RESEARCH PAPER

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

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Abstract

The effect of Pi on the properties of phosphoenol-pyruvate carboxylase (PEPC) from *Amaranthus hypochondriacus*, a NAD-ME type C$_4$ plant, was studied in leaf extracts as well as with purified protein. Efforts were also made to modulate the Pi status of the leaf by feeding leaves with either Pi or mannose. Inclusion of 30 mM Pi during the assay enhanced the enzyme activity in leaf extracts or of purified protein by >2-fold. The effect of Pi on the enzyme purified from dark-adapted leaves was more pronounced than that from light-adapted ones. The K_i for malate increased >2.3-fold and >1.9-fold by Pi in the enzyme purified from dark-adapted leaves and light-adapted leaves, respectively. Pi also induced an almost 50–60% increase in K_m for PEP or K_a for glucose-6-phosphate. Feeding the leaves with Pi also increased the activity of PEPC in leaf extracts, while decreasing the malate sensitivity of the enzyme. On the other hand, Pi sequestering by mannose marginally decreased the activity, while markedly suppressing the light activation, of PEPC. There was no change in phosphorylation of PEPC in leaves of *A. hypochondriacus* due to the feeding of 30 mM Pi. However, feeding with mannose decreased the light-enhanced phosphorylation of PEPC. The marked decrease in malate sensitivity of PEPC with no change in phosphorylation state indicates that the changes induced by Pi are independent of the phosphorylation of PEPC. It is suggested here that Pi is an important factor in regulating PEPC *in vivo* and could also be used as a tool to analyse the properties of PEPC.

Introduction

Phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) is a key enzyme involved in primary carbon fixation of C$_4$ plants (Andreo *et al*., 1987; Rajagopalan *et al*., 1994; Chollet *et al*., 1996; Vidal and Chollet, 1997; Nimmo, 2000). The enzyme is regulated by factors, such as light, temperature or inorganic nutrients. 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; Doncaster and Leegood, 1987; Parvathi *et al*., 2000a). These changes during light activation are mostly due to the phosphorylation of the enzyme (Chollet *et al*., 1996; Vidal and Chollet, 1997; Parvathi *et al*., 2000a).

The C$_4$ plants are tolerant to heat but quite sensitive to cold temperatures (Phillips and McWilliam, 1971). At low temperature, the activity of PEPC decreased but its malate sensitivity increased in C$_4$ plants, while at high temperature the activity of the enzyme increased and malate sensitivity decreased. The changes in PEPC induced by temperature appear to be due to conformational changes in the protein (Wu and Wedding, 1987; Chinthapalli *et al*., 2003).

Among the inorganic nutrients, nitrogen has profound influence on PEPC, at both the levels of *de novo* synthesis of enzyme and the phosphorylation of PEPC in C$_3$, C$_4$ as well as in CAM plants. Ammonium ions promote the

biosynthesis of PEPC (Sugiyama and Sakakibara, 2002) and stimulate PEPC activity *in vitro* (Gayathri and Raghavendra, 1994). The extent of light activation of PEPC was increased in the presence of NO_3^- or NH_4^+ (Vidal *et al*., 2002). The stimulation by nitrate or ammonia ions appears to be through the enhancement of PEPC-PK activity and an increase in the phosphorylation status of PEPC (Sugiyama and Sakakibara, 2002; Vidal *et al*., 2002).

Pi also plays an important role in plant metabolism, not only as the substrate for oxidative and photophosphorylation but also as a key component of several metabolic processes (Theodoron and Plaxton, 1993). Pi modulates the activity of several enzymes such as, fructose and sedoheptulose *bis*phosphatases, ribulose-1,5-*bis*phosphate carboxylase and sucrose phosphate synthase (Iglesias *et al*., 1993; Rao, 1997). The role of Pi in C_3 photosynthesis has been extensively studied (Cséke and Buchanan, 1986), but the role of Pi in C_4 photosynthesis has yet to be examined in detail.

The effect of Pi on PEPC is interesting, as Pi is an end-product of the PEPC reaction. However, studies on the direct effects of Pi on PEPC are quite limited (Wong and Davies, 1973; Podestá *et al*., 1990; Meyer *et al*., 1989; Salahas and Gavalas, 1997). There are contradictory reports in the literature on the effects of Pi on the activity of C_4 PEPC: inhibition of enzyme activity (O'Leary, 1982; Doncaster and Leegood, 1987), marginal or marked activation (Podestá *et al*., 1990; González *et al*., 1987) or no effect (Wong and Davies, 1973). Usuda and Shimogawara (1992) reported a decrease in PEPC activity in maize when plants were grown a Pi-deprived condition. Further, the material used in these experiments also varied, from extracts of etiolated or illuminated leaves to purified PEPC, making it difficult for comparisons to be made.

PEPC is also regulated by metabolites. L-Malate, a product of carboxylation, is a competitive inhibitor of PEPC and a key player of feedback regulation not only in C_4 but also in C_3 and CAM plants (Vidal and Chollet, 1997). G-6-P is an allosteric activator of PEPC that increases the V_{max} of the enzyme and decreases the K_m for PEP (Andreo *et al*., 1987; Vidal and Chollet, 1997). The K_i (malate) and K_a (G-6-P) values would reflect the modulation of regulatory properties of PEPC.

The present study is an attempt to re-evaluate critically the effect of Pi on the properties of PEPC from *A. hypochondriacus,* a C_4 plant. Experiments were conducted at different levels; Pi effects were analysed *in vitro* by its addition during assay, while using leaf extracts or purified PEPC, and *in vivo* by measuring the effect of Pi fed to intact leaves. Initially, the effect of Pi on the kinetic and regulatory properties of PEPC was studied in leaf extracts and with purified protein. The Pi status of leaves was modulated by feeding with either Pi or mannose, a Pi sequester (Loughman *et al*., 1989). The phosphorylation status of PEPC was evaluated after feeding the leaves with Pi. The presence of Pi increased the activity of the enzyme (particularly the dark-form) and decreased the malate sensitivity. The changes caused by Pi appear to be independent of phosphorylation of PEPC.

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. They were grown outdoors in the field under a natural photoperiod of approximately 12 h and temperatures of 30–40/25–30 °C day/night. The fully expanded upper leaves were harvested about 2–3 h after sunrise and the leaf discs were then prepared.

Extraction and assay of PEPC

Thirty leaf discs (each of *c*. 0.2 cm^2 and total weight of 125 mg) were quickly extracted in a prechilled mortar and pestle with 500 μl of extraction medium containing 100 mM HEPES-KOH, pH 7.3, 5 mM $MgCl_2$, 2 mM KH_2PO_4, 1 mM EDTA, 10% (v/v) glycerol, 10 mM β-mercaptoethanol, 10 mM NaF, 2 mM PMSF, 5 mM DTT, and 2% (w/v) PVP. The homogenate was centrifuged at 15 000 *g* for 5 min. The supernatant was desalted through a small Sephadex G-25 column and was used for enzyme assays.

The activity of PEPC was assayed coupling to NAD malic dehydrogenase (MDH) and monitoring NADH oxidation at 340 nm in a Shimadzu UV-Vis Spectrophotometer (Parvathi *et al*., 2000*a*). The assay mixture (1 ml) contained 50 mM HEPES-KOH, pH 7.3, 5 mM $MgCl_2$, 0.2 mM NADH, 2 units of MDH, 2.5 mM PEP, 10 mM $NaHCO_3$, and leaf extract (equivalent to 1 μg of chlorophyll). The sensitivity of PEPC to malate was checked using 0.5 mM malate in a separate assay. The ranges of concentration used for various parameters were as follows: K_m (PEP), 0.1–5 mM, K_i (malate), 0.1–2 mM, K_a (G-6-P), 0.5–5 mM.

Light activation of PEPC

Leaf discs (each of *c*. 0.2 cm^2 and total weight of about 125 mg) were floated on 10 ml distilled water (unless otherwise specified) in a 5 cm diameter Petri dishes. They were illuminated under white light (Philips Comptalux R95 flood bulbs) at an intensity of 1000 μE m^{-2} s^{-1}. The light was passed through a water filter of 10 cm thickness to prevent heating. After 30 min of illumination (or dark incubation for comparison), the leaf discs were extracted quickly for PEPC assay.

Purification of PEPC and preparation of anti-PEPC antiserum

The PEPC protein was purified from *A. hypochondriacus* leaves as described in detail by Gayathri *et al*. (2000). Leaves of *A. hypochondriacus* were taken in the early morning before sunrise for dark-adapted leaves and at midday for light-adapted leaves. 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).

Estimation of chlorophyll and protein

Chlorophyll was estimated by extraction with 80% (v/v) acetone (Arnon, 1949) and protein was estimated by the method of Lowry *et al*. (1951).

Evaluation of the effects of Pi or mannose

Whenever needed, Pi was added in the form of K_2HPO_4 (pH adjusted to 7.3 with HCl). The leaves were fed through the petiole, with either 30 mM Pi or 10 mM mannose, under low light (200 μE $m^{-2} s^{-1}$). After feeding for the required time, leaf discs were prepared and either kept in darkness or illuminated (1000 μE $m^{-2} s^{-1}$) for 30 min. Then the discs were homogenized and their extract was used for assaying PEPC.

In vivo *labelling of PEPC with* [32]*Pi*

Labelling of PEPC with [32]Pi *in vivo* was carried out using the procedure described by Bakrim *et al.* (1992) and modified slightly by Parvathi *et al.* (2000*b*).

Excised leaves were fed through petiole with 100 μl (60 μCi) of $KH_2{}^{32}PO_4$ (specific activity of 10 mCi $mmol^{-1}$) under moderate illumination (200 μE $m^{-2} s^{-1}$) for 3 h. The leaves were left in darkness for 3 h to ensure that the PEPC was dephosphorylated. A set of leaves was used for preparing leaf discs either to be illuminated (1000 μE $m^{-2} s^{-1}$) or kept in darkness for 60 min (Parvathi *et al.*, 2000*a*). These leaf discs were then extracted with 0.5 ml of extraction buffer as described above. Another set of leaves were fed with Pi or mannose for 2 h. The leaf extracts were examined for the protein levels and the phosphorylation status of PEPC as described (Parvathi *et al.*, 2000*b*).

Replication

The experiments were repeated 3–5 times on different days. The average values ±SE are reported.

Results

Effect of Pi during assay on PEPC in leaf extracts

The activity of PEPC was enhanced in the presence of Pi in the leaf extracts (Fig. 1A). The activity of the enzyme increased by >2.5-fold at 15 or 20 mM Pi, while the sensitivity of PEPC to malate decreased (Fig. 1B) from about 90% (no Pi) to about 70% (in the presence of 30 mM Pi). Due to the marked stimulation of PEPC activity by Pi, even in extracts from dark-adapted leaves, the extent of light activation got masked as the Pi concentration was increased in the assay medium. As a result, the L/D ratio of PEPC in leaf extracts decreased (Fig. 2).

Effect of Pi during the assay on the properties of purified PEPC

Studies were extended to the purified form of PEPC and its response to 30 mM Pi during the enzyme assay. The V_{max} of the purified PEPC increased upon addition of Pi (Fig. 3A) and there was a drastic decrease in its malate sensitivity (Fig. 3B).

Table 1 summarizes the response to Pi of catalytic and regulatory properties of purified PEPC from dark-adapted and light-adapted leaves. The presence of Pi increased V_{max} by >1.8-fold and 1.6-fold whereas K_i (malate) increased by >2.3-fold and >1.9-fold in dark-adapted and light adapted leaves, respectively. The K_m (PEP) increased by about >1.5-fold and 1.3-fold, respectively. K_a (G-6-P) increased by about 1.7-fold and >1.6-fold in dark-adapted and light-adapted leaves, respectively. Thus, the effects of
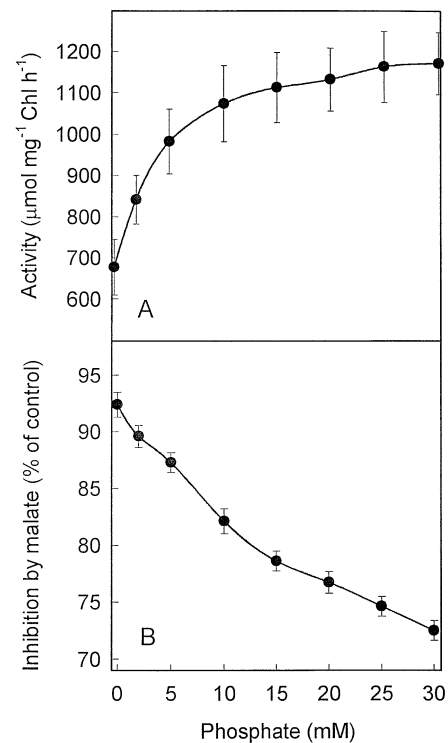

Fig. 1. The effect of addition of Pi during the enzyme assay on the activity (A) or the extent of inhibition by 0.5 mM malate (B) of PEPC in extracts from dark-adapted leaves of *A. hypochondriacus*. The average values of 3–5 experiments are represented as ±SE. The changes caused by Pi were all statistically significant (P <0.01).
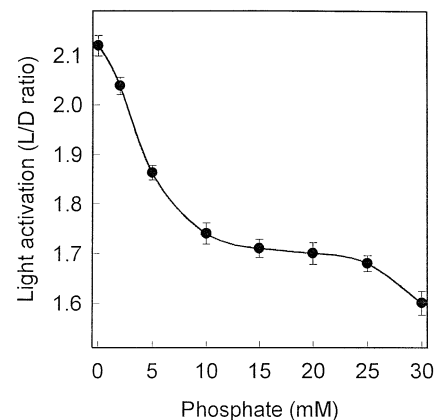

Fig. 2. The extent of light activation of PEPC in leaf extracts, in relation to different concentration of Pi in the assay medium. The average values of 3–5 experiments are represented as ±SE. The changes caused by Pi were all statistically significant (P <0.01).

Pi were much more pronounced in purified PEPC from dark-adapted leaves than from light-adapted ones.

Effect of feeding Pi or mannose to leaves

Feeding leaves with Pi through the transpiration stream increased PEPC activity and decreased the extent of
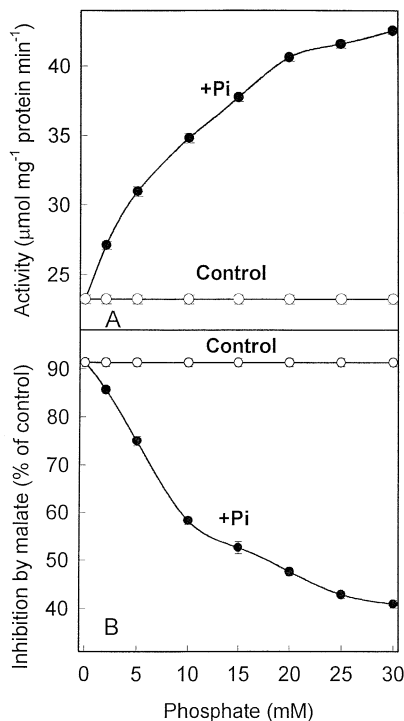
Fig. 3. The effect of added Pi on the activity (A) or the extent of inhibition by 0.5 mM malate (B) of purified PEPC from dark-adapted leaves of *A. hypochondriacus*. The average values of 3–5 experiments are represented as ±SE. Some errors are within the symbols. The changes caused by Pi were all statistically significant (P <0.01).

inhibition by malate (Fig. 4). The effect of Pi was dependent on the duration of Pi feeding. By about 3 h of feeding, there was >2-fold increase in PEPC activity, while there was a decrease in malate sensitivity, from 92% to 76% in the dark-adapted leaves (Fig. 4B), in all extracts from dark-adapted leaves. Again, the effects of Pi were low on PEPC in extracts from illuminated leaves.

Mannose, a Pi sequestering agent, was fed to the leaves for 90 min and the properties of PEPC extracted from these leaves (after illumination or dark-adaptation) were examined. The effect of mannose was concentration-dependent and peaked at 10 mM mannose. Feeding of leaves with 10 mM mannose decreased the activity of PEPC; the decrease being more pronounced in illuminated leaves than in the dark-adapted ones (Fig. 5A). There was little effect on the extent of malate inhibition, in illuminated or dark-adapted leaves (Fig. 5B).

Effect of Pi on the phosphorylation status of PEPC

The phosphorylation status of PEPC was examined, with or without pretreatment with Pi and after illumination of leaves (Fig. 6). There was a marked phosphorylation of PEPC in extracts from illuminated leaves. However, there was no change in PEPC phosphorylation due to the presence of Pi. The phosphorylation of PEPC was suppressed in the presence of mannose, particularly in

illuminated leaves. No phosphorylation was detected in dark, either in the control or Pi-treated leaves (Fig. 6).

Discussion

These results demonstrate the marked regulation of PEPC by Pi *in vivo* (leaves) as well as *in vitro* (purified protein). The presence of Pi not only increased PEPC activity but also decreased the malate sensitivity of the enzyme from *A. hypochondriacus* (Figs 1, 3). This study endorses the earlier suggestions that Pi can stabilize PEPC (Podestá *et al.*, 1990; Salahas and Gavalas, 1997; Salahas *et al.*, 1997b). High levels of Pi (~100 mM) can protect PEPC against heat denaturation (Salahas *et al.*, 1997a; Jensen *et al.*, 1995) as well as cold temperature (Salahas *et al.*, 1997a). It is therefore important that PEPC is extracted in Pi buffer for maximum activity and stability.

The reports on the effect of Pi on PEPC have been quite contradictory. Doncaster and Leegood (1987) reported >40% inhibition of enzyme activity, whereas Podestá *et al.* (1990) reported an increase of 150% in PEPC activity. The activity of Pi was unaffected, as per Wong and Davies (1973). These results, therefore, clear the ambiguities and confirm that the PEPC from *A. hypochondriacus* is stimulated by Pi. The concentration of Pi in mesophyll cells of plants is expected to be around 20 mM (Mimura *et al.*, 1998) and can be an important factor.

The internal levels of Pi in leaves increase after feeding with external Pi (Mimura *et al.*, 1990). When the leaves of *A. hypochondriacus* were fed with Pi, the activity of PEPC increased, while the extent of malate inhibition decreased (Fig. 4). These results suggest that the changes in Pi levels in the leaves would lead to a marked modulation *in vivo* of PEPC activity and its malate sensitivity. Marked changes in PEPC, during growth in the presence or absence of Pi were noticed in maize seedlings (Usuda and Shimogawara, 1992) and suspension cell cultures of *Brassica napus* (Moraes *et al.*, 2000).

Rapid utilization of cytoplasmic Pi occurs upon the addition of sequestering agents like mannose (Loughman *et al.*, 1989). Mannose is phosphorylated in cytoplasm and hence decreases not only the cytoplasmic Pi concentration (Lee and Ratcliffe, 1993) but also the levels of ATP (Van Quy and Champigny, 1992). Therefore, an attempt was made to decrease the level of Pi by feeding mannose to leaves. Mannose feeding in the dark has not only decreased the PEPC activity but has also markedly suppressed the light activation of PEPC (Fig. 5). In a similar study, Van Quy and Champigny (1992) found that mannose feeding had inhibited kinase activity and restricted light activation of PEPC in wheat leaves. This could be the reason for the inhibition of phosphorylation in mannose-fed leaves even upon illumination (Fig. 6).

Mannose at high concentration may affect various metabolites by sequestering Pi into mannose-6-phosphate,

Table 1. *Changes in the kinetic and regulatory properties of purified PEPC from dark-adapted and light-adapted leaves of* Amaranthus hypochondriacus, *respectively, in response to 30 mM Pi during enzyme assay*

The average values of 3–5 experiments are represented as ±SE. The changes caused by Pi were all statistically significant ($P < 0.01$).

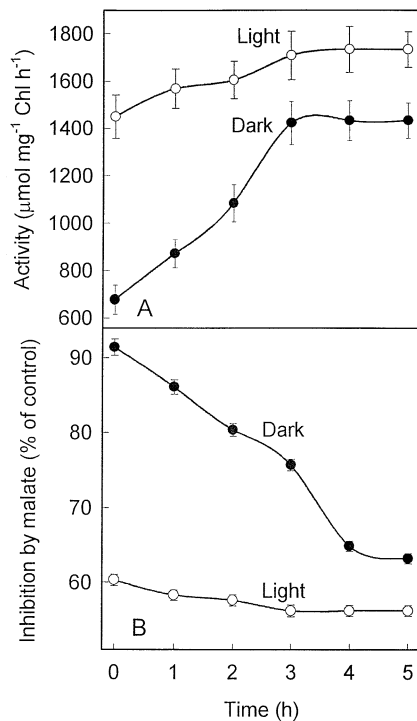| Parameters	PEPC from dark-adapted leaves		Δ Change (-fold)	PEPC from light-adapted leaves		Δ Change (-fold)
	Control	+30 mM Pi		Control	+30 mM Pi	
V_{max} (μmol mg^{-1}protein min^{-1})	22.6±0.63	41.4±0.52	1.8	29.7±0.47	49.9±0.62	1.6
K_m (PEP) (mM)	0.33±0.01	0.49±0.01	1.5	0.14±0.01	0.18±0.02	1.3
K_i (malate) (mM)	0.13±0.01	0.34±0.02	2.6	0.21±0.02	0.40±0.08	1.9
K_a (G-6-P) (mM)	0.21±0.01	0.38±0.01	1.8	0.29±0.02	0.47±0.02	1.6

Fig. 4. The effect on the activity (A) or the extent of inhibition by 0.5 mM malate (B) of PEPC, extracted from illuminated or dark-adapted leaves of *A. hypochondriacus*, after varying periods of feeding 20 mM Pi through petioles. The average values of 3–5 experiments are represented as ±SE. Some errors are within the symbols. Further details are described in Materials and methods. The changes caused by Pi were all statistically significant ($P < 0.01$).

Fig. 5. Effect of feeding mannose to leaves of *A. hypochondriacus* on the activity (A) or the malate sensitivity by 0.5 mM malate (B) of PEPC extracted from dark-adapted or illuminated leaves. The average values of 3–5 experiments are represented as ±SE. Some errors are within the symbols. Further details are described in Materials and methods.

and may cause an imbalance in metabolism (Herold and Lewis, 1977; Brouquisse *et al.*, 2001). The properties of PEPC may, therefore, be affected due to the altered level of the metabolites. However, this possibility would reaffirm the importance of Pi *in vivo*, as these effects of mannose are consequences of lowered Pi.

It is interesting to note that the leaves treated with mannose still exhibit limited light activation of PEPC and a decrease in malate inhibition of the enzyme. Obviously, these changes must be due to a process other than phosphorylation. It would be interesting to examine
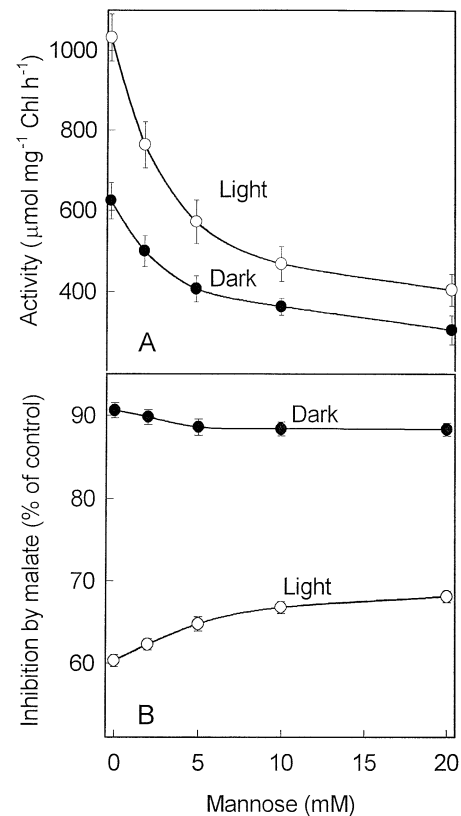
light-activation of PEPC in mannose-treated leaves. For example, the cytosolic alkalization of mesophyll cells of C$_4$ leaves (Raghavendra *et al.*, 1993) could mediate some of these changes in PEPC.

The phenomenon of the increase in PEPC activity as well as the decrease in malate sensitivity due to Pi is quite similar to the effects of light on PEPC in C$_4$ leaves. The light activation of PEPC is achieved by post-translational
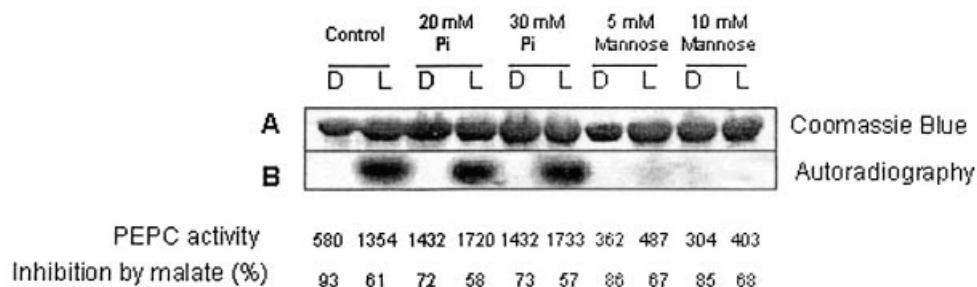
Fig. 6. The pattern of protein levels and the phosphorylation of PEPC in leaves of *A. hypochondriacus* exposed to either dark (D), light (L) treatment or fed with either Pi (20 and 30 mM) or mannose (5 and 10 mM). The leaves of *A. hypochondriacus* labelled with ^{32}Pi were extracted after the required treatment. The PEPC protein was immunoprecipitated from leaf extracts, separated on 10% SDS-PAGE and examined for either protein levels by using Coomassie Blue (A) or radioactivity, by using autoradiography (B). The amount of protein in each lane was 15 µg. The activity and sensitivity to malate (0.5 mM) of PEPC are shown for a ready comparison.

Table 2. *Relative changes in kinetic and regulatory properties of PEPC to various regulatory factors from leaves of* Amaranthus hypochondriacus

The illumination was carried out for 30 min at 1000 µE m^{-2} s^{-1} and PEPC was incubated for 45 min at 45 °C. During enzyme assay 1.25% (w/v) PEG or 30 mM Pi was added. The values represented are as compared with control (100%). Dark, 15 °C, zero PEG and zero Pi are taken as controls.

Regulatory factor/transition	Parameters				Reference
	V_{max}	K_m (PEP)	K_i (malate)	K_a (G-6-P)	
Dark→Light	234	70	430	315	Parvathi, 1998; Parvathi *et al.*, 2000
Temperature (15 °C→45 °C)	165	22	155	52	Chinthapalli *et al.*, 2003
PEG (0→1.25%)	169	60	151	140	Parvathi, 1998
Pi (0→30 mM)	183	130	233	160	Present work

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). It is, therefore, possible that there is a change in the phosphorylation status of PEPC in the presence of Pi. The extent of PEPC phosphorylation was much higher in illuminated discs than in dark-adapted leaves. However, there was no change in phosphorylation status of PEPC due to Pi treatment (Fig. 6). Feeding mannose or Pi could affect the specific radioactivity of leaf ATP. Thus, these results may be termed as qualitative indications. However, the high level of PEPC phosphorylation under light, even after feeding Pi, indicated that the process of PEPC phosphorylation was stable and similar. Therefore, it is concluded that the changes in PEPC induced by Pi are not dependent on phosphorylation.

This study's results suggest that the Pi-induced decrease in malate sensitivity is not related to the phosphorylation status of PEPC. A similar situation has recently been reported during the temperature-modulated decrease in malate sensitivity of PEPC (Chinthapalli *et al.*, 2003). Although the suggestion is speculative at this stage, the presence of Pi could either stabilize the tetrameric shape of PEPC or change the conformational status of protein or both. Further experiments on fluorescence and the circular dichroism spectra of PEPC are necessary to assess this possibility.

Table 2 summarizes the changes in properties of PEPC from *A. hypochondriacus* due to light or temperature and compares them with the effects of PEG-6000 or Pi. The increases in V_{max}, K_i (malate) and K_a (G-6-P) on illumination are all due to the phosphorylation of the enzyme (Parvathi, 1998; Parvathi *et al.*, 2000a). However, the changes in V_{max} or K_i (malate) induced by warm temperatures were due to a process other than phosphorylation, possibly changes in the oligomerization or conformation of the enzyme (Chinthapalli *et al.*, 2003). The effects of Pi as recorded in the present work are quite interesting as the presence of Pi caused an increase in V_{max} or K_i (malate), but had no significant effect on K_m (PEP) or K_a (G-6-P). It is speculated that the effects of Pi on the kinetic properties and stability of PEPC are due to a shifting of the dimer/tetramer equilibrium towards the tetramer. Further experiments are necessary to test this hypothesis.

These results establish that cytoplasmic Pi is an important factor in regulating PEPC of C$_4$ plants such as

A. hypochondriacus. Further, Pi could be used as a tool to analyse the properties of C₄ PEPC.

Acknowledgements

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