Chapter 11

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

There has been remarkable progress in understanding certain aspects of biochemistry and molecular biology of C₄-PPPC, for e.g. cloning and expression of native and recombinant PEPC in E. coli; characterization of PEPC phosphorylation and PEPC-PK, evolutionary tendency of PEPC based on deduced amino acid sequences (Chollet et al., 1996; Vidal and Chollet, 1997). Yet there are several gaps in our knowledge of regulation of this key enzyme of C4 pathway: e.g. interaction of PEPC with bicarbonate, basis of Ca²⁺-dependence of PEPC phosphorylation. The present investigation used the leaves of Amaranthus hypochondriacus (a NAD-ME type C4 plant) for studies on purification, characterization and regulation of PEPC.

Among the aspects studied were:

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

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

3. The influence of bicarbonate on the catalytic and regulatory properties of PEPC.
4. The pattern and consequences of light activation of PEPC on the affinity of enzyme to bicarbonate.

5. The response of PEPC and the pattern of PEPC phosphorylation to calcium (by using CaCl₂, Ca²⁺-chelators and activators/inhibitors of CDPK or CCaMK) in crude and desalted extracts.


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

Polyclonal antibodies were raised in rabbits against purified PEPC from A. hypochondriacus. The monospecificity of anti-PEPC antiserum was confirmed by Ouchterlony double diffusion. Anti-PEPC antiserum could be used for Western blots, immunoprecipitation and ELISA. Anti-PEPC IgG were purified from the anti-PEPC antiserum. These IgG were employed to purify rapidly PEPC from Amaranthus leaves by using an immunoabsorbent column. This procedure was quite useful for the rapid purification of PEPC from leaves during light/dark transitions (Arrio-Dupont et al., 1992). An ELISA technique was standardized to detect and determine PEPC. ELISA can therefore be used as a diagnostic kit for the detection and quantification of PEPC-protein in plants.
The extraction from leaves and assay of activity are invariably linked to dilution of enzymes and thus deprive the enzymes from their natural physico-chemical environment. Such dilution of the medium (e.g. encountered during enzyme assays) affects the quaternary structure of PEPC by inducing dissociation and loss of activity (Willeford and Wedding, 1992). The inclusion of compatible solutes like glycerol and PEG can provide a favorable microenvironment against dilution (Reinhart, 1980; Karabourniotis et al., 1983). The effects of these solutes during extraction and assay of PEPC were therefore studied.

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

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

Compared to the extensive literature on the modulation of malate-sensitivity of PEPC, the studies on changes in kinetic characteristics of the enzyme and in particular the affinity to bicarbonate are quite limited. It has recently been reported
that bicarbonate plays an important role in the allosteric regulation of PEPC (Ogawa et al., 1997; Dong et al., 1997a). Such modulation of PEPC by one of the substrates, bicarbonate is extremely interesting and warrants further studies. Therefore, the interaction of kinetic characteristics of PEPC-enzyme with bicarbonate was studied.

Bicarbonate stimulated the activity of PL-PC extracted from dark- as well as light-adapted leaves of Amaranthus hypochondriacus. The stimulation by bicarbonate of PEPC activity was complex. The sensitivity of the enzyme to malate decreased with increasing concentration of bicarbonate. The stimulation by Glc-6-p was more at low concentration of HCO₃⁻ (0.05 mM) than that at high concentration (10 mM). The catalytic and regulatory properties of the enzyme were thus modulated markedly by bicarbonate.

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

Light activation of photosynthetic enzymes is a cardinal event in regulation of photosynthesis in both C₃ and C₄ plants (Buchanan, 1980; Selinio et al., 1986). As PEPC is a key enzyme involved in CO₂ fixation in C₄ and CAM plants, the regulation of PEPC by light is a topic of interest. It is obvious that the light-signal
has to be perceived by the Chloroplast and the effect is transmitted to the cytosol, the site of PEPC in C4 mesophyll cells. Although, the kinetic and regulatory properties of PEPC are modulated by light/dark transitions in vivo there is very limited information on the mechanism of transduction of light-signal.

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

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

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

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

An attempt was made to characterize the Ca\(^{2+}\)- or CaM-independent protein kinase (CDPK) in leaf extracts of A. hypochondriacus. Anti-CCaMK(maize)-antibodies cross reacted with two proteins (75 and 80 kD) in leaf extracts of A. hypochondriacus. One of them (75 kD) appears to be a CDPK, since it also binds to CaM as indicated by immunoblot analysis. The synthesis of 75 kD increased upon illumination particularly in C4 leaves. The phosphorylation of substrates histone I11S as well as syntide-2 suggested the presence of an active CDPK in A. hypochondriacus leaf extracts. Further, the phosphorylation of histone HIS was dependent on the availability of Ca\(^{2+}\), but not on CaM and was suppressed significantly by a wide range of inhibitors related to Ca\(^{2+}\)- and CaM-dependent protein kinases, but at a high concentration. These results suggest the presence of CDPK rather than CCaMK in A. hypochondriacus leaf extracts. As PEPC was a poor substrate for CDPK, we feel that this CDPK may be involved in phosphorylating PEPC-PK and leads to the upstream regulation of PEPC.
phosphorylation by Ca\(^{2+}\). Further experiments are needed to reveal the complete chain of the components involved in the light-induced phosphorylation of PEPC.

**Major conclusions/outcome from the present study are:**

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

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

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

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

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

6. Illumination enhanced the affinity of PEPC to bicarbonate by at least two times, besides the 2 to 5-fold increase in \(V_{\text{max}}\) and 3 to 4-fold increase in \(K_j\) for malate. *In vitro* phosphorylation of dark-form PEPC by protein kinase A decreased the \(K_m\) (HCO\(_3^-\)) of the enzyme, besides increasing \(K_j\) (malate). Thus, phosphorylation of PEPC is important during the sensitization of enzyme to HCO\(_3^-\) by illumination in leaves. A marked interaction between light and bicarbonate appears to be an important component in the regulation of C\(_4\) PEPC.
7. Ca$^{2+}$ inhibited PEPC at higher concentration (>50 µM) and stimulated at low concentrations (≤20 µM). The inhibitory effect of Ca$^{2+}$ was mainly due to the competition with Mg$^{2+}$.

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

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

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

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