Chapter 2

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

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

Native leaf PEPC is highly susceptible to limited proteolysis near the N-terminal during extraction and subsequent purification steps (Chollet et al., 1996). The purified PEPC also is unstable, if proper precautions are not taken. The enzyme loses frequently its N-terminal end (during either extraction or storage) and becomes malate-insensitive (McNaughton et al., 1989; Chollet et al., 1996). Therefore, 50% glycerol was included along with PEPC and the mixture was stored in liquid nitrogen as small aliquots. The stability of purified PEPC was examined with or without glycerol.
Polyclonal antibodies were raised in rabbits against the purified PEPC from *Amaranthus hypochondriacus* leaves using the procedure of Nimmo et al. (1986). Ouchterlony double immunodiffusion method was employed to check the titer value of antiserum. Immunoprecipitation using the antibody confirmed that the PEPC was precipitated effectively in the crude extracts from leaves. Further, the cross reactivity of antiserum was checked and assessed by using Western blot analysis and Enzyme-Linked Immunosorbent Assay (ELISA) using alkaline phosphatase.

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

The organic cosolutes, glycerol and PEG, have been shown to stabilize the activity and integrity of several enzymes, including PEPC, during adverse conditions of extraction (Selinioti et al., 1987; Rhodes and Hanson. 1993; Drilias et al., 1994; Podesta and Plaxton. 1994; Law and Plaxton, 1995). Although the reaction media containing organic cosolutes are not physiological, they resemble an environment closer to the conditions in vivo, than those which are associated with the extensive dilution that occurs during extraction and assays. The organic cosolutes promote self association of proteins and stabilize their structure by being preferentially excluded from contact with the protein surface (Timasheff, 1992). Under such a high protein concentration, the stability of oligomeric enzymes is enhanced. The interaction of compatible solutes on the stability and oligomeric status of the enzyme was studied in presence of PEG or glycerol or both by using gel filtration chromatography.
The uniqueness of C4-PEPC is that the enzyme in leaves is markedly regulated by light. The activity of PEPC in leaves of C4 plants is activated, by two to three fold by light, compared to that in dark-adapted ones. The kinetic and regulatory properties of PEPC are also modulated by light. We have therefore studied the kinetic and regulatory properties of PEPC in illuminated leaf samples and the interaction between the increased affinity for bicarbonate and light.

In spite of the extensive literature on the modulation of malate-sensitivity of the enzyme, the studies on changes in kinetic characteristics of PEPC, and in particular the affinity to \( \text{HCO}_3^- \) are quite limited (O'Leary, 1982; Chollet et al., 1996). One of the likely reasons is the practical difficulty in the complete removal of dissolved bicarbonate during PEPC assays. We have also examined the kinetic and regulatory properties of PEPC at two concentrations of bicarbonate: low or limiting (0.05 mM) and high or saturating (10 mM). Low bicarbonate (0.05 mM) reflects the physiological environment of the cell, while most of the literature on PEPC, used 10 mM bicarbonate.

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

The effect of calcium on PEPC and PEPC-PK activity were re-examined critically in the present work. As per the present results, \( \text{Ca}^{2+} \) inhibits PEPC at high concentration and such inhibition is due to competition with \( \text{Mg}^{2+} \) (Mukerji, 1977;
Gavalas and Manetas, 1980b; Gayathri and Raghavendra, 1994). Ca$^{2+}$ also acts as a stabilizer at low concentration. Therefore, we have studied the mechanism of the effect of calcium on PEPC activity.

Experiments were conducted to examine the phosphorylation of PEPC using both direct and indirect methods. An indirect method is to incubate the crude extracts with ATP and MgCl$_2$ and determining the extent of activation of PEPC after incubation, whereas in direct method, the incorporation of $^{32}$P into PEPC in vitro is monitored. Incubation of crude extracts prepared from illuminated leaf discs with ATP and MgCl$_2$ stimulated the activity and decreased the malate sensitivity. Indirect way was used first to check the effect of Ca$^{2+}$ on the extent of phosphorylation of PEPC. In both direct and indirect methods phosphorylation of PEPC was observed with extracts prepared from only illuminated leaves but not from the dark-adapted ones. Further experiments were designed to evaluate the involvement of calcium and calmodulin as secondary messengers during the phosphorylation of PEPC.

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

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

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

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

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

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

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

7. To investigate the PEPC phosphorylation pattern in presence of typical inhibitors or activators of different types of protein kinases: BAPTA and EGTA ($Ca^{2+}$-chelators); trifluoperazine and W7 (Calmodulin antagonists); $Ca^{2+}$, Phosphatidyl serine and diacylglycerol (Protein kinase C activators); H7 (Protein kinase C inhibitors); staurosporine (CaM-dependent kinase inhibitor); $Ca^{2+}$ and calmodulin (CaMK and MLCK activators); and ML7 (MLCK inhibitor).

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