Changes in properties of phosphoenolpyruvate carboxylase with induction of Crassulacean Acid Metabolism (CAM) in the C₄ plant *Portulaca oleracea*

A.M.A. MAZEN

*Department of Botany, Faculty of Science at Sohag, South Valley University, Sohag, Egypt*

**Abstract**

Aiming at understanding the odd case of CAM expression by a C₄ plant, some properties of phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31, orthophosphate:oxaloacetate carboxylase, phosphorlylating) were comparatively studied in leaves of CAM-expressing and non-expressing *Portulaca oleracea* L. plants. CAM expression was induced by growing plants under an 8-h photoperiod and under water-stress. CAM induction in leaves of these plants (designated as CAM) is indicated by the nocturnal acidification and by the clear diurnal oscillation pattern and amplitude of acidity, malic acid, and PEPC activity characteristic of CAM plants. Treatment of the other plant group (designated as C₄) by growth under a 16-h photoperiod and well-watered conditions did not induce expression of the tested criteria of CAM in plants. In these C₄ plants, the mentioned CAM criteria were undetectable. PEPC from CAM and C₄ *Portulaca* responded differently to any of the studied assay conditions or effectors. For example, extent and timing of sensitivity of PEPC to pH change, inhibition by maleate, activation by glucose-6-phosphate or inorganic phosphate, and the enzyme affinity to the substrate PEP were reversed with induction of CAM from the C₄-*P. oleracea*. These contrasting responses indicate distinct kinetic and regulatory properties of PEPC of the two modes. Thus by shifting to CAM in the C₄ *Portulaca* a new PEPC isoenzyme may be synthesized to meet CAM requirements. Simultaneous occurrence of both C₄ and CAM is suggested in *P. oleracea* when challenged with growth under stress.

**Additional key words**: malic acid.

**Introduction**


In contrast to the situation in C₃ plants, PEPC plays an essential role in photosynthetic C metabolism of both C₄ and CAM plants. PEPC (EC 4.1.1.31) catalyses the carboxylation of PEP using HCO₃⁻ as a substrate in a reaction that yields oxaloacetate and P₇ (for review see Chollet et al. 1996, Svensson et al. 1997, Vidal and Chollet 1997, Gonzalez et al. 1998, Cushman and Bohnert 1999, Cushman et al. 1999). Therefore, induction of CAM characteristics in a C₄ plant may be accompanied by special metabolic changes linked to PEPC.

During shift from C₃ to CAM, there is increase in PEP carboxylase extractable activity (Queiroz and Morel 1974, Greenway et al. 1978, Winter 1979, Holtum and Winter 1982, Winter et al. 1982) and in its protein quantity (Pierre and Queiroz 1978, Foster et al. 1982). CAM induction is also accompanied by changes in PEP carboxylase properties (Brulft et al. 1979, 1982, Brulft and Queiroz 1982, Winter 1982, Winter et al. 1982). Induction of CAM is probably linked to changes in the PEPC isoenzym pattern through the synthesis of a protein that is functionally responsible for CAM operation and shows kinetic and regulatory properties different from those of the enzyme existing under non CAM mode (Winter 1981, Brulft et al. 1982, Brulft-
and Queiroz 1982, Ting 1985, Chollet et al. 1996, Vidal and Chollet 1997). There is no information on changes of PEPC properties during shift from C₃ to CAM. In the first part of this series, PEP carboxylase was shown to increase in both activity and quantity during expression of CAM characteristics in the C₃ plant *P. oleracea* (Mazen 1996). Increase in PEPC activity may be due to an increase in enzyme protein quantity, modification of enzyme properties, or both. Therefore, to understand the mechanism of CAM induction from C₃ metabolism it is essential to have information on properties of PEPC in plants that operate in both modes of photosynthetic metabolism. This paper is an extension of the studies started on PEPC-related changes that arise during development of CAM characteristics in *P. oleracea*. It reports changes in some PEPC properties when *P. oleracea* was induced to express CAM characteristics.

**Materials and methods**

**Plants**: Conditions, similar to those previously employed (Mazen 1996), which led to minimum or maximum CAM characteristics, were followed. Seeds of *P. oleracea* L. were germinated and grown in plastic pots filled with a mixture of garden soil and sand in the greenhouse. Plants were left to grow until 2 weeks old, five uniform plants in each pot. Plants were then transferred to a growth chamber for 5 weeks before experimental use. Incandescent bulbs and fluorescent tubes (Osram, HWL [MBTF], Germany) provided an irradiance of 380 μmol(photon) m⁻² s⁻¹ at plant level. Day/night temperatures of 30/15 °C were maintained. Plants were then divided into two groups (designated as C₃ and CAM). Plants of the C₃ group were grown under long days with a 16-h photoperiod (06:00-22:00) and well-watered (watering every 2 d during light period). Plants of the CAM group were grown under short days with an 8-h photoperiod and water stress conditions (left without watering for 14 d before a final single watering). Irradiation was from 06:00 until 14:00 every day. Study of PEPC properties in plants of both groups started 10 d after watering the plants of the CAM group. For estimation of diurnal changes in titratable acidity, of malate content, or PEPC activity as affected by pH or malate, samples were taken every 6 h starting at 06:00. In experiments in which properties of PEP carboxylase were **compared in extracts obtained during the late light period** (tissue decarboxylated) or during the first half of the dark period (tissue acidifying), samples were taken at 13:00 and 23:00 for the CAM plants and at 19:00 and 23:00 for the C₃ plants, respectively. In experiments in which properties of PEP carboxylase under the effect of activators or inhibitors were studied, samples were taken at 23:00 for both groups.

**Enzyme extraction**: The extraction and assay procedures followed Winter (1982) with slight modification. Leaves were sliced with a razor blade after the midrib had been removed. Slices of 3 g fresh mass were ground for 20 s with mortar and pestle in the presence of 0.5 g washed sand, 10 mg Polyclar AT, and 10 cm³ extraction buffer. To compare properties of PEPC in extracts obtained during the late light period (tissue deacidified) or during the first half of the dark period (tissue acidifying), the extraction mixture consisted of 200 mM HEPES-KOH, 0.2 mM EDTA, 0.5 mM DTT, pH of either 7.00 for extractions in the light or 7.15 for extractions in the dark. The slightly increased pH of the extraction buffer for preparation of leaf extracts in the dark compensated for the increase in the malic acid content of the tissue. The homogenate was passed through two layers of Miracloth (Calbiochem, La Jolla, CA, USA). Two cm³ of crude extract were rapidly desalted under pressure using a Sephadex G-25 (medium) column (0.9-2.0 cm). This procedure required 30 s. The first 2 cm³ of the eluate were usually used for enzyme assays. Desalting buffers were at pH 7.0 and contained 50 mM HEPES-KOH, 0.2 mM EDTA, and 0.5 mM DTT. Aliquots of leaf extracts, after passage through Sephadex G-25, were used for determination of total soluble proteins. All materials were cooled at 4 °C before use.

**PEPC** was assayed at 25 °C. Activity of PEPC (50 mm⁻³ desalted extract per test) was measured spectrophotometrically 2 min after homogenisation of the leaves. The assay was based on the oxidation of NADH due to oxaloacetate reduction coupled to malate dehydrogenase. Reaction mixtures (total volume 3 cm³) contained 2 mM KHCO₃, 5 mM MgCl₂, 3 units of malate dehydrogenase (desalted), and varying amounts of PEPC; reaction mixtures were buffered with 25 mM HEPES-KOH, pH 8.0, 7.5, or 7.0. KHCO₃ was added immediately prior to enzyme assays that were started by addition of enzyme extracts.

**Titratable acidity**: Samples of known mass were extracted in 20% boiling ethanol for 15 min, and the extracts were titrated with 10 mM NaOH to pH 7.

**Leaf malate content** was determined according to the method of Kraybill and Martin (1996) in which leaf tissue was sliced upon thawing, and cell sap (up to 10% of total tissue liquid) was extracted by centrifugation. Malate concentrations of the sap were determined using the
enzymatic/spectrophotometric method of Gutmann and Wahlefeld (1974) and a standard curve based upon known malate concentrations.

Results

Induction of CAM: As predicted from earlier results (Koch and Kennedy 1980, 1982, Kraybill and Martin 1996, Mazen 1996), CAM was induced in *P. oleracea* treated by water stress and short day. CAM induction was indicated by the diurnal rhythms of acidity (solid line in Fig. 1A), malic acid content (solid line in Fig. 1B), and PEPC activity (solid line in Fig. 1C). The fluctuation amplitudes of acidity and malic acid were ca. 150 meq kg\(^{-1}\) (fresh mass) and 100 mmol kg\(^{-1}\) (dry mass), respectively. These high values are characteristic of CAM plants (Milburn et al. 1968). Fluctuation amplitude of PEPC activity was only ca. 25 mmol kg\(^{-1}\) (protein) s\(^{-1}\). These CAM criteria were practically lacking in *Portulaca* plants not treated by the mentioned conditions (labelled as C\(_4\)), and therefore considered as non-CAM performing. The difference of the two groups in CAM performance is important in the context of comparative enzymological studies.

![Fig. 1. Diurnal fluctuations of titratable acidity (A), malate content (B), and PEPC activity (C) in leaves from CAM (solid lines) and C\(_4\) (broken lines) plants of *Portulaca oleracea*, over 24-h cycle. Error bars represent standard deviations. The mean of each data point is significantly different (p<0.05) from the mean at 06:00 except otherwise marked by a little circle. Horizontal black bars designate dark period.](image)

![Fig. 2. PEPC activity in desalted leaf extracts from CAM (A) and C\(_4\) (B) *Portulaca* plants in the absence (solid lines) and presence (broken lines) of 2 mM malate in the assay medium at various time intervals over the 24-h cycle. Error bars represent standard deviations. The mean of each data point in case of malate absence is significantly different (p<0.05) from its counterpart one in case of malate presence except otherwise marked by a little circle. Horizontal black bars designate dark period.](image)

Sensitivity to inhibition by malate: The pattern of activity of PEPC, measured at pH 8.0, 2 mM PEP, and in the absence of malate, remained unchanged in CAM (Fig. 2A) and C\(_4\) (Fig. 2B) *Portulaca* plants during the light/dark cycle. Addition of 2 mM malate caused the PEPC activity of CAM-*Portulaca* to show a diurnal oscillatory behaviour that is characteristic of CAM plants. In these plants, the presence of malate reduced PEPC activity during the light period stronger than during the dark period. In C\(_4\) plants the effect of malate addition was opposite to that in CAM-*Portulaca*. In other words, in C\(_4\)
plants the malate-caused suppression of PEPC activity was stronger during the dark period than during the light period.

Fig. 3. PEPC activity in desalted leaf extracts from CAM (A) and C₄ (B) Portulaca plants at pH 8 (solid lines) and pH 7 (broken lines) at frequent intervals over a 24-h cycle. The extraction buffer was at pH 7.00 for extractions in the light and at 7.15 for extractions in the dark. Error bars represent standard deviations. The mean of each data point in case of pH 8 is significantly different (p<0.05) from its counterpart one in case of pH 7 except otherwise marked by a little circle. Horizontal black bars designate dark period.

pH response of PEPC during a diurnal cycle. PEPC activity was assayed in desalted leaf extracts from CAM- and C₄-Portulaca plants, which were obtained at frequent intervals over a 24-h cycle. The assay pH was either 8.0 or 7.0. Reduction of pH from 8 (Fig. 3A, solid line) to 7 (Fig. 3A, broken line) caused a significant reduction in PEPC activity of CAM-Portulaca at all times of the diurnal cycle with stronger reduction at light than at dark. Compared to that of CAM-plants, PEPC activity of C₄-plants (Fig. 3B) was only slightly reduced in response to reduction of the reaction pH from 8.0 (solid line) to 7.0 (broken line). This reduction was significant only during the dark period.

Effect of PEP concentration on PEPC activity: Increase in PEP content in the assay buffer caused a parallel increase in PEPC activity in day (deacidification time) and dark (maximum acidification) samples from leaves of both CAM- and C₄-Portulaca (Fig. 4A,B). The difference was only in magnitude of increase. In CAM-Portulaca plants (Fig. 4A) the PEPC activity increase was stronger in dark samples than in the light ones. This effect was reversed in C₄-Portulaca plants, although the magnitude was not as large as in CAM-Portulaca.

Fig. 4. Effect of different PEP concentration in the assay medium on PEPC activity in CAM (A) and C₄ (B) Portulaca leaf extracts sampled at the time of maximum rate of CAM acidification (solid lines) and at the time of deacidification (broken lines). Error bars represent standard deviations. The mean of each data point in case of acidification is significantly different (p<0.05) from its counterpart one in case of deacidification.

PEPC response to effectors (Fig. 5): Activation by glucose-6-phosphate and inorganic phosphate, and inhibition by malate were compared for PEPC in extracts of leaves sampled at the time of maximum rate of acidification (23:00) in Portulaca operating in either CAM or C₄. Effectors were applied at several concentrations in presence of 2 mM PEP at pH 7.0. In general, PEPC from CAM-Portulaca was much more sensitive to activation by G-6-P or P; than that from C₄-Portulaca. With regard to inhibition by malate, PEPC from C₄-plants was more sensitive than its counterpart one from CAM-plants.
Discussion

The present results establish the inducibility of CAM in the leaves of the C₄ species *P. oleracea*. The water stress-short day conditions induced CAM in this species which confirmed and extended my previous study (Mazen 1996). This also supports other studies on CAM inducibility in C₄ species (Koch and Kennedy 1980, 1982, Ku et al. 1981, Guralnick and Jackson 1993, Kraybill and Martin 1996). CAM was not detectable in plants of the C₄ control group that were not water-stressed. Criteria regarding PEPC from plants of this group were, therefore, used for comparison with those from plants of the CAM operating group.

![Graphs](image)

**Fig. 5.** Effect of the activators G-6-P (A) and P₁ (B) and the inhibitor malate (C) in the assay medium on PEPC activity in CAM (solid lines) and C₄ (broken lines) *Portulaca* leaf extracts sampled at the time of maximum rate of acidification in CAM (23:00). Effectors were applied in presence of 2 um PEP at pH 7.0. Error bars represent standard deviations. The mean of each data point in case of CAM is significantly different (p<0.05) from its counterpart one in case of C₄.

In the previous paper (Mazen 1996) I showed that during the CAM induction there was a parallel progressive increase in PEPC activity and protein quantity in this C₄ plant. Metabolic control of enzyme activity can occur as a result of other factors, for example, changes in the concentration of regulatory molecules or modification of the enzyme. Therefore the possibility of contribution of change in PEPC to the increase in activity was not ruled out, but it seemed essential to meet requirements of the new metabolism. Therefore I checked whether a co-ordinate change in PEPC properties also takes place as a consequence of CAM induction.

Changes in both enzyme amount and properties are documented in case of CAM induction from C₃ plants (Quercz and Morel 1974, Greenway et al. 1978, Pierre and Queiroz 1978, Bruflert et al. 1982, Bruflert and Queiroz 1982, Foster et al. 1982, Winter 1982, Winter et al. 1982). Similarly, this report shows that besides the increase in PEPC activity and amount (Mazen 1996), changes in PEPC properties also take place during CAM induction in C₄-*P. oleracea*. Examples are: (1) In contrast to PEPC from C₄-*Portulaca*, the malate-inhibited activity of PEPC from CAM-*Portulaca* was stronger during the day than during the night. (2) The reduction of pH in the assay buffer from 8 to 7 caused a pronounced reduction in CAM-plant PEPC activity at all times of the diurnal cycle, with the strongest effect during day. This effect was reversed in C₄-plant PEPC activity, which was slightly affected and the highest reduction in response to reduction of pH in the assay buffer took place during the dark period. (3) PEPC increase in the assay buffer caused a parallel increase in PEPC activity in day and dark samples from both CAM- and C₄-*Portulaca*. The difference was only in magnitude of increase. In CAM-*Portulaca*, for example, PEPC activity increase was stronger in dark samples than in the light period. This effect was reversed in C₄-*Portulaca*, although the magnitude was not as high as in CAM-*Portulaca*. (4) PEPC from CAM-*Portulaca* was much more sensitive to activation by G-6-P and P₁ than that from the C₄-operating plant.

Contrasting responses mentioned in the preceding paragraph indicate existence of PEPC in two distinct isozymes with distinct kinetic and regulatory properties of the two modes and different in their susceptibility to malate inhibition during a day/night cycle. These results are in line with current view of PEPC activation in C₄ and CAM plants, where events of metabolic regulation in C₄ are in opposite direction to those in CAM. In both modes, these events are achieved through reversible phosphorylation (Carter et al. 1991, McNaughton et al. 1991, Carter et al. 1995a,b, Chollet et al. 1996, Hartwell et al. 1996, Lillo et al. 1996, Smith et al. 1996, Vidal and Chollet 1997, Walker and Leeood 1996). In CAM plant, the enzyme is dephosphorylated, strongly inhibited by malate, becomes less sensitive to activation by G-6-P, and hence is deactivated during the day, while at night it is phosphorylated and becomes much less sensitive to malate and much more sensitive to activation by G-6-P.
and hence activated. Diurnal timing of phosphorylation state is reversed in C₄-plants, where phosphorylation and all mentioned consequences take place during the light and vice versa during the dark. Phosphorylation prevents inhibition of PEPC by the build-up of malate in the mesophyll that sustains operation of the C₄ pathway during the light period. The phosphorylation state of PEPC, and thereby its activity, is controlled by the activity of a novel protein kinase termed PEPC kinase, which is controlled directly by synthesis/degradation in response to a circadian rhythm in CAM, and to light/dark in C₄ (Chollet et al. 1996, Vidal and Chollet 1997).

Change of PEPC properties can be induced in two different ways. A new CAM-PEPC isoenzyme synthesised to replace the old C₄-PEPC is one possibility. The other possibility is that the new CAM isozyme is added to the old C₄ one. In other words, the two isoforms coexist under the new situation, and in this case both C₄ and CAM might be working together in the same plant. I support the second possibility for the following reasons. The magnitude of fluctuation amplitude of PEPC activity in case CAM induced from C₄-Portulaca (Fig. 1C) was not as high as it was in the case of shift from C₃ to CAM, for example, in Mesembyranthemum crystallinum (Foster et al. 1982, Holm and Winter 1982, Winter et al. 1982). This may be explained on the basis that an increase in PEPC activity starts from an already high level in the C₄ operating Portulaca plants. The values of criteria tested on PEPC are thus the nets of activity of the two isoforms.

By comparison, increase in activity from C₃ to CAM in M. crystallinum starts from a very low activity level. The high acidity observed in this report (Fig. 1A) in non-CAM plants (broken line) as well as results presented earlier (Mazen 1996) support this view.

Whether a new CAM-PEPC replaced the old C₄ one or the CAM and C₄ isoforms are coexisting during CAM is still to be studied. If both isoforms coexist during CAM, the two metabolisms may work simultaneously. In other words, during the night, malate accumulates from fixation of respiratory CO₂ catalysed by CAM-PEPC isoenzyme, while atmospheric CO₂ is fixed by C₄-PEPC isoenzyme during the daytime. Night-accumulated malate and night-fixed-malate are decarboxylated in the bundle sheath cells. Results of Kraybill and Martin (1996) on the pattern of diurnal CO₂ exchange in P. oleracea support this assumption. Net CO₂ uptake occurs during the day despite of the diurnal CAM acid fluctuations. More than one photosynthetic activity in the same leaf is not an unusual case. Differential photosynthetic activity in the same leaf was proven in Peperomia (Nishio and Ting 1987, Ting et al. 1994). In this plant, the leaf palisade has most of the C₄-photosynthetic activity, while the CAM activity is predominating in the spongy mesophyll and in the upper multiple epidermis of the same leaf.

Full understanding of the biochemical regulation is necessary to understand the ecological significance of CAM expression by C₄ plants. This paper represents the second contribution toward this goal.

References


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