Changes in properties of phospho*enol*pyruvate carboxylase with induction of Crassulacean Acid Metabolism (CAM) in the C_4 plant *Portulaca oleracea*

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Abstract

Aiming at understanding the odd case of CAM expression by a C_4 plant, some properties of phospho*enol*pyruvate carboxylase (PEPC, EC 4.1.1.31, orthophosphate: oxaloacetate carboxylyase, phosphorylating) 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 C_4) 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 C_4 plants, the mentioned CAM criteria were undetectable. PEPC from CAM and C_4 *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 malate, activation by glucose-6-phosphate or inorganic phosphate, and the enzyme affinity to the substrate PEP were reversed with induction of CAM from the C_4-*P. oleracea*. These contrasting responses indicate distinct kinetic and regulatory properties of PEPC of the two modes. Thus by shifting to CAM in the C_4 *Portulaca* a new PEPC isoform may be synthesised to meet CAM requirements. Simultaneous occurrence of both C_4 and CAM is suggested in *P. oleracea* when challenged with growth under stress.

Additional key words: malic acid.

Introduction

Although shifts from C_3 to CAM occur in a number of succulents (*e.g.*, Hanscom and Ting 1978, Winter and Troughton 1978, Schuber and Kluge 1981, Olivares *et al.* 1984, Chu *et al.* 1990, Herppich *et al.* 1992, Holthe *et al.* 1992, Fernández *et al.* 1999), only some species of C_4 plants, namely *Portulaca. oleracea*, *Portulaca grandiflora*, *Portulaca mundula*, *Orcuttia californica*, and *Orcuttia viscida* express CAM characteristics (Koch and Kennedy 1980, 1982, Ku *et al.* 1981, Guralnick and Jackson 1993, Kraybill and Martin 1996, Mazen 1996, Keeley 1998).

In contrast to the situation in C_3 plants, PEPC plays an essential role in photosynthetic C metabolism of both C_4 and CAM plants. PEPC (EC 4.1.1.31) catalyses the carboxylation of PEP using HCO_3 as a substrate in a reaction that yields oxaloacetate and P_i (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 C_4 plant may be accompanied by special metabolic changes linked to PEPC.

During shift from C_3 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 (Brulfert *et al.* 1979, 1982, Brulfert and Queiroz 1982, Winter 1982, Winter *et al.* 1982). Induction of CAM is probably linked to changes in the PEPC isoform 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, Brulfert *et al.* 1982, Brulfert-

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Abbreviations: CAM, Crassulacean acid metabolism; G-6-P, glucose-6-phosphate, LD-WW, long days and well-watered conditions; P_i, inorganic phosphate; PEP, phospho*enol*pyruvate; PEPC, phospho*enol*pyruvate carboxylase; SD-WS, short days and water stress conditions.

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 C_4 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 C_4 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 C_4 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 C_4 and CAM). Plants of the C_4 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 deacidified) 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 C_4 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 cm^3 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 cm^3 of crude extract were rapidly desalted under pressure using a *Sephadex G-25* (medium) column (0.9×20 cm). This procedure required 30 s. The first 2 cm^3 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 cm^3) contained 2 mM $KHCO_3$, 5 mM $MgCl_2$, 3 units of malate dehydrogenase (desalted), and varying amounts of PEP; reaction mixtures were buffered with 25 mM HEPES/KOH, pH 8.0, 7.5, or 7.0. $KHCO_3$ 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.

Total soluble proteins were determined according to Bradford (1976).

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. 1*A*), malic acid content (*solid line* in Fig. 1*B*), and PEPC activity (*solid line* in Fig. 1*C*). 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.

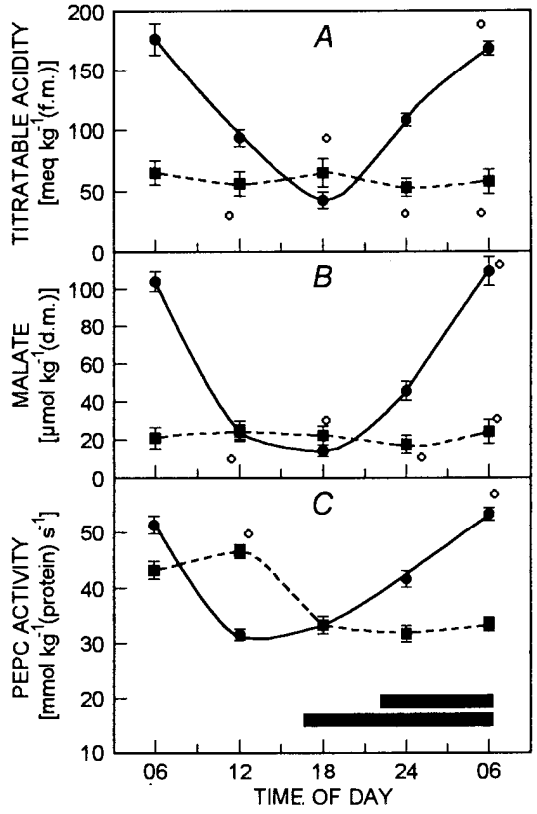

Fig. 1. Diurnal fluctuations of titratable acidity (*A*), malate content (*B*), and PEPC activity (*C*) in leaves from CAM (*solid lines*) and C_4 (*broken lines*) plants of *Portulaca oleracea*, over 24-h cycle. *Error bars* represent standard deviations. The mean of each data point is significantly different ($p<0.05$) from the mean at 06:00 except otherwise marked by a *little circle*. *Horizontal black bars* designate dark period.

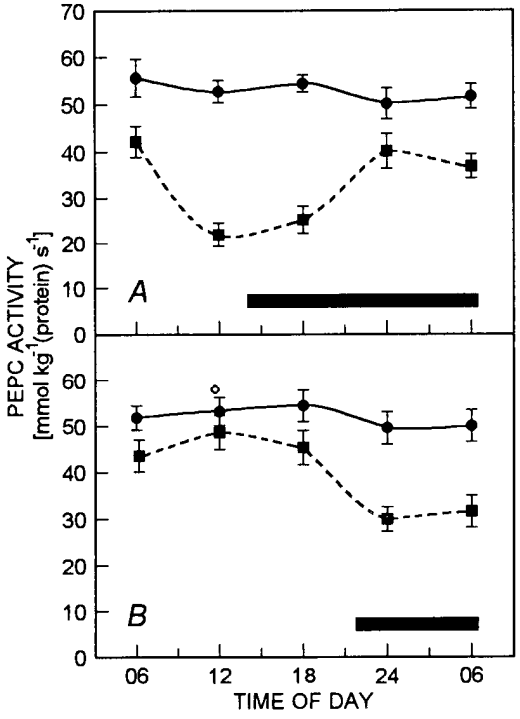

Fig. 2. PEPC activity in desalted leaf extracts from CAM (*A*) and C_4 (*B*) *Portulaca* plants in the absence (*solid lines*) and presence (*broken lines*) of 2 mM malate in the assay medium at various time intervals over the 24-h cycle. *Error bars* represent standard deviations. The mean of each data 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.

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. 2*A*) and C_4 (Fig. 2*B*) *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.

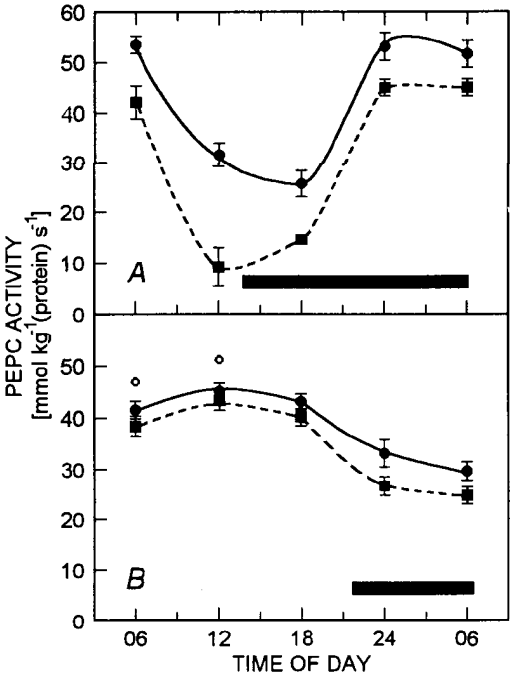

Fig. 3. PEPC activity in desalted leaf extracts from CAM (*A*) and C_4 (*B*) *Portulaca* plants at pH 8 (*solid lines*) and pH 7 (*broken lines*) at frequent intervals over a 24-h cycle. The extraction buffer was at pH 7.00 for extractions in the light and at 7.15 for extractions in the dark. *Error 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 C_4-*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. 3*A*, *solid line*) to 7 (Fig. 3*A*, *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 C_4-plants (Fig. 3*B*) 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 (de-acidification time) and dark (maximum acidification) samples from leaves of both CAM- and C_4-*Portulaca* (Fig. 4*A,B*). The difference was only in magnitude of increase. In CAM-*Portulaca* plants (Fig. 4*A*) the PEPC activity increase was stronger in dark samples than in the light ones. This effect was reversed in C_4-*Portulaca* plants, although the magnitude was not as large as in CAM-*Portulaca*.

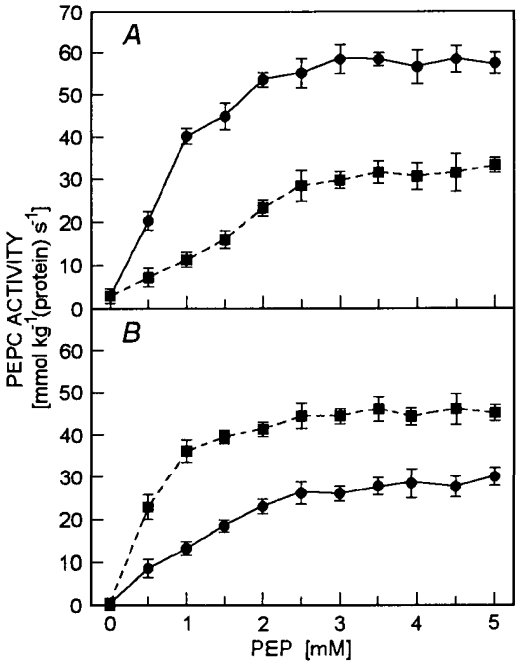

Fig. 4. Effect of different PEP concentration in the assay medium on PEPC activity in CAM (*A*) and C_4 (*B*) *Portulaca* leaf extracts sampled at the time of maximum rate of CAM acidification (*solid lines*) and at the time of deacidification (*broken lines*). *Error bars* represent standard deviations. 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 C_4. 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_i than that from C_4-*Portulaca*. With regard to inhibition by malate, PEPC from C_4-plants was more sensitive than its counterpart one from CAM-plants.

Discussion

The present results establish the induciblity of CAM in the leaves of the C_4 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 C_4 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 C_4 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.

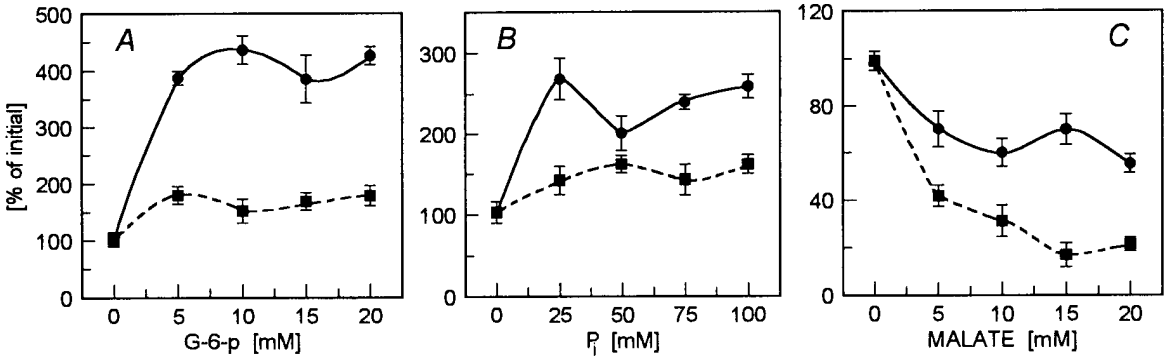

Fig. 5. Effect of the activators G-6-P (*A*) and P_i (*B*) and the inhibitor malate (*C*) in the assay medium on PEPC activity in CAM (*solid lines*) and C_4 (*broken lines*) *Portulaca* leaf extracts sampled at the time of maximum rate of acidification in CAM (23:00). Effectors were applied in presence of 2 mM 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 C_4.

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 C_4 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 C_3 plants (Queiroz and Morel 1974, Greenway *et al.* 1978, Pierre and Queiroz 1978, Brulfert *et al.* 1982, Brulfert 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 C_4-*P. oleracea*. Examples are: (*1*) In contrast to PEPC from C_4-*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 C_4-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*) PEP increase in the assay buffer caused a parallel increase in PEPC activity in day and dark samples from both CAM- and C_4-*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 C_4-*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 P_i than that from the C_4-operating plant.

Contrasting responses mentioned in the preceding paragraph indicate existence of PEPC in two distinct isoforms 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 C_4 and CAM plants, where events of metabolic regulation in C_4 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 Leegood 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 C_4-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 C_4 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 C_4 (Chollet *et al.* 1996, Vidal and Chollet 1997).

Change of PEPC properties can be induced in two different ways. A new CAM-PEPC isoform synthesised to replace the old C_4-PEPC isoform is one possibility. The other possibility is that the new CAM isoform is added to the old C_4 one. In other words, the two isoforms coexist under the new situation, and in this case both C_4 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 C_4-*Portulaca* (Fig. 1*C*) was not as high as it was in the case of shift from C_3 to CAM, for example, in *Mesembryanthemum crystallinum* (Foster *et al.* 1982, Holtum 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 C_4 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 C_3 to CAM in *M. crystallinum* starts from a very low activity level. The high acidity observed in this report (Fig. 1*A*) 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 C_4 one or the CAM and C_4 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 CO_2 catalysed by CAM-PEPC isoform, while atmospheric CO_2 is fixed by C_4-PEPC isoform 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 CO_2 exchange in *P. oleracea* support this assumption. Net CO_2 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 C_3-photosynthetic activity, while the CAM activity is predominatingly 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 C_4 plants. This paper represents the second contribution toward this goal.

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