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PHOSPHORYLATION - DEPHOSPHORYLATION PROCESS AS A PROBABLE MECHANISM FOR THE DIURNAL REGULATORY CHANGES OF PHOSPHOENOLPYRUVATE CARBOXYLASE IN CAM PLANTS

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Day and night forms of phosphoenolpyruvate carboxylase (EC 4.1.1.31) (PEPC) were extracted from leaves of the CAM plants Kalanchoe daigremontiana, K. tubiflora and K. blossfeldiana previously fed with [^{32}P] labelled phosphate solution. A one-step immunochemical purification followed by SDS polyacrylamide gel electrophoresis and autoradiography showed that, in all species, the night form of the enzyme was phosphorylated and not the day form. Limited acid hydrolysis of the night form and two-dimensional separation identified predominantly labelled phosphoserine and phosphothreonine. In vitro addition of exogenous acid phosphatase (EC 3.1.3.2) to desalted night form-containing extracts resulted within 30 min in a shift in PEPC enzymic properties similar to the in vivo changes from night to day form. It is suggested that phosphorylation - dephosphorylation of the enzyme could be the primary in vivo process which might explain the observed rhythmicity of enzymic properties.

Phosphoenolpyruvate carboxylase (E.C. 4.1.1.31, PEPC) is considered to be a key enzyme in the regulation of the diurnal cycle of Crassulacean Acid Metabolism (CAM) (1,2). It has been shown that in CAM plants the regulatory properties of the enzyme change during the diurnal cycle (3-8): PEPC extracted during the night has high affinity towards the substrate PEP and is only weakly sensitive to inhibition by malate and to activation by glucose-6-phosphate (G-6-P); in contrast, PEPC extracted during the

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day has low affinity towards PEP and high sensitivity to inhibition by malate and activation by G-6-P. The night form of PEPC is supposed to be the physiologically active enzyme permitting high rates of nocturnal CO_2 fixation and subsequent malate synthesis. The day form is physiologically inactive. Thus futile recycling by PEPC of CO_2 deriving from malate decarboxylation during the day would be prevented.

The mechanism bringing about the mentioned changes of PEPC properties is, in spite of several hypotheses (9-11), still unknown. Recently it has been shown by Nimmo et al. (12) that in Bryophyllum fedtschenkoi the night form of PEPC was phosphorylated, not the day form. These authors therefore proposed that the changes in the regulatory PEPC properties were the consequence of phosphorylation and dephosphorylation of the enzyme. Since until now only a small number of plant enzymes are known to be regulated by phosphorylation/dephosphorylation processes (13), the case of PEPC is per se of general interest. In the context of CAM regulation, checking the existence of this mechanism in several species is necessary in order to afford generalization on its physiological role.

This paper reports experiments providing further insights on the mechanism and physiological meaning of PEPC phosphorylation in CAM plants. Results obtained for 3 Kalanchoe species showed that exogenously supplied [^{32}P] was incorporated into the enzyme when extraction was performed during the night at time of maximum enzyme capacity; in contrast no label was found in the day-form extracted at time of minimum enzyme capacity. Moreover, it was established that in the night-form label was localized in serine and threonine residues of the enzyme. Connection between the phosphorylated state of the enzyme and its

functional and regulatory properties is discussed in relation to the physiological meaning of CAM.

MATERIAL AND METHODS

Plant Material. Plants of Kalanchoe daigremontiana and K. tubiflora were cultivated as previously described (4); leaf discs (⌀ 0.6 cm) and phyllodia were respectively sampled at the 5th node from the apex.

Short-day-treated Kalanchoe blossfeldiana were obtained as described in (4); cuttings with 3 pairs of leaves were used as experimental material.

Incubation with [^{32}P]. Label was incorporated into floating discs of K. daigremontiana and phyllodia from K. tubiflora dipping in an incubation medium containing 0.2 mCi ^{32}P ([^{32}P] orthophosphate, Amersham). Different experimental protocoles were as follows: 1) tissues were sampled 1h before the beginning of night and incubated during 1h light + 8h darkness; this span corresponds to the transition from phase IV to phase I and the main part of phase I of the CAM cycle (14); 2) tissues were sampled 1h before the beginning of day and were incubated during 1h darkness + 8h light; this corresponds to the transition from phase I to phase II, phase II and III of the CAM cycle.

Cuttings of K. blossfeldiana were dipped in 0.2 ml solution containing 1 mCi [^{32}P]; incubation was carried out during 3 hours from the 2nd to the 5th hour of the night or of the day.

Extraction and immunoprecipitation of PEPC. Leaf extracts were prepared as previously described (4) and desalted through Sephadex G-25 columns. At suited time points, aliquotes of the extracts were immunoprecipitated by convenient amounts of antibodies (raised against PEPC of K. daigremontiana or K. blossfeldiana). Immunoprecipitates were thoroughly washed and analysed by SDS-PAGE. After electrophoresis, the gels were stained by Coomassie blue and subjected to autoradiography.

Measurement of PEPC capacity and properties. PEPC capacity in crude and desalted extracts was measured in an optimal reaction medium, at saturating PEP concentrations, pH 7.0. Inhibition by malate was tested at 0.5 mM malate and 1 mM PEP (non saturating) for 0.04 and 0.03 enzyme unit (EU) of PEPC activity extracted from K. daigremontiana and K. tubiflora respectively; in the case of PEPC extracted from K. blossfeldiana, 0.02 EU were subjected to inhibition by 3 mM malate and 2 PEP concentrations were used, 1.2 mM (non saturating) or 7.5 mM (saturating). Activation by glucose-6-P (10 mM) was tested on 0.02 EU of PEPC extracted from K. blossfeldiana at 1.2 mM PEP concentration. All assays were performed at pH 7.0.

In vitro incubation of extracts with acid phosphatase. Aliquots of desalted extracts, containing 1 EU of PEPC, were added 3 EU of acid phosphatase from wheat germ (E.C. 3.1.3.2, Flucka, Switzerland) and kept at constant temperature of 17°C; PEPC properties in these aliquots and in controls without acid phosphatase were tested throughout 12 hours.

Determination of phosphorylated amino acids (15,16). Six EU of PEPC from K. blossfeldiana extracted after 3 hours of [^{32}P] labelling in darkness were immunoprecipitated. The immunoprecipitates were hydrolysed by HCl 6 N, at 100°C, during 3 hours, then dried overnight. The hydrolysate was redissolved in H_2O and subjected to two-dimensional separation on a cellulose plate (DC - Plastikfolien cellulose, 0.1 mm, Merck), together with 10 µg of DL-serine-O-P acid form (Serva, FRG), DL-threonine-2P (Serva, FRG), L-tyrosine-O-P (generous gift from Dr J.C. Robert, Hopital Bichat, Paris) and Pi. The cellulose plate was subjected to electrophoresis (1.5h, 900v) in formic acid/acetic acid/H_2O (25:87:887), then to ascending chromatography in pyridine M/isobutyric acid (3:5), during 4 hours. Amino-acids were detected by ninhydrine spray, phosphates and ester-phosphates were determined by the ferric acid/sulfo salicylic acid reagent (17) and [^{32}P] labelled compounds by autoradiography (-80°C for two weeks using a XRay Film, Kodak XAR-5).

RESULTS AND DISCUSSION

Diurnal pattern of PEPC phosphorylation

Results in Fig.1 show SDS gel electrophoretic patterns and the corresponding autoradiograms of PEPC immunoprecipitates obtained after extraction of the enzyme from leaves of 3 Kalanchoe species (K. blossfeldiana, daigremontiana and tubiflora) following incubation with [^{32}P]. In all cases, incubation during the night resulted in labelled PEPC monomers. In contrast no labelling could be observed when leaves had been incubated during the day. This

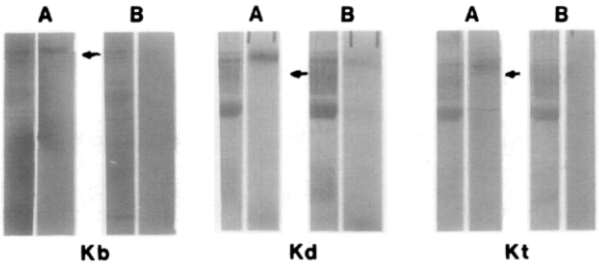

Fig.1. SDS gel electrophoretic patterns (a) and corresponding autoradiograms (b) of PEPC immunoprecipitates. Extracts from Kalanchoe blossfeldiana (Kb), K. daigremontiana (Kd) and K. tubiflora (Kt) prepared during the night (A) or during the day (B) after incorporation of [^{32}P] by the plants. Arrows indicate the phosphorylated monomer.

TABLE I: Sensitivity to malate and G-6-P, of the night-form (phosphorylated) and the day-form (non phosphorylated) of PEPC

	Inhibition by malate		Activation by G-6-P	
	Night form	Day form	Night form	Day form
K. daigr.	45	70	-	-
K. tubi.	25	85	-	-
K. bloss.	75	89	140	200

Extraction 9h after beginning of the night, 6h after beginning of the day. Conditions of assays: K. daigremontiana and K. tubiflora, PEP 1 mM, L-malate 0.5 mM, PEPC 0.04 and 0.03 EU, respectively, pH 7.0; K. blossfeldiana, PEP 1.2 mM, L-malate 3 mM, G-6-P 10 mM; pH 7.0. Results expressed as % of inhibition or activation of the activity without effector.

absence of PEPC labelling did not result from a lack in [^{32}P] incorporation into the proteins: the specific radioactivity of the total protein fraction (obtained after precipitation by trichloroacetic acid) was similar or even higher for extracts prepared during the day than for those prepared during the night. Results shown in Table I indicate that differences in enzyme characteristics of the labelled and non-labelled PEPC were similar to those previously established (3-8) for the night- and the day-forms of the enzyme: the non-labelled enzyme is more sensitive to inhibition by malate and to activation by G-6-P.

These results are in agreement with those published by Nimmo et al. (12) for Bryophyllum fedtschenkoi, and contain some degree of generalization owing to the fact that they concern 3 species, various incubation times and modes of incorporation (via petioles, excised phyllodia or floating leaf discs) . It should be noted that in the case of K. daigremontiana a slight [^{32}P] incorporation into PEPC was observed after incubation during the day.

Phosphorylation of the night-form of PEPC was confirmed by checking the localization of label in the enzyme molecule. Labelled enzyme was obtained from K. blossfeldiana, purified immunochemically and subjected to limited acid hydrolysis. Phosphorylated amino-acids were separated by high voltage electrophoresis and TLC, and the chromatograms were autoradiographed as described in Material and Methods. As shown in Fig.2, major spots on chromatograms and autoradiograms were associated with phosphoserine and phosphothreonine. These data show that PEPC was phosphorylated during night, mainly on serine and threonine residues; as a consequence, upon night/day transition, an endogenous phosphatase might remove the serine- and threonine-bound phosphate.

In vitro changes of PEPC properties in presence of exogenous phosphatase

Analyses performed in extracts obtained during day and night showed that sensitivity of PEPC to malate and G-6-P

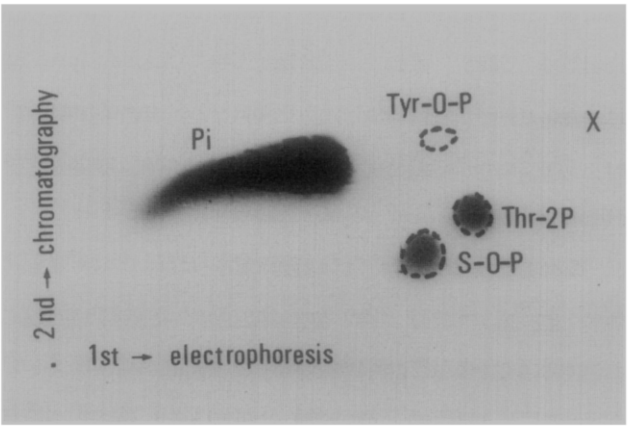

Fig.2. Two-dimensional separation of phosphorylated amino-acids from [^{32}P]- labelled PEPC. Enzyme was extracted from K. blossfeldiana leaves during the night after 3h incorporation of [^{32}P]-orthophosphate, and purified immunochemically. Dotted circles indicate the standard phosphoserine (Ser-O-P), phosphothreonine (Thr-2P) and phosphotyrosine (Tyr-O-P); Pi: orthophosphate; x: non determined spot.

TABLE II: Changes in sensitivity to malate and to G-6-P of the night-form of PEPC in absence (control) or presence of exogenous acid phosphatase

	PEP 1.2 mM		PEP 7.5 mM
	Inhibition by malate	Activation by G-6-P	Inhibition by malate
Control			
Night-form extraction 23h00			
0h00	77 ± 4	147 ± 12	66 ± 4
0h30	68 ± 1	155 ± 5.9	53 ± 5
↓ 12h00	56 ± 6	179 ± 16	46 ± 10
Day form extraction 12h00	85 ± 4	223 ± 36	73 ± 1
Night form (Extraction 23h30)			
0h00	77 ± 4	147 ± 12	66 ± 4
↓ phosphatase			
0h30	86 ± 2	197 ± 14	71 ± 6
↓ 12h00	82 ± 2	231 ± 9	63 ± 2

Extracts from K. blossfeldiana leaves obtained at 23h30 (night-form) or 12h00 (day-form), desalted and kept during 12 hours under constant conditions. Results expressed as % of inhibition or activation of the activity without effector; PEPC, 0.02 EU ; malate, 3 Mm; G-6-P 10 mM; pH 7.1; mean number of 4 experiments ± s.d.

increased upon night/day transition (4,8). These differences in enzyme properties were confirmed by the results presented in Table II (control, night-form 23h30, day-form 12h00). When the 23h30-extract is maintained during 12 hours under constant conditions (darkness, 17°C, pH 7.1), sensitivity to G-6-P increased spontaneously, but sensitivity to malate decreased (both at saturating and non-saturating PEP concentrations in the reaction medium). Addition of acid phosphatase (Table II) immediately after extraction hastened (within 30 min) the increase in sensitivity to G-6-P which was maintained higher than the control during the 12 hours-

experiment. In presence of phosphatase an increase in the sensitivity to malate in these extracts could be observed only at non-saturating PEP concentrations.

To sum up, acid phosphatase *in vitro* shifted within 30 min the sensitivity of PEPC to G-6-P and to malate (at 1.2 mM PEP) in a way which is consistent with the simultaneous evolution of these properties *in vivo*.

It is known that G-6-P modifies the Km(PEP) and that malate acts as a mixed-type inhibitor. The *in vivo* coherence of changes in sensitivity towards G-6-P and malate can be mimicked by the effect of phosphatase *in vitro* only if measurements are performed at non-saturating PEP. This would suggest that phosphorylation-dephosphorylation process results in modifying the affinity of the enzyme for the substrate PEP.

Present results show that the observed day/night rhythmicity in the properties of PEPC extracted from *Kalanchoe* leaves could be due to a phosphorylation-dephosphorylation process exerted on the enzyme; this post-translational process would imply the existence of coupled protein kinase/phosphatase activities whose antagonistic action could result in the *in vivo* modification of PEPC structure. Wu and Wedding recently established (10) a relationship between the properties of PEPC extracted from a CAM plant and the state of aggregation of the enzyme. It could be hypothesized that a phosphorylation-dephosphorylation process would account for the change in the quaternary structure of PEPC.

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