ethoxyzolamide**	0.82 ± 0.03	0.29 ± 0.02	0.02 ± 0.002

* preincubation of leaf discs.

** included during the assay.

Table 7.2. *Characteristics of PEPC in extracts prepared from dark-adapted or illuminated leaves of* Amaranthus hypochondriacus.

PEPC activity was assayed at pH 7.3 and 2.5 mM PEP. The L/D ratios reflect the effect of illumination on each parameter. Further details are described in "Materials and Methods".

Bicarbonate/ Exposure of leaves	Parameter	
	Velocity	K_i (malate)
	µmol min^{-1} mg^{-1} protein	mM
0.05 mM HCO3-		
Light (L)	0.72 ± 0.026	0.08 ± 0.002
Dark (D)	0.15 ± 0.021	0.02 ± 0.001
L/D	4.8	4.0
10 mM HCO_3^-		
Light(L)	0.83 ±0.035	0.22 ±0.01
Dark (D)	0.42 ± 0.019	0.07 ± 0.01
L/D	2.0	3.1

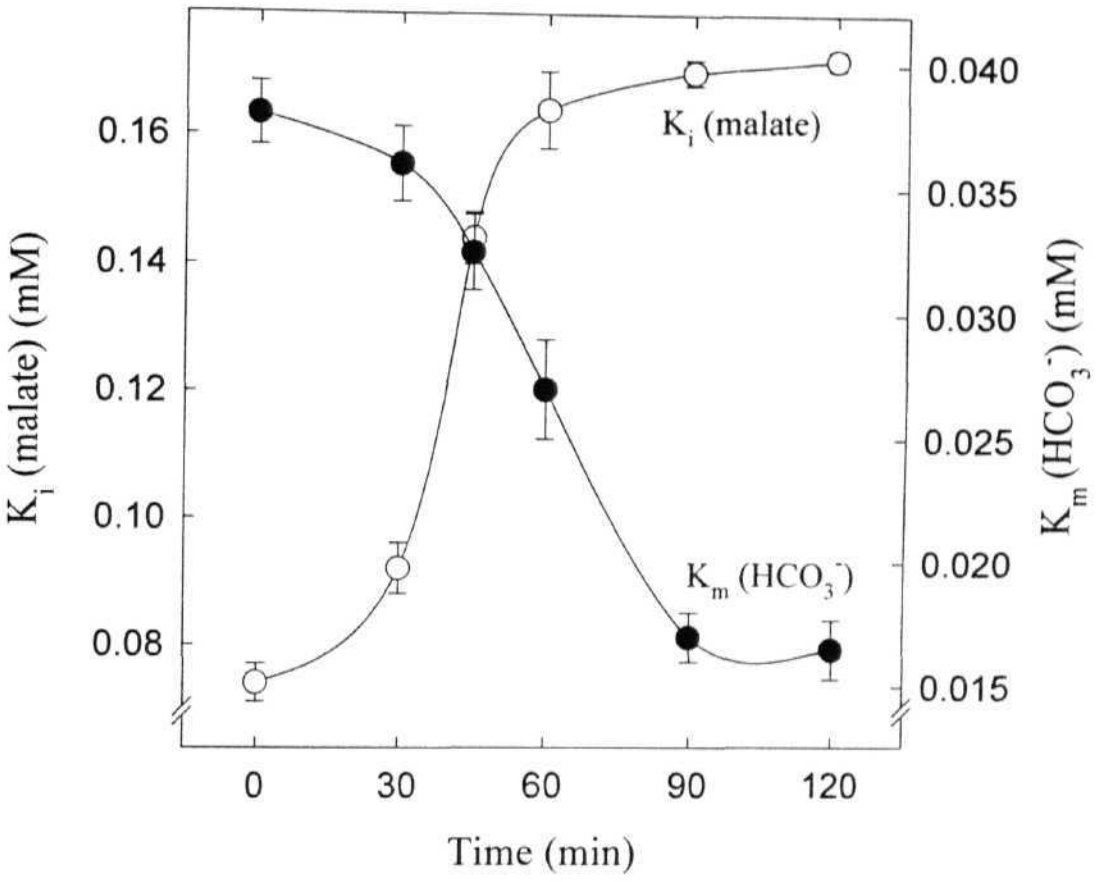


Figure 7.5. The time course of changes in Kj (malate) and K_m (HCO3") of purified PEPC from dark-adapted leaves of *Amaranthus hypochondriacus* during *in vitro* phosphorylation by incubation with mammalian PKA and Mg.ATP. Further details are described in "Materials and Methods".

changes occurred when ATP and/or PKA were omitted from the *in vitro* phosphorylation mixture (data not shown).

There was about four fold increase in K_i (malate) and about two-fold decrease in K_m (HCO_3^-), on incubation of wild type PEPC with PKA. Such changes were not observed when mutant (S15D) PEPC was incubated with PKA (Table 7.3).

Discussion

Since it is impossible to remove completely the dissolved bicarbonate, we have evaluated our data by assuming that the endogenous bicarbonate, in spite of the precautions, is about 100 μM (Bauwe, 1986) (Fig. 7.2). If we consider the gross activities of PEPC without the background correction by activity in the absence of added bicarbonate, the values of V_{max} were much higher, but the K_m (HCO3") were only slightly higher than those of corrected values (Fig. 7.3-inset). When gross-activity of PEPC was considered, there was still a two-fold increase in the affinity of PEPC to bicarbonate on illumination (Fig. 7.1). We therefore emphasize that the marked (two-fold) increase in the affinity of PEPC to bicarbonate on illumination (Figs. 7.2 and 7.3) can be seen readily, despite the technical limitation of inability to remove the dissolved bicarbonate.

Our observations establish that on illumination, the K_m for HCO3- of PEPC is dramatically lowered, when compared to that of the PEPC from dark-adapted leaves (Fig. 7.3). The increase in the affinity of PEPC to bicarbonate can be seen while considering the activity of PEPC in relation to only externally added bicarbonate or by taking into account the observed total activity, in presence of endogenous bicarbonate. We therefore suggest that illumination of leaves leads to the sensitization of PEPC to low levels of bicarbonate.

Table 7.3. *Characteristics of two forms of maize PEPC (wild type and mutant, S15D) purified from* E. coli *transformants.*

The purified PEPC was incubated with mammalian PKA and Mg.ATP. The characteristics were studied before (Control) or al the end of 2-h incubation with PKA. PEPC activity was assayed at pH 7.3 and 2.5 mM PEP. Further details are described in "Materials and Methods".

Parameter	Wild Type		Mutant (S15D)	
	Control	After 2 h	Control	After 2 h
V_{max} (μmolmin^{-1} mg^{-1} protein)	12.4 ±0.5	12.4 ±0.8	14.2 ±0.4	14.0 ±0.9
K_i (malate) (mM)	0.35 ±0.01	1.39 ±0.1	1.2 ±0.1	1.3 ±0.2
$K_m(HCO_3^-)$ (mM)	0.10 ±0.01	0.06 ±0.02	0.05 ±0.01	0.06 ±0.01

PEPC uses HCO3- for carboxylation of PEP (O'Leary, 1982). When in equilibrium with air, the concentration of HCO_3^-, in the C4 mesophyll cytosol with a pH of 7.4, is expected to be about 80 μM (Jenkins et al. 1989). The K_m (HCO_3^-) of the dark form of PEPC (about 50 uM) from *Amaranthus hypochondriacus* (Table 7.1) is below the expected physiological concentration. This value is within the range of K_m for HCO_3^- (25-100 μM) reported for the C4 PEPC in a pH range of 7 to 8 (O'Leary, 1982; Bauwe, 1986). However, the dramatic decrease on illumination of the K_m (HCO3-) of PEPC (Fig. 7.3), suggests that the activity of PEPC can easily be enhanced, at least by two-fold, due to the increased affinity of the enzyme to bicarbonate.

Most of the literature on the changes in the properties of PEPC in leaves of C4 plants indicates marked changes in V_{max} of the enzyme and sensitivity to malate and Glc-6-P (Echevarria et al.. 1994; Duff et al., 1995; Chollet et al., 1996; Vidal and Chollet, 1997). While confirming a similar trend, we emphasize that a significant increase in the affinity of PEPC to bicarbonate occurs on illumination (Fig. 7.3).

Atmospheric CO_2 entering mesophyll cells is rapidly converted to HCO_3^-, the substrate for the carboxylation reaction of C4 photosynthesis, catalyzed by PEPC (O'Leary, 1982). This reaction is catalyzed by carbonic anhydrase, confined to the cytosol of mesophyll cells, where PEPC is also located (Gutierrez et al., 1974; Ku and Edwards, 1975; Burnell and Hatch, 1988). In a long-term response, carbonic anhydrase activity is higher (>100-fold) in illuminated leaves than in dark adapted ones (Burnell et al., 1990).

It is possible that the apparent decrease in K_m for HCO_3^- of PEPC in light may partly be due to the increase in the activity of carbonic anhydrase. We have therefore tested the possible involvement of carbonic anhydrase, by including EZ, an inhibitor of carbonic anhydrase (Badger and Pfanz, 1995) during the assay of

PEPC. If the observed increase in the activity and decrease in K_m (HCO3") of PEPC were due to carbonic anhydrase, both these changes should be suppressed by EZ. However the decrease in K_m (HCO_3^-) of PEPC induced by illumination was not affected by EZ (Table 7.1). Further, we add bicarbonate directly during our experiments. We therefore rule out the possibility of the involvement of carbonic anhydrase in light-induced increase in the affinity of PEPC to HCO_3^-.

One of the reasons for light activation of PEPC in C4 leaves is due to the enhanced phosphorylation of the enzyme on illumination by a PEPC-protein kinase (Jiao et al., 1991b; Bakrim et al., 1992, 1993). CHX, an inhibitor of cytosolic protein synthesis, is capable of restricting the light activation of PEPC, by blocking the PEPC-protein kinase activity (Jiao et al., 1991b; Bakrim et al., 1992, 1993; Vidal and Chollet, 1997).

The sensitivity to malate of PEPC is a reflection of its phosphorylation status. The light induced decrease in malate sensitivity of PEPC [indicated by increase in Kj (malate)] was prevented completely by pretreatment of leaf discs with CHX (Table 7.1). On the other hand, the light-induced decrease in K_m (HCO3-) of PEPC was significantly suppressed by CHX (Table 7.1). The phosphorylated form of PEPC appears to have a lower K_m for HCO3" than that of the dephosphorylated form.

In vitro phosphorylation of PEPC by incubation with mammalian protein kinase A is a very useful technique to assess the changes in regulatory properties of PEPC. The phosphorylation of PEPC is associated with a marked decrease in malate sensitivity of the enzyme. This phenomenon has already been documented with purified PEPC from maize and sorghum (Terada et al., 1990; Echevarria et al., 1994; Duffet al., 1995).

The successful *in vitro* phosphorylation of PEPC from *Amaranthus hypochondriacus* is indicated the marked increase in Kj (malate) with increasing

duration of incubation in presence of PKA (Fig. 7.5). Along with progress in the increase in K_i (malate), there was steady decrease in K_m (HCO_3^-). The two-fold decrease in K_m (HCO_3^-) on *in vitro* phosphorylation of the enzyme (Fig. 7.5) was quite similar to that found in light form of PEPC (Fig. 7.3). On the other hand, the replacement of seryl group with aspartate, which makes the enzyme non-phosphorylatable, prevented not only the increase in Kj (malate) but also the decrease in K_m (HCO3-), found in wild-type of PEPC (Table 7.3). These results suggest that phosphorylation of PEPC plays a major role during the light-induced decrease in K_m (HCO3-).

Illumination causes cytosolic alkalization in mesophyll cells of particularly C_4 plants (Raghavendra et al., 1993; Yin et al., 1993). Such a rise in cytosolic pH can lead to an increase in PEPC activity through several factors: synthesis and activation of PEPC-protein kinase (Chollet et al., 1996), rise in cytosolic calcium and possible rise in the extent of the PEPC phosphorylation (Pierre et al., 1992); direct increase in PEPC activity due to the optimal pH in alkaline range (Andreo et al., 1987). Our observations add another dimension to the biochemical basis of promotion of PEPC activity on illumination. On illumination there is a simultaneous increase in the availability of dissolved HCO_3^- due to the rise in cytosolic pH (Hatch and Burnell, 1990), as well as the affinity of enzyme to bicarbonate (Table 7.2, Figs. 7.1 and 7.2). We propose that besides the phosphorylation-dephosphorylation cascade, the combined effect of rise in dissolved HCO3" (due to cytosolic alkalization) and increase in affinity of PEPC to bicarbonate can be a significant factor during the light activation of PEPC in C4 leaves.

The marked decrease in the K_m (HCO3-) of PEPC on illumination (Fig. 7.3), raises an important question whether 10 mM HCO_3^-, as used in most of the cases, is justified to examine the regulation of PEPC. The responses of the activity and regulatory properties of PEPC to illumination were indeed more

pronounced at **0.05** mM HCO_3^- than that at 10 mM HCO3- (Fig. **7.4, Table** 7.2; also Parvathi et al., 1998a). We therefore suggest that the regulatory properties **of** PEPC **and their** modulation by factors such as light or temperature **should** be reexamined at 0.05 mM HCO_3^-. The physiological level of bicarbonate **in** cytosol of C4 mesophyll cells, the site of PEPC, is indeed expected to be about 80 μM at a pH of 7.4 (Jenkins et al., 1989).

Light is known to induce a rise in pH of the cytosol in mesophyll cells and thereby increases the dissolved bicarbonate. This in turn allows the enzyme to be less sensitive to feed back inhibition by malate, which is expected to be 35 mM in mesophyll cytosol in steady state photosynthesis (Doncaster and Leegood, 1987). Thus, PEPC may function smoothly in presence of high levels of malate during illumination.

This is the first report indicating the sensitization of PEPC in C4 leaves to bicarbonate by light. We propose that the marked interaction between light and bicarbonate can be an important component in the regulation of C4 PEPC.

Major conclusions from the results presented in this chapter are:

1. Illumination increased the affinity of PEPC to bicarbonate by at least two times, besides the 2 to 5 fold increase in V_{max} and 3 to 4 fold increase in K_i for malate.

2. Light to dark ratio (L/D) decreased with increasing concentration of HCO_3^-.

3. Carbonic anhydrase was not involved in the modulation of kinetic and regulatory properties of the enzyme by bicarbonate during illumination.

4. CHX, an inhibitor of cytosolic protein synthesis suppressed the light enhnaced decrease **in** K_m (HCO3").

5. *In vitro* phosphorylation by protein kinase A of dark-form PEPC from *Amaranthus* and a recombinant WT (but not mutant, S15D) PEPC from maize leaves decreased the K_m (HCO_3^-) of the enzyme, besides increasing Kj (malate).

6. We conclude that phosphorylation of PEPC is important during the sensitization of PEPC to HCO_3^- by illumination in C4 leaves. The marked interaction between PEPC, light and bicarbonate is an important component in the regulation of C_4 PEPC.

Chapter 8

Effect of Calcium on the Activity and Phosphorylation of PEPC

Chapter 8

Effect of Calcium on the Activity and Phosphorylation of PEPC

Introduction

Calcium plays a key role in plant growth and development because changes in cytosolic free Ca^{2+} regulate a surprising large variety of cellular processes through Ca^{2+}-modulated proteins and their targets (Bush, 1995). For e.g., Ca^{2+}-, Ca^{2+}/CaM- and Ca^{2+}/phospholipid-dependent kinases have been identified in plants (Roberts and Harmon, 1992; Stone and Walker, 1995). There are evidences indicating the occurrence of also Ca^{2+}-dependent phosphatases in plant tissues (Luan, 1998).

C_4 plants maintain soluble Ca^{2+} at low levels in their leaves, therefore they are called as calciophobes. The apparent ability of C4 plants in keeping soluble Ca^{2+} at low level in their leaf tissues opens up interesting possibility of the regulation of C4 photosynthesis by inorganic cations. The significance of Ca^{2+} in the operation of the C4 pathway is not yet understood completely. It remains to be seen whether low soluble Ca^{2+} is a prerequisite for the optimal functioning of C4 pathway. Gavalas and Manetas (1980a) speculated that C4 photosynthesis and mineral nutrition are closely interconnected.

Phosphorylation-dephosphorylation cascade systems represent a major mechanism of cellular regulation. During this cascade, the target enzyme or protein is phosphorylated by protein kinase and dephosphorylated by protein phosphatase. PEPC is one of the best examples of plant enzymes regulated by phosphorylation-dephosphorylation cascade. PEPC in leaves of C4 plants is activated in light and de-activated in dark due to phosphorylation of PEPC in light by PEPC-PK and dephosphorylation in dark by type 2A protein phosphatase. Phosphorylated form is

more active and less sensitive to malate **than the dephosphorylated form. The reversible** phosphorylation of PEPC occurs on a serine residue (Lepiniec et al., 1994; Rajagopalan et al., 1994; Chollet et al., 1996; Vidal and Chollet, 1997).

PEPC from C3 plants also undergoes phosphorylation, **the pattern** being different from that of C4- and CaM plants. In barley mesophyll protoplasts PEPC was always in phosphorylated condition, which contrasts with C4-PEPC (phosphorylated during illumination) and CAM-PEPC (phosphorylated during darkness) (Smith et al., 1996). Thus, the PEPC and PEPC-PK appear to be regulated in a different manner in C3 protoplasts than in C4 protoplasts or leaf tissue (Smith et al., 1996).

There are reports describing the involvement of Ca^{2+}-independent PEPC-PK in C4 plants (Carter et al., 1991; Jiao and Chollet 1991; Bakrim et al., 1992; Wang and Chollet, 1993; Li and Chollet, 1994). On the contrary, some authors reported that PEPC phosphorylation was mediated by Ca^{2+}-dependent PEPC-PK (Echevarria et al., 1988; Bakrim et al., 1992; Ogawa and Izui, 1992; Ogawa et al., 1992; Pierre et al., 1992). It has also 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). There are suggestions that cytosolic calcium, pH and photosynthetic metabolites (such as PGA or pyruvate) may act as secondary messengers in the light activation of C4 PEPC-PK (Pierre et al., 1992; Duff et al., 1996; Giglioli-Guivarc'h et al., 1996). However, the evidences of involvement of secondary messengers such as calcium, calmodulin and cAMP during the phosphorylation of PEPC are still ambiguous. Recently, it has been reported by using maize **mutant** *{bundle sheath defective2-mutable 1)* that Calvin cycle is not indispensable for the light activation of C4 PEPC-PK (Smith et al., 1998).

The present work was designed to reevaluate the role of calcium on PEPC activity in crude and desalted extracts *of Amaranthus hypochondriacus*, a NAD-malic enzyme type C4 plant. Attempts were made to assess the activity of PEPC in leaf extracts in the presence or absence of calcium. It is possible that the response of PEPC to calcium is modulated, when leaves are illuminated, since light is known to stimulate markedly the activity of PEPC in C4 leaves. We have therefore checked the effect of calcium chloride on PEPC activity in leaf extracts prepared from illuminated or dark-adapted leaves. Later, the experiments were extended to study the pattern of PEPC phosphorylation in the presence or absence of calcium.

Results

Ca^{2+}, at concentrations of 50 μM or above, inhibited significantly PEPC activity in extracts from leaves of *Amaranthus hypochondriacus* (Fig. 8.1). The inhibition of PEPC by high concentrations of calcium (50-500 μM) was observed only at pH 7.8 (optimal pH), but not at pH 7.3 (suboptimal pH). The effect of Ca^{2+} on PEPC was marginal and almost negligible at pH 7.3 (Fig. 8.1). All the subsequent experiments were therefore conducted at pH 7.8. The extent of inhibition by Ca^{2+} was more at 5 mM $MgCl_2$ than that at 15 mM $MgCl_2$ (Figs. 8.1 and 8.2). Further, the inhibition by calcium of PEPC activity was more pronounced in extracts from illuminated leaves than that from dark-adapted ones.

The primary reason for inhibition by 100 μM $CaCl_2$, was the competition with Mg^{2+} as indicated by an increase in K_{m_1} for Mg^{2+}, with no change in V_{max} (Fig. 8.3). On the other hand, the presence of 500 μM $CaCl_2$ caused a mixed type of inhibition of PEPC in relation to Mg^{2+} (Fig. 8.4). 15 mM Mg^{2+} was able to reverse the inhibition of PEPC by 100 μM calcium, but not at 500 μM calcium.

There was a slight stimulation of PEPC activity at low concentrations of $CaCl_2$ (20 μM), particularly when PEPC was assayed at pH 7.8. The extent of

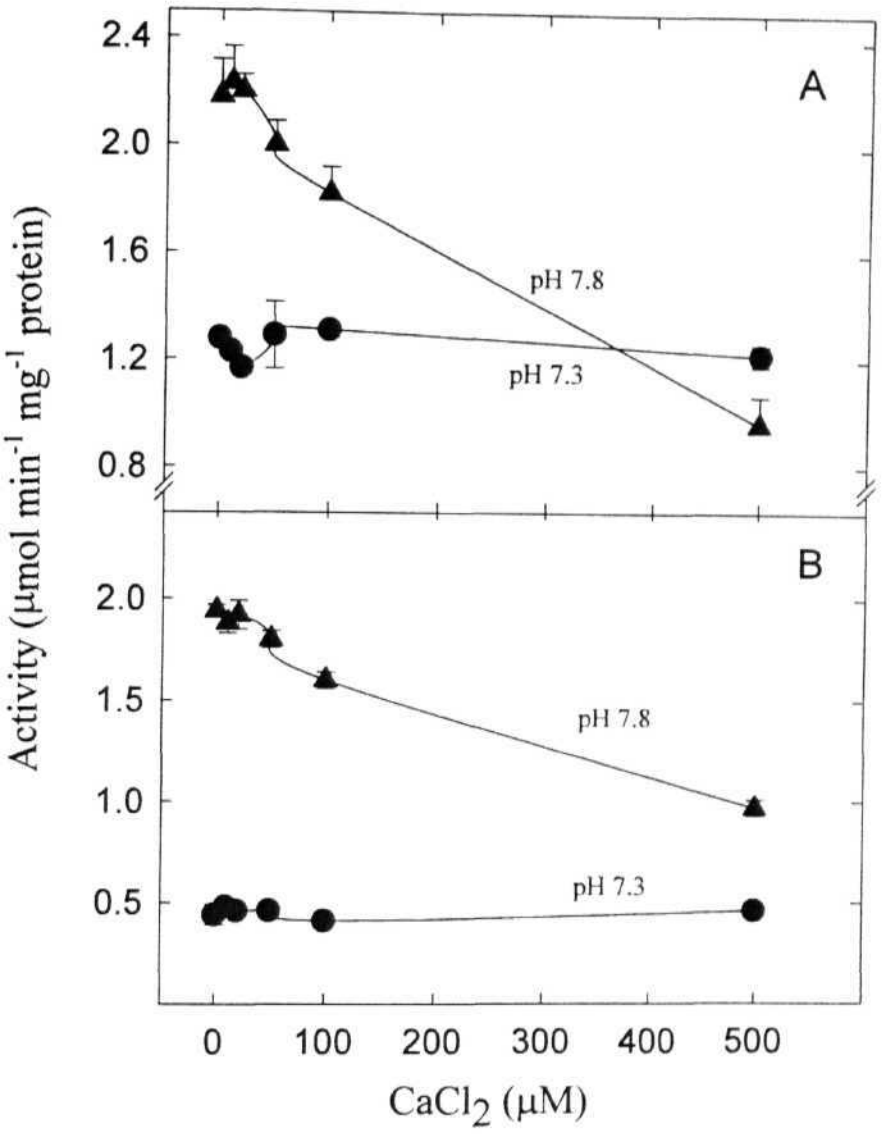


Figure 8.1. Effect of $CaCl_2$ on the activity of PEPC in crude extracts from illuminated (A) or dark-adapted (B) leaf discs of *Amaranthus hypochondriacus.* PEPC was assayed at either suboptimal pH (7.3) or optimal pH (7.8). $CaCl_2$ was included in the assay medium at the concentrations indicated, while measuring PEPC activity. PEPC was assayed with 2.5 mM PEP and 5 mM $MgCl_2$. Further details are described in "Materials and Methods".

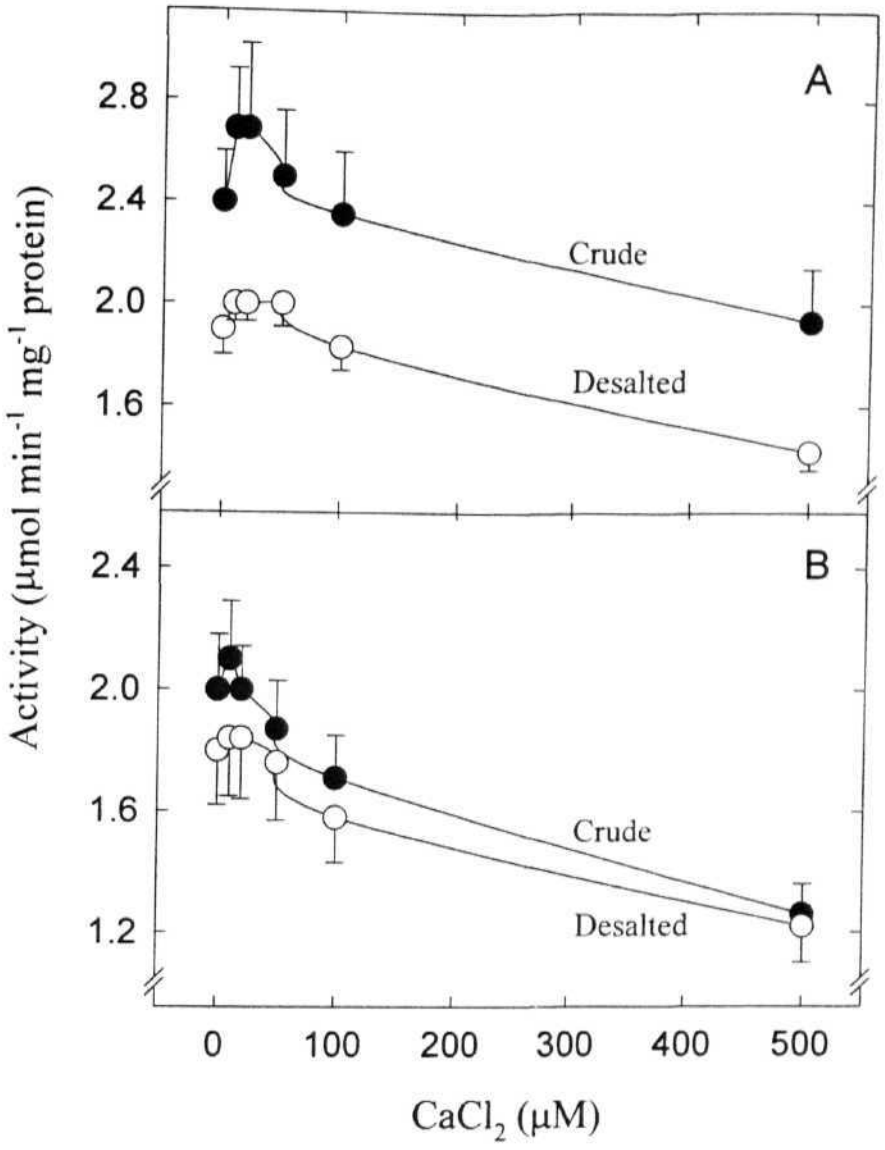


Figure 8.2. Effect of $CaCl_2$ on PEPC activity in crude or desalted extracts from illuminated (A) or dark-adapted (B) leaf discs of *Amaranthus hypochondriacus*. PEPC was assayed at pH 7.8. $CaCl_2$ was included in the assay medium at the concentrations indicated, while measuring the PEPC activity. PEPC was assayed with 2.5 mM PEP and 15 mM $MgCl_2$. Further details are described in "Materials and Methods".

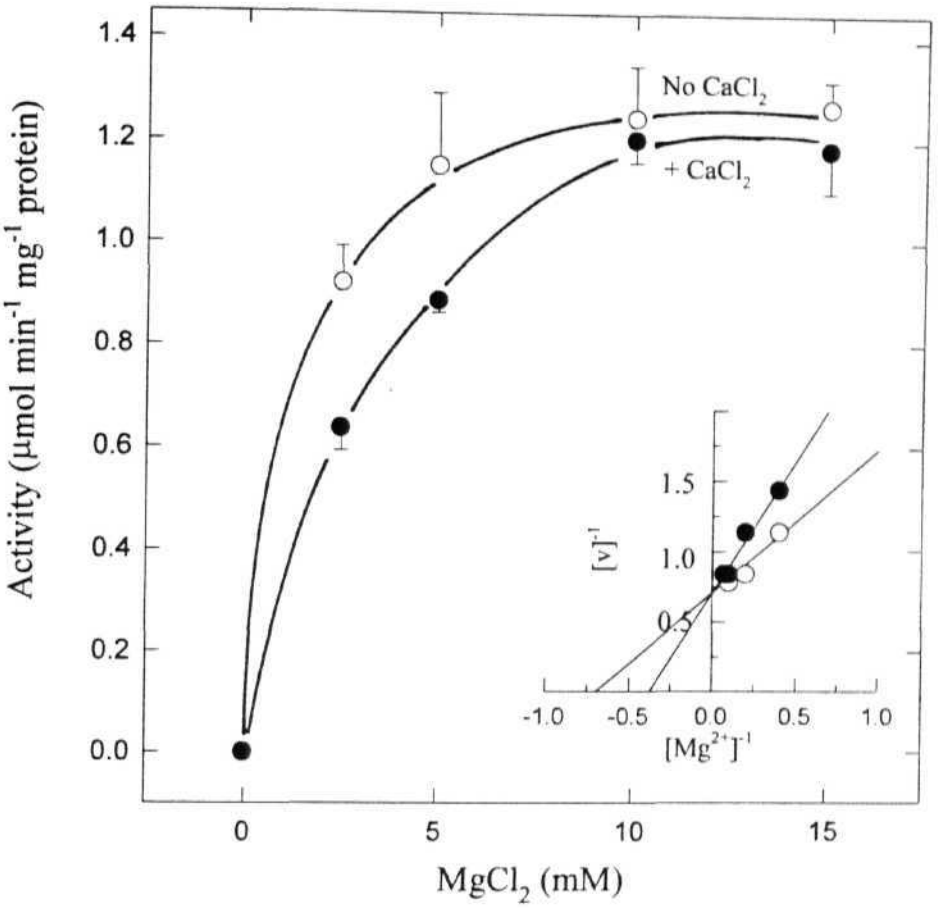


Figure 8.3. The response of PEPC to varying levels of $MgCl_2$ in desalted extracts from dark-adapted leaf discs. The enzyme was assayed in absence or presence of 100 μM $CaCl_2$ at pH 7.3. The inset is a reciprocal plot of PEPC activity vs concentration of $MgCl_2$. K_m for Mg^{2+} increased from 1.45 mM (in absence of Ca2+) to 2.69 mM (in presence of 100 μM Ca^{2+}), while V_{max} (1.43 μmol min"^{1} mg^{-1} protein) did not change. Further details are described in "Materials and Methods".

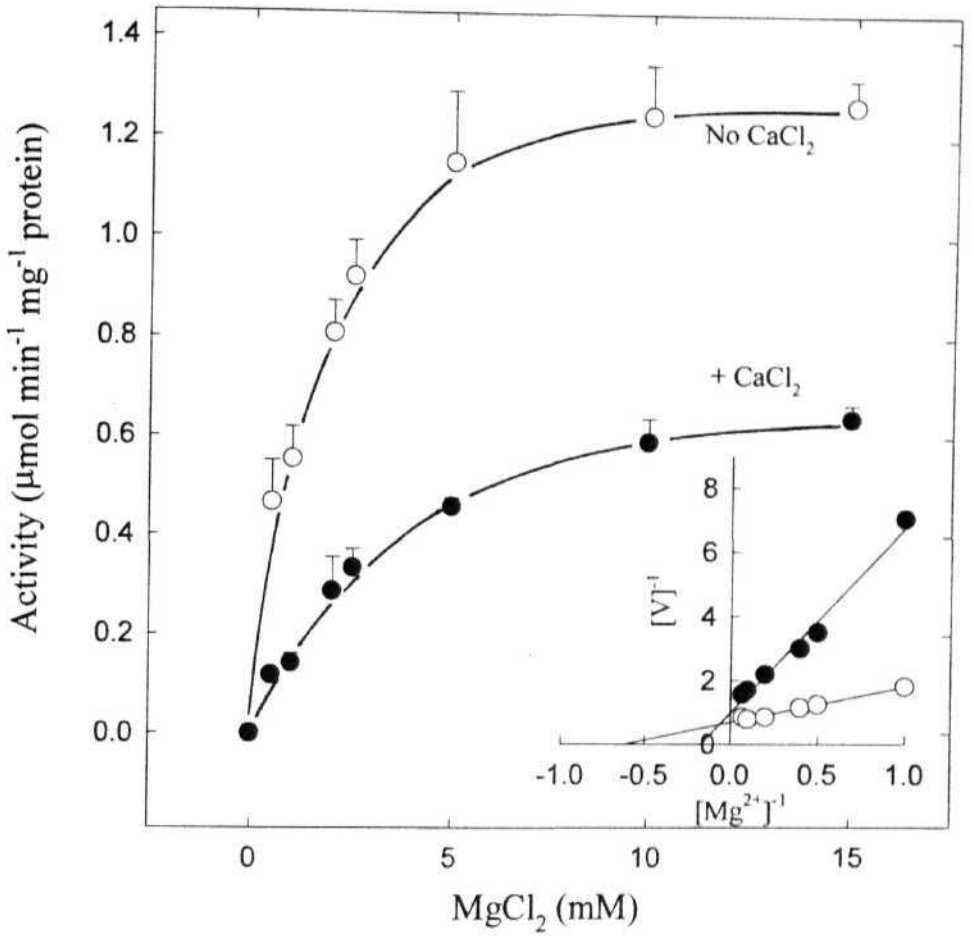


Figure 8.4. The response of PEPC to varying levels of $MgCl_2$ in desalted extracts from dark-adapted leaf discs. The enzyme was assayed in absence or presence of 500 μM $CaCl_2$ at pH 7.3. The inset is a reciprocal plot of PEPC activity vs concentration of $MgCl_2$. K_m for Mg^{2+} increased from 1.6 mM (in absence of Ca^{2+}) to 6.2 mM (in presence of 500 μM Ca^{2+}), whereas V_{max} decreased from 1.45 μmol min^{-1} mg^{-1} protein to 1.06 μmol min^{-1} mg^{-1} protein. Further details are described in "Materials and Methods".

stimulation by Ca^{2+} was more in illuminated extracts of crude than that in desalted extracts (Fig. 8.5). The stimulation by Ca^{2+} was particularly visible when the activities were expressed as percent of control (Fig. 8.6). Similar type of stimulation (15%) was observed when 15 mM $MgCl_2$ was used during assay (Fig. 8.7). Again the effect of calcium was pronounced in crude extracts from illuminated leaves.

EGTA inhibited PEPC in a concentration-dependent manner. Inhibition of PEPC by EGTA was more in extracts from illuminated leaves (30% at 2 mM) than those of dark-adapted ones (20% at 2 mM) (Fig. 8.8). EGTA had no effect on PEPC, when assayed at pH 7.3 (data not shown).

Since there could be some internal calcium already available in the leaf, we used desalted extracts to study the effect of calcium on PEPC, and compared with the results obtained with the crude extracts. The kinetics of PEPC were examined in presence or absence of 100 and 500 μM Ca^{2+}. The K_m (PEP) of PEPC was not effected, while K_m for Mg^{2+} or K_i (malate) increased in presence of 100 μM $CaCl_2$ (Table 8.1). The K_m (PEP) of PEPC was lowered, while K_m for Mg^{2+} or K_i (malate) increased in presence of 500 μM $CaCl_2$.

The PEPC-PK activity was measured indirectly incubating the leaf extracts with ATP and determining stimulation in PEPC activity due to preincubation with ATP. The stimulation by ATP of PEPC activity was more in illuminated leaves than that in dark-adapted ones (data not shown). The stimulation of PEPC activity on incubation with ATP indicates the ATP-dependent phosphorylation of PEPC and is used as an indirect measure of PEPC-PK activity. Such approach is adapted when monitoring phosphorylation of PEPC in root nodules, maize, wheat, sorghum and amaranthus leaves (Jiao and Chollet, 1989; Van Quy et al., 1991a; Bakrim et al., 1992; Pierre et al., 1992; Manh et al. 1993; Schuller and Werner, 1993; Echevarria et al., 1994; Duff et al., 1995; Gao and Woo, 1996a; Colombo et al., 1998).

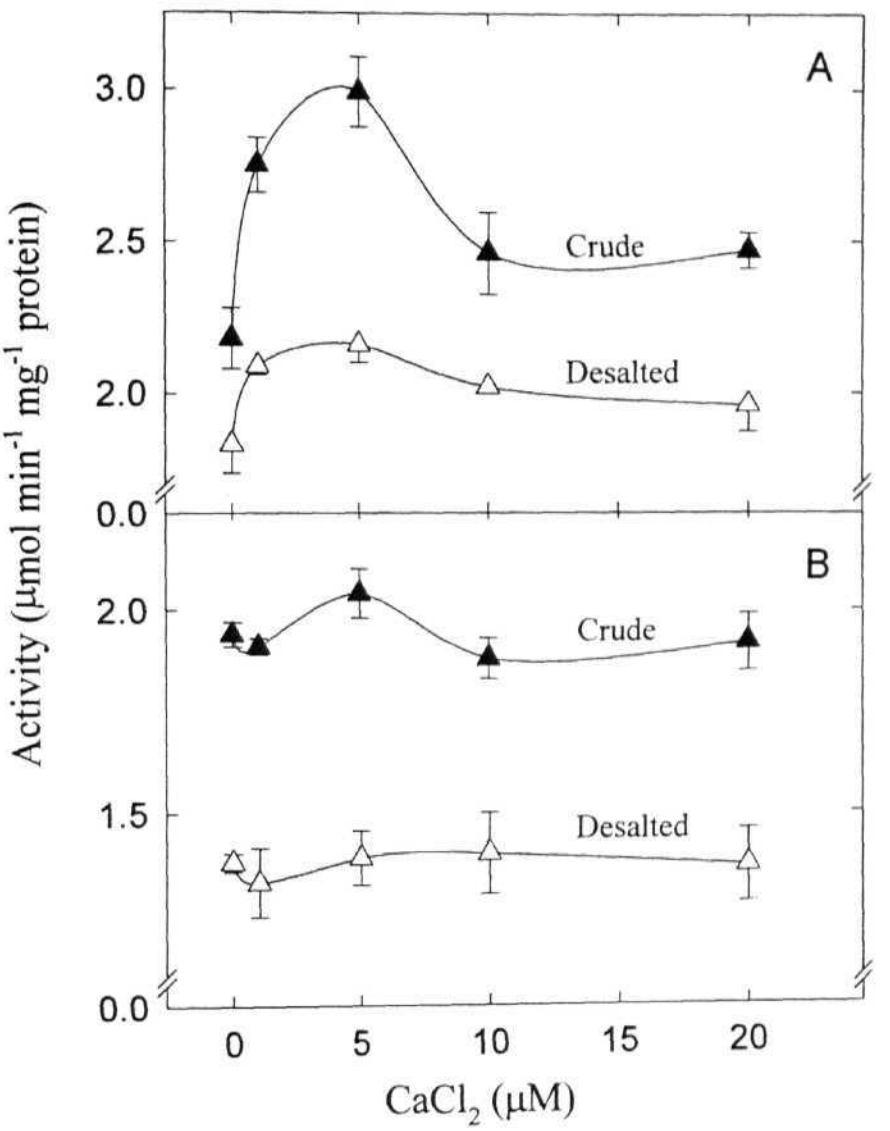


Figure 8.5. The effect of $CaCl_2$ at low range of 0-20 μM on PEPC activity in crude or desalted extracts prepared from illuminated (A) or dark-adapted (B) leaf discs. The enzyme was assayed at optimal pH (7.8) with 5 mM $MgCl_2$. Further details are as in Figure 8.1.

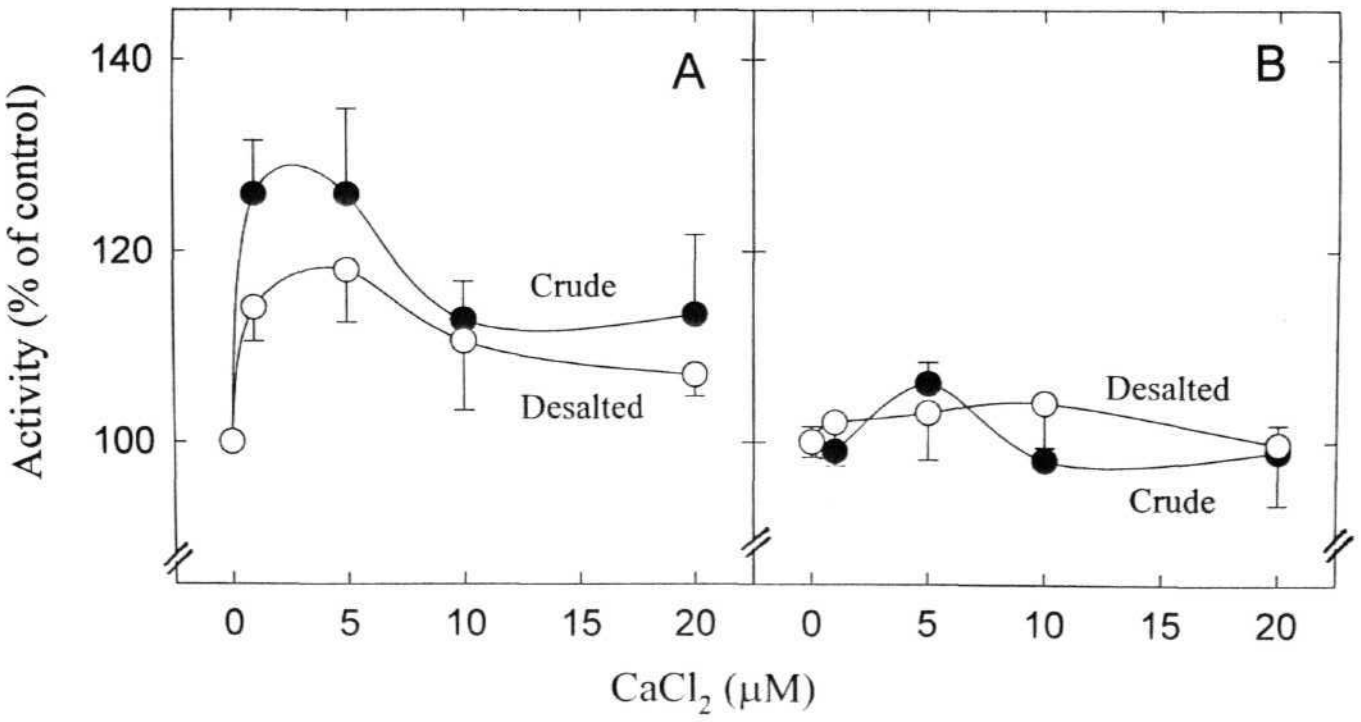


Figure 8.6. The response to $CaCl_2$ of PEPC activity expressed as % of respective controls (no $CaCl_2$) in crude or desalted extracts prepared from illuminated (A) or dark-adapted (B) leaf discs. PEPC was assayed at pH 7.8. Further details are as in Figure 8.1.

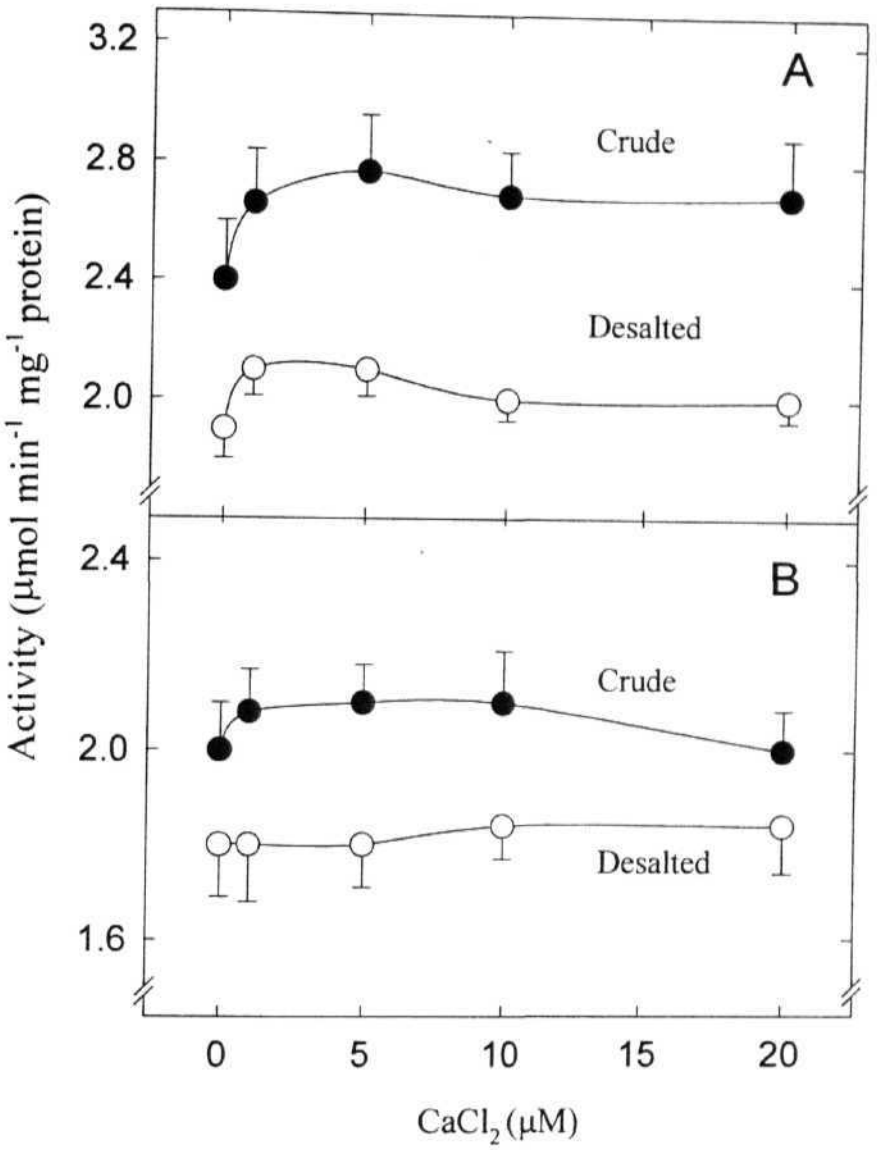


Figure 8.7. The effect of $CaCl_2$ at low range of 0-20 μM on PEPC activity in crude or desalted extracts prepared from illuminated (A) or dark-adapted (B) leaf discs. The enzyme was assayed at pH 7.8 with 15 mM $MgCl_2$. Further details are as in Figure 8.1.

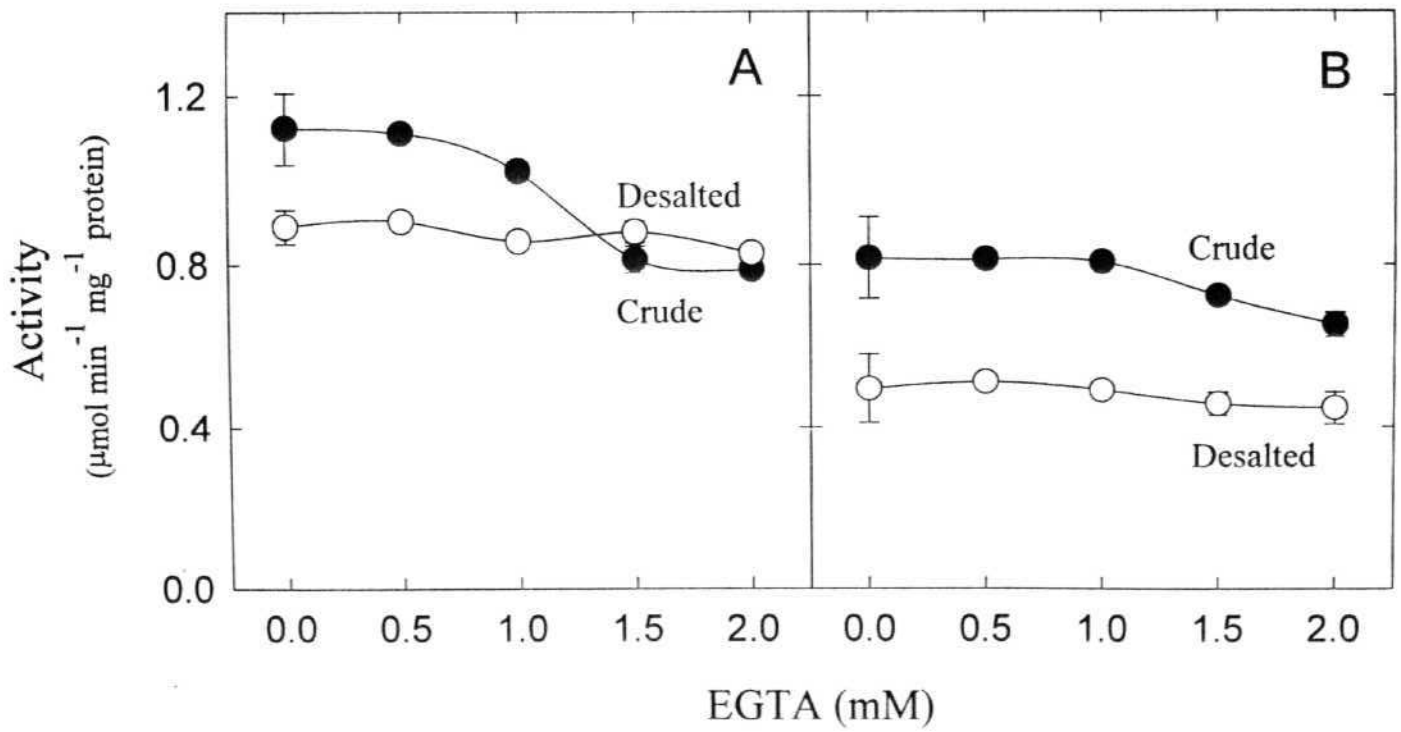


Figure 8.8. Effect of EGTA on PEPC activity in crude or desalted extracts prepared from illuminated (A) or dark-adapted (B) leaf discs. PEPC was assayed at pH 7.8. Further details are as in Figure 8.1.

Table 8.1. *Kinetic characteristics of PEPC from desalted leaf extracts of* Amaranthus hypochondriacus *in the absence or presence of 100 $\mu M\ CaCl_2$ or 500 $\mu M\ CaCl_2$.*

PEPC activity was assayed at pH 7.8 in presence of 15 mM $MgCl_2$. Further details are described in "Materials and Methods".

Extract/Parameter	Control	100 μM $CaCl_2$	500 μM $CaCl_2$
V_{max} (μmol min^{-1} mg^{-1} protein)	1.43 ±0.07	1.40 ±0.02	1.06 ±0.02
K_m (PEP) (mM)	0.16 ±0.04	0.15 ±0.03	0.09 ±0.03
K_i (malate)(mM)	0.53 ± 0.1	0.92 ± 0.2	0.8 ± 0.1
K_m (Mg^{2+}) (mM)	1.45 ± 0.2	2.69 ± 0.6	6.2 ± 0.6

ATP-dependent activation of PEPC was initiated by adding 1 mM ATP + 5 mM $MgCl_2$ to leaf extracts. Aliquots of extracts were removed at specific times after the addition of ATP and assayed immediately for PEPC activity at suboptimal pH (7.3) (Fig. 8.9). The increase in the activity of PEPC and decrease in malate sensitivity on incubation with ATP and $MgCl_2$ confirm PEPC phosphorylation and give an indirect measure of PEPC-PK activity (Table 8.2). The stimulation by ATP of PEPC activity was much more when assayed in presence of malate than that in the absence of malate. We have therefore chosen to indicate the increase in the extent of stimulation by ATP of PEPC activity in presence or absence of malate as a measure of PEPC phosphorylation/PEPC-PK activity. The stimulation of PEPC activity after incubation with ATP (for 60 min) was more at pH 7.8 (81%) than that at pH 7.3 (44%) (Table 8.2). This indicates that ATP-dependent PEPC phosphorylation preferred an alkaline pH.

Various concentrations of Ca^{2+} or EGTA were added to the preincubation medium containing Mg.ATP and extract. Low concentrations of Ca^{2+} (up to 20 μM) increased (>20%) the ATP-dependent activation of PEPC in crude and desalted extracts. 10 μM seemed to be optimal for such stimulation and stimulation by Ca^{2+} was pronounced when malate was present in the assay medium (Fig. 8.10A).

EGTA decreased the ATP-dependent stimulation of PEPC activity in the leaf extracts. When malate was included in the assay a slight inhibition by EGTA was observed in desalted extracts, much less than the marked inhibition in crude extracts (Fig. 8.1 OB). There were consistent differences between crude and desalted leaf extracts, in the activity of enzyme, and response to Ca2+ or EGTA (Table 8.3).

The sensitivity to Ca^{2+} of PEPC or PEPC-PK was more in crude than that in desalted extracts. We have therefore chosen crude extracts for studies on inhibitor specificity of *in vitro* phosphorylation of PEPC. ATP-dependent phosphorylation of

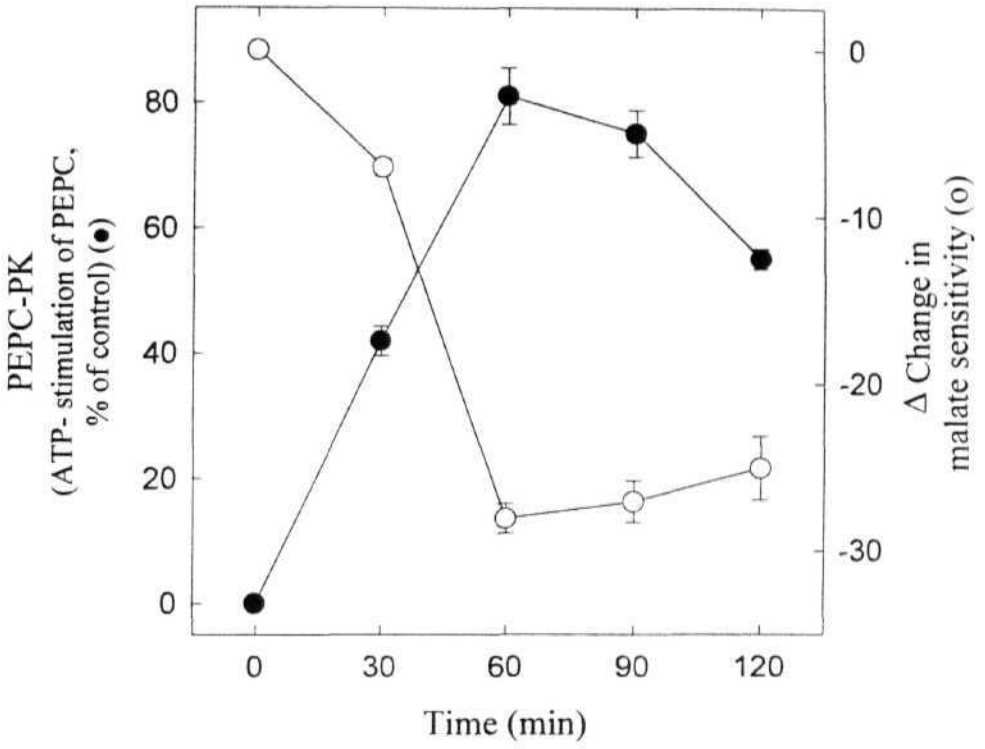


Figure 8.9. Stimulation of PEPC activity in extracts from illuminated leaf discs on incubation with 1 mM ATP and 5 mM $MgCl_2$ at pH 7.8. Aliquots were taken at different time intervals and assayed for PEPC activity in presence or absence of 0.5 mM malate. The stimulation by ATP was taken as an indirect measure of PEPC-PK activity. Further details are described in "Materials and Methods".

Table 8.2. *Stimulation by ATP of PEPC activity' in extracts prepared from illuminated leaf discs of* Amaranthus hypochondriacus.

The crude extracts were incubated for 60 min with 1 mM ATP, 5 mM $MgCl_2$ and 10 μM $CaCl_2$, before assaying the aliquots for PEPC activity at pH 7.8. Further details are described in "Materials and Methods".

pH of PEPC incubation	Parameter	Incubation with ATP: Before incubation	After incubation	% Stimulation or A change
7.3	PEPC activity	μmol min^{-1} mg^{-1} protein		
	No malate	0.36 ± 0.06	0.52 ±0.01	44%
	+ malate	0.15 ±0.05	0.30 ± 0.05	100%
	Inhibition by malate	58%	42%	Δ -16
7.8	PEPC activity			
	Nomalate	0.52 ± 0.06	0.94 ± 0.03	81%
	+ malate	0.21 ± 0.04	0.56 ± 0.05	167%
	Inhibition by malate	60%	40%	Δ -20

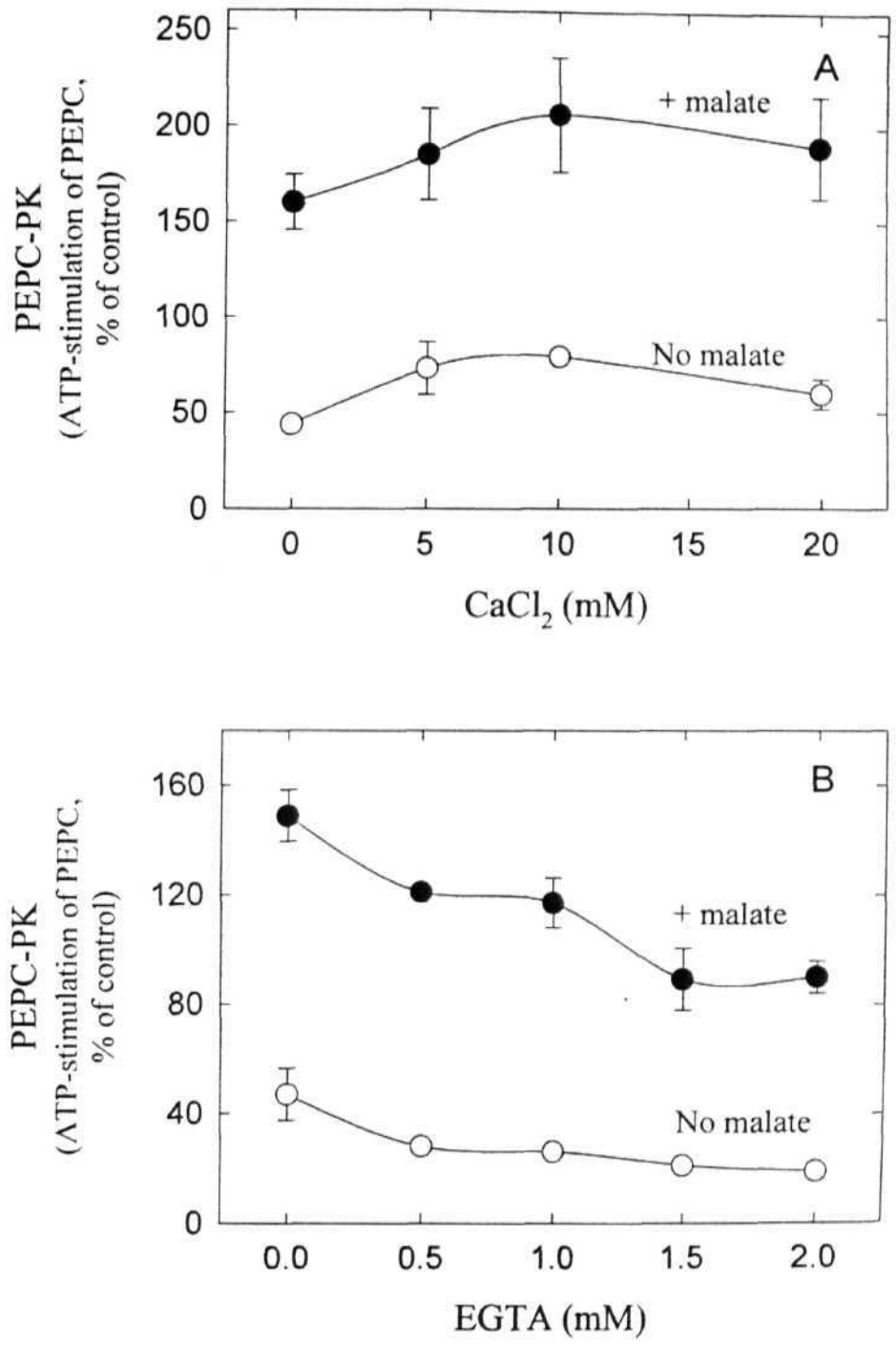


Figure 8.10. Effect of $CaCl_2$ on PEPC-PK activity, as indicated by ATP-dependent activation of PEPC, in leaf extracts from illuminated leaf discs. The crude extracts from illuminated leaf discs were incubated with 1 mM ATP, 5 mM $MgCl_2$ and $CaCl_2$ (A) or EGTA (B) at pH 7.8 for 1 h. PEPC was assayed in presence or absence of 0.5 mM malate at pH 7.3. Further details are described in "Materials and Methods".

Table 8.3. *The effect of $CaCl_2$ and EGTA on the stimulation by ATP of PEPC activity in crude or desalted extracts prepared from illuminated leaf discs of* Amaranthus hypochondriacus.

The crude or desalted extracts were incubated for 60 min with 1 mM ATP, 5 mM $MgCl_2$ and 10 μM $CaCl_2$ or 2 mM EGTA, before assaying the aliquots for PEPC activity at pH 7.3. The activity of PEPC in leaf crude extracts (control) was 0.94 ± 0.03 μmol min^{-1} mg^{-1} protein and malate inhibition was 42%, whereas in desalted leaf crude extracts PEPC activity was 0.76 ± 0.04 μmol min^{-1} mg^{-1} protein and malate inhibition was 47%. The pattern of calculation is illustrated in Table 8.2.

Parameter	Crude		Desalted	
	% Stimulation by ATP	A change in malate sensitivity	% Stimulation by ATP	A change in malate sensitivity
Control (None)	44 ± 0.3	-22 ± 1.2	33 ±5.3	-19 ± 2.3
$CaCl_2$ (10 μM)	79 ± 0.9	-33 ±2.5	44 ±5.0	-25 ±3.1
EGTA (2 mM)	19 ±0.7	-8 ± 1.1	27 ±0.7	-6 ± 0.8

PEPC was restricted by various Ca^{2+}-CaM dependent protein kinase inhibitors, such as CaM antagonists (TFP and W7), MLCK inhibitor (ML7) and protein kinase C inhibitor (H7) (Table 8.4). After using ATP-stimulation of PEPC activity as a measure of PEPC phosphorylation, we have attempted to examine directly the phosphorylation of PEPC and its response to calcium.

In vitro phosphorylation assays were performed using rapidly prepared crude extracts of illuminated or dark-adapted *Amaranthus* leaves as the source of crude PEPC-PK. In most of these experiments, the assays were supplemented with the purified dark-form PEPC which acts as the typical substrate, a practice adapted by others earlier (Nimmo et al., 1987; Jiao and Chollet, 1988). After the phosphorylation reaction was completed the protein was precipitated with anti-PEPC antiserum and resolved on SDS-PAGE (Plates 8.1 A, C). The autoradiographs illustrate that the phosphorylation of PEPC can be documented only with extracts from illuminated leaves, but not with the corresponding dark-adapted leaves (Plates 8.1B, D, E).

The phosphorylation of PEPC was strongly dependent on the concentration of Ca^{2+} included in the assay mixture (Plate 8.2). The extent of phosphorylation was maximum at 10 μM $CaCl_2$. In contrast, the presence of BAPTA (a Ca^{2+} chelator) suppressed the extent of phosphorylation, otherwise stimulated by Ca^{2+} (Plate 8.3). The inhibition by BAPTA was relieved by the addition of Ca^{24}, but not by the inclusion of Mg^{2+}.

The effect of several kinase inhibitors and activators on PEPC phosphorylation was studied so as to identify the type of protein kinase involved in PEPC phosphorylation. Plate 8.4 illustrates the effects of these kinase inhibitors and activators on PEPC-PK. Phosphorylation of PEPC was promoted by the addition of Ca^{2+} and to a limited extent by presence of CaM (Plate 8.4). There was only marginal effect by the addition of Phosphatidyl serine and diacylglycerol.

Table 8.4. *The effect of inhibitors TFP, W_7 (CaM antagonists), ML7 (MLCK inhibitor) and H7 (protein kinase C inhibitor) on the stimulation by ATP of PEPC activity in extracts prepared from illuminated leaf discs of* Amaranthus hypochondriacus.

The crude extracts were incubated for 60 min with 1 mM ATP, 5 mM $MgCl_2$ and 10 μM $CaCl_2$ in presence or absence of different inhibitors, before assaying the aliquots for PEPC activity at pH 7.3. The activity of PEPC in leaf crude extracts (control) was 0.94 ± 0.03 μmol min^{-1} mg^{-1} protein and malate inhibition was 40%. Further details are as in Table 8.2.

Inhibitor	A change in malate sensitivity	Stimulation in activity (%)
Control (None)	-23 ± 6.0	80 ±8.7
TFP (100 μM)	-12.2 ± 1.6	18.1 ±2.0
W_7 (100 μM)	-11.9 ± 2.1	39.6 ±3.2
ML_7 (10 μM)	-12.7 ± 1.9	29.9 ± 1.3
H_7 (100 μM)	-9.4 ±3.1	52.3 ± 5.2

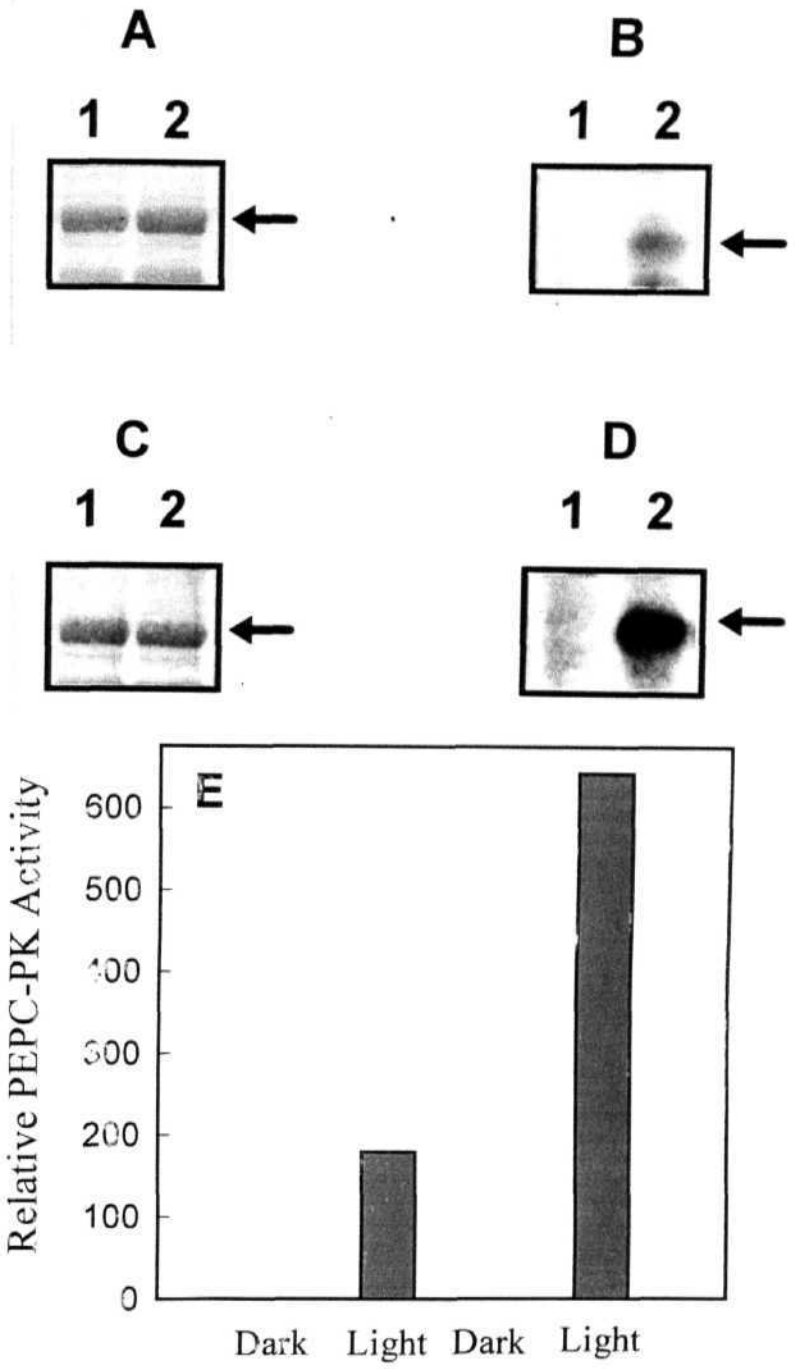


Plate 8.1. *In vitro* phosphorylation of PE?C in crude extracts from dark-adapted or illuminated leaves of *Amaranthus hypochondriacus.* Dark-adapted or illuminated leaf crude extracts were incubated with phosphorylation mixture containing γ-($AT^{32}P$). PEPC-protein was separated by immunoprecipitation using anti-PEPC antiserum. The proteins in immunoprecipitates were resolved on 10% SDS-PAGE and stained for Coomassie brilliant blue R-250. The gels were destained, dried under vacuum and autoradiographed at -80°C for 4 days (B, D). Lane 1: Extracts from dark-adapted leaves, Lane 2: Extracts from illuminated leaves. A, B or C, D correspond to the samples from different days. (A, C) Coomassie blue-stained gels; (B, D) Autoradiographs; (E) Corresponding histogram, representing the relative PEPC-PK activity. The area of PEPC bands on the autoradiographs were quantitated by image analysis. Further details are described in "Materials and Methods".

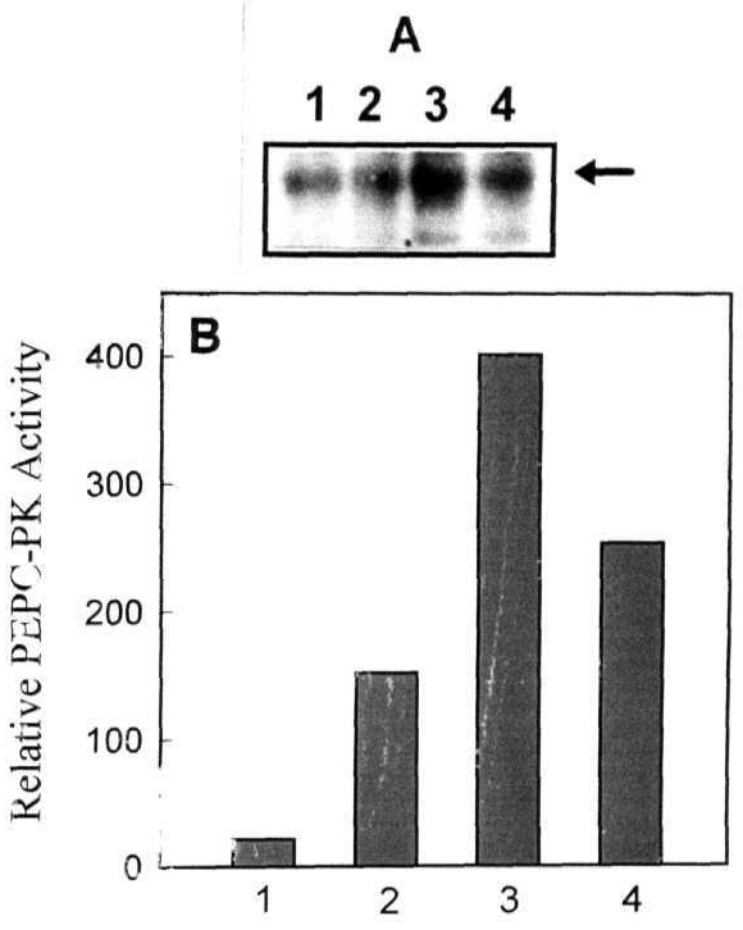


Plate 8.2. Effect of $CaCl_2$ on *in vitro* phosphorylation of PEPC. Leaves were extracted after illumination and phosphorylation was carried out in the absence or presence of different concentrations of $CaCl_2$ in the reaction mixture. Lane 1: Control (no $CaCl_2$), Lane 2: 5 μM $CaCl_2$, Lane 3: 10 μM $CaCl_2$, Lane 4: 20 μM $CaCl_2$. (A) Autoradiograph, (B) Corresponding histogram of relative PEPC-PK activity. Further details are as described for Plate 8.1 and in "Materials and Methods".

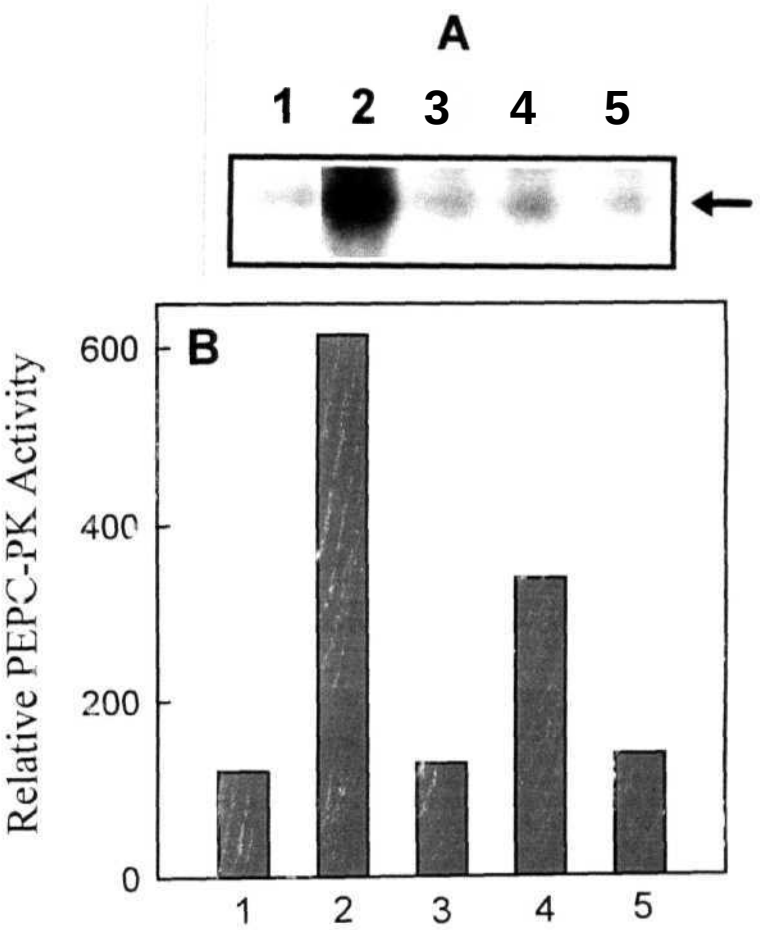


Plate 8.3. The sensitivity of PEPC-PK activity to BAPTA (Ca2+ chelator). Lane 1: Control (no $CaCl_2$), Lane 2: 10 μM $CaCl_2$, Lane 3: 1 mM BAPTA, Lane 4: 1 mM BAPTA + 10 μM$CaCl_2$, Lane 5: 1 mM BAPTA + 10 μM $MgCl_2$. (A) Autoradiograph, (B) Corresponding histogram of relative PEPC-PK activity. Further details are as described for Plate 8.1 and in "Materials and Methods".

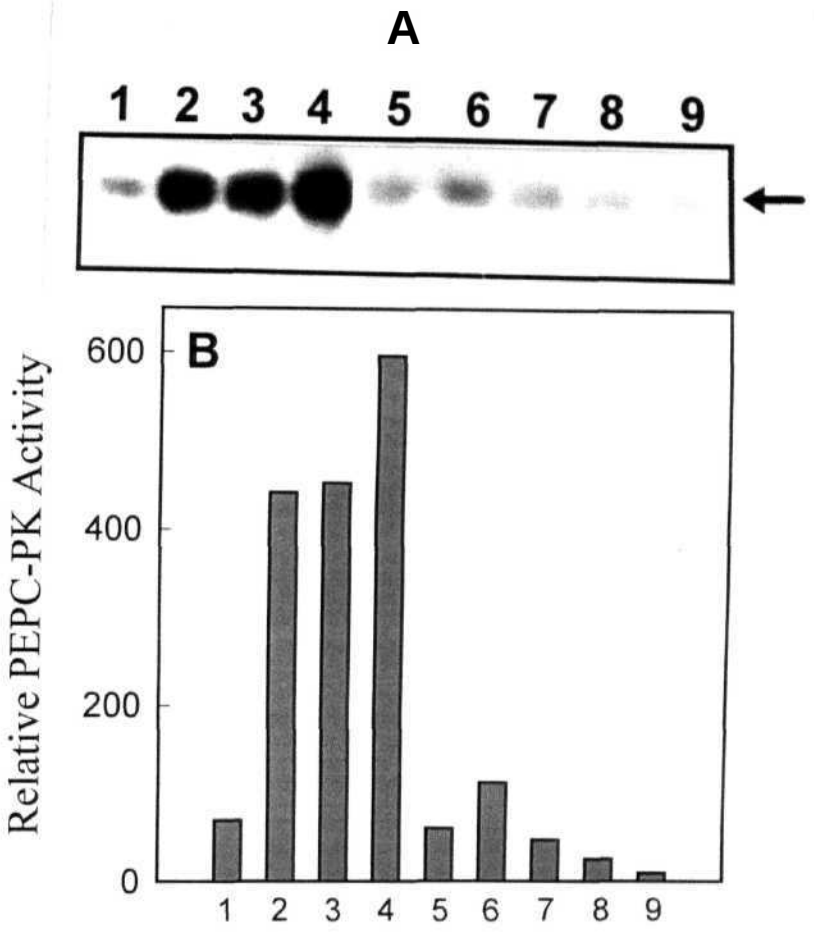


Plate 8.4. The sensitivity of PEPC phosphorylation to a range of activators or inhibitors of protein kinases. Lane 1: Control (no $CaCl_2$), Lane 2: 10 μM $CaCl_2$, Lane 3: 10 μM $CaCl_2$ + Phosphatidyl serine (20 μg/ml) + diacylglycerol (200 μM), Lane 4: 10 μg CaM + 10 μM $CaCl_2$, Lane 5: 500 μM U7, Lane 6: 50 μM staurosporine, Lane 7: 500 μM TFP, Lane 8: 500 μM W7 and Lane 9: 100 μM ML7. (A) Autoradiograph, (B) Corresponding histogram of relative PEPC-PK activity. Further details are as described for Plate 8.1 and in "Materials and Methods".

W7 and TFP, two well known CaM antagonists, decreased the extent of phosphorylation of PEPC. H7 (protein kinase C inhibitor), staurosporine (CaM kinase inhibitor) and ML_7 (an inhibitor of myosin light chain kinase, MLCK) restricted the phosphorylation of PEPC (Plate 8.4).

Discussion

The present results demonstrate that calcium exerts a significant effect directly on the activity of PEPC, besides the modulation of PEPC phosphorylation (which also may modulate PEPC, indirectly). The preliminary evidences suggest a definite involvement of Ca^{2+} (and likely of CaM) during the phosphorylation of PEPC.

Even moderate levels of Ca^{2+} (>50 µM) inhibited PEPC activity and such inhibitor}' effects of calcium on PEPC depended on the pH. The high inhibition of PEPC by Ca^{2+} particularly at pH 7.8 (Fig. 8.1) may be due to the higher affinity of PEPC to Mg^{2+} at pH 7.8 than that at lower pH (González et al., 1984; Rajagopalan et al., 1994). An examination of kinetics of PEPC in presence or absence of calcium revealed that the inhibition of PEPC by calcium was due to competition with Mg^{2+} at 100 µM $CaCl_2$ (Fig. 8.3), while the inhibition at 500 µM was of mixed type (Fig. 8.4). These data confirm the earlier reports of inhibition of PEPC by calcium ion and that the inhibition by Ca^{2+} was due to competition with Mg^{2+} (Mukerji, 1977; Gavalas and Manetas, 1980b; Gayathri and Raghavendra, 1994). Since there is a marked change in the kinetic properties of PEPC due to 100 µM $CaCl_2$, even at saturating level of Mg^{2+} (Table 8.1), it is possible that Ca^{2+} is not only competing with Mg^{2+} for binding site but also changing the conformation of the enzyme.

Although high concentrations of Ca^{2+} were inhibitory at pH 7.8 (Fig. 8.1), the stimulation of PEPC at low concentrations of Ca^{2+} (10-20 µM) was quite surprising. Ca^{2+} stimulation was more at pH 7.8 than that at pH 7.3 and was more

pronounced in extracts from illuminated leaves than in dark-adapted leaves (Figs. 8.5-8.7). In view of the stimulation by Ca^{2+}, the inhibition of PEPC by EGTA (Fig. 8.8) appears to be logical. The stimulation by Ca^{2+} (at 10 μM) and inhibition by EGTA confirm that low level (<20 μM) of Ca^{2+} is beneficial for PEPC activity, particularly in the extracts from illuminated leaves.

The observations on the stimulation by Ca^{2+} of PEPC extracted from illuminated leaf tissue and the inhibition by calcium (>50 μM) at alkaline pH of 7.8 may be physiologically quite relevant. Illumination induces marked cytosolic alkalization in mesophyll cells of C4 plants (Raghavendra et al., 1993). The increase in cytosolic pH can raise cytosolic calcium and lead to an increase in the activity of PEPC. However, cytosolic calcium at concentrations of >50 μM could be inhibitory. A fine tuning of cytosolic calcium therefore necessary for optimal activity of PEPC. Thus, our results indicate that Ca^{2+} plays a dual role in the modulation of PEPC activity.

Our observations confirm that PEPC is phosphorylated in leaf extracts by an endogenous protein kinase and this process can further be stimulated by the addition of ATP to the medium. The involvement of calcium was suggested by the stimulation by Ca^{2+} (Fig. 8.10A) and marked inhibition by EGTA of PEPC-PK activity (Fig. 8.1 OB). However, Ca^{2+} promoted PEPC-PK activity, but was not essential, since PEPC-PK activity was seen even in absence of calcium, but in presence of calcium the activity of PEPC-PK was much more pronounced.

Our observations on the regulation of PEPC activity by Ca^{2+} are important in view of the conflicting reports on the role of Ca^{2+} in the regulation of PEPC-PK activity. The addition of EGTA to phosphorylation assays inhibited the *in vitro* phosphorylation and this inhibition was reversed by the addition of external calcium to the assay (Echevarria et al., 1988; Ogawa et al., 1992). The use of different protein kinase inhibitors during phosphorylation assay demonstrated that

Ca^{2+}- or Ca^{2+}/CaM-dependent protein kinase was involved in phosphorylation of PEPC (Vidal et al., 1990; Ogawa and Izui, 1992; Ogawa et al., 1992). On the other hand, there are reports suggesting that PEPC-PK is Ca^{2+}-independent *in vitro* (Chollet et al., 1990; Echevarria et al., 1990; Jiao and Chollet, 1991; McNaughton et al., 1991; Duff et al., 1996; Giglioli-Guivarc'h et al., 1996). Ca^{2+}-dependent and Ca^{2+}-independent protein kinases were isolated from sorghum leaves (Bakrim et al., 1992). Recent reports using mesophyll protoplasts of *Digitaria sanguinalis* and *Sorghum* indicated that the stimulation by Ca^{2+} can be seen only during *in situ* phosphorylation of PEPC (Duff et al., 1996; Gigioli-Guivarc'h et al., 1996; Nhiri et al., 1998).

There was a marked difference between crude and desalted extracts in their maximal activity and properties of PEPC. PEPC activity was more in crude than that in desalted extracts. The sensitivity to EGTA or stimulation by Ca^{2+} was also more in crude than that in desalted extracts (Figs. 8.2, 8.5-8.8). The reasons for such significant difference between crude and desalted extracts are not known, at present, but could be due to the presence of low molecular weight compounds. Low molecular weight compounds may even protect PEPC from inactivation or to the higher enzyme concentration present in the crude extracts compared to desalted extracts (Rodriguez-Stores and Muñoz-Clares, 1987). There was a significant difference between crude and desalted extracts in the extent of ATP-dependent phosphorylation of PEPC and the response of PEPC-PK to Ca^{2+} or EGTA (Table 8.3). Studies on C4 mesophyll protoplasts suggested that Ca^{2+} regulated PEPC-PK activity only *in situ* but not *in vitro* (Duff et al., 1996; Giglioli-Guivarc'h et al., 1996). There is a possibility that PEPC is sensitized by a low molecular weight compound and only such sensitized PEPC responds to Ca^{2+}. Further experiments are necessary to confirm and identify regulatory metabolite/compound. However, phosphorylation of PEPC was not affected by a number of putative light-modulated cytosolic effectors including calcium/calmodulin, fructose 2,6-

bisphosphate, PPi, and thioredoxin h (Jiao and Chollet, 1989; Chollet et al., 1990; McNaughton et al., 1991).

Plates 8.2-8.4 illustrate that phosphorylation of PEPC is dependent on Ca^{2+} and to some extent CaM. The inhibition pattern was similar to that of MLCK, which belongs to the Ca^{2+}-CaM-dependent PK family (Table 8.4, Plate 8.4). The presence of Ca^{2+}-CaM dependent PEPC-PK in sorghum leaf crude extracts was previously reported by Echevarria et al. (1988). Ogawa et al. (1992) reported that MLCK type of kinase was involved in the phosphorylation of PEPC in maize leaves.

Ogawa et al. (1992) have reported that partial proteolysis of PEPC-PK can cause desensitization to calcium. The phosphorylation of PEPC activity was inhibited by the additions of EGTA in standard phosphorylation assays (Ogawa and Izui, 1992). The kinase responsible for PEPC phosphorylation can therefore be attributed to the calcium-dependent protein kinases rather than calcium-independent kinases. The group of Ogawa suggested that at least four protein kinases or its catalytic subunit in the PEPC-PK preparation, may be present and two of them are calcium-dependent (Ogawa et al., 1992). Immunopurified C4 PEPC from sorghum and maize could be phosphorylated *in vitro* by various kinases, including calcium- or magnesium-dependent protein kinase, both in crude extract and in a reconstituted system (Bakrim et al., 1992) or catalytic-subunit of cAMP-dependent protein kinase from bovine heart (Terada et al., 1990; Bakrim et al., 1992).

On the other hand, PEPC-PK preparations partially purified through Blue-dextran agarose either from maize or sorghum were active even under calcium depleted conditions (Chollet et al., 1990; Echevarria et al., 1990; McNaughton et al., 1991). Vidal et al. (1990) isolated a calcium-dependent protein kinase(s) from sorghum leaves that could phosphorylate dark-form PEPC *in vitro*, but phosphorylation did not change the properties of the target enzyme, i.e., PEPC.

Thus, the reports on regulation of C4 PEPC-PK by calcium have been controversial. Further experiments are needed to purify PEPC-PK from C4 leaves and elucidate its exact nature by performing reconstitution assays *in vitro.*

Our results endorse the opinion that PEPC phosphorylation occurs in **a** Ca^{2+}-CaM-dependent manner in crude leaf extracts *of Amaranthus.* The PEPC-PK was inhibited by the CaM antagonist (W_7) and MLCK inhibitor (KT5926). but did not require Ca^{2+}-binding protein (CaM) for activity. The responses of this enzyme was reminiscent of CDPK, a protein kinase not found in animals, which has an intrinsic, CaM-like, Ca^{2+}-binding regulatory domain, with four typical EF-hand motifs, linked to an N-terminal catalytic domain by an intervening junction domain (Roberts and Harmon, 1992).

Recent reports however lead to a conclusion that a Ca^{2+}-independent PEPC-PK phosphorylates C_4 PEPC in leaves (Chollet et al., 1996; Vidal and Chollet, 1997). We therefore suggest that the regulation by Ca^{2+} or CaM would be al an upstream level of regulation of PEPC-PK. For e.g. a CDPK like protein-kinase may modulate the Ca^{2+}-independent PEPC-PK. CDPK from maize leaves has recently been purified and characterized (Ogawa et al., 1998). A fine-tuning of cytosolic Ca^{2+} is anyway essential for regulating the activity or phosphorylation or both of PEPC.

Major conclusions from the results presented in **this chapter are:**

1. Calcium inhibited PEPC activity at pH 7.8 in extracts from light- and dark-adapted leaves of *Amaranthus hypochondriacus.* The inhibitory effect of calcium was due to its competition with Mg^{2+}.

2. Low concentrations of calcium (1-20 µM) stimulated PEPC activity, while being inhibited by EGTA, in extracts from light-adapted leaves. Thus low

concentration of calcium is beneficial for PEPC activity of particularly the light-form.

3. The stimulation by Ca^{2+} or inhibition by EGTA was more in crude extracts than that in desalted extracts. These results suggest that unidentified low molecular weight compounds are involved in the stimulation of PEPC activity by Ca^{2+}.

4. ATP-dependent phosphorylation of PEPC was indicated by the increase in PEPC activity on incubation of leaf extracts (particularly from illuminated leaves) with ATP and $MgCl_2$. The extent of ATP stimulation, indicating the PEPC phosphorylation, was more at pH 7.8 than that at pH 7.3.

5. $CaCl_2$ (10 μM) enhanced markedly (by 81%) the stimulation by ATP of PEPC activity, while being stimulated further by the presence of CaM. However, the phosphorylation of PEPC was markedly restricted by various Ca^{2+} as well as CaM dependent protein kinase inhibitors. These results suggest that phosphorylation of PEPC is regulated by a CDPK or a Ca^{2+}-CaM dependent protein kinase.

6. A fine tuning of calcium levels in mesophyll cytosol is essential for maximal activity of PEPC and PEPC-PK particularly at alkaline pH in illuminated leaves of C4 plants. Since recent reports suggest PEPC-PK itself may not be Ca^{2+}-dependent, a CDPK may be regulating the PEPC-PK at an upstream level.

Chapter 9

Preliminary Studies on the Occurrence of a Calcium-Dependent Protein Kinase in Leaf Extracts

Chapter 9

Preliminary Studies on the Occurrence of a Calcium-Dependent Protein Kinase in Leaf Extracts

Introduction

Plant cells respond to a wide range of stimuli through the generation of a Ca^{2+} signal (i.e. a stimulus-induced increase in $[Ca^{2+}]cyt$). These Ca^{2+} signatures have to be transduced by other elements in the signaling cassette to elicit the final response (McAinsh and Hetherington, 1998). Changes in Ca^{2+} levels are perceived by various Ca^{2+} binding proteins (such as CaM and CaM like proteins) and the signals are transduced usually to protein kinases and/or protein phosphatases (Roberts and Harmon, 1992; Poovaiah and Reddy, 1993; Snedden and Fromm. 1998;Zielinski, 1998).

The reversible cascade of phosphorylation/dephosphorylation of proteins leads to the sequence of related physiological events (Roberts and Harmon, 1992; Bootman and Berridge, 1995; Hunter, 1995). There are two major types of protein kinases which are regulated by Ca^{2+}: Ca^{2+}/phospholipid-dependent (PKC) and Ca^{2+}/CaM-dependent (CCaMK) (Nishizuka, 1992; Braun and Schulman, 1995; Sugita et al., 1994; Nairn and Picciotto, 1994). There are very few reports on presence of Ca^{2+}/phospholipid-dependent protein kinases and Ca^{2+}-CaM-dependent protein kinases in plants, for e.g., PKC from *Brassica campestris* seedlings (Nanmori et al., 1994) and CCaMK from maize etiolated coleoptiles (Pandey and Sopory, 1998). A third group of protein kinases in plants is getting increased attention: Ca^{2+}-dependent protein kinases (CDPK), which do not require CaM, phospholipid or diacylglycerol for their activation. Thus CDPKs differ from both the CCaMK and PKC families prevalent in animals (Roberts and Harmon, 1992; Stone and Walker, 1995).

All the CDPKs have the same general structure, consisting of three adjacent functional domains - catalytic, autoinhibitory and CaM-like. These domains run from the amino- to the carboxyl terminus (Harper et al., 1991; Roberts and Harmon, 1992). The catalytic domain of CDPK shows similarity with animal PKC and Ca^{2+}-CaM-dependent protein kinase (Roberts and Harmon, 1992). Sequence comparisons suggest that the CDPK kinase domain originated from the fusion of a gene encoding a Ca^{2+}/CaM-dependent kinase with a CaM-like gene. As a result, CDPK may be capable of a fast and versatile response to Ca^{2+} because direct Ca^{2+} binding relieves limitations due to the relatively slow diffusion of a larger Ca^{2+}-CaM complex (Harper et al., 1991). The CDPK enzyme is found throughout the plant, from leaves to roots (Harper et al., 1991). However, the role of CDPK isoforms in specific signal transduction pathways remains uncertain. Similarly, the function and *in vivo* substrates of CDPK are still unknown.

There are strong evidences of the importance of Ca^{24} during phosphorylation of PEPC *in situ,* particularly in mesophyll protoplast system (Duff et al., 1996; Giglioli-Guivarc'h et al., 1996). However, the exact site of action of Ca^{2+}, during light activation of PEPC has remained ambiguous, since the PEPC-PK itself does not show an essential requirement for Ca^{24} or CaM. It has been proposed that a CDPK may be regulating the PEPC-PK at an upstream level (Chollet et al., 1996; Vidal and Chollet, 1997), for e.g., phosphorylating either PEPC-PK or a protein modulator of PEPC-PK.

One of the major objectives of present work is to evaluate the pattern of PEPC phosphorylation in *Amaranthus hypochondriacus.* The results described in previous Chapter emphasize the dependence of PEPC phosphorylation on Ca^{2+}. This dependence is likely to be due to the involvement of a CDPK. It is therefore necessary to detect and characterize CDPK in leaf extracts of *Amaranthus hypochondriacus.* The present Chapter is a preliminary attempt to evaluate **the** occurrence **and** activity of CDPK in *Amaranthus hypochondriacus* leaves.

Results

Probing with anti-CCaMK antibodies raised against etiolated maize coleoptiles revealed two positively reacting protein-bands in leaf extracts of *Amaranthus hypochondriacus* (Plate 9.1). Two distinct bands corresponding to 75 and 80 kD were detected on Western blot. The levels of 75 kD protein increased upon illumination. Similar trend was observed in pea leaf extracts (Plate 9.2). The presence of the kinase was determined in C_4, C3-C4 and C3 species of *Alternanthera* (Plate 9.3). The increase in the intensity of 75 kD protein-band with illumination depended on the duration of illumination and was maximum at 30 min (Plate 9.4).

When PVDF membrane (with proteins of leaf extracts or purified PEPC) was probed with biotinylated CaM, two protein-bands were recognized to be CaM-binding proteins (Plate 9.5). One of them was of 75 kD while the other was 30 kD.

The leaf extracts were able to actively phosphorylate histone **HIS,** a typical characteristic of CDPK. This kinase activity was saturated at a concentration of 50 μg of histone IIIS (Fig. 9.1). *In vitro* phosphorylation of histone HIS was dependent and stimulated by the addition of Ca^{2+}. A stimulation of about 8-fold of CDPK occurred at 20 μM Ca^{2+} (Fig. 9.2). In contrast, the presence of CaM during kinase assay had only a very small effect on the Ca^{2+}-dependent phosphorylation of histone (Fig. 9.3).

Among the different protein substrates tested, syntide-2 (animal CaM kinase specific substrate) and histone HIS (PKC or CDPK specific substrate) were phosphorylated at a better rate than several others, for e.g., BSA, casein, MBP and even PEPC (Table 9.1). Further the presence of CaM had no significant effect on the extent of phosphorylation of these protein substrates. There was a significant increase (50-90% over dark) in the kinase activity (with substrates other than syntide) in extracts from illuminated leaves. The extent of phosphorylation was

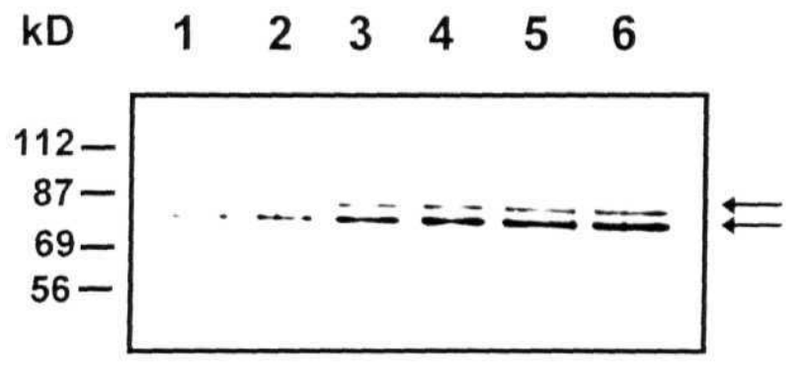


Plate 9.1. Detection of proteins in leaf extracts of *Amaranthus hypochondriacus* showing cross-reactivity with anti-CCaMK (from maize) antibodies. Proteins, after separation by SDS-PAGE, were transferred onto PVDF membrane and probed with anti-CCaMK antibodies and the complex was visualized with an alkaline phosphatase reaction. Two proteins correspond to 75 kD and 80 kD were observed on the blot. Lanes 1, 3, 5: Extracts from dark-adapted leaves, Lanes 2, 4, 6: Extracts from illuminated leaves. Lanes 1 and 2 contained 2 μg, lanes 3 and 4 contained 5 μg, and lanes 5 and 6 contained 10 ug of protein. The levels of 75 kD protein increased upon illumination, while that of 80 kD was not changed. Further details are as described in "Materials and Methods".

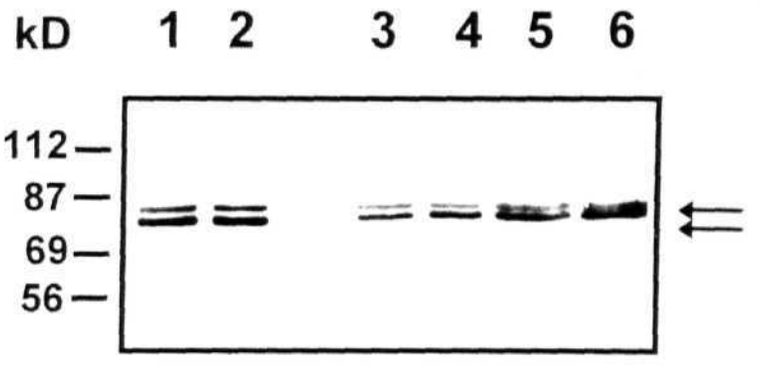


Plate 9.2. Western blot, illustrating a comparison of cross reactivity to anti-CCaMK antibodies of proteins in leaf extracts of *Amaranthus hypochondriacus* and pea. Lanes 1, 3, 5: Extracts from dark-adapted leaves, Lanes 2, 4, 6: Extracts from illuminated leaves. Lanes 1 and 2 contained 10 μg protein of *Amaranthus hypochondriacus* leaf extracts, lanes 3 and 4 contained 5 μg protein of pea leaf extracts, and lanes 5 and 6 contained 10 ug protein of pea leaf extracts. **The levels of** 75 **kD protein** in ***A.*** *hypochondriacus* extracts increased upon **illumination.** Further details **are** as described for Plate 9.1 and as in **"Materials and Methods".**

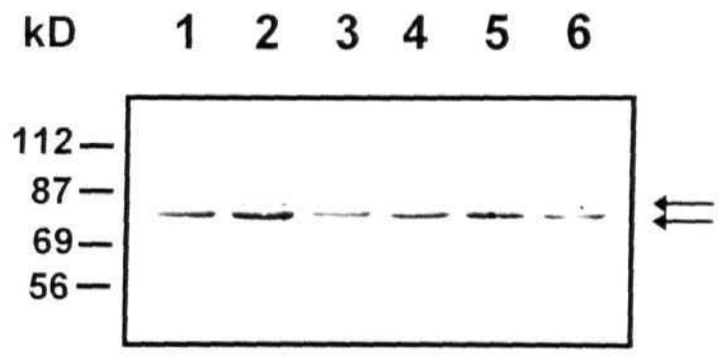


Plate 9.3. CCaMK like proteins, as indicated by the Western blots, in leaf extracts of *Altemanthera pungens* (a C_4 species), *Alternanthera tenella* (a C3-C4 intermediate) and *Altemanthera sessilis* (a C_3 species). Lanes 1,3,5: Extracts from dark-adapted leaves, Lanes 2, 4, 6: Extracts from illuminated leaves. Lanes 1 and 2 contained leaf extracts of *A. pungens*, lanes 3 and 4 contained leaf extracts of *A. tenella,* and lanes 5 and 6 contained leaf extracts of *A. sessilis.* All the lanes contained 10 μg of protein. The protein band of 75 kD was more in extracts from illuminated leaves of particularly *A. pungens.* Further details are as described for Plate 9.1 and as in "Materials and Methods".

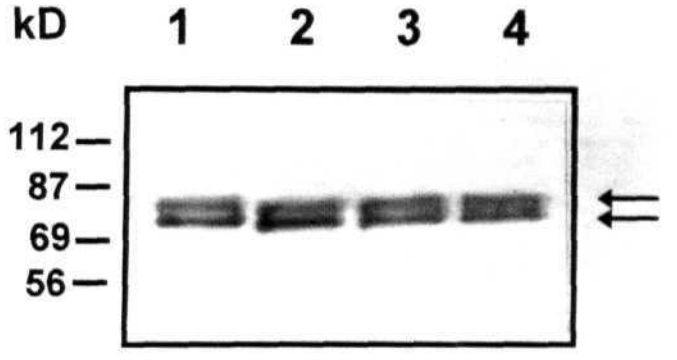


Plate 9.4. Effect of duration of illumination on the abundance of CCaMK like proteins in *Amaranthus* leaves. Lane 1: Extracts from dark-adapted leaves, Lanes 2-4: Extracts from leaves illuminated for 10, 20 and 30 min, respectively. All the lanes contained 10 μg of protein. The protein band of 75 kD increased on illumination, but not the 80 kD. Further details are as in Plate 9.1 and as described in "Materials and Methods".

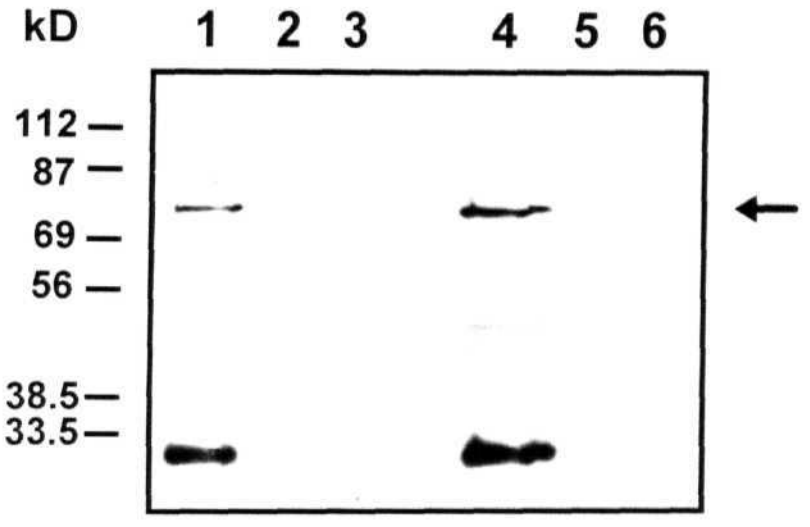


Plate 9.5. The presence of CaM-binding proteins in leaf extracts of *Amaranthus hypochondriacus* as revealed by Western blot analysis. Lanes 1 and 4: Leaf extracts (5 and 7.5 μg protein), Lanes 2 and 5: Partially purified PEPC (2 and 4 μg protein), Lanes 3 and 6: Purified PEPC (1 and 3 μg protein). The proteins on the PVDF membrane were probed with biotinylated CaM. The two major bands correspond to 75 and 30 kD proteins. Further details are described in "Materials and Methods".

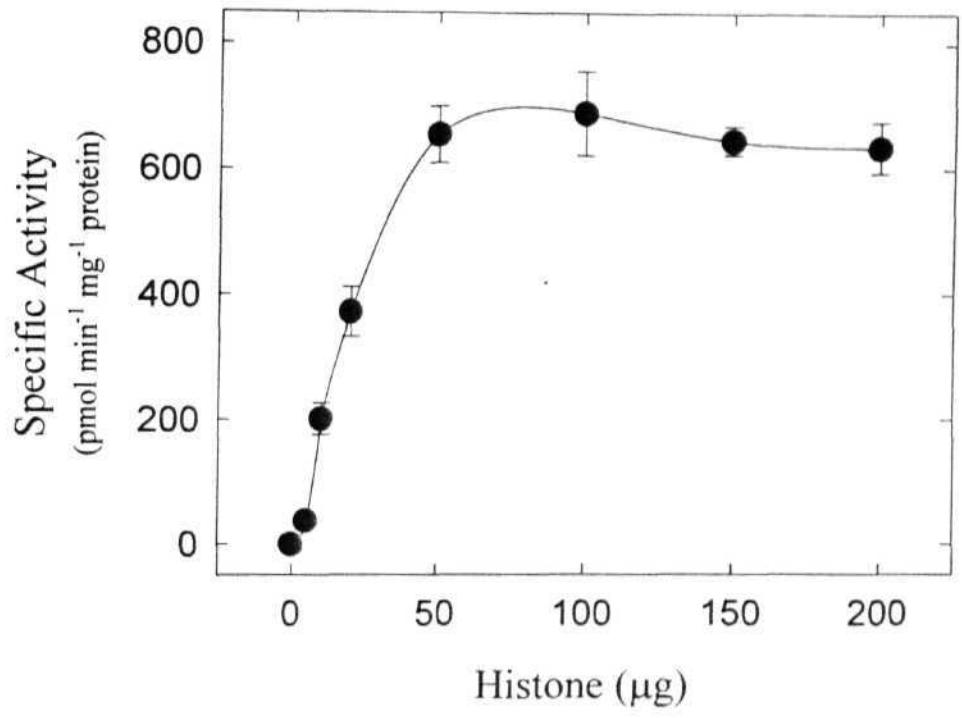


Figure 9.1. The dependence of kinase activity on the concentrations of histone HIS in the assay medium. Further details are as described in "Materials and Methods".

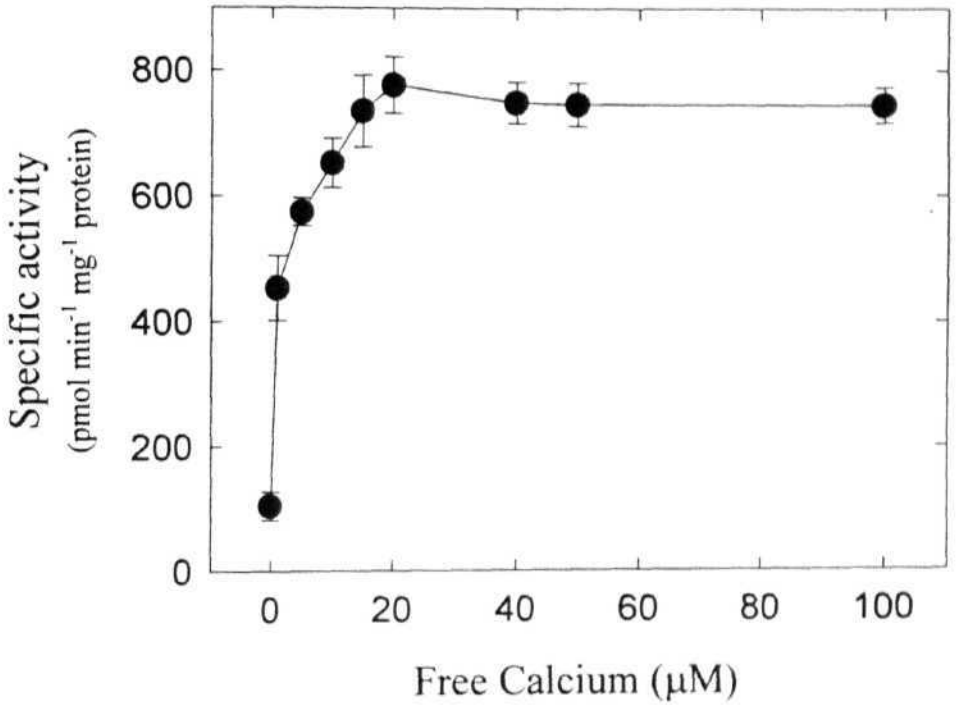


Figure 9.2. Marked stimulation of protein kinase activity by free Ca^{2+} in leaf extracts of *Amaranthus hypochondriacus* with histone HIS (50 μg) as the substrate. Free Ca^{2+} concentrations in the assay were modulated with the help of CaCl2-EGTA buffers according to Veluthambi and Poovaiah (1986). Further details are described in "Materials and Methods".

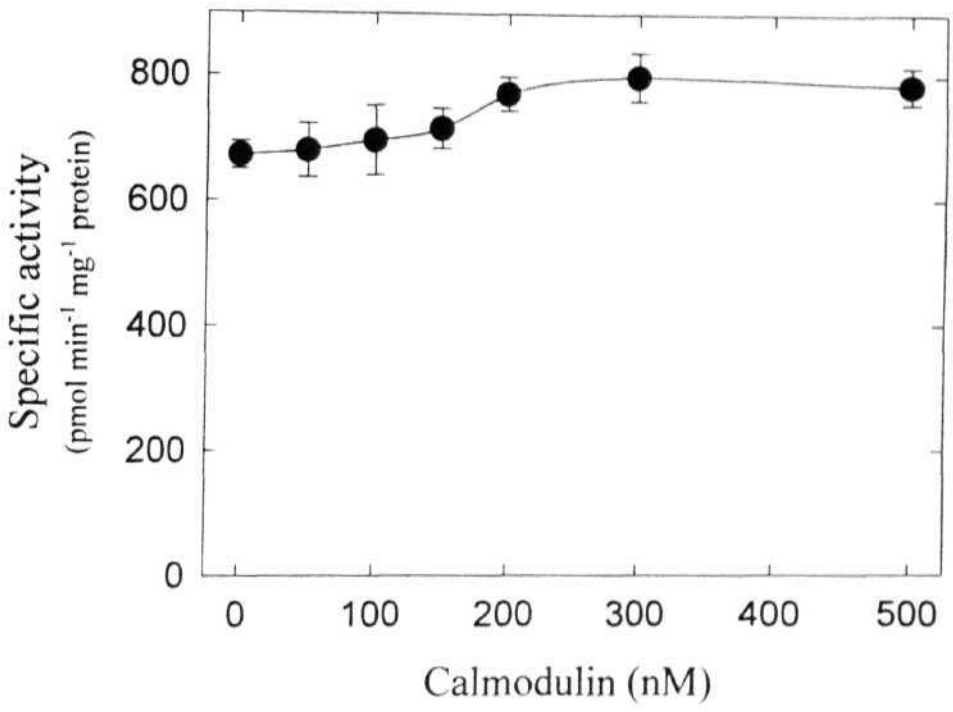


Figure 9.3. Marginal dependence on CaM of CDPK in leaf extracts of *A. hypochondriacus*. The substrate was histone HIS (50 (ig) and the concentration of free Ca^{2+} was 20 μM. Please note that the scale of Y axis is the same as that of Figure 9.2. Further details are described in "Materials and Methods".

Table 9.1. *Substrate specificity of Ca^{2+}-dependent protein kinase in crude extracts of dark-adapted leaves from* Amaranthus hypochondriacus.

Protein kinase assays were performed in absence or presence of 300 nM of CaM. All assays were performed in presence of 20 μM free Ca^{2+}. Further details are described in "Materials and Methods".

Protein substrate	Protein kinase activity	
	No CaM	+ CaM (300 nM)
	pmol min^{-1} mg^{-1} protein	
Histone HIS (50 μg)	759 ± 57	786 ± 46
Syntide-2 (2 μg)	887 ±71	890 ± 98
MBP (20 μg)	57 ± 5	54 ± 4
Casein (50 μg)	113 ±9	160 ±10
BSA (50 μg)	115±7	163 ± 8
PEPC *{Amaranthus)* (5 (Ag)	130 ±5	133 ± *1*
PEPC (WT, from maize) (3 μg)	48 ± 3	48 ± 6
PEPC (S15D, from maize) (3 μg)	21 ±2	29 ±4

low, with no further stimulation by light of kinase activity when a mutant form of PEPC was used (S15D, which mimics the phosphorylated form) as the substrate (Table 9.2).

The sensitivity of the CDPK activity to different activators and inhibitors was examined. CaM exerted a small stimulatory (11%) effect on the Ca^{2+}-stimulated CDPK activity. Similarly, diacylglycerol and Phosphatidyl serine also marginally enhanced the Ca^{2+}-enhanced phosphorylation of histone (Table 9.3). EGTA at 1 mM inhibited the kinase activity very strongly (almost 85%, Table 9.3). Calmidazolium, W7, and TFP (CaM antagonists) inhibited the phosphorylation of histone, while H7 (protein kinase C inhibitor) had marginal inhibitory effect on kinase activity. Similarly, ML7 (MLCK inhibitor) and staurosporine (CaM kinase inhibitor) also suppressed strongly the phosphorylation of histone (Table 9.3).

Discussion

Two distinct protein-bands in *Amaranthus hypochondriacus* leaf extracts corresponding to 75 and 80 kD cross reacted strongly with polyclonal antibodies raised against CCaM kinase purified from maize etiolated colcoptiles (Plates 9.1 and 9.2). One of them (75 kD) was capable of binding to CaM (Plate 9.5). However, the biochemical evidence suggested that the protein kinase in leaf extracts of *A. hypochondriacus* is more of a CDPK rather than CCaMK-type. We therefore suggest that 75 kD protein is likely to be CDPK-like one. Further, the leaf tissues are reported to have CDPK with a molecular weight of about 80 kD (Roberts and Harmon, 1992).

The existence of multiple isoforms of CDPK in plants is known (Roberts and Harmon, 1992). The cross reactivity of C4, C3-C4 and C3 species of *Alternanthera* further confirm the ubiquitous presence of this kinase in plants (Plate 9.3). The intenstiy of 75 kD increased upon illumination (Plates 9.1 and 9.2). Although, we

Table 9.2. *The response of Ca^{2+}-dependent protein kinase (with different substrates) in crude extracts to illumination of leaves from* Amaranthus hypochondriacus *compared to the dark-adapted ones.*

Protein kinase assays were performed in presence of 20 uM free Ca^{2+}. Further details are described in "Materials and Methods".

Protein substrate	Protein kinase activity		
	Dark	Light	L/D
	pmol min^{-1} mg^{-1} protein		
Histone HIS (50 ug)	711 ±63	1047 ±82	1.5
Syntide-2 (2 ug)	832 ±72	1063 ± 113	1.3
MBP (20 ug)	93 ± 13	172 ± 10	1.9
Casein (50 ug)	123 ±1	183 ± 9	1.5
BSA (50 ug)	**136** ± 7	**213** ± 10	1.6
PEPC *{Amaranthus)* (5 ug)	133 ±4	220 ±9	1.7
PEPC (WT, from maize) (3 ug)	48 ±3	88 ±7	1.8
PEPC (S15D, from maize) (3 ug)	29 ±2	21 ±3	0.7

Table 9.3. *Effect of different protein kinase activators or inhibitors on the activity of* Ca^{2+}*-dependent protein kinase in crude extracts from dark-adapted leaves of* Amaranthus hypochondriacus.

Protein kinase assays were performed in presence of 20 μM free Ca^{2+}. Further details are described in "Materials and Methods". Figures in parentheses represent the values of % of control.

Activator/inhibitor	Histone HIS	Syntide-2
	Protein kinase activity	
	pmol min^{-1} *mg*$^{-1}$ *protein*	
Control (None)	739 ± 62(100)	900 ±93 (100)
CaM (300 nM)	819 ±89 (111)	923 ±79 (103)
DAG (200 μM) + PS (20 μg/ml)	833 ±67 (113)	ND
EGTA(1 mM)	104i 7(14)	189 ± 9 (21)
TFP(1 mM)	325 ± 34 (44)	198 ±41 (22)
W7(l mM)	192 ± 10(26)	252 ± 12(28)
Calmidazolium (10 μM)	274 ±31 (37)	252 ± 32 (28)
H7 (200 μM)	577 ± 11 (78)	603 ± 42 (67)
ML7 (200 μM)	340 ±21 (46)	333 ± 36 (37)
Staurosporine (10 μM)	228 ± 10(31)	243 ±9 (27)

ND = Not determined.

do not have an explanation for such phenomenon, the synthesis of 75 kD CDPK is obviously promoted in light. It is well known that intracellular concentrations of cytosolic Ca^{2+} increased upon illumination. We feel that such Ca^{2+} signature may induce the synthesis or activity of CDPK and which in turn may result in protein phosphorylation. Further experiments are needed to explain this phenomenon.

Leaf extracts of *A. hypochondriacus* phosphorylated actively several substrates, including histone, demonstrating the existence of a protein kinase. The positive dependence of this kinase activity on Ca^{2+}, but not CaM (Fig. 9.2) and the preference for histone (Table 9.1) suggested that the activity is of a CDPK. CDPKs have some specific biochemical properties such as dependence of activity on micromolar concentrations of free Ca^{2+}, direct binding of Ca^{2+} and no requirement for CaM or Phosphatidyl serine for activity, ability to phosphorylate histone H1 and ability to bind to hydrophobic matrixes in a Ca^{2+}-dependent manner (Roberts and Harmon, 1992). We therefore conclude that an active CDPK occurs in *A. hypochondriacus* leaves.

The present priliminary report forms an addition to the extremely limited literature on the properties of CDPK in plant tissues. CDPK has been purified to homogeneity from soybean cell cultures (Putnam-Evans et al., 1990). The activity of CDPK *in vitro* is stimulated 50- to 100-fold by micromolar free Ca^{2+} and in the presence of millimolar magnesium. Partially purified CDPK from oat plasma membrane is activated 10-fold by calcium alone and another 20-fold by the addition of a crude soybean lipid preparation (Roberts and Harmon, 1992). Unlike other protein kinases, soybean cell CDPK can use either Mg^{2+}-ATP or Mg^{2+}-GTP as the phosphoric group donor. CDPK phosphorylates the substrate histone H1 on serine residue and undergoes autophosphorylation on serine or threonine residues (Putnam-Evans et al., 1990).

Syntide-2 and histone IIIS were the best substrates for CDPK (Table 9.1), Among the protein substrates, lysine-rich histone (histone H1 from calf thymus or plants and histone IIIS) is the best substrate for most CDPKs. The substrates of CDPK are phosphorylated also by other protein kinases. For e.g., lysine-rich histone is a good substrate for protein kinase C but a poor substrate for CaM-dependent protein kinase (Roberts and Harmon, 1992). A protein kinase C specific substrate, 4-14 myelin basic protein, is phosphorylated by *Dunaliella* CDPK. Similarly peptides derived from rnyosin light chains are phosphorylated by CDPK as well as myosin light-chain kinase, a CaM-dependent protein kinase. This suggests that CDPK has a broad substrate specificity, overlapping with that of protein kinase C and the Ca^{2+}/CaM-dependent protein kinases (Roberts and Harmon, 1992).

Several protein kinase inhibitors related to CaM or CaM kinase or PKC inhibited the phosphorylation of histone (Table 9.3). However, the concentrations required for such inhibition were more than that required for CaM or CaM kinase or PKC inhibition. This is a typical characteristic of CDPK (Hetherington and Trewavas, 1982; Roberts and Harmon, 1992).

CDPK contains a hydrophobic domain similar to the one found in CaM, which is exposed upon binding of Ca^{2+} (Roberts and Harmon, 1992). As a result, the interaction of CDPKs with numerous hydrophobic and amphipathic structures is similar to that of CaM. Since most of these inhibitors are amphiphilic, they may be interacting with CDPK by the satne mechanism as the CaM antagonists (Roberts and Harmon, 1992). Thus, the Observation on sensitivity of kinase activity to different inhibitors has to be treated with caution.

Tables 9.2 and 9.3 demonstrate that PEPC is a poor substrate for CDPK. In contrast, PEPC phosphorylation occurs in a Ca^{2+}-dependent manner (Chapter 8). A recent report by Ogawa et al. (1998) suggested that PEPC from maize is a poor

substrate for CDPK. Although these appear to be contradictory to each other, a logical explanation would be that the PEPC-PK (but not PEPC itself) is modulated by a Ca^{2+}-dependent protein kinase. Thus. PEPC-PK can be expected to be a better substrate than the purified PEPC. We therefore endorse the suggestion that a CDPK acts at an upstream end of the light-induced PEPC-PK activity or PEPC phosphorylation, as per the current model of PEPC regulation (Vidal and Chollet, 1997). Incidentally, the synthesis of 75 kD protein which is likely to be a CDPK is markedly enhanced on illumination, particularly in leaves of C4 plants (Plates 9.1-9.4).

Further experiments are needed to establish if CDPK regulates the PEPC-PK in a Ca^{2+} dependent manner. These results would then reveal/confirm the chain of signaling elements involved in the light-signal transduction, which leads to PEPC phosphorylation.

Major conclusions from the results presented in this chapter are:

1. Two proteins of a very close molecular weight: 75 kD and 80 kD in leaf extracts of *A. hypochondriacus* cross reacted strongly with anti-CCaMK (from maize) antibodies. One of them (75 kD) appears to be a CDPK, since it binds to also CaM, as indicated by the Western blots.

2. The levels of 75 kD protein (which seems to be a CDPK) increased upon illumination particularly in leaves of C4 plants, indicating a light induced synthesis of CDPK.

3. Experiments with leaf extracts of *Amaranthus hypochondriacus* confirmed the presence of an active CDPK, with a broad substrate specificity. Histone HIS and syntide-2 were the best substrates for CDPK among the several substrates tested. PEPC was a poor substrate for phosphorylation by this CDPK.

4. Phosphorylation of histone IIIS or syntide-2 **required** an optimal Ca^{2+} of 20 μM but did not depend on CaM. This suggests the occurrence of CDPK in *A. hypochondriacus* leaves.

5. Phosphorylation of histone IIIS or syntide-2 was suppressed significantly by a wide range of inhibitors of Ca^{2+}- and CaM-dependent protein kinases, but at a high concentration of inhibitors. The substrate specificity and inhibition pattern are similar to other Ca^{2+}-dependent protein kinases.

6. Further experiments are needed to characterize CDPK and to establish its role in the regulation of PEPC and/or PEPC-PK in *A. hypochondriacus* leaves.

Chapter 10

General Discussion

Chapter 10

General Discussion

PEPC is widely distributed in plant tissues, green algae and microorganisms and even is used as a molecular marker to visualize phylogenetic trends (Gehrig et al., 1998). PEPC, a cytosolic enzyme, catalyzes the irreversible exergonic β-carboxylation of PEP by HCO_3^- in the presence of Mg^{2+} to yield OAA and Pi (Rajagopalan et al., 1994; Chollet et al., 1996; Vidal and Chollet, 1997). PEPC has been purified from several photosynthetic and nonphotosynthetic tissues (Chollet et al., 1996). In present work, PEPC was purified to homogeneity from the leaves of *Amaranthus hypochondriacus,* PEPC was purified by conventional method involving four purification steps, such as 40-60% ammonium Sulfate, DEAE-Sepharose, phenyl-Sepharose and HAP. Finally the pure PEPC, eluted from HAP, was concentrated with PEG 20,000 and stored in liquid N_2. The purity of the enzyme was confirmed by the appearance of a single band of about 100 kD on SDS-PAGE (Plate 4.1). The purified PEPC had a very high specific activity of >55 U mg^{-1} protein (Table 4.1). This is one of the highest activities reported for PEPC from C4 plants in the literature (O'Leary, 1982).

The stability of the enzyme during storage is an important factor to maintain the maximum activity and high malate sensitivity, since purified PEPC is unstable. The N-terminal end of PEPC is very susceptible to proteolytic cleavage. The enzyme after such loss of N-terminus exhibits much less sensitivity of the enzyme to malate. The inclusion of protease inhibitors and glycerol during extraction and the process of purification protects the N-terminus (McNaughton et al., 1989; Baur et al., 1992). The intactness of the enzyme in our preparations was assessed by checking the sensitivity of the enzyme to malate. The kinetic characteristics of **the** enzyme (Table 4.4) were similar to the reported values in the literature indicating

the enzyme was stored with a good shape and intact N-terminus. Purified enzyme was stored in liquid N_2 along with 50% glycerol without altering kinetic characteristics of the enzyme for about 4 months.

Polyclonal antibodies were raised in rabbits against purified PEPC and the anti-PEPC antiserum was used for the immunological characterization of PEPC. The cross reactivity of the anti-PEPC antiserum was assessed by Ouchterlony double diffusion and Western blot analysis (Plates 4.4 and 4.5). Anti-PEPC antiserum could be used for quantitative estimation of PEPC by ELISA. The absorbance at 405 nm was linear with increase in concentration of PEPC We therefore suggest that ELISA can be used as a diagnostic kit to detect the amount of PEPC present in leaf extracts or given samples.

PEPC could be purified rapidly from *Amaranthus hypochondriacus* leaves also by using immunoadsorbent column chromatography (Fig. 4.6). PEPC was purified by 120 fold with about 10% yield (Table 4.3). Although the recovery of the enzyme was poor, the enzyme was pure (Plate 4.7) and with very good catalytic and regulatory properties (Table 4.4). The high quality of the preparation and the short time required for the purification arc advantages of immunoadsrobent chromatography. The rapid purification protocol based on immunoaffinity columns can be useful particularly for examining the properties of PEPC from leaves during light/dark transitions (Arrio-Dupont et al.. 1992).

PEPC is an oligomeric enzyme. The enzyme exists predominantly as a tetramer along with the dimeric and monomeric forms. However, the terameric form of the enzyme is the most active form (Wu et al., 1990). The dilution of the enzyme (a common consequence of extraction) leads to the dissociation of PEPC from its quaternary structure and subsequently leads to the loss of activity (Angelopoulos and Gavalas, 1988). The concentration of PEPC *in vitro* assay conditions is far below to its expected concentration *in vivo.* Therefore the addition

of compatible solutes during *in vitro* conditions maintain the quaternary structure of the enzyme by mimicking its *in vivo* situation. Although, glycerol or high concentration of protein can shift the equilibrium towards its active tetrameric form of PEPC (Podestá and Andreo. 1989). the significance of the oligomeric transition of PEPC during light/dark transition is debatable since oligomerization/ dissociation have mostly been observed *in vitro* (Rajagopalan et al., 1994).

Our observations suggest that the inclusion of PEG and glycerol during extraction and assay of the enzyme optimizes the activity of PEPC in leaf crude extracts (Fig. 5.1). The maximal activity of PEPC (in crude extracts as well as in purified form) increased, whereas K_m (PEP) decreased in presence of PEG and/or glycerol. On the other hand. the sensitivity of the enzyme to malate decreased, but the K_m (HCO_3^-) was not much effected (Tables 5.1 and 5.2). These altered kinetic characteristics of the enzyme arc due to the shift of the enzyme towards the tetrameric state promoted by compatible solutes. It has already been reported that the changes in the aggregation of the enzyme leads to the altered kinetic properties of PEPC (Willeford et al., 1990).

Gel filtration can be used to check the oligomeric status of the enzyme upon dilution (McNaughton et al.. 1989). Podestá and Andreo (1989) reported that PEPC was eluted as a tetramer with glycerol. whereas tetramer and dimer in absence of glycerol. Three types of oligomers were noticed when the molecular size of PEPC was assessed in presence or absence of PEG and/or glycerol, with gel filtration on Sephadex G-200 column (Fig. 5.4). The activity of PEPC was detected only in the fractions corresponding to the tetramer, irrespective of the presence of glycerol or PEG (Fig. 5.6). Addition of compatible solutes helped to maintain the quaternary structure of the enzyme by promoting the existence of tetrameric state. There is a continuous debate about the activity of monomer or dimer of PEPC (McNaughton et al., 1989; Wu et al., 1990; Meyer et al., 1991). We can conclude from these results that only tetrameric form of the enzyme is active.

The regulatory properties of the enzyme have been extensively studied in response to light. The sensitivity of the enzyme to malate and/or Glc-6-P is modulated by factors such as light, temperature and metabolites, whereas the affinity of the enzyme to substrates was not much studied. The present work, reports for the first time that on illumination the enzyme affinity to HCO3" increased indicating the marked interaction of light, bicarbonate and PEPC.

The studies on changes in kinetic properties of the enzyme, such as the affinity of the enzyme to HCO_3^-, are limited due to the difficulty in the complete elimination of dissolved HCO_3^- during the assays (Chollet et al., 1996). Therefore, there is a lot of variation in the reported K_m (HCO_3^-) values (Uedan and Sugiyama, 1976; Bauwe, 1986; Gao and Woo. 1995, 1996b; Dong et al.. 1997a). So far HCO_3^- has been known only as a substrate for PEPC activity, but our results demonstrate that bicarbonate can be also a regulatory metabolite. A few reports have recently appeared indicating that HCO3" caused significant changes in the regulatory properties of PEPC (Dong et al., 1997a; Ogawa et al.. 1997; Parvathi et al., 1998a).

The sensitivity of PEPC to malate and Glc-6-P was decreased with increasing concentration of HCO3- in the assay medium (Figs.6.3 and 6.4). On the other hand, the affinity of the enzyme to Mg^{2+} increased at higher HCO3- concentration (Table 6.1)- This is the first detailed report indicating the marked desensitization by HCO3" of PEPC to malate and Glc-6-P, and sensitization to Mg^{2+}.

The relative response of PEPC to HCO_3^- changed on illumination of leaves and saturated at much more lower concentration of HCO3" compared to dark (Figs. 7.1 and 7.2). On illumination the affinity of PEPC to HCO3- increased by at least two times, besides the 2 to 5-fold increase in V_{max} and 3 to 4-fold increase in K_i for malate (Fig. 7.3; Table 7.1). These results suggest that illumination of leaves

sensitizes PEPC to HCO_3^-. On the other hand, **the extent** of light activation was drastically decreased with increasing concentration of HCO_3^- (Fig. 7.4). There are many reports on the changes in the kinetic and regulatory properties of PEPC, such as marked changes in V_{max} and sensitivity to malate or Glc-6-P, on illumination (Chollet et al., 1996; Vidal and Chollet, 1997). While confirming a similar trend, we emphasize that a marked increase in the affinity of PEPC to bicarbonate occurs on illumination.

The interconversion of CO_2 to HCO_3^- is catalyzed by carbonic anhydrase in cytosol of mesophyll cells, where also PEPC is located (Burncll and Hatch, 1988). The activity of carbonic anhydrase is higher (> 100-fold) in light-adapted leaves than that in dark-adapted ones (Burnell et al.. 1990). It is possible that the apparent decrease in K_m for HCO_3^- of PEPC on illumination may partly be due to the light-induced activity of carbonic anhydrase. However, the inclusion of an inhibitor of carbonic anhydrase, ethoxyzolamide during assay did not prevent the light-induced decrease in K_m (HCO_3^-) of PEPC. We therefore rule out the possibility of the involvement of carbonic anhydrase in light-induced increase in the affinity of PEPC to HCO3-(Table 7.1).

One of the reasons for light activation of pE PC in C4 leaves is due to the stimulation of PEPC phosphorylation on illumination by a PEPC-PK. The sensitivity of PEPC to malate is a reflection of its phosphorylation status. CHX (cytosolic protein synthesis inhibitor) inhibits the light activation of PEPC by blocking the synthesis of PEPC-PK (Jiao et al., 1991b, Bakrim et al., 1992, 1993). Pretreatment of leaf discs with CHX completely prevented the light-induced changes of PEPC, such as increase in the Kj for malate and decrease in K_m for HCO_3^- (Table 7.1). Further. *in vitro* phosphorylation by mammalian PKA of PEPC from *Amaranthus* and a recombinant WT, but not the mutant (S15D) PEPC from maize leaves decreased the K_m (HCO3") of the enzyme, besides increasing

K_i (malate) (Fig. 7.5, Table 7.3). These results suggest that phosphorylation plays an important role during the light-induced sensitization of PEPC to bicarbonate.

On illumination there is a simultaneous increase in the availability of dissolved HCO3" due to the cytosolic alkalization of mesophyll cells, as well as the affinity of enzyme to PEPC (Table 7.2. Figs. 7.1-7.3). Thus, our observations add another dimension to the biochemical basis of light activation of PEPC. This is the first report indicating the marked interaction between light, bicarbonate and PEPC in C4 plants.

Cytosolic Ca^{2+} acts as a secondary messenger in a variety of physiological responses. PEPC is also localized in the cytosol of mesophyll cells. The interaction of Ca^{2+} and PEPC within the cytosol is therefore a topic of great interest. The changes in the intracellular levels of Ca^{2+} are perceived and the signal is transduced downstream by other secondary messengers to elicit the final response. Protein kinases are among such secondary messengers of signal transduction.

C4 plants maintain soluble Ca^{2+} at low levels in their leaves and are called as calciophobes. It is however not clear if low levels of Ca^{2+} is a prerequisite for the smooth functioning of C4 pathway (Gavalas and Manetas, 1980a). Therefore the effects of Ca^{2+} on the activity of PEPC and pattern of PEPC phosphorylation in leaf extracts were examined.

Ca^{2+} inhibited the activity of PEPC at higher concentrations (>50 μM). The extent of inhibition was more in illuminated leaves compared to dark-adapted leaves, particularly pH 7.8 (Fig. 8.1)- The inhibitory effect of Ca^{2+} (100 μM) was due to the competition with Mg^{2+} (Fig. 8.3). K_m (PEP) and V_{max} were not much effected, whereas Kj (malate) and K_m (Mg^{2+}) increased when 100 μM $CaCl_2$ was present in the assay medium along with optimal Mg^{2+} (15 mM) (Table 8.1). The marked changes in the kinetic properties of the enzyme suggest that Ca^{2+} is not

only competing with Mg^{2+} for its binding site, but also changing the confirmation of the enzyme.

Low levels of Ca^{2+} (≤20 |iM) stimulated PEPC activity, particularly at pH 7.8. The extent of stimulation by Ca^{2+} was more in illuminated leaves compared to dark-adapted ones (Figs. 8.5-8.7). These results suggest that low level of Ca^{2+} is essential for the optimal activity of PEPC.

Protein phosphorylation/dephosphorylation cascade is an important mode of post-translational modification of enzyme in plants as well as in animals. PEPC is one of such enzymes and is phosphorylated in light by PEPC-PK and dephosphorylated in dark by type 2A PEPC-protcin phosphatase (Vidal and Chollet, 1997). Phosphorylated form of the enzyme is more active and less sensitive to malate compared to dephosphorylated form. The regulatory phosphorylation occurs on serine residue of the enzyme. for e.g. Ser15 in maize and Ser8 in sorghum. In PEPC *of Amaranthus hypochondriacus* the serine residue located at 11^{th} position (Rydzik and Berry. 1996) is expected to be phosphorylated by PEPC-PK on illumination. The next phase of our experiments were designed to check the effect of Ca^{2+} on the activity of PEPC-PK in crude and desalted leaf extracts.

A typical consequence of phosphorylation of PEPC is the marked decrease in the sensitivity of enzyme to malate. Phosphorylation of PEPC can therefore be studied by two different methods: indirect or direct assays. An indirect measure of PEPC-PK activity is the stimulation of PEPC activity on incubation with ATP indicates the ATP-dependent phosphorylation of PEPC, along with the decrease in the malate sensitivity of the enzyme confirm PEPC phosphorylation. The stimulation by ATP was more at pH 7.8 compared to pH 7.3. These results reveal that ATP-dependent phosphorylation of PEPC prefers alkaline pH. The extent of stimulation by ATP was pronounced when malate was added during assay (Table 8.2). The stimulation of ATP-dependent activation of PEPC by Ca^{2+} and

marked inhibition by EGTA (Fig. 8.10) suggest that PEPC phosphorylation is dependent on the availability of Ca^{2+}.

The next set of our experiments involved the direct assay of PEPC phosphorylation. Phosphorylation of PEPC was observed only in illuminated leaves. There was no detectable phosphorylation in dark-adapted ones (Plate 8.1). Phosphorylation of PEPC required Ca^{2+} during the assay, and was strongly inhibited by BAPTA. The inhibition by BAPTA of PEPC phosphorylation was partially reversed by the addition of Ca^{2+} but not with Mg^{2+} (Plates 8.2 and 8.3). There was only a marginal stimulation by CaM of PEPC (Plate 8.4). We therefore suggest that the PEPC phosphorylation is dependent on Ca^{2+}, but CaM may not be an absolute requirement.

The pattern of the phosphorylation of PEPC was further checked by using a wide range of inhibitors of protein kinases (Plate 8.4). Phosphorylation was strongly restricted by H7 (protein kinase C inhibitor). W7 and TFP (CaM antagonists), staurosporinc (CaMK inhibitor) and ML7 (MLCK inhibitor). While confirming the involvement of Ca^{2+} during PEPC phosphorylation, we suggest that a fine tuning of Ca^{2+} is needed for the optimal activity of both PEPC and PEPC-PK.

Although the results from present study on PEPC phosphorylation *in vitro* by $AT^{32}P$ suggest the involvement of Ca^{2+}, recent reports emphasized that PEPC-PK is a Ca^{2+}-independent protein kinase (Chollet et al., 1996: Vidal and Chollet, 1997; Nhiri et al., 1998). A compromise suggestion linking these two apparently contradictory observations is that PEPC-PK is upregulated by another Ca^{2+}-dependent protein kinase, possibly CDPK. We have therefore made an attempt to examine the occurrence and properties of Ca^{2+}-dependent protein kinase in leaf extracts of *Amaranthus hypochondriacus*.

Two (75 and 80 kD) proteins in *A. hypochondriacus* leaf extracts cross reacted strongly with anti-CCaMK (maize) antibodies (Plate 9.1), suggesting the occurrence of Ca^{2+}-dependent protein kinase in *A. hypochondriacus* leaf extracts. However, one of them (75 kD) showed also strong CaM binding activity, a character of CDPKs (Plate 9.5). Further, the direct assays of phosphorylation indicated that kinase activity' was dependent strongly on Ca^{2+}, but not on CaM (Fig. 9.3, Table 9.3). Among all the protein substrates tested, histone IIIS and syntide-2 were the best substrates for this kinase (Table 9.1). The phosphorylation was Ca^{2+}-dependent but CaM-independent (Figs. 9.2 and 9.3). The phosphorylation of histone IIIS was strongly inhibited by the CaM antagonists (calmidazolium, TFP and W7) and CaM kinase inhibitors (ML7 and staurosporine), whereas H7 had marginal effect on the phosphorylation of histone (Table 9.3). However, these inhibitors were required at much higher concentrations for inhibition of CDPK. These are all typical characteristics of the CDPK (a Ca^{2+}-dependent/CaM-independent protein kinase) reported in plants (Roberts and Harmon, 1992). A light-induced synthesis of CDPK-like protein (75 kD) in leaves of *A. hypochondriacus* appeared to occur (Plates 9.1. 9.2 and 9.4). This observations can explain partly the stimulation of PEPC activity on illumination in leaves of C4 plants, such as *A. hypochondriacus* and *Alternanthera pungens* (Plates 9.2 and 9.3).

PEPC was a poor substrate for CDPK in *A. hypochondriacus* leaf extracts (Tables 9.1 and 9.2). Very recently. Ogawa et al. (1998) also reported that PEPC was a poor substrate for maize CDPK. An obvious explanation is the involvement of CDPK at an upstream end of the regulation of PEPC-PK (Ca^{2+}-independent), but not direct involvement in the catalysis of PEPC phosphorylation.

There are three major contributions of the present work, which expand our current knowledge of the regulation of C4-PEPC: (i) The involvement of Ca^{2+} during the phosphorylation of PEPC promoted in illuminated leaves either at the upstream or downstream end of PEPC-PK, (ii) Occurrence and properties of a

CDPK in leaves *of A. hypochondriacus,* a C4 plant, and (iii) The phosphorylated form of PEPC is not only less sensitive to malate, but also more sensitive to HCO_3^-, compared to dephosphorylated form, indicating a marked interaction between phosphorylation of PEPC and HCO3-. The likely course of events during the light signal transduction chain in mesophyll cells of C4 plants, which leads to phosphorylation of PEPC and increase in enzyme activity is illustrated in Figure 10.1. The observations from the present work are integrated into the model. It is well known that cytosolic pH as well as free Ca^{2+} rises upon illumination (Pierre et al., 1992). Such elevated Ca^{2+} could promote the activity and/or synthesis of 75 kD protein (CDPK) which may phosphorylate PEPC-PK. The activated PEPC-PK subsequently stimulates phosphorylation of PEPC. Phosphorylated PEPC is more active, less sensitive to malate. and needs less bicarbonate than the dephosphorylated PEPC.

Further experiments are required to establish the sequence of components involved in the light-signal transduction chain, which finally results in PEPC phosphorylation.

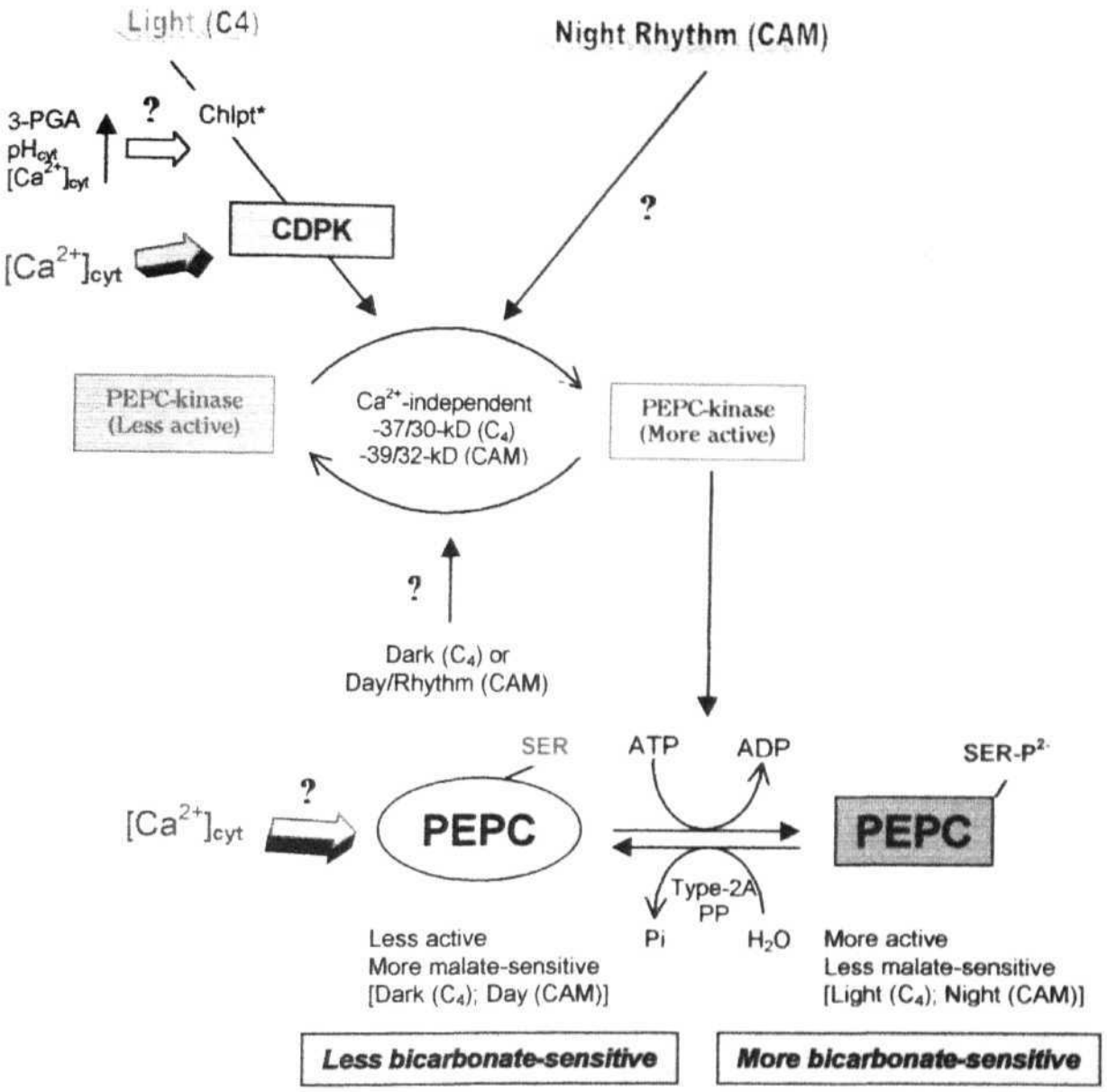


Figure 10.1. Schematic working model of the molecular mechanism of the light-activation of PEPC in the mesophyll cells by reversible phosphorylation of a single target serine near the N-terminus. The light-signal is transduced through a series of secondary messengers: pH_{cyt}, $[Ca^{2+}]_{cyt}$, CDPK and PEPC-PK. Chlpt*, illuminated Chloroplast; pH_{cyt} and $[Ca^{2+}]_{cyt}$, mesophyll cytosolic pH and [Ca2+], respectively; 3-PGA, 3-phosphoglycerate; CDPK, Ca^{2+}-dependent protein kinase; PP, protein phosphatase.

Chapter 11

Summary and Conclusions

Chapter 11

Summary and Conclusions

PEPC is ubiquitous in plants and localized in cytosol of mesophyll cells. PEPC is an important enzyme mediating the primary carbon assimilation (catalyzes the irreversible β-carboxylation of PHP to OAA and Pi) in C4 and CAM plants. The enzyme is allosteric and is highly regulated by light, temperature and metabolites (Andreo et al., 1987; Rajagopalan et al., 1994).

There has been remarkable progress in understanding certain aspects of 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 deduced amino acid sequences (Chollet et al., 1996: Vidal and Chollet, 1997). Yet there are several gaps in our knowledge of regulation of this key enzyme of C4 pathway: e.g. interaction of PEPC with bicarbonate, basis of Ca^{2+}-dependence of PEPC phosphorylation. The present investigation used the leaves of *Amaranthus hypochondriacus* (a NAD-ME type C4 plant) for studies on purification, characterization and regulation of PEPC.

Among the aspects studied were:

1. Protocols for purification of PEPC from the leaves of *Amaranthus hypochondriacus* by conventional method as well as immunoadsorbent chromatography.

2. The effect of compatible solutes on PEPC and interaction of these solutes with stability and oligomeric status of the enzyme.

3. The influence of bicarbonate on the catalytic and regulatory properties of PEPC.

4. The pattern and consequences of light activation of PEPC on the affinity of enzyme to bicarbonate.

5. The response of PEPC and the pattern of PEPC phosphorylation to calcium (by using $CaCl_2$, Ca^{2+}-chelators and activators/inhibitors of CDPK or CCaMK) in crude and desalted extracts.

6. A preliminary examination of the occurrence and properties of CDPK in leaf extracts *of Amaranthus hypochondriacus.*

The first set of our experiments were performed to purify PEPC from the leaves of *Amaranthus hypochondriacus* and to evolve the protocol for rapid purification of PEPC. PEPC has been purified to homogeneity from several photosynthetic and non-photosynthetic tissues (Rajagopalan et al.. 1994; Chollet et al., 1996). Nevertheless, a method of rapid purification along with long-term storage, is extremely useful for detailed studies of the enzyme. Our protocol of PEPC purification (involving 40-60% ammonium Sulfate fractionation, followed by chromatography on DEAE-Sepharose, phenyl-Sepharose and HAP columns) yielded an enzyme with specific activity of 55 U mg^{-1} protein. Thus, this preparation had one of the highest specific activities reported so far in the literature.

Polyclonal antibodies were raised in rabbits against purified PEPC from *A. hypochondriacus.* The monospecificity of anti-PEPC antiserum was confirmed *Vy* Ouchterlony double diffusion. Anti-PEPC antiserum could be used for Western blots, immunoprecipitation and ELISA. Anti-PEPC IgG were purified from the anti-PEPC antiserum. These IgG were employed to purify rapidly PEPC from *Amaranthus* leaves by using an immunoadsorbent column. This procedure was quite useful for the rapid purification of PEPC from leaves during light/dark transitions (Arrio-Dupont et al., 1992). An ELISA technique was standardized to detect and determine PEPC. ELISA can therefore be used as a diagnostic kit for the detection and quantification of PEPC-protein in plants.

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

The addition of PEG and glycerol during both extraction and assay stabilized PEPC and helped to maintain high activities of the enzyme. The inclusion of solutes in assay medium stimulated the activity of even purified pEPC. In the presence of PEG, the affinity for PEP (of PEPC in leaf extracts or purified PEPC) decreased. while the Kj (malate) increased suggesting that the regulation of PEPC is dampened without affecting catalytic characteristics of the enzyme. The extent of light activation of PEPC was marginally decreased in presence of PEG and glycerol during extraction and assay.

Gel filtration of purified PEPC on Scphadcx G-200 column showed the existence of three different forms: monomer. dimer and tetramer. The presence of glycerol and PEG during elution of PEPC from Sephadcx G-200 column, resulted in predominance of tetramers. The absence of these solutes during elution resulted in the formation of dimers and monomers. This suggests that the inclusion of PEG and glycerol helps to maintain quaternary structure of the enzyme. The present results suggest and recommend that PEG and glycerol be included during both extraction and assay so as to optimize the activity and stability of PEPC.

Compared to the extensive literature on the modulation of malate-sensitivity of PEPC, the studies on changes in kinetic characteristics of the enzyme and in particular the affinity to bicarbonate are quite limited. It has recently been reported

that bicarbonate plays an important role in the allosteric regulation of PEPC (Ogawa et al., 1997; Dong et al., 1997a). Such modulation of PEPC by one of the substrates, bicarbonate is extremely interesting and warrants further studies. Therefore, the interaction of kinetic characteristics of PEPC-enzyme with bicarbonate was studied.

Bicarbonate stimulated the activity of PL-PC extracted from dark- as well as light-adapted leaves of *Amaranthus hypochondriacus*. The stimulation by bicarbonate of PEPC activity was complex. The sensitivity of the enzyme to malate decreased with increasing concentration of bicarbonate. The stimulation by Glc-6-p was more at low concentration of HCO_3^- (0.05 mM) than that at high concentration (10 mM). The catalytic and regulatory properties of the enzyme were thus modulated markedly by bicarbonate.

Illumination significantly increased the affinity of PEPC to bicarbonate by at least two times, besides the 2 to 5-fold increase in V_{max} and 3 to 4-fold increase in K_i for malate. While light to dark ratio (L/D) decreased with increasing concentration of HCO_3^-. The inclusion of ethoxyzolamide, an inhibitor of carbonic anhydrase, during the assay had no effect on the modulation of kinetic and regulatory properties of the enzyme. On the other hand, CHX, an inhibitor of cytosolic protein synthesis suppressed the light enhanced decrease in K_m (HCO_3^-). *In vitro* phosphorylation of dark-form PEPC by protein kinase A decreased the K_m (HCO_3^-) of the enzyme, besides increasing Kj (malate). These results suggest that phosphoryl at ion of PEPC is important during the sensitization of PEPC to HCO3" by illumination in C4 leaves.

Light activation of photosynthetic enzymes is a cardinal event in regulation of photosynthesis in both C3 and C4 plants (Buchnan, 1980; Selinioti et al., 1986). As PEPC is a key enzyme involved in CO_2 fixation in C4 and CAM plants, the regulation of PEPC by light is a topic of interest. It is obvious that the light-signal

has to be perceived by the Chloroplast and the effect is transmitted to the cytosol, the site of PEPC in C4 mesophyll cells. Although, the kinetic and regulatory properties of PEPC are modulated by light/dark transitions *in vivo* there is very' limited information on the mechanism of transduction of light-signal.

C4 plants maintain soluble Ca^{2+} at low levels in their leaves and therefore appear to be calciophobes (Gavalas and Manctas. 1980a). It remains to be seen whether low soluble Ca^{2+} is a prerequisite for the smooth functioning of this biosynthetic route. The effects of Ca^{2+} on the activity of PEPC in crude and desalted extracts were therefore re-examined in detail.

Illumination of plant cells is known to rise the cytosolic pH and possibly the free Ca^{2+} (Pierre et al., 1992; Raghavendra et al., 1993). Therefore, the effect of Ca^{2+} on the activity of PEPC was assessed at sub-optimal (7.3) or optimal (7.8) pH. Ca^{2+} at a concentration of 100 μM or above inhibited PEPC activity (Gayathri and Raghavendra, 1994). The extent of inhibition was more at pH 7.8 than that at pH 7.3. Such inhibition was due to the competition with Mg^{2+}. On the other hand, low concentrations of Ca^{2+} stimulated the activity of PEPC. The extent of inhibition caused by EGTA was more in light than that of dark-form. This suggests that low concentration of calcium is beneficial for PEPC activity of particularly the light-form while high concentrations are inhibitory for both dark- and light-forms. These results further confirm that Ca^{2+} is an important component of regulatory mechanisms involved in the modulation of PEPC activity.

Phosphorylation/dephosphorylation cascade is one of the most important modes of post-translational modification of enzymes in both plants and animals. PEPC is one of the enzymes in the plant tissue, that undergoes regulatory phosphorylation and has become a model system to study the regulatory changes brought out by protein phosphorylation in plant cells. However, there is

considerable debate about the nature of regulation of PEPC-PK by calcium (Vidal and Chollet, 1997).

Phosphorylation of PEPC was stimulated by low concentrations of Ca^{2+} (10 μM), whereas inhibited by Ca^{2+} chclators EGTA or BAPTA. This confirms that low level of Ca^{2+} is optimal for PEPC phosphorylation. CaM stimulated the extent of phosphorylation, while diacylglyccrol and phosphatidylserine had marginal effect. Phosphorylation of PEPC was strongly inhibited by protein kinase C inhibitor (H7), CaM antagonists (W7 and TFP), CaMK inhibitor (staurosporine), and MLCK inhibitor (ML7). These results indicate that phosphorylation of PEPC occurs in a Ca^{2+}-CaM-dependent manner. As per the literature PEPC-PK itself is calcium-independent (Chollet et al., 1996; Vidal and Chollet. 1997; Nhiri et al., 1998). We therefore suggest that the Ca^{2+}-CaM dependence may be at an upstream level, for e.g. another kinase or a regulatory protein modulating the PEPC-PK activity in a Ca^{2+}-dependent manner.

An attempt was made to characterize the Ca^{2+}- or CaM-independent protein kinase (CDPK) in leaf extracts of *A. hypochondriacus.* Anti-CCaMK(maize)-antibodies cross reacted with two proteins (75 and 80 kD) in leaf extracts of *A. hypochondriacus.* One of them (75 kD) appears to be a CDPK, since it also binds to CaM as indicated by immunoblot analysis. The synthesis of 75 kD increased upon illumination particularly in C4 leaves. The phosphorylation of substrates histone IIIS as well as syntide-2 suggested the presence of an active CDPK in *A. hypochondriacus* leaf extracts. Further, the phosphorylation of histone HIS was dependent on the availability of Ca^{2+}, but not on CaM and was suppressed significantly by a wide range of inhibitors related to Ca^{2+}- and CaM-dependent protein kinases, but at a high concentration. These results suggest the presence of CDPK rather than CCaMK in *A. hypochondriacus* leaf extracts. As PEPC was a poor substrate for CDPK. we feel that this CDPK may be involved in phosphorylating PEPC-PK and leads to the upstream regulation of PEPC

phosphorylation by Ca^{2+}. Further experiments are needed to reveal the complete chain of the components involved in the light-induced phosphorylation of PEPC.

Major conclusions/outcome from the present study are:

1. PEPC was purified from leaves *of Amaranthus hypochondriacus* with a specific activity of 55 U mg^{-1} protein. This is one of the highest specific activities of PEPC reported in the literature.

2. Polyclonal antibodies were raised in rabbits against purified PEPC and were used for Western blots, immunoprecipitation and ELISA.

3. Immunoaffinity chromatography for the rapid isolation of highly active PEPC was standardized.

4. The addition of PEG and glycerol during both extraction and assay helped to stabilize the enzyme by promoting its tetrameric state.

5. The marked modulation of regulatory and kinetic properties of PEPC by bicarbonate reveals that bicarbonate is not only a substrate, but also an important regulator of PEPC.

6. Illumination enhanced the affinity of PEPC to bicarbonate by at least two times, besides the 2 to 5-fold increase in V_{max} and 3 to 4-fold increase in K_i for malate. *In vitro* phosphorylation of dark-form PEPC by protein kinase A decreased the K_m (HCO_3^-) of the enzyme, besides increasing Kj (malate). Thus, phosphorylation of PEPC is important during the sensitization of enzyme to HCO_3^- by illumination in leaves. A marked interaction between light and bicarbonate appears to be an important component in the regulation of C_4 PEPC.

7. Ca^{2+} inhibited PEPC at higher concentration (>50 μM) and stimulated at low concentrations (≤20 μM). The inhibitory effect of Ca^{2+} was mainly due to **the** competition with Mg^{2+}.

8. *In vitro* phosphorylation of PEPC was stimulated by Ca^{2+} and inhibited by EGTA or BAPTA. Various inhibitors of Ca^{2+}-CaM-dependent protein kinases suppressed PEPC phosphorylation. Although phosphorylation of PEPC seems to be Ca^{2+}-CaM-dependent, such modulation by Ca^{2+}/CaM may be of a protein-kinase or a regulatory protein.

9. Two proteins of 75 and 80 kD in leaf extracts of *A. hypochondriacus* cross reacted with anti-CCaMK antibodies raised against purified CCaMK from maize etiolated coleoptiles. One of them (75 kD) was a CaM-binding protein.

10. Phosphorylation of histone HIS in a Ca^{2+}-dependent, but CaM-indcpcndcnt manner. Phosphorylation of histone IIIS was inhibited by a wide range of inhibitors related to both Ca^{2+}- and CaM-dependent protein kinases. Finally, PEPC was a poor substrate for the kinase activity. We conclude that the *A, hypochondriacus* leaf extracts contain an active CDPK, and suggest that this may be involved in an upstream regulation of PEPC-PK.

These results are discussed in relation to the relevant literature. Some of the results are published or in press (Parvathi et al., 1998a, b; Raghavendra et al., 1998).

Chapter 12

Literature Cited

Chapter 12

Literature Cited

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Appendix

List of Publications in Refereed Science Journals/ Proceedings of Photosynthesis Congress:

1. **K. Parvathi** and A.S. Raghavendra (1995) Bioenergetic processes in guard cells related to stomatal function. *Physiol Plant* **93:** 146-154

2. **K. Parvathi** and A.S. Raghavendra (1997) Both rubisco and phospho*enol* pyruvate Carboxylase are beneficial for stomatal function in epidermal strips *of Commelina benghalensis. Plant Sci* **124:** 153-157

3. **K. Parvathi** and A.S. Raghavendra (1997) Blue light-promoted stomatal opening in abaxial epidermis of *Commelina bcnghalensis* is **maximal** at low calcium. *Physiol Plant* **101:** 861-864

4. **K. Parvathi,** A.S. Bhagwat and A.S. Raghavendra (1998) Enhanced affinity of C4 PEP Carboxylase to bicarbonate on illumination in *Amaranthus hypochondriacus* leaves. *In* Proceedings of XIth International Photosynthesis Congress. G. Garab, ed, Kluwer Academic Publishers, The Netherlands (in press)

5. A.S. Raghavendra. **K. Parvathi** and J. Gayathri (1998) Modulation by calcium of PEP Carboxylase and PEPC-protein kinase from leaves of *Amaranthus hypochondriacus*, an NAD-ME type C4 plant. *In* Proceedings of XIth International Photosynthesis Congress. G. Garab, ed, Kluwcr Academic Publishers, The Netherlands (in press)

6. **K. Parvathi,** A.S. Bhagwat and A.S. Raghavendra (1998) Modulation by bicarbonate of catalytic and regulatory properties of C4 phospho*enol*pyruvate Carboxylase from *Amaranthus hypochondriacus:* Desensitization to malate and glucose 6-phosphate and sensitization to Mg^{2+}. *Plant Cell Physiol* **39:**(in press) 1294 - 1298

First pages of articles are attached.

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Modulation by Bicarbonate of Catalytic and Regulatory Properties of C_4 Phospho*enol*pyruvate Carboxylase from *Amaranthus hypochondriacus*: Desensitization to Malate and Glucose 6-Phosphate and Sensitization to Mg^{2+}

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The catalytic and regulatory properties of phospho*enol*pyruvate (PEP) Carboxylase (PEPC) are modulated remarkably by the increase in the level of bicarbonate in the assay medium. The activity of PEPC increased by two-fold as the concentration of bicarbonate was raised from 0.05 to 10 mM. During this state, there was only marginal effect on K_m for PEP, while the affinity of PEPC to Mg^{2+} increased by >2 fold. In contrast, the sensitivity of PEPC to malate decreased with increasing concentration of HCO_3^-. Similarly, the stimulation by glucose 6-phosphate (G-6-P) at optimal concentration (10 mM) of HCO_3^- was much less than that at suboptimal concentration (0.0S mM). A', for malate increased by about 3 fold and A, for G-6-P rised by four-fold as bicarbonate concentration was rised from 0.05 to 10 mM. These results suggest that HCO_3^- desensitizes PEPC to both malate and G-6-P. Further, these changes were manifested in both dark- as well as light-forms of (he enzyme. Similar results were obtained with PEPC in leaf extracts or in purified form. We therefore propose that bicarbonate-induced changes are independent of phosphorylation and possibly through a significant change in the conformation of the enzyme. This is the firs t detailed report indicating marked modulation of regulatory and catalytic properties of PEPC by bicarbonate, one of its substrate.

Key words: *Amaranthus hypochondriacus* — Bicarbonate — Malate sensitivity — Phospho*enol*pyruvate Carboxylase — Regulatory properties.

Phospho*enol*pyruvate Carboxylase (PEPC), a key enzyme mediating the primary carbon assimilation, is highly regulated in leaves of C_4 plants. The activity of PEPC in C_4 leaves is enhanced on illumination. The enzyme is also markedly modulated by metabolites, for e.g., PEPC is activated by glucose 6-phosphate (G-6-P) and feed-back inhibited by L-malate (Andreo et al. 1987, Rajagopalan et a). 1994, Chollet et al. 1996, Vidal and Chollet 1997).

The sensitivity of PEPC to malate is influenced by various factors like light, pH and G-6-P. When leaves are illuminated, there is a marked decrease in sensitivity of PEPC to malate besides an increase in activity (Rajagopalan et al. 1993). Malate inhibition is competitive at pH 7.0, and non-competitive at pH 8.0 (Gonzalez et al. 1984). G-6-P is an allosteric activator of PEPC, decreases K_m for PEP (Uedan and Sugiyama 1976) and protects the enzyme from malate inhibition (Gupta et al. 1994).

Compared to the extensive literature on the modulation of malate-sensitivity of PEPC, the studies on changes in kinetic characteristics of the enzyme, and in particular the affinity to HCO_3^- are quite limited (O'Leary 1982, Chollet et al. 1996). This may be mainly due to the difficulty in the complete removal of dissolved bicarbonate during PEPC assays. There is also a lot of discrepancy for the K_m value for HCO_3^-, which ranges from 0.02 to 0.1 mM in C_4 plants (at a pH range of 7 to 8) (Uedan and Sugiyama 1976, Bauwe 1986, Gao and Woo 1995, 1996, Dong et al. 1997). Ogawa et al. (1997) and Dong et al. (1997) have recently been reported that bicarbonate plays a major role in the allosteric regulation of PEPC. Such, modulation of PEPC by one of the substrates, HCO_3^- is extremely interesting.

Most of the studies on PEPC have been made using 5 or 10 mM HCO_3^- (Doncaster and Leegood 1987, Ausenhus and O'Leary 1992, Bakrim et al. 1993, Rajagopalan et al. 1993, Gupta et al. 1994, Podesta et al. 1995, Gao and Woo 1996, Svensson et al. 1997) in the assay medium, while the level of bicarbonate in the cytosol of C_4 mesophyll cells (the site of PEPC) is expected to be 80 μM (Jenkins et al. 1989). We have therefore examined the kinetic and regulatory properties of PEPC at two concentrations of HCO_3^-: low or limiting (0.05 mM) bicarbonate and high or saturating (10 mM) bicarbonate.

Materials and Methods

Plant material—Plants of *Amaranthus hypochondriacus* L. (cultivar AG-67) were raised from seeds. The plants were grown outdoor under a natural photoperiod of approximately 12 h and temperature of 30–40°C day/25–30°C night. Leaves were harvest-

Abbreviations: MDH, NAD-malate dehydrogenise, PEP, phospho*enol*pyruvate; PEPC, phospho*enol*pyruvate Carboxylase; G-6-P, glucose 6-phosphate.

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MODULATION BY CALCIUM OF PEP CARBOXYLASE AND PEPC-PROTEIN KINASE FROM LEAVES OF *AMARANTHUS HYPOCHONDRIACUS*, AN NAD-ME TYPE C_4 PLANT

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Key words: Calmodulin. CO_2 uptake, inhibitors, light activation, phosphorylation/ dephosphorylation, regulatory mechanism

1. Introduction

Phospho*enol*pyruvate Carboxylase (PEPC; EC 4.1.1.31) is a principal enzyme in primary CO_2 fixation in C_4 plants. PEPC undergoes reversible phosphorylation by PEPC-protein kinase (PEPC-PK) in light. The phosphorylated form is more active and less sensitive to L-malate than the dephosphorylated form (1,2,3). The involvement of Ca^{2+} during the PEPC phosphorylation is a matter of debate. Two types of PEPC-PKs: Ca^{2+}-dependent and Ca^{2+}-independent are reported to occur in leaf extracts of C_4-species (1,2,3). Duff et al. (4) and Giglioli-Guivarc'h et al. (5) suggested that Ca^{2+} was involved in the *in situ* phosphorylation of PEPC in *Digitaria sanguinalis*, but an upstream level of regulation. Multiple forms of protein kinases could be involved in the regulation of PEPC phosphorylation (5). The present article is an attempt to re-evaluate the role of Ca^{2}* on PEPC-PK activity.

2. Materials and Methods

Plants of *Amaranthus hypochondriacus* L. (cultivar AG-67) were grown in field (approximate photoperiod of 12 h and temperature of 30-40 ^{0}C day/25-30 ^{0}C night). Leaves were harvested from 3 to 4 week-old plants. PEPC was purified from dark-adapted leaves to homogeneity by a modified procedure of Iglesias et al. (6).

Leaf discs were illuminated at 1000 $\mu E\ m^{-2}\ min^{-1}$ for 30 min and extracted in chilled mortar and pestle with extraction medium containing 100 mM HEPES-KOH. pH 7.3, 10 mM $MgCl_2$, 2 mM K_2HPO_4, 1 mM EDTA, 10% (v/v) glycerol, 10 mM β-mercaptoethanol and 2 mM PMSF. The homogenate was centrifuged at 7000 g for 5 min. The supernatant was used as a PEPC-PK source, whereas purified PEPC from dark-adapted leaves was used as the substrate for PEPC-PK. The activity of PEPC

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ENHANCED AFFINITY OF C_4 PEP CARBOXYLASE TO BICARBONATE ON ILLUMINATION IN *AMARANTHUS HYPOCHONDRIACUS* LEAVES

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Key words: Carbonic anhydrase, light activation, malate sensitivity, pH, phosphorylation/dephosphorylation, protein synthesis

1. Introduction

Phospho*enol*pyruvate Carboxylase (PEPC, EC 4.1.1.31) mediates the primary carbon assimilation in C_4 and CAM plants (1). The activity of PEPC is highly regulated by light. Illuminated leaves exhibit two- to three- fold higher activities than that of dark-adapted ones. Besides an increase in the activity, there is a marked decrease in the sensitivity to L-malate in the light-form of PEPC (2), mainly due to the light-induced phosphorylation of the enzyme. Most of the attention was focused on the effect of external factors such as pH or temperature on regulatory properties of PEPC, i.e., the sensitivity of the enzyme to either malate or Glc-6-P (3,4). We report for the first time that the affinity of PEPC to HCO_3^- in C_4 leaves markedly increases upon illumination.

2. Materials and Methods

2.1. Extraction, assay and light activation

Plants of *Amaranthus hypochondriacus* L. (cultivar AG-67) were raised from seeds and grown in field (approximate photoperiod of 12 h and temperature of 30-40 °C day/25-30 ^{0}C night). Leaves were harvested from three to four-week old plants.

Light activation of PEPC in leaf discs by illumination at 1000 µmol m^{-2} s^{-1} for 30 min as well as feeding with cycloheximide (CHX) were carried out, as described earlier (5).

Leaf discs were extracted with a medium containing 100 mM HEPES-KOH, pH 7.3, 10 mM $MgCl_2$, 2 mM K_2HPO_4, 1 mM EDTA, 10% (v/v) glycerol, 10 mM β-mercaptoethanol, 2 mM PMSF and 10 µg/ml leupeptin and 100 jig/ml chymostatin were also present. The homogenate was cleared by centrifugation and the supernatant was used for desalting on a Sephadex G-25 column (1 x 3 cm).

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Blue light-promoted stomatal opening in abaxial epidermis of *Commelina benghaiensis* is maximal at low calcium

K. Parvathi and A. S. Raghavendra

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The requirement for calcium in blue light-promoted stomatal opening, in comparison with that in red light, was studied in epidermal strips of *Commelina benghaiensis* L. Blue light promoted stomatal opening in the presence of low level of calcium, whereas in red light opening was relatively tolerant to calcium. Stomatal opening under blue light was restricted by external calcium (above 5 μM) or abscisic acid. When present in the incubation medium, EGTA increased the extent of stomatal opening under blue light. Verapamil (a calcium-channel blocker) and trifluoperazine (TIT, a calmodulin antagonist) reduced the stimulation of stomatal opening by blue light. Lanthanum, an external calcium-channel antagonist, had no significant effect on stomatal opening under either blur or red light. These observations indicate that blue light-promoted stomatal opening preferentially occurs at low levels of calcium, and modulation by calmodulin is strongly suggested. We conclude that a fine-tuning of the calcium level within guard cells is essential during the transduction of the blue light signal.

Key words - Blue light, calcium, *Commelina benghaiensis*, guard cells, red light, signal transduction, stomata.

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Introduction

Stomatal guard cells are used as a model system to study signal transduction in plants because, within a few minutes, these cells respond to hormones or light (Parvathi and Raghavendra 1995). Blue light plays a major role in plants, but the mechanism by which it is perceived and transduced is not clearly understood (Short and Briggs 1994). Several secondary messengers are possibly involved in the transduction of blue light by different plant tissues, including guard cells (Assmann 1993, Kaufman 1993). Recent reports suggest the involvement of carotenoids, particularly zeaxanthin, in blue light perception by guard cells (Srivastava and Zeiger 1995, Quinones et al. 1996, Tallman et al. 1997) and corn coleoptiles (Quinones and Zeiger 1994).

Calcium acts as a secondary messenger in stomatal function as well as in several other developmental processes in plants (Trewavas and Gilroy 1991, Bush 1995, Chasan 1995). Under experimental conditions, stomatal closure occurs due to the elevated levels of cytosolic Ca^{2+} caused by photolysis of microinjected caged Ca'' (Trewavas and Gilroy 1991) or the presence of abscisic acid (ABA) (Trewavas and Gilroy 1991) or increases in external Ca^{7+} (McAinsh et al. 1995).

The literature on the interaction between Ca^{3+} and light effects on stomata is intriguing. Blue light-promoted stomatal opening has been found to be calcium dependent possibly involving calcium- and calmodulin (CaM)-dependent protein kinases of the myosin light-chain kinase (MLCK) type (Shimazaki et al. 1992, 1993). On the other hand, the presence of calcium causes

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Both rubisco and Phosphoenolpyruvate Carboxylase are beneficial for stomatal function in epidermal strips of *Commelina benghalensis*

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Abstract

The relative importance of the Calvin cycle and β-carboxylation, through Phosphoenolpyruvate carboxylase (PEPC), pathways was assessed in relation to stomatal opening in epidennal strips of *Commelina benghalensis*, using D,L-glyceraldehyde (an inhibitor of the Calvin cycle) and 3,3-dichloro-2-dihydroxyphosphinoyl-methyl-2-propenoate (DCDP, an inhibitor of PEPC) or metabolites related to the Calvin cycle or PEPC. Stomatal opening was markedly inhibited by both DCDP and D,L-glyceraldehyde. In the absence of these inhibitors, exogenous metabolites such a* PEP or ribose-5-P (R-5-P) had little or no effect on stomatal opening. On the other hand, in the presence of DCDP or D,L-glyceraldehyde, metabolites related to not only PEPC (PEP or malate) but also the Calvin cycle (R-5-P or PGA) relieved the inhibition of stomatal opening. We suggest that both Calvin cycle and β-carboxylation activity are beneficial for stomatal opening, particularly when cither of these pathways is restricted. © 1997 Elsevier Science Ireland Ltd.

Keywords: **Guard cells; Rubisco; *Commelina benghalensis*, PEP Carboxylase, Slomata**

1. Introduction

The mode of carbon fixation by guard cells has been a topic of intense debate, despite the presence of conspicuous chloroplasts [1,2]. The rates of photosynthetic carbon assimilation by guard cells are usually less than those of mesophyll cells. The carbon assimilation in guard cells appears to occur predominantly, if not exclusively, through

Abbreviations: **DCDP,** 3,3-dichloro-2-dihydroxyphosphinoyl-methyl-2-propenoate; **PEP, Phosphoenolpyruvate; PEPC, Phosphoenolpyruvate Carboxylase; PGA.** 3-phosphoglyceric **acid; R-5-P,** ribose-5-phosphate; **Rubisco, nbulose** 1,5-bisphosphate carboxylase/oxygenase.

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Minireview

Bioenergetic processes in guard cells related to stomatal function

K. Parvathi and A. S. Raghavendra

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The energy required for ion uptake in guard cells is provided by two important bioenergetic processes, namely respiration and photosynthesis. The blue light-sensitive plasma membrane redox system is considered as the third bioenergetic phenomenon, since it uses blue light to create a proton gradient across the membrane. The unique features of respiration and photosynthesis in guard cells and then role in stomatal function are emphasized. Evidence for and against the blue light-sensitive components on plasma membrane (ATPase/distinct redox chain) and the photoreceptors (flavins, carotenoids, ptenns) in guard cells are presented. The information on ion channels and their response to various kinds of secondary messengers including G-proteins, phov phoinositides, diacylglycerol, calcium, cAMP and protein kinases are reviewed. A model is presented indicating the possible mechanism of perception and transduction by guard cells of external signals and their interaction with different bioenergetic components.

Key words - **Guard cells, ion channels, photosynthesis, plasma membrane ATPase, proton pump, redox system, respiration, secondary messenger*, signal transduction.**

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Introduction

Stomata open when the guard cell (GC) turgor increases as a result of cation (particularly K^+) uptake, malate synthesis, and formation of soluble sugar. The energy required for proton efflux, the earliest event during stomatal opening, is derived principally from two important metabolic pathways: respiration and photosynthesis. A third process of blue light-sensitive redox system generates a proton gradient sufficient to mediate signal transduction. Each or all of these three bioenergetic phenomena would be essential for stomatal opening, depending on the GC microenvironment.

This review focuses on the unique features of respiration and photosynthesis in guard cells, distinct from those of mesophyll cells (MC), and the effects of blue light on the former. Ion-channels form an important link between cellular energy and ion-uptake. The recent literature on ion-channels in guard cells is also reviewed with emphasis on secondary messengers and the mechanism of signal transduction.

As this article describes recent literature, readers interested in details of stomatal function and guard cell metabolism may consult recent reviews (Tallman 1992, Assmann 1993, Kaufman 1993, Reams and Assmann 1993). Due to limitation of space, reference is often made to a review instead of the original article.

Abbreviations - CaM, calmodulin; DG, 1,2-diacylglycerol; DHAP, dihydroxyacetonephosphate; **FC, fusicoccin, GAP. glyceraldehyde-3-phosphate, *GC.* guard cells. GCC, guard cell chloroplasts. GCP, guard cell protoplasts, IP_3,** inositol 1,4,5-triphosphate; **LHCP, light harvesting chlorophyll pigment-protein; MC. mesophyll cells, MCC, mesophyll cell chloroplasts; MCP. mesophyll cell protoplasts, MLCK, myosin light chain kinase,** PEPC, phospho*enol*pyruvate carboxylase; **PGA. 3-phosphoglycerate; PKC, protein kinase C,** Rubisco, **ribulose 1,5-bisphosphate carboxylase/oxygenase.**

Respiration

Respiration is the major source of energy for stomatal movement. Guard cell respiration is essential for not only stomatal opening but also stomatal closure as indicated by the arrest of closure under anoxia and prevention of

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