Interaction of polyethylene glycol-6000 with C₄ phosphoenolpyruvate carboxylase in crude leaf extracts as well as in purified protein form from *Amaranthus hypochondriacus* L.: evidence for oligomerization of PEPC *in vitro* and *in vivo*

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ABSTRACT

The interaction of phosphoenolpyruvate carboxylase (PEPC) with a compatible solute, PEG-6000, was examined using crude leaf extracts as well as the purified protein from leaves of *Amaranthus hypochondriacus*, a NAD-malic enzyme type C₄ plant. The inclusion in the assay medium of PEG-6000 stimulated the activity of purified PEPC by about 2.5-fold over control. The addition of PEG during both extraction and assay, stimulated PEPC activity by almost 5.0 fold in crude extracts. The stimulation by PEG of the dark-form of PEPC (2.4 fold) was more than that of the light-form (1.7 fold). Gel filtration of PEPC in leaf extracts on Sephadex G-200, showed the existence of three different oligomeric forms: tetramer, dimer and monomer. The exclusion of PEG and glycerol during extraction and elution on Sephadex resulted in a marked shift of the enzyme into dimer and/or monomer, with a very small proportion of tetramer but on the contrary, the inclusion of PEG and glycerol resulted in the enzyme maintaining predominantly a tetrameric shape. Thus, the activity and the structural properties of PEPC can be influenced by the presence or absence of compatible solutes (PEG or glycerol), obviously due to changes in the microenvironment of the enzyme. [Physiol. Mol. Biol. Plants 2008; 14(3) : 227-234] E-mail : asrsl@uohyd.ernet.in

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Abbreviations : MDH: NAD-malate dehydrogenase, PMSF: Phenylmethyl sulphonyl fluoride, PEP: Phosphoenolpyruvate, PEPC: Phosphoenolpyruvate carboxylase, PEG: Polyethylene glycol, PVP: Polyvinyl pyrrolidone.

Phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) is a ubiquitous cytosolic enzyme which occurs in the photosynthetic and non-photosynthetic tissues of C₃, C₄ and CAM plants. PEPC plays a vital role in primary carbon fixation in the leaves of C₄ and CAM plants. PEPC is highly regulated by several external factors, such as light, temperature, water stress or inorganic nutrients (Andreo *et al.*, 1987; Rajagopalan *et al.*, 1994; Chollet *et al.*, 1996; Chinthapalli *et al.*, 2003; Murmu *et al.*, 2003). PEPC is subjected to feed back inhibition by oxaloacetate, malate and aspartate. PEPC is activated by G-6-P and related phosphorylated metabolites, which reduce the K_m for phosphoenolpyruvate (PEP). The

structural biology and the molecular evolution of PEPC have been reviewed (Izui *et al.*, 2004; Westhoff *et al.*, 2004).

PEPC is a homotetramer of about 400 kD consisting of four identical subunits, each with a molecular mass of 95-110 kD (Chollet *et al.*, 1996). The tetrameric form of PEPC is reported to be the most active form while dissociation of the enzyme results in a marked decrease in the catalytic activity of the enzyme (Walker *et al.*, 1986; Wagner *et al.*, 1987; Podestá *et al.*, 1990; Willeford *et al.*, 1990; Wu *et al.*, 1990). The oligomeric status of the enzyme in solution depends on conditions such as pH (Walker *et al.*, 1986), ionic strength (Wagner *et al.*, 1987) or temperature (Wu and Wedding, 1987). *In vitro* experiments have indicated that dilution of the enzyme may be an important factor while inducing dissociation of PEPC into dimer and/or monomer (Wu and Wedding,

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1987; Angelopoulos and Gavalas, 1988; Kleczkowski and Edwards, 1990). Thus, the dilution of the enzyme leads to a marked instability of PEPC.

Several authors have noted that the instability of PEPC activity in extraction or assay media could be overcome by addition of glycerol (Uedan and Sugiyama 1976; Karabourniotis *et al.*, 1983; Manetas *et al.*, 1986; Selinioti *et al.*, 1987). Besides glycerol, other organic solutes (PEG, proline, betaine) also promote self association of proteins and stabilize their structure (Timasheff *et al.*, 1982). Besides PEPC, other cytosolic enzymes, known to be activated by PEG include pyruvate kinase (Podestá and Plaxton, 1993) and fructose-1, 6-bisphosphatase (Hodgson and Plaxton, 1995) from the endosperm of germinating castor seeds. In agreement with above, we also have observed that PEPC is unstable when extracted in a simple buffered medium, primarily due to dilution.

Detailed studies were therefore made with purified enzyme and later with crude extracts by using PEG to keep up the high activity. Initially, experiments were performed with purified enzyme and the enzyme was incubated with PEG *in vitro*. In the next phase, the stimulation of PEPC activity by including PEG during both extraction of enzyme from leaves of *Amaranthus hypochondriacus* and subsequent assay of reaction were monitored. Inclusion of PEG during the assay medium may reflect closely the physiological situation and is likely to be a way of mimicking the intracellular environment *in vivo*. Assays were run at pH 7.3, which seems to be close to the microenvironment of PEPC *in vivo*, i.e., cytosol (Huber and Edwards, 1975; Karabourniotis *et al.*, 1985). Attempts were also made to assess the oligomeric status, during the interaction of PEPC with PEG and glycerol, to see if any changes occur *in vivo*.

MATERIALS AND METHODS

Plant material

Plants of *Amaranthus hypochondriacus* L. (cv. AG-67) were raised from seeds. The plants were grown in earthen pots, filled with soil supplemented with farmyard 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 to 40°C day/25 to 30°C night. The upper fully expanded leaves from 4 to 6 week-old plants were harvested, about 2 to 3 h after sunrise. Leaf discs of ca. 0.2 cm² were punched under water with the help of a sharp paper punch and dark-

adapted for 2 h. When required the dark-adapted leaf discs were illuminated at 1000 $\mu\text{E m}^{-2}\text{s}^{-1}$ for 20 min.

Extraction, purification and assay of PEPC

The extraction and assay of PEPC were as already described (Parvathi *et al.*, 2000; Chinthapalli *et al.*, 2003). Thirty leaf discs were quickly extracted using a chilled mortar and pestle with 1 mL of extraction medium containing 100 mM Tris-HCl (pH 7.3), 10 mM MgCl₂, 2 mM K₂HPO₄, 1 mM EDTA, 10% (v/v) glycerol, 10 mM β -mercaptoethanol, 10 mM NaF, 2 mM PMSF, 10 $\mu\text{g mL}^{-1}$ chymostatin and 2% (w/v) insoluble PVP. The homogenate was centrifuged at 15,000 g for 5 min and the supernatant was used as "crude extract". A small aliquot was kept aside, prior to centrifugation for chlorophyll estimation. The procedure used for the purification of PEPC was as described in detail by Gayathri *et al.* (2000). Chlorophyll was estimated by extraction with 80% (v/v) acetone (Arnon, 1949). Protein was estimated by the method of Lowry *et al.* (1951).

PEPC activity was assayed by coupling to MDH and monitoring NADH oxidation at 340 nm in a Shimadzu 1601 UV-Visible spectrophotometer at a temperature of 30°C. The assay mixture (1 mL) contained 50 mM Tris-HCl (pH 7.3), 5 mM MgCl₂, 0.2 mM NADH, 2 U MDH, 2.5 mM PEP, 10 mM NaHCO₃ and leaf extract equivalent to 1 μg of chlorophyll or purified enzyme equivalent to 2 μg (or as described). One unit of enzyme activity is the capacity of the enzyme to catalyze the formation of 1 μmol of oxaloacetate min^{-1} .

Interaction of PEPC with PEG during assay and/or extraction

The effect of PEG-6000 (Sigma Chemical Co., USA) on PEPC was checked by incubating 2 $\mu\text{g mL}^{-1}$ of purified PEPC (unless otherwise specified) with different concentrations of PEG-6000. The stock solutions (50%, w/v) of these solutes were prepared in distilled water. The purified enzyme or the extract was left in 1 mL of the assay medium ($\pm$ PEG-6000) for 30 s and reaction was started by the addition of 50 μL of 50 mM PEP. The reaction was linear up to 5 min with purified enzyme. To ascertain the response of PEG in relation to PEPC concentration in the medium, 1 to 5 μg of PEPC-protein mL^{-1} was used in 1 mL of the assay medium.

In a further extension to these experiments, leaf extracts were prepared without or with PEG. For this 30 leaf discs were extracted with extraction buffer in the presence of different concentrations (0-10%, w/v) of PEG-6000. Thus, PEPC activity was examined by

including PEG-6000 during extraction alone, assay alone or during both extraction and assay.

Gel filtration on Sephadex G-200

Gel filtration of leaf extracts on Sephadex G-200 (1 x 30 cm) column, was performed to assess the molecular size of PEPC. Leaf crude extracts were prepared with or without solutes (20% (v/v) glycerol or 5% (w/v) PEG or both) in 100 mM Hepes-KOH, pH 7.3, containing 10 mM MgCl₂, 2 mM K₂HPO₄, 1 mM EDTA, 2 mM PMSF and 10 mM 2-mercaptoethanol. The standard buffer used for all gel chromatographic experiments was 50 mM Hepes/NaOH (pH 7.3) with or without PEG and/or glycerol, as mentioned in text. The column was washed and equilibrated with 50 mM Hepes/NaOH (pH 7.3) with additions as described. A standard quantity of 500 μ L of protein containing 450–500 μ g of protein or the leaf extract was applied to the column. All runs were made at a flow rate of 10 mL/h at 30°C. Fractions of 1 mL were collected and the pattern of PEPC was checked by assaying the enzyme activity. The column was equilibrated by using four protein-standards: thyroglobulin (669 kD), apoferritin (443 kD), alcohol dehydrogenase (150 kD), BSA (66 kD). The void volume, V₀ was determined with blue dextran (2000 kD).

Replications

All experiments were repeated 3 to 5 times on different days. The average values $\pm$ SE are presented.

RESULTS

Effect of PEG on purified PEPC

The first sets of experiments were conducted to know the effect of PEG-6000 on the purified enzyme during the assay. The stimulation by PEG-6000 (1.25%, w/v) of PEPC activity was 2.4 fold over control. The specific activities of PEPC on stimulating by PEG-6000 are shown in Fig. 1. The activities are directly expressed for an easy comparison. The activity of PEPC in the control sets (without PEG) was $46 \pm 0.2 \mu\text{mol min}^{-1} \text{mg}^{-1}$ protein and with PEG-6000, the activity increased to $120 \pm 0.1 \mu\text{mol min}^{-1} \text{mg}^{-1}$ protein. Since, a concentration of 1.25% (w/v) PEG was optimal for stimulation of PEPC activity, this indicates that PEPC is stimulated at lower concentrations of PEG but gets suppressed at higher concentrations. A PEPC protein concentration of $2 \mu\text{g mL}^{-1}$ was optimal for eliciting a maximal stimulation of 2.2 fold by 1.25% (w/v) PEG-6000 (Fig. 2). The activity

Fig. 1. Stimulation of purified PEPC when the enzyme was assayed in the presence of different concentrations of PEG-6000. PEPC activity in the control sets (without PEG) was $46 \pm 0.2 \mu\text{mol min}^{-1} \text{mg}^{-1}$ protein. The average values of 3 to 5 different replications $\pm$ SE are represented. Error bars if not seen are within the symbols.

in the control sets (without PEG) was $46 \pm 0.2 \mu\text{mol min}^{-1} \text{mg}^{-1}$ protein.

Effect of PEG-6000 when included during the extraction and/or assay of PEPC

We have also examined the effect of PEG-6000 on PEPC activity in leaf crude extracts. The activity of PEPC was determined while including PEG-6000 during either extraction alone, or only during assay or both during extraction and assay. Inclusion of PEG-6000 during

Fig. 2. Stimulation of purified PEPC by 1.25% (w/v) PEG-6000 as a function of PEPC concentration. PEPC activity in the control sets (without PEG) was $46 \pm 0.2 \mu\text{mol min}^{-1} \text{mg}^{-1}$ protein. The average values of 3 to 5 different replications $\pm$ SE are represented. Error bars if not seen are within the symbols.

extraction alone led to a maximum of 1.8 fold increase in PEPC activity, but when included only in the assay medium, PEG-6000 stimulated PEPC activity by a maximum of 2.2 fold. However, the inclusion of PEG-6000 during both extraction and assay resulted in maximum catalytic activity of PEPC (4.8 fold stimulation) (Fig. 3). Maximum stimulation of PEPC activity occurred at 5% (w/v) PEG. (PEPC activity in control set was $606 \pm 18 \mu\text{mol mg}^{-1} \text{Chl h}^{-1}$). Thus, there was a slight variation in the optimal concentrations of PEG-6000 needed for maximal stimulation of PEPC in crude extracts or in the purified form. We have no immediate explanation for this phenomenon. However our observations demonstrate that inclusion of PEG during assay as well as extraction is necessary to maintain maximal activity of PEPC.

In some of the initial experiments, PEPC protein was examined by using antibodies/western blots. The amount of PEPC protein in leaf extracts was similar.

Effect of light on the stimulation of PEPC activity in leaves by PEG-6000

The influence of PEG-6000 on light- and dark-form of PEPC in crude leaf extracts was examined. When included only in assay, at an optimal concentration of 2% (w/v), PEG-6000 stimulated PEPC activity by 2.4 fold in dark-adapted leaf discs, but by only 1.7 fold in light treated leaf discs. Thus, the dark-form of enzyme showed a more marked stimulation by PEG than the light-form (Fig. 4). The activities in the control sets (without PEG-

Fig. 3. Stimulation by PEG-6000 of PEPC in extracts from dark-adapted leaf discs of *Amaranthus hypochondriacus*. PEPC activity in control sets (without PEG) was $606 \pm 18 \mu\text{mol mg}^{-1} \text{Chl h}^{-1}$. The average values of 3 to 5 different replications $\pm$ SE are represented. Error bars if not seen are within the symbols.

Fig. 4. Stimulation by PEG-6000 of PEPC in extracts from dark-adapted (●) and light treated (○) leaf discs of *Amaranthus hypochondriacus*. PEPC activities in control sets (without PEG-6000) were $1113 \pm 14.5 \mu\text{mol mg}^{-1} \text{Chl h}^{-1}$ for dark-adapted and $1597 \pm 18 \mu\text{mol mg}^{-1} \text{Chl h}^{-1}$ for light treated leaf discs. The average values of 3 to 5 different replications $\pm$ SE are represented. Error bars if not seen are within the symbols.

6000) were $1113 \pm 14.5 \mu\text{mol mg}^{-1} \text{Chl h}^{-1}$ in dark-adapted leaf discs and $1597 \pm 18 \mu\text{mol mg}^{-1} \text{Chl h}^{-1}$ in light-treated leaf discs.

Effect of PEG-6000 on the oligomerization state of PEPC in crude leaf extracts

To gain further insight into the possible interaction of PEPC with compatible solutes, we have examined the quaternary structure of PEPC by gel filtration in crude leaf extracts of PEPC on Sephadex G-200 column. The elution profile of the enzyme in crude extracts was compared with the calibration of the column with standard proteins of known molecular weights—thyroglobulin (669 kD), apoferritin (443 kD), alcohol dehydrogenase (150 kD) and BSA (66 kD) (Fig. 5). The calibration curve obtained by elution pattern of four protein standards was used to assess the oligomeric status of PEPC and to determine the form of PEPC: tetramer, dimer, or oligomer. The number of the fraction at which a peak of elution occurs can be taken as the indication of its apparent molecular weight. Thus the protein peaks eluting at the fraction Nos. 10, 18 and 25 correspond to molecular weights of about 450 kD, 225 kD and 100 kD, respectively. These molecular weights correspond approximately to the tetramer, dimer and monomeric forms of PEPC in leaf extracts.

When the leaves of *A. hypochondriacus* were extracted with a medium containing neither glycerol nor

Fig. 5. Elution profile of four protein molecular weight standards from Sephadex G-200 column during calibration.

PEG, PEPC eluted at two peaks numbering 18 and 25 (Fig. 6A). These peaks correspond to dimer and monomers. If the extracts were prepared with glycerol and/or PEG in the extraction medium and elution buffer, PEPC eluted mainly at fraction No. 12, i.e. as a tetramer of about 450 kD (Fig. 6B & 6C). These results provide a clear demonstration of variability in oligomeric status of PEPC in leaf extracts, and its modulation by PEG and glycerol.

DISCUSSION

In this study, PEG-6000, particularly at a concentration of 1.25% (w/v) has been found to be highly effective in modulating PEPC activity (Figs. 1 and 2). Stimulation of PEPC by PEG at 1.25 mM PEP was reported by Huber and Sugiyama (1986). However, high concentrations of PEG may cause a precipitation of the protein which may result in the apparent decrease in the activity as observed in our experiments. A similar decrease in the extent of activation of cytosolic pyruvate kinase under high concentration (>10% w/v PEG) has been reported by Podesta and Plaxton (1993). High concentrations of PEG up to 40% (w/v) have been used for precipitation of PEPC (Stamatakis *et al.*, 1988; Angelopoulos and Gavalas, 1991). The effect of this solute on enzyme activity does not seem to depend upon the osmotic potential as the osmotic potentials of PEG at concentrations capable of PEPC stimulation are very low. Therefore, the activation of the enzyme by PEG can best be interpreted to be due to “exclusion-volume theory” which suggests that solutes like PEG promote self-association of enzyme-protein, and thereby mimic the intracellular situation where the enzyme is usually

Fig. 6. Elution profile from Sephadex G-200 column of PEPC in leaf extracts of *Amaranthus hypochondriacus* when; neither glycerol nor PEG-6000 were included in the extraction medium and elution buffer (A); 20% (w/v) glycerol was included in both the extraction medium and elution buffer (B); and when 20% (w/v) glycerol and 5% (w/v) PEG-6000 were included in both the extraction medium and elution buffer (C).

highly concentrated. Stimulation of PEPC activity was thus, possibly due to microconcentration of enzyme (or) removal of water/hydrophobic interaction. Stamatakis *et al.* (1988) have tested low concentrations of different PEGs: 200 to 20000 (0.005 M to 0.28 M), on PEPC from desalted extracts of *Cynodon dactylon* and have shown that stimulation of PEPC is not due to osmotic potential but by self-promotion of homologous protein molecules in solution.

It is a common experience that during the extraction and the assay of PEPC as per the normal procedures, a marked dilution of the enzyme occurs in leaf extracts. Such dilution can affect adversely the quaternary structure of PEPC due to the dissociation of the enzyme (Willeford and Wedding, 1992). These adverse effects can be counteracted either by increasing PEP concentration (Wagner *et al.*, 1987; Willeford and Wedding, 1992) or by addition of solutes (Selinioti *et al.*, 1987; Stamatakis *et al.*, 1988; Podesta and Andreo, 1989). Hence, we extended our studies on interaction of PEPC with PEG to crude leaf extracts. This serves two purposes: first to evaluate the effects of PEG on PEPC activity in crude leaf extracts and second, to identify the best way of extraction/assay of enzyme. Our results indicate that the inclusion of PEG during both extraction and assay provides protection of the enzyme against dilution and leads to higher extractable PEPC activities. We suggest that PEG could be a valuable additive to the medium to determine maximum catalytic efficiency of the enzyme. Further studies are needed to elucidate the exact molecular mechanism involved in the regulation of PEPC by PEG *in vivo*. We are sure that the effects of PEG are not due to the possible changes in malate levels or phosphorylation status of the protein, as these are seen with desalted extracts as well as purified protein.

Gel filtration technique has frequently been employed to assess the molecular size of PEPC from a few C₃-, C₄- and CAM species. While many of these attempts were with purified enzyme, some of the reports used crude extracts. Jones *et al.* (1978) reported dissociation, on dilution, of purified PEPC from a CAM plant, *Bryophyllum fedtschenkoi*, using a Sepharose CL-6B column. McNaughton *et al.* (1989) detected changes in the oligomeric status of the PEPC, extracted from illuminated and dark-adapted leaves of maize when passed through a Superose 6 column. Willeford *et al.* (1990) have also reported that PEPC predominantly exists as tetramer in presence of saturating substrate concentrations and as dimer under sub-saturating (<0.4 mM PEP) substrate level. Podesta and Andreo (1989)

observed that purified PEPC from maize eluted as a tetramer when the elution buffer contained glycerol. However in the absence of glycerol in elution buffer the enzyme appeared as both tetramer and dimer at pH 7.0. Although the existence of different oligomers (mono, di- and tetramers) of PEPC is well documented, the information on the activity of monomer or dimer of PEPC is ambiguous and controversial. The gel filtration patterns on Sephadex G-200 from the present study, demonstrate that the presence of PEG-6000 and glycerol in both the extraction and elution medium promotes the association of the less active PEPC monomers into highly active tetramers (Fig. 6). Experiments were not done by adding proteins, such as globin or gelatin. However the efficacy of 20% glycerol and Sephadex elution pattern demonstrate that PEG or glycerol results in the aggregation of protein.

Most of the observations on changes in oligomeric status of PEPC were made *in vitro* (Wu and Wedding, 1985; Wagner *et al.*, 1987; Podesta and Andreo, 1989; Willeford *et al.*, 1990). Attempts to record such changes *in vivo* of oligomerization or dissociation of PEPC have so far not been successful (Wagner *et al.*, 1987; Podesta and Andreo, 1989; Weigend and Hinch, 1992). As a result, the physiological significance of changes in oligomeric status of PEPC has been questioned (McNaughton *et al.*, 1989; Wu *et al.*, 1990). Our results demonstrate that inclusion of PEG or glycerol helps in keeping PEPC in its native active tetrameric form even in crude leaf extracts. These studies are quite relevant to the situation *in vivo*, and may be extended further to assess changes in oligomerization of PEPC, for e.g. on illumination. The physiological relevance of such oligomerization is reflected in responses of PEPC to temperature. Chinthapalli *et al.* (2003) reported that the marked modulation of malate sensitivity of PEPC in C₄ leaves at low temperature is indicative of folding or aggregation of protein. Our findings confirm that the activity and structural properties of PEPC are influenced by the microenvironment. For example, the concentration of PEPC while being assayed *in vitro*, is far below its expected concentration *in vivo*. Precautions must therefore be taken while studying the properties and regulation of PEPC. A similar situation may exist in case of also other plant enzymes, particularly cytosolic ones, such as pyruvate kinase (Podesta and Plaxton, 1993). Further studies are necessary to establish the changes in the microenvironment of the protein, including the folding or surface properties. Experiments are planned to study the intrinsic tryptophan fluorescence of PEPC, to be taken up in the next part of the study.

Apart from PEPC, several oligomeric enzymes are markedly influenced by the presence of PEG or glycerol. Examples for such enzymes are: Rubisco activase and PPI-dependent phosphofructokinase (Salvucci, 1992; Podestá and Plaxton, 1993; Moorehead *et al.*, 1994), cytosolic pyruvate kinase (Podestá and Plaxton, 1993) and cytosolic FBPase (Hodgson and Plaxton, 1995). The addition of PEG or glycerol shifted the enzyme to active tetrameric form, in case of pyruvate kinase (Podestá and Plaxton, 1993) or FBPase (Hodgson and Plaxton, 1995). In the absence of PEG or glycerol, the enzymes appeared mostly in an inactive form.

CONCLUSIONS

PEG-6000 stimulated PEPC activity not only in its purified form but also in crude extracts. Inclusion of PEG and glycerol during extraction and assay is most beneficial in terms of increasing the extractable PEPC activity. This effect can possibly be due to the prevention of the dilution of enzyme by such compounds thereby maintaining the enzyme in its most active tetrameric shape. Inclusion of PEG and glycerol during extraction and elution from Sephadex G-200 provides evidence that these compounds promote the association of the PEPC monomers and dimers into the active tetramers. We suggest that the inclusion of PEG and glycerol in the extraction and assay media can also improve the light activation and stability of PEPC during short term storage.

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