Chapter 8

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PEPC is an important enzyme, mediating the primary carbon fixation in C₄ and CAM plants. In C₃ plants and algae, the enzyme plays an auxiliary role (O'Leary, 1982; Latzko and Kelly, 1983; Andreo et al., 1987). Due to its strategic importance, the properties, regulation, molecular biology and evolution of C₄ PEPC have been studied extensively (Lepiniec et al., 1994; Rajagopalan et al., 1994; Toh et al., 1994). The main objective of present work is to study the properties and regulation of C₄-PEPC using the leaves of Amaranthus hypochondriacus, a NAD-ME type C₄ plant, as the source of the enzyme.

The first set of experiments were designed to purify PEPC from leaves of A. hypochondriacus and to examine the stability of PEPC under different conditions of storage. As per literature, PEPC has been purified from several C₄ plants (Andreo et al., 1987; Rajagopalan et al., 1994). Our purification method involved 40-60% ammonium sulphate fractionation, followed by DEAE-Sepharose, HAP chromatography and finally through a Seralose 6-B column. The protocol was efficient and resulted in a high yield of nearly 50% and with PEPC having one of the highest specific activities reported for C₄ plants (54 Units mg⁻¹ protein). The purified PEPC was homogenous and appeared as a single band on 10% SDS-PAGE. Purified PEPC appeared as two bands on native gels and stained positively when examined for PEPC activity. Two-dimensional electrophoresis of these two bands resulted in a single band on SDS-PAGE indicating that PEPC is composed of only one-type of subunits, with a molecular mass of about 100-kD. We suggest that these two bands may be isozymes of a homotetramer, since dimer or monomer are not expected to stain for PEPC activity.
Purified PEPC is highly unstable and loses its N-terminal. The loss of the N-terminal is reflected in the loss of its malate sensitivity (McNaughton et al., 1989). Proteolysis of PEPC during purification is observed, particularly in the absence of protease inhibitors like PMSF or chymostatin (McNaughton et al., 1989). We have used PMSF as the protease inhibitor, so as to avoid proteolysis and to maintain the stability of the enzyme during extraction and purification.

In literature, there are several reports that the instability of PEPC could be prevented by inclusion of glycerol under in vitro conditions (Uedan and Sugiyama, 1976; Manetas et al., 1987; Selinioti et al., 1987; Podesta and Andreo, 1989). Hence, we studied the properties of the purified enzyme after storing at different temperatures (i.e., room temperature, 4 °C, -20 °C, liquid nitrogen) in the presence or absence of glycerol. High activity of PEPC was maintained for up to four months, in liquid nitrogen when stored along with 50% (v/v) glycerol. A study of the purified PEPC, after storage at different temperatures, revealed that the enzyme stored in liquid nitrogen with 50% (v/v) glycerol maintained all the three key characteristics, even after 4 months: high V_{max}, stimulation by G-6-P, and inhibition by malate.

Although extensive literature is available on purification, properties and regulation of PEPC, studies on immunological properties of PEPC are quite limited. An attempt has therefore been made to study the immunological properties of PEPC and assess its cross-reactivity with the enzyme in leaf extracts of a few C_3- or C_4-type dicots, or C_3-C_4 intermediates or C_4-monocots.

Antibodies were raised in rabbits against the purified PEPC from A. hypochondriacus. The antibody exhibited a titer value of 1/100 against the PEPC of A. hypochondriacus (in leaf extracts and purified enzyme) and Alternantherapungens. The antibody showed limited cross-reactivity with the PEPC from C_4 monocot (Zea mays), as indicated by the intensity of precipitation. This was confirmed further by Western blot analysis.
Immunoprecipitation experiments were conducted to assess the specificity of the antiserum. A fixed volume of leaf extract was mixed with different volumes of antibody solution. In the control samples, a serum of non-immunised rabbit was used. When the concentration of the antibody was increased, the amount of PEPC precipitated from the supernatants increased. In contrast, there was no PEPC precipitation with non-immunised serum. There was a proportionate decrease in PEPC activity in the supernatants with increase in PEPC precipitation. The immunoprecipitates were later analysed by 10% SDS-PAGE to confirm the identity and quantity of PEPC protein.

Single radial immunodiffusion was performed to quantitate the amount of PEPC protein in crude leaf extracts using the anti-PEPC antiserum of *Amaranthus hypochondriacus*. There was a proportionate increase in the diameter of the ring with the increase in the antigen concentration.

The cross-reactivity between PEPC of C$_3$, C$_3$-C$_4$ and C$_4$ plants was examined by Ouchterlony double diffusion. The anti-PEPC antiserum raised against PEPC from *Amaranthus hypochondriacus* showed very strong immunoreaction with PEPC in leaf extracts of *Amaranthus viridis*, *Alternanthera pungens* (C$_4$ dicots) and faint reaction with *Zea mays*, a C$_4$ monocot. However, we could not detect much cross-reactivity with PEPC in leaf extracts of C$_3$ plants or C$_3$-C$_4$ intermediates. Hence we evaluated the immunological identity with also Western blots.

Anti-PEPC antiserum raised against *A. hypochondriacus* enzyme showed high cross-reactivity with purified PEPC from *A. hypochondriacus*, or the enzyme from leaf extracts of *Amaranthus viridis* or *Alternanthera pungens* (all three C$_4$ dicots), but limited reactivity with that of C$_4$ monocots: maize, sorghum or pearl millet. PEPC in leaf extracts of C$_3$ plants (*Pisum sativum*, *Commelina benghalensis*, *Alternanthera sessiles*) and C$_3$-C$_4$ intermediates (*Parthenium hysterophorus*, *Alternanthera tenella*, *Alternanthera ficoides*), exhibited stronger cross-reactivity with anti-PEPC antiserum of
A. *hypochondriacus* than that with the anti-PEPC antiserum against maize-PEPC. All these results indicated that C₄ dicot PEPC was closer to C₃ species, or C₃-C₄ intermediates than with C₄ monocot PEPC.

Using ELISA, it was possible to quantitate very low levels (ng) of PEPC protein in crude leaf extracts of *A. hypochondriacus*, as indicated by the linear relationship between immuno-precipitate-linked absorbance and PEPC protein in leaf crude extracts. In contrast, μg amounts of PEPC protein was required for detecting PEPC in pea leaf extracts indicating that the cross-reactivity of C₃ dicot PEPC with C₄-PEPC was less than that of C₄ dicot PEPC.

The tetrameric form of PEPC is reported to be the most active form (Walker et al., 1986; Podesta et al., 1990 Wu et al., 1990). Organic solutes such as PEG, proline, betaine play an important role in maintaining the integrity of the enzyme, even in adverse conditions (Drilias et al., 1994). The presence of solute helps in maintenance of the homologous interaction of the protein and thus increase enzyme stability. Normally, the enzyme in crude leaf extracts tends to be in diluted state. Several authors have reported that the instability of PEPC in extraction or assay media could be overcome by addition of glycerol (Manetas, 1982; Selinioti et al., 1987). Besides glycerol, other organic solutes (PEG, proline, betaine) also promote self-association and stabilize the structure of proteins (Timasheff et al., 1982). We have therefore studied in detail the interaction of PEG with PEPC from *A. hypochondriacus* purified form as well as in crude leaf extracts.

The effect of three different PEGs (PEG-6000, 8000 and 20,000) were studied on purified enzyme at either saturating (2.5 mM) or sub-saturating (0.5 mM) concentration of PEP. The activity of PEPC was markedly enhanced by PEG and such stimulation by PEG-6000 was more than that by PEG-8000 or PEG-20,000, irrespective of substrate concentration. The extent of activation by (1.25% w/v) PEG-6000 was more at 0.5 mM PEP, than at 2.5 mM PEP. There was a decrease in PEPC activity as the PEG concentration
increased above 10% (w/v). This may be due to the possible precipitation of the enzyme at higher levels of PEG. The activity of PEPC increased also in presence of ethylene glycol, or glycerol but the extent of activation, when compared to that with PEGs, was low. Sorbitol had only a marginal effect on PEPC.

In presence of PEG, the affinity for PEP of PEPC decreased and the enzyme was less malate sensitive. This indicates that PEG relieved malate inhibition of PEPC while activating the enzyme. The $K_A$ for G-6-P increased in the presence of PEG. G-6-P and PEG were reported to shift PEPC from maize to an active state (Huber and Sugiyama, 1986).

Further experiments were taken up to assess the effect of PEG on PEPC during enzyme extraction from leaves. Different concentrations of PEG were used during either extraction, or assay, or both. Maximal activation was obtained when PEG was present during both extraction and assay.

In the next set of experiments, the effect of PEG was studied on the light or dark-form of PEPC. Light activation of PEPC was achieved by illuminating leaf discs at $1500 \mu$E m$^{-2}$ for 20 min (after pre-darkening for 2 h). The light-form of PEPC was 2 to 3 times more active and less sensitive to malate than the dark-form. The activation by PEG of the light-form was less than that of the dark-form, particularly at sub-optimal PEP.

Glycerol is usually included while extracting leaves for studies on PEPC. Efforts were therefore made to study the effect of a combination of glycerol and PEG during extraction and/or assay on the activity, stability and light activation of PEPC. The activity of PEPC remained high even after 2 h of extraction if both PEG and glycerol were present. Further, the extent of light activation also improved in presence of PEG and glycerol. Of all the combinations, inclusion of glycerol plus PEG during extraction as well as in assay medium was the best for maintaining high stability and maximum light activation of PEPC.
From the above experiments we have observed that activity of PEPC was enhanced in presence of organic solutes. However the changes in the oligomeric structure of PEPC in presence of organic solutes will allow us to gain further insight into the possible interaction of PEPC with compatible solutes. Hence we examined the quaternary structure of PEPC in crude leaf extracts by gel filtration on Sephadex G-200 column.

In the absence of either PEG or glycerol, PEPC eluted as a mixture of dimer and monomers. If the extracts were prepared with glycerol and/or PEG in buffered medium, PEPC appeared predominantly as a tetramer. On the other hand, if PEG or glycerol were omitted while eluting the column with only buffer, PEPC eluted as a mixture of tetramer and dimer. The presence of 0.3 M sorbitol in both extraction and elution buffer resulted in the appearance of enzyme as a mixture of tetramer, dimer and monomers. These results provide a good demonstration of variability in oligomeric status of PEPC in leaf extracts and its modulation by glycerol and PEG.

PEPC is located in cytosol of mesophyll cells in leaves of C₄ plants. The reduction of nitrate to ammonium also occurs in C₄ mesophyll cells (Hatch, 1987). Biosynthesis of PEPC in C₄ leaves is modulated by the extent and form of nitrogen available to the plant. For e.g. the levels of PEPC increased when leaves of maize were fed with nitrate or ammonium (Sugiharto and Sugiyama, 1992). The effectiveness of ammonium salt as an inducer of PEPC biosynthesis was two fold greater than that of nitrate.

When a range of different salts were tested, PEPC in leaf extracts of A. hypochondriacus was stimulated by ammonium salts and to some extent by potassium ions. Acetate and sulphate salts of ammonium, besides chloride, stimulated the activity of PEPC. On the other hand, monovalent ions like lithium, sodium and rubidium had only a marginal effect on PEPC. The stimulation by potassium was significant, but not as marked as that by ammonium. The presence of calcium chloride decreased the activity of PEPC.
The inhibition of PEPC by calcium has been reported earlier (Gavalas and Manetas, 1980).

Although ammonium ions could stimulate in vitro the activity of PEPC (dark-form of the enzyme), the light-activated form of PEPC was not stimulated, but was slightly inhibited. The light-activated form of PEPC is known to differ from the dark-form in several characteristics: malate sensitivity, phosphorylation status and $K_m$ for PEP (Rajagopalan et al., 1994). The absence of stimulation by ammonium chloride of PEPC from illuminated leaves indicates that only the dark-form of the enzyme has the conformational status capable of responding to ammonium ions.

In the presence of ammonium chloride, the $V_{\text{max}}$ increased but the affinity to PEP was not altered. Double reciprocal plots revealed that the $K_m$ for PEP, in the presence or absence of ammonium chloride, was similar. Also, there was no significant change in the sensitivity of the enzyme to malate in the presence of ammonium chloride. However, the response to G-6-P was quite different in the presence of ammonium chloride. There was only a marginal stimulation of G-6-P in the presence of ammonium chloride. Further, there was a decrease in PEPC activity at high concentrations of G-6-P in presence of ammonium chloride.

Our observations demonstrate that ammonium ion is an allosteric activator of PEPC from $C_4$ plants in vitro. Allosteric activation by ammonium of PEPC fits well with intercellular enzymic distribution. An increase in the availability of ammonium ions can stimulate PEPC and promote carbon amino acid metabolism. These results form an additional basis of a better nitrogen use efficiency in $C_4$ plants than that in $C_3$ species (Hatch, 1987).

PEPC is subjected to phosphorylation-dephosphorylation cascade during light/dark transitions in vivo (Jiao and Chollet, 1991; Nimmo, 1993; Rajagopalan et al., 1994). A protein-serine kinase phosphorylates PEPC, while a type 2A protein phosphatase dephosphorylates the enzyme. Experiments with
mesophyll protoplasts of maize (Devi and Raghavendra, 1992) and sorghum (Pierre et al., 1992) have shown that pH and calcium are important factors during light activation and phosphorylation of PEPC. Yet, the mechanism of action of calcium and its interaction with pH during PEPC phosphorylation are not clear. Experiments were therefore designed to investigate if calcium modulates PEPC or PEPC-PK or both.

The effect of calcium on PEPC was examined at either optimal (pH 7.8) or sub-optimal (pH 7.3) levels of pH. The presence of calcium inhibited PEPC, the inhibitory effect being more at pH 7.8 than at pH 7.3. The effect of calcium on PEPC was dependent on the light- or dark-form of the enzyme. The extent of inhibition by calcium (at pH 7.8) was only marginal in light-form of PEPC compared to dark-form. At pH 7.3, the degree of inhibition was marginal in case of both light and dark-forms. Low concentration of calcium was necessary for optimal activity of PEPC at both pH 7.3 and 7.8 in extracts from illuminated leaves.

The activity of PEPC-PK or PEPC-phosphorylation were studied either directly or indirectly. An indirect measure of PEPC-PK is the stimulation of PEPC activity on incubation of leaf extracts with ATP. Incubation with ATP stimulated PEPC activity and decreased the malate sensitivity particularly in extracts from illuminated leaves. As a result of marked stimulation by ATP of PEPC activity in extracts from illuminated leaves, there was an increase in L/D ratio. The effect of ATP was dependent on pH. The extent of stimulation by ATP was more at pH 7.8 than at pH 7.3. Calcium (even at a low concentration of calcium 20-50 μM) stimulated the PEPC activity during ATP incubation. Such stimulation by calcium was more at pH 7.3 than at pH 7.8. Pretreatment of leaves with EGTA in vivo decreased the extent of stimulation by ATP of PEPC activity (a measure of PEPC-PK) particularly in leaves exposed to light. Pretreatment with EGTA sharpened further the response to calcium.
A major conclusion from these experiments is that calcium affects PEPC and PEPC-kinase in different ways. Presence of calcium inhibits PEPC, particularly at pH 7.8. On the other hand, calcium is essential for PEPC-PK and promotes phosphorylation of PEPC.

Direct evidence of PEPC-phosphorylation was obtained by either incubation of leaf extracts with $\gamma$-ATP (in vitro) or feeding intact leaves with $\text{Pi}$ (in vivo). In vivo labelling of leaves with $^{32}\text{Pi}^{}$ showed that the phosphorylation of PEPC was more in light than that in dark treated leaves. Most of the experiments were therefore conducted with illuminated leaves. Pretreatment of leaves with EGTA in vivo decreased the extent of PEPC phosphorylation. With 2 mM EGTA, phosphorylation of PEPC was reduced to almost nil, but could be restored by addition of calcium during incubation with $\gamma$-ATP. The stimulatory effect of calcium (20 $\mu$M being optimal) was amplified due to the pretreatment of leaves with EGTA. Besides calcium, magnesium also was necessary for PEPC phosphorylation and both had a synergistic effect.

A few experiments were undertaken to study if calcium-related secondary messengers, such as calmodulin, play any role in PEPC-phosphorylation. When leaves were fed through petiole, with metabolic inhibitors such as TFP (a calmodulin antagonist), phosphorylation of PEPC in light decreased. Phosphorylation of PEPC was also inhibited by verapamil, lanthanum and diltiazem (calcium channel blockers). These results suggest that besides calcium, calmodulin and calcium channel-activity play an important role phosphorylation of PEPC in vitro.

To summarize, major conclusions from the present study are:

1. PEPC purified from *Amaranthus hypochondriacus*, had one of the highest activities reported in $C_4$ plants. The enzyme was stable for more than 4 months, if stored in liquid nitrogen along with 50% (v/v) glycerol.
2. The antibody of PEPC from *A. hypochondriacus* (a C\textsubscript{4}-dicot) reacted with PEPC from both C\textsubscript{3} and C\textsubscript{4} plants, but with a large variation in the extent of cross-reaction. The antibody of PEPC from *A. hypochondriacus*, a C\textsubscript{4} dicot showed much stronger cross-reaction with the enzyme of *A. viridis* and *A. pungens* (C\textsubscript{4} dicots), than with that of C\textsubscript{3} species or C\textsubscript{3}-C\textsubscript{4} intermediates or *Z. mays* (C\textsubscript{4} monocot). Similarly, anti-PEPC antiserum from *Z. mays* showed faint cross-reactivity with the enzyme in leaf extracts of C\textsubscript{4} dicots, or C\textsubscript{3} species or C\textsubscript{3}-C\textsubscript{4} intermediates.

3. The presence of PEG, a compatible solute, enhanced markedly the activity of enzyme, when checked with either purified enzyme or crude leaf extracts. Maximum activation of PEPC was obtained, when PEG was present during both extraction from leaves and assay of the enzyme. The affinity for PEP decreased, the response to G-6-P was enhanced and the enzyme became less malate sensitive in presence of PEG.

4. The oligomeric status of PEPC (tetramer, dimer and monomer) was examined by gel filtration in presence or absence of compatible solutes during enzyme extraction from leaves.

5. Presence of both PEG and/or glycerol both during extraction and/or elution helped in maintaining PEPC predominantly in tetrameric form.

6. In the absence of PEG or glycerol and sorbitol during extraction and elution, PEPC was present as a dimer and/or monomer, with a small proportion of tetramer.

7. Ammonium ions stimulated the PEPC activity *in vitro* from C\textsubscript{4} plants. The activation by ammonium of PEPC was at the allosteric site of enzyme.

8. Calcium affected PEPC and PEPC-PK in different ways and the effect was dependent on pH. The presence of calcium inhibited PEPC activity, particularly at pH 7.8, but had very little effect at pH 7.3. Calcium promoted PEPC phosphorylation, both *in vitro* and *in vivo*.
9. Marked stimulation by ATP in the presence of calcium either at pH 7.3 or pH 7.8 indicated that calcium promoted phosphorylation of PEPC. Both calcium and magnesium were necessary for PEPC-phosphorylation and have a synergistic effect on PEPC-PK.

10. Pretreatment of leaves with EGTA \textit{in vivo} decreased the activity and phosphorylation of PEPC in leaves exposed to light (but not in darkness). Pretreatment with EGTA sharpened the response to calcium.

11. Labelling of leaves with $^{32}\text{Pi} \textit{in vivo}$ revealed that the phosphorylation of PEPC was more in light than that in dark-adapted leaves.

12. TFP (a calmodulin antagonist) as well as verpamil, lanthanum, diltiazem (calcium channel blockers) decreased phosphorylation of PEPC in light. These results indicate that both calcium and calmodulin are involved during PEPC phosphorylation enhanced under illumination.