Journal of Experimental Botany, Vol. 54, No. 383, pp. 707–714, February 2003
DOI: 10.1093/jxb/erg078

Journal of Experimental Botany

RESEARCH PAPER

Dramatic difference in the responses of phospho*enol*pyruvate carboxylase to temperature in leaves of C_3 and C_4 plants

Bhaskarrao Chinthapalli, Jhadeswar Murmu and Agepati S. Raghavendra[1]

Department of Plant Sciences, School of Life Sciences, University of Hyderabad, Hyderabad 500 046, India

Received 13 September 2002; Accepted 17 October 2002

Abstract

Temperature caused phenomenal modulation of phospho*enol*pyruvate carboxylase (PEPC, EC 4.1.1.31) in leaf discs of *Amaranthus hypochondriacus* (NAD-ME type C_4 species), compared to the pattern in *Pisum sativum* (a C_3 plant). The optimal incubation temperature for PEPC in *A. hypochondriacus* (C_4) was 45 °C compared to 30 °C in *P. sativum* (C_3). *A. hypochondriacus* (C_4) lost nearly 70% of PEPC activity on exposure to a low temperature of 15 °C, compared to only about a 35% loss in the case of *P. sativum* (C_3). Thus, the C_4 enzyme was less sensitive to supra-optimal temperature and more sensitive to sub-optimal temperature than that of the C_3 species. As the temperature was raised from 15 °C to 50 °C, there was a sharp decrease in malate sensitivity of PEPC. The extent of such a decrease in C_4 plants (45%) was more than that in C_3 species (30%). The maintenance of high enzyme activity at warm temperatures, together with a sharp decrease in the malate sensitivity of PEPC was also noticed in other C_4 plants. The temperature-induced changes in PEPC of both *A. hypochondriacus* (C_4) and *P. sativum* (C_3) were reversible to a large extent. There was no difference in the extent of phosphorylation of PEPC in leaves of *A. hypochondriacus* on exposure to varying temperatures, unlike the marked increase in the phosphorylation of enzyme on illumination of the leaves. These results demonstrate that (i) there are marked differences in the temperature sensitivity of PEPC in C_3 and C_4 plants, (ii) the temperature induced changes are reversible, and (iii) these changes are not related to the phosphorylation state of the enzyme. The inclusion of PEG-6000, during the assay, dampened the modulation by temperature of malate sensitivity of PEPC in *A. hypochondriacus*. It is suggested that the variation in temperature may cause significant conformational changes in C_4-PEPC.

Key words: Cold sensitivity, conformational change, malate sensitivity, PEPC, PEG-6000, temperature.

Introduction

Phospho*enol*pyruvate carboxylase (EC 4.1.1.31) is a ubiquitous enzyme occurring in the cytosol of photosynthetic and non-photosynthetic tissues of C_3, C_4 and CAM plants (Andreo *et al*., 1987; Rajagopalan *et al*., 1994; Chollet *et al*., 1996; Vidal and Chollet, 1997; Nimmo, 2000). C_4 plants differ from C_3 plants in several features, including their light and temperature responses (Berry and Björkman, 1980; Sugiyama *et al*., 1979). The temperature optima for photosynthesis and growth in C_4 plants are usually higher than those for C_3 plants (Berry and Björkman, 1980). The C_4 plants, in general, are tolerant to heat, but are quite sensitive to cold temperatures (Du *et al*., 1999*a*). The cold sensitivity of the C_4 pathway has been suggested to be related to the cold sensivitity of key C_4 enzymes, such as pyruvate phosphate dikinase (PPDK) or PEPC (Potvin and Simon, 1990; Burnell, 1990; Du *et al*., 1999*a*).

The cold sensitivity of PPDK in C_4 plants is well established and the mechanism of cold inactivation of PPDK is studied in detail (Krall *et al*., 1989; Burnell, 1990; Du *et al*., 1999*b*). By contrast, the reports on cold sensitivity of PEPC have been conflicting. There are

[1] To whom correspondence should be addressed. Fax: +91 40 23010145. E-mail: asrsl@uohyd.ernet.in
Abbreviations: MDH, NAD-malate dehydrogenase; NAD-ME, NAD-malic enzyme; PEG-6000, polyethylene glycol 6000; PEPC, phospho*enol*pyruvate carboxylase; PMSF, phenylmethylsulphonyl fluoride; PPDK, pyruvate phosphate dikinase.

reports which suggest PEPC is sensitive to cold temperature (Phillips and McWilliam, 1971; Selinioti *et al*., 1986), while others could not detect any significant change in the properties of PEPC at cold temperature (Du *et al*., 1999*b*; Krall and Edwards, 1993). In addition, some of these experiments involved the long-term exposures of plants or the short-term exposure of purified enzymes and so diverse experimental material has been used.

On illumination, the activity of PEPC in leaves of C_4 plants is enhanced 2–3-fold, with a marked decrease in the malate sensitivity of the enzyme. These changes during light activation are due to the phosphorylation of the enzyme (Chollet *et al*., 1996; Vidal and Chollet, 1997; Parvathi *et al*., 2000*a*). The work of Selinioti *et al*. (1986) indicated that an amplification of the light effect by temperature difference could occur. Such interaction of light and temperature would be very important for C_4 plants. Compared to the extensive literature on the properties and mechanism of light activation of PEPC, in C_4 plants, the literature on the regulation by temperature of PEPC is quite limited (Rajagopalan *et al*., 1994).

The present study is an attempt to characterize the temperature responses of PEPC from a typical C_4 plant, *Amaranthus hypochondriacus* and compare them with those of a C_3 plant pea (*Pisum sativum*). Experiments were conducted on leaf discs so as to simulate the physiological situation *in vivo*. Studies were extended to a few more species to establish the similar nature of PEPC responses in C_3 and C_4 plants. Attempts were then made to understand the mechanism of temperature-induced changes in the properties of PEPC in *A. hypochondriacus* (C_4) by examining the changes in the phosphorylation status, protein levels or the modulation by PEG-6000.

Materials and methods

Plant material

Plants of *Amaranthus hypochondriacus* L. (cv. AG-67) and *Pisum sativum* L. (cv. Arkel) 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 °C day/25–30 °C night. The upper fully expanded leaves were harvested, about 2–3 h after sunrise. Leaf discs were prepared from 4–6-week-old plants of *A. hypochondriacus* and 8–10-d-old plants of *P. sativum*.

Extraction and assay of PEPC

Thirty leaf discs (each of *c*. 0.2 cm^2 and a total weight of 125 mg) were extracted in a chilled mortar and pestle with 500 µl of extraction medium containing 100 mM TRIS-HCl, pH 7.3, 5 mM $MgCl_2$, 2 mM KH_2PO_4, 1 mM EDTA, 20% (v/v) glycerol, 10 mM β-mercaptoethanol, 10 mM NaF, 2 mM PMSF, 5 mM DTT, and 2% (w/v) insoluble PVP. The homogenate was centrifuged at 15 000 *g* for 5 min. The supernatant was used as crude extract.

In some of the experiments, 10 µg ml^{-1} chymostatin was used, instead of PMSF. However, PMSF was used routinely, as it was found to be quite effective in avoiding proteolysis in the case of *A. hypochondriacus*.

The activity of PEPC was assayed by coupling to NAD malic dehydrogenase (MDH) and monitoring NADH oxidation at 340 nm in a Shimadzu UV-Vis Spectrophotometer (Parvathi *et al*., 2000*b*). The assay was performed at 30 °C (irrespective of the preincubation temperature of the leaf discs). The assay mixture (1 ml) contained 50 mM TRIS-HCl, pH 7.3, 5 mM $MgCl_2$, 0.2 mM NADH, 2 U 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 either 0.5 mM malate (C_4 species) or 2 mM malate (C_3 species). In some of the experiments (as specified), 1.25% (w/v) PEG-6000 was also present during the assay.

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).

Incubation of leaf discs at different temperatures

Thirty leaf discs were floated on distilled water in a 5 cm diameter Petri dishes and were left in darkness for 2 h. After predarkening, the leaf discs were incubated 30 min at required temperature in the range of 15 °C to 50 °C in a thermostatically controlled water bath.

The temperature range used for all species was 15 °C to 50 °C. At the end of 30 min in each temperature, the leaf discs were extracted (as described above) and the extract was examined for PEPC activity.

Preparation of anti-PEPC antiserum

Anti-PEPC antiserum was raised in 6-month-old white rabbits following the principles of Nimmo *et al*. (1986) as described in Gayathri *et al*. (2001).

In vivo *labelling of PEPC with* $^{32}P_i$

Labelling of PEPC with ^{32}Pi *in vivo* was done by following the procedure described by Bakrim *et al*. (1992).

Excised leaves were fed through the petiole with 100 µl (60 µCi) of $KH_2{}^{32}PO_4$ (specific activity of 10 mCi $mmol^{-1}$) under moderate illumination (400 µE m^{-2} s^{-1}) for 4 h. The leaves were left in darkness for 2 h to ensure that the PEPC is dephosphorylated. A set of leaf discs were exposed to different temperatures for 30 min. In a parallel treatment, leaf discs were illuminated (1000 µE m^{-2} s^{-1}) or kept in darkness for 45 min (Parvathi *et al*., 2000*b*).

The incubated leaf discs were extracted with 5 ml of extraction buffer described above. The leaf extracts were cleared by centrifugation for 15 000 *g* for 5 min and the supernatants were assayed for PEPC. Extracts containing 0.2 units of PEPC-activity (approximately 300 µl) was mixed with the volume of anti-PEPC antiserum (200 µl) and left overnight at 4 °C. The immunoprecipitated PEPC was pelleted by centrifugation at 15 000 *g* for 5 min. The supernatant was removed and the pellet washed twice with 0.5 M TRIS-HCl, pH 8.0, 1.5 M NaCl and 1% Triton X-100, and once with 0.1 M TRIS-HCl, pH 8.0. The pellet was finally suspended in SDS-PAGE sample buffer and subjected to 10% SDS-PAGE. The gels were stained with Coomassie brilliant blue R-250, destained and dried (Biorad gel dryer). The dried gels were used for autoradiography at –80 °C, as described in detail by Parvathi *et al*. (2000*a*).

Western blotting

The levels of PEPC protein were assessed by using western blots (Betz and Dietz, 1991), after transferring the proteins electrophoretically from the gel onto the polyvinylidene difluoride (PVDF) membranes (Towbin *et al*., 1979). Anti-PEPC antiserum prepared

against PEPC of *A. hypochondriacus* was used. The blots were visualized by using 5-bromo-4-chloro-3-indolylphosphate (BCIP) and *p*-nitroblue-tetrazolium chloride (NBT). Further details are described by Gayathri *et al*. (2001).

Replication

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

Results

Changes in the activity and malate sensitivity of PEPC with temperature

The optimal incubation temperature for PEPC in *Amaranthus hypochondriacus* (C_4) was 45 °C, compared to 30 °C in *Pisum sativum* (C_3 species). The response of the enzyme to temperature was quite dramatic when plotted as the percentage of maximum activity (Fig. 1). The decrease in PEPC activity at 15 °C was much higher in the case of *A. hypochondriacus* (C_4) than that of *P. sativum* (C_3). Similarly, the decrease in activity of PEPC at a high temperature was much greater in *P. sativum* (C_3) than in *A. hypochondriacus* (C_4). Thus, the C_4 PEPC was more sensitive to sub-optimal temperatures and less sensitive to supraoptimal temperatures than that of C_3 species.

As the temperature was raised from 15 °C to 50 °C, there was a marked decrease in the malate sensitivity of PEPC in *A. hypochondriacus* (from 85% to 39%). Such a decrease was more than that in *Pisum sativum* (from 66% to 34%) (Fig. 2). The extent of malate inhibition was always higher in the case of *P. sativum* than of *A. hypochondriacus*.

In *Amaranthus hypochondriacus*, the use of chymostatin during extraction did not make a great difference. For example, the inhibition by malate of PEPC at 15 °C was 69% and 76%, respectively, in the presence of PMSF and chymostatin. The malate inhibition at the incubation temperature of 50 °C fell to 31% and 40%, in the presence of PMSF and chymostatin, respectively. Thus, the pattern of PEPC response to a rise in temperature (increase in activity and decrease in malate sensitivity) was similar, irrespective of the presence of either chymostatin or PMSF. As chymostatin was expensive, PMSF was used routinely in all the experiments.

Reversibility of the effect of temperature on PEPC

The next set of experiments were designed to determine if the changes, induced in the properties of PEPC at various temperatures, were reversible. The leaf discs incubated at the optimal incubation temperatures of 45 °C (*A. hypochondriacus*) and 30 °C (*P. sativum*) were taken

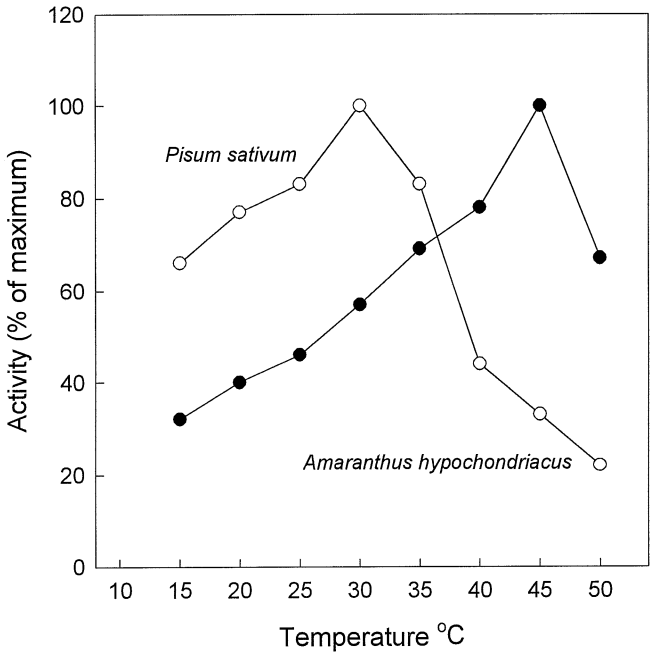

Fig. 1. The activity of PEPC in extracts from leaf discs of *Amaranthus hypochondriacus* (C_4 plant) and *Pisum sativum* (C_3 species) after exposure to varying temperatures. The activity of PEPC is represented as a percentage of its maximum. The preincubation time for leaf discs was 30 min for each temperature. The experiments were done on at least three different days and the average values ±SE are represented. The maximal activity of PEPC in *A. hypochondriacus* and *P. sativum* were 1365±106 and 27.1±2.4 μmol mg^{-1} Chl h^{-1}, respectively.

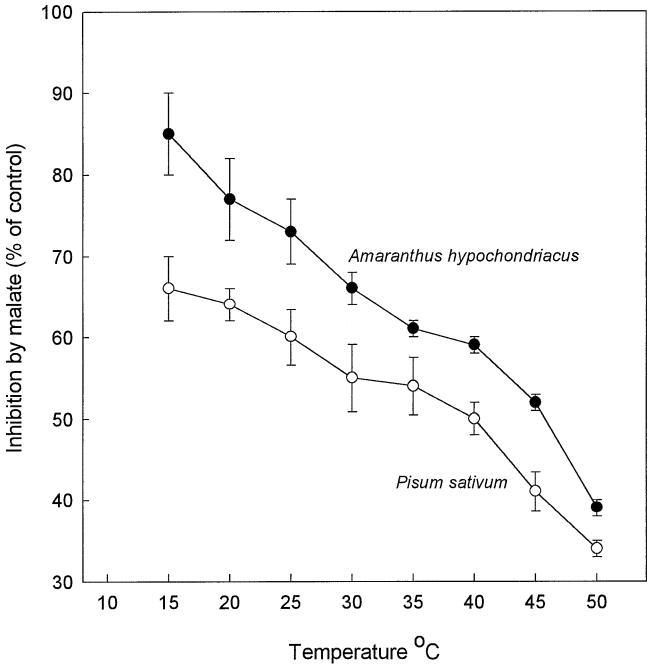

Fig. 2. Effect of temperature on the malate sensitivity of PEPC in extracts prepared from the leaf discs of *Amaranthus hypochondriacus* (C_4 plant) or *P. sativum* (C_3 species), exposed to different temperatures. The activity was measured at 30 °C and assayed in the absence or presence of either 0.5 mM malate (*A. hypochondriacus*) or 2 mM malate (*Pisum sativum*). Further details were as described in Fig. 1.

as controls. The leaf discs were exposed to various temperatures (for 30 min) and then transferred to their respective optimal incubation temperature (for 30 min). The decrease in the activity of PEPC at either suboptimal or supraoptimal temperature was significantly reversed in both *P. sativum* and *A. hypochondriacus* (Fig. 3A, B).

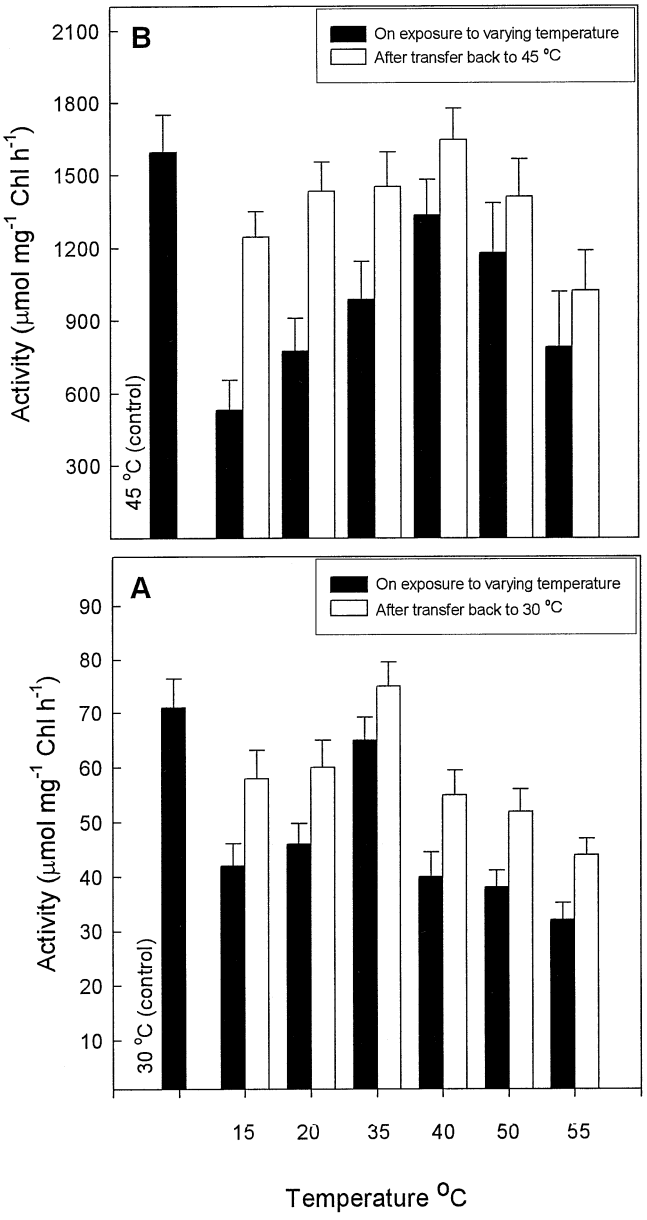

Fig. 3. The reversibility of the effects of temperature on PEPC. The leaf discs were preincubated at the optimal incubation temperature (control), exposed to varying temperatures and then transferred back to the control temperature. (A) *Pisum sativum* (C_3 species), (B) *Amaranthus hypochondriacus* (C_4 plant).

Comparison of the effects of temperature on PEPC in a range of C_3 and C_4 plants

The effect of temperature on PEPC activity and sensitivity to L-malate were studied in leaf discs of a few C_3 and C_4 plants (Tables 1, 2). When leaf discs were incubated at different temperatures, the average PEPC activity in C_4 plants fell to 40% and 62% at suboptimal and supraoptimal temperatures, respectively, compared to the control (optimal incubation temperature). In C_3 species, the average PEPC activity at suboptimal and supraoptimal temperature was 65% and 38%, respectively, compared to the control. The sensitivity to L-malate of PEPC in C_4 plants increased by almost 2-fold, at suboptimal temperature, while there was only about 20% increase at the suboptimal temperature, in C_3 species. Similarly, the decrease in the extent of malate inhibition of PEPC as the temperature was raised from 15 °C to 50 °C was higher in C_4 plants than in C_3 species. Thus, the C_4 PEPC was more tolerant to high temperature and more susceptible to low temperature than C_3 plants.

Changes in the phosphorylation state and protein levels of PEPC

Attempts were made to examine if the variation in temperature caused any modulation of either the phosphorylation status or the protein levels of PEPC. Since illumination is known to increase the phosphorylation of PEPC, light/dark treatment of leaf discs was included for comparison. The extent of PEPC phosphorylation was much higher in illuminated discs than in dark-adapted leaves. However, there was no significant change in the status of PEPC phosphorylation in leaf discs exposed to different temperatures (Fig. 4).

Western blot analysis indicated that the levels of PEPC-protein in leaf discs of *A. hypochondriacus*, were almost similar in control (dark-treated), illuminated discs as those at varied temperature (Fig. 4). Thus, neither a change in temperature nor illumination, caused any significant change in the PEPC-protein levels.

The effects of temperature on PEPC in the presence of PEG-6000

Since there was no change in the phosphorylation status of PEPC on exposure to temperature, the other possibility was a change in the conformational status of the enzyme. Compatible solutes like PEG-6000 promote the oligomerization of PEPC, increase the enzyme activity and decrease its malate sensitivity (Huber and Sugiyama, 1986). The effect of temperature on PEPC from leaf discs of *A. hypochondriacus* (C_4) was therefore assessed with or without PEG-6000 in the assay medium (Table 3). In the absence of PEG-6000, the activity of PEPC decreased at sub- or supraoptimal temperature and the extent of inhibition by malate decreased steeply at 50 °C compared

Table 1. *Effect of varying temperature on the activity of PEPC in different species of C_4 and C_3 plants*

The values in parentheses represent the activity at suboptimal or supraoptimal temperatures as a percentage of that at the optimal incubation temperature.

Type/species	PEPC activity (μmol mg^{-1} Chl h^{-1})		
	Temperature		
	Optimal	Suboptimal	Supraoptimal
C_4 species	45 °C	15 °C	50 °C
Euphorbia hirta	1490 (100)	793 (53)	951 (64)
Portulaca oleracea	2690 (100)	1025 (38)	1180 (43)
Trianthema portulacastrum	1586 (100)	543 (34)	1131 (71)
Amaranthus spinosus	1503 (100)	645 (42)	997 (66)
Amaranthus hypochondriacus	1749 (100)	566 (32)	1188 (67)
Average	1804 (100)	714 (40)	1089 (62)
C_3 species	30 °C	15 °C	50 °C
Lycopersicum esculentum	27 (100)	17 (62)	12 (44)
Arachis hypogaea	23 (100)	14 (61)	10 (43)
Euphorbia pulcherrima	31 (100)	23 (74)	17 (54)
Tridax procumbens	27 (100)	17 (62)	7 (26)
Pisum sativum	34 (100)	23 (67)	11 (32)
Average	29 (100)	19 (65)	11 (38)

Table 2. *Effect of varying temperature on the extent of inhibition by malate of PEPC in different species of C_4 and C_3 plants*

Type/species	Inhibition by malate (% of control)		
	Temperature		
	Suboptimal	Optimal	Supraoptimal
C_4 species	15 °C	45 °C	50 °C
Euphorbia hirta	63	24	21
Portulaca oleracea	69	28	17
Trianthema portulacastrum	74	40	35
Amaranthus spinosus	80	53	38
Amaranthus hypochondriacus	85	52	39
Average	74	39	30
C_3 species	15 °C	30 °C	50 °C
Lycopersicum esculentum	88	72	54
Arachis hypogaea	89	75	56
Euphorbia pulcherrima	75	68	45
Tridax procumbens	88	71	55
Pisum sativum	66	55	34
Average	81	68	49

to that at the optimal incubation temperature of 45 °C. The temperature-induced changes in malate sensitivity of PEPC were dramatically reduced with the addition of PEG in the assay medium.

Discussion

The results from this study demonstrate that a change in temperature can induce quite dramatic changes, not only in the activity but also the malate sensitivity of PEPC in both C_3 and C_4 plants. The changes were caused by a short duration of exposure to temperatures and were reversible to a large extent. The temperature-induced changes can therefore be physiologically relevant and important.

It may be argued that other factors could possibly affect the physiology of leaf discs at different temperatures, for example, concentration of dissolved CO_2, oxidative stress or induction of heat shock proteins. The marked reversibility of the temperature effects of PEPC support the concept that leaf discs are able to simulate the conditions *in vivo*. Experiments are in progress to examine the temperature responses of purified PEPC-protein *in vitro*. These results will be presented later and would naturally have limited implication.

That the optimal incubation temperature for PEPC in *A. hypochondriacus* (C_4) is 45 °C, compared to 30 °C in *P. sativum* (C_3 species), is not surprising. The data in Fig. 1 and Table 1 confirm that the C_4 PEPC is quite sensitive to

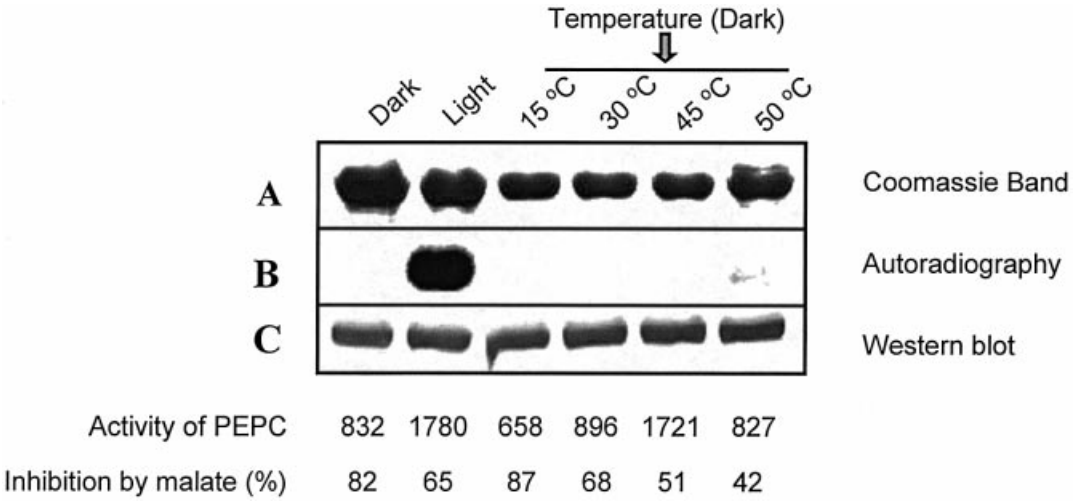

Fig. 4. The pattern of protein levels and the phosphorylation of PEPC in leaf discs of *Amaranthus hypochondriacus* exposed either to the dark–light treatment or to varying temperatures of 15 °C to 50 °C (only dark-incubation). The extracts from leaves of *A. hypochondriacus* labelled with ^{32}Pi, were prepared after the given treatments. The PEPC enzyme was immunoprecipitated from leaf discs, separated on SDS-PAGE and examined for protein levels as well as radioactivity. The figure includes gels (A) stained with Coomassie Blue; (B) exposed to autoradiography; (C) western blots. Extracts equal to 0.2 units of PEPC were used in each lane of rows (A) and (B). The amount of protein in each lane was 25 μg, in row (C).

Table 3. *The effect of including PEG-6000 in the assay medium on the response of PEPC from leaf discs of* Amaranthus hypochondriacus *to temperature*

The extracts were prepared from leaf discs exposed to varying temperature and the characteristics of PEPC were determined in the absence or presence of 1.25% (w/v) PEG-6000.

Temperature and parameter	–PEG	+PEG
PEPC activity (μmol mg^{-1} Chl h^{-1})		
Suboptimal 15 °C	585±45(40)[a]	668±57(39)
Optimal 45 °C	1441±112(100)	1732±139(100)
Supraoptimal 50 °C	734±53(51)	827±68(47)
Inhibition by malate (% of control)		
Suboptimal 15 °C	70±4	58±4
Optimal 45 °C	46±3	49±3
Supraoptimal 50 °C	40±3	56±3

[a] The figures in parentheses represent the activity as percentage of that at optimal incubation temperature.

suboptimal temperatures compared to the PEPC of C_3 species. Thus, these results endorse the suggestion that PEPC is one of the cold-sensitive enzymes in C_4 plants (Phillips and McWilliam, 1971; Selinioti *et al*., 1986).

The sharp increase in the activity of PEPC with temperature, particularly above 15 °C, could be physiologically significant, as the temperature is expected to rise from about 10–15 °C in the morning to 35–40 °C at midday, on a typically clear and sunny day. A combination of light and warm temperature could amplify the photoactivation of the PEPC, as observed in the case of *Egeria densa* (Casati *et al*., 2000) and *Amaranthus paniculatus* (Selinioti *et al*., 1986). The marked reversibility of the effect of temperature on PEPC in both C_4 and C_3 plants (Fig. 3) is an additional indication of the possible physiological relevance of temperature effects on PEPC.

These results demonstrate clearly the dramatic changes induced by temperature in the sensitivity of PEPC to malate. As the temperature was raised from 15 °C to 50 °C, there was a marked decrease in malate sensitivity of PEPC. The extent of such a decrease in C_4 plants (85% to 39%) was more than that in C_3 species (66% to 34%) (Fig. 2; Table 2). Thus, PEPC appeared to be highly sensitive to malate at cold temperatures, while becoming relatively insensitive to malate at warm temperatures. The extent of malate inhibition is quite high in *P. sativum* compared with *A. hypochondriacus*. Again the limited reports in the literature had conflicting observations. At low temperature, the sensitivity of PEPC to malate was very high in maize (Wu and Wedding, 1987), but was quite low in *Bryophyllum fedtschenkoi* (Carter *et al*., 1995). Lowering the temperature from 25 °C to 3 °C not only decreased the

catalytic capacity of PEPC, but also caused a considerable reduction (about 10-fold) in the sensitivity of PEPC to malate (Carter *et al*., 1995).

The decrease in malate sensitivity of PEPC can also occur due to the proteolysis of the enzyme. However, the authors are confident that this is not the reason during these observations. There was no detectable change in the protein levels as indicated by the western blots (Fig. 4C). The changes in activity of PEPC due to temperature were reversible to a marked extent (Fig. 3). The inclusion of chymostatin did not cause any change in the pattern of results.

The regulation of PEPC is achieved by post-translational 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). The phosphorylation of the enzyme leads to an increase in the activity of the enzyme and a decrease in malate sensitivity. The changes induced by a rise in temperature, namely the increase in the activity and the decrease in the extent of malate inhibition are quite similar to the changes effected during light activation of PEPC. It is therefore, quite possible that there is a change in phosphorylation status of PEPC on exposure to temperature. However, these experiments rule out the possible role of phosphorylation of the enzyme in the temperature effects of PEPC (Fig. 4). This is the first report that the temperature-induced changes in PEPC of C_4 species is independent of phosphorylation.

An attractive and alternative possibility is the change in the conformational status of the enzyme. PEPC is very active when it is in a tetrameric shape, while its activity and malate sensitivity decreases when the enzyme dissociates into a monomer or dimer (Shi *et al*., 1981; Walker *et al*., 1986; Willeford *et al*., 1990). Temperature may affect the oligomeric status of the enzyme PEPC. A rise in temperature causes the aggregation of PEPC in the case of C_4 and dissociation in case of CAM (Wu and Wedding, 1987). Cold/chilling may make the enzyme dissociate from the active tetrameric shape to less active dimers or monomers. However, this is not well corroborated in the case of C_4-PEPC (Walker *et al*., 1986). McNaughton *et al*. (1989) reported that changes in the oligomerization state of PEPC may not be related to malate sensitivity and light-induced changes. It is possible that other types of conformational changes would still occur, for example, in the hydrophobic microenvironment of the protein.

Compatible solutes like PEG-6000 protect the enzymes against a variety of adverse conditions *in vitro* by increasing the local concentration of protein (Timasheff, 1992). This study's experiments suggest that the temperature may modulate the conformation of PEPC, presumably by changing the local hydrophobic microenvironment of the enzyme. The marked damping of the temperature responses of PEPC when PEG-6000 was included during the assay (Table 3) is an indication that temperature may be causing reversible changes in the conformational status of PEPC. It would be of great interest to examine such changes further.

Another important observation from these results is that the high activity of PEPC is not always associated with a low level of malate sensitivity during the temperature responses (Figs 1, 2) and exposure to PEG-6000 (Table 3). Such an absence of a relationship between PEPC activity and malate sensitivity has already been noticed by Carter *et al*. (1995) in the case of PEPC from *Bryophyllum fedtschenkoi*. These observations suggest that another significant mode of regulation of PEPC that is different from phosphorylation, occurs with varying temperature. This could be largely due to a change in the conformational status of the enzyme involving either the oligomeric status and/or the microenvironment of protein, for example, folding hydrophobic regions.

Acknowledgements

This work is supported by a grant (to ASR) from the University Grants Commission (No. 3-174/2001/SR-II), New Delhi. JM is a recipient of a Junior Research Fellowship from the Council of Scientific and Industrial Research, New Delhi.

References

Andreo CS, González DH, Iglesias AA. 1987. Higher plant phospho*enol*pyruvate carboxylase. *FEBS Letters* **213,** 1–8.

Arnon DI. 1949. Copper enzymes in isolated chloroplasts. Polyphenol oxidase in *Beta vulgaris*. *Plant Physiology* **24,** 1–15.

Bakrim N, Echevarría C, Crétin C, Arrio-Dupont M, Pierre JN, Vidal J, Chollet R, Gadal P. 1992. Regulatory phosphorylation of *Sorghum* leaf phospho*enol*pyruvate carboxylase: identification of the protein-serine kinase and some elements of the signal-transduction cascade. *European Journal of Biochemistry* **204,** 821–830.

Berry JA, Björkman O. 1980. Photosynthetic responses and adaptation to temperature in higher plants. *Annual Review of Plant Physiology* **31,** 491–543.

Betz M, Dietz K-J. 1991. Immunological characterization of two dominant tonoplast polypeptides. *Plant Physiology* **97,** 1294–1301.

Burnell JN. 1990. A comparative study of the cold sensitivity of pyruvate, Pi dikinase in *Flaveria* species. *Plant and Cell Physiology* **31,** 295–297.

Carter PJ, Wilkins MB, Nimmo HG, Fewson CA. 1995. Effects of temperature on the activity of phospho*enol*pyruvate carboxylase and on the control of CO_2 fixation in *Bryophyllum fedtschenkoi*. *Planta* **196,** 375–380.

Casati P, Lara MV, Andreo CS. 2000. Induction of a C_4-like mechanism of CO_2 fixation in *Egeria densa*, a submerged aquatic species. *Plant Physiology* **123,** 1611–1621.

Chollet R, Vidal J, O'Leary MH. 1996. Phospho*enol*pyruvate carboxylase: a ubiquitous, highly regulated enzyme in plants. *Annual Review of Plant Physiology and Plant Molecular Biology* **47,** 273–298.

Du Y-C, Nose A, Wasano K. 1999*a*. Effects of chilling

temperature on photosynthetic rates, photosynthetic enzyme activities and metabolite levels in three sugarcane species. *Plant, Cell and Environment* **22,** 317–324.

Du Y-C, Nose A, Wasano K. 1999*b*. Thermal characteristics of C_4 photosynthetic enzymes from leaves of three sugarcane species differing in cold sensitivity. *Plant and Cell Physiology* **40,** 298–304.

Gayathri J, Parvathi K, Chinthapalli B, Westhoff P, Raghavendra AS. 2001. Immunological characteristics of PEP carboxylase from leaves of C_3-, C_4- and C_3–C_4 intermediate species of *Alternanthera*: comparison with selected C_3- and C_4-plants. *Indian Journal of Experimental Biology* **39,** 643–649.

Huber SC, Sugiyama T. 1986. Changes in sensitivity to effectors of maize leaf phospho*enol*pyruvate carboxylase during light/dark transitions. *Plant Physiology* **81,** 674–677.

Krall JP, Edwards GE. 1993. PEP carboxylase from two C_4 species of *Panicum* with markedly different susceptibilities to cold inactivation. *Plant and Cell Physiology* **34,** 1–11.

Krall JP, Edwards GE, Andreo CS. 1989. Protection of pyruvate, Pi dikinase from maize against cold lability by compatible solutes. *Plant Physiology* **89,** 280–285.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. 1951. Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* **193,** 265–275.

McNaughton GAL, Fewson CA, Wilkins MB, Nimmo HG. 1989. Purification, oligomerization state and malate sensitivity of maize leaf phospho*enol*pyruvate carboxylase. *Biochemical Journal* **261,** 349–355.

Nimmo GA, Nimmo HG, Hamliton ID, Fewson CA, Wilkins MB. 1986. Purification of the phosphorylated night form and dephosphorylated day form of phospho*enol*pyruvate carboxylase from *Bryophyllum fedtschenkoi*. *Biochemical Journal* **239,** 213–220.

Nimmo HG. 2000. The regulation of phospho*enol*pyruvate carboxylase in CAM plants. *Trends in Plant Science* **5,** 75–80.

Parvathi K, Gayathri J, Muralihalli GB, Bhagwat AS, Raghavendra AS. 2000*a*. Modulation of phospho*enol*pyruvate carboxylase phosphorylation in leaves of *Amaranthus hypochondriacus*, a NAD-ME type of C_4 plant. *Photosynthetica* **38,** 23–28.

Parvathi K, Bhagwat AS, Ueno Y, Izui K, Raghavendra AS. 2000*b*. Illumination increases the affinity of phospho-*enol*pyruvate carboxylase to bicarbonate in leaves of a C_4 plant, *Amaranthus hypochondriacus*. *Plant and Cell Physiology* **41,** 905–910.

Phillips PJ, McWilliam JR. 1971. Thermal responses of the primary carboxylating enzymes from C_3 and C_4 plants adapted to contrasting temperature environments. In: Hatch MD, Osmond CB, Slatyer RO, eds. *Photosynthesis and photorespiration*. New York: Wiley-Interscience, 97–104.

Potvin C, Simon JP. 1990. The evolution of cold temperature adaptation among a population of a widely distributed C_4 weed: barnyard grass. *Evolutionary Trends in Plants* **4,** 98–105.

Rajagopalan AV, Devi MT, Raghavendra AS. 1994. Molecular biology of C_4 phospho*enol*pyruvate carboxylase: structure, regulation and genetic engineering. *Photosynthesis Research* **39,** 115–135.

Selinioti E, Manetas Y, Gavalas NA. 1986. Co-operative effects of light and temperature on the activity of phospho*enol*pyruvate carboxylase from *Amaranthus paniculatus* L. *Plant Physiology* **82,** 518–522.

Shi J-N, Wu M-X, Zha J-J. 1981. Studies on plant phospho*enol*pyruvate carboxylase. V. A reversible cold-inactivation of sorghum leaf carboxylase. *Acta Phytophysiologica Sinica* **7,** 317–326.

Sugiyama T, Schmitt MR, Ku SB, Edwards GE. 1979. Differences in cold lability of pyruvate, Pi dikinase among C_4 species. *Plant and Cell Physiology* **20,** 965–971.

Timasheff SN. 1992. A physicochemical basis for the selection of osmolytes by nature. In: Somero GN, Osmond CB, Bolis CL, eds. *Water and life*. Berlin, Heidelberrg, New York: Springer-Verlag, 70–84.

Towbin H , Staehlin T, Gordon J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets. Procedure and some applications. *Proceedings of the National Academy of Sciences, USA* **76,** 4350–4354.

Vidal J, Chollet R. 1997. Regulatory phosphorylation of C_4 PEP carboxylase. *Trends in Plant Science* **2,** 230–237.

Walker GH, Ku MSB, Edwards GE. 1986. Catalytic activity of maize leaf phospho*enol*pyruvate carboxylase in relation to oligomerization. *Plant Physiology* **80,** 848–855.

Willeford KO, Wu M-X, Meyer CR, Wedding RT. 1990. The role of oligo-merization in the regulation of maize leaf phospho*enol*pyruvate carboxylase activity. Influence of Mg-PEP and malate on the oligomeric equilibrium of PEP carboxylase. *Biochemical and Biophysical Research Communications* **168,** 778–785.

Wu M-X, Wedding RT. 1987. Temperature effects on phospho*enol*pyruvate carboxylase from a CAM and C_4 plant. *Plant Physiology* **85,** 497–501.