In Vivo Properties of Phosphoenolpyruvate Carboxylase in Crassulacean Acid Metabolism Plants —Is Pineapple CAM Not Regulated by PEPC Phosphorylation?—

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The in vivo sensitivity to inhibition by L-malate of phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) in rapidly prepared crude extracts (within 2 min) was investigated for two CAM plants *Kalanchoë pinnata* and pineapple (*Ananas comosus* (L.) Merr. cv. Smooth-cayenne N67-10). The phosphorylation process may not occur in pineapple PEPC. Variations were also observed in apparent kinetic properties and molecular mass as well as subunit composition of PEPC from these plants. Two different subunits (major polypeptide 112 kDa and minor polypeptide 119 kDa) were separated from day and night forms of PEPC from *K. pinnata* but only one identical subunit (107 kDa) from pineapple. The integrity of both forms of in vivo PEPC from these plants was demonstrated by Western blot analyses using anti-N-terminus antibody. *K. pinnata* lost malate sensitivity about 87% whereas pineapple PEPC lost only 21% sensitivity at night in the presence of 2 mM L-malate. The apparent K_i (L-malate) was 6.4-fold higher in phase I (5.8 mM) than that in phase III (0.9 mM) from *K. pinnata* but it was only 1.0 and 0.5 mM for day and night PEPC respectively from pineapple. These findings suggest phosphorylation process may not be the major regulatory mechanism in pineapple PEPC.

Keywords: CAM, malate inhibition, phosphoenolpyruvate carboxylase, phosphorylation/dephosphorylation

INTRODUCTION

Crassulacean acid metabolism plants show diurnal rhythms of CO₂ fixation. In these plants phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) catalyzes the key step of CO₂ fixation into malate at night. During the day CO₂ is released from malate and refixed photosynthetically. Regulation of PEPC plays a central role in the control of CAM (Nimmo et al., 1990) and also PEPC is highly regulated in vivo. PEPC properties also regulated by photoperiod, day-night transition, temperature fluctuations, salt and water stress, which are paralleled by the classical physiological changes in the activity of CAM e.g. CO₂ fixation and titratable acidity (Osmond and Holtum, 1981; Jiao and Chollet, 1991; Winter and Smith, 1996). PEPC is modulated by L-malate and glucose-6-phosphate, which act as negative and positive allosteric effectors, respectively. Moreover, the enzyme is regulated by phosphorylation of a single serine residue, which is located in its N-terminus, results in an increase in catalytic activity and more notably, by feedback inhibition by malate (Nimmo et al., 1987;

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Chollet et al., 1996 ; Vidal and Chollet, 1997).

It is now amply documented that native leaf and recombinant forms of PEPC are highly susceptible to limited proteolysis near the N-terminus during extraction and subsequent purification (Nimmo et al., 1986 ; McNaughton et al., 1989 ; Ausenhus and O'Leary 1992 ; Baur et al., 1992 ; Wang et al., 1992 ; Duff et al., 1995). Removal of this plant-invariant N-terminal domain markedly decreases the in vitro phosphorylatability and sensitivity of PEPC to its negative allosteric effector L-malate (Chollet et al., 1996). The simultaneous loss of both N-terminus and malate sensitivity of PEPC due to proteolytic degradation challenges the significance of changes in oligomeric status of the enzyme during the diurnal regulation in vivo (McNaughton et al., 1989 ; Weigerid and Hinch, 1992). It is therefore, necessary to protect the enzyme from detrimental effects of proteolysis during extraction and purification (McNaughton et al., 1989 ; Arrio-Dupont et al., 1992 ; Baur et al., 1992 ; Weigerid and Hinch, 1992). Thus, many earlier kinetic analyses of purified or commercial plant PEPC have probably been compromised by this N-terminal truncation (Jiao et al., 1991 ; Lepiniec et al., 1994 ; Duff et al., 1995).

Despite the interest of its role in temporal regulation of the pathway in CAM plants, the information on the in vivo regulation of PEPC from plants having crassulacean acid metabolism is scant. Also there have been no reports about the in vivo PEPC properties of *Kalanchoë* species specially *K. pinnata* and one of the major CAM fruit plant, the pineapple. We have therefore, investigated the possibility of modification of the kinetic properties of the enzyme during extraction with particular reference to inhibition by L-malate and also the apparent phosphorylation status of *K. pinnata* and pineapple, since our knowledge of this phenomenon is still rather fragmentary in relation to its assumed regulatory importance in CAM plants. The present work reveals that (1) the structural and kinetic properties immediately after extraction is strikingly different in vivo day and night form of PEPC from these two CAM plants and (2) apparently the phosphorylation process may not play an important role in pineapple CAM.

MATERIALS AND METHODS

Plant materials. Pineapple (*Ananas comosus* (L.) Merr. cv. Smooth-cayenne N67-10) and *Kalanchoë pinnata* were vegetatively propagated and grown in pots in a green house with heating under natural photoperiod. Leaf materials were collected after being kept for 2 weeks in a growth chamber (KG-50 HLA, Koito Industrial Co., Ltd., Japan) with a photoperiod of 11 h (6:00 to 17:00) light and 13 h (17:00 to 6:00) dark. Conditions in the growth chamber were 30°C during the light period at a light intensity of 420–450 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD with fluorescent lamps, and 25°C during the dark period, and a relative humidity of 70% during both periods. In the case of pineapple, fully expanded, mature leaves and for *K. pinnata*, 4th to 6th leaf pair from the apex of the shoot were collected. Leaf samples were collected at 11:00 (phase III) and 23:00 (phase I), fixed in liquid nitrogen, and kept at -80°C before use.

Reagents. L-Malate dehydrogenase (L-MDH, EC 1.1.1.37), PEP and NADH were from Roche Diagnostics GmbH Mannheim, Germany ; Chymostatin, Pepstatin A, *trans*-Epoxysuccinyl-L-Leucylamido (4-Guanidino)-Butane (E-64) and DTT from SIGMA-ALDRICH CHEMIE, GmbH Germany ; IgG fraction of anti-phosphoenolpyruvate carboxylase (maize) (rabbit) from Rockland, USA ; anti-phosphorylation site antibody (APS-IgG, raised against a synthetic peptide encompassing the phosphorylation domain of the C₄ PEPC from sorghum) was the generous gift of Dr. Jean Vidal, Université de Paris-Sud, ORSAY Cedex, France ; Bio-Rad Protein Assay Dye Reagent Concentrate, SDS-PAGE Standards and

Immun-Blot Assay Kit from Bio-Rad Laboratories, USA ; Coomassie blue R-250 from Nacalai Tesque Inc., Japan ; whereas PMSF and Triton X-100 were from Wako Pure Chemical Industries, Ltd., Japan. All other reagents were of analytical grade and from Katayama Chemicals, Japan.

Enzyme extraction. Leaf samples (4 cm²) were ground using a chilled mortar and pestle in the presence of 5 mL of ice-cold extraction medium containing 50 mM Bicine-KOH (pH 8.2), 0.2 mM EDTA-NaOH (pH 7.0), 5.0 mM MgCl₂, 5 mM DTT, 1% (w/v) Triton X-100, 20% (v/v) glycerol, 2 mM MIA, and 200 μ M PMSF. The brei was filtered through one layer of Miracloth (Calbiochem-Novabiochem, La Jolla, CA, USA) and centrifuged for 20 s in a microcentrifuge (MICRO 6 CFM-100, IWAKI Glass Co., Ltd., Japan) at full speed to remove the particulate material. The supernatant was used immediately for enzyme assay. Care was taken to handle all steps quickly and preparation of extracts took no more than 2 min.

PEPC activity assays. PEPC was assayed at 30°C in a 3.0 mL final volume by following the oxidation of NADH at 340 nm using a UV-visible recording spectrophotometer (UV-160A, Shimadzu, Japan). The standard assay conditions were 50 mM Tris-HCl (pH 8.2 for pineapple and 8.6 for *K. pinnata*) containing 20% (v/v) glycerol, 5 mM MgCl₂, 2 mM NaHCO₃, 0.2 mM NADH, 2.0 mM PEP, 0.1 mg mL⁻¹ BSA, 10.0 IU L-MDH. Assays were initiated by the addition of an aliquot of rapidly prepared and centrifuged crude enzyme extract. All reported rates are from linear portions of absorbance vs. time curves (usually between 0 and 2 min). The range of L-malate concentrations used in this study was chosen to encompass the inhibitor concentration which causes 50% inhibition of initial PEPC activity (K_i). The corresponding K_i values were estimated from a plot of PEPC activity vs. L-malate concentration. PEPC assays for K_i (L-malate), K_m (PEP) and K_a (Glc-6-P) were performed immediately after extraction in case of day PEPC from *K. pinnata* but rest of the forms of PEPC from pineapple and *K. pinnata* were performed within 45 min after extraction. Chlorophyll content was determined according to Arnon (1949).

SDS-PAGE and Western blotting. For SDS-PAGE, 10% (w/v) gels were run according to Laemmli (1970) in an Atto electrophoresis cell (AE-6400, Atto Corporation, Japan). After electrophoretic separation, proteins on the gels were stained with Coomassie blue R-250 and electroblotted onto a nitrocellulose membrane for immunoblotting using the Trans-Blot Semi-Dry Electrophoretic Transfer Cell (Bio-RAD, 170-3940). The membrane was blocked at room temperature by a 30 min incubation in TBS (20 mM Tris, 500 mM NaCl, pH 7.5) containing 3% gelatine. PEPC was immunochemically labeled for 105 min incubation of the membrane at room temperature in 10 mL TTBS (20 mM Tris, 500 mM NaCl, 0.05% Tween-20 pH 7.5) containing 1/20 000 fold diluted rabbit anti-(maize leaf PEPC) immune serum. Also, another electroblotted nitrocellulose membrane was blocked and incubated with anti-phosphorylation site antibody (APS-IgG) raised against a synthetic peptide encompassing the phosphorylation domain of the C₄ PEPC from sorghum according to Bakrim et al. (1998). Immunoreactive proteins were visualized using an alkaline phosphatase-linked secondary antibody (goat anti-rabbit IgG from Bio-RAD) according to the manufacturer's instructions. The molecular masses of the proteins were estimated by comparison with molecular mass standard. Protein concentration was determined according to Bradford (1976).

Reproducibility of data. All experiments have been performed at least in triplicate ; in each case, similar trends were observed.

RESULTS

The in vivo sensitivity to inhibition by L-malate of PEPC

The in vivo sensitivity to inhibition by L-malate of day (phase III) and night form (phase

I) of PEPC in rapidly prepared crude extracts (within 2 min) of two CAM plants, *K. pinnata* and pineapple was observed. When we used the extraction medium without glycerol, PMSF and BSA in assay medium, the rapidly prepared crude extract of both day and night form of PEPC from those plants showed rapid loss of its L-malate sensitivity within 10 min (Fig. 1). However, the addition of 20% (v/v) glycerol, PMSF and MIA in extraction medium, along with standard assay medium could maintain the loss of sensitivity to inhibition by L-malate at nighttime in both plants and in daytime in pineapple (Fig. 2). But rapid loss of malate sensitivity was observed in the extracts of leaves prepared during the day in *K. pinnata*, 83% loss of malate sensitivity was observed within 45 min after extraction though initial loss of sensitivity was 32% (Fig. 2A).

K. pinnata showed highly apparent phosphorylated state of PEPC, lost its sensitivity to inhibition by L-malate about 87% at nighttime (Fig. 2B). But pineapple PEPC was highly sensitive to inhibition by L-malate and lost its sensitivity only about 21% at night in the

Fig. 1 In vivo sensitivity to inhibition by L-malate of day (A) and night form (B) of PEPC from *K. pinnata* and pineapple in the absence of glycerol, PMSF and BSA.

PEPC activity was measured in the absence of 2 mM L-malate (○) and in the presence of 2 mM L-malate (●), and sensitivity (△) based on control activity as 100%.

Fig. 2 In vivo sensitivity to inhibition by 2 mM L-malate of day (A) and night form (B) of PEPC from *K. pinnata* and pineapple in the presence of 20% (v/v) glycerol, 200 μ M PMSF and 0.1 mg mL⁻¹ BSA.

PEPC activity was measured in the absence (○) and in the presence (●) of 2 mM L-malate and sensitivity (△) based on control activity as 100%.

presence of 2 mM L-malate (Fig. 2B), also its PEPC activity was lower than that of *K. pinnata*. Moreover, no marked difference was observed between the day and night form of PEPC sensitivity to inhibition by L-malate from pineapple (Figs. 1, 2).

*Apparent K_i (L-malate) of day and night form of PEPC from pineapple and *K. pinnata**

The in vivo sensitivity of day and night form of PEPC to inhibition by L-malate from pineapple and *K. pinnata* was examined. A striking difference in the sensitivity of the enzyme in these two forms of PEPC was observed. The concentration of malate required for

Fig. 3 Inhibition by L-malate of day (○) and night form (●) of in vivo PEPC from *K. pinnata* and pineapple.

Enzyme activity is expressed as a percentage of the activity in the absence of L-malate.

Fig. 4 SDS polyacrylamide (10% w/v) mini-gel electrophoresis of rapidly prepared (within 2 min) crude extracts (100 µg protein) of day and night form of PEPC from *K. pinnata* and pineapple.

Lane 1, molecular mass standards; lanes 2 and 3, day and night form of PEPC from pineapple respectively; lanes 4 and 5, day and night form of PEPC from *K. pinnata* respectively. Proteins were stained with Coomassie blue R-250.

50% inhibition of day and night PEPC from pineapple was only 0.5 and 1.0 mM respectively (Fig. 3), where as apparent K_i (L-malate) of night PEPC from *K. pinnata* was very high, 5.8 mM and day PEPC was only about 0.9 mM (Fig. 3).

SDS-PAGE and Western blotting

When examined by polyacrylamide-gel electrophoresis in the presence of SDS, rapidly prepared (within 2 min) crude extracts of day and night *K. pinnata* and pineapple leaves gave four major protein staining bands (Fig. 4). Western blots of the same preparations were probed with anti-phosphoenolpyruvate carboxylase (maize)(rabbit) immune serum and revealed two bands, one is minor and another is major band for both day and night form of PEPC from *K. pinnata* and only one identical band for both forms of pineapple PEPC (Fig. 5). Similar results were also obtained when (1) crude extracts were incubated for 45 min, (2) 200 μ M PMSF, 1 μ g mL⁻¹ chymostatin, 2 μ g mL⁻¹ pepstatin A and 10 μ g mL⁻¹ E-64 in rapidly prepared (within 2 min) crude extracts, (3) 1 μ g mL⁻¹ chymostatin both in rapidly prepared (within 2 min) crude extracts and rapid extraction of leaves frozen in liquid N₂ which accomplished in a few seconds (data not shown). The apparent subunit molecular mass of minor and major band of *K. pinnata* was identified as 119 and 112 kDa respectively and 107 kDa of pineapple PEPC (Fig. 4). No difference between the day and night form of PEPCs apparent subunit molecular mass was observed in *K. pinnata* and pineapple. Western blot with anti-phosphorylation site antibody (APS-IgG, raised against a synthetic peptide encompassing the phosphorylation domain of the C₄ PEPC from sorghum) also revealed the integrated form of the same subunit molecular masses from these plants (Fig. 6).

Substrate affinity and activation by allosteric effector

The affinity towards the substrate PEP of day and night form of PEPC indicating a modest difference exists in both forms of enzyme from *K. pinnata* and pineapple (Table 1). However, about 3-fold higher affinity toward this substrate of day and night form of PEPC from *K. pinnata* than pineapple was observed. Table 1 also shows that V_{max} for this substrate did not change markedly in these forms of PEPC in both of the plants though about 2-fold

Fig. 5 Western blot of rapidly prepared (within 2 min) crude extracts (100 μ g protein) of day and night form of PEPC from *K. pinnata* and pineapple.

Proteins were resolved by SDS-PAGE and blot-transferred onto a nitrocellulose membrane. Western analysis was performed using 1 : 20 000 dilution of rabbit anti-(maize leaf PEPC) immune serum. Immunoreactive proteins were visualized using an alkaline phosphatase-linked secondary antibody (goat anti-rabbit IgG). Lanes 1 and 2, day and night form of PEPC from pineapple respectively; lanes 3 and 4, day and night form of PEPC from *K. pinnata* respectively.

Fig. 6 Western blot using anti N-terminus antibody (APS-IgG) of rapidly prepared (within 2 min) crude extracts (100 μ g protein) of day and night form of PEPC from *K. pinnata* and pineapple.

Proteins were resolved by SDS-PAGE and blot-transferred onto a nitrocellulose membrane. Western analysis was performed using 20 μ L of anti N-terminus antibody (APS-IgG) immune serum. Immunoreactive proteins were visualized using an alkaline phosphatase-linked secondary antibody (goat anti-rabbit IgG). Lanes 1 and 2, day and night form of PEPC from *K. pinnata* respectively; lanes 3 and 4, day and night form of PEPC from pineapple respectively.

Table 1 Apparent kinetic parameters of PEPC from rapidly prepared crude extracts of *K. pinnata* and pineapple.

	PEP		Glc-6-P	
	K_m (mM)	V_{max} ($\mu\text{mol mg chl}^{-1} \text{min}^{-1}$)	K_a (mM)	V_{max} ($\mu\text{mol mg chl}^{-1} \text{min}^{-1}$)
<i>K. pinnata</i>				
Day form	0.69 ± 0.06	8.45 ± 0.45	0.51 ± 0.10	5.02 ± 0.88
Night form	0.48 ± 0.17	7.58 ± 1.44	0.12 ± 0.03	6.88 ± 1.06
Pineapple				
Day form	1.95 ± 0.30	4.22 ± 1.44	0.69 ± 0.07	4.29 ± 0.34
Night form	1.40 ± 0.36	4.91 ± 0.24	0.68 ± 0.06	3.57 ± 0.50

Values are expressed as the means of three separate determinations $\pm$ SD.

higher maximum velocity was observed in day and night forms of *K. pinnata* than pineapple. K_a (Glc-6-P) of night PEPC from *K. pinnata* was low but 4.3-fold higher K_a (Glc-6-P) of day PEPC was observed, however, maximum velocity for this effector did not change markedly in this plant. Also no marked difference was observed for K_a (Glc-6-P) and V_{max} for this allosteric effector of both forms of PEPC in pineapple, despite 5.7-fold higher K_a (Glc-6-P) was found at night than that of *K. pinnata*. Also, 1.2- and about 2-fold lower maximum velocity for Glc-6-P of day and night forms respectively was observed in pineapple than that from *K. pinnata*.

DISCUSSION

In vivo sensitivity to inhibition by L-malate of day and night form of PEPC in rapidly prepared crude extracts from two CAM plants, *K. pinnata* and pineapple was observed, considering that the changes in the sensitivity are an indirect indication of the phosphorylation state of the enzyme (Jiao and Chollet, 1991; Duff et al., 1995). The apparent K_i of PEPC for L-malate was high in *K. pinnata* (5.8 mM) at night. But in daytime it was about 6.4-fold lower (0.9 mM) in this plant (Fig. 3). Similarly, in *K. daigremontiana*, the apparent K_i increased from 0.5 to 5.0 mM during night (Borland et al., 1999), compared with the range of 0.3–3.0 mM for *Bryophyllum fedtschenkoi* in earlier work (Nimmo et al., 1984). However, the apparent K_i (L-malate) of day and night form of PEPC from pineapple was low, 1.0 and 0.5 mM (Fig. 3) respectively compared to *Kalanchoë* species. Similar results were also obtained with a cocktail of protease inhibitors used in extraction medium (data not shown). Moreover, night value of pineapple K_i (L-malate) is similar to that of day form of *Kalanchoë* species. As the apparent K_i of PEPC for L-malate reflects the phosphorylation state of PEPC (Carter et al., 1991), our result suggests that at night pineapple PEPC may not be phosphorylated.

K. pinnata showed highly apparent phosphorylated state (as judged by its malate sensitivity) of PEPC, lost its sensitivity to inhibition by 2 mM L-malate about 87% at nighttime. But pineapple PEPC was highly sensitive to inhibition by L-malate, and it only lost its sensitivity about 20% during day and night (Fig. 2), also its PEPC activity was lower than that of *K. pinnata*. Moreover, no marked difference was observed between the day and night form of PEPC sensitivity to inhibition by L-malate from pineapple (Figs. 1, 2). Furthermore, according to Jiao and Chollet (1991), the phosphorylated PEPC is highly active and less sensitive to inhibition by L-malate like night form of *K. pinnata* but dephosphorylated PEPC is less active and more malate sensitive which is observed in both forms of pineapple PEPC and day form of *K. pinnata* (Figs. 1, 2). PEPC-Kinase phosphorylates PEPC near its N-terminus and

thereby decreases the malate sensitivity of this allosteric target enzyme (Jiao and Chollet, 1991 ; Li and Chollet, 1993 ; Wang and Chollet, 1993). Since decreases in the malate sensitivity of plant PEPC can be due not only to regulatory phosphorylation but also to N-terminal proteolysis of the protein (Wang et al., 1992 ; Lepiniec et al., 1994 ; Duff et al., 1995). In this context, our results again suggest that, apparently PEPC from pineapple might not phosphorylate and also, that the high L-malate sensitivity of pineapple PEPC showed conserving the N-terminal. Perhaps, PEPC-Kinase might be inhibited by high concentration of malate in this plant. However, the day form from *Crassula argentea* was about 10 times less sensitive to malate (Wu and Wedding, 1985a, b) than the day form from *B. fedtschenkoi*. The night form from *C. argentea* was only 25% inhibited by saturating malate concentrations (Wu and Wedding, 1985a, b), whereas the night form from *B. fedtschenkoi* was 100% inhibited. May be the properties of phosphoenolpyruvate carboxylase differ greatly from one crassulacean plant to another and also phosphorylation and proteolysis of the enzyme may not occur in all CAM plants.

When we used the extraction medium without glycerol, PMSF and BSA in assay medium, the rapidly prepared crude extract of both day and night form of PEPC from those plants showed loss of L-malate sensitivity within 10 min (Fig. 1). However, the addition of 20% (v/v) glycerol, PMSF and MIA in extraction medium and using standard assay medium could prevent the change of sensitivity to inhibition by L-malate at night time in both plants and in day time in pineapple (Fig. 2). The inclusion of other protease inhibitor like lupeptine in extraction buffer was not effective to prevent the change of L-malate sensitivity (data not shown), perhaps hydrophobic nature of PMSF associated with hydrophobic regions of PEPC, which could stabilize it. Several authors have also been reported the marked instability of PEPC activity in aqueous extraction or assay media could be overcome by the addition of glycerol (Uedan and Sugiyama 1976 ; Manetas et al., 1986 ; Selinioti et al., 1987 ; Podestá and Andreo 1989 ; Wedding et al., 1990).

Changes in the sensitivity of PEPC to inhibition by L-malate is affected by the seryl phosphorylation and several other processes, including proteolysis of N-terminus and possible changes in the aggregation state of the holoenzyme (Jiao and Chollet, 1991 ; Duff et al., 1995). However, the integrity of N-terminus of in vivo PEPC of both day and night forms from *K. pinnata* and pineapple also demonstrated by Western blot analyses using anti-N-terminus antibody (Fig. 6). PEPC extracted from *Mesembryanthemum crystallinum* during the light period rapidly lost its high sensitivity after isolation unless the enzyme extracts were kept at acid pH and/or in the presence of malate (Winter, 1981). Nimmo et al. (1986) also observed in *Bryophyllum (Kalanchoë) fedtschenkoi* that day form was more susceptible to proteolysis of PEPC. They also observed that the malate sensitivity of PEPC in crude day extracts declined more rapidly than in night extracts, but this was slowed by the inclusion of 10 mM malate in the buffers and by chromatography on hydroxyapatite. At this point the malate sensitivity of the night form of the *B. fedtschenkoi* PEPC was fairly stable but that of the day form was not. After chromatography on DEAE-cellulose, both forms were fairly stable. In contrast to these results, the enzyme extracted from *K. pinnata* during the day, 6 mM L-malate could not prevent the loss of malate sensitivity (data not shown). However, inclusion of 20% (v/v) glycerol, PMSF and MIA in extraction medium along with standard assay medium could prevent the loss of sensitivity to inhibition by L-malate at nighttime in both plants though day form of PEPC from *K. pinnata* could not (Fig. 2). The loss of malate sensitivity by 83% was observed during 45 min after extraction in *K. pinnata* though, initial loss was 32% in daytime. The inclusion of PMSF and MIA in the extraction buffer as well as glycerol and BSA might prevent the N-truncation (Fig. 6). However, Nimmo et al. (1986) postulated that, the main factor in this irreversible decrease in malate sensitivity appeared to be the proteolysis

of the enzyme, not related to the phosphorylation process in *B. fedtschenkoi*. But our results of Western blot analyses with anti-N-terminal antibody showed the integrity of N-terminal end of PEPC from day form of *K. pinnata* (N-terminal proteolysis was not occurred in day form of *K. pinnata*) (Fig. 6), lead us to speculate that, perhaps some other reasons are responsible for this phenomena and/or may be PEPC-Kinase and N-terminus of PEPC in CAM plants are functioning differently than other plants.

PEPC assayed in rapidly prepared leaf extracts of *K. pinnata* showed marked changes in K_a (Glc-6-P) in day and night form but not for K_m (PEP) (Table 1), but no marked difference was observed for these kinetic parameters in pineapple. K_a (Glc-6-P) of night PEPC from *K. pinnata* was low but 4.3-fold higher K_a (Glc-6-P) of day PEPC was observed, however, maximum velocity for this effector did not change markedly in this plant. Glc-6-P also caused a 5-fold increase in dephospho-PEPC activity at pH 7.1 in sorghum (Pacquit et al., 1995). But no marked difference was observed for K_a (Glc-6-P) and V_{max} for this allosteric effector of both forms of PEPC in pineapple. However, these striking differences in apparent kinetic properties in both forms of this enzyme between pineapple and *K. pinnata*, assuming that the above phenomena allow for an efficient control of PEPC activity in vivo and might play roles in its regulation in these two CAM plants differently. Moreover, the potent feedback inhibitor L-malate and allosteric activator Glc-6-P are present in the same cell compartment as the enzyme (Stitt and Heldt, 1985) and believed to play a significant role in the in vivo metabolic control of PEPC (O'Leary, 1982 ; Andreo et al., 1987 ; Echevarria et al., 1994).

We also separated two subunits of both day and night forms of PEPC from *K. pinnata* which contain a major polypeptide (apparent subunit molecular mass 112 kDa) and a minor polypeptide (apparent subunit molecular mass 119 kDa) whereas in pineapple only one polypeptide (apparent subunit molecular mass 107 kDa) was present in vivo states (Fig. 4). Western blot of this enzyme also revealed the two bands of *K. pinnata* in both day and night form of PEPC but single band from pineapple (Fig. 5). Those enzyme subunits were shown to be intact by Western blot experiments after probing the membrane with N-terminal (ASP-IgG) antibody raised against the corresponding synthetic epitopes (Pacquit et al., 1995) (Fig. 6). In these experiments APS-IgG was shown to bind equally well to the N-terminal domain of either day and night form of the enzyme from both plants (Fig. 6). The two forms of the enzyme from both plants behaved identically with each other on electrophoresis (Fig. 4). Nimmo et al. (1986) separated two related subunits of purified PEPC from *B. fedtschenkoi* which contained a major polypeptide (subunit molecular mass 112 kDa) and a minor polypeptide (subunit molecular mass 123 kDa) in both day and night forms which were consistent with our results, lead us to speculate that *Kalanchoë* species PEPC has two subunits, one is major and another is minor polypeptide and the apparent molecular mass of the minor polypeptide is higher than the major polypeptide though the relationship of these two polypeptides in vivo is unclear (Nimmo et al., 1986). However, another plausible explanation is the minor band has the capability of regulatory properties of phosphorylation and malate sensitivity of PEPC. Moreover, in these two CAM plants both the day and night enzyme forms were found to be in the identical constitution of molecular subunit, but still displayed the characteristic differential sensitivity to inhibition by L-malate, apparent phosphorylation states and activation by Glc-6-P. Also variations were observed in apparent kinetic properties of PEPC from these two CAM plants.

It is now well established that PEPC phosphorylation occurs during the dark period according to a circadian rhythm in CAM plants (Hartwell et al., 1996) and phosphorylation states of PEPC is highly controlled by the PEPC-Kinase and protein phosphatase type 2A in CAM plants (Carter et al., 1990). To our knowledge, the present study provides the first in

vivo suggestion for the absence of apparent phosphorylation in pineapple, and the phosphorylation/dephosphorylation process may not be the only major regulatory mechanism of PEPC in this particular CAM plant. The present results have paved a new path for further studies on photoperiod, day night transition, temperature fluctuations, and water status of pineapple to elucidate the *in vivo* role of this ubiquitous enzyme in regulation of CAM.

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〈和文抄録〉

CAM 植物における PEP カルボキシラーゼの in vivo 特性 ——パインアップルの CAM は PEPC のリン酸化で制御される？——

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2 種類の CAM 植物；セイロンベンケイソウ (*Kalanchoë pinnata*) とパインアップル (*Ananas comosus* (L.) Merr. cv. Smooth cayenne N67-10) の葉から昼間と夜間に 2 分以内で抽出したホスホエノールピルビン酸カルボキシラーゼ (PEPC, EC4.1.1.31) について、リンゴ酸による阻害の検討を行った。パインアップルの PEPC ではリン酸化が生じていないと考えられた。また、見かけのキネテック特性や分子量、特に PEPC サブユニット構成は 2 種類の CAM 植物で異なっていた。セイロンベンケイソウの PEPC では 2 種類のサブユニット (多量ポリペプチド 112 kDa と微量ポリペプチド 119 kDa) が昼夜ともに観察された。しかし、パインアップルの PEPC では単一のポリペプチド (見かけの分子量は 107 kDa) のみが観察された。ウエスタンブロット解析でも同様の結果が確認された。両植物から抽出した各々の PEPC サブユニットの N 末端領域の保存状態についても、N 末端抗体を用いたウエスタン解析で確認された。セイロンベンケイソウの PEPC はリンゴ酸感受性が 13% 減少したことより明らかにリン酸化された状態が示唆されたが、パインアップルでは夜間においてもリンゴ酸感受性があまり減少しない (相対活性が 79% の低下)、すなわち高い活性阻害が生じる状態を維持した。in vivo 状態の見かけの K_i (malate) は、セイロンベンケイソウでは昼型 (0.9 mM) にくらべ夜型 (5.8 mM) で 6.4 倍高かったが、パインアップルでは昼型と夜型ではそれぞれ 1.0 mM と 0.5 mM であった。以上の結果は、パインアップルの PEPC においてはリン酸化が必ずしも活性制御の主因ではないことを示唆している。