Regulation by Temperature of Phosphoenolpyruvate carboxyiase in *Amaranthus hypochondriacus*, a NAD-ME type C_4 plant

Thesis submitted to the University of Hyderabad

for the Degree of

DOCTOR OF PHILOSOPHY

By

Chinthapalli Bhaskar Rao

Supervisor

Professor A.S. Raghavendra *FNA, FASC FNASC, FAAS*

Department of Plant Sciences
School of Life Sciences
University of Hyderabad
Hyderabad 500 046, INDIA

September 2003

Enrol No. 97LPPH04

DEPARTMENT OF PLANT SCIENCES
SCHOOL OF LIFE SCIENCES
UNIVERSITY OF HYDERABAD
HYDERABAD-500 046
INDIA

DECLARATION

I hereby declare that the work presented in this thesis entitled **"Regulation by Temperature of Phosphoenolpyruvate carboxylase in *Amaranthus hypochondriacus*, a NAD-ME type C4 plant"** has been carried out by me under the supervision of Professor A. S. Raghavendra in the Department of Plant Sciences, School of Life Sciences, University of Hyderabad and this work has not been submitted for any degree or diploma of any other University or Institute.

Chinthapalli Bhaskar Rao (Candidate)
Enrol. No. 97LPPH04

Professor A.S. Raghavendra
FNA, FASc, FNASC, FAAS
(Supervisor)

DEPARTMENT OF PLANT SCIENCES
SCHOOL OF LIFE SCIENCES
UNIVERSITY OF HYDERABAD
HYDERABAD-500 046
INDIA

CERTIFICATE

This is to certify that **Mr. Chinthapalli Bhaskar Rao** has carried out the research work embodied in the present thesis entitled **"Regulation by Temperature of Phosphoenolpyruvate carboxylase in *Amaranthus hypochondriacus*, a NAD-ME type C4 plant"** for the degree of Doctor of Philosophy under my supervision in the Department of Plant Sciences. School of Life Sciences, University of Hyderabad.

Supervisor

Prof. A.S. RAGHAVENDRA
Dept. of Plant Sciences
School of Life Sciences
University of Hyderabad
HYDERABAD-500 046, INDIA

Head
Department of Plant Sciences

Head
Dept. of Plant Sciences
School of Life Sciences
University of Hyderabad
Hyderabad-500 134, (India)

25/9/03

Dean
School of Life Sciences

Dean, School of Life Sciences
University of Hyderabad,
Hyderabad-500 134, (India)

To

My Parents

ACKNOWLEDGEMENTS

I wish to express my deep felt gratitude and appreciation to my supervisor Professor A.S. **Raghavendra** *FNA, FASC, FNASC, FAAS* for his encouragement, keen **interest** in this **work,** constant help and support. He has been more than a guide **and** I am privileged to have associated myself with him all these years.

My sincere thanks to Prof. T. Suryanarayana, Dean, School of Life Sciences, Prof. M.N.V. Prasad, Head. Department of Plant Sciences, **Prof. A.R. Reddy** and **Prof.** R.P. Sharma, former Deans and Prof. A.S. Raghavendra, former Head, for providing necessary facilities for the research.

I wish to thank Prof. Hans Walter **Heldt** (University of Gottingen, Germany), Prof. Carlos S Andrco (Universidad Nacional de Rosario, Argentina), Dr. Alain Vavasseur (Commissariat a I'F.nergie Atomique, France) for their helpful discussions and suggestions during their visit to our lab.

My sincere thanks arc due to Dr. T.P. Radhakrishnan, Dr. A. **Samantha** (School of Chemistry) for allowing me to use the **Spectrofluorimeter.** 1 extend my sincere to Dr. Abbani K. **Bhuyan** for his valuable discussions and suggestions during the **Fluorescence** and CD spectral data. I also thank Mr. **Raghunath** Reddy of the same department for his help in doing Spectrofluorimeter experiments.

I owe my thanks to Dr K.V. Reddy, Principal Scientific Officer, CIL, for his kind help to use central facilities during my experiments. I also thank Mr. Suresh Kumar for his help in doing experiments with circular dichroism **spectropolarimeter.**

This thesis had been the dream of my father (Late) Sri Ch. **Appanna,** who was my major source of inspiration and encouragement. Today even in his absence his blessings and words have impelled me to pursue my work despite several hurdles. To my mother **Smt Ch. Bullamma,** I have no words to express my gratitude to make me what I am today.

My heartfelt gratitude to 'My **friend'** Mr. **Jhadeswar Murmu** who has not only been my friend, but also a fountain head of all the best that has occurred to me in this campus. Many a times when I have yielded to the stress of the circumstances, he has taken **every** pain to patiently transform all my shattered hopes carefully into a perfect mould. He has always contributed his best to the success of all my present endeavors.

My heartfelt gratitude to Mr. R. **Samual** (Childhood friend) for his never failing friendship who was always there when 1 needed help.

I fondly **thank** Dr. Vijaya Chitra with whom 1 had long interactions and who extended complete cooperation to help me during all phases of my Ph.D work.

I would like to acknowledge the help provided by my senior colleagues Dr. K. **Parvathi** (Massachusetts Institute of Technology, USA) and **Dr. K.P.M.S.V. Padmasree** (Assistant Professor, University of Hyderabad). I also thank my junior lab mates Ms. L. **Padmavathi, Mr.** Jhadeswar **Murmu,** Ms. **D. Suhita, Mr. Venkat Apparao Kolla,** Ms. **K. Riazunnisa, Mr. D. Sudhakara** Rao for the good times **we** spent together in the lab. I am also thankful to Mr. Narasing **Rao** and **Mr.** Venu for their assistance in the lab and also in monitoring the plants in the field that was regularly needed for my Ph.D. work.

Friends are always a source of inspiration and they have made me vigilant through their discussions. Mr. Syed **Gulam** Hussain and Mr. **Perem Ravindra Babu** are few to mention in this regard. Dr. B. **Laxminarayana (NIH,** USA) and Mr. B. Chandrasekhar (Genetec, India) have always provided their companion ship and emotional support.

My sincere mentions are due to my friends of yesteryears who refreshed me with their wit and words. Besides, there are many friends, some their presence and some otherwise, who kept me fresh with fun and frolic through all these years.

My brother and sister-in-law, who have always extended unwavering support and encouragement in my academic career. My sisters and brothers-in-law who immensely helped in accomplishing this endeavor. My second **brother** deserves my sincere thanks for being with me always. 1 thank the recreating company of little ones, Satish Kumar, Sirisha, Srikanth, Prasuna, Anjali and Appaji who made life simple and enjoyable at home.

Heartfelt thanks to all the members of Sri S. Adinaraya (my uncle) and **Smt. Leela** Shantha **kumari** (My Aunt) for making me feel at home, welcoming me at all times and treating me as one of their family members. I am very much thankful to Mr. S. Ravi and Mrs. **Malathi** (Advocates. High Coun). Mr. S. Giri and Mr. S. **Hari,** for their practical words that always boosted the morale in me during frustrating situations.

1 feel fortunatc to have a very good hostel wingmates like Mr. Perem Ravindra Babu, Mr. B. Chandrasekhar, Dr. B. Laxminarayana, Mr. Troy **Baily,** Mr. Sridhar who always made me feel at homc.

The financial assistance from **Volkswagen-Stiftüng Research** Project (Germany) and Department of Biotechnology Research Project (New Delhi) is gratefully acknowledged.

Chinthapalli Bhaskar Rao

ABBREVIATIONS

BCIP	=	5-bromo-4-chloro-3-indolyl phosphate
CAM	=	crassulacean acid metabolism
CD	=	circular dichroism
CDPK	=	calmodulinlike-domain protein kinase or Ca^{2+}-dependent protein kinase
FBPase	=	fructose 1,6-phosphate
FPLC	=	fast-protein liquid chromatography
Glc-6-P	=	glucosc-6-phosphate
HAP	=	hydroxylapatite
MDH	=	NAD malic dehydrogenase
NBT	=	nitro blue tetrazolium
OAA	=	oxaloacetate
PEP	=	phosphoenolpyruvate
PEPC	=	PEP carboxylase
PEPC-PK	=	PEPC-protein kinase
PMSF	=	phenylmethylsulphonyl fluoride
PPDK	=	pyruvate Pi dikinase
PEG	=	polyethylene glycol

All the remaining abbreviations are all standard ones, and as per Plant Physiology issue, 2003, Instructions for contributors, website:http://www.aspb.org

Contents

Chapter 1

Introduction and Review of Literature

Chapter 1

Introduction and Review of Literature

Phospho*enol*pyruvate carboxylase (PEPC)

Phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) is a very important enzyme, involved in the primary carbon fixation in leaves of C_4 and CAM plants (Andreo et al., 1987). The enzyme plays an important role also in leaves of C_3 plants, and tissues other than leaves (e.g. root nodules, fruit pods, seeds and cotton fibers), since PEPC is responsible for channeling of carbon into keto-acids required for amino acid synthesis and ensuing protein synthesis.

Stupendous progress has been made in our knowledge of biochemistry and molecular biology of PEPC in not only C_4 plants, but also C_3 species and legume root nodules. Several authors have periodically reviewed the literature on the properties, regulation and functions of C_4 PEPC particularly in the past decade. The properties and regulation of PEPC are summarized in several recent reviews (Lepiniec et al., 1994; Rajagopalan et al., 1994; Toh et al., 1994; Chollet et al., 1996; Vidal and Chollet. 1997; Nimmo, 2000; Matsuoka et al., 2001; Chinthapalli et al., 2002; Vidal et al., 2002; Kai et al., 2003; Svensson et al., 2003). The earlier literature can be found in two excellent reviews (O'Leary, 1982; Andreo et al., 1987).

Occurrence

PEPC is ubiquitous and is distributed widely in photosynthetic and non-photosynthetic tissues of higher plants, green algae, bacteria and legume root

nodules and is believed to be absent in animal tissues, yeast or fungi (Andreo et al., 1987; Vance and Gantt, 1992; Lepiniec et al., 1994; Kai et al., 2003). The activities of PEPC levels in leaves of C_3 plants are about 2 to 5% of that found in C_4 plants (Edwards and Walker, 1983; Latzko and Kelly, 1983).

PEPC constitutes about 15% of total soluble protein in maize (Hague and Sims, 1980). The enzyme is confined to the cytoplasm of mesophyll cells in C_4 and CAM plants. In C_3 plants, PEPC may be localized in both cytosol and chloroplasts of the leaves (Perrot-Rechenmann et al., 1982, Latzko and Kelly. 1983). Thus, PEPC is considered as a typical marker enzyme for cytosol and for C_4 mesophyll cells.

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

Purification

Since the partial purification for the first time from spinach leaves (Bandurski and Greiner, 1953), PEPC has been purified from a wide variety of sources: cotton, *Pennisetum*, *Sorghum* and maize (all leaves), lupin and soybean

root nodules, maize root tips, *Vicia faba* guard cells and epidermis of *Commelina communis* (O'Leary, 1982; Rajagopalan et al., 1994). Among the recent reports are the purification of PEPC from *Amaranthus hypochondriacus* (Gayathri et al., 2000), *Brassica napus* (Moraes and Plaxton. 2000), *Selenastrum minutum* (Rivoal et al., 2002) and castor oilseed (Blonde and Plaxton, 2003).

The native structure and recombinant forms of PEPC are highly susceptible to limited proteolysis and as a result their N-terminus is frequently lost during extraction and purification (Chollet et al., 1996). The integrity of the enzyme can be maintained during isolation of PEPC by the inclusion of glycerol, L-malate and protease inhibitors (especially chymostatin) and by the use of rapid purification protocols using FPLC, HPLC or immunoaffinity chromatography (Wang and Chollet, 1993; Duff et al., 1995; Zhang et al.. 1995). With proper precautions and suitable protocols of rapid purification, the preparation of PEPC with an intact N-terminal region is possible from leaves (C4, CAM and C_3) and root nodules.

The cloning and expression of recombinant PEPC in *E. coli* has been successfully employed with the enzyme from *Sorghum* (Cretin et al., 1991) or maize (Yanagisawa and Izui, 1990) or *Flaveria* (Westhoff et al., 1997). The recombinant DNA technology made it possible to produce large amounts of C4-type or C_3-type PEPC in *E. coli* (Pacquit et al., 1993; Svensson et al.. 1997; Biasing et al., 2000).

PEPC Isoforms and Molecular Evolution

In higher plants, four types of PEPC isoforms have been reported so far, namely, C_4-, C3-, CAM- and dark/non-autotrophic forms. Chromatographic, immunological and kinetic properties of PEPC can be used to distinguish these isoforms (O'Leary, 1982; Andreo et al., 1987; Rajagopalan et al., 1994).

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

There is a lot of variation in the number of PEPC isoforms reported from the leaves of C_3 plants and CAM species. Four major isoforms of PEPC were reported in leaves of a C_3 plant *Flaveria conquistii*, C3-C4 intermediate *Flaveria floridana* and a C_3 performing *Mesembryanthemum crystallinum* (Adams et al.. 1986; Slocombe et al., 1993). Three isoforms were noticed in leaves of two C3 species: *Gossypium hirsutum* and *Vicia faba* (Mukerji and Ting. 1971; Schulz et

al., 1992). Two kinetically and immunologically distinct isoforms of PEPC were identified in castor oilseeds (Blonde and Plaxton, 2003).

The four isoforms of PEPC are encoded by different genes in C_4 plants (Hermans and Westhoff, 1990). Genomic, cDNA sequences and Southern hybridizations showed that both *F. trinervia* (C_4) and *F. pringlei* (C3) contain four distinct classes *of ppc* genes which are named *ppcA* to *ppcD*. The classes of *ppc* genes identified in *F. trinervia* and *F. pringlei* relate to one another by gene-to-gene relationship (Hermans and Westhoff, 1990). Recently, Biasing et al. (2002). has shown the existence of four PEPC gene class in grasses. The hypothesis that the grass C4 PEPC gene could have derived from root pre-existing PEPC gene was further substantiated by analyzing the amino acid sequence of PEPC from different plant families (Biasing et al., 2002).

C_4 plants have evolved several times independently from ancestral C3 plants due to selective environmental conditions during the evolution of higher plants (Monson, 1999). According to Sanchez and Cejuda (2003) that both plant-type and bacterial-type PEPCs diverged early during the evolution of plants from a common ancestor, probably the PEPC from gamma-proteobacteria. The dicotyledonous genus *Flaveria*, comprises not only C_3 and C_4 species but also a large number of C3-C4 intermediates which makes *Flaveria* a good model system for the studying evolution of PEPC (Westhoff et al., 1997; Biasing et al., 2000; Svensson et al., 2003).

Protein Structure

PEPC is a homotetrameric enzyme of about 400-kD with four identical subunits, each with a molecular mass of 95 to 110-kD (Andreo et al., 1987). However, its quarternary structure depends on protein and effector concentrations.

The primary structure of PEPC was first deduced from a cloned DNA of *E. coli* in 1984 (Fugita et al.. 1984). Since then. more than 75 molecular species of PEPC have been established from their primary structure, including the enzymes from maize (Matsumura et al., 2002; Kai et al., 2003), *Anacystis nidulans*. a cyanobacterium (Katagiri et al., 1985), and *Thermus* sp., an extreme thermophilic bacterium (Nakamura et al., 1995; Chen et al., 2002). A list of selected plant sources for which the amino acid sequences of PEPC are available is presented in Table 1.

The alignment of all the deduced amino acid sequences and the construction of related phylogenetic trees showed that several PEPCs had evolved from the same ancestral origin. The amino acid similarities between various pairs of enzyme forms were more than 50% (Lepiniec et al., 1994; Toh et al., 1994; Kai et al., 2003; Svensson et al., 2003). The C-terminus of PEPC is quite conserved in prokaryotes and eukaryotes and may be involved in catalytic activity (Toh et al.. 1994; Dong et al., 1999).

Chemical modification of amino acid residues have shown that Arg, His. and Lys residues are essential for the catalytic activity of PEPC (Podesta et al.. 1986; Wagner et al.. 1988). Some of these conserved residues or regions are

Table 1. A list of selected plant sources for which the amino acid sequences **of** PEP carboxylase are available

Plant species	Form of PEPC	No. of amino acids	Reference
Amaranthus hypochondriacus	C_4 (leaf)	964	Rydzik and Berry, 1996
Zea mays	C_4 (leaf)	970	Hudspeth and Grula, 1989
Sorghum vulgare	C_4 (leaf)	952	Cretin et al., 1990
Flaveria trinervia	C_4 (leaf)	966	Poetsch et al., 1991
Solatium tuberosum	C_3 (leaf)	956	Merkelback et al., 1993
Nicotiana tabacum	C_3 (CC*)	964	Koizumi et al., 1991
Flaveria pringlei	C_3 (leaf)	966	Hermans and Westhoff, 1992
Glycine max	C_3 (seed)	967	Sugimoto et al., 1992
Aloe arborescens	CAM (leaf)	964	Honda et al., 1996
Anacystis nidulans	Prokaryote	1016	Katagiri et al., 1985
Escherichia coli	Prokaryote	883	Fugita et al., 1984
Corynebacterium glutamicum	Prokaryote	919	O'Regan et al., 1989
Anacystis variabilis	Prokaryote	1025	Luinenberg and Coleman, 1992

* Culture cells .

expected to be associated with substrate binding and catalytic function. The conserved positively-charged amino acid residues (11 arginine, 2 histidine and 2 lysine) may be particularly important, since both the substrates, PEP and HCO_3^-, are anions at physiological pH (Toh et al., 1994). Using site-directed mutagenesis and PEPC of *Flaveria trinervia,* Gao and Woo (1996b) observed that Arg^{450} and Arg ' were essential for PEPC function.

The structural elements that give the C_4 PEPC its specific kinetic and regulatory properties were examined by following the strategy of domain swapping and making C_3-C_4 chimerical enzymes (Westhoff et al., 1997; Biasing et al., 2000; Svensson et al., 2003). The C3 enzyme (FP966) was progressively interchanged with corresponding parts of the C_4 enzyme (FT966) starting from the amino terminus, while the reciprocal strategy was applied, i.e. regions of the C4 enzyme were swapped with corresponding segments of the C_3 PEPC. Detailed studies on these chimeric C_3/C_4 enzymes indicated that region 2 (position 296 to 437) and region 5 (position 645 to 966) contain the major determinants for C_4 specific kinetic and regulatory properties. Region 2 was essential for the allosteric regulation by Glc-6-P, where as Region 5 was the key factor for Km PEP of the nonactivated enzymes. The central determinant in this region was amino acid position 774, which held a serine in all C_4 enzymes but an alanine in C_3 or CAM enzyme (Biasing et al., 2000; Svensson et al.. 2003).

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

Crystallization and Three-Dimensional Structure

Plant PEPC is similar to the *E. coli* enzyme in primary structure except that the *E. coli* enzyme lacks the part of N-terminus region, that is involved in regulatory phosphorylation of plant enzyme. Thus *E. coli* PEPC can be a good model to study the three-dimensional structure and can be con-elated with plant PEPC. Kai et al. (1999a) have successfully crystallized the PEPC from *E. coli.* The X-ray diffraction study revealed "dimer-of-dimer" form with respect to subunit contact. Despite the continious efforts, the progress in crystallization of C_4 plant PEPC has been slow (Matsumura et al., 1999a, 2002) but recently the maize PEPC has been crystallized and characterized (Kai et al., 2003).

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

Properties of PEPC

PEPC, a cytosolic enzyme located in mesophyll cells of C_4 and CAM plants, catalyzes the carboxylation **of** PEP to yield oxaloacetate (OAA) and

inorganic phosphate (Pi). β-Carboxylation of PEP by PEPC occurs in a two step mechanism (O'Leary, 1982; Andreo et al., 1987). The first step involves the reversible, rate-limiting formation of carboxyphosphate and the enolate of pyruvate from the substrates. The second step would be the carboxylation of the enolate with the formation of oxaloacetate and Pi (Andreo et al.. 1987; Rajagopalan et al., 1994; Chollet et al., 1996; Vidal and Chollet, 1997). First Mg^{2+} binds to PEPC, and when this Mg-Enzyme complex is at equilibrium; phosphoenolpyruvate (PEP) binds onto it followed by HCO_3^- binding. Mg, PEP and HCO_3^- have to be present on PEPC before the enzyme reaction begins (Chollet et al., 1996).

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

PEPC is subjected to feedback inhibition by oxaloacetate, malate and aspartate (Rajagopalan et al., 1994; Chollet et al., 1996; Vidal and Chollet, 1997). Both OAA and malate are competitive inhibitors while aspartate may exert non-

competitive inhibition (Figure 1). PEPC is an allosteric enzyme and exhibits a hyperbolic response to the increasing concentration of PEP and Mg^{2+}.

The optimal pH for the activity of PEPC is around 8.0. The activity of PEPC therefore depends on the cytosolic pH (Andreo et al., 1987; Rajagopalan et al., 1993). Changes in cytosolic pH may modulate the catalytic activity of PEPC either directly or indirectly through regulation of PEPC-PK or PEPC-protein phosphatase or both (Rajagopalan et al., 1993).

Regulation of PEPC

PEPC is regulated by external environmental factors (such as light, high temperature, and photoperiod) as well as internal factors (metabolites, Pi, and cytosolic pH). Nutrition, particularly nitrogen, can mediate long-term regulation of PEPC. Nitrate, ammonium ions, glutamine and amino acids (i.e. glycine and alanine) promote the biosynthesis of PEPC protein, and lead to an increase in PEPC activity.

Light

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). On illumination, the activity of PEPC in leaves of C_4 plants is enhanced by 2-3 fold along with a marked decrease in the malate sensitivity of the enzyme (Huber and Sugiyama, 1986; Rajagopalan et al., 1993; Parvathi et al., 2000b). These changes during the light activation are due to the phosphorylation of the enzyme by PEPC-

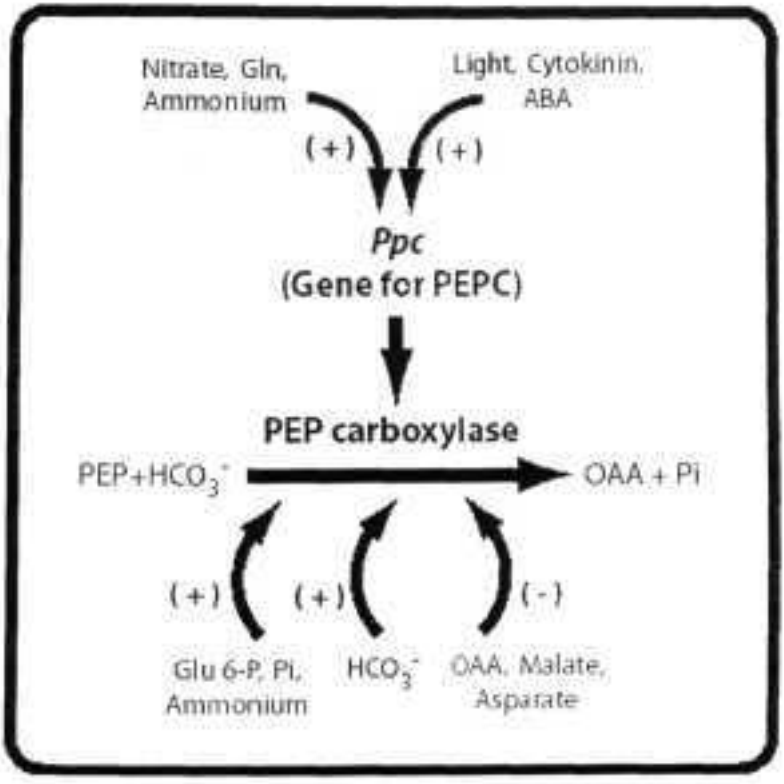


Figure 1. The regulation of PEPC by effectors at the enzyme or gene level. PEPC catalyses the carboxyiation of PEP to yield OAA and Pi. Among the effectors, which can regulate the enzyme are those which can either inhibit the reaction (-) or stimulate (+) the reaction. The levels of enzyme can be modulated also by stimulating (+) **the** expression of *Ppc* (gene for PEPC) and biosynthesis **of PEPC.**

protein kinase and dephosphorylation in dark by a type 2A protein phosphatase (Chollet et al., 1996, Vidal and Chollet, 1997; Parvathi et al., 2000b). 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 response of PEPC in C_3 leaves to light is much less than that in C_4 plants. The activation of PEPC on exposure to light is marginal (about 10-15%) in C_3 species (Rajagopalan et al., 1993). A marginal increase on exposure to light activation 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 faba* L. (Schnabl et al., 1992), although no light activation of PEPC could be detected in guard cell protoplasts of *Commelina communis* L. (Willmer et al., 1990).

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 C_4 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. 1998).

Light is also known to induce marked alkalization of cytosol in mesophyll cells of C_4 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 C_4 plants (Selinioti et al., 1986). Attempts have been made to correlate the poor rate of C_4 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).

The temperature optimum of the C_4 PEPC is around 40-45°C and its activity sharply drops below the optimal temperature. At low temperature, the sensitivity of PEPC to malate was very high in maize (Wu and Wedding. 1987) and *Amaranthus hypochondriacus* (Chinthapalli et al., 2003). Lowering the temperature from 25°C to 3°C not only reduced the catalytic cativity of PEPC but also caused a considerable reduction in the sensitivity of PEPC to malate (Carter et al., 1995). The temperature dependent regulation of PEPC was shown to be independent of phosphorylation, particularly at warm temperature (Chinthapalli et al., 2003). Further experiments are needed to establish if other possibilities like changes in conformational status are involved during temperature regulation in C_4 PEPC.

Inhibitors and Activators

PEPC is modulated by metabolites such as malate, aspartate, glycine and Glc-6-P (Andreo et al., 1987; Chollet et al., 1996; Vidal and Chollet, 1997). L-malate, a product of carboxylation, is a competitive inhibitor of PEPC (Huber and Edwards, 1975). Malate inhibits not only C_4-PEPC, but also the C_3 and CAM forms (Kluge et al., 1988; Echevarria et al., 1990; Jaio and Chollet, 1990). Aspartate inhibits the enzyme (Iglesias et al., 1986), and can also protect PEPC against thermal inactivation (Mares and Leblova, 1980). Several other analogues of PEP/pyruvale are powerful inhibitors of C_4 enzyme and are used to study the reaction mechanism of the enzyme (Gonzalez and Andreo, 1989; Janc et al., 1992).

Glc-6-P is an allosteric activator of PEPC, decreases Km for PEP (Uedan and Sugiyama, 1976), protects the enzyme from malate inhibition (Gupta et al., 1994). and can induce aggregation of PEPC into the tetrameric form (Willeford and Wedding, 1992; Wu and Wedding, 1994). Phosphorylation of PEPC has no effect on its response to Glc-6-P, therefore it is likely that the activation of PEPC by Glc-6-P involves a more complex path than the inhibition by malate. A list of selected inhibitors and activators of PEPC is given in Table 2.

Glycine activates the PEPC of C_4 monocots (Bandarian et al., 1992; Gillinta and Grover, 1995; Gao and Woo, 1996a; Tovar-Mendez et al., 1998). Surprisingly, glycine has no effect on PEPC from C_4 dicots. The reason for such specific response of glycine on PEPC from monocots is not known.

Table 2. A **list of** selected inhibitors and activators of C_4-PEP carboxylase. Most of these studies were done using the PEPC from either maize or *Amaranthus*.

Inhibitors/Activators	**K_I /K_A (mM)**	**Reference**
Inhibitors	K,	
L-malate	0.07	Parvathi et al., 2000a
Oxalacetate	1.0	O'Leary, 1982
1-Hydroxycyclopropane	0.01	O'Leary, 1982
DCDP	0.04	Jenkins et al., 1987
E-Methyl phosphoenolpyruvate	0.1	Gonzalez and Andreo, 1988
Z-bromoPEP	0.26	Díaz et al., 1988
Z-3-fluroPEP	0.09	Díaz et al., 1988
Z-Phosphoenolbutyrate	0.02	Gonzalez and Andreo, 1988
E-Phosphoenolbutyrate	0.11	Gonzalez and Andreo, 1988
Z-3-ChloroPEP	0.06	Liu et al., 1990
Methyl PEP	0.02	Gonzalez and Andreo, 1988
Phosphoenol 3-fluropyruvate	0.004	Gonzalez and Andreo, 1988
Activators	**K_A**	
Phenylphosphate	0.2	O'Leary et al., 1982
Glucose-6-phosphate	0.4	Parvathi et al., 1998
Dihydroxyacetone phosphate	2.0	Doncaster and Lcegood, 198769
Glycine	3.1	Tovar-Mendez et al., 2000
Inorganic phosphate	0.4	Podesta et al., 1990

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

Nitrogen Source

The biosynthesis of PEPC in leaves of C_3, C_4 and CAM plants is highly regulated by the availability and source of nitrogen (Foyer et al., 1994; Murchie et al., 2000). The rise in level of PEPC-mRNA and PEPC protein was more pronounced in maize plants supplemented with NH_4 or glutaminc than those with NO_3^-. Ammonium salts induced a 2-fold greater PEPC biosynthesis than that by nitrate ions (Sugiyama and Sakakibara, 2002). Recent studies have demonstrated that roots may sense nitrogen signals, by producing cytokinins, which are transported to leaves to activate gene expression in C_4 plants (Sakakibara el al., 1997; Sugiyama 1998).

Ammonium ions also stimulate activity *in vitro* (Gayathri and Raghavendra. 1994). The extent of light activation of PEPC is increased in presence of ammonium and this is probably due to implication of **PEPC-PK** activities (Duff and Chollet, 1995; Giglioli-Guivarc'h et al., 1996; Murchie et al., 2000). The effect of ammonium on PEPC was at the regulatory allosteric site on the enzyme. Where as the modulation of PEPC by glutamine is due to increase in phosphorylation of PEPC protein kinase (Manh et al., 1993).

Induction of PEPC

Submerged aquatic macrophytes employ a CO_2 concentrating mechanisms so as to use effectively dissolved HCO_3^- (Raven, 1970; Bowes and Salvucci, 1989). An appreciable shift from C3 photosynthesis to a Kranz-less C_4 acid metabolism has been observed in at least three members of the Hydrocharitaceae. *Hydrilla verticillata*, *Elodea canadensis* and *Egeria densa*, when plants were grown with air levels CO_2, high temperature, and long photoperiods (Bowes and Salvucci, 1989; Reiskind et al., 1997; Casati et al., 2000; Rao et al., 2002). Two isoforms of PEFC were highly expressed under high temperature and high light. Under these conditions, an increase in total PEPC activity was due to the expression of lower molecular weight isoform that was strongly phosphorylated in the light. The changes in kinetic and regulatory properties of PEPC were correlated with changes in the phosphorylation state of enzyme (Lara et al., 2001).

In another interesting instance, the amphibious leafless sedge *Eleocharis vivipara* developed Kranz anatomy and shifted to C_4 photosynthesis (including high activity of PEPC) under terrestrial conditions, but retained C_3-like traits and operated C_3 photosynthesis when submerged in water (Ueno, 1996, 1998). The transition from water to land could signal a water-deficient condition, therefore anatomical development and new gene expression could represent an adaptational response to water stress. Abscisic acid (ABA), induced Kranz anatomy, a new form of C4-PEPC and expression of other C_4 genes in *Eleocharis vivipara* (Uchino

et al., 1998). This observed transition is unique because normally ABA represses the expression of genes involved in C_4 photosynthesis (Sheen, 1999).

ABA also promotes the induction of PEPC, along with CAM in succulent plants (Dai et al., 1994; Taybi et al., 1995). The patterns of accumulation of these photosynthetic enzymes in ABA induced plants were similar to those after temperature induction (Casati et al., 2000). Another pattern of induction was noticed in ***Portulaca*** *oleracea*, a succulent C_4 plant during the exposure to short photoperiods or water-stress, which induce CAM. During such induction, a new form **of** CAM-form of PEPC was synthesized, besides the original CVform of PEPC. These two forms of PEPC (C_4 and CAM) were quite distinct in their kinetic and regulatory properties (Mazen, 2000).

Posttranslational Modification

The characteristics of PEPC are modulated by posttranslational modification of the enzyme. One of the strongest modes is the reversible phosphoiylation **of** single Ser residue near the N terminus resulting in a striking up- or down-regulation of the enzyme's allosteric properties (Chollet et al.. 1996: Vidal and Choilet, 1997; Nimmo, 2000). The second type of posttranslational modification is the change in oligomeric state of the enzyme, studied mostly *in vitro* (Jiao and Choilet. 1991; Rajagopalan et al., 1994; Choilet et al., 1996). A third possibility is the regulation by modulation of redox states of the enzyme, and reduction in cysteine residues. However, the significance of the latter two *in vivo* is not yet clear.

Phosphorylation

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

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

for some step(s) in the transduction pathway, particularly for protein synthesis related to PEPC protein kinase (Vidal and Chollet, 1997). A model is presented in Figure 2 showing the likely events during the transduction of light signal to control C_4-PEPC-PK and subsequently PEPC phosphorylation.

Parvathi et al. (2000a) reported that the regulation by Ca^{2+} or CAM could be al an upstream level of regulation of PEPC-PK. For example, a CDPK-like protein kinase may modulate the Ca^{2+}-independent PEPC-PK. In a recent study, a Ca^{2+}-dependent phosphoinositide-specific phospholipase C (PI-PLC) has been found to participate in the light dependent cascade leading to C4-PEPC phosphorylation in mesophyll cell protoplasts of *Digitaria sanguinalis* (Coursol et al., 2000).

The transcription of the PEPC kinase gene and the abundance of PEPC kinase mRNA responds to photosynthesis in C_3 and C_4 plants (Hartwell et al., 1996) and metabolic triggers in maize (Hartwell et al., 1999). Intense efforts were made to isolate, purify and clone the C4-PEPC-PK from maize, *Sorghum, Flaveria* and two CAM plants: *Kalanchoe fedtschenkoi* and *Mesembryanthemum crystallinum* (Tsuchida et al., 2001; Vidal et al., 2002; Nimmo, 2003). Ogawa et al. (1998) suggested that at least four types of protein kinases could be detected in their PEPC-PK preparation, of which two of them were calcium-dependent. It has been suggested that multiple forms of PEPC-PK (both Ca^{2+}-dependent and Ca^{2+}-independent) are involved in the regulation of PEPC phosphorylation (Bakrim et al., 1992; Giglioli-Guivarc'h et al., 1996; Nhiri et al., 1998).

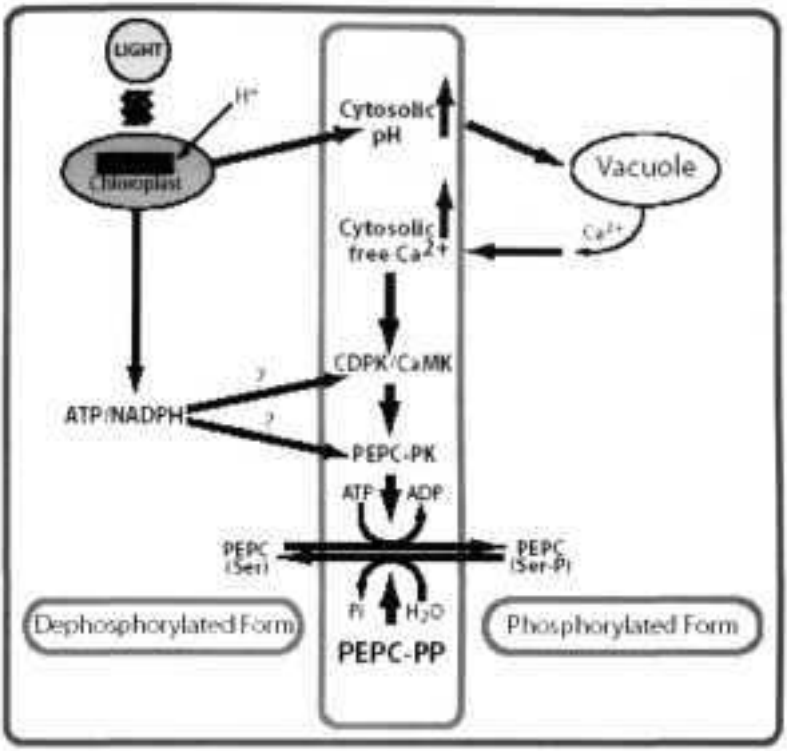


Figure 2. An over view of the transduction of light signal by a series of secondary messengers during activation of PEPC. Cytosolic free Ca^{2+} is a key factor, which can stimulate CDPK and/or PEPC-PK and enhance the phosphorylation status of PEPC. Besides the modulation of cytosolic pH, ATP and NADPH generated by chloroplasts may mediate the activation of CDPK or PEPC-PK or both.

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

Oligomerization

PEPC is a homotetramer. The oligomerization/dissociation of PEPC has been shown to regulate PEPC *in vitro,* but so far, experimental evidence *in vivo* is lacking.

The active form of PEPC is tetramer. PEPC is very active when it is in tetrameric shape, while the activity and malate sensitivity decreases when the enzyme dissociated into monomer or dimer (Walker et al., 1986; Willeford et al.. 1990). But the enzyme can exist as a dimer or monomer depending on several factors: pH. ionic strength (Wagner et al., 1987), temperature (Wu and Wedding, 1987) and concentration of PEPC (Willeford and Wedding, 1992). The presence of PEP, Mg^{2+}, Glc-6-P, malate or compatible solutes like PEG-6000 or glycerol

promoted the aggregation of the PEPC (Podesta and Andreo, 1989; Manetas, 1990; Wedding et al., 1994). It is suggested that Pi can shift the dimer/tetramer equilibrium towards tetramer (Salahas and Gavalas, 1997).

Regulatory phosphorylation may not always be involved during the increase in PEPC activity and subsequent decrease in malate sensitivity of the enzyme. The temperature dependent changes in PEPC activity and malate sensitivity are independent of phosphorylation, and possibly due to changes in aggregation status of PEPC (Wu and Wedding, 1987; Chinthapalli et al., 2003). There are also reports that reversible dissociation/association PEPC is not the reason for the diurnal variation of PEPC activity (Weigend and Hincha, 19921-

Reduction of-SH Groups

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

Conformational Changes

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

Some of the Points to be Resolved

In the past decade, a significant progress has been made on the biochemistry and molecular biology of PEPC. however, there is still scope for further studies on C_4-PEPC. For example, there are only a few reports on the regulation on PEPC by temperature. PEPC being a characteristic feature of tropical plants (which are exposed to not only high light, but also to warm temperature), the enzyme is bound to be modulated by temperature. The modulation of PEPC by temperature at the level of leaf and purified enzyme needs urgent attention. Further, the effect of temperature on PEPC seems to be quite different in C_4 and CAM, thus, examination of this phenomenon would be able to offer a logical explanation of contrasting difference in temperature response in PEPC in C_4 and CAM plants.

In tropical climates there is usually a huge fluctuation of both light and temperature along with changes in nutrient availability. It is therefore important and study of these interactions between light, temperature and nitrogen nutrition on PEPC. For e.g. the nitrogen requirements may differ among C_4 monocots and C4 dicots particularly in relation to the sub-classification of C_4 plants, namely NADP-ME, NAD-ME and PEP-CK. An attempt was made to study the effect of temperature during light activation of PEPC in leaves of *Egeria densa* (Casati et al., 2000; Lara et al., 2001). Experiments are essential **to** examine such interactions in a diverse range of C_4 plants.

The extent of carbon fixation is very high during the day. It is, however, not clear if the activity of PEPC follows a strong diurnal **rhythm.** It is possible that the levels of PEPC change (protein and mRNA) during day/night cycles. In a related study, Hartwell et al. (1996) found that the mRNA levels of PEPC-protein kinase start increasing well before the sunrise in leaves of maize. **Further,** studies are needed to determine mRNA and protein levels of PEPC as well as the post-translational modification of PEPC, if any.

The cloning and expression of PEPC in *E. coli* is a simple, elegant and extremely versatile system. Production of recombinant PEPC in *E. coli* has already been achieved using the cDNA from *Sorghum* (Cretin et al., 1991), maize (Dong et al.. 1997) and *Flaveria* (Westhoff et al.. 1997). **This** system was also used to study the properties of chimeric PEPC made from C_3 and C_4 PEPC and also to analyze the importance of different amino acids using site directed

mutagenesis (Biasing et al., 2000). Such studies can be extended to probe the properties of PEPC from interesting plant systems like C_3-C_4 intermediates or transgenic plants.

Present work

The present investigation focuses on the temperature effects of PEPC in leaves as well as purified PEPC from *Amaranthus hypochondriacus*, a NAD-malic enzyme type C_4 plant. The approach and objectives are further elaborated in the next chapter.

Chapter 2

Approach and Objectives

Chapter 2

Approach and Objectives

Amaranthus hypochondriacus, a NAD-ME type of C_4 plant, is an important grain crop and leafy vegetable, grown in semi-arid, sub-tropical and tropical regions. Our laboratory has been using *A. hypochondriacus* as a model system to study the properties and regulation of C4-PEPC (Parvathi et al., 2000a, b; Gayathri et al., 2000). The present study is undertaken with *A. hypochondriacus*, for studies on PEPC using leaf discs, and purified enzyme. Pea (*Pisum sativum*) is a typical C3 plant, is used for the comparison.

Among the environmental factors, light and temperature modulate dramatically the activity and properties of PEPC. Compared to the extensive literature on the properties and mechanism of light activation of PEPC, in C_4 plants, the literature on the regulation by temperature of PEPC is quite limited (Rajagopalan et al.. 1994). The present study is an attempt to characterize the temperature responses of PEPC from typical C_4 plant, *A. hypochondriacus* and compare with that of a C_3 plant pea *(P. sativum).* Experiments were conducted on leaf discs so as to stimulate physiological situation *in vivo.* Studies were extended to a few more species to establish if the nature of PEPC responses is similar in a range of C3 and C_4 plants.

Phosphorylation of PEPC is regulated by light/dark transitions *in vivo* (Rajagopalan et al.. 1994; Chollet et al.. 1996; Vidal and Chollet, 1997; Parvathi et al., 2000b). On illumination, the phosphorylation of PEPC on a serine residue is catalyzed by protein kinase and dephosphorylation occurs during darkness by type

2A protein phosphatases. *De novo* synthesis of PEPC-PK is an important component during PEPC phosphorylation. Attempts were therefore made to examine if variation in temperature caused any modulation of either the phosphorylation status or the protein levels of PEPC leaf discs.

The organic cosolutes, such as glycerol and PEG, stabilize the activity and integrity of several enzymes, including PEPC, during the dilution of enzyme, which occurs during extraction medium (Podesta and Plaxton, 1994; Law and Plaxton, 1995). Although the reaction media containing organic cosolutes are not exactly physiological, they resemble an environment closer to the conditions *in vivo*, than those during extraction and assay. 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 oligomeric status and conformational changes of the purified C_4-PEPC with temperature were studied in presence of PEG-6000.

Studies were extended with purified PEPC of *A. hypochondriacus* (C_4) to assess the changes induced by temperature of PEPC *in vitro* compared to those *in vivo*. Purification of PEPC was done from leaves of *A. hypochondriacus* by conventional techniques, using the protocol described already (Gayathri et al., 2000).

Polyclonal antibodies were raised in rabbits against the purified PEPC from *A. hypochondriacus* leaves using the procedure of Nimmo et al. (1986). and the

anti-PEPC antiserum was used in experiments involving western-blots and protein phosphorylation.

Experiments were conducted to examine the conformational changes of PEPC protein through fluorescence and CD-spectra. Studies were made on the intrinsic and extrinsic fluorescence of PEPC protein at different temperature. These studies were extended to check the effect of PEG-6000 (compatible solute) and urea (dcnaturant) on PEPC protein. Further, CD-spectra were used, to evaluate the secondary structure of PEPC of *A. hypochondriacus*. The mean residue ellipticity of PEPC at different temperatures were estimated.

The specific Objectives of the Present Study are:

1. Investigate the effect of varying temperature on the activity and properties of PEPC in leaves of C_4 and C_3 plants.
2. Assess if the temperature induced changes in PEPC are reversible.
3. Examine if PEG-6000 (a compatible solute) can modulate the temperature responses of purified C_4-PEPC.
4. Assess the changes in protein level or phosphorylation status of PEPC on exposure to temperature or light.
5. Determine the changes induced by temperature in catalytic and regulatory properties of PEPC protein purified from leaves *A. hypochondriacus*.
6. Study the temperature induced conformational changes in PEPC protein reflected in its fluorescence properties and CD-spectra.

Chapter 3

Materials and Methods

Chapter 3

Materials and Methods

Plant Material

Plants of *Amaranthus hypochondriacus* L. cv AG-67. and *Pisum sativum* L. cv Arkel. were raised from seeds. The plants were grown in 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 temperatures of 30 - 40°C day/25 - 30°C night. The upper fully expanded leaves were harvested, about 2 - 3 h after sunrise. Leaf discs were prepared from 4- to 6- week-old plants of *A. hypochondriacus* (Fig. 3.1) and 8- to 10-day-old plants of *P. sativum* (Fig. 3.2).

In some experiments, four each of C_3 and C_4 plants were used so as to ascertain that the responses are characteristic of C_3- or C_4-type. The plants used for these studies, which were grown in the field, are as follows:

C3 species:

Euphorbia hirta L. *Trianthema portulacastrum* L.

Portulaca oleracea L. *Amaranthus spinosus* L.

C_4 species:

Lycopersicum esculentum Mill. *Euphorbia pulcherrima* Willd.

Arachis hypogaea L. cv ICGS 44. *Tridax procumbens* L.

Fig. 3.1

Fig. 3.2

Fig. 3.1. A view of 4- to 6- old plants of *Amaranthus hypochondriacus* AG-67, grown in the field (outdoors).

Fig. 3.2. A view of 8- to 10- old plants of *Pisum sativum* L. cv. Arkel, grown in the field (outdoors).

Preparation of Leaf Discs

Discs of *ca.* 0.2 cm^2 were punched from leaves, under water, with the help of a paper punch. Thirty discs were kept in a Petri dish (5-cm diameter) containing 10 mL water and left in darkness for 2 h. Leaf discs incubated at different temperatures were extracted and assayed accordingly as described in detail (Parvathi et al., 2000b; Chinthapalli et al., 2003).

In some of the experiments 1.25% (w/v) PEG-6000 was included during the incubation and/or assay. During experiments involving light-activation, the predarkened discs were illuminated as described in the following pages.

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 Tris-HCl [pH 7.3], 10 mM $MgCl_2$, 2 mM K_2HPO_4, 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 7000g 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.

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 Tris-HCl, [pH 7.3], 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 leaf 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 the 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:

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

Incubation of Leaf Discs at Different Temperature

Thirty leaf discs were floated on distilled water in a 5 cm diameter Petri dishes and were left in darkness for 2 h. After predarkening, the leaf discs were incubated 30 min at required temperature in the range of 15°C to 50°C in a themostatically controlled water bath. At the end of 30 min in each temperature, the leaf discs were extracted (as described above) and the extract was examined for PEPC activity.

In experiments to check the reversibility of temperature effects, the leaf discs were exposed to various temperatures (for 45 min) and then transferred to optimal temperature (for 45 min). At the end of 45 min, the leaf discs were extracted (as described above) and the extract was examined for PEPC activity.

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 bulbs) at an intensity of 1000 $\mu mol\ m^{-}\ s^{-1}$ (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 45 min illumination (or dark-incubation for comparison), the leaf discs were extracted (as described above) for studies on PEPC.

Kinetic and Regulatory Properties of the Enzyme

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

Exposure of Purified PEPC to Varying Temperature

The purified PEPC (50 μg mL^{-1}) was incubated at different temperature (15°C to 50°C) for required time intervals (0 to 60 min) prior to the assay at room temperature. An aliquot (containing 0.1 μg protein) of PEPC was used for assaying the activity. Unless otherwise specified, PEPC was incubated for 45 min at different temperature.

In experiments to check reversibility, the purified PEPC incubated at optimal temperature of 40°C was treated as control. The PEPC protein was exposed to various temperatures (for 45 min) and then transferred back to the optimal temperature of 40°C, for the next 45 min. Immediately the protein was assayed for PEPC at room temperature (30°C).

Interaction of PEPC with PEG or Glycerol

The effect of PEG or other compatible solutes on PEPC was checked by incubating purified PEPC (50 $\mu g\ mL^{-1}$, unless otherwise specified) with 1.25% (w/v) PEG-6000 or 10% (v/v) glycerol, at required temperature. An aliquot of PEPC protein was taken out and used for enzyme assay. In some of the experiments, the concentration of PEPC protein was raised to 200 $\mu g\ mL^{-1}$, during prcincubation at varying temperature.

In some of the experiments (with leaf extracts), 1.25% (w/v) PEG-6000 was present during the assay.

Purification of PEPC

Extraction and Ammonium Sulfate Fractionation

Leaves (40 g) of *Amaranthus hypochondriacus* were picked from the field-grown plants (exposed to sunlight for 2-3 h). washed, chopped into small pieces and suspended in 160 mL of buffer containing 100 mM phosphate buffer [pH 7.3], 25% (v/v) glycerol, 5 mM DTT, 10 mM $MgCl_2$, 1 mM EDTA, 2 mM PMSF, 50 $\mu g\ mL^{-1}$ chymostatin, 10 mM NaF and 2% (w/v) solid insoluble PVP. The

leaves were then homogenized using a mixie [1.5 min; maximum speed, Remi Equipments (Ato-mix Blender)]. The homogenate was filtered through four layers of cheese cloth and the filtrate was centrifuged at 40,000g for 10 min.

The supernatant (160 mL) was brought to 40% saturation with saturated ammonium sulfate solution. The suspension was stirred slowly for 30 min and then centrifuged at 40,000*g* for 40 min. The precipitate was discarded, the supernatant was brought to 60% saturation by further addition of saturated ammonium sulfate solution and the precipitate was collected by centrifugation at 40,000g for 30 min.

The extraction and $(NH_4)_2SO_4$ precipitation were performed at 4°C and the subscquent steps were carried out in an air-conditioned room with a temperature of 15-20°C.

***DEAE-Sepharose** Chromatography*

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

and the enzyme was precipitated with 60% (v/v) saturated ammonium sulfate solution.

Hydroxylapatite (HAP) Chromatography

The precipitate from the above step, after **ammonium sulfate precipitation,** was dissolved in 200 mM phosphate buffer [pH 7.3] containing 10% (v/v) glycerol, and dialyzed against 20 mM phosphate buffer [pH 7.3] containing 10% (v/v) glycerol. The dialyzed sample was applied onto a 1 x 12 cm **HAP** column.

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

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

Concentration and Storage

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

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

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

The electrode buffer contained 25 mM Tris-HCl, 192 mM glycinc, [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) β–mercaptoethanol, 0.04% (w/v) bromophenol bluc] 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.

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

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

Non-denaturing PAGE

Native gel was run as described by Law and Plaxton (1995). A 6% (w/v) polyacrylamide gel (8 x 8 cm) was polymerized without SDS, using only 375 mM Tris-HCl buffer [pH 8.8]. 4% (w/v) 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.

Immunological Characteristics of PEPC

Preparation of Anti-PEPC Antiserum

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

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

(250 µl) of 50% Freund's incomplete adjuvant. After 2 weeks, blood was collected from the ear vein. The blood was allowed to coagulate and the antiserum was collected by centrifugation at 10,000*g* for 30 min. The antiserum was split into several small aliquots and stored at -20°C.

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

Western Blotting

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 difluoride (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 rain, 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 $MgCl_2$.

In Vivo **Labelling** of **PEPC with** ^{32}Pi

Labelling of PEPC with ^{32}Pi *in vivo* was done by following the procedure described by Bakrim et al. (1992).

Excised leaves were fed through petiole with 100 μL (60 μCi) of $KH_2{}^{32}PO_4$ (Specific activity of 10 mCi/mmol) under moderate illumination (400 $\mu E\, m^{-2}$ s") for 4 h. The leaves were left in darkness for 2 h to ensure that the PEPC is dephosphorylated. A set of leaf discs was exposed to different temperatures for 30 min. In a parallel treatment, leaf discs were either illuminated (1000 $\mu E\, m^{-2}\, s^{-1}$) or kept in darkness for 45 min (Parvathi et al., 2000b).

The incubated leaf discs were extracted with 5 mL of extraction buffer described above. The leaf extracts were cleared by centrifugation for 15,000 g for 5 min and supernatants were assayed for PEPC. Extracts containing 0.2 units of PEPC-activity (approximately 300 μL) was mixed with the volume of anti-PEPC antiserum (200 μL) and left overnight at 4 °C. The immunoprecipitated PEPC was pelleted by centrifugation at 15,000 g for 5 min. The supernatant was removed and the pellet washed twice with 0.5 M Tris-HCl [pH 8.0], 1.5 M NaCl and 1% **Triton** X-100, and once with 0.1 M **Tris-HCl** [pH 8.0].

SDS-PAGE **and** Autoradiography

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

Gels were stained with Coomassie brilliant blue R-250 and destained thoroughly by using destaining solution with constant shaking. These gels were dried under vacuum with a gel dryer (Bio-Rad Laboratories, USA). The X-ray **film**

was cut to the gel-size and was placed on top of the gel. The gel and X-ray film were placed between two intensifiers, inside an X-ray cassette. The gel position was marked by cutting the corner of the X-ray film. The cassette was left in a Deep-Freezer (-80°C). After 4 to 5 days, the X-ray film was developed using X-ray film-developer and fixed with X-ray film-fixer. The developed and fixed X-ray film was washed thoroughly with water and was allowed to dry.

Intrinsic and Extrinsic Fluorescence of PEPC

The intrinsic fluorescence of PEPC protein was studied by monitoring the fluorescence pattern, typical of amino acid residues. The samples were prepared by taking PEPC (10 $\mu g\ mL^{-1}$) into 50 mM Tris-HCl [pH 7 .3] and 10% glycerol (buffer A). The mixture was incubated at different temperatures for 45 min in a thermo-regulated water bath. The fluorescence emission spectrum of each sample was recorded at room temperature with a spectrofluorimeter (Spec Fluoromax 3). The samples were excited at 280 nm and the emission spectrum was measured between 300 to 400 nm, using a light path of 1 cm. Further details are indicated while describing the results.

The extrinsic fluorescence was monitored using 1-anilinonaphthalene-8-sulfonic acid (ANS). The PEPC protein (10 $\mu g\ mL^{-1}$ in buffer A) was incubated at different temperatures for 45 min. Immediately, ANS solution (2 uM of ANS in buffer A) was added to the treated protein to attain a final concentration of 1 uM ANS. After incubating the samples for 2 h, the fluorescence emission of each

sample was recorded at room temperature with a spectrofluorimeter (Spec Fluoromax 3). The excitation was at 350 nm and emission spectra were measured between 400 to 600 nm. The spectra of buffer (as well as buffer+ANS) were collected and subtracted from the spectra of enzyme with buffer (+ANS).

The effects of 0.5 mM malate (competitive inhibitor) and 2 mM Glc-6-P (an allosteric activator) were also examined on the pattern of intrinsic and extrinsic fluorescence of PEPC.

Circular Dichroism (CD) Spectra

All the circular dichroism (CD) measurements were recorded with a spectropolarimeter (Jasco J-810) using 1.0 cm path length cell and an average of 3 repetitive scans between 250 and 200 nm. The spectra were recorded with a scan speed of 20 nm min^{-1} and with a response time of 1 s.

The purified PEPC (50 $\mu g\ ml^{-1}$) protein taken in a buffer (Tris-HCl, **pH** 7.3 and 10% (v/v) glycerol) was incubated at different temperatures in thermo-regulated water bath for 45 **min** prior to recording the spectra at room temperature. The spectra of buffer were subtracted from the respective spectra of enzyme with buffer, which gives the spectra of the enzyme.

The CD results were expressed as mean residue ellipticity (θ), obtained by the equation,

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

in which M_{MRW} (the mean **amino acid residue** weight), *d* is **the cell path in** cm. **and** c is the concentration of the protein **in mg** mL^{-1} (Sievers. 1978). These values were M_{MRW} = 414.93. d = 0.1, **and** c = 0.05.

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

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

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

Replication and Statistical Analysis

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

Chemicals/Materials

Most of the biochemicals, were from either Sigma (Sigma Chemical Company. St Louis. USA) or Bochringer Mannheim (Boehringer Mannheim Company, **Germany).** PVDF membranes (Millipore) were obtained from Sigma-Aldrich Chemical Company, St Louis. USA. while DEAE-Sepharose **CL-6B** and Sephadex G-25. from Pharmacia. Sweden.

[^{32}P]-labelled KH_2PO_4 was procured from Board of Radiation and Isotope Technology, Bombay. The X-ray film was from Konica Photofilms Mfg. Co. Ltd., X-ray Developer and Fixer from Allied Photographers India Limited.

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

Chapter 4

Dramatic Difference in the Responses of PEPC to Temperature in Leaves of C_3 and C_4 Plants

Chapter 4

Dramatic Difference in the Responses of PEPC to Temperature in Leaves of C_3 and C_4 Plants

C_4 plants differ from C3 plants in several features, including their light and temperature responses (Berry and Bjorkman, 1980; Sugiyama et al., 1979). The temperature optima for photosynthesis and growth in C_4 plants are usually higher than those for C_3 plants (Long, 1999). However, the C_4 plants are quite sensitive to cold temperatures. The cold sensitivity C_4 pathway has been suggested to be related to the cold sensitivity of key C_4 enzymes, such as pyruvate phosphate dikinase (PPDK) or PEPC (Potvin and Simon, 1990; Burnell, 1990; Du et al., 1999a).

The cold sensitivity of PPDK in C_4 plants is well established and the mechanism of cold inactivation of PPDK is studied in detail (Krall et al., 1989; Burnell, 1990; Du et al., 1999b). In contrast, the reports on cold sensitivity of PEPC have been quite conflicting. There are reports which suggest PEPC is sensitive to cold temperature (Phillips and Mc William, 1971; Selinioti et al., 1986), while others could not detect any significant change in the properties of PEPC at cold temperature (Du et al., 1999b; Krall and Edwards, 1993). Further, some of these experiments involved either long term exposures of plants or short term exposure of purified en/ymes and thus involve diverse experimental material.

On illumination, the activity of PEPC in leaves of C_4 plants is enhanced by 2-3-fold along with a marked decrease in the malate sensitivity of the enzyme.

These changes during the light activation are due to the phosphorylation of the enzyme (Chollet et al., 1996, Vidal and Chollet, 1997, Parvathi et al., 2000a). The work of Selinioti et al. (1986) indicated that the amplification of the light effect by temperature difference could occur. Such interaction of light and temperature would be very important for C_4 plants. Compared to the extensive literature on the properties and mechanism of light activation of PEPC, in C_4 plants, the literature on the regulation by temperature of PEPC is quite limited (Rajagopalan et al., 1994).

The present study is an attempt to characterize the temperature responses of PEPC from a typical C_4 plant, *Amaranthus hypochondriacus* and compare with that of a C_3 plant pea *(Pisum sativum)*. Experiments were conducted on leaf discs so as to simulate physiological situation *in vivo.* Studies were extended to a few more species to establish the nature of PEPC responses to be similar in C_3 and C_4 plants. Attempts were then made to understand the mechanism of temperature induced changes in the properties of PEPC in *A. hypochondriacus* (C4) by examining the changes in the phosphorylation status, protein levels or the modulation by PEG-6000.

Results

Changes in the activity and malate sensitivity of PEPC with temperature

The optimal temperature for PEPC in *Amaranthus hypochondriacus* (C_4) was 45°C, compared to 30°C in *Pisum sativum* (C_3 species) (Fig. 4.1). The response of enzyme to temperature was, quite dramatic when represented as either

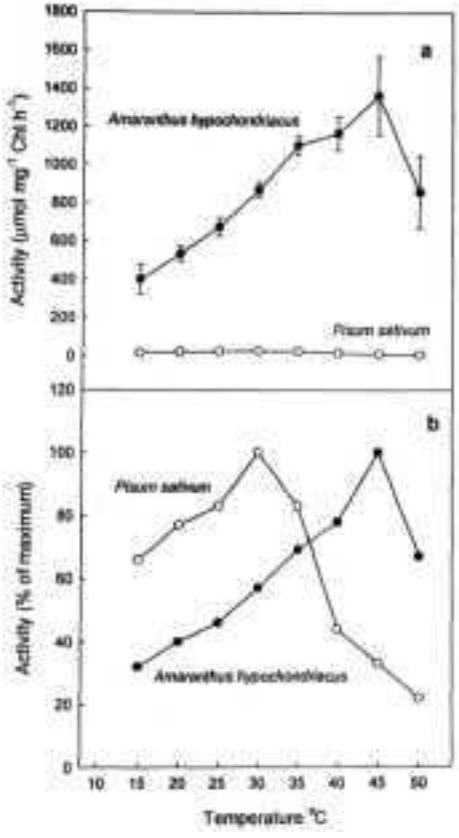


Fig. 4.1. The activity of PEPC in extracts from leaf discs of ***Amaranthus hypochondriacus*** (C_4 plant) and ***Pisum sativum*** (C_3 species) after exposure to varying temperatures. The activity of PEPC is represented as either enzyme units of $\mu mol\ mg^{-1}\ Chl\ h^{-1}$ (a) or % of its maximum (b). The preincubation time for leaf discs was 30 **min** for each temperature. The experiments were done on at least three different days and the average values ± SE are represented. For *Pisum sativum*, the SE values were within the symbols.

enzyme **units of μmol mg^{-1} Chl h^{-1} (Fig.** 4.1A) or % of maximum activity (Fig. 4.1B). The decrease in PEPC activity at 15°C was much higher in case of *A. hypochondriacus* (C_4) than that of *P. sativum* (C3). Similarly, the decrease in activity of PEPC at high temperature of 50°C was much greater in the case of *P. sativum* (C_3) than that of *A. hypochondriacus* (C_4). Thus, the C_4 PEPC was more sensitive to sub-optimal temperatures and less sensitive to supra-optimal temperatures than that of C_3 species.

As the temperature was raised from 15°C to 50°C, there was a marked decrease in malate sensitivity of PEPC in *A. hypochondriacus,* C_4 plant (from 85% to 39%). Such decrease was more than that in *Pisum sativum,* C_3 species (from 66% to 34%) (Fig. 4.2). The extent of malate inhibition was always higher in case of *P. sativum* (C_3 species) than that of *A. hypochondriacus* (C_4 plant). In case of *Amaranthus hypochondriacus,* the use of chymostatin during extraction did not make a great difference. For e.g. the inhibition by malate of PEPC at 15 °C was 69% and 76%, respectively, in presence of PMSF and chymostatin. The malate inhibition at incubation temperature of 50 °C fell to 31% and 40%, in presence of PMSF and chymostatin, respectively. Thus, the pattern of PEPC response to rise in temperature (increase in activity and decrease in malate sensitivity) was similar, irrespective of the presence of either chymostatin or PMSF (Table 4.1). As chymostatin was expensive, PMSF was used routinely in all the experiments.

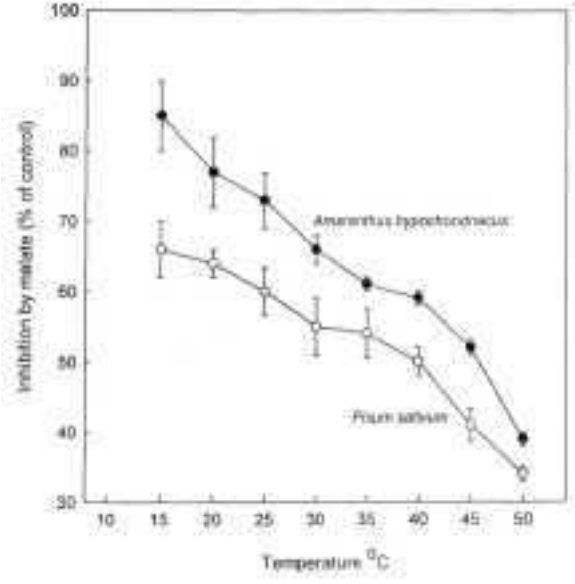


Fig. 4.2. Effect of temperature on the malate sensitivity of PEPC in extracts prepared from the leaf discs of *Amaranthus hypochondriacus* (C4 plant) or *Pisum sativum* (C_3 species), exposed to different temperatures. The activity was measured at $30^{o}C$ and assayed in the absence or presence of either 0.5 mM malate (in case of *A. hypochondriacus)* or 2 mM malate (in case of *Pisum sativum).* Further details were as described in Fig. 4.1.

Table 4.1. The effect of including either PMSF or chymostatin during the extraction, on the responses of PEPC to temperature. The extracts were prepared from leaf discs of *Amaranthus hypochondriacus* exposed to varying temperature for 45 min and the characteristics of PEPC were determined. The concentration of 2 mM PMSF and 10 μg/mL chymostatin were used.

Temperature (°C)	PEPC Activity ($\mu mol\ mg^{-1}\ Chl\ h^{-1}$)		Inhibition by malate (% of control)	
	PMSF	Chymostatin	PMSF	Chymostatin
15	619	580	69	76
30	1064	1045	51	59
35	1103	1064	43	54
45	1587	1548	37	47
50	1180	1103	31	40

Arrhenius plots

Arrhenius plots were constructed by **plotting the activity** of PEPC against the reciprocal of temperature in the absence (Fig. 4.3A) or presence of malate (Fig. 4.3B). These enzyme activities were measured after exposing the leaf discs to a range of 10°C to 45°C in case of *A. hypochondriacus* (C_4) and 10°C to **35°C** in case of *P. sativum* (C_3). Arrhenius plots exhibited abrupt changes or "break-points" at only one point of 17°C in *A. hypochondriacus* (C_4) while at two points corresponding 17°C and 27°C in case of *P. sativum* (C3). The patterns of Arrhenius curves in presence of malate were quite similar to those in the absence, with similar breaks in the slope.

The activation energy of PEPC from *A. hypochondriacus* (C_4) was less than that from *P. sativum* (C_3 species) in the temperature range of 10 to 27°C (Table 4.2). However, the activation energy of PEPC from *A, hypochondriacus* (C_4) was less than that of *P. sativum* (C_3) above the temperature of 27°C. The activation energy increased by 2 to 4 fold at temperatures below 17°C, in case of both *A. hypochondriacus* C_4) and *P. sativum* (C3)-

Reversibility of the effect of temperature on PEPC

The next set of experiments were designed to determine if the changes, induced in the properties of PEPC at various temperatures, were reversible to a large extent. The leaf discs incubated at optimal temperatures of 45 °C in case of *A. hypochondriacus* and 30°C in case of *P. sativum* were taken as controls. The leaf discs were exposed to various temperatures (for 30 min) and then transferred

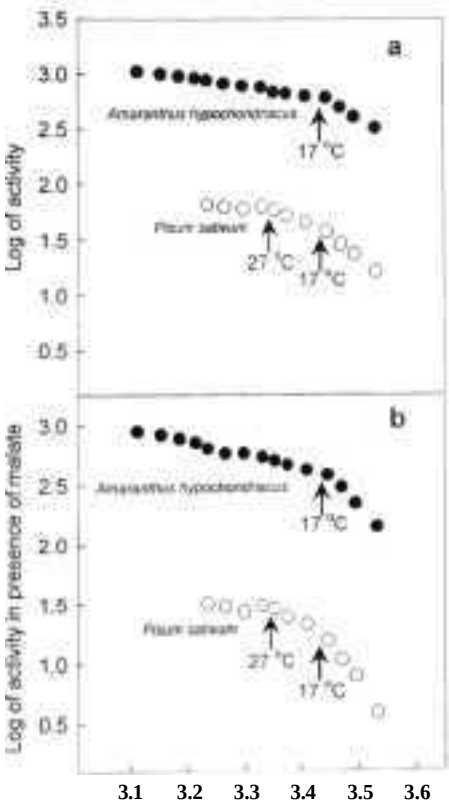


Fig. 4.3. Arrhenius plots of PEPC **activity** in the absence of malate or in the presence of malate during the assay. The points are averages of three to five seperate experiments. The activity of PEPC was determined in the extracts prepared from leaf discs of *Amaranthus hypochondriacus* (C_4 plant) or *Pisum sativum* (C_3 species), exposed to varying temperatures. Further details were as in Figs. 4.1 and 4.2. The 'break-points' are indicated by arrows.

Table 4.2. Activation energy (kcal mol^{-1}) of PEPC in extracts from leaf discs of *Amaranthus hypochondriacus* (C_4 plant) or *Pisum sativum* (C3 **species) exposed to** different temperatures.

Temperature range (°C)	*Amaranthus hypochondriacus* (C_4)	*Pisum sativum* (C3)
	Activation energy (kcal **mol"**)	
Enzyme activity in the absence of malate		
10-17	13.8	18.3
17-27	3.8	9.1
27-35 or 27-45 [a]	2.9	1.23
Enzyme activity in the presence of malate		
10-17	23.0	31.0
17-27	6.1	12.5
27-35 or 27-45 [a]	4.5	1.03

[a] The range was 27-35°C for *Pisum sativum* and 27-45°C for *Amaranthus hypochondriacus*

to their respective optimal temperature (for 30 min). The decrease in the activity of PEPC at either suboptimal or supraoptimal temperature was significantly reversed in case of both *A. hypochondriacus* and *P. sativum* (Fig. 4.4A, B).

Comparison of the effects of temperature on PEPC in a range of C_3 and C_4 plants

The effect of temperature on PEPC activity and sensitivity to L-malate were studied in leaf discs of a few C_3 and C_4 plants (Tables 4.3; 4.4). When leaf discs were incubation at different temperatures, the average PEPC activity in C_4 plants fell to 40% and 62% at suboptimal and supraoptimal temperatures respectively, compared to control (optimal incubation temperature). In C_3 species, the average PEPC activity at sub-optimal and supra-optimal temperature was 65% and 38% respectively, compared to the control. The sensitivity to L-malate of PEPC in C_4 plants increased by almost 2-fold, at suboptimal temperature, while there was only about 20% increase at suboptimal temperature, in C_3 species. Similarly, the decrease in the extent of malate inhibition of PEPC as the temperature was raised from 15 °C to 50 °C was higher in case of C_4 plants than that in C_3 species. Thus, the C_4 PEPC was more tolerant to high temperature and more susceptible to low temperature than that of C_3 plants.

Changes in the phosphorylation state and protein levels of PEPC

Attempts were made to examine if variation in temperature caused any modulation of either the phosphorylation status or the protein levels of PEPC. Since illumination is known to increase the phosphorylation of PEPC. light/dark treatment of leaf discs was included for comparison. The extent of PEPC

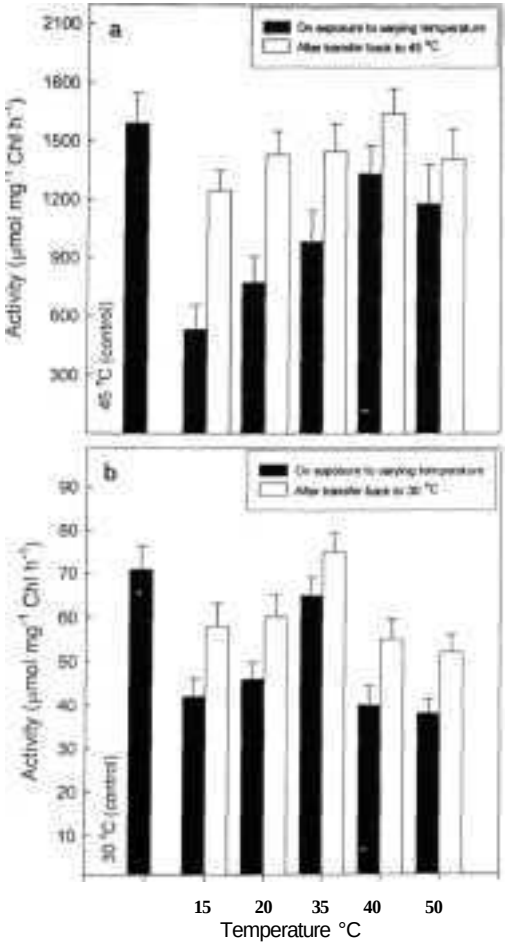


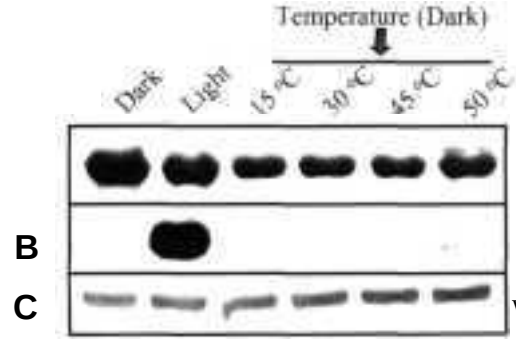
Fig. 4.4. The reversibility of the effects of temperature on PEPC. The leaf discs were pre-incubated at optimal temperature (control), exposed to varying temperatures and then transfered back to the control temperature. (a) *Amaranthus hypochondriacus* (C_4 plant) (b) *Pisum sativum* (C3 species).

Table 4.3. Effect of varying temperature on the activity of PEPC in different species of C_4 and C_3 plants. The values in parentheses represent the activity at sub-optimal or supra-optimal temperatures as % of that at optimal temperature.

Type/Species	PEPC Activity ($\mu mol\ mg^{-1}\ Chl\ h^{-1}$) Temperature		
	Optimal	Suboptimal	Supraoptimal
C_4 species	45°C	15°C	50°C
Euphorbia hirta	1490 ±79 (100)	793 ± 54 (53)	951 ± 68 (64)
Portulaca oleracea	2690 ± 134(100)	1025 ± 69 (38)	1180 ± 75 (43)
Trianthema portulacastrum	1586 ±96 (100)	543 ± 42 (34)	1131 ±71 (71)
Amaranthus spinosus	1503 ±91 (100)	645 ± 45 (42)	997 ± 65 (66)
Amaranthus hypochondriacus	1749±94(100)	566 ±41(32)	1188 ± 79 (67)
Average	**1804 ±84 (100)**	**714 ± 43 (40)**	**1089 ±61 (62)**
C_3 species	30°C	**15°C**	**50°C**
Lycopersicum esculentum	27 ±0.8 (100)	17 ± 0.5 (62)	12 ± 0.3 (44)
Arachis hypogaea	23 ±0.5 (100)	14 ± 0.4 (61)	10 ± 0.2 (43)
Euphorbia pulcherrima	31 ±0.9 (100)	23 ± 0.6 (74)	17 ± 0.4 (54)
Tridax procumbens	27 ±0.7 **(100)**	17 ± 0.3 (62)	7 ± 0.2 (26)
Pisum sativum	34 ±1.1 **(100)**	23 ± 0.7(67)	11 ± 0.2 (32)
Average	**29 ±0.7 (100)**	**19** ± 0.5 (65)	**11 ± 0.2 (38)**

Table 4.4. Effect of varying temperature on the extent of inhibition by malate of PEPC in different species of C_4 and C_3 plants. Further details were as in Table 4.2.

Type/Species	Inhibition by malate (% of control)			
	Temperature			Decrease with rise in temperature
C_4 species	15°C	**45°C**	**50°C**	**15°C *minus* 50°C**
Euphorbia hirta	63 ± 3.2	24 ± 1.5	21 ± 1.3	**42**
Portulaca oleracea	69 ± 3.4	28 ± 1.7	17 ± 1.1	52
Trianthema portulacastrum	74 ± 3.5	40 ± 2.0	35 ± 2.1	**39**
Amaranthus spinosus	80 ± 3.8	53 ± 3.1	38 ± 2.2	**42**
Amaranthus hypochondriacus	85 ± 3.9	52 ± 2.8	39 ± 2.1	**46**
Average	74 ± 3.5	**39 ± 1.9**	**30 ± 1.7**	44
C3 species	15°C	**30°C**	**50°C**	**15°C *minus* 50°C**
Lycopersicum esculentum	88 ± 3.5	72 ± 3.2	54 ± 2.5	34
Arachis hypogaea	89 ± 3.5	75 ± 3.1	56 ± 2.6	33
Euphorbia pulcherrima	**75** ± 3.1	68 ± 2.5	45 ± 2.0	30
Tridax procumbens	**88** ± 3.4	**71 ± 3.1**	55 ± 2.1	33
Pisum sativum	66 ± 3.0	55 ± 2.1	34 ± 1.5	32
Average	**81** ± 3.3	**68 ± 2.6**	**49** ± 1.9	**32**



Activity of PEPC	832	1780	658	896	1721	827
Inhibition by malate (%)	82	65	87	68	51	42

Fig. 4.5. The pattern of protein levels and the phosphorylation of PEPC in leaf discs of *Amaranthus hypochondriacus* exposed to either dark-light treatment or varying temperature of 15°C to 50°C (only dark-incubation). The extracts from leaves of *A. hypochondriacus* labeled with ^{32}Pi, were prepared after the given treatments. The PEPC enzyme was immunoprecipitated from leaf discs, separated on SDS-PAGE and examined for protein levels as well as radioactivity. The Figure includes gels (A) Stained with Coomassie Blue; (B) exposed to autoradiography; (C) Western blots. Extracts equal to 0.2 units of PEPC were used in each lane of rows A and B. The amount of protein in each lane was 25 μg, in case of row C (Western Blots).

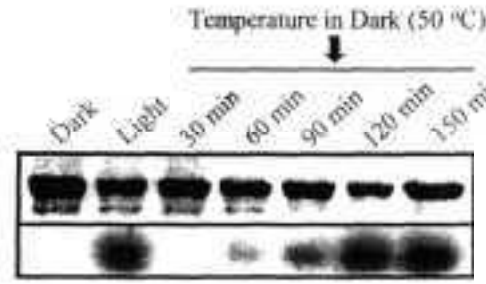
phosphorylation was much higher in illuminated discs than that in dark adapted leaves. However, there was no significant change in the status of PEPC phosphorylation in leaf discs exposed to different temperatures (Fig. 4.5A;B).

Western blot analysis indicated that the levels of PEPC-protein in leaf discs of *A. hypochondriacus*, were almost similar in control (dark treated), illuminated discs as well as those at varied temperature (Fig. 4.5C). Thus, neither a change in temperature nor illumination, caused any significant change in the PEPC-protein levels.

While at 50 °C (dark treated), PEPC is phosphorylated indicating a small signal at 45 min of incubation with low activity and malate sensitivity. Studies were extended to check the level of phosphorylation in PEPC at 50°C of incubation over a period of 150 min.

Changes in the phosphorylation state of PEPC over a long period

Exposure of leaves detached from *A. hypochondriacus* plants and incubated at 50°C (dark adapted) for a period over 150 min time course along with illuminated as well as dark adapted leaves. There was a rapid decline of PEPC activity and malate sensitivity compared to dark and illuminated leaves. In addition, this treatment caused a significant increase in phosphorylation only after about 120 min, while the inhibition by malate is apparently by 30 min (Fig. 4.6A, **B).**



Autoradiography

Activity of PEPC	789	1610	989	842	691	585	509
Inhibition by malate (%)	84	61	45	40	32	27	19

Fig. 4.6. The pattern of phosphorylation of PEPC in leaf discs of *Amaranthus hypochondriacus* exposed to either dark-light treatment or temperature of 50°C (only dark-incubation) with over a period of two and half hours. The extracts from leaves of *A. hypochondriacus* labeled with ^{32}Pi, were prepared after the given treatments. The PEPC enzyme was immunoprecipitated from leaf discs, separated on **SDS-PAGE** and examined for protein levels as well as radioactivity. The Figure includes gels (A) Stained with Coomassie Blue; (B) exposed to **autoradiography.** Extracts equal to 0.2 units of PEPC were used in each lane of rows A and B.

The effects of temperature on PEPC in presence of PEG-6000

Since there was no change in the phosphorylation status of PEPC on exposure to temperature, the other possibility was a change in the conformational status of enzyme. Compatible solutes like PEG-6000 promote the oligomerization of PEPC, increase the enzyme activity and decrease its malate sensitivity (Huber and Sugiyama, 1986). The effect of temperature on PEPC from leaf discs of *A. hypochondriacus* (C_4) was therefore assessed with or without PEG-6000 in the assay medium (Table 4.5). In the absence of PEG-6000, the activity of PEPC decreased at sub- or supraoptimal temperature and the extent of inhibition by malate decreased steeply at 50°C compared to that at optimal temperature of 45°C. The temperature induced changes in malate sensitivity of PEPC were dramatically dampened with the addition of PEG in the assay medium.

Discussion

The results from this study demonstrate that a change in temperature can induce quite dramatic changes in not only the activity but also the malate sensitivity of PEPC in both C_3 and C_4 plants. The changes were caused by a short duration of exposure to temperatures and were reversible to a large extent. The temperature induced changes can therefore be physiologically relevant and important.

It may be argued that other factors may possibly affect the physiology of leaf discs at different temperatures for e.g., concentration of dissolved CO2, oxidative stress, or induction of heat shock proteins. The marked reversibility

Table 4.5. The effect of including PEG-6000 in the assay medium on the response of PEPC from leaf discs of *Amaranthus hypochondriacus* to temperature. The extracts were prepared from leaf discs exposed to varying temperature and the characteristics of PEPC were determined in the absence or presence of 1.25% (w/v) PEG-6000.

Temperature and parameter	No PEG	+ PEG
PEPC Activity (μmol mg^{-1} Chl h^{-1})		
Sub-optimal 15°C	585 (40)*	668 (39)
Optimal 45°C	1441 (100)	1732(100)
Supra-optimal 50°C	734(51)	827 (47)
Inhibition by malate (% of control)		
Sub-optimal 15°C	70	58
Optimal 45°C	46	49
Supra-optimal 50°C	40	56

The figures in parentheses represent the activity as % that at optimal temperature

temperature effects of PEPC support the concept that leaf discs are ideal to simulate the conditions *in vivo*.

The optimal temperature for PEPC in *A. hypochondriacus* (C4) as 45°C, compared to 30°C in *P. sativum* (C3 species), is not surprising. The data in Fig. 4.1 and Table 4.3 confirm that the C_4 PEPC is quite sensitive to sub-optimal temperatures compared to the PEPC of C_3 species. Thus, our results endorse the suggestion that PEPC is one of the cold sensitive enzymes in C_4 plants (Phillips and McWilliam, 1971; Selinioti et al., 1986).

The sharp increase in the activity of PEPC with temperature, particularly above 15°C, could be physiologically significant, as the temperature is expected to rise from about 10 to 15°C in the morning to 35 to 40°C at midday, on a typically clear and sunny day. A combination of light and warm temperature could amplify the photoactivation of the PEPC, as observed in case of *Egeria densa* (Casati et al., 2000) and *Amaranthus paniculatus* (Selinioti et al., 1986). The marked reversibility of the effect of temperature on PEPC in case of both C_4 and C_3 plants (Fig. 4.4) is an additional indication of the possible physiological relevance of temperature effects on PEPC.

These results demonstrate clearly the dramatic changes induced by temperature in the sensitivity of PEPC to malate. As the temperature was raised from 15°C to 50°C, there was a marked decrease in malate sensitivity of PEPC. The extent of such decrease in C_4 plants (85 to 39%) was more than that in C_3 species (66 to 34%) (Fig. 4.2; Table 4.4). Thus, PEPC appeared to be highly

sensitive to malate at cold temperature, while becoming relatively insensitive **to** malate at warm temperature. **The extent of malate inhibition** is quite high in case of *P. sativum* (C3 species) **than that of** *A. hypochondriacus* (C4 plant). Again the limited reports in the literature had conflicting observations. At low temperature, the sensitivity of PEPC to malate was very high in maize (Wu and Wedding. 1987) but was quite low in case of *Bryophyllum fedtschenkoi* (Carter et al., 1995). Lowering the temperature from 25 to 3°C not only decreased the catalytic capacity of PEPC but also caused a considerable reduction (about 10-fold) in the sensitivity of PEPC to malate (Carter et al., 1995).

The decrease in malate sensitivity of PEPC can also occur due to the proteolysis of enzyme. However, we are confident that this is not the reason during our observations. There was no detectable change in the protein levels as indicated by the western blots (Fig. 4.5C). The changes in activity of PEPC due to temperature were reversible to a marked extent (Fig. 4.4). The inclusion of chymostatin did not cause any change in the pattern of results (Table 4.1).

Arrhenius plots revealed interesting differences between not only the C_3 and C_4 plants, but also the pattern in presence or absence of malate (Fig. 4.3). As the temperature was raised, the activation energy was lowered in both *A. hypochondriacus* (C_4 plant) and in *P. sativum* (C3 species). The changes in activation energy as indicated by discontinuities ("breakpoints") in Arrhenius plots at a critical temperature can be an indication of the cold lability of PEPC from different species (McWilliam and Ferrar, 1974; Graham et al., 1979; **Selinioti** et

al., 1986). The break at 27°C in case of C_3 plants suggests that the C_3 enzyme does not respond much to temperatures, above 27°C. In contrast, the absence of such break and the continuation of slope indicate that the activation energy for continues to decrease C4-PEPC as the temperature rises from 27°C to 45°C. A similar trend is reflected in the activation energies calculated from these Arrhenius curves. The activation energy of C_4 PEPC was 3 to 4 in the range 17 to 45°C, while the activation energy of C_3 PEPC fell to about 1, above the temperature of 27°C (Table 4.2).

The presence of malate increased significantly the activation energy in both C_3 and C_4 species (Table 4.2). Such increase in activation energy of PEPC in presence of malate, an inhibitor, is logical as malate being an effective inhibitor may slow down the thermodynamic responses of PEPC. But in presence of malate the activation energy increased nearly two-fold over that in the absence of malate, during the temperature range of 10°C to 27°C in both *A. hypochondriacus* (C_4 plant) and *P. sativum* (C_3 species). The limited studies made earlier on the activation energy of PEPC again were conflicting. Some of the reports indicate discontinuity in the Arrhenius plots of PEPC (Phillips and McWilliam, 1971) while others did not observe such break points (Du et al., 1999b).

The regulation of PEPC is achieved by posttranslational modification of the enzyme, by phosphorylation of a serine residue near the N-terminus of PEPC (Rajagopalan et al.. 1994; Chollet et al., 1996; Vidal and Chollet, 1997). The phosphorylation of the enzyme leads to an increase in the activity of enzyme and

decrease in the malate sensitivity. The changes **induced by rise in temperature,** namely **the** increase in the activity and decrease in **the extent of malate inhibition** are quite similar to the changes effected during light **activation of** PEPC. It is therefore, quite possible that there is a change in phosphorylation status of PEPC on exposure to temperature. However, our experiments **rule** out the possible role of phosphorylation of enzyme in temperature effects of PEPC (Fig. 4.5). This is the first report that the temperature induced changes in PEPC of C_4 species is independent of phosphorylation.

An attractive and alternative possibility is the change in the conformational status of the enzyme. PEPC is very active when it is in a tetrameric shape, while its activity and malate sensitivity decreases when the enzyme dissociates into a monomer or dimer (Shi et al., 1981; Walker et al., 1986; Willeford et al., 1990). Temperature may affect the oligomeric status of the enzyme PEPC. Rise in temperature causes the aggregation of PEPC in case of C4 and dissociation in case of CAM (Wu and Wedding, 1987). Cold/chilling may make the enzyme to dissociate from active tetrameric shape to less active dimers or monomers. However, this is not well corroborated in case of C4-PEPC (Walker et al., 1986). McNaughton et al. (1989) reported that changes in oligomerization state of PEPC may not be related to malate sensitivity and light induced changes. It is possible that other types of conformational changes would still occur, for e.g. in the hydrophobic microenvironment of the protein.

Compatible solutes like PEG-6000 protect the enzymes against a variety **of adverse conditions *in vitro* by increasing the local concentration of protein** (Timasheff, 1992). **Our experiments suggest that the temperature** may **modulate the conformation** of **PEPC, presumably** by **changing** the **local** hydrophobic microenvironment of enzyme. The **marked** damping of temperature responses of PEPC when PEG-6000 was **included during** the assay (Table 4.5) is an indication that temperature may be causing reversible changes in the conformational status of PEPC. It would be of great interest to examine further such changes.

Another important observation **from** our results is that the high activity of PEPC is not always associated **with low level of** malate sensitivity, during temperature responses (Fig. 4.1; 4.2) and exposure to PEG-6000 (Table 4.5). Such no-relationship of PEPC activity and malate sensitivity has been noticed earlier by Carter et al. (1995), in case of PEPC from *Bryophyllun fedtschenkoi.*

These observations suggest that another significant mode of regulation of PEPC. different from phosphorylation, occurs with varying temperature. This could be largely due to the change in conformational status of enzyme involving either the oligomeric status and/or the microenvironment of protein, e.g., folding hydrophobic regions.

Major conclusions from the results presented in this chapter are:

1. Temperature caused a dramatic modulation of PEPC in leaf discs of *A. hypochondriacus* (C_4) as well as *Pisum sativum* (C3). There was a strong

contrast in the **temperature optima** for PEPC activity in leaf discs of *A. hypochondriacus* (C_4 plant) **and** *P. sativum* (C3 species).

2. The steep increase in activity of PEPC with rise in temperature could be physiologically significant, as the temperature is expected to rise from about 10 to 15°C in the **morning** to 35 to 40°C at midday, on a clear and sunny day.

3. Temperature induced changes in the properties of PEPC were reversible to large extent. **This** marked reversibility of the effect of temperature of PEPC in case of both C_4 and C_3 is an additional indication of the physiological relevance of temperature effects on PEPC.

4. During illumination, the phosphorylation of the protein leads to an increase in the activity of PEPC and decrease in the malate sensitivity. The changes induced by rise in temperature (increase in the activity and decrease in the extent of malate inhibition) are quite similar to the changes during light activation of PEPC. However, our results indicate that the phosphorylation is not the main reason for the temperature effects **on** PEPC.

5. In the presence of PEG-6000 during the assay, which undergoes marked damping of temperature responses suggesting that the temperature may be causing reversible changes in the conformational status of PEPC.

In the next chapter, studies were done with purified PEPC *of Amaranthus hypochondriacus* (C_4) to check the changes induced by temperature of PEPC properties *in vitro* compared to *in vivo.*

Chapter 5

Modulation by Temperature of Purified C_4 PEPC: Protection by PEG-6000 Against Changes in Malate Sensitivity of the Enzyme

Chapter 5

Modulation by Temperature of Purified PEPC: Protection by PEG-6000 Against Changes in Malate Sensitivity of the Enzyme

Many C_4 species are cold sensitive and one of the factors associated with this phenomenon is the modulation by cold temperature of key enzymes of C4 pathway, such as PPDK or PEPC (Leegood and Walker, 1999; Du et al., 1999a). At low temperature, PPDK, a chloroplastic enzyme is inactivated due to dissociation and such inactivation/dissociation is prevented by glycerol, sucrose or sorbitol (Shirahashi et al., 1978). We have already shown that C_4-PEPC is extremely sensitive to cold temperature (Chinthapalli et al., 2003). It is possible that solutes such as glycerol or PEG-6000 can protect PEPC against temperature induced changes.

The sensitivity of PEPC to malate is influenced by various factors, such as light, temperature, pH and Glc-6-P. When leaves are illuminated, there is a marked decrease in sensitivity of PEPC to malate besides an increase in the enzyme activity. The changes during light activation are all due to the phosphorylation of the enzyme. We have recently demonstrated that there is a marked decrease in the sensitivity to malate of PEPC in leaf discs, as the temperature was raised from 15°C to 50°C. However, there was no difference in the extent of phosphorylation of PEPC at varying temperature in leaves of *Amaranthus hypochondriacus* (Chinthapalli et al., 2003).

Compatible solutes, like PEG-6000, promote the oligomerization of PEPC, increase the enzyme activity and decrease its malate sensitivity (Huber and Sugiyama, 1986). Protection of enzyme activity against high temperature was shown also in presence of glycerol, although PEG was more effective than glycerol (Drilias et al., 1994). Besides PEPC, other cytosolic enzymes know to be activated by PEG are: pyruvate kinase from germinating castor oil seed endosperm (Podesta and Plaxton, 1993) and fructose 1,6-phosphatase (FBPase) (Hodgson and Plaxton, 1995).

The previous chapter described the characteristics of modulation by temperature of PEPC in leaf discs of *A. hypochondriacus* (C_4 species) compared with that in *P. sativum* (C_3 plant). The work described in this chapter is undertaken to study the properties of PEPC protein purified from the leaves of *Amaranthus hypochondriacus* and preincubated to varying temperature. We have also attempted to assess the role of PEG-6000 in modulating the responses of PEPC protein to varying temperature.

Results

Purification of PEPC by conventional method

Purification of PEPC from leaves is done, as already described (Gayathri et al., 2000).

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 and Hydoxylapatite (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 mg^{-1} protein (Fig. 5.1). When eluted from the hydrophobic interaction chromatography using DEAE-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 34.3 U mg^{-1} protein from HAP column (Fig. 5.2). The most notable property observed in the course of purification procedure was that the enzyme strongly bound on hydrophobic columns. Finally, the enzyme was concentrated by using solid PEG 20,000.

After the above three steps, PEPC was purified by 93-fold, with a final specific activity of 34 U mg^{-1} protein, and an overall recovery of about 59% (Table 5.1). The purity of the enzyme was confirmed by the appearance of a single band of about 100-kD on SDS-PAGE (Fig. 5.3).

Duration of incubation at different temperatures

The activity of purified PEPC changed gradually on exposure to varying temperatures, and the responses stabilized by 15 to 30 min, except in case of 45°C (Fig. 5.4A). The activity increased at 45°C, was stable at 30°C and decreased at 15°C or 50°C. Similar trend in the speed of response was noticed in the sensitivity to malate of PEPC (Fig. 5.4B). By 45 min of exposure, the malate sensitivity of PEPC increased at 15°C, was stable at 30°C and decreased at 45°C as well as at 50°C. The optimal incubation of 45 min was used for all subsequent experiments.

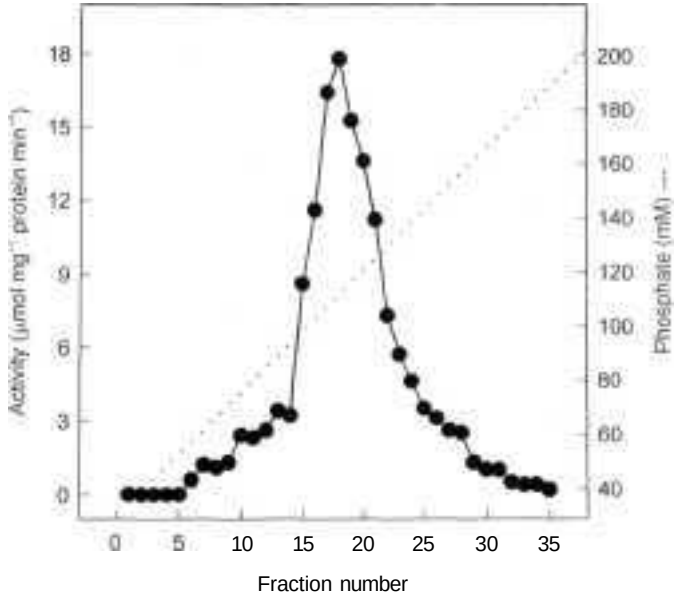


Fig. 5.1: The pattern of PEPC activity during elution from a DEAE-sepharose column. The enzyme from 40-60% ammonium sulfate fraction of leaf extracts was loaded after dialysis, onto a DEAE-sepharose (1 x 12 cm) column. preequilibrated with 20 mM potassium phosphate buffer (pH 7.3) containing 10% (v/v) glycerol. The column was washed with the same buffer and was eluted with a linear gradient from 40-200 mM phosphate buffer (pH 7.3) 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".

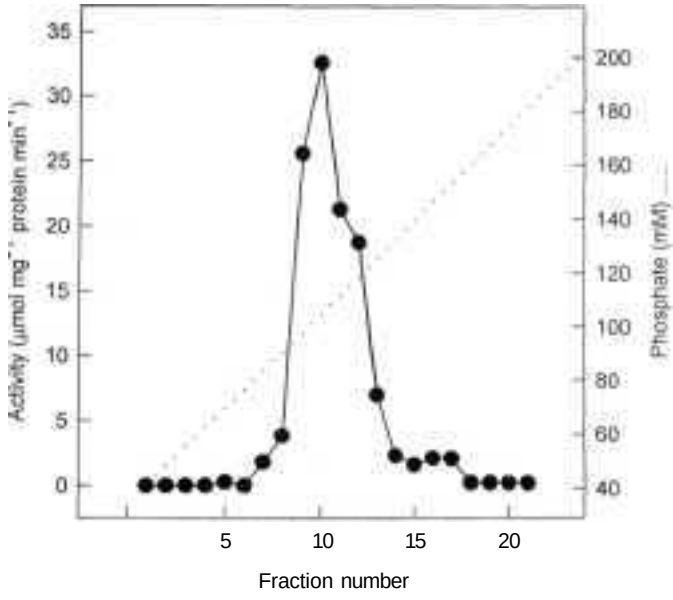


Fig. 5.2: The pattern of elution profile of PEPC from hydroxylapatite column. The active fractions obtained from DEAE-sepharose column were pooled and precipitated by 60% ammonium sulphate. The precipitate was dissolved in 200 mM phosphate buffer (pH 7.3) containing 10% (v/v) glycerol and dialyzed against 20 mM phosphate buffer (pH 7.3) containing 10% 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 5.1. The purification pattern of PEPC from leaves of *Amaranthus hypochondriacus* by conventional methods involving three steps.

Step	Total activity	Total protein	Specific activity	Purification	Yield
	(μmol min^{-1})	*(mg)*	*(μmol mg^{-} protein miri)*	*(Fold)*	*(%)*
Crude extracts	957	2487	0.37	1.0	100
40-60% $(NH_4)_2SO_4$	895	527	1.7	**4.5**	**93**
DEAE-Sepharose	818	46.2	17.8	48.2	85
Hydroxylapatite	567	16.4	34.3	93.5	59

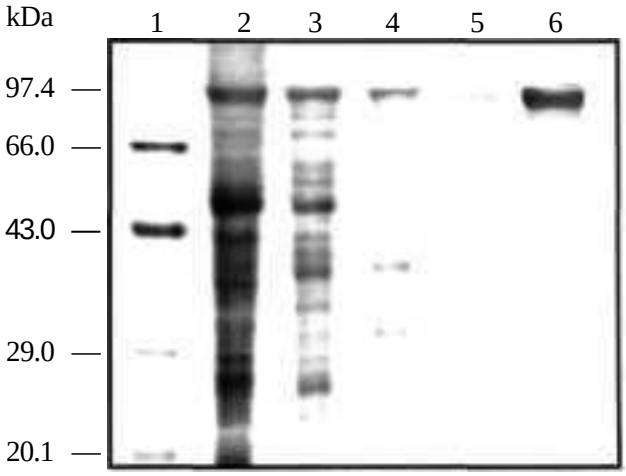


Fig. 5.3: Purification of PEPC from *Amaranthus hypochondriacus* leaves by conventional method involving three steps. Samples from various steps were analyzed by SDS-PAGE and the gel was stained with silver nitrate. Lane 1: Molecular weight markers; Lane 2: Crude extract; Lane 3: 40-60% ammonium sulphate precipitate; Lane 4: Pool of active fractions from DEAE-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 position 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 8 μg protein, while Lane 4 and 5 contained 2 (ig and lane 6 contained 4 μg protein. Further details are described in "Materials and Methods".

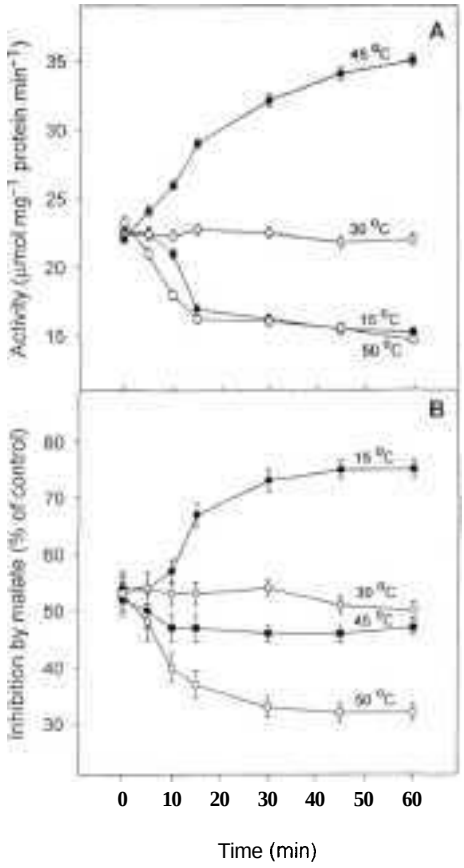


Fig. 5.4: The activity and malate sensitivity of PEPC purified from *Amaranthus hypochondriacus* leaves as a function of preincubation time. The enzyme is preincubated at varying temperatures for different periods as shown and the enzyme was assayed at 30°C. The activity of PEPC (A) and the inhibition by 0.5 mM malate (B) are represented.

Changes in the activity and malate sensitivity of PEPC at varying temperature

The activity of PEPC was maximal at 40°C. The response of enzyme to temperature was quite dramatic when plotted as % of maximum activity (Fig. 5.5A). The decrease in the activity of PEPC at 15°C was much greater than that at 50°C upon incubation. Thus, the purified PEPC protein from *Amaranthus hypochondriacus* (a C_4 species) was more sensitive to suboptimal temperature than that to supraoptimal temperature. As the temperature was raised from 15°C to 50°C, there was marked decrease in the extent of inhibition by malate (from 68 to 33%). The inclusion of PEG-6000 during preincubation, decreased slightly the response of PEPC activity to temperature, but dramatically desensitized PEPC to temperature induced changes in the malate sensitivity. The extent of malate inhibition stayed in a narrow range of 51 to 48%, as the temperature was raised from 15°C to 50°C (Fig. 5.5B).

Reversibility of the effect of temperature

The purified PEPC was incubated at optimal temperature of 40°C and treated as control. The PEPC protein was exposed to various temperatures (for 45 min) and then transferred back to the optimal temperature of 40°C, the next (for 45 min). The changes in the activity as well as malate inhibition of PEPC at either suboptimal or supraoptimal temperature, were significantly reversed (Fig. 5.6A, B). The reversibility was partial when the PEPC protein was exposed to supraoptimal temperatures 50°C. Thus, changes, induced by temperature, in the activity and malate sensitivity of PEPC were reversible to a large extent.

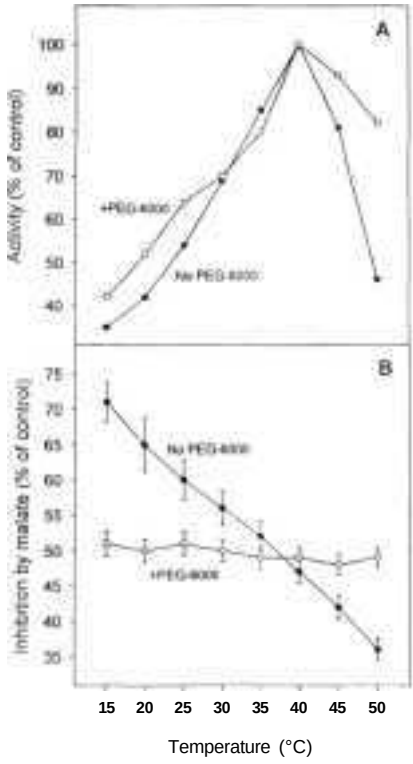


Fig. 5.5: The activity and malate sensitivity of PEPC purified from *Amaranthus hypochondriacus* (C_4 plant) after exposure to varying temperature for 45 min with and without 1.25% (w/v) PEG-6000. The malate sensitivity is assessed by checking the PEPC activity in presence of 0.5 mM malate. The preincubated time for purified protein was 45 min for each temperature. The maximal activity of PEPC with or without PEG-6000 was 31 ± 0.42 and 54 ± 0.52 μmol mg^{-1} protein min" , respectively.

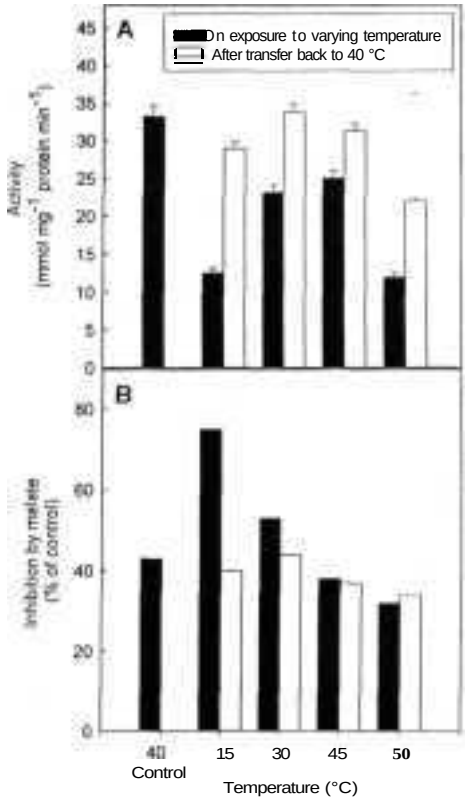


Fig. 5.6: The reversibility of the effects of temperature on purified PEPC. The enzyme exposed to varying temperature for 45 min and then transferred back to the 40°C (optimal temperature-control) and kept for 45 min. The activity of PEPC (A) and the inhibition of enzyme by 0.5 mM malate (B) are represented.

*Temperature induced changes in the properties of **PEPC***

The modulation by **temperature** of **kinetic and regulatory properties of** PEPC was examined in **detail** (Table 5.2). **At low or suboptimal temperature, the** velocity of enzyme decreased by 40%, while **the K_m for** PEP increased by >4.5-fold, compared to that at the optimal temperature. Similarly, K_i for malate decreased by about 35% and the K_a for G-6-P increased by almost 2-fold, as the temperature was lowered from 40°C to 15°C. **When** PEG-6000 was included during the assay, changes in (V_{max}) or K_a (G-6-P) of the enzyme increased due to suboptimal or supraoptimal temperatures were retained. In a strong contrast, the changes in K_m (PEP) or K_i (malate) were markedly dampened.

Effect of PEG-6000 or glycerol during the preincubation at varying temperature

The effect of temperature on purified PEPC was therefore assessed with or without PEG-6000 during preincubation at different temperatures. The treatments of glycerol and increased protein were included for further comparison. In the absence of PEG-6000, the activity decreased at sub- or supraoptimal temperature, while the extent of inhibition by malate decreased steeply from 15°C to 50°C. When PEPC was assayed in presence of PEG-6000 in preincubation medium, the activity increased with the rise in temperature from 15°C to 50°C, but there was only a marginal change in malate sensitivity (Table 5.3). Glycerol did not exert such dramatic effect. There were only marginal differences In the temperature induced changes in the activity and malate sensitivity of PEPC in presence or absence of glycerol. Similarly, there was no detectable change in pattern of

Table 5.2. Changes in the kinetic and regulatory properties **of PEPC protein** after exposure to the required temperature in *Amaranthus hypochondriacus,* in the presence or absence 1.25% (w/v) PEG-6000, during the assay. The changes caused by PEG-6000 were all statistically significant ($P<0.01$).

Parameter	Temperature		
	Suboptimal (15°C)	Optimal (40°C)	Supraoptimal (50°C)
No PEG-6000			
V_{max} (μmol mg^{-1} protein min^{-1})	19.4±0.12	32.1±0.20	24.7±0.20
K_m (PEP) (mM)	0.78±0.04	0.17±0.01	0.45±0.02
K_i (malate) (mM)	0.55±0.02	0.87±0.04	1.17±0.08
K_a (G-6-P) (mM)	1.05±0.07	0.55±0.04	0.76±0.06
1.25% (w/v) **PEG-6000**			
V_{max} (μmol mg^{-1} protein min^{-1})	22.3±0.14	42.5±0.24	34.2±0.18
K_m (PEP) (mM)	0.47±0.02	0.22±0.01	0.28±0.01
K_i (malate) (mM)	0.87±0.04	1.37±0.08	1.40±0.09
K_a (G-6-P) (mM)	1.21±0.08	0.58±0.03	0.62±0.04

Table 5.3. The effect of including PEG-6000 **or glycerol or high protein** concentration, **during** preincubation, on the response **of PEPC to temperature. The** purified **PEPC** (50 $\mu g\ mL^{-1}$) protein exposed to varying **temperature in the absence** or **presence of** 1.25% (w/v) PEG-6000, 10% (v/v) glycerol or 200 $\mu g\ mL^{-1}$ protein (in stead of 50 $\mu g\ mL^{-1}$) and the characteristics of PEPC protein were determined.

Parameter/Addition during the preincubation	Temperature		
	Suboptimal (15°C)	Optimal (40°C)	Supraoptimal (50°C)
Activity ($\mu mol\ mg^{-}$ protein min^{-1})			
No PEG-6000 nor Glycerol	11.2 ± 0.40 (34)[a]	32.3 ±0.44 **(100)**	13.9 ±0.41 (42)
+ PEG-6000	23.2 ± 0.42 (42)	54.1 ±0.51 **(100)**	44.4 ± 0.42 (82)
+ Glycerol	16.1 ±0.39(40)	39.4 ±0.47 (100)	**19.6** ±0.40 (49)
4X Protein	13.5 ±0.41 (38)	35.0 ±0.48 **(100)**	15.1 ±0.40(43)
Inhibition by malate (% of control)			
No PEG-6000 nor Glycerol	68 + 3	50 ±2	33 ±2
+ PEG-6000	51 ±3	49 ±2	49 ± 1
+ Glycerol	70 ±3	53 ±3	47 ± 1
4X Protein	70 + 3	51 +2	33 + 1

The figures in parentheses represent the % of maximum (at 40°C).

temperature reponses, when the amount of PEPC protein was raised 4 times (from 50 $\mu g\ mL^{-1}$ to 200 $\mu g\ mL^{-1}$).

Effect of PEG-6000 or glycerol on oligomerization at varying temperature

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 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 45 min with PEG or glycerol and the nature of protein was examined on native PAGE (Fig. 5.7). Native PAGE analysis shows that cold temperature tends to shift the equilibrium of PEPC protein towards tetramer, where as at higher temperature there is a loss of tetramer. Glycerol had only small effect at higher temperature.

Discussion

The present results establish that temperature modulates the properties of PEPC *in vitro* (Fig. 5.4) as well as *in vivo* (Chinthapalli et al., 2003). The temperature induced changes in the activity as well as malate sensitivity were reversible. An interesting feature is that at low temperature, there is not only a marked decrease in the activity of the enzyme, but also a decrease in the malate sensitivity of PEPC (Fig. 5.4). These results endorse the earlier observations that C_4- PEPC is sensitive to cold temperatures (Angelopoulos and Gavalas, 1988. Wu and Wedding, 1987, Krall and Edwards, 1993. Du et al., 1999b, Chinthapalli et al., 2003).

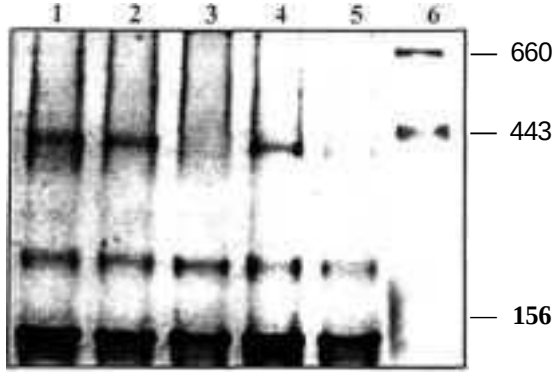


Fig. 5.7: Effect of PEG or glycerol or both on the structure of PEPC during native PAGE. Lanes 1 to 3: Protein incubated at 15, 30 and 50°C in **50** mM **TRIS-HC1,** pH 7.3; lane 4: Protein incubated at 50°C with 1.25% PEG-6000; lane 5: Protein incubated at 50°C with 10% glycerol; lane 6: Molecular weight markers. PEPC was incubated at 50°C with these compatible solutes for 45 min and subjected to nondenaturing PAGE and were stained with silver nitrate.

Inclusion of PEG-6000 in the preincubation mixture not only increased the activity of PEPC protein, but also dampened changes in malate inhibition of the enzyme at both low and high temperatures (Fig. 5.5B and Table 5.3). The marked protection by PEG-6000 of PEPC against malate inhibition, particularly at low temperature (Fig. 5.5B) is extremely interesting. Krall and Edwards (1993) reported that the presence of compatible solutes favors the existence of PEPC in tetrameric form. We (Chinthapalli et al.. 2003) have recently observed that temperature induced reversible changes in PEPC of leaf discs may involve conformational changes in PEPC. The sharp increase in the activity of PEPC with temperature, with no change in malate sensitivity, in presence of PEG-6000, suggests that these changes are independent of phosphorylation. A change in subunit interactions and/or quaternary structure can influence both PEPC activity as well as malate inhibition, and this may be the basis for the temperature modulation observed. This phenomenon needs to be studied further.

The reversibility of the effect of temperature on PEPC (Fig. 5.6) is quite significant. The reversibility was partial, when the PEPC protein was exposed to supraoptimal temperatures 50°C. This is quite similar to the previous report in leaf discs of *Amaranthus hypochondriacus* (Chinthapalli et al., 2003). This marked reversibility is an additional indication of the physiological significance of temperature effects on PEPC.

The changes in kinetic characteristics of PEPC with varying temperature are interesting. As the temperature was raised from 15°C to 40°C, there is an

increase in V_{max} and decrease in K_m (PEP), with further enhancement in presence of PEG-6000 (Table 5.2). The decrease in K_m (PEP) due to a shift of the enzyme equilibrium towards the tetrameric form, can be caused by either PEG or glycerol (Podesta and Andreo, 1989; Stamatakis et al., 1988). An increase of K_m for PEP due to temperature rise (15°C to 35°C) was also found for PEPC in *Kalanchoe diagremontiana*, a CAM plant (Buchanan et al., 1984, Osmond and Holtum, 1981). However, the effects of temperature or PEG-6000 reported herein could be also due to conformational changes of the tetrameric form itself.

There is striking influence of temperature on sensitivity of PEPC to malate. The present results (Fig. 5.4B, Table 2 and 3) illustrate that, with the rise in temperature, there is an increase in K_i (malate) and decrease in K_a (G-6-P). However, in presence of PEG-6000, there was no significant increase in K_i (malate) (Table 2). Similar effects are observed in maize by Wu and Wedding (1987). It is noticed that PEG-6000 reduces malate inhibition of PEPC in both crude extracts as well as purified preparation in light/dark (Huber and Sugiyama 1986). However, low level of malate inhibition was not always associated with the high activity of PEPC, particularly in presence of PEG-6000 (Fig. 5.5A; B). It would be interesting to reexamine the correlation between the activity and the malate sensitivity of PEPC, under varied experimental conditions.

The information on the activity of monomer or dimer of PEPC is ambiguous. The activity of tetramer was much greater in presence of PEG and/or glycerol than that in their absence. It is obvious that the presence of PEG or

glycerol is effective in maintaining the tetrameric structure of the enzyme and keeping up high specific activity (Fig. 5.7). PEG has been found to promote the self association and/or activation a number of regulatory enzymes in dilute solutions (Salvucci, 1992; Podesta and Plaxton, 1993). V/hereas, 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). However the present study demonstrate for the first time that PEG can also markedly influence the structural properties of PEPC with regard to temperature. Native PAGE analysis shows that cold temperature tends to shift the equilibrium of PEPC protein towards tetramer. where as at higher temperature there is a loss of tetramer. These results endorse with the above finding that the addition of PEG-6000 to the PEPC protein during incubation for 45 min at higher temperature tends to promote the association of PEPC and regain the tetrameric form. Glycerol had only small effect at higher temperature.

The present stud}' emphasizes that PEG-6000 can be an excellent tool to study the properties of PEPC and their response to temperature. While PEG-6000 may promote the tetrameric status of the enzyme, the temperature appears to cause additional conformational changes in the PEPC protein. Further experiments are necessary to study and establish the physiological relevance of these responses.

Major conclusions from the results presented in this chapter are:

1. PEPC was purified from leaves of *A. hypochondriacus* with a specific activity of 34 U mg^{-1} protein.
2. Temperature caused marked and reversible changes in the properties of PEPC protein purified from *A. hypochondriacus* leaves, implicating the physiological significance of temperature effects and PEPC could be one of the biochemical basis of temperature responses of C_4 plants.
3. As the temperature was raised from 15°C to 50°C, there was an increase in V_{max} upto 40°C, while there was a marked decrease in malate sensitivity of PEPC.
4. Temperature caused marked and reversible changes in the properties of PEPC protein purified from *A. hypochondriacus* leaves. Since these experiments were all done *in vitro* with purified PEPC, the changes are obviously independent of phosphorylation.
5. The presence of PEG-6000 was effective in protecting PEPC against cold inactivation, indicating the importance of the conformation of PEPC.
6. Nondenaturing PAGE of purified PEPC showed the existence of three different forms with proportionally increasing molecular weight: monomer, dimer and tetramer. Cold temperature tends to shift the equilibrium of PEPC protein towards tetramer. The presence of PEG and/or glycerol resulted in predominance of tetramer.

In the next chapter, studies were extended to check the temperature induced conformational changes by monitoring intrinsic and extrinsic fluorescence.

Chapter 6

Effect of Temperature on Conformational Changes in Purified PEPC as Indicated by Intrinsic and Extrinsic Fluorescence

Chapter 6

Effect of Temperature on Conformational Changes in Purified PEPC, as Indicated by Intrinsic and Extrinsic Fluorescence

PEPC has an average molecular weight of 400-kD and is a homotetramer (Andreo et al., 1987; Chollet et al., 1996). The enzyme is very active when it is in tetrameric shape, while the activity and malate sensitivity decreases when the enzyme dissociates into monomer or dimer (Walker et al., 1986; Willeford et al., 1990).

In several studies on plant PEPC, it has been observed that stability of enzymes is strongly associated with the maintenance of multimeric structure, whereas the monomeric or dimeric structures tend to lose enzyme activity (Jensen et al., 1995; Willeford and Wedding, 1992; Willeford et al., 1990). The presence of PEP, Mg^{+}, G-6-P, malate or compatible solutes like PEG-6000 or glycerol can promotc the aggregation of the PEPC (Podesta and Andreo, 1989; Manetas, 1990; Wedding et al., 1994; Chinthapalli et al., 2003).

PEPC undergoes posttranslational modification by reversible phosphorylation (Chollet et al., 1996; Vidal and Chollet, 1997) or shift in oligomeric state of the enzyme (Rajagopalan et al., 1994; Chollet et al., 1996). Compared to the extensive literature on posttranslational modification of enzyme in C_4 PEPC, studies on conformational changes other than phosphorylation and oligomerization of PEPC are limited. There are events where regulatory phosphorylation is not involved for the increase in PEPC activity and subsequent

decrease in malate sensitivity of the enzyme (Chinthapalli et al., 2003). At low temperature the activity of the enzyme decreases and malate sensitivity increased in C_4 plants, and at high temperature enzyme activity increased and malate sensitivity decreased. These changes appear to be due to aggregation of PEPC rather than phosphorylation (Wu and Wedding, 1987; Chinthapalli et al., 2003).

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

The present study is an attempt to characterize the conformational changes, if any, of PEPC from a typical C_4 plant, *A. hypochondriacus* on exposure to varying temperature. The conformational changes are monitored by recording either intrinsic or extrinsic fluorescence with the help of ANS. Studies were

further extended to check these temperature induced changes with those of effectors, compatible solutes, and denaturants like urea.

Results

Studies on intrinsic fluorescence:

Fluorescence emission spectra of PEPC purified from *A. hypochondriacus* were examined, using different concentrations of protein in the medium (Fig. 6.1). When the protein was excited at 280 nm, the emission of fluorescence had a maximum at 335 nm. The intensity of fluorescence emission increased, as the concentration of protein was raised from 5 μg to 30 μg mL '. Further experiments were done with 10 μg mL^{-1} of PEPC protein.

Effect of temperature

The intrinsic fluorescence spectra of PEPC decreased markedly with the increase in temperature from 15°C to 45°C (Fig. 6.2). In addition, there was a shift in emission maxima from 339 nm, to 335 nm and to 333 nm at 15°C, 30°C and 45°C respectively.

The reversibility of temperature effects

The next set of experiments was designed to assess if the changes induced in the fluorescence of PEPC protein at varying temperatures, were reversible. The purified PEPC protein was preincubated at 15°C, 30°C or 45°C for 45 min and then the samples were transferred back to 30°C. The fluorescence emission spectra were recorded over a period of 120 min at an excitation of 280 nm.

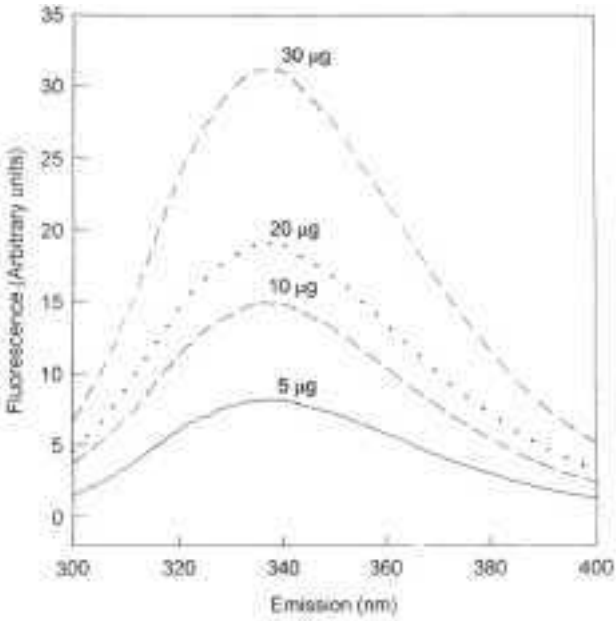


Fig. 6.1. Intrinsic fluorescence spectra of PEPC purified from *A. hypochondriacus* leaves after exposure to 30°C for 45 min. Different concentrations of protein in the incubation medium were used. Excitation of protein was at 280 nm.

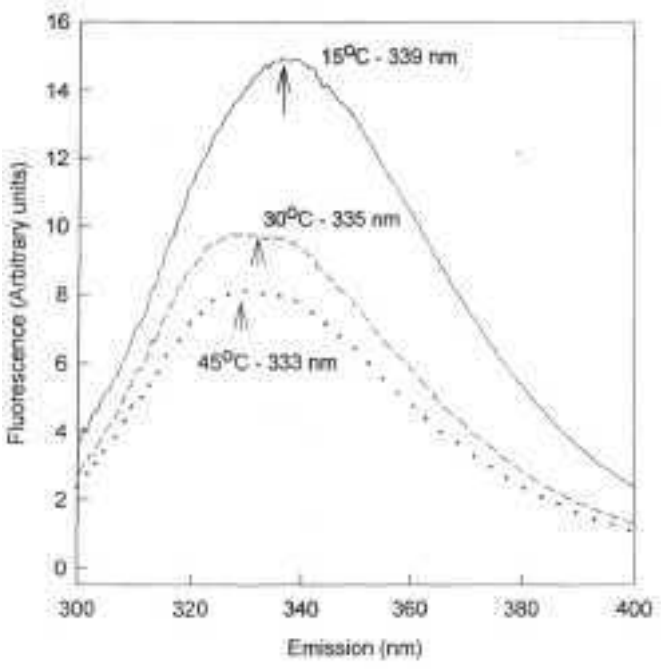


Fig. 6.2. Intrinsic fluorescence spectra of PEPC purified from *A. hypochondriacus* leaves (10 μg PEPC/ml in buffer A, see 'Materials and Methods') after exposure to different varying temperatures of 15°C, 30°C or 45°C for 45 min. The spectrum was then recorded immediately at 30°C using excitation wavelength of 280 nm. The intensity of fluorescence decreased with increase in temperature with a slight shift in emission maxima (indicated by arrows): from 339 nm (15°C), to 335 nm (30°C) or 333 nm (45°C).

Soon after the exposure to varying temperature, i.e., at 0 min after treatment, there was a decrease in the intensity of fluorescence at 45°C and increase at 15°C, compared to that 30°C (Fig. 6.3). This pattern is similar to the trend already shown in Fig. 6.2. However, after 120 min exposure to the normal temperature of 30°C, these differences were minimized, indicating the temperature induced changes were reversible.

Influence of effectors on temperature induced changes

The pattern of fluorescence emission can also provide a measure of qualitative changes in the enzyme in response to the binding of malate or Glc-6-P. In the presence of L-malate, the competitive inhibitor and Glc-6-P, the allosteric activator of the enzyme PEPC exhibited a significant difference between the emission spectra at different temperatures was observed (Table 6.1).

The signal around 340 nm was enhanced in presence of Glc-6-P at 15°C whereas the intensity of fluorescence at 30°C and 45°C were almost similar. There was a progressive quenching of intrinsic fluorescence in presence of malate at all temperature tested (Fig. 6.4).

PEG-6000 promote oligomerization of PEPC (Huber and Sugiyama, 1986) and urea is a well known denaturant and dissociates proteins (Encinas et al., 2002). Attempts were made, therefore to study the fluorescence in presence of PEG-6000 and urea. The presence of PEG increased the intrinsic fluorescence of PEPC and such increase was more pronounced at 15°C than that at 30°C or 45°C (Fig. 6.5).

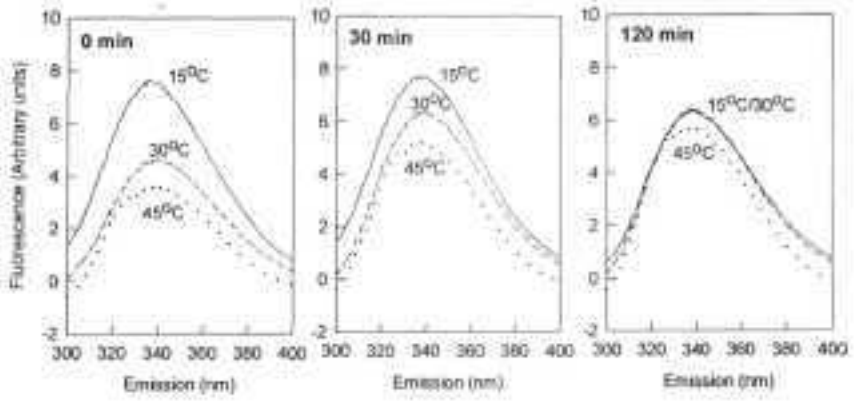


Fig. 6.3. The reversibility of the effects of temperature on purified PEPC. The enzyme was exposed to varying temperature of 15°C, 30°C and 45°C for 45 min, and then transferred to 30°C. The fluorescence emission was monitored at 30 min intervals. Further details were as in Fig. 6.2.

Table 6.1. Effect of PEG-6000, urea, Glc-6-P or malate on the intrinsic fluorescence of *A. hypochondriacus* PEPC. The intrinsic fluorescence was measured as described under "Materials and Methods". The values in the parenthesis are % of that at 30°C. The enzyme was exposed to varying temperature in the absence or presence of 1.25% (w/v) PEG-6000, 3 M urea, 2 mM Glc-6-P and 0.5 mM malate and the characteristics of PEPC protein were determined.

Effector	Temperature		
	15°C (Low)	30°C (Moderate)	45°C (High)
	Intrinsic fluorescence (Arbitrary units)		
None (Control)	17.40(129)	13.40(100)	9.46(70)
+ Malate	7.56 (110)	6.83(100)	6.28(92)
+ Glc-6-P	14.19(272)	5.22(100)	4.21(80)
+ PEG-6000	29.48(125)	23.58(100)	21.44(91)
+ Urea	7.12(111)	6.41(100)	4.27(66)

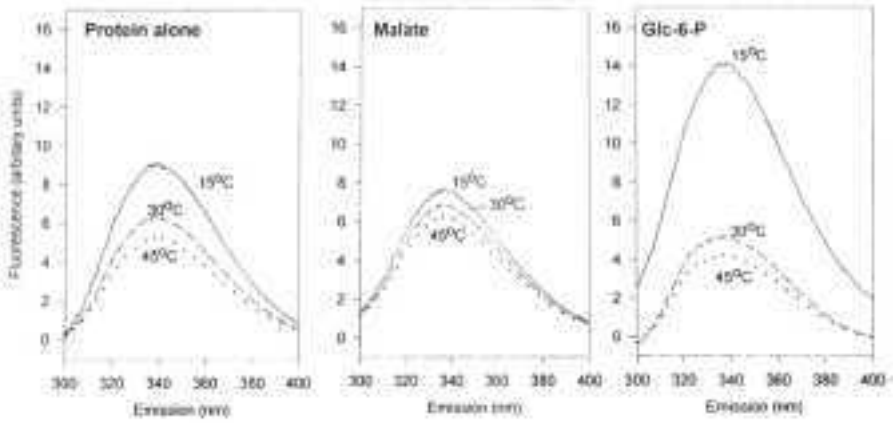


Fig. 6.4. The intrinsic fluorescence of PEPC upon incubation at varying temperatures with or without effectors like 0.5 mM malate or 2 mM Glc-6-P. Further details were as in Fig. 6.2.

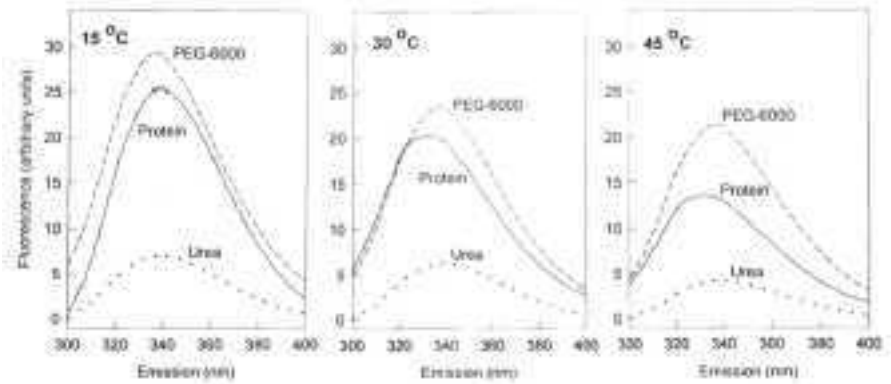


Fig. 6.5. The effect of 1.25% (w/v) PEG-6000 or 3 M urea on the intrinsic fluorescence of PEPC from *A. hypochondriacus*, after exposure to varying temperatures. Further details were as in Fig. 6.2.

In contrast, the intrinsic fluorescence emission of PEPC was markedly quenched in presence of urea at 15°C. The observations are summarized in Table 6.1.

*Studies on extrinsic or **ANS-fluorescence:***

ANS is frequently used as a probe to monitor the conformational status of proteins, as the dye binds to the hydrophobic regions and increases the fluorescence emission of the proteins (Takai et al., 1997). We applied this approach to C_4 PEPC to study the structural changes at different temperatures. In the absence oi' any protein, ANS exhibits a weak fluorescence with the maximum emission maxima at 524 nm. When the dye binds to a protein, the fluorescence of ANS increases, and the peak of emission shifting to 462 nm (Fig. 6.6). The fluorescence of ANS-protein complex increased with time. Irrespective of the pretreatment of protein at 15°C, 30°C or 45°C. The fluorescence increased along with the time by incubation with ANS from 1 to 240 min (Fig. 6.7).

Effect of temperature

The extrinsic fluorescence of PEPC (due to ANS) increased markedly as the temperature was raised from 15°C to 45°C (Fig. 6.8). There was also a slight shift in the peak of emission from 454 nm at 15°C to 456 nm at 30°C and 459 nm at 45°C.

Influence of malate, Glc-6-P, PEG-6000 or urea

L-malate, a competitive inhibitor of the enzyme, increased the extrinsic fluorescence of PEPC. while Glc-6-P, an allosteric activator, had no significant effect on the fluorescence (Fig. 6.9).

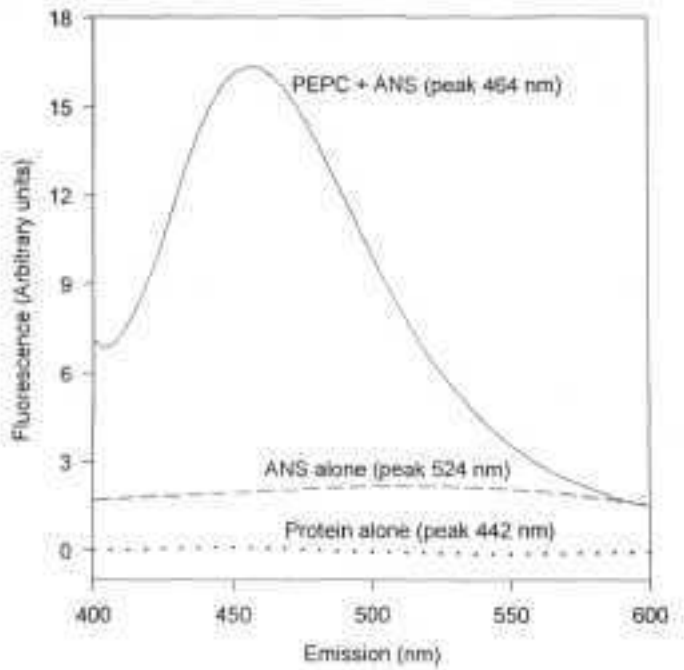


Fig. 6.6. Fluorescence emission spectra of ANS (peak at 524 nm), ANS+PEPC (462 nm) or PEPC protein (442 nm). The enzyme (10 μg PEPC/ml in buffer A) was pretreated at 30°C for 45 min. The ANS was then added to the incubated solution at a final concentration of 1 μM ANS. Immediately the samples were measured at room temperature with and without ANS and ANS alone. The excitation was at 350 nm. The details are described in 'Materials and Methods'.

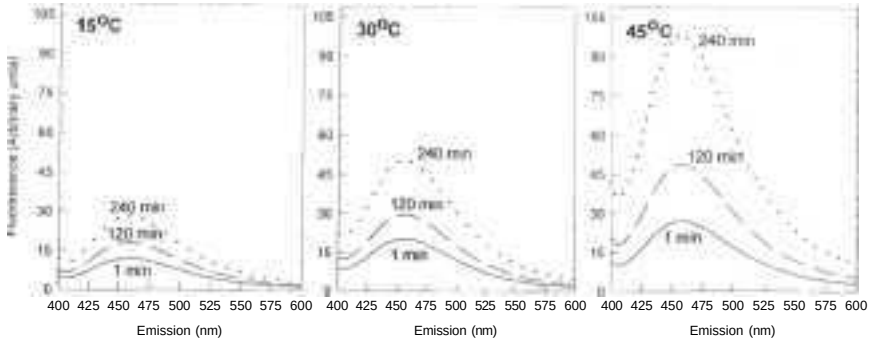


Fig. 6.7. The extrinsic fluorescence emission spectra of PEPC in presence of ANS over varying periods of incubation. The enzyme (10 μg PEPC/ml in buffer A) was pretreated at different temperature for 45 min. The ANS was then added to the incubated solution at a final concentration of 1 μM ANS. Fluorescence was measured at every 30 min interval up to 240 min with an excitation of 350 nm and the emission was recorded from 400 to 600 nm. Further details were as in Fig. 6.6.

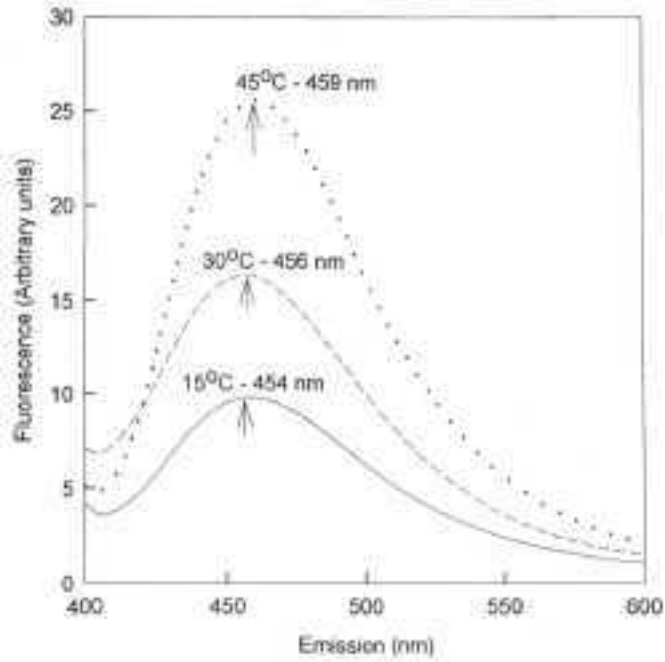


Fig. 6.8. Extrinsic fluorescence spectra of PEPC purified from *A. hypochondriacus* leaves after exposure to different varying temperature. The intensity of fluorescence increased and the peak of emission shifted from 454 to 459 nm. Further details were as in Fig. 6.6.

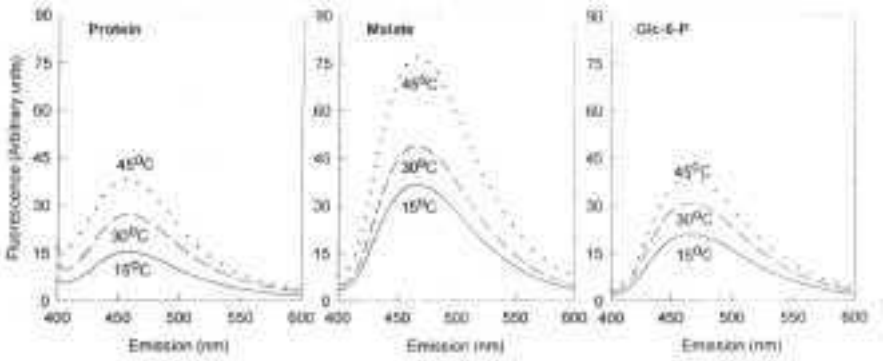


Fig. 6.9. The extrinsic fluorescence of PEPC upon incubation at varying temperature with or without effectors like 0.5 mM malate or 2 mM Glc-6-P. Further details were as in Fig. 6.6.

The presence of 1.25% PEG 6000 decreased the fluorescence of PEPC, while urea increased the extrinsic fluorescence. These effects were more pronounced at 45°C than those at 15°C (Fig. 6.10). These observations are summarized in Table 6.2.

Discussion

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

The PEPC protein from *A. hypochondriacus* exhibited high intrinsic fluorescence. The peak of fluorescence at 335 nm suggests that most of the protein's intrinsic fluorescence is possibly due to tryptophan residues. The marked changes in PEPC with temperature in the intrinsic (as well as the extrinsic fluorescence) demonstrate that marked changes are occurring in the tertiary structure of the enzyme. A similar phenomenon was found in PEPC of *Rhodothermus obamensis* during thermo denaturation at different temperatures (Takai et al., 1997). The *Rhodothermus* PEPC showed a decrease in intrinsic fluorescence during denaturation at warm temperature, and a shift in the peak

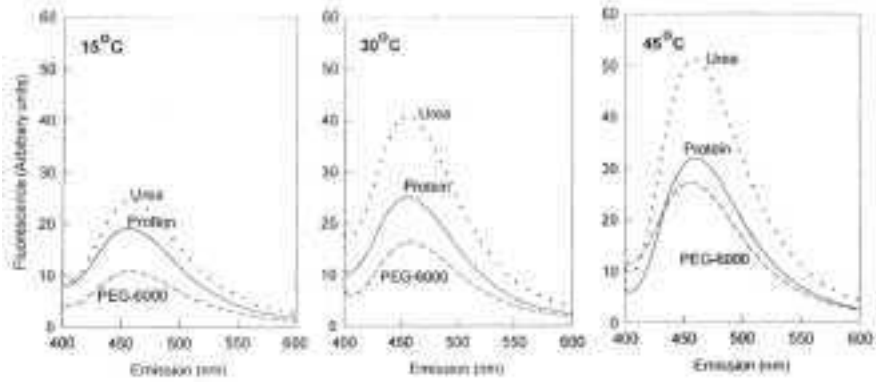


Fig. 6.10: The effect of 1.25% (w/v) PEG-6000 and 3 M urea on the extrinsic fluorescence of PEPC from *A. hypochondriacus*, exposed to varying temperatures. Further details were as in Fig. 6.6.

Table 6.2. Effect of PEG-6000, urea. Glc-6-P or malate on the extrinsic fluorescence of *A. hypochondriacus* PEPC. The ANS fluorescence was measured as described under "Materials and Methods". The values in the parenthesis are % of that at 30°C. The enzyme was exposed to varying temperature in the absence or presence of 1.25% (w/v) PEG-6000, 3 M urea, 2 mM Glc-6-P and 0.5 mM malate and the characteristics of PEPC protein were determined.

Effector	Temperature		
	15°C (Low)	30°C (Moderate)	45°C (High)
	ANS fluorescence (Arbitrary units)		
None (Control)	17.41 (66)	26.30(100)	35.27(134)
+ Malate	36.61 (75)	48.41 (100)	77.04(159)
+ Glc-6-P	20.92 (68)	30.51 (100)	38.54(126)
+ PEG-6000	10.91 (66)	16.35(100)	27.27(166)
+ Urea	24.45 (59)	40.91 (100)	51.27(125)

(Takai et al., 1997). A decrease in the intrinsic fluorescence of protein was noticed in case of also GAPDH of *Thermatogo maritime* and RNase Rs from *Rhizosporastolonifer* (Wrba et al., 1990; Desphande et al., 2003).

The nature of changes in protein could be compared and ascertained by using factors, which were earlier used for studies on protein conformation. For e.g., PEG-6000 is known to promote the aggregation of several enzymes, including PEPC, pyruvate kinase and FBPase (Huber and Sugiyama, 1986; Podesta and Plaxton, 1993; Hodgson and Plaxton, 1995). While urea, at concentration of 3 M or higher dissociates the proteins effectively (Encinas et al., 1998; Encinas et al., 2002). Thus, the increase in intrinsic fluorescence by PEG is an indication of aggregation of the protein. In contrast, the decrease in intrinsic fluorescence in presence of urea suggests the dissociation/denaturation of the enzyme. By drawing an anology, the increase in intrinsic fluorescence of PEPC at 15°C, and the decrease at 45°C indicates that PEPC gets dissociated at warm temperature (e.g., 45°C) and tends to be aggregated or oligomerized at cold temperatures (e.g., 15°C).

ANS presents an emission maximum at 524 nm that decreases to 462 nm in presence of C_4 PEPC. indicating binding of the probe to the protein. This is similar to the previous results with PEP carboxykinase from *E. coli* (Encinas et al., 1998) or *Saccharomyces cerevisiae* (Encinas et al., 2002). With a rise in temperature from 15°C to 45"C, there was an enhancement of fluorescence intensity and the shift in emission maxima of ANS. Such increase in extrinsic

fluorescence suggests an increase in the binding of dye due to exposure of hydrophobic regions to the surface on thermal denaturation. Similarly, Deshpande et al. (2003), observed that blue shift in emission and enhancement of fluorescence intensity of ANS at 70°C in RNase Rs from *Rhizopus stolonifer* indicated the presence of solvent exposed hydrophobic surfaces as a result of heat denaturation.

The reversibility of changes, induced in intrinsic fluorescence of PEPC protein emphasizes the physiological significance of temperature induced changes in PEPC. According to Schultes and Jaenicke (1991), the changes occur in GAPDH from *Thermatoga maritime* are reversible, which exhibits intrinsic thermostability with denaturation temperature. Similarly, in the enzyme lysozyme the changes conformation were reversible (Chryssomallis et al., 1981). However, 3-isophenylmalate dehydrogenase from *thermus thermophillus* showed irreversible changes (Imada et al.. 1995).

PEG promotes the tighter folding in case C_4 PEPC, which is an agreement with results obtained with castor oil seed FBPase (Hodgson and Plaxton, 1995). By contrast, denaturation of PEPC with 3 M urea caused the fluorescence emission intensity to substantially decreases, as the temperature increased without any pronounced shifts in its emission maximum. However, in case of yeast PEP carboxykinase, the presence of urea caused not only a quenching of the intrinsic fluorescence, but also a shift in the emission maxima towards red (Encinas et al.. 2002). Similar changes were obtained in presence of urea-induced unfolding of

tetrameric PEP carboxykinase from *E. coli* and *Saccharomyces cerevisiae* (Encinas et al., 1998; 2002).

Further, the studies were extended to check the influence of allosteric effectors on intrinsic fluorescence. The extrinsic fluorescence of PEPC, with ANS was enhanced in presence of Glc-6-P (an activator) and decrease in presence of malate (an inhibitor). Similar results were observed in case of *E. coli* with both activators and inhibitor (Yoshinaga, 1976) and allosteric activators in *Rhodothermusobamensis* (Takai et al., 1997). L-aspartate, an inhibitor of PEPC, also markedly quenched the fluorescence of PEPC from *E. coli* (Yoshinaga, 1976). This suggests that the effectors produce a pronounced change in the enzyme conformation of PEPC from *A. hypochondriacus.*

The present work is the first detailed report on conformational changes in C4-PEPC, studied at varying temperature and in presence of effectors, PEG-6000 and urea. On exposure to varying temperatures PEPC altered its conformation, as indicated by changes in both intrinsic and extrinsic fluorescence. Further experiments were therefore undertaken to study the protein conformations at the secondary structure level, using the circular dichroism spectra. These are described in the next chapter.

Major conclusions from the results presented in this chapter are:

1. The marked modulation of intrinsic fluorescence provides a qualitative indication of conformational changes in PEPC, due to varying temperature. The reversibility of these changes suggests that the

temperature induced conformational changes in PEPC could be of physiological significance.

2. PEG-6000 and urea are known to promote aggregation and dissociation of the protein, respectively. Thus, the PEPC protein is expected to unfold in presence of urea and fold in presence of PEG-6000. Increase in intensity of intrinsic fluorescence emission spectrum at 45°C and decrease at 15°C, suggest that the PEPC protein gets folded at cold temperature and is unfolded at warm temperature.

3. The temperature induced conformational changes can be visualized using also a hydrophobic dye, ANS. The increase in the extrinsic fluorescence of PEPC suggests that the increase in the binding of the dye, obviously due to the unfolding of protein, appears to increase at warm temperatures

4. Additional conformational changes are induced by malate or Glc-6-P in PEPC. as indicated by changes in both intrinsic and extrinsic fluorescence.

Chapter 7

Conformational Changes in Purified PEPC Studied by Circular Dichroism Spectroscopy

Chapter 7

Conformational Changes in Purified PEPC Studied by Circular Dichroism Spectroscopy

Detailed molecular analysis of the structure-function relationships in PEPC have been hampered by the lack of information on its three-dimensional structure. A partial X-ray crystallographic analysis has recently been accomplished for PEPC from *E. coli* (Inoue et al., 1989; Kai et al., 1999b; Matsumura et al., 1999a) and C_4 plant, *Zea mays* (Matsumura et al., 1999b; Matsumura et al., 2002).

Relative analysis of the various structure of *E. coli* PEPC in its inactive state (T state) with maize PEPC in its active (R state) shows that the relative orientations of the two subunits in the basal "dimer" are different, implicating an allosteric transition (Kai et al., 2003). Comparison of the inactive (T state) *E. coli* PEPC and active (R state) *Zea mays* PEPCs, revealed dynamic movements in Zm PEPC around two loops near the C-terminal side of the β-barrel, where catalytic side is located (Matsumura et al., 2002). Information derived from these three-dimensional structures combined with related biochemical studies, has established models for the reaction mechanism and allosteric regulation of this important carbon-fixing enzyme (Kai et al., 2003).

Circular dichroism (CD) spectroscopy measures the difference in absorption **of** left- and right- circularly polarized light as it passes through optically active or chiral samples. Spectra in the far UV-CD wavelength range

(about 190 nm to 250 ran) can provide information on the polypeptide conformation of protein (Wallace and Janes, 2001).

The previous chapter presented the information on the conformational changes of PEPC protein of *Amaranthus hypochondriacus*, visualized through intrinsic and extrinsic fluorescence. In this chapter, focus is made on the conformational changes of PEPC protein using CD-spectra. The Percentage contents of α-helix, (3-sheet and random coil of PEPC protein were estimated from CD-spectra (Greenfield and Fasman, 1969). All the references to CD-spectra herein pertain to far-UV region.

Results

Effect of temperature on far UV-CD spectra of PEPC

The far UV-CD spectrum of PEPC showed a broad negative band from about 210 to 220 nm, which is characteristic of a protein having α-helices. The mean residue negative ellipticity of PEPC decreased markedly as the temperature was raised from 15°C to 45°C (Fig. 7.1). At 45°C, there was a shift in negative band at 227 nm. Estimates of helical content of the PEPC protein showed that the % of a-helices decreased with increase in temperature (Table 7.1). Similarly, there was an increase in p-sheet with increase in temperature.

Changes in secondary structure of PEPC was monitored as a function of increasing temperature over a period of time at varying temperature in C_4 PEPC protein. The negative ellipiticity of the spectra increased at 15°C and decreased at

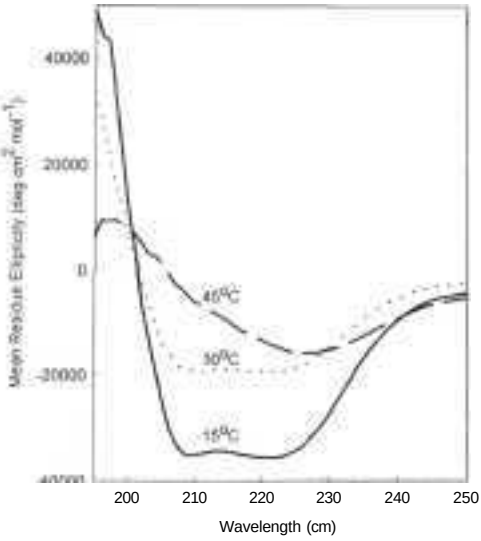


Fig. 7.1. Circular dichroism spectra of PEPC purified from *A. hypochondriacus* leaves after exposure to varying temperature for 45 min (50 μg PEPC mL^{-1} in buffer A, see 'Materials and Methods' for further details). The spectrum was then recorded immediately at 30°C.

Table 7.1. Secondary structure content of PEPC protein from *Amaranthus hypochondriacus* incubated at different temperatures for 45 min as indicated by the estimated percentages of a-helix, β-sheet and random coil from CD curves. The details of measurement and calculations are described in 'Materials and Methods'.

Temperature	a-helix	P-sheet	Random coil
		Percentage (%)	
15°C	80.7	6.82	12.4
30°C	47.2	9.47	43.3
45°C	28.3	11.8	59.9

45°C, as the protein was incubated **for 120 min** (Fig. 7.2). There was no much change in the spectra at 30°C.

Reversibility of temperature effects

The next set of experiments was designed to assess if the changes induced in CD-spectra of PEPC protein by varying temperature were reversible. The purified PEPC protein was preincubated at 15°C, 30°C or 45°C for 45 min and then the samples were brought back to the 30°C. The spectra were recorded over a period of 120 min. Soon after the exposure to varying temperature i.e., at 0 min after treatment, there was a decrease in the negative ellipticity at 15°C and increase at 45°C, compared to that at 30°C (Fig. 7.3).

Effect of PEG-6000 or urea

PEG-6000 induces the protection of enzymatic activity against high temperature (Drilias et al., 1994), and promote oligomerization of PEPC (Huber and Sugiyama, 1986). Urea is a well known denaturant and dissociates proteins (Encinas et al., 2002). Attempts were made to study the far UV-CD spectra in presence of either PEG-6000 or urea. PEG, increased the negative ellipticity of PEPC protein at 222 nm, and such increase was more pronounced with protein pretreatment at 15°C than that at 30°C or 45°C (Fig. 7.4). In contrast, the mean residue negative ellipticity of PEPC decreased markedly in presence of urea, with a maximum loss of secondary structure of PEPC protein at 45°C. The estimated α-helicity of PEPC increased in presence of PEG, and decrease in presence of urea (Table 7.2).

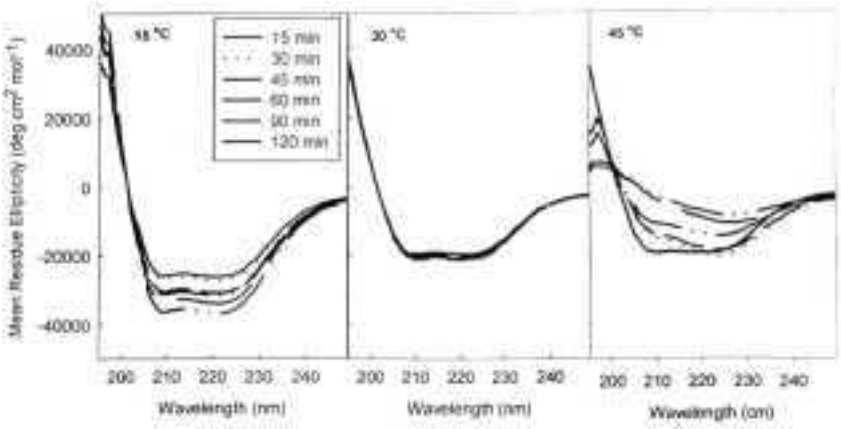


Fig. 7.2. CD spectra of PEPC purified after exposure to different varying temperatures of 15°C, 30°C or 45°C over a period of 120 min. The spectrum was recorded at every 15 min intervals at 30°C.

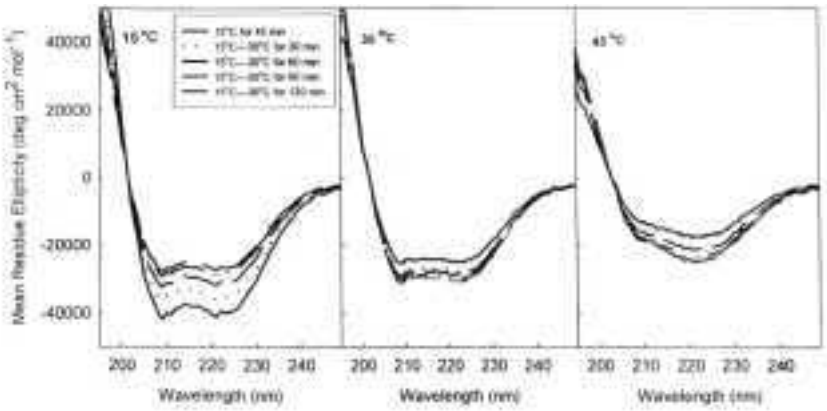


Fig. 7.3. **The** reversibility of the effects of temperature on purified PEPC. The enzyme was exposed to varying temperature of 15 °C, 30 °C and 45 °C for 45 min, and then by transfer 30°C. The CD spectra was monitored at 30 min interval. Further details were as in Fig. 7.1.

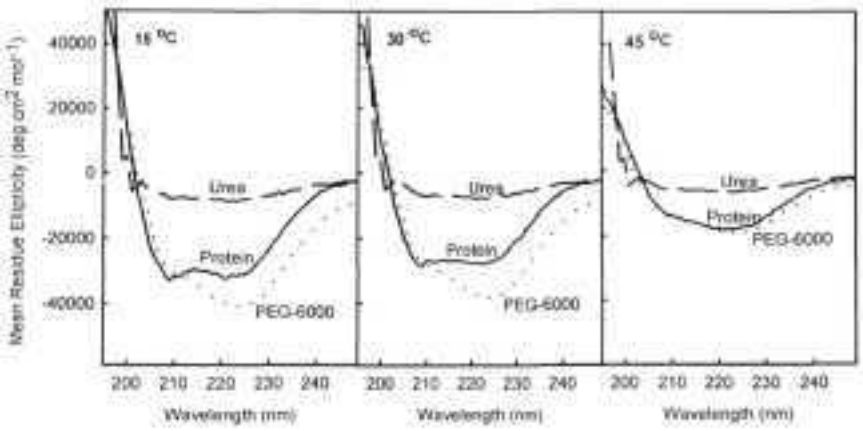


Fig. 7.4. The effect of 1.25% (w/v) PEG-6000 and 3 M urea on the CD spectra of PEPC from *A. hypochondriacus*, exposed to varying temperatures. Further details were as in Fig. 7.1.

Table 7.2. The effect of including PEG-6000 or urea during preincubation, on the response of PEPC to varying temperature. The percentages of α-helix, P-sheet and random coil of PEPC protein were estimated from CD-spectra. Further details were as in Table 7.1.

Modulator	Temperature (°C)		
	15°C	30°C	45°C
		a-helix (%)	
Protein only	79.2	55.4	31.6
+ 1.25% PEG-6000	99.4	97.2	48.1
+ 3M urea	15.7	7.3	4.2
		P-sheet (%)	
Protein only	8.4	11.03	13.44
+ 1.25% PEG-6000	0.3	0.7	4.47
+ 3 M urea	3.4	4.5	4.9
		Random coil (%)	
Protein only	12.4	33.57	54.96
+ 1.25% PEG-6000	0.3	2.1	47.43
+ 3 M urea	80.9	88.2	90.9

Influence of effectors

The pattern of CD-spectra can also provide a measure of qualitative changes in the enzyme in response to the binding of malate or Glc-6-P. In the presence of L-malate, the competitive inhibitor and Glc-6-P, the allosteric activator of the enzyme PEPC exhibited a significant difference between the negative ellipticity at different temperatures was observed.

The negative residue ellipticity of PEPC increase in presence of Glc-6-P, such effect of Glc-6-P being the maximum at 30°C. In contrast, negative ellipticity decrease in presence of malate, with maximum effect being at 45°C (Fig. 7.5). The estimated α-helicity of PEPC increased marginally in presence of Glc-6-P and decreased marginally in presence of malate (Table 7.3).

Discussion

Far UV circular dichroism reflects the secondary structure of a protein and is hence used frequently as tool to monitor protein conformation. It was therefore very useful to study the CD-spectra of PEPC subjected to different temperature, to understand the nature of changes in protein on exposure to temperature.

The crystal structure of maize PEPC (C_4 form) confirmed the tetrameric nature of protein, made of dimers. The monomer contained a eight standard β-barrel with an abundance of α-helices (Matsumura et al., 2002). According to Kai et al. (2003) *E. coli* PEPC monomer consisted of an eight standard β-barrel and approximately 40 a-helices comprising of 65% of the polypeptide, whereas

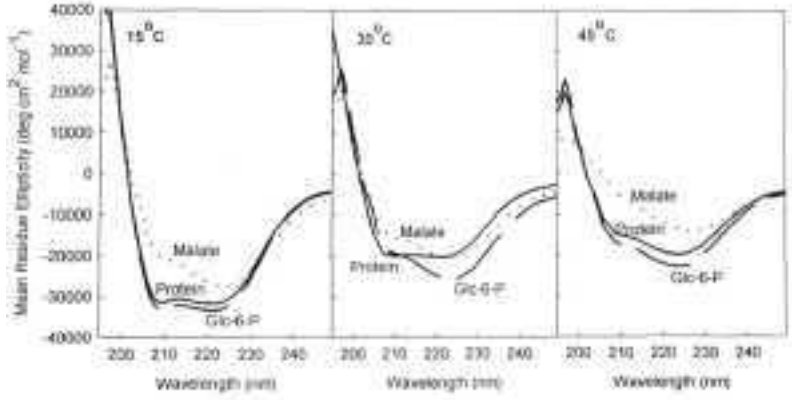


Fig. 7.5. The CD spectra of PEPC upon incubation at varying temperature with or without effectors like 0.5 mM malate or 2 mM Glc-6-P. Further details were as in Fig. 7.1.

Table 7.3. The effect of **including Glc-6-P or malate during preincubation, on the** response of PEPC to varying temperature. The percentages of α-helix, p-sheet and random coil of PEPC protein were estimated from CD-spectra. Further details were as in Table 3.

Effector	Temperature		
	15°C	30°C	45°C
		a-helix (%)	
Protein only	75.4	48.2	36.1
+ 2 mM Glc-6-P	84.7	61.8	56.1
+ 0.5 mM malate	68.1	50.6	21.2
		P-sheet (%)	
Protein only	9.7	10.8	12.7
+ 2 mM Glc-6-P	4.7	5.1	6.3
+ 0.5 mM malate	5.6	8.2	10.7
		Random coil (%)	
Protein only	14.9	41.0	51.2
+ 2 mM Glc-6-P	10.6	33.1	37.6
+ 0.5 mM malate	26.3	41.2	68.1

P-strands comprised only 5%. The **secondary structure** of *Zea mays* PEPC is almost similar to that of *E. coli* PEPC, except **for** certain regions (Kai et al., 2003). The CD-spectra of C_4 PEPC protein **from** *A. hypochondriacus* shows characteristic dual peak at 208 and 222 nm (Fig. 7.1) suggesting that the major part of protein is in α-helical conformation.

Quantitative analysis of the CD spectra revealed that the PEPC of *A. hypochondriacus* too contained significant proportion of α-helices, while the β-sheets were <10%, at 30°C (fable 7.1 to 7.3). The use of CD-spectra have revealed interesting changes in several proteins with factors such as temperature: For e.g., rapid changes in magnitude and shape of CD- spectra in case of barstar with increasing temperature, suggested marked changes in protein under thermal denaturing conditions (Nolting et al., 1997). However, Kamal and Behere (2002). observed that the heat induced denaturation was rather mild in case of soyabean peroxidase. In another interesting study, recombinant PEPC from *Thermus* species was subjected to limited proteolysis and the generated major fragments were used **for** CD spectral analysis. Peptide cleavage had no substantial effect on the PEPC quaternary secondary structure and thermostability (Nakamura et al.. 2002).

The CD spectrum of PEPC from *A. hypochondriacus* showed a pronounced dual peak, after the pretreatment at 15°C. indicating a marked folding of the protein with a rigid a helical conformation. In contrast, when pretreated. at warm

temperature 45°C, the curve showed a significant **reduction of dual peak, thus** revealing a marked reduction in a-helicity (Fig. 7.1; Table 7.1). These results are similar to the findings of Nolting et al. (1997), who observed a significant loss of α-helix and increase in random coil with thermal denaturation of barstar protein. However, Nolting et al. (1997) employed subzero temperatures, while we recorded an increase in a-helicity of PEPC at even 15°C (compared to that at 30°C) (Table 7.1 to 7.3).

The changes in CD-spectra of PEPC after exposure to 15°C were reversed, when the protein was transferred back to 30°C (Fig. 7.3). This point is an indication of physiological significance of changes in CD-spectra of PEPC. However, the changes in CD-spectra induced at 45°C were not completely reversed at 30°C, suggesting of irreversible aggregation of unfolded protein at high temperature. Kamal and Behere (2002) showed that thermal and denaturant induced unfolding of soyabean peroxidase and apo-soyabean peroxidase was largely reversible. However, a small part of the total protein molecules underwent irreversible thermal aggregation.

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

caused **a marked** decrease in the a-helicity **and** increased **random coil percent of** PEPC, indicating a complete disruption of secondary structure of the protein. Further studies were extended to check the effect of Glc-6-P (an allosteric activator) and malate (a competitive inhibitor). The negative **ellipiticity** of CD-signal of PEPC was enhanced in presence of Glc-6-P irrespective of temperature: 15°C, 30°C or 45°C. On the other hand, malate caused limited disruption of secondary structure and the effect of malate was more pronounced at 30°C and 45°C than that at 15°C (Fig. 7.5). Matsumura et al. (2002) reported that in the vicinity of negative affector binding site in *Zea mays* PEPC structure, large conformational changes are observed compared with the aspartate bound T-state.

The present study emphasizes that the PEPC protein undergoes marked conformational changes in its secondary structure, after treatment at different temperature. Changes can also been seen in presence of compatible solutes like PEG-6000 or urea (a protein denaturant), or effectors like Glc-6-P (allosteric activator) and malate (competitive inhibitor). The a-helicity of PEPC is stabilized at cold temperature and destabilized at warm temperature.

Major conclusions from the results presented in this chapter are:

1. There are marked changes in the patterns of CD-spectra of PEPC, with varying temperature as well as by the presence of PEG-6000 or urea.
2. The α-helical percentage increased markedly at cold temperature 15°C (compared to that 30°C), while decreasing at 45°C, suggesting stabilization of

the secondary structure at cold temperature and destabilization at warm temperature.

3. The change in α-helical conformation of PEPC reversed on transfer from 15°C back to 30°C. However, the changes induced by 45°C were not much reversible.
4. Urea of 3 M induced almost complete disruption of secondary structure of the protein. However, PEG increased the a-helical content, and obviously stabilized the protein.
5. Negative ellipticity increased in presence of Glc-6-P with temperature indicating an increase in the a-helical content, while the presence of malate, decreased negative ellipiticity of the PEPC protein.

A brief general discussion is presented in the next chapter.

Chapter 8

General Discussion

Chapter 8

General Discussion

The pattern of enzyme response to variation in temperature can be a possible biochemical basis for adaptation of C_4 plants to different thermal environments (Phillips and McWilliam, 1971). The present study was undertaken to examine the temperature induced changes in the properties of PEPC in C_4 plants. The results from this study demonstrate that a change in temperature can induce quite dramatic changes in not only the activity but also the malate sensitivity of PEPC in both C_3 and C_4 plants. The changes were caused after even a short duration of exposure to varying temperature and were reversible.

The temperature optimum for PEPC from *Amaranthus hypochondriacus* (C_4) was 45°C compared to 30°C in *Pisum sativum* (C3 species) (Fig. 4.1). The activity of PEPC decreased on exposure to either suboptimal or supraoptimal temperature. The decrease in PEPC activity at 15°C (cold temperature) was much higher in case of *A. hypochondriacus* (C_4) than that of *P. sativum* (C_3). Similarly, at high temperature of 50°C the decrease in activity of PEPC was much greater in the case of *P. sativum* (C_3) that of *A. hypochondriacus* (C_4). Thus, the C_4 PEPC was more sensitive to sub-optimal temperatures and less sensitive to supra-optimal temperatures than that of C_3 species. The marked sensitivity of C_4 PEPC to sub-optimal temperatures, endorses the suggestion that PEPC is one of the cold sensitive enzymes in C_4 plants (Phillips and McWilliam, 1971; Wu and Wedding. 1987).

There was a marked decrease **in malate sensitivity** of PEPC, as **the** temperature was raised from 15°C to 50°C (Fig. 4.2). Thus, PEPC was highly sensitive to malate at cold temperature, **while** becoming relatively malate-insensitive at warm temperature. This is the first **detailed** report to demonstrate that a change in temperature can induce quite dramatic changes in not only the activity but also the malate sensitivity of PEPC in both C_3 and C_4 plants. The changes in PEPC induced were caused by a short duration of exposure to temperatures and were reversible to a large extent (Fig. 4.4), indicating physiological significance of temperature induced changes in C4-PEPC.

Arrhenius plots revealed interesting differences between PEPC of not only the C_3 and C_4 plants, but also the pattern in presence or absence of malate. As the temperature was raised, the activation energy was lowered in both *A. hypochondriacus* (C_4 plant) and in *P. sativum* (C_3 species) (Table 4.2). The break at 27°C in case of C_3 plants suggested that the C_3 enzyme did not respond much to temperatures, above **27°C.** In contrast, the absence of such break and the continuation of slope indicated that the activation energy for C_4-PEPC continued to decrease as the temperature rises from 27°C to 45°C (Fig. 4.3). The abrupt changes in activation energy were previously reported for PEPC (McWilliam and **Ferrar,** 1974; Uedan and Sugiyama, 1976, Vidal and Gadal, 1983, Selinioti et al., 1986). But there were no breakpoints during similar studies on PEPC from sugarcane (Du et al.. 1999a), wheat or Johnson grass (Phillips and McWilliam. 1971).

On illumination, the activity of PEPC in leaves of C_4 plants is enhanced by 2-3 fold along with a **marked** decrease in **the malate sensitivity of the enzyme.** These changes during the **light activation are due to** the **phosphorylation** of **the** enzyme (Rajagopalan et al., 1993; Chollet et al., 1996; Vidal **and Chollet, 1997;** Nimmo, 2000; Nimmo, 2003). The phosphorylation of the enzyme leads to an increase in the activity of enzyme and decrease in the malate sensitivity. The temperature induced changes in PEPC, particularly the increase in the activity and decrease in the extent of malate inhibition, are quite similar to the changes effected during light activation of PEPC. Therefore, it is quite possible that there is a change in phosphorylation status of PEPC on exposure to temperature. However, our experiments revealed that the phosphorylation of enzyme is not the major factors in temperature induced effects on PEPC (Fig. 4.5). This is the first report to demonstrate that the temperature induced changes in PEPC of C_4 species is independent of phosphorylation.

Attempts were then made to identify changes other than the phosphorylation **on** exposure of PEPC to temperature. An interesting possibility is a change in **the** conformational status of enzyme. Compatible solutes like PEG-6000 promote the oligomerization of PEPC. increase the enzyme activity and decrease its malate sensitivity (Huber and Sugiyama, 1986). The temperature induced changes in malate sensitivity of PEPC were dramatically dampened with the addition of PEG in the assay medium (Table 4.5). Such marked dampening of malate sensitivity is an indication that temperature causes reversible changes in the

conformational status of PEPC. involving either the oligomeric status and/or the microenvironment of the protein.

Studied with purified PEPC from *A. hypochondriacus* revealed similar responses of enzyme to temperature in leaves. The optimal incubation temperature for purified PEPC of *A. hypochondriacus* was 40°C. As the temperature was raised from 15°C to 50°C, there was a steep increase in activity and decrease in malate sensitivity of PEPC and these changes were reversible (Fig. 5.6). At low temperature, the sensitivity of PEPC to malate was very high in maize and *A. hypochondriacus* (Wu and Wedding, 1987; Chinthapalli et al., 2003). Recently. Crafts-Brandner and Salvucci (2002) reported that PEPC extracted from heat-treated leaf of maize was significantly more sensitive to malate than the normal one.

Inclusion of PEG in the preincubation mixture increased the activity of PEPC protein at all temperatures and dampened malate sensitivity of the enzyme (Fig. 5.5; Table 5.3). The presence of compatible solutes, such as PEG, protects oligomeric enzymes such as PEPC and PPDK (Salahas et al., 1990). PEG-6000 was effective in protecting PEPC from maize leaves, against cold temperature (Huber and Sugiyama, 1986). Our results also suggest that PEG protects PEPC against cold temperature obviously due to the oligomerization of the enzyme, in both leaf discs of *A. hypochondriacus* and in purified form (Table 4.5).

PEPC exists predominately as a tetramer along with dimer or monomeric forms (Walker et al.. 1986). Analysis by native PAGE showed that cold

temperature shifted **the equilibrium of PEPC protein towards tetramer, where as at higher temperature the proportion of tetramer decreased (Fig. 5.7). The present** study **is the** first **report** that **temperature** can **markedly influence the structural** properties of PEPC with regard to temperature.

Proteins exhibit significant intrinsic fluorescence due to mainly their aromatic aminoacids: tryptophan, tyrosine and phenylalanine. The C_4-PEPC exhibited a peak of fluorescence at 335 nm, possibly tryptophan residues (Fig. 6.1). The intrinsic fluorescence decreased with the rise in temperature. Similar decrease in intrinsic fluorescence during denaturation at warm temperature was also noticed in bacterial PEPC, GAPDH and RNase RS (Takai et al., 1997; Schultes and Jaenicke, 1991; Deshpande et al., 2003). Our data suggest that the structural integrity of PEPC is stabilized at 15°C, while being destabilized at 45°C. The changes in intrinsic fluorescence of PEPC protein with temperature were also reversible, emphasizing the physiological significance of temperature induced changes in PEPC.

PEG-6000 promotes *oligomerization* of PEPC (Drillias et al., 1995; Huber and Sugiyama, 1986), while urea is a well known denaturant (Encinas et al., 1998; 2002). The inclusion of PEG and urea during incubation appears to promote folding and unfolding, respectively, of PEPC (Fig. 6.5). Increase in intrinsic fluorescence of PEPC protein such increase was more with the protein pretreatment at 15°C than that at 30°C or 45°C in presence of PEG. In contrast, there was a dramatic decrease in presence of urea, and such decrease was more at

45°C than that at 15°C or 30°C (Table 6.1). The increase in fluorescence in presence of PEG was an indication of aggregation of PEPC protein and decrease in fluorescence suggested the denaturation/or dissociation of the enzyme.

The status of protein conformation can be monitored by also intrinsic fluorescence with the help of suitable probes. ANS is a polycyclic aromatic fluorescent probe that interacts with hydrophobic sites on the proteins (Semisotnov et al., 1991). As the temperature was raised, there was an increase in intrinsic fluorescence of PEPC (due to ANS). The extrinsic fluorescence was higher at 45°C than that at 30°C or 15°C. Similar observations were found in PEPC from *E. coli* and *Rhodothermus obamensis* (Yoshinaga. 1976; Takai et al., 1997). The changes, induced in the fluorescence of PEPC protein at different temperatures, were reversible to a large extent. The patterns of extrinsic fluorescence also suggested that the PEPC protein was getting aggregated at cold temperature and was dissociated at warm temperatures.

Further studies were carried out to examine the secondary structure of PEPC of *A. hypochondriacus*. Recently. Kai et al. (2003) reported that *E. coli* PEPC monomer consisted of an eight standard β–barrel and approximately 40 α–helices comprising of 65% of the polypeptide, whereas P-strands comprised only 5%. The CD spectra of PEPC revealed a decrease in % of α-helicities as the temperature increased. In contrast, % of β-sheet structures, and random coil increased at warmer temperature (Fig.7.1: Table 7.1 to 7.3). These results are

similar to the observed loss of α-helix and increase in random coil with thermal denaturation of barstar protein (Nölting et al., 1997).

Kamal and Behere (2002) have shown that thermal and denaturant induced unfolding of soyabean peroxidase and apo-soyabean peroxidase is largely reversible. Similarly, Nolting et al. (1997) also have shown that the temperature induced changes of barstar protein arc highly reversible. There was a significant reversibility of changes in PEPC protein induced at 15°C (Fig. 7.3). The reversibility indicates that temperature induced changes in conformation of protein could be physiological significant.

The effects of PEG-6000 and urea on the PEPC protein after exposing the enzyme to different temperature were quite drastic. The native enzyme showed less α-helicity than the PEG protected enzyme (Fig. 7.4; Table 7.3). In contrast, urea treated enzyme showed, less α-helicity and more P-sheet structure than that of native or PEG-treated PEPC. It is known that PEG-6000 stabilizes proteins, while urea denatures/dissociates the proteins. Many proteins need high concentrations of urea (above 3 M) for denaturing (Encinas et al., 2002). However, marked denaturation occurred in PEPC at even 3 M urea. This point indicates the relatively low energetic stability of PEPC.

Further studies were extended to check the effect of Glc-6-P (an allosteric activator) and malate (a competitive inhibitor). The negative ellipiticity was apparently enhanced in presence of Glc-6-P at 15°C, 30°C or 45°C (Fig. 7.5).

However, there was decrease in presence of malate and simultaneously loses the dual nature of negative ellipticity suggesting that Glc-6-P somehow induces the α-helical conformation at low temperature (Table 7.4).

Further experiments are required to establish the detected mechanism of folding and unfolding of C_4-PEPC in response to temperature.

Chapter 9

Summary and Conclusions

Chapter 9

Summary and Conclusions

Phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) is a ubiquitous enzyme occurring in cytosol of photosynthetic and non-photosynthetic tissues of C_3, C_4 and CAM plants (Andreo et al., 1987; Rajagopalan et al., 1994; Chollet et al., 1996; Vidal and Chollet, 1997; Nimmo, 2000; Kai et al., 2003; Svensson et al.. 2003). The C_4 plants in general are tolerant to heat but are quite sensitive to cold temperatures (Long, 1999). The cold sensitivity C_4 pathway has been suggested to be related to the cold sensitivity of key C_4 enzymes, such as PPDK (Potvin and Simon, 1990; Burnell, 1990). The mechanism of cold inactivation of PPDK is therefore studied in detail (Krall et al., 1989; Burnell, 1990; Du et al., 1999b), but the sensitivity of PEPC to cold temperature has been a matter of debate.

On illumination, the activity of PEPC in leaves of C_4 plants is enhanced by 2-3 fold along with a marked decrease in the malate sensitivity of the enzyme. These changes during the light activation are due to the phosphorylation of the enzyme (Chollet et al., 1996, Vidal and Chollet, 1997). Compared to extensive information on the regulation by light, the literature on temperature induced changes in PEPC is quite limited and is often contradictory, particularly the effect of temperature on the activity and malate sensitivity of PEPC. The present investigation used the leaves of *Amaranthus hypochondriacus* (a NAD-ME type C_4 plant) for studies initially on leaf discs and compared with that of *Pisum sativum* (C_3 species) and other C_3 and C_4 species. Further studies were extended

to purified PEPC to ascertain if temperature modulated PEPC *in vitro* **and then examine if** there **are any changes in protein structure.**

Among the aspects studied are:

1. The effect of varying temperature **on** the activity and properties of PEPC in leaves of C_4 and C3.
2. Reversibility of the temperature induced changes.
3. Modulation by PEG-6000 (a compatible solute) or temperature responses of purified C_4-PEPC.
4. Changes in protein level or phosphorylation status of PEPC on exposure to varying temperature or light.
5. Changes induced by temperature in catalytic and regulatory properties of purified PEPC protein.
6. Detailed examination of the temperature induced conformational changes in PEPC protein, as reflected in its fluorescence properties and CD-spectra.

Temperature caused dramatic modulation of PEPC in leaf discs of *A. hypochondriacus,* compared to the pattern in *P. sativum* (C_3 plant). The optimal incubation temperature for PEPC in *A. hypochondriacus* (C_4) as 45°C, compared to 30°C in *P. sativum* (C_3 species), is not surprising. The marked sensitivity of C_4 PEPC to sub-optimal temperatures, endorses the suggestion that PEPC is one of the cold sensitive enzymes in C_4 plants (Phillips and McWilliam, 1971).

The steep increase in the activity of PEPC with temperature, particularly above 15°C, could be physiologically significant, as the temperature is expected to

rise from about 10 to 15°C in the morning to 35 to 40°C at midday, on a typically clear and sunny day. A combination of light and warm temperature could amplify the photoactivation of the PEPC, as observed in case of *Egeria densa* (Casati et al., 2000) and *Amaranthus paniculatus* (Selinioti et al., 1986).

Temperature induced dramatic changes in the sensitivity of PEPC to malate. As the temperature was raised from 15°C to 50°C, there was a marked decrease in malate sensitivity of PEPC (Fig. 4.2). *A. hypochondriacus* (C_4) lost nearly 70% of PEPC activity on exposure at low temperature of 15°C, compared to only about 35% loss in case of P. *sativum* (C_3). The extent of such decrease in C_4 plants (about 45%) was more than that in C_3 species (about 30%). Maintenance of high enzyme activity at warm temperatures, along with a steep decrease in the malate sensitivity of PEPC was noticed in also other C_4 plants. Thus, PEPC appeared to be highly sensitive to malate at cold temperature, while becoming relatively insensitive to malate at warm temperature. The results from this study demonstrate that a change in temperature can induce quite dramatic changes in not only the activity but also the malate sensitivity of PEPC in both C3 and C_4 plants. The changes were caused by a short duration of exposure to temperatures and were reversible to a large extent (Fig. 4.4). The temperature induced changes can therefore be physiologically relevant and important.

Arrhenius plots revealed interesting differences between not only the C_3 and C_4 plants, but also the pattern in presence or absence of malate. As the temperature was raised, the activation energy was lowered in both *A.*

hypochondriacus (C_4 plant) and in *P. sativum* (C_3 species). The changes in activation energy as indicated by discontinuities ("breakpoints") in Arrhenius plots at a critical temperature can be an indication of the cold lability of PEPC from different species. The break at 27°C in case of C_3 plants suggested that the C_3 enzyme did not respond much to temperatures, above 27°C. In contrast, the absence of such break and the continuation of slope indicated that the activation energy for C_4-PEPC continued to decrease as the temperature was raised from 27°C to 45°C. The limited studies made earlier on the activation energy of PEPC again having conflicting observations. Some of the reports indicate discontinuity in the Arrhenius plots of PEPC (Phillips and McWilliams 1971) while others did not observe such break points (Du et al., 1999a).

The regulation of PEPC is mediated by mostly posttranslational modification of the enzyme, by phosphorylation of a serine residue near the N-terminus of PEPC (Rajagopalan et al., 1994; Chollet et al., 1996; Vidal and Chollet, 1997). The extent of PEPC phosphorylation was much higher in illuminated discs of *A. hypochondriacus* than that in dark adapted leaves. However, there was no significant change in the status of PEPC phosphorylation in leaf discs exposed to different temperatures. Western blot analysis indicated that the levels of PEPC-protein in leaf discs of *A. hypochondriacus*, were almost similar in control (dark treated), illuminated discs as well as those at varied temperature. Thus, these observations suggest that neither a change in temperature nor illumination caused any significant change in the PEPC-protein

levels. The changes induced by rise in temperature, particularly the increase in the activity and decrease in the extent of malate inhibition are quite similar to the changes effected during light activation of PEPC. However, our experiments rule out the possible role of phosphorylation of enzyme in temperature effects of PEPC. This is the first report to demonstrate that the temperature induced changes in PEPC of C_4 species is independent of phosphorylation.

Since there was no change in phosphorylation status of PEPC on exposure to temperature, an attractive and alternative possibility is the change in the conformational status of enzyme. PEPC is very active when it is in a tetrameric shape, while its activity and malate sensitivity decreases when the enzyme dissociates into a monomer or dimer (Walker et al., 1986). Temperature may affect the oligomeric status of the enzyme PEPC. Rise in temperature causes the aggregation of PEPC in case of C_4 and dissociation in case of CAM (Wu and Wedding, 1987).

Compatible solutes like PEG-6000 can promote the oligomerization of PEPC, increase the enzyme activity and decrease its malate sensitivity (Huber and Sugiyama, 1986). Experiments were therefore designed to study the temperature induced changes in PEPC in relation or presence of PEG-6000. The temperature induced changes in malate sensitivity of PEPC were dampened with the addition of PEG in the assay medium. The marked dampening of malate sensitivity is an indication that temperature may be causing reversible changes in the conformational status of PEPC. This could be largely due to the change in

conformational status of enzyme involving either the oligomeric status and/or the microenvironment of protein, e.g., folding hydrophobic regions.

Studies were done with purified PEPC of *A. hypochondriacus* (C_4) to check the changes induced by temperature of PEPC properties *in vitro* and compare them with those *in vivo* (Chinthapalli et al., 2003). The optimal incubation temperature for C_4-PEPC was 40°C. As the temperature was raised from 15°C to 50°C, there was an increase in the activity of PEPC, along with a steep decrease in the malate sensitivity of the enzyme. Thus, the purified C_4 PEPC protein was more sensitive to malate at 15°C, than that at 40°C. The temperature induced changes in PEPC were reversible. The inclusion of PEG-6000 during preincubation, decreased slightly the response of PEPC activity to temperature, but dramatically desensitized PEPC to temperature induced changes in the malate sensitivity. A change in subunit interactions and/or quaternary structure can influence both PEPC activity as well as malate inhibition, and this may be the basis for the temperature modulation observed.

The studies were extended to observe the changes in kinetic characteristics of PEPC with varying temperature. As the temperature was raised from 15°C to 40°C, there was an increase in V_{max} and K_i (malate), while there was a decrease in K_m (PEP) and K_a (Glc-6-P). In presence of PEG-6000, there was no change in either K_i for malate or K_a (Glc-6-P). Glycerol (10%, v/v) also did not exert any such effect. An increase of K_m for PEP due to temperature rise (15°C to 35°C) was also found for PEPC in *Kalanchoe diagremontiana*, a CAM plant (Buchanan

et al., 1984; Osmond and Holtum, 1981). However, the effects of temperature or PEG-6000 reported herein could be also due to conformational changes of the tetrameric form itself.

Attempts were then made to check the oligomerization of PEPC at different temperature in presence of PEG-6000 or glycerol. PEPC exists predominately as a tetramer along with dimer or monomeric forms (Walker et al., 1986). PEG is shown to promote the self association and/or activation a number of regulatory enzymes in dilute solutions (Podesta and Plaxton, 1993). Native PAGE analysis showed that cold temperature tends to shift the equilibrium of PEPC protein towards tetramer, where as at higher temperature there is a loss of tetramer. The present study is the first report that PEG can markedly influence the structural properties of PEPC with regard to temperature.

Studies were extended to check the conformational changes in the PEPC protein through fluorescence and circular dichroism (CD) spectra. When excited at 280 nm, the intrinsic fluorescence of PEPC protein from *A. hypochondriacus* (C_4) exhibited a maximum at 335 nm (Fig. 6.1)- The intrinsic fluorescence of PEPC decreased markedly with the increase in temperature from 15°C to 45°C (Fig. 6.2). In addition, the maxima of fluorescence emission shifted from 339 nm to 335 nm and to 333 nm at 15°C, 30°C and 45°C, respectively. These data revealed that the temperature could stabilize the structural integrity of the enzyme much better at 15°C than at 30°C or 45°C. These changes are quite similar to the pattern of PEPC in *Rhodothermus obamensis* (Takai et al., 1997). The changes in

fluorescence were reversible upon **exposure of protein to normal temperature** of 30°C. The reversibility of changes **induced by intrinsic** fluorescence of PEPC protein emphasizes the physiological significance of temperature induced changes.

ANS is a polycyclic **aromatic fluorescent** probe **that** interacts with hydrophobic sites on the proteins (Takai et **al.**, 1997). The extrinsic fluorescence of PEPC (due to ANS) increased markedly as the temperature was raised from 15°C to 45°C. There was also a slight shift in the peak of emission from 454 nm at 15°C to 456 nm at 30°C and 459 nm at 45°C. Similar observations were found in **PEPC** from *E. coli* and *Rhodothermus obamensis* (Yoshinaga, 1976; Takai et al., 1997).

The intrinsic and extrinsic fluorescence PEPC protein from *A. hypochondriacus* was modulated significantly by the presence of PEG or urea. PEG-6000, which causes an aggregation of PEPC, increased the intensity of the fluorescence emission spectra, without affecting the maxima at 15°C. However, the intensity **of** fluorescence decreases with a small shift in the emission maxima when PEPC was kept at 30°C or 45°C. These results established that gross conformational changes occur in PEPC, with a shift towards hydrophobic environment, in presence of PEG-6000 (Lakowicz, 1983). By contrast, denaturation of PEPC with 3 M urea decreased the fluorescence emission of PEPC at 15°C. Obviously unfolding of the protein by urea causes a lowering of fluorescence emission at warm temperatures. Increasing concentration of urea, exposed more hydrophobic surfaces, leading to the unfolding of the enzyme, in

case of PEP carboxykinase (Encinas et al., 1998). Results with ANS emission spectra reveal that these changes were quite reverse when compared with the intrinsic fluorescence in the presence of PEG-6000 and urea. The effect was more pronounced, as we increased the temperature from 15°C to 45°C.

Further studies were made to check the influence of allosteric effectors on intrinsic and extrinsic fluorescence of PEPC from *A. hypochondriacus*. The fluorescence of PEPC at 340 nm was enhanced in presence of Glc-6-P (an activator) at 45°C when compared to 30°C and 15°C and decreased in presence of malate (an inhibitor). Similar effects of activators and inhibitors were observed in case of PEPC from *E.coli* and *Rhodothermus obamensis* (Yoshinaga, 1976; Takai et al., 1997). Fluorescence of enzyme PEPC with ANS was markedly quenched by addition of L-asparate without significant change in the position of emission maxima and produces a pronounced change in the PEPC conformation in *E. coli* (Yoshinaga, 1972). The fluorescence emission of ANS with PEPC also increased in the presence of malate, an inhibitor, and decreased in presence of Glc-6-P an activator. These observations suggest that the allosteric effectors, produce a pronounced change in the enzyme conformation.

Studies on circular dichioism (CD) spectra were conducted to evaluate the secondary structure of PEPC of *A. hypochondriacus*. The CD-spectra of C4-PEPC of *A. hypochondriacus* at room temperature showed broad negative band, with dual peaks at around 208 nm and 222 nm. suggesting that the major part of the protein was in α-helical conformation. The intensification of dual peak, after the

pretreatment at 15°C, indicated a marked folding of the protein with a rigid helical conformation. In contrast, at warm temperature when protein is pretreated, there was a significant reduction of dual peak, suggesting a significant reduction in a-helicity. These results are similar to the observation that there was a significant loss of α-helix and increase in random coil with thermal denaturation of barstar protein (Nolting et al., 1997).

The changes in CD-spectra after exposure at different temperature were reversible, particularly after the pretreatment at lower temperature, reflecting the physiological significance of changes in CD-spectra. However, the changes induced at high temperature (45°C) were not completely reversible, obviously because of the iiTeversible aggregation of unfolded protein at high temperature. Also, in case of soyabean peroxidase, the thermal and denaturation and associated unfolding of the protein were not reversible (Kamal and Behere, 2002).

PEG and urea are known to induce aggregation and dissociation of proteins. In presence of PEG-6000 the negative ellipticity of PEPC increased, as indicating that the protein attained a high α-helical conformation. In contrasi, 3 M urea caused a marked decrease in the α-helicity and thus increased the % of random coil. Further studies were extended to check the effects of Glc-6-P (an allosteric activator) and malate (a competitive inhibitor) on the CD-spectra of PEPC protein. The changes in CD-spectra suggested that Glc-6-P induced a marked stability in the a-helicity of PEPC. while malate caused disruption in the extent of a-helicity.

This is the first detailed report on the structural changes induced by temperature in C_4 PEPC and its changes with temperature, studied by using diverse techniques of PAGE, fluorescence emission and CD spectra.

Major conclusions from the present study are:

1. Temperature caused a dramatic modulation of PEPC in leaf discs of *A. hypochondriacus* (C_4) as well as *Pisum sativum* (C3). The steep increase in activity of PEPC with rise in temperature could be physiologically significant, as the temperature is expected to rise from about 10 to 15°C in the morning to 35 to 40°C at midday, on a clear and sunny day.
2. Temperature induced changes in the properties of PEPC were reversible to large extent. This marked reversibility of the effect of temperature of PEPC in case of both C_4 and C_3 is an additional indication of the physiological relevance of temperature effects on PEPC.
3. During illumination, there was an increase in the phosphorylation of the protein, increase in the activity of PEPC and decrease in the malate sensitivity. The changes induced by rise in temperature (increase in the activity and decrease in the extent of malate inhibition) are quite similar to the changes during light activation of PEPC. However, there was no change in the phosphorylation of protein, indicating protein phosphorylation was not the main reason for the temperature effects on PEPC.
4. Temperature caused marked and reversible changes in the properties of PEPC protein purified from *A. hypochondriacus* leaves. Since these experiments

were **all** done ***in*** *vitro* with **purified** PEPC, the changes were obviously independent of phosphorylation.

5. The presence of PEG-6000 protected PEPC against cold inactivation, indicating the importance of the conformation of PEPC.
6. The marked changes in intrinsic fluorescence (obviously from tiyptophan and tyrosine residues) demonstrated qualitative (conformational) changes in PEPC protein at different temperature and these changes were reversible.
7. Increase in intrinsic fluorescence in presence of PEG and decrease in presence of urea demonstrated the aggregation and dissociation of protein, respectively.
8. The temperature induced confonnational changes could also be visualized using a hydrophobic dye, ANSA. The extrinsic fluorescence indicated by ANS increased at warm temperature and decreased at cold temperature.
9. The changes in intrinsic and extrinsic fluorescence suggested unfolding of PEPC at warm temperature and folding at cold temperature.
10. The recording of the CD-spectra revealed that the mean residue negative ellipticity of PEPC decreased with a rise in temperature, suggesting significant changes in the secondary structure of protein. The changes induced by cold temperature were reversible.
11. The PEPC of *A. hypochondriacus* contained significant amounts of α-helices but the β-chains were of only a minor proportion. The α-helical

percentages of PEPC increased marked at 15°C (compared to 30°C), while decreasing at 45°C.

12. The presence of PEG-6000 increased the negative ellipticity of CD-spectra and led to marked increase in α-helices. In contrast, the presence of urea disrupted the secondary structure.

It would be extremely interesting to examine in detail the molecular changes in purified PEPC with temperature *in vitro* and the interaction of light and temperature effects of PEPC in leaves *in vivo*. Leaves of *A. hypochondriacus* offer a good model for studies on C_4-PEPC *in vivo* as well as *in vitro*.

Chapter 10

Literature Cited

Literature Cited

Adams CA, Leung F, Sun SSM (1986) Molecular **properties of** phospho*enol*pyruvate carboxylase from C_3, C3-C4 intermediates **and** C4 *Flaveria* species. Planta 167: 216-225

Alvarez R, Garcia-Maurino S, Feria AB, Vidal J, Echevarria C (2003) **A** conserved 19-amino acid synthetic peptide from the carboxy terminus of phosphoenolpyruvate carboxylase inhibits the *in vitro* phosphorylation of the enzyme by the calcium-independent phosphoenolpyruvate carboxylase kinase. Plant Physiol 132: 1097-106.

Andreo CS, **Gonzalez DH, Iglesias AA** (1987) Higher plant phosphoenolpyruvate carboxylase. Structure and regulation. FEBS Lett 213: 1-8

Angelopoulos **K,** Gavaias NA (1988) Reversible cold inactivation of C4-phospho*enol*pyruvate carboxylase: Factors affecting reactivation and stability. J Plant Physiol 132: 714-719

Angelopoulos K, Zervondakis G, Vonquiouklakis S, Gavalas NA (1990) Differences among C_4 species in cold inactivation of phospho*enol*pyruvate carboxylase and the effect of pH on it (Abstract No.2). Physiol Plant 79: Suppl., 58

Arnon DI (1949) Copper enzymes in isolated chloroplasts. Polyphenol oxidase in *Beta vulgar is.* Plant Physiol 24: 1-15

Bakrim N, **Echevarría** C, **Cretin C, Arrio-Dupont M, Pierre JN, Vidal J, Chollet R, Gadal P** (1992) Regulatory phosphorylation of *Sorghum* leaf phospho*enol*pyruvate carboxylase: identification of the protein-serine kinase and some elements of the signal transduction cascade. Eur J Biochem 204: 821-830

Bandarian V, Poehner WJ, Grover SD (1992) Metabolite activation of crassulacean acid metabolism and C_4 phospho*enol*pyruvate carboxylase. Plant Physiol 100: 1411-1416

Bandurski RS, Greiner CM (1953) The enzymatic synthesis of oxaloacetate from phospho*enol*pyruvate and carbon dioxide. J Biol Chem 204: 781-786

Berry **JA,** Bjorkman **O** (1980) Photosynthetic responses and adaptation to temperature **in** higher plants. Annu Rev of Plant Physiol 31: 491-543

Betz M, Dietz K-J (1991) Immunological characterization of two dominant tonoplast polypeptides. Plant **Physiol** 97: 1294-1301

Biasing OE, Ernst K, Streubel M, Westhoff P, Svensson P (2002) The **non-**photosynthetic phosphoenolpyruvate carboxylases of the C_4 dicot *Flaveria trinervia* **Implications for** the evolution of C_4 photosynthesis. Planta 215: 448-456

Biasing OE, Westhoff P, Svensson P (2000) Evolution of C_4 phosphoenolpyruvate carboxylase in *Flaveria,* a conserved serine residue in the carboxyl-terminal part of the enzyme is a major determinant for C_4-specific characteristics. J Biol Chem 275: 27917-27923

Blonde JD, **Plaxton** WC (2003) Structural and kinetic properties of high and low molecular mass phosphoenolpyruvate carboxylase isoforms from the endosperm of developing castor oil seeds. J Biol Chem 278: 11867-11873

Blum H, Beier **H,** Grass HJ (1987) Improved silver stain of plant proteins. RNA and DNA in polyacrylamide gels. Electrophoresis 8: 93-97

Bowes G, Salvucci ME (1989) Plasticity in the photosynthetic carbon metabolism of submerged aquatic macrophytes. Aquat Bot 34: 233-266.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248-254

Brooks **SPJ** (1992) A simple computer program with statistical tests for analysis of enzyme kinetics. Biotechniques 13: 906-911

Buchanan-Bollig IC, Kluge M, Müller D (1984) Kinetic changes with temperature of PEP carboxylase from a CAM plant. Plant Cell Environ 7: 63-70

Burnell JN (1990) A comparative study of the cold sensitivity of pyruvate Pi dikinase in *Flaveria* species. Plant Cell Physiol 31: 295-297

Carter PJ, Wilkins MB, **Nimmo** HG, Fewson CA (1995) Effects of temperature on the activity of phosphoenolpyruvate carboxylase and on the control of CO_2 fixation in *Bryophyllum fedtschenkoi.* Planta 196: 375-380

Casati P, Lara MV, Andreo CS (2000). Induction of a C_4 like mechanism of CO_2 fixation in *Egeria densa,* a submerged aquatic species. Plant Physiol 123: 1611-1621

Cavaille D, Combes D (1995) Effect of temperature and pressure on yeast invertase stability: a kinetic **and** conformational study. **J** Biotechnol 43: 221-228

Chardot TP, Wedding RT (1992) Regulation of *Crassula argentea* phosphoenolpyruvate carboxylase **in relation** to temperature. **Arch** Biochem Biophys 293: **292-297**

Chen LM, Omiya T, Hata S, Izui K (2002) Molecular characterization of a phosphoenolpyruvate carboxylase from a thermophilic cyanobacterium, *Synechococcus vulcanus* **with** unusual allosteric properties. Plant Cell Physiol 43: 159-69

Chinthapalli B, Murmu J, Raghavendra AS (2003) Dramatic difference in the responses of phosphoenolpyruvate carboxylase to temperature in leaves of C_3 and C_4 plants. J Exp Bot 54: 707-14

Chinthapalli B, Raghavendra AS, Rishi AR, Goyal, A (2002) Phosphoenolpyruvate carboxylase from C_4 plants: Properties and regulation. *In* A Goyal, S L Mehta, M L Lodha, eds, Vol. 1, Reviews in Plant Biochemistry and Biotechnology, New Delhi, pp 143-160

Chollet R, Vidal J, O'Leary MH (1996) Phospho*enol*pyruvate carboxylase: a ubiquitous, highly regulated enzyme in plants. Annu Rev Plant Physiol Plant Mol Biol 47: 273-298

Chryssomallis GS, Torgerson PM, Drickamer HG, Weber G (1981) Effect of hydrostatic pressure on lysozyme and chymotrypsinogen detected by fluorescence polarization. Biochemistry 20: 3955-3959

Coursol S, Giglioli-Guivarc'h N, Vidal J, Pierre JN (2000) An increase in phosphoinositide-specific phospholipase C activity precedes induction of C_4 phosphoenolpyruvate carboxylase phosphorylation in illuminated and NH_4Cl-treated protoplasts from *Digitaria sanguinalis*. Plant J 23: 497-506

Crafts-Brandner SJ, Salvicci ME (2002) Sensitivity of photosynthesis in a C_4 plant, maize, to heat stress. Plant Physiol 129: 1773-1780

Cretin C, Keryer E, Tagu D, Lepiniec L, Vidal J, Gadal P (1990) Complete cDNA sequence of *Sorghum* phospho*enol*pyruvate carboxylase involved in C_4 photosynthesis. Nucl Acid Res 18: 658

Cretin C, Santi S, Keryer E, Lepiniec L, Tagu D, Vidal J, Gadal P (1991) The phospho*enol*pyruvate carboxylase gene family of sorghum: Promoter structures, amino acid sequences and expression of genes. Gene 99: 87-94

Dai Z, Ku MSB, Edwards GE (1994) Effects of growth regulators on the induction of crassulacean **acid metabolism in the facultative halophyte** *Mesembryanthemum crystallinum* L. **Planta 192: 287-294**

Deshpande RA, Khan MI, Shankar V (2003) Equilibrium unfolding of RNase Rs from *Rhizopus stolonifer*: **pH dependence of chemical and** thermal denaturation. Biochim Biophys **Acta 1648: 184-94**

Devi MT, Raghavendra AS (1992) Light activation of phosphoenolpyruvate carboxylasc in maize **mesophyll** protoplasts. **J Plant Physiol 139:** 431-435

Diaz E, O'Laughlin JT, O'Leary MH (1988) Reaction of phosphoenolpyruvate **carboxylase** with (Z)-3 **bromophosphoenol-pyruvate** and (Z)-3-fluorophosphoenolpyruvate. Biochemistry 27: 1336-1341

Doncaster HD, Leegood RC (1987) Regulation of **phospho*enol*pyruvate** carboxylase activity in maize leaves. **Plant** Physiol **84: 82-87**

Dong L, Ermolova NV, Chollet R (2001) Partial purification and biochemical characterization of a heteromeric protein phosphatase 2A **holoenzyme** from maize (*Zea mays* L.) leaves that dephosphorylates **C4** phosophoenolpyruvate carboxylase. Planta 213: 379-89

Dong L, Patil S, Condon SA, Haas EJ, Chollet R (1999) The conserved C-terminal tetrapeptide of *Sorghum* C_4 phosphoenolpyruvate carboxylasc is indispensable for maximal catalytic activity, but not for homotetramer formation. Arch **Biochem** Biophys 371: 124-128

Dong L-Y, Ueno Y, Hata S, Izui K (1997) Effects of site-directed **mutagenesis** of conserved Lys^{606} residue on catalytic and regulatory functions of maize C_4-form **phospho*enol*pyruvate** carboxylase. Plant Cell Physiol 38: 1340-1345

Drilias P. Gousias II, Manetas Y, Gavalas NA (1994) Temperature dependence of **phospho*enol*pyruvate** carboxylase activity in the presence of cosolutes. Photosynthetica 30: 225-232

Drilias P, Manetas Y, Gavalas NA (1997) Detecting **amino** acid residues in the active site of **phospho*enol*pyruvate** carboxylase at low and high (physiological) enzyme concentrations. **Photosynthetica** 33: 151-157

Du Y-C, Nose A, Wasano K (1999a) Thermal characteristics of C4 photosynthctic enzymes from leaves of three sugarcane species differing in cold sensitivity. Plant Cell Phvsiol 40: 298-304

Du YC, Nose A, Wasano K (1999b) Effects of chilling temperature on photosynthetic rates, photosynthetic enzyme activities and metabolite levels in three sugarcane species. Plant Cell Environ 22: 317-324

Duff SMG, Andreo CS, Pacquit V, Lepiniec L, Sarath G, Condon SA, Vidal J, Gadal P, Chollet R (1995) Kinetic analysis of the non-phosphorylated, *in vitro* phosphorylated, and phosphorylation-site-mutant (Asp8) forms of intact recombinant C_4 phosphoenolpyruvate carboxylase from *Sorghum.* Eur J Biochem 228: 92-95

Duff SMG, Chollet R (1995) *In vivo* regulation of wheat leaf phosphoenolpyruvate carboxylase by reversible phosphorylation. Plant Physiol 107: 775-782

Echevarria C, Vidal J, **Jiao** J-A, **Chollet** R (1990) Reversible light activation of phosphoenolpyruvate carboxylase protein-serine kinase in maize leaves. FEBS Lett 275: 25-28

Edwards GE, **Walker** DA (1983) C_3, C_4: Mechanisms and Cellular and Environmental Regulation of Photosynthesis. Blackwell Scientific, Oxford, UK.

Encinas **MV,** Evangelio **JA, Andreu JM, Goldie H, Cardemil** E (1998) Stability of *E. coli* phosphoenolpyruvate carboxykinase against urea-induced unfolding and ligand effects. Eur J Biochem 255: 58-63

Encinas MV, **González-Nilo** FD, Andreu JM, Alfonso C, Cardemil E (2002) Urea induced unfolding studies of free and ligand bound tetrameric ATP-dependent *Saccharomyces cerevisiae* phosphoenol-pyruvate carboxykinase: Influence of quarternary structure on protein conformational stability. Int J Biochem Cell Biol 34: 645-656

Foyer CH, Noctor G, Lelandais **M,** Lescure JC, **Valadier MH, Boutin JP,** Horton **P** (1994) Short-term effects of nitrate, nitrite and ammonium assimilation on photosynthesis, carbon partitioning and protein phosphorylation in maize. Planta 192: 211-220

Frank J, Clarke RJ, Vater J, **Holzwarth** JF (2001) Influence of allosteric effectors on the kinetics and equilibrium binding of phosphoenolpyruvate (PEP) to phosphoenolpyruvate carboxylase (PEPC) from *Zea mays.* Biophys Chem 92: 53-64.

Fugita N, **Miwa** T, Ishijima S, **Izui** K, Katsuki H (1984) The primary structure of phosphoenolpyruvate carboxylase of *Escherichia coli.* Nucleotide sequence of the ppc gene and deduced amino acid sequence. J Biochem (Tokyo) 95: 909-916

Gao Y, **Woo** KC (1996a) Regulation of phospho*enol*pyruvate carboxylase in *Zea mays* by protein phosphorylation and metabolites and their roles in photosynthesis. Aust J Plant Physiol 23: 25-32

Gao Y, **Woo** KC (1996b) Site-directed mutagenesis of *Flaveria trinervia* phospho*enol*pyruvate carboxylase: Arg^{450} and Arg^{767} are essential for catalytic activity and Lys^{829} affects substrate binding. FEBS Lett 392: 285-288

Garcia-Mourino S, Monreal JA, Alvarez R, Vidal J, Echiverria C (2003) Characterization of salt stress enhanced phosphoenolpyruvate carboxylase kinase activity in leaves *of Sorghum vulgare*: independence from osmotic stress, involvement of ion toxicity and significance of dark phosphorylation. Planta 216: 648-655

Gayathri J, Parvarthi K, Raghavendra AS (2000) Purification and stability during storage of phosphoenolpyruvate carboxylase from leaves of *Amaranthus hypochondriacus,* a NAD-ME type C_4 plant. Photosynthetica 38: 45-52

Gayathri J, Parvathi K, Chinthapalli B, Westhoff P, Raghavendra AS (2001) Immunological characteristics of PEP carboxylase from leaves of C3-, C4- and C3-C4 intermediate species of *Alternanthera* - Comparison with selected C_3-and C_4- plants. Indian J Exp Biol 39: 643-649

Gayathri J, **Raghavendra** AS (1994) Ammonium ions stimulate *in vitro* the activity of phospho*enol*pyruvate carboxylase from leaves of *Amaranthus hypochondriacus,* a C_4 plant: evidence for allosteric activation. Biochem Mol Biol Intl 33: 337-344

Giglioli-Guivarc'h N, Pierre J-N, Brown S, Chollet R, Vidal J, Gadal P (1996) The light-dependent transduction pathway controlling the regulatory phosphorylation of C_4-phospho*enol*pyruvate carboxylase in protoplasts from *Digitaria sanguinalis.* Plant Cell 8: 573-586

Gillinta J, Grover SD (1995) Kinetic interactions of glycine with substrates and effectors of phospho*enol*pyruvate carboxylase from maize leaves. Photosynth Res 45: 121-126

Gonzalez DH, Andreo CS (1988) Carboxylation and dephosphorylation of phosphoenol-3-fluoropyruvate by maize leaf phosphoenolpyruvate carboxylase. Biochem J 253: 217-22

Gonzalez DH, **Andreo** CS (1989) The use of substrate analogues to study the active-site structure and mechanism of PEP carboxylase. Trends Biochem Sci 14: 24-27

Gonzalez DH, Iglesias AA, Andreo CS (1987) Interaction of acetylphosphate and carbamyl phosphate with plant phospho*enol*pyruvate carboxylase. Biochem J 241: 543-548

Graham D, Hockley DG, Patterson BD (1979) Temperature effects on phosphoenolpyruvate carboxylase from chilling-sensitive and chilling resistant plants. *In i* M Lyons, JK Raison eds, Low Temperature Stress in Crop Plants: The Role of the Membrane, Academic Press, New York pp 187-202

Greenfield NJ, Fasman GD (1969) Computed circular dichroism spectra for the evaluation of protein conformation. Biochemistry 8: 4108-4116

Greenspan P, Mayer EP, Fowler SD (1985) Nile red: a selective fluorescent stain for intracellular lipid droplets. J Cell Biol 100: 965-973

Gupta SK, Ku MSB, Lin JH, Zhang D, Edwards GE (1994) Light/dark modulation of phosphoenolpyruvate carboxylase in C_3 and C_4 species. Photosynth Res 42: 133-143

Hague DR, Sims TL (1980) Evidence for light-stimulated synthesis of phospho*enol*pyruvate carboxylase in leaves of maize. Plant Physiol 66: 505-509

Hartwell J, Gill A, Nimmo GA, Wilkins MB, Jenkins GI, Nimmo HG (1999) Phosphoenolpyruvate carboxylase kinase is a novel protein kinase regulated at the level of expression. Plant J 20: 333-342

Hartwell J, Smith LH, Wilkins MB, Jenkins GI, Nimmo HG (1996) Higher plant phosphoenolpyruvate carboxylase kinase is regulated at the level of translatable mRNA in response to light or a circadian rhythm. Plant J 10: 1071-1078

Hermans J, Westhoff P (1990) Analysis of expression and evolutionary relationships of phospho*enol*pyruvate carboxylase genes in *Flaveria trinervia* (C_4) and *F. pringlei* (C_3). Mol Gen Genet 224: 459-468

Hermans J, Westhoff P (1992) Homologous genes for the C_4 isoforms of phospho*enol*pyruvate carboxylase in a C_3 and C_4 *Flaveria* species. Mol Gen Genet 234: 275-284

Hodgson RJ, Plaxton WC (1995) Effect of polyethylene glycol on the activity, intrinsic fluorescence and oligomeric structure of castor seed cytosolic fructose 1,6-bisphosphatase. FEBS Lett 368: 559-562

Honda H, Okamoto T, Shimada H (1996) Isolation of a cDNA for a phosphoenolpyruvate carboxylase from a monocot CAM-plant, *Aloe*

arborescens: structure and its gene expression. Plant Cell Physiol 37: 881-888

Huber SC, Edwards GE (1975) Inhibition of phosphoenolpyruvate carboxylase from C_4 plants by malate and aspartate. Can J Bot 53: 1925-1933

Huber SC, Sugiyama T (1986) Changes in sensitivity to effectors of maize leaf phospho*enol*pyruvate carboxylase during light/dark transitions. Plant Physiol 81: 674-677

Hudspeth RL, Grula JW (1989) Structure and expression of the maize gene encoding the phospho*enol*pyruvate carboxylase isozyme involved in C_4 photosynthesis. Plant Mol Biol 12: 579-589

Iglesias AA, Andreo CS (1984) On the molecular mechanism of maize phospho*enol*pyruvate carboxylase activation by thiol compounds. Plant Physiol 75: 983-987

Iglesias AA, Gonzalez DH, Andreo CS (1986) Purification and molecular and kinetic properties of phospho*enol*pyruvate carboxylase from *Amaranthus viridis* L. leaves. Planta 168: 239-244

Imada K, Sato M, Tanaka N, Katabe Y, Matsuura Y, Oshima T (1995) Three-dimensional structure of a highly thermostable enzyme, 3-isopropylmalate dehydrogenase of *Thermus thermophilus* at 2.2 A resolution. J Mol Biol 222: 725-738

Inoue M, Hayashi M, Sugimoto M, Harada S, Kai Y, Kasai N, Terada K, Izui K (1989) First crystallization of a phosphoenolpyruvate carboxylase from *Escherichia coli*. J Mol Biol 208: 509-510

Izui K, Yabuta IN, Ogawa N, Ueno Y, Furumoto T, Saijo Y, Hata S, Sheen J (1995) Enzymological evidence for involvement of a calcium protein kinase in regulatory phosphorylation of PEP-carboxylase in maize. Arch Biochem **Biophys** 269: 526-535

Janc JW, Urbauer JL, O'Leary MH, Cleland WW (1992) Mechanistic studies of phosphoenolpyruvate carboxylase from Zea mays with (Z)- and (E)-3-fluorophosphoenolpyruvate as substrates. Biochemistry 1992 31:6432-6440

Jenkins CLD, Harris RLN, McFadden HG (1987) 3.3-Dichloro-2-dihydroxyphosphinoylmethyl-2-propenoate. a new, specific inhibitor of phosphoenolpyruvate carboxylase. Biochem Int 14: 219-226

Jensen WA, Armstrong JM, De Giorgio J, Hearn MT (1995) Stability studies on maize leaf phosphoenolpyruvate carboxylase: **the** effect of salts. Biochemistry 34: 472-480

Jiao JA, Chollet R (1989) Regulatory **seryl-phosphorylation of C_4** phosphoenolpyruvate carboxylase by a **soluble protein kinase from** maize leaves. Arch Biochem Biophys 269: 526-35

Jiao JA, Chollet R (1990) Regulatory phosphorylation of **serine-15** in maize phosphoenolpyruvate carboxylase by a **C_4-leaf** protein-serine kinase. Arch Biochem Biophys 283: 300-305

Jiao JA, Chollet R (1991) Posttranslational regulation of phosphoenolpyruvate carboxylase in C_4 and Crassulacean acid metabolism plants. Plant Physiol 95: 981-985

Kai Y, Matsumura H, Inoue T, Terada K, Nagara Y, Yoshinaga T, Kihara A, Tsumura K, Izui K (1999a) Three-dimensional structure of phosphoenolpyruvate carboxylase: a proposed mechanism for allosteric inhibition. Proc Natl Acad Sci USA 96: 823-828

Kai Y, Matsumura Shirakata S, Inoue T, Yoshinaga Y, Ueno Y, Izui K (1999b) Crystal structure of phosphoenolpyruvate carboxylase: **the** reaction mechanism and regulation. Acta Cryst 58:103

Kai Y, Matsumura H, Izui K (2003) Phosphoenolpyruvate carboxylase: three-dimensional structure and molecular mechanisms. Arch Biochem Biophys 414: 170-179

Kamal JKA, Beliere DV (2002) Thermal and conformational stability of seed coat soyabean peroxidase. Biochemistry 41: 9034-9042

Katagiri F, Kodaki T, Fujita N, Izui K, Katsuiki H (1985) Nucleotide sequence of the phosphoenolpyruvate carboxylase gene of cyanobacterium *Anacystis nidulans.* Gene 38: 265-269

Kluge M, Maier P, Brulfert J, Faist K, Wollny, B (1988) Regulation of phospho*enol*pyruvate carboxylase in crassulacean acid metabolism: ***in*** *vitro* phosphorylation of the enzyme. J Plant Physiol 133: 252-256

Koizumi N, Sato F, Terano Y, Yamada Y (1991) Sequence analysis of cDNA encoding phosphoenolpyruvate carboxylase from cultured tobacco cells. Plant Mol Biol 17: 535-539

Krall JP, Edwards GE (1993) PEP carboxylase from two C_4 species of *Panicum* with markedly different, susceptibilities to cold inactivation. Plant cell Physioi 34: 1-11

Krall JP, Edwards GE, Andreo CS (1989) Protection of **pyruvate, Pi dikinase** from maize against **cold liability by compatible solutes. Plant Physiol** 89:280-285

Laemmli UK (1970) Cleavage **of structural** proteins **during the assembly of the** head of bacteriophage T_4. **Nature** 227: 680-685

Lakowicz JR (1983) Principles of fluorescence spectroscopy, **Plenum Press,** New York.

Lara MV, Casati P, Andreo CS (2001) *In vivo* phosphorylation of phosphoenolpyruvate carboxylase in *Egeria densa*, a submersed aquatic species. Plant Cell Physiol 42(4): 441-445

Latzko E, **Kelly GJ** (1983) The many-faceted function of phosphoenolpyruvate carboxylase in C_3 plants. Physiol Veg 21: 805-815

Law RD, Plaxton WC (1995) Purification and characterization of a novel phosphoenolpyruvate carboxylase from banana fruit. Biochem J 307: 807-816

Leblova S, Strakosova A, Vojtechová M (1991) Regulation of activity of phospho*enol*pyruvate carboxylase isolated from germinating maize *(Zea mays* L.) seeds by some metabolites. Biol Plant (Praha) 33: 66-74

Leegood **RC, Walker RP** (1999) Regulation of C_4 pathway. *In* RF Sage, RK Monson, eds, C_4 Plant Biology. Academic Press, San Diego pp 89-131

Lepiniec L, Vidal J, Chollet R, Gadal P, Cretin C (1994) Phosphoenolpyruvate carboxylase: Structure, regulation and evolution. Plant Sci 99: 111-124

Liu J, Peliska JA, O'Leary MH (1990) Synthesis and study of (Z)-3-chlorophosphoenolpyruvate. Arch Biochem Biophys 277:143-8

Long SP (1999) Environmental responses. *In* RF Sage and RK Monson, eds. C_4 Plant Biology. Academic Press, San Diego pp 215-249

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the folin phenol reagent. J Biol Chem 193: 265-275

Luinenberg I, Coleman JR (1992) Identification, characterization and sequence analysis of the gene encoding phospho*enol*pyruvate carboxylase in *Anabaena* Sp. **PCC** 7120. J Gen Microbiol 138: 685-691

Manetas Y (1990) **A re-examination of NaCl** effects on phosphoenolpyruvate carboxylase at high (physiological) enzyme concentrations. **Physiol** Plant 78: 225-229

Manh CT, Bismuth E, Boutin J-P, Provot M, Champigny M-L (1993) Metabolite effectors **for short-term** nitrogen-dependent **enhancement of** phosphoenolpyruvate carboxylase activity and decrease of net sucrose synthesis in wheat leaves. **Physiol** Plant 89: 460-466

Maralihalli G, Bhagwat AS (2001) Limited proteolysis by trypsin influences activity of maize phosphoenolpyruvate carboxylase. Indian J Biochem Biophys 38: 361-367

Mares J, Leblova S (1980) Phosphoenolpyruvate carboxylase from leaves of maize, sorghum and millet. Photosynthetica 14: 25-31

Matsumura H, Nagata T, Terada M, Shirakata S, Inoue T, Yoshinaga T, Ueno Y, Saze H, Izui K, Kai Y (1999a) Plausible phosphoenolpyruvate binding site revealed by 2.6 A^0 structure of Mn^+-bound phosphoenolpyruvate carboxylase from *Escherichia coli.* FEBS Lett 458: 93-96

Matsumura H, Nagata T, Terada M, Shirakata S, Inoue T, Yoshinaga Y, Ueno Y, Saze H, Izui K, Kai Y (1999b) Crystallization and preliminary X-ray diffraction studies of C_4-form phospho*enol*pyruvate carboxylase from maize. Acta Cryst 55: 1937-1938

Matsumura H, Xie Y, Shirakata S, Inoue T, Yoshinaga T, Ueno Y, Izui K, Kai Y (2002) Crystal structures of C_4 form maize and quaternary complex of *E. coli* phosphoenolpyruvate carboxylases. Structure 10: 1721-30

Matsuoka M, Furbank RT, Fukayama H, Miyao M (2001) Molecular engineering of C_4 photosynthesis. Annu Rev Plant Physiol Plant Mol Biol 52: 297-314

Mazen AMA (2000) Changes in properties of phosphoenolpyruvate carboxylase with induction of Crassulacean Acid Metabolism (CAM) in the C_4 plant *Portulaca oleracea.* Photosynthetica 38: 385-391

McNaughton GAL, Fewson CA, Wilkins MB, Nimmo HG (1989) Purification, oligomerization state and malate sensitivity of maize leaf phospho*enol*pyruvate carboxylase. Biochem J 261: 349-355

Mc William JR, Ferrar PJ (1974) Photosynthetic adaptation of higher plants to thermal stress. *In* RL Bieleski, AR Ferguson, MM Cresswell. eds,

Mechanisms of Regulation of Plant Growth. Bulletin 12, Royal Society of New Zealand, Wellington pp 467-476

Merkelback S, **Gehlen J, Denecke M, Hirsch HJ, Kreuzaler F** (1993). Cloning, sequence analysis and expression of a cDNA encoding active phosphoenolpyruvate carboxylase of the C_3 plant *Solarium tuberosum*. Plant MolBiol. 23:881-888.

Monson RK (1999) The origins of C_4 genes and evolutionary pattern in the C_4 metabolic phenotype. *In* RF Sage. RK Monson. eds, C_4 Plant Biology. Academic Press. San Diego pp 377-410

Moraes TF, **Plaxton** WC (2000) Purification and characterization of phosphoenolpyruvate carboxylase from *Brassica napus* (rapeseed) suspension cell cultures: implications for phosphoenolpyruvate carboxylase regulation during phosphate starvation, and the integration of glycolysis with nitrogen assimilation. Eur J Biochem 267(14): 4465-4476

Mukerji SK (1977) Corn leaf phospho*enol*pyruvate carboxylase. Purification and properties of two isoenzymes. Arch Biochem Biophys 182: 343-351

Mukerji SK, Ting IP (1971) Phospho*enol*pyruvate carboxylase isoenzymes. Separation and properties of three forms from cotton leaf tissue. Arch Biochem Biophys 143: 297-317

Murchie EH, **Ferarrio-Mery** S, **Valadier** MH, Foyer CH (2000) Short-term nitrogen-induced modulation of phosphoenolpyruvate carboxylase in tobacco and maize leaves. J Exp Bot 51: 1349-1356

Nakamura T, **Izui** K, **Yumoto** N (2002) Thermostable and active phosphoenolpyruvate carboxylase from *Thermus sp.* even after proteolytic cleavage. J Mol Cata 17: 215-222

Nakamura T, Yoshioka I, Takahashi M, Toh H, Izui K (1995) Cloning and sequence analysis of the gene for phosphoenolpyruvate carboxylase from an extreme thermophile, *Thermus sp.* J Biochem 118: 319-324

Nhiri M, **Bakrim** N, Pacquit V, Hachimi-Messouak ZE, **Osuna** L, **Vidal** J (1998) Calcium-dependent and independent phosphoenolpyruvate carboxylase kinases in *Sorghum* leaves: Further evidence for the involvement of the calcium-independent protein kinase in the *in situ* regulatory phosphorylation of C_4 phosphoenolpyruvate carboxylase. Plant Cell Physiol 39: 241-246

Nimmo GA, **Nimmo** HG, **Hamliton** ID, **Fewson** CA, **Wilkins** MB (1986) Purification of the phosphorylated night form and dephosphorylated day

form of phospho*enol*pyruvate carboxylase from *Bryophyllum fedtschenkoi*. Biochem J 239: 213-220

Nimmo HG (2000) **The regulation** of phosphoenolpyruvate **carboxylase in** CAM **plants. Trends Plant** Sci 5: 75-80

Nimmo HG (2003) **Control** of **the** phosphorylation of **phosphoenolpyruvate** carboxylase in higher plants. Arch Biochem Biophys 414: 189-196

Nolting B, Globik R, Soler-González AS, Fersht AR (1997) Circular dichroism of denatured barstar residual structure. Biochemistry 36: 9899-9905

O'Leary MH (1982) Phospho*enol*pyruvate carboxylase: an enzymologists's view. Annu Rev Plant Physiol 33: 297-315

O'Regan M, Thierbach G, Bachmann B, Villeval D, Lepage P, Viret JF, Lemoine Y (1989) Cloning and nucleotide sequence of the phosphoenolpyruvate carboxylase coding gene of *Corynebacterium glutamicum* ATCC13032. Gene 77: 237-251

Ogawa N, Yabuta N, Ueno Y, Izui K (1998) Characterization of a maize Ca^{2+}-dependent **protein kinase** phosphorylating phosphoenolpyruvate carboxylase. Plant Cell Physiol 39: 1010-1019

Oishi M (1971) The separation of T-even bacteriophage DNA from host DNA by hydroxylapatite chromatography. Methods Enzymol 21: 140-147

Osmond CB, Holtum JAM (1981) Crassulacean acid metablism. *In* MD Hatch and NK Boardman, eds, The Biochemistry of Plants. A Comprehensive Treatise. Vol 8, Academic Press, New York pp 283-328

Pacquit V, Santi S, Cretin C, Bui VL, Vidal J, Gadal P (1993) Production and properties of recombinant C_3-type phosphoenolpyruvate carboxylase from *Sorghum vulgare*: *In vitro* phosphorylation by leaf and root PyrPC protein serine kinases. Biochem Biophys Res Commun 197: 1415-1423

Parvathi K, Bhagwat AS, Raghavendra AS (1998) Modulation by bicarbonate of catalytic and regulator)' properties of C_4 phosphoenolpyruvate carboxylase from *Amaranthus hypochondriacus*: Desensitization to malate and glucose-6-phosphate and sensitization to Mg^{2+}. Plant Cell Physiol 39: 1294-1298

Parvathi K, Gayathri J, Muralihalli, GB, Bhagwat AS, Raghavendra AS (2000a) Modulation of phosphoenolpyruvate carboxylase

phosphorylation in leaves of *Amaranthus hypochondriacus*, a NAD-ME type of C_4 plant. *Photosynthetica* 38: 23-28

Parvathi K, Bhagwat AS, **Ueno** Y, **Izui** K, **Raghavendra** AS (2000b) Illumination increases the affinity of phosphoenolpyruvate carboxylase to bicarbonate in leaves of a C_4 plant, *Amaranthus hypochondriacus*. Plant Cell Physiol 41: 905-910

Perrot-Rechenmann C, **Vidal J, Brulfert J, Brulet** A, **Gadal P** (1982) A comparative immunocytochemical localization study of phospho*enol*-pyruvate carboxylase in leaves of higher plants. Planta 155: 24-30

Phillips PJ, **Mc** William **JR** (1971) Thermal responses of the primary carboxylating enzymes from C3 and C_4 plants adapted to contrasting temperature environments. *In* MD Hatch. CB Osmond and RO Slatyer, eds, Photosynthesis and Photorespiration, Wiley-Interscience. New York pp 97-104

Pierre JN, **Pacquit** V, Vidal J, Gadal P (1992) Regulatory phosphorylation of phospho*enol*pyruvate carboxylase in protoplasts of *Sorghum* mesophyll cells and the role of pH and Ca^{2+} as possible components of the light-transduction pathway. Eur J Biochem 210: 531-537

Podesta FE, Andreo CS (1989) Maize leaf phospho*enol*pyruvate carboxylase. Oligomeric state and activity in the presence of glycerol. Plant Physiol 90: 427-433

Podesta FE, Gonzalez DH, Iglesias AA (1990) Phosphate activates phospho*enol*pyruvate carboxylase from the C4 plant *Amaranthus viridis* L. Bot Acta 103:266-269

Podesta FE, **Iglesias** AA, Andreo CS (1986) Modification of an essential amino group of phosphoenolpyruvate carboxylase from maize leaves by" pyridoxal phosphate and by pyridoxal phosphate-sensitized photooxidation. Arch Biochem Biophys 246: 546-53

Podesta FE, Plaxton WC (1993) Activation of cytosolic pyruvate kinase by polyethylene glycol. Plant Physiol 103: 285-288

Podesta FE, Plaxton WC (1994) Regulation of cytosolic carbon metabolism in germinating *Ricinus communis* cotyledons. I. Developmental profiles for the activin concentration and molecular structure of the pyrophosphate and ATP-dependent phosphofructokinases, phospho*enol*pyruvate carboxylase and pyruvate kinase. Planta 194: 374-380

Poetsch W, Hermans J, Westhoff P (1991) Multiple cDNA of phospho*enol*-pyruvate carboxylase in the C_4 dicot *Flaveria trinervia*. FEBS Lett 292: 133-136

Potvin C, Simon JP (1990) The evolution of cold temperature adaptation among population of a widely distributed C_4 weed: barnyard grass. Evol Trends Plants 4: 98-105

Potvin C, Simon JP, Strain BR (1986). Effect of low temperature on the photosynthetic metabolism of the C_4 grass *Echinochloa crusgalli*. Oecologia 69: 499-509.

Raghavendra AS, Yin Z-H, Heber U (1993) Light-dependent pH changes in leaves **of C_4** plants. **Comparison** of the **pH response to carbon dioxide** and oxygen with that **of C_3** plants. Planta 189: 278-287

Rajagopalan AV, Devi MT, Raghavendra AS (1993) Patterns of phospho*enol*pyruvate carboxylase activity **and cytosolic pH during light** activation and dark **deactivation in C_3** and **C_4 plants. Photosynth** Res 38: 51-60

Rajagopalan AV, Devi MT, Raghavendra AS (1994) Molecular biology of **C_4 phospho*enol*pyruvate** carboxylase: structure, regulation and genetic engineering. Photosynth Res 39: 115-135

Rajagopalan AV, Gayathri J, Raghavendra AS (1998) Modulation by weak bases or weak acids of the pH of cell sap and phosphoenolpyruvate carboxylase activity in leaf discs of **C_4** plants. Physiol **Plant 104: 456-462**

Rao SK, Magnin NC, Reiskind JB, Bowes G (2002) **Photosynthetic and other** phosphoenolpyruvate carboxylase isoforms in the single-cell, facultative **C_4** system **of** *Hydrilla verticillata.* Plant Physiol 130: 876-86

Raven, JA (1970) Exogenous inorganic carbon sources **in plant** photosynthesis. Biol Rev **45:** 167-221

Reiskind JB, Madsen TV, Van Ginkel LC, Bowes G (1997). Evidence that inducible C_4-type photosynthesis is a chloroplastic CO2-concentrating mechanism in *Hydrilla.* a submersed monocot. Plant Cell Environ 20: 211-220

Rivoal J, Plaxton WC, Turpin DH (1998) Purification and characterization of high- and low-molecular-mass isoforms of **phosphoeno/pyruvate** carboxylase from *Chlamydomonas reinhardtii*. Kinetic, structural and immunological evidence that **the** green algal enzyme is distinct from the **prokaryotic** and higher plant enzymes. Biochem J 331: 201-209

Rivoal J, Turpin DH, Plaxton WC (2002) *In vitro* phosphorylation of phosphoenolpyruvate carboxylase **from the** green **alga** *Selenastrum minutum*. **Plant Cell Physiol 43:** 785-792

Rustin P, Meyer C, Wedding R (1988) The effect of **adenine** nucleotides **on** purified phospho*enol*pyruvate carboxylase from **the CAM** plant *Crassula argentea*. Plant Physiol 88: 153-157

Rydzik E, Berry J (1996) The C_4 photosynthetic phospho*enol*pyruvate carboxylase from grain amaranth. Plant Physiol (PGR 95-135) 110: 713

Sakakibara H, Kobayashi K, Deji A & Sugiyama T (1997) Partial characterization of the signaling pathway for the nitrate-dependent expression of genes for nitrogen-assimilatory enzymes using detached maize leaves. Plant Cell Physiol 38: 37-843.

Salahas G, Gavalas NA (1997) Effects of phosphate on the activity, stability and regulatory properties of phospho*enol*pyruvate carboxylase from the C_4 plant *Cynodon dactylon*. Photosynthetica 33: 189-197

Salvucci ME (1992) Subunit interactions of Rubisco activase: polyethylene glycol promotes self-association, stimulates ATPase and activation activities, and enhances interactions with Rubisco. Arch Biochem Biophys 298: 688-696

Sanchez R, **Cejudo** FJ (2003) Identification and expression analysis of a gene encoding a bacterial-type phosphoenolpyruvate carboxylase from Arabidopsis and rice. Plant Physiol 132: 949-57

Saze H, Ueno Y, Hisabori T, Hayashi H, Izui K (2001) Thioredoxin-mediated reductive activation of a protein kinase for the regulatory phosphorylation of C_4-form phosphoenolpyruvate carboxylase from maize. Plant Cell Physiol 42: 1295-1302

Schnabl H, Denecke M, Schulz M (1992) *In vitro* and ***in*** *vivo* phosphorylation of stomatal phospho*enol*pyruvate carboxylase from *Vicia faba* L. Bot Acta 105: 367-369

Schultes V, Jaenicke R (1991) Folding intermediates of hyperthermophilic D-glyceraldehyde-3-phosphate dehydrogenase from *Thermototga maritima* are trapped at low temperature. FEBS Lett 290: 235-238

Schulz M, Hunte C, Schnabl H (1992) Multiple forms of phospho*enol*py ruvate carboxylase in mesophyll, epidermal and guard cells of *Vicia faba*. Physiol Plant 86: 315-321

Selinioti E, Manetas Y, Gavalas NA (1986) Cooperative effects of light and temperature on the activity of phospho*enol*pyruvate carboxylase from *Amaranthus paniculatus* L. Plant Physiol 82: 518-522

Semisotnov GV, Rodionova NA, Razgulyaev OI, Uversky VN, Gripas AF, Gilmanshin RJ (1991) Study of the molten globule intermediate state in protein folding by a hydrophobic fluorescent probe. Biopolymers 31: 119-128

Shaffner AR, Sheen J (1992) Maize C_4 photosynthesis involves differential regulation of phosphoenolpyruvate carboxylase genes. Plant J 2: 221-32

Sheen J (1999). C_4 gene expression. Annu Rev Plant Physiol Plant Mol Biol 50: 187-217

Shi J-J, Wu M-X, Zha J-J (1981) Studies **on plant** phospho*enol*pyruvate carboxylase. V. A reversible cold-inactivation of **sorghum leaf PEP carboxylase. Acta Phytophysiol Sinica 7:** 317-326

Shi L, Palleros DR, Fink AL (1994) Protein conformational changes induced by 1,1'-bis(4-anilino-5-naphthalenesulfonic acid): preferential binding to the molten globule **of DnaK. Biochemistry 33: 7536-7546**

Shirahashi K, Hayakawa S, Sugiyama T (1978) Cold lability of pyruvate, orthophosphate dikinase in the maize leaf. Plant Physiol 62: 826-830

Sievers G (1978) Circular dichroism studies on cytochrome C peroxidase from baker yeast (*Saccharomycescerevisiae*). **Biochem Biophys Acta 536:** 212-225

Sims TL, Hague DR (1981) Light-stimulated increase of translatable mRNA **for** phospho*enol*pyruvate **carboxylase in leaves of maize. J Biol** Chem 256: 8252-8255

Slocombe SP, Whitelam GC, Cockburn W (1993) Investigation of phospho*enol*pyruvate carboxylase (PEPcase) in *Mesembryanthemum crystallinum* L. **in C_3 and CAM photosynthetic** states. **Plant Cell Environ** 16: 403-411

Stamatakis K, Gavalas NA, Manetas Y (1988) Organic cosolutes increase the catalytic efficiency of phospho*enol*pyruvate **carboxylase, from** *Cynodon dactylon* (L.) Pers, apparently through self-association of the enzymic protein. Aust J Plant Physiol 15: 621-631

Sugimoto K, Kawasaki T, Kato T, Whittier RF, Shibata D, Kawamura Y (1992) cDNA sequence and expression of a phospho*enol*pyruvate carboxylase gene from soybean. Plant Mol Biol 20: **743-747**

Sugiyama T (1998) Nitrogen-responsive expression of C_4 photosynthesis genes in maize. *In* K Satoh, and N Murata, eds, Stress Responses of Photosynthetic Organism. Elsevier Scientific Publishers, Tokyo pp. 167-180.

Sugiyama T and Sakakibara H (2002) Regulation of carbon and nitrogen assimilation **through** gene **expression. *In* CH** Foyer **and** G **Noctor,** eds, Advances **in** Photosynthesis **and Respiration.** Voll **12, Kluwer** Academic Publishers, The Netherlands **pp** 227-238

Sugiyama T, Schmitt MR, Ku SB, Edwards GE (1979) Differences in cold liability of pyruvate, Pi dikinase among C_4 species. Plant Cell Physiol 20:965-971

Svensson P, Biasing OE, Westhoff P (1997) Evolution of the enzymatic characteristics of C_4 phospho*enol*pyruvate carboxylase. A comparision of the orthologous *ppcA* phospho*enol*pyruvate carboxylases of *Flaveria trinervia* (C_4) and *Flaveria pringlei* (C_3). Eur J Biochem 246: 452-460

Svensson P, Biasing OE, Westhoff P (2003) Evolution of C_4 phosphoenolpyruvate carboxylase. Arch Biochem Biophys 414: 180-188

Takai K, Sako Y, Uchida A (1997) Extrinsic thermostablization factors and thermodenaturation mechanisms for Phosphoenolpyruvate carboxylase (PEPC) from an extremely thermophilic bacterium *Rhodothermus obemensis*. J Ferment Bioeng 84: 291-299

Taybi T, Sotta B, Gehrig H, Güelü S, Kluge M, Brulfert J (1995) Differential effects of abscisic acid on phospho*enol*pyruvate carboxylase and CAM operation in *Kalanchoe blossfeldiana.* Bot Acta 108: 240-246

Timasheff SN (1992) A physicochemical basis for the selection of osmolytes by nature. *In* GN Somero, CB Osmond and CL Bolis, eds, Water and Life. Springer-Verlag, Berlin-Heidelberg, New York pp 70-84

Ting IP, Osmond CB (1973a) Photosynthetic phospho*enol*pyruvate carboxylases. Characteristics of alloenzymes from leaves of C_3 and C_4 plants. Plant Physiol 51: 439-447

Ting IP, Osmond CB (1973b) Multiple forms of plant phospho*enol*pyruvate carboxylase associated with different metabolic pathways. Plant Physiol 51: 448-453

Toll H, Kawamura T, Izui K (1994) Molecular evolution of phospho*enol*pryuvate carboxylase. Plant Cell Environ 17: 31-43

Tovar-Mendez A, Rodríguez-Sotres R, Lopez-Valentin D, Muñoz-Clares RA (1998) Re-examination of the roles of PEP and Mg^{2+} in the reaction catalysed by the phosphorylated and non-phosphorylated forms of phospho*enol*pyruvate carboxylase from leaves of *Zea mays:* effects of the activators glucose 6-phosphate and glycine. Biochem J **332:** 633-642

Towbin H, Staehlin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets. Procedure and some applications. Proc Natl Acad Sci USA 76: 4350-4354

Tsuchida Y, Furumoto T, Izumida A, Hata S, Izui K (2001) Phosphoenolpyruvate carboxylase kinase involved in C_4 photosynthesis in *Flaveria trinervia:* cDNA cloning and characterization. FEBS Lett 507:318-322

Uchino A, Sentoku N, **Nemoto** N, **Ishii R, Samejima M,** Matsuoka M (1998) C_4-type gene expression is not directly dependent on Kranz anatomy in an amphibious sedge *Eleocharis vivipara.* Plant J 14: 565-572

Uedan K, **Sugiyama** T (1976) Purification and characterization of phospho*enol*pyruvate carboxylase from maize leaves. Plant Physiol 57: 906-910

Ueno **O** (1996) Structural characterization of photosynthetic cells in an amphibious sedge, *Eleocharis vivipara,* in relation to C_3 and C4 metabolism. Planta 199: 382-393

Ueno **O** (1998) Induction of kranz anatomy and C_4-like biochemical characteristics in a submerged amphibious plant by abscisic acid. Plant Cell 10:571-584

Vance CP, Gantt JS (1992) Control of nitrogen and carbon metabolism in root nodules. Physiol Plant 85: 266-274

Vidal J, **Chollet** R (1997) Regulatory phosphorylation of C_4 PEP carboxylase. Trends Plant Sci 2: 230-237

Vidal J, Coursol S, Pierre J-N (2002) Reversible phosphorylation in the regulation *of* photosynthetic phosphoenolpyruvate carboxylase in C_4 plants. *In* E-M. Aro and B Andersson, eds. Advances in Photosynthesis and Respiration: Regulation of Photosynthesis. Vol. 11, Kluwer Academic Publishers, The Netherlands pp 363-375

Vidal J, **Gadal** P (1983) Influence of light on phospho*enol*py ruvate carboxylase in *Sorghum* leaves. Identification and properties of two isoforms. Physiol Plant 57: 119-123

Wagner R, Gonzalez DH, Podesta FE, Andreo CS (1987) Changes in the quaternary structure of phospho*enol*pyruvate carboxylase induced by ionic strength affect its catalytic activity. Eur J Biochem 164: 661-666

Wagner R, Podesta FE, Gonzalez DH, Andreo CS (1988) Proximity between fluorescent probes attached to four essential lysyl residues in phosphoenolpyruvate carboxylase. A resonance energy transfer study. Eur J Biochem 173: 561-568

Walker GH, Ku MSB, Edwards GE (1986) Catalytic activity of maize leaf phospho*enol*pyruvate carboxylase in relation to oligomerization. Plant Physiol 80: 848-855

Wallace BA, Janes RW (2001) Synchrotron radiation circular dichroism spectroscopy of proteins: secondary structure, fold recognition and structural genomics. Curr Opin Chem Biol. 5: 567-571

Wang YH, Chollet R (1993) Partial purification and characterization of phosphoenolpyruvate carboxylase protein-serine kinase from illuminated maize leaves. Arch Biochem Biophys 304: 496-502

Wedding RT, O'Brein CE, Kline K (1994) Oligomerization and its affinity of maize phospho*enol*pyruvate carboxylase for its substrate. Plant Physiol 104:613-616

Weigend M, Hincha DK (1992) Quaternary structure of phospho*enol*pyruvate carboxylase from CAM, C_4- and C_3- plants: No evidence for diurnal changes in the oligomeric state. J Plant Physiol 140: 653-660

Westhoff P, Svensson **P, Ernst K, Biasing O, Burscheidt J, Stockhaus J** (1997) Molecular evolution of C_4 phosphoenolpyruvate carboxylase in the genus *Flaveria*. Aust J Plant Physiol 24: 429-436

Willeford KO, Wedding RT{1992) Oligomerization and regulation of higher plant phospho*enol*pyruvate carboxylase. Plant Physiol 99: 755-758

Willeford KO, Wu M-X, Meyer CR, **Wedding RT** (1990) The role of oligomerization in regulation of maize leaf phospho*enol*pyruvate carboxylase activity. Influence of Mg-PEP and malate on the oligomeric equilibrium of PEP carboxylase. Biochem Biophys Res Commun 168: 778-785

Willmer CM, Petropoulou Y, Manetas Y (1990) No light activation and high malate sensitivity **of** phospho*enol*pyruvate carboxylase in guard cell protoplasts of *Commelina communis* L. J Exp Bot 41:1103-1107

Wrba A, Schweiger A, Schultes V, Jaenicke R, Zavodsky P (1990) Extremely thermostable **D-glyceraldehyde-3** -phosphate dehydrogenase from the eubacterium ***Thermotoga maritima***. Biochemistry 29: 7584-7592

Wu M-X, Wedding RT (1987) Temperature effects on **phospho*enol*pyruvate** carboxylase from a CAM and a **C_4** plant. A comparative study. Plant Physiol 85: 497-501

Wu M-X, Wedding RT (1994) Modification of maize leaf **phospho*enol*py**ruvate carboxylase with fluorescein isothiocyanate. Plant Cell Physiol 35: 569-574

Yanagisawa S, **Izui** K (1990) Multiple interaction between tissue-specific nuclear proteins and the promoter of the **phospho*enol*pyruvate** carboxylase gene for C4 photosynthesis in *Zea mays*. **Mol** Gen Genet 224: **325-332**

Yin Z-H, Heber U, Raghavendra AS (1993) Light-dependent pH changes in leaves of **C_4** plants. Comparison **of** cytosolic **alkalinization** and vacuolar acidification with that of **C_3** plants. Planta 189: 267-277

Yoshinaga T (1976) Phosphoenolpyruvate carboxylase of *E. coli* studies on multiple conformation states elicited by allosteric effectors with a fluorescent probe, **1-anilinonapthalene-8-sulphonate**. Biochem Biophys **Acta** 452: 566-579

Zhang XQ, **Chollet** R (1997) Phosphoenolpyruvate carboxylase protein kinase from soybean root nodules: partial purification, **characterization**, and **up/down-regulation** by photosynthate supply from the shoots. Arch Biochem Biophys 343: 260-268

Zhang XQ, Li B, Chollet R (1995) *In vivo* regulatory phosphorylation of soybean nodule **phospho*enol*pyruvate** carboxylase. Plant Physiol 108: 1561-1568

* * * * *

Appendix

Research papers published

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

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

2. **Bhaskarrao Chinthapalli** and A.S. Raghavendra. (2003) Phenominal Difference in the response of Phosphoenolpyruvate carboxylase to Temperature in leaves of C_3 and C_4 plants ***Journal*** *of Experimental Botany* 54: 707-714.

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

4. **Bhaskarrao Chinthapalli,** A.S. Raghavendra and Arun Goyal (2002) Phosphoenolpyruvate Carboxylase from C_4 Plants: Properties and Regulation (*A Minireview*). *Reviews of* ***Plant*** *Biochemistry and Biotechnology* Vol I: 143-159.

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

6. J. Gayathri. K. **Parvathi, Bhaskarrao Chinthapalli,** P. Westhoff and A.S. Raghavendra. 2001. Immunological characteristics of PEP carboxylase from leaves of C_3-, C_4- and C3-C4 intermediate species of *Alternanthera* - Comparison **with** selected C3- and C_4- plants. *Indian Journal Experimental Biology* 39: 643-649.

7. **Bhaskarrao Chinthapalli,** C. Raghavan, O. Biasing, P. Westhoff and A.S. Raghavendra. (2000). Phosphoenolpyruvate carboxylase purified from leaves of C3, C_4 and C3-C4 intermediate species of *Alternanthera:* Properties at limiting and saturating bicarbonate. ***Photosynthetica*** 38: 415-419.

8. **P. Komaraiah, Ch. Bhaskar Rao, A.S. Raghavendra, S.V. Ramakrishna and P. Reddanna** (2001) **Production of fungicidal sesquiterpenes from Potato Hairy Root cultures.** ***Proceedings of BIOHORIZON.*** **3rd National Symposium on Biochemical Engineering and Biotechnology. Department of Biochemical Engineering and Biotechnology, Indian Institute of Technology, New Delhi,** Feb 23-24. p.BE 16.

9. **Bhaskarrao Chinthapalli and A.S. Raghavendra. (2001) Marked modulation by temperature of phosphoenolpyruvate carboxylase from leaves of** *Amaranthus hypochondriacus*, a NAD-ME type C_4 **plant.** ***PS2001, Proceedings of The 12"' International Congress on Photosynthesis***, **CSIRO** publishing. Melbourne. Australia

10. M.T. Devi,, **Bhaskarrao Chinthapalli** and A.S. Raghavendra. (2000). Responses of photosynthesis, **photoinhibition and dark respiration to** CO_2 **in** leaf discs **of** C_3-C_4 intermediate species: **Reduced sensitivity to external** CO_2 **in** C3-C4 intermediates compared to that in C_3 species. ***Journal Plant Biology*** **26:** 125-131.

Journal of Experimental Botany, Vol. 54. No. 383, pp. 707-714, February 2003
DOI: 10.1093/jxb/erg078

Journal of Experimental Botany

RESEARCH PAPER

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

Bhaskarrao Chinthapalli, **Jhadeswar Murmu** and Agepati S. Raghavendra[1]

Department of Plant Sciences, School of Life Sciences, University of Hyderabad, Hyderabad 500 046, India

Received 13 September 2002; Accepted 17 October 2002

Abstract

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

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

Introduction

Phospho*enol*pyruvate carboxylase (EC 4.1.1.31) is a ubiquitous enzyme occurring in the cytosol of photosynthetic and non-photosynthetic tissues of C3, C_4 and CAM plants (Andreo *et al.*, 1987; Rajagopalan *et al.*, 1994; **Cbollel *et al.*,** 1996; Vidal and Chollet, 1997; Nimmo, 2000). C_4 plants differ from C_3 plants in several features, including their light and **temperature** responses (Berry and Björkman, 1980; **Sugiyama** *et al.*, 1979). The **temperature** optima **for** photosynthesis and growth in C_4 plants **are** usually higher than those **for** C_3 **plants** (Berry and Bjorkman, 1980). The C_4 plants, in general, are tolerant **to** heat, but **are** quite sensitive to cold temperatures (Du *et al.,* 1999*a*). The cold sensitivity of the C_4 pathway has been suggested to be related to the cold sensivitity **of** key C_4 enzymes, such as pyruvate phosphate dikinase (PPDK) or PEPC (Potvin **and Simon,** 1990; Burnell, 1990; Du *et al.*, 1999*a*).

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

[1] To whom correspondence should be addressed. Fax: +91 40 23010145. E-mail asrsl@uohyd.ernet.in

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

J. Plant Physiol. 160. 51 - 56 (2003)
© Urban & Fischer Verlag
http://www.urbanfischer.de/journals/jpp

JOURNAL OF PLANT PHYSIOLOGY

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

Jhadeswar Murmu, Bhaskarrao Chinthapalli, Agepati S. Raghavendra*

Department of Plant Sciences, School of Life Sciences, University of Hyderabad, Hyderabad b00046, India

Received May 27, 2002 • Accepted July 15, 2002

Summary

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

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

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

* E-mail corresponding author. asrsl@uohyd.ernet.in

0176-1617/03/160/01-51 $ 15.00/0

Reviews in Plant Biochemistry and Biotechnology
Edited by: **Arun Goyal, S.L. Mehta, M.L. Lodha**
Volume **I,** pp. **143-159,** 2002

Phosphoenolpyruvate Carboxylase from C_4 Plants: Properties and Regulation

Bhaskarrao Chinthapalli[1], A.S. Raghavendra[1]*, A.S. Rishi[2], and Arun Goyal[2]
[1]Department of Plant Sciences, School of Life Sciences, University of Hyderabad, Hyderabad-500 046, India; [2]Department of Biology, College of Science and Engineering, Department of Biochemistry and Molecular Biology, School of Medicine, and Center for Cell and Molecular Biology, University of Minnesota Duluth. Duluth, MN 55812, USA

Phophoenolpyruvate carboxylase (PEPC) is a key enzyme involved in primary carbon assimilation in C_4 and Crassulacean Acid Metabolism (CAM) plants. PEPC mediates the irreversible β-carboxylation of phosphoenolpyruvate (PEP) to form oxaloacetate (OAA) and Pi. Besides carboxylation, PEPC also catalyzes a HCO_3^- - dependent hydrolysis of PEP to yield pyruvate and Pi. PEPC is regulated by the post-translational modification of the enzyme, by either phosphorylation or oligomerization or both. PEPC is also regulated by external/environmental factors (such as light, temperature, photoperiod) as well as internal factors (water status, Pi, cytosolic pH). Attempts to crystallize PEPC from C_4 plants have been partially successful. Nevertheless, the PEPC from *E. coli* has been crystallized, and its three-dimensional model is constructed. The mechanism and signaling pathways that control expression and regulation of C_4 photosynthesis gene(s) have been studied in plants such as maize, *Flaveria, Amaranthus,* Sorghum and an amphibious sedge *Eleocharis vivipara.* A major break through in genetic engineering of C_4-PEPC is the success in transforming rice, a C_3 crop, with the C_4-PEPC gene from maize, with a >100-fold increase in the activity of PEPC. There is still a wide scope for further research on PEPC, particularly the modulation of enzyme by temperature, interaction between light, temperature and nitrogen nutrition during the regulation of PEPC, and diurnal rhythms in enzyme activity. With the availability of novel technologies in the areas of genomics, proteomics and metanomics, we may learn a great deal from the molecular analysis of PEPC and other enzymes associated with its regulation; and dissect signal-transduction pathways in various plant cell types and other microorganism. This review summarizes the recent advances, particularly of the last 5-10 years, on the physiology, biochemistry, molecular biology and genetic engineering of PEPC from C_4 plants.

Key words: CO_2 fixation, C_4 pathway, enzyme regulation, PEPC, photosynthesis.

Phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) is a ubiquitous cytosolic enzyme, involved in the primary carbon fixation in leaves of C_4 and CAM plants. The enzyme also

*Corresponding author. E-mail: asrsl@uohyd.ernet.in
Abbreviations: ABA, Abscisic acid; CAM, Crassulacean acid metabolism; CDPK, Ca^{2+} dependent protein kinase: NAD-ME, NAD-malic enzyme; OAA, oxalacetate; PEP, Phosphoenolpyruvate; PEPC. PEP carboxylase; PEPC-PK, PEPC-protein kinase; PI-PLC, Phosphoinositide-specific phospholipase C.

Cytosolic pH as a Secondary Messenger During Light Activation of Phosphoenolpyruvate Carboxylase in Mesophyll Cells of C_4 Plants

CH. BHASKAR RAO, NASSER SYED, JHADESWAR MURMU, AND A. S. RAGHAVENDRA

Department of Plant Sciences, School of Life Sciences, University of Hyderabad. Hyderabad 500 046, India

1. INTRODUCTION

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

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

Signal Transduction in Plants: Current Advances.
Edited by Sopory *et al*, Kluwer Academic/Plenum Publishers, 2001.

Indian Journal of Experimental Biology
Vol. 39, July 2001, pp. 643-649

Immunological characteristics of PEP carboxylase from leaves of C_3-, C_4- and C_3-C_4 intermediate species of *Alternanthera* - Comparison with selected C_3- and C_4- plants

J Gayathri, K Parvathi, Bhaskarrao Chinthapalli, Peter Westhoff* & A S Raghavendra**
Department of Plant Sciences, School of Life Sciences, University of Hyderabad, Hyderabad 500 046, India
*Universität Düsseldorf, Institut für Entwicklungs- und Molekularbiologie der Pflanzen, Universitätsstrasse 1, D-40225 Düsseldorf, Germany

Received 14 August 2000; revised 12 March 2001

Immunological cross-reactivity of phosphoenolpyruvate carboxylase (PEPC) in leaf extracts of C_3-, C_4- and C_3-C_4 intermediate species of *Alternanthera* (along with a few other C_3- and C_4- plants) was studied using anti-PEPC antibodies raised against PEPC of *Amaranthus hypochondriacus* (belonging to the same family as that of *Alternanthera*, namely Amaranthaceae). Antibodies were also raised in rabbits against the purified PEPC from *Zea mays* (C_4- monocot – Poaceae) as well as *Alternanthera pungens* (C_4- dicot – Amaranthaceae). Monospecificity of PEPC-antiserum was confirmed by immunoprecipitation. Amount of PEPC protein in leaf extracts of *A. hypochondriacus* could be quantified by single radial immunodiffusion. Cross- reactivity of PEPC in leaf extracts from selected C_3-, C_4-, and C_3-C_4 intermediate species (including those of *Alternanthera*) was examined using Ouchterlony double diffusion and Western blots. Anti-PEPC antiserum raised against *A. hypochondriacus* enzyme showed high cross-reactivity with PEPC in leaf extracts of *A. hypochondriacus* or *Amaranthus viridis* or *Alternanthera pungens* (all C_4 dicots), but limited cross-reactivity with that of *Zea mays*, *Sorghum* or *Pennisetum* (all C_4 monocots). Interestingly, PEPC in leaf extracts of *Alternanthera tenella*, *A. ficoidea*, *Parthenium hysterophorus* (C_3-C_4 intermediates) exhibited stronger cross-reactivity (with anti-serum raised against PEPC from *Amaranthus hypochondriacus*) than that of *Pisum sativum*, *Commelina benghalensis*, *Abutilon asiaticum* (C_3 plants). Further studies on cross-reactivities of PEPC in leaf extracts of these plants with anti-PEPC antisera raised against PEPC from leaves of *Zea mays* or *Alternanthera pungens* confirmed two points - (i) PEPC of C_3-C_4 intermediate is distinct from C_3 species and intermediate between those of C_3- and C_4-species; and (ii) PEPC of C_4-dicots was closer to that of C_3-species or C_3-C_4 intermediates (dicots) than to that of C_4-monocots.

Being a key enzyme, properties of PEPC can be used as an interesting tool to assess the evolutionary relationships between C_3 and C_4 plants and C_3-C_4 intermediates[1-3]. Our studies on biochemical properties of the enzyme demonstrated that PEPC from C_3-C_4 intermediates of *Alternanthera* (*A. tenella and ficoidea*) exhibits an intermediate status between those of C_3- (*A. sessilis*) and C_4-species (*A. pungens*)[4].

A rapid conventional method for purification of PEPC from the leaves of *Amaranthus hypochondriacus* (another C_4-dicot and belonging to the same family of *Alternanthera*, namely Amaranthaceae) has been developed in our lab[5]. We have raised anti-PEPC (*Amaranthus*) antibodies in rabbit and used anti-PEPC antiserum to check the cross-reactivity with PEPC of *Alternanthera* species.

**Corresponding author. Fax: +91-40-3010145. E-mail: asrsl@uohyd.ernet.in

In contrast to the extensive literature on purification and storage of PEPC[1-3,5], the studies on immunological properties of PEPC are quite limited. Since the topic is of general interest, a comprehensive study has been attempted, by including a few more C_3 and C_4 plants. The photosynthetic status of C_3- and C_4 plants and C_3-C_4 intermediates used in this study has already been established[4-6]. It is well known that there is a significant difference between PEPC of C_4-dicots and C_4-monocots[2,3]. We have therefore included in the test plants, a few monocots as well. Anti-serum prepared against PEPC of maize (a monocot) was also used to confirm the differences, if any, between monocots and dicots. Finally, the cross-reactivity was confirmed by using anti-PEPC antibodies raised against the enzyme from *Alternanthera pungens* (a C_4-species).

Materials and Methods

Plant material— The details of the plant material and their mode of multiplication/growth have been reported earlier[4-6].

S17-008

Marked modulation by temperature of phosphoenolpyruvate carboxylase from leaves of *Amaranthus hypochondriacus*, a C_4 plant, compared to that in *Pisum sativum*, a C_3 species

Bhaskarrao Chinthapalli, AS Raghavendra

Department of Plant Sciences. School of Life Sciences. University of Hyderabad, Hyderabad 500046. India. Fax: +91-40-3010145, email: asrslrs@uohyd.ernet.in

Keywords: Arrhenius plots, Cold sensitivity. C_4 enzymes. PEPC, Temperature

Introduction

The temperature optima for photosynthesis and growth in C_4 plants are higher than those for C_3 plants. The cold sensitivity C_4 pathway has been suggested to be related to the cold sensivitity of key C_4 enzymes, such as pyruvate phosphate dikinase or PEP carboxylase, PEPC (Kingston-Smith *et al.* 1997; Du *et al.* 1999). However, McWilliam and Ferrai (1974) have suggested that the high temperature tolerance of C_4 plants is due to not the greater thermostability of their PEP carboxylase, but heat-resistant protein synthesizing machinery, which replaces the PEP carboxylase denatured by high temperature. Selinioti *et al.* (1986) observed that light and temperature interact during the activation of PEPC and this might be important for C_4 plants, such as *A. paniculatus.*

On illumination, the activity of PEPC is enhanced by 2-3 fold along with a marked decrease in the malate sensitivity of the enzyme. These changes during the light activation are duc mainly to the phosphorylation of the enzyme (Chollet *et al.* 19%, Vidal and Chollet 1997, Parvathi *et al.* 2000a). Compared to the extensive literature on the properties and mechanism of light activation of PEPC, in C« plants, the literature on the regulation by temperature of PEPC is quite limited (Rajagopalan *et al* 1994). The present study is an attempt to characterize the temperature responses of PEPC from a typical C_4 plant, *Amaranthus hypochondriacus* and compared with those of a C3 plant *Pisum sativum.* Experiments were conducted on leaf discs so as to simulate physiological situation *in vivo* and to also assess the reversibility of temperature effects.

Materials and Methods

Plants of *Amaranthus hypochondriacus* L. (cv. AG-67) and *Pisum sativum* L. (cv. Arkel) were raised from seeds. The plants 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 were harvested, about 2 - 3 h after sunrise. Leaf discs (each of *ca.* 0.2 mm") were prepared from 4- to 6- week-old plants of *Amaranthus hypochondriacus* and 8- to 10-day-old plants of *Pisum sativum.*

The preparation of leaf extracts and the assay of PEPC are all described in detail elsewhere (Parvathi *et al.* 2000b). The sensitivity of PEPC to malate was checked using either 0.5 mM malate (in case of C4 species) or 2 raM malate (in C_3 species).Chlorophyll was estimated by extraction with 80% acetone (Arnon 1949).

Thirty leaf discs were floated on distilled water in a 5 cm diameter Petri dishes and were left in darkness for 2 h. After predarkening, the leaf discs were incubated 30 min at

PHOTOSYNTHETICA 38 (3): 415-419, 2000

Phosphoenolpyruvate carboxylase purified from leaves of C_3, C_4, and C3-C4 intermediate species of *Alternanthera*: Properties at limiting and saturating bicarbonate

Bhaskarrao CHINTHAPALLI*, Chitra RAGHAVAN*, O. BLÄSING**, P. WESTHOFF**, and A.S. RAGHAVENDRA****

Department of Plant Sciences, School of Life Sciences, University of Hyderabad, Hyderabad 500 046, India'
Institut für Entwicklungs- und Molekularbiologie der Pflanzen, Heinrich-Heine-Universität, Universitätsstrasse 1, D-40225 Düsseldorf, Germany"

Abstract

Phosphoenolpyruvate carboxylase (PEPC) was purified from leaves of four species of *Alternanthera* differing in their photosynthetic carbon metabolism: *Alternanthera sessilis* (C3), *A. pungens* (C4), *A. ficoides* and *A. tenella* (C3-C4 intermediates or C3-C4). The activity and properties of PEPC were examined at limiting (0.05 mM) or saturating (10 mM) bicarbonate concentrations. The V_{max} as well as K_m values (for Mg^{2+} or PEP) of PEPC from *A. ficoides* and *A. tenella* (C3-C4 intermediates) were in between those of C_3 (*A. sessilis*) and C_4 species (*A. pungens*). Similarly, the sensitivity of PEPC to malate (an inhibitor) or G-6-P (an activator) of *A. ficoides* and *A. tenella* (C3-C4) was also of intermediate status between those of C_3 and C_4 species of *A. sessilis* and *A. pungens*, respectively. In all the four species, the maximal activity (V_{max}), affinity for PEP (K_m), and the sensitivity to malate (K_I) or G-6-P (K_A) of PEPC were higher at 10 mM bicarbonate than at 0.05 mM bicarbonate. Again, the sensitivity to bicarbonate of PEPC from C_3-C_4 intermediates was in between those of C_3- and C4-species. Thus the characteristics of PEPC of C3-C4 intermediate species of *Alternanthera* are intermediate between C3- and C_4-type, in both their kinetic and regulatory properties. Bicarbonate could be an important modulator of PEPC, particularly in C_4 plants.

Additional key words: glucose-6-phosphate; malate; proteins; species differences in enzyme activity.

Introduction

The C3-C4 intermediates constitute a group of plants with a syndrome of physiological, anatomical, and biochemical traits that fall between the features of C_3 and C_4 plants (Rawsthorne 1992, Raghavendra and Das 1993, Rawsthorne and Bauwe 1998). The extensive literature on PEPC established that the C_4 enzyme is distinct from that of C_3, as indicated by their biochemical properties and amino acid sequences. The genes for C_4, C3, or CAM-PEPC are also different, both in structure and phylogeny (Lepiniec *et al.* 1994, Rajagopalan *et al.* 1994, Chollet *et al.* 1996).

The activities of PEPC in C3-C4 intermediates are significantly higher than in C_3 species (Ku *et al.* 1983, Edwards and Ku 1987), but the molecular properties of PEPC from C3-C4 intermediates were not studied in detail. The limited literature suggests that the PEPC from C_3-C_4 intermediates may be of C_3 type such as in *Panicum*, *Moricandia*, and *Flaveria* (Holaday *et al.* 1981, Adams *et al.* 1986, Bauwe and Chollet 1986), or intermediate type such as in *Flaveria* (Nakamoto *et al.* 1983). Thus, the nature of PEPC in C_3-C_4 intermediates is yet to be resolved clearly.

Alternanthera offers a unique and ideal opportunity of having species of C_3, C_4 as well as C3-C4 intermediates within the same genus (Rajendrudu *et al.* 1986, Raghavendra and Das 1993). We have been

Received 25 May 2000, *accepted* 23 October 2000.
*** Author for correspondence; fax; +91-40-3010120 (or) 3010145; e-mail: asrsl@uohyd.ernet.in
Abbreviations: G-6-P, glucose-6-phosphate; K_A, activator constant; K_I, inhibitor constant; MDH, malate dehydrogenase; PEP, phosphoenolpyruvate; PEPC, PEP carboxylase
Acknowledgements: This work was supported by a grant from Volkswagen-Stiftung (No 1/71-371) for a collaborative project between Prof Peter Westhoff (Universitat Düsseldorf, Germany) and Prof A S Raghavendra (University of Hyderabad. India) C.R. was recipient of a Senior Research Fellowship from the Indian Council of Agricultural Research (New Delhi, India)

J. Plant Biol., Vol. 26 <2). August. 2000. pp. 125–131.
© Society for Plant Physiology and Biochemistry

ARTICLE

Responses of Photosynthesis, Photoinhibition and Dark Respiration to CO_2 in Leaf Discs of C_3–C_4 Intermediate Species: Reduced Sensitivity to External CO_2 in C3-C4 Intermediates Compared to that in C_3 species

M. Tirumala Devi, Bhaskar Rao Chinthapalli and A. S. Raghavendra*
Department of Plant Sciences. School of Life Sciences, University of Hyderabad. Hyderabad 500 046. India

MS received 10 February 2000; accepted 23 June 2000

The patterns of photosynthesis, photoinhibition and dark respiration in C_3–C_4 intermediates (*Alternanthera*flcoida, *A. tenella* and *Parthenium hysterophorus*)were studied at 25°C in comparison with those of C, (A. *sessilis* and *Pisum sativum*)and C_4 (A. *pungens* and *Amaranthus viridis)* species. The photosynthetic rates of C_3–C_4 intermediates were similar to those of C_3 species. Under limiting CO, (0.03%), photosynthesis decreased at high light intensities both in C_3 and C_3–C_4 intermediate species. Such photoinhibition at low CO, (0.03%) in C_3–C_4 intermediates was (significantly) less than that in C_3 species and was relieved at high CO, levels (e.g. 5%). The relative sensitivity to 3,3-dichloro-2-dihydroxyphosphinoylmethyl-2-propeonate (DCDP, an inhibitor of PEP carboxylase) indicated that PEP-carboxylase has an important role in photosynthetic CO, fixation by intermediates particularly at limited CO_2. During these measurements, it was noticed that there was a marked decrease in dark respiration as the external CO, concentration was raised. The inhibitory effect of CO, on dark respiration was significantly less in C_3–C_4 intermediates than that in C_3 plants and was least in C_4 species. We propose that an alteration in mitochondiral metabolism and reduced sensitivity to external CO, (compared to C_3 species) are important features of C_3–C_4 intermediates.

Keywords: C_3–C_4 intermediates, PEP carboxylase. CO_2 sensitivity, dark respiration, photoinhibition, *Ct* plants.

Introduction

Higher plants are grouped into three categories based on their mode of photosynthesis, viz. C3, C_4 and CAM species (Edwards and Walker, 1983). However, there are several plants with anatomical, biochemical and physiological characteristics intermediate to those of C_3 and C_4 species (Reviews: Edwards and Ku, 1987; Monson and Moore, 1989; Rawsthorne, 1992; Raghavendra and Das, 1993), grouped as C_3–C_4 intermediates. At least 24 species belonging to eight genera of six families are identified as naturally occurring C3-C4 intermediates (Rawsthorne, 1992).

*For correspondence. (e-mail: asrsl@uohyd.ernet.in)

The C_3–C_4 intermediate species offer a good model to examine the biochemical, molecular and evolutionary aspects of C_4 pathway (Moore, 1982; Monson and Moore, 1989; Raghavendra and Das, 1993). The photosynthetic and photorespiratory characteristics of the intermediates of *Flaveria*, *Panicum*, *Moricandia* and *Neurachne* have been studied in detail (Holaday *et al.*, 1982; Holaday and Chollet, 1984; Hattersley *et al.*, 1986; Huber *et al.*, 1989; Brown *et al.*, 1991; Ku *et al.*, 1991).

The information on the photosynthetic features of these intermediates of *Alternanthera* and *Parthenium* is quite limited (Rajendrudu *et al.*, 1986; Moore *et al.*, 1987) The C_3–C_4 intermediates of *Alternanthera* and *Parthenium* appear to have no functional C_2 cycle (Rajendrudu *et al.*, 1986; Moore *et al.*, 1987; Devi and Raghavendra, 1992, 1993a). Photosynthe-