The EMBO Journal vol.10 no.8 pp.2063 – 2068, 1991

Circadian rhythms in the activity of a plant protein kinase

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Communicated by P.Cohen (Dundee)

Bryophyllum fedtschenkoi **is a Crassulacean acid metabolism plant whose phosphoenolpyruvate carboxylase is regulated by reversible phosphorylation in response to a circadian rhythm. A partially purified protein kinase phosphorylated phosphoenolpyruvate carboxylase *in vitro* with a stoichiometry approaching one per subunit and caused a concomitant 5- to 10-fold decrease in the sensitivity of the carboxylase to inhibition by malate. The sites phosphorylated *in vitro* were identical to those phosphorylated in intact tissue. The activity of the protein kinase was controlled in a circadian fashion. During normal diurnal cycles, kinase activity appeared between 4 and 5 h after the onset of darkness and disappeared 2–3 h before the end of darkness. Kinase activity displayed circadian oscillations in constant environmental conditions. The activity of protein phosphatase 2A, which dephosphorylates phosphoenolpyruvate carboxylase, did not oscillate. Treatment of detached leaves with the protein synthesis inhibitors puromycin and cycloheximide blocked the nocturnal appearance of the protein kinase activity, maintained phosphoenolpyruvate carboxylase in the dephosphorylated state and blocked the circadian rhythms of CO_2 output that is observed in constant darkness and CO_2-free air. The simplest explanation of the data is that there is a circadian rhythm in the synthesis of phosphoenolpyruvate carboxylase kinase.**

Key words: Bryophyllum fedtschenkoi/circadian rhythm/Crassulacean acid metabolism/phosphoenolpyruvate carboxylase/protein phosphorylation

Introduction

Crassulacean acid metabolism (CAM) is a metabolic adaptation of plants to arid environments in which there is a diurnal cycle of CO_2 metabolism. External CO_2 is fixed at night, when the stomata are open, by the actions of phosphoenolpyruvate carboxylase (EC 4.1.3.1, PEPc) and malate dehydrogenase, yielding malate which is stored in the cell vacuole. During the day, the stomata are closed, malate is released from the vacuole and decarboxylated, and the resulting CO_2 is assimilated via the Calvin cycle (Osmond and Holtum, 1981). Regulation of PEPc plays a central role in the control of CAM. The enzyme is controlled both by feedback inhibition by malate and by phosphorylation of a serine residue (e.g. Nimmo *et al.*, 1990). The purified, dephosphorylated 'day form' of PEPc is substantially more sensitive to malate than is the phosphorylated 'night form' (Nimmo *et al.*, 1986).

Detached leaves of the CAM plant *Bryophyllum fedtschenkoi* can show persistent circadian rhythms of CO_2 metabolism (e.g. Wilkins, 1959, 1960, 1973, 1983, 1984). For example, leaves in continuous darkness and CO_2-free air at 15°C exhibit a circadian rhythm of CO_2 output. This rhythm is directly attributable to changes in flux through PEPc, which periodically fixes internal CO_2 that would otherwise be released (Warren and Wilkins, 1961). Two lines of evidence indicate that the reversible phosphorylation of PEPc is involved in the generation of these rhythms and is controlled by an endogenous clock. First, during the normal diurnal cycle, the period during which PEPc is phosphorylated (as judged both by its K_i for malate and by immunoprecipitation of the enzyme from $^{32}P_i$-labelled leaves) corresponds to the period during which malate is accumulated. However, this is not simply a response to light because dephosphorylation of the 'night form' of PEPc occurs 1–2 h before the start of the light period and phosphorylation begins several hours after its end (Nimmo *et al.*, 1984). Secondly, the phosphorylation state of PEPc shows a pronounced circadian rhythm in constant conditions; in continuous darkness the periods during which PEPc is phosphorylated correlate with periods during which there is flux through the enzyme (Nimmo *et al.*, 1987).

These results lead to the question of how the phosphorylation state of PEPc is controlled by an endogenous clock. It is already known that PEPc can be dephosphorylated by protein phosphatase 2A (PP2A) (Carter *et al.*, 1990). In this paper we identify the protein kinase that is responsible for the phosphorylation of PEPc *in vivo*, and show that the activity of this enzyme, but not that of PP2A, is controlled by an endogenous rhythm in a process that appears to involve protein synthesis.

Results

Purification and properties of phosphoenolpyruvate carboxylase kinase

Preliminary work showed that *B.fedtschenkoi* leaves contained detectable PEPc kinase activity during the middle of the dark period (see also below). PEPc kinase was partially purified from such leaves by a three-step procedure as described in Materials and methods. The kinase was separated from PEPc itself by chromatography on blue dextran–agarose (Jiao and Chollet, 1989), to which PEPc does not bind (not illustrated).

Further purification of the kinase was obtained by chromatography on Mono Q, as illustrated in Figure 1. Accurate quantification of PEPc kinase activity in crude extracts is difficult because of the presence of protein phosphatase activity and of proteases that can cleave PEPc to generate a non-phosphorylatable form. As a consequence, the yield and purification of the kinase were not estimated.

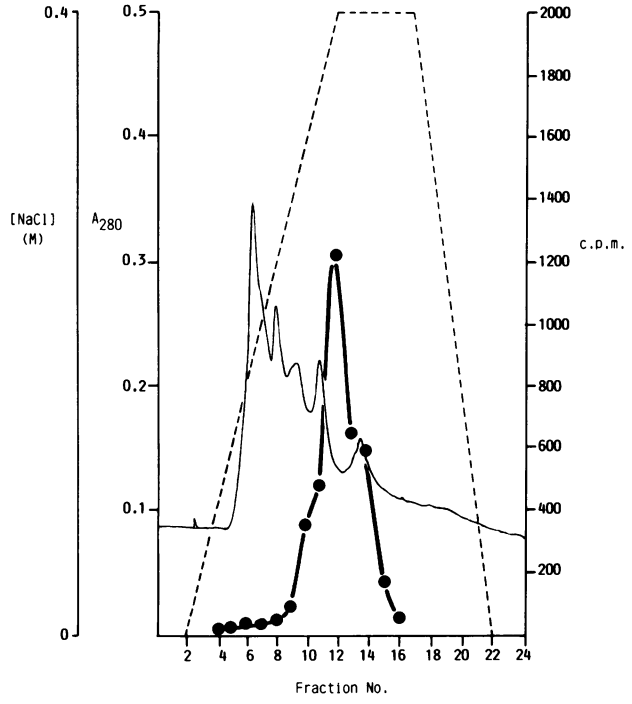

Fig. 1. Chromatography of PEPc kinase on Mono Q. PEPc kinase activity from 60 g leaves was purified on Mono Q as described in Materials and methods; (———), A_{280}; (--------), NaCl gradient; (●), PEPc kinase activity (^{32}P-radioactivity incorporated into PEPc/10 min).

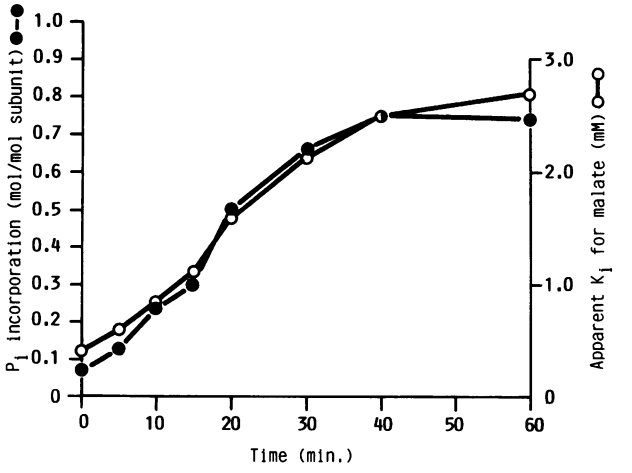

Fig. 2. Relationship between phosphorylation and malate sensitivity of PEPc. Purified 'day form' PEPc was phosphorylated with partially purified PEPc kinase as described in Materials and methods; (●), phosphate incorporation into PEPc estimated by precipitation with trichloracetic acid; (○), apparent K_i for malate.

Similar observations have been made for PEPc kinase activity in the C_4 plant maize (Jiao and Chollet, 1989), in which PEPc becomes phosphorylated in response to light (e.g. Nimmo *et al.*, 1990).

When partially purified PEPc kinase was incubated with purified dephosphorylated ('day form') PEPc and [γ-^{32}P]ATP, the added PEPc was the only protein that became phosphorylated. *B.fedtschenkoi* PEPc contains two related subunits, with apparent M_r values 112 000 and 123 000, in a ratio of approximately 10:1 (Nimmo *et al.*, 1986). Both subunit types became phosphorylated and the kinase did not appear to discriminate between them (not illustrated). The functions of, and the relationship between, the two subunit types are not clear.

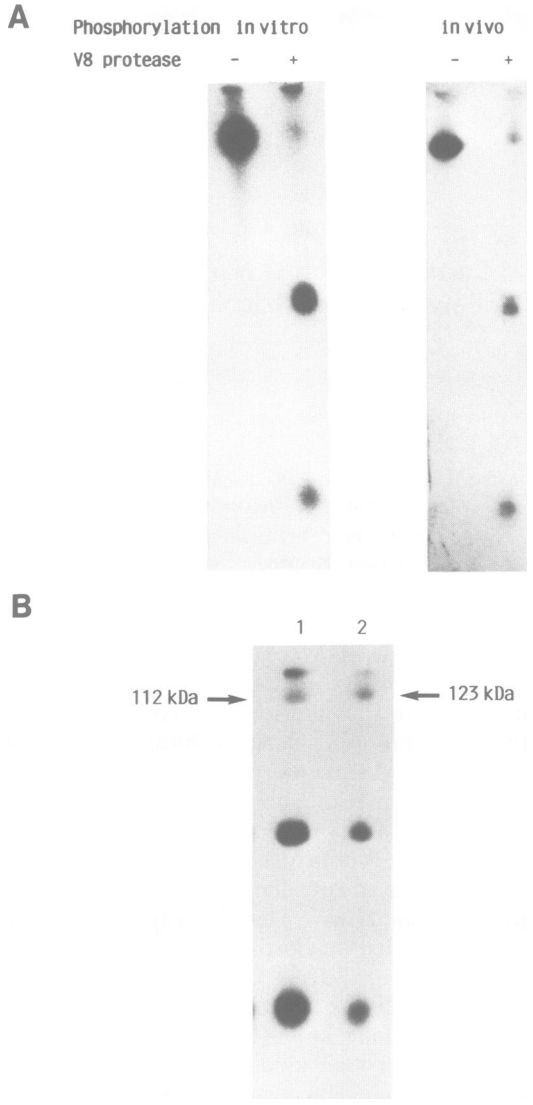

Fig. 3. Peptide mapping of phosphorylated PEPc. (**A**) PEPc was either phosphorylated *in vitro* or phosphorylated *in vivo* and isolated by immunoprecipitation. The M_r 112 000 and 123 000 subunits from 0.03 units of PEPc activity were pooled and digested for 30 min with V8 protease (25 ng), and the peptides were separated on a 15% SDS–polyacrylamide gel. The figure shows an autoradiograph of the dried gel. (**B**) PEPc was phosphorylated *in vitro* and the M_r 112 000 and 123 000 subunits were excised and digested separately in (A). Lane 1, M_r 112 000 subunit from 0.03 units of PEPc activity. Lane 2, M_r 123 000 subunit from 0.06 units of PEPc activity. The figure shows an autoradiograph of the dried gel. The arrows indicate the undigested M_r 112 000 and 123 000 subunits.

When phosphorylation was allowed to go to completion, the maximum stoichiometry obtained was in the range 0.7–0.9 mol incorporated/mol of PEPc subunits. A typical time course of phosphorylation is shown in Figure 2, which indicates that phosphorylation resulted in an increase in the apparent K_i of the PEPc for malate from 0.48 mM to 2.7 mM. These values are similar to those of the 'day form' and 'night form' of PEPc respectively (Nimmo *et al.*, 1984, 1986). The sites phosphorylated by the purified kinase *in vitro* were compared with those phosphorylated in intact tissue by one-dimensional phosphopeptide mapping. The 'night form' of PEPc was isolated by immunoprecipitation from $^{32}P_i$-labelled tissue and compared with PEPc

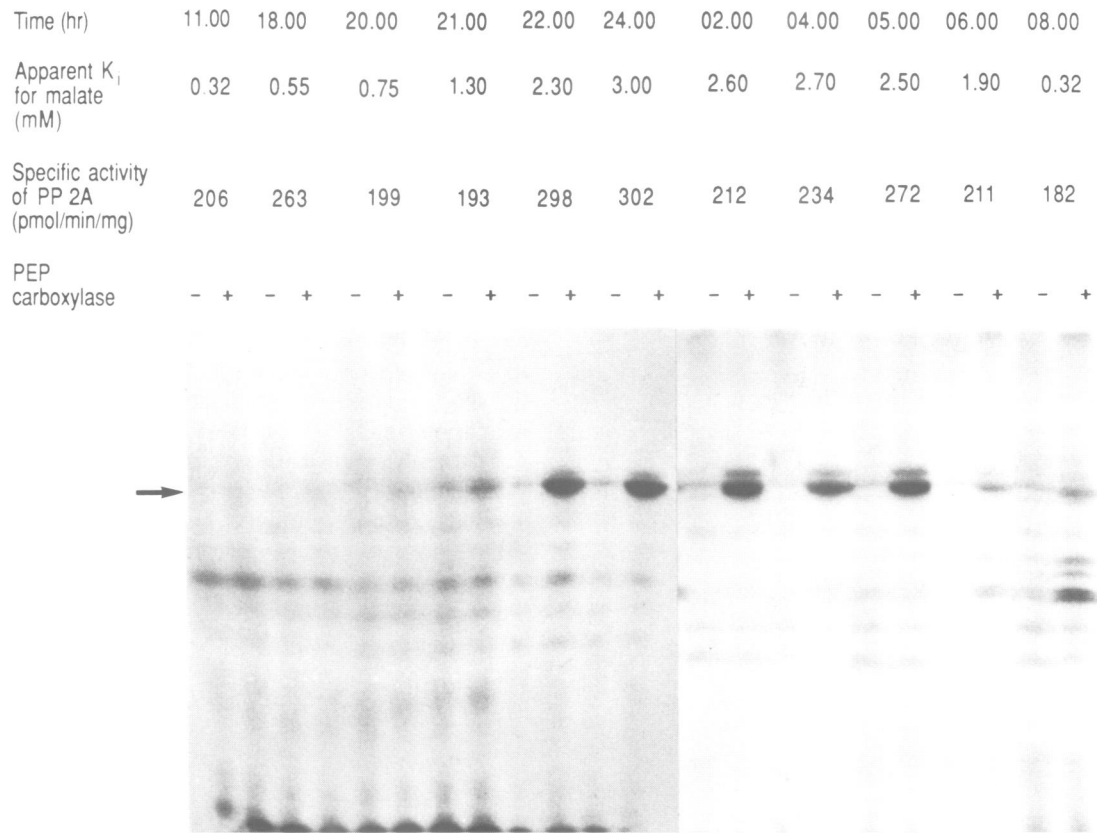

Fig. 4. The activities of PEPc kinase and PP2A throughout the normal diurnal cycle. Extracts were prepared from pairs of leaves using 3 ml of extraction buffer/g, fresh tissue. Extracts were desalted and assayed as described in Materials and methods. The top three rows show the time of extraction, the apparent K_i of PEPc for malate and the specific activity of PP2A in the extract. To detect PEPc kinase activity, samples of the desalted extracts were incubated without (−) or with (+) added 'day form' PEPc. The photograph shows the autoradiographs of dried SDS−polyacrylamide gels. The arrow indicates the position of the M_r 112 000 subunit of PEPc. The samples taken between 02.00−08.00 h and 11.00−24.00 h were analysed on separate gels.

phosphorylated *in vitro*. The resulting maps (Figure 3A) are essentially identical and show that the same site(s) are phosphorylated both *in vitro* and in intact leaves. Figure 3B indicates that the sites of phosphorylation in the M_r 112 000 and 123 000 subunits are very similar or identical. In combination, these data provide very strong evidence that the protein kinase isolated in this work is responsible for the phosphorylation of PEPc *in vivo*.

The effects of potential regulators of PEPc kinase were investigated using the partially purified enzyme. $CaCl_2$ (1 mM), EGTA (5 mM) and Ca^{2+}/EGTA buffers (free Ca^{2+} 0.1−10 μM) had no significant effect on PEPc kinase activity. The enzyme was not affected by the presence of 1 μM okadaic acid but NaF (5 mM) gave 50% inhibition. Kinase activity was affected by ligands of PEPc itself. Activity was reduced to 30 ±11% (n = 4) by 5 mM L-malate, to 15 ± 15% (n = 4) by 10 mM glucose 6-phosphate, which activates PEPc, and to 61 ± 14% (n = 3) by 5 mM phosphoenolpyruvate. These effects could result from binding of the effector to the kinase or to PEPc or both. The effect of malate was not absolutely stereospecific, as D-malate gave 30% inhibition at 5 mM.

Diurnal regulation of phosphoenolpyruvate carboxylase kinase

B.fedtschenkoi leaf extracts were prepared and desalted at intervals throughout the normal diurnal cycle as described in Materials and methods. They were then assayed for PEPc kinase activity and for PP2A, the enzyme that is responsible for dephosphorylation of PEPc (Carter *et al.*, 1990). The kinase was assayed by incubating desalted extracts with [γ-^{32}P]ATP, either without or with added purified dephosphorylated ('day form') PEPc. Phosphorylation was detected by SDS−PAGE and autoradiography. The results (Figure 4) show that PEPc kinase activity appeared between 20.00 h and 22.00 h, remained high until 05.00 h, and then disappeared by 06.00 h. The apparent K_i values of the PEPc in these extracts for malate are also shown in Figure 4. It can be seen that the presence of PEPc kinase activity correlates with the times at which PEPc is in the phosphorylated form, with a high K_i for malate. In contrast, the specific activity of PP2A remains fairly constant throughout the diurnal cycle (Figure 4). These data indicate that the major factor that controls the phosphorylation state of PEPc is the presence or absence of PEPc kinase activity.

The data in Figure 4 show that the activity of PEPc kinase is not controlled directly by illumination/darkness during the normal diurnal cycle. To investigate control of the enzyme in constant conditions, leaves were placed in continuous darkness and CO_2-free air at 15°C at 16.00 h. Such leaves do not initially contain PEPc kinase activity (Figure 4). Extracts prepared after 7 h in constant conditions did contain PEPc kinase activity whereas those prepared after 21.00 h did not (not illustrated). These times correspond to the first trough and the first peak, respectively, of CO_2 output (e.g. see below, Figure 6). These results show that PEPc kinase activity, like the phosphorylation state of PEPc, is controlled by an endogenous clock.

To investigate the mechanism underlying the appearance of PEPc kinase activity during the dark period, detached *B.fedtschenkoi* leaves were allowed to take up solutions of the protein synthesis inhibitors cycloheximide or puromycin from early in the light period. Extracts were prepared, desalted and assayed for PEPc kinase activity at 02.00 h in the following dark period. Representative results are shown in Figure 5. Control leaves, allowed to take up water or dilute ethanol, contained PEPc kinase activity and PEPc with a high K_i for malate. Leaves allowed to take up the inhibitors contained no detectable PEPc kinase activity and their PEPc had a low K_i for malate, characteristic of the dephosphorylated form.

Detached *B.fedtschenkoi* leaves kept in continuous darkness and CO_2-free air at 15°C show persistent circadian rhythms of CO_2 output, due to periodic fixation of internal CO_2 by PEPc (see Introduction). Bollig and Wilkins (1979) have already shown that cycloheximide can block these rhythms. Similar results were obtained in the present work. The effects of puromycin are shown in Figure 6. Control leaves, allowed to take up water, exhibited a circadian rhythm of CO_2 metabolism: troughs correspond to periods of CO_2 fixation by PEPc. Increasing concentrations of puromycin over the range $10^{-5}-10^{-3}$ M gave increasing inhibition of these rhythms. To assess the metabolic status of the leaves after these treatments, they were illuminated for 6 h after three days. The control leaves and those treated with lower concentrations of puromycin responded with a pulse of increased CO_2 output. This is probably due to decarboxylation of accumulated malate by malic enzyme. Leaves treated with higher concentrations of

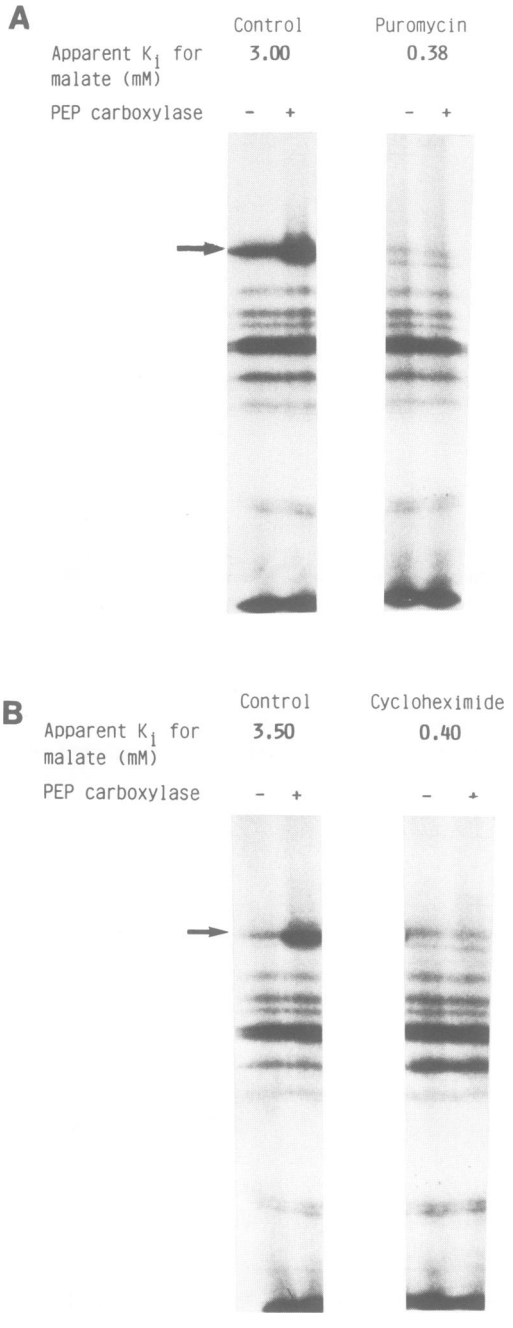

Fig. 5. Effects of pretreatment of leaves with protein synthesis inhibitors on PEPc kinase activity. Leaves were pretreated with either (**A**) 1 mM puromycin or (**B**) 50 μM cycloheximide as described in Materials and methods. Control leaves were treated with (A) water or (B) 0.01% (v/v) ethanol. The apparent K_i for malate and the activity of PEPc kinase were assessed as in Figure 4.

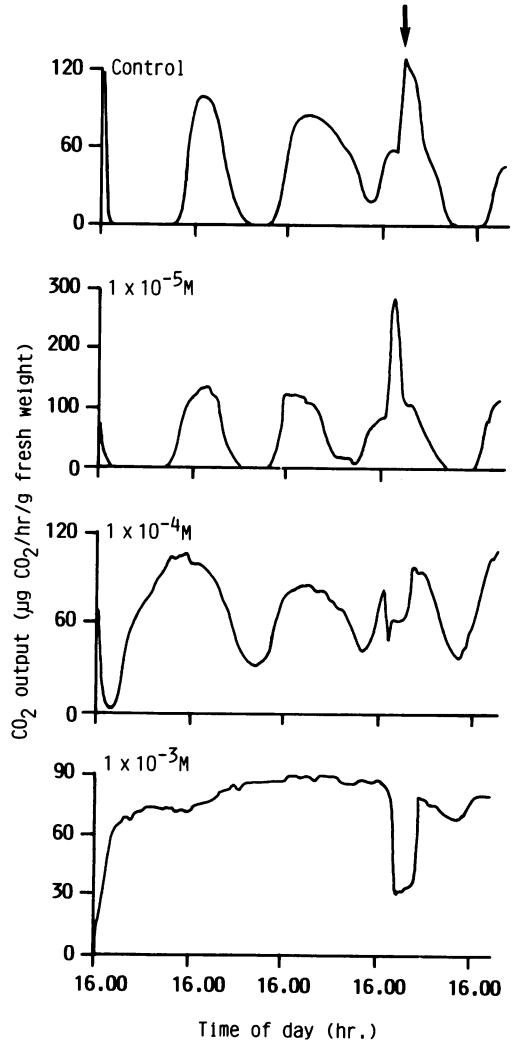

Fig. 6. The effects of puromycin on the circadian rhythm of CO_2 output in constant darkness. Leaves were placed in constant conditions and allowed to take up the indicated concentration of puromycin. The traces show CO_2 output determined as described in Materials and methods. Leaves were illuminated for 6 h starting at the point indicated by the arrow.

puromycin exhibited a trough in CO_2 output on illumination. These leaves presumably cannot accumulate malate because their PEPc is dephosphorylated (Figure 5). The trough in CO_2 output is probably due to fixation of CO_2 by ribulose-1,5-bisphosphate carboxylase. These observations indicate that leaves are still capable of carrying out photosynthesis, in spite of prolonged treatment with puromycin. Similar observations were made for leaves treated with cycloheximide (not shown).

Discussion

The protein kinase isolated in this work is responsible for the phosphorylation of PEPc in *B.fedtschenkoi* leaves at night, as judged by the fact that it phosphorylates the same site(s) that is (are) phosphorylated in intact tissue. The stoichiometry of phosphorylation suggests that only a single serine residue per subunit becomes phosphorylated. Although the sequence of *B.fedtschenkoi* PEPc is not known, it is likely that the phosphorylation site is equivalent to Ser15 of maize leaf PEPc. Phosphorylation of this residue by a maize leaf protein kinase results in a decrease in the malate sensitivity of the enzyme (Jiao and Chollet, 1990). Complete phosphorylation of the *B.fedtschenkoi* PEPc causes a change in the apparent K_i for malate that is very similar to the difference between the 'day' and 'night' forms of the enzyme (Nimmo *et al.*, 1984, 1986). This suggests that in intact tissue PEPc may be essentially fully dephosphorylated during the day but fully phosphorylated at night.

The phosphorylation state of PEPc exhibits an endogenous rhythm (Nimmo *et al.*, 1984, 1987). The data presented here suggest that this is largely due to fluctuations in the activity of PEPc kinase which match those in the K_i of PEPc for malate. The appearance and disappearance of PEPc kinase activity are not directly related to changes in illumination, and indeed persist in constant environmental conditions. Hence the activity of this protein kinase exhibits an endogenous circadian rhythm. In contrast to PEPc kinase, the activity of PP2A does not change throughout the diurnal cycle. However, it is not yet clear whether the activity of this enzyme against phosphorylated PEPc *in vivo* can be affected by metabolites, nor have we formally proved that it is the only phosphatase active against PEPc. It is therefore possible that short-term control of phosphatase activity, specific to PEPc, also plays a role in regulating the phosphorylation state of PEPc.

Experiments involving the use of presumed inhibitors of protein synthesis in higher plants must be interpreted with great care since such compounds can affect processes other than protein synthesis (e.g. Ellis, 1977). Indeed, Bollig and Wilkins (1979) interpreted the abolition of circadian rhythms of CO_2 output by cycloheximide in terms of an effect of the drug on the permeability of the tonoplast. However, in the present work we found that two structurally unrelated compounds which affect protein synthesis in different ways had very similar effects: both cycloheximide and puromycin blocked the nocturnal appearance of PEPc kinase activity, maintained PEPc in the dephosphorylated (low K_i for malate) form and blocked the circadian rhythm of CO_2 output by periodic fixation of CO_2 via PEPc. Neither inhibitor affected endogenous protein phosphorylation in leaf extracts (Figure 5). Even after three days of treatment with the highest inhibitor concentrations used in this work, leaves were still capable of photosynthesis, as judged by the reduction of CO_2 output in response to illumination (Figure 6). This indicates that the inhibitors had not radically disrupted leaf cell metabolism. Hence it seems reasonable to suggest that the effects of the two compounds do indeed flow from an inhibition of protein synthesis. The simplest interpretation of our data is therefore that PEPc kinase is synthesized and destroyed periodically, under the control of a circadian rhythm. However, more complex explanations cannot be ruled out. For example, PEPc kinase protein may be present throughout the circadian cycle, but its activity may be controlled by activating and inactivating proteins at least one of which is controlled at the level of protein synthesis by the circadian rhythm. Involvement of a protein synthesis step in the regulation of a protein kinase is unusual but not unprecedented. For example, activation of the $p34^{cdc2}$ protein kinase that regulates the onset of M phase involves its association with a cyclin which is synthesized during the cell cycle and destroyed at the end of M phase (e.g. Nurse, 1990). Purification of PEPc kinase to homogeneity will be required to distinguish between the various possibilities.

Several cases of the control of specific protein synthesis by circadian clocks have already been described. The *period* (*per*) gene of *Drosophila melanogaster* is thought to be part of the circadian oscillator that controls eclosion and adult locomotor activity (e.g. Hall and Rosbash, 1987). Concentrations of both *per* mRNA and the *per*-encoded protein oscillate in constant conditions (Siwicki *et al.*, 1988; Hardin *et al.*, 1990). Genes that are presumably downstream of a circadian oscillator and whose synthesis is controlled at the level of transcription include the light harvesting chlorophyll *a/b* protein (e.g. Taylor, 1989) and the liver transcriptional activator protein DBP (Wuarin and Schibler, 1990). On the other hand, the luciferin-binding protein of the dinoflagellate *Gonyaulax polyedra* is controlled by a circadian rhythm at the level of translation (Morse *et al.*, 1990). It is not yet clear whether the activity of *Bryophyllum* PEPc kinase is controlled at the level of transcription or translation or both. Since the list of proteins whose activities are controlled by circadian rhythms include both a transcription factor (Wuarin and Schibler, 1990) and a protein kinase (this work), it is tempting to speculate that control of regulatory proteins may be a common feature of circadian clocks.

Materials and methods

Materials

Bryophyllum (*Kalanchoe*) *fedtschenkoi* Hamet et Perrier plants were maintained and harvested as described by Nimmo *et al.* (1984). The photoperiod was 8 h, from 08.00 h until 16.00 h.

Okadaic acid was from Moana Bioproducts, blue dextran–agarose, puromycin and cycloheximide were from Sigma and [γ-^{32}P]ATP was from Amersham. The dephosphorylated 'day form' of PEPc was purified as described by Nimmo *et al.* (1986) and [^{32}P]casein was prepared as given in McGowan and Cohen (1988).

Preparation of leaf extracts

Leaves were homogenized in extraction buffer (100 mM Tris–HCl, pH 8.0, 2 mM EDTA, 10 mM malate, 1 mM benzamidine hydrochloride, 1 mM dithiothreitol, 2% (w/v) polyethylene glycol 20 000) (1 ml/g fresh tissue unless stated otherwise) with 50 mg sodium bicarbonate/g and a few drops of octanol for 30 s at low speed in a Waring blender at 5°C. The homogenate was filtered through two layers of muslin and its pH was adjusted to 7.5. The extract was centrifuged at 15 000 *g* for 15 min to remove particulate material. Prior to assays of PEPc, PEPc kinase and PP2A, extracts were desalted into 50 mM Tris–HCl, pH 7.8, 1 mM benzamidine hydrochloride, 1 mM dithiothreitol, 5% (v/v) glycerol using Sephadex G25 columns.

Purification of PEPc kinase

Leaves (60 g), taken during the middle of the night (midnight–02.00 h) were homogenized and centrifuged as described above. The supernatant was brought to 50% saturation with ammonium sulphate, stirred for 30 min and centrifuged at 15 000 *g* for 15 min. The precipitate was resuspended in 4 ml Buffer A (50 mM Tris–HCl, pH 7.5, containing 1 mM benzamidine hydrochloride, 1 mM dithiothreitol and 5% (v/v) glycerol), desalted into this buffer by passage through a Sephadex G25 column and clarified by centrifugation at 11 600 *g* for 5 min. The material was loaded onto a 2.7 × 4.5 cm column of blue dextran–agarose equilibrated in Buffer A. Unbound protein was removed by washing with Buffer A, and PEPc kinase was then eluted with Buffer A containing 0.5 M NaCl (Jiao and Chollet, 1989). Peak fractions were pooled, desalted into Buffer A by passage through a Sephadex G25 column and loaded onto a Mono Q HR5/5 column equilibrated in Buffer A at a flow rate of 1 ml/min. Unbound protein was removed by washing with Buffer A and the column was developed with a linear 10 ml gradient of 0–400 mM NaCl in Buffer A. Fractions (1 ml) were dialysed individually overnight into Buffer B (50 mM Tris–HCl, pH 7.8, containing 1 mM benzamidine hydrochloride, 1 mM dithiothreitol and 20% (v/v) glycerol). Fractions containing PEPc kinase activity were pooled, concentrated to ~1 ml using Centricon 30 filters and stored at −20°C. The Mono Q column was run at room temperature and all other steps were carried out at 0–5°C.

Assay procedures

The PEPc kinase activity of desalted extracts was assayed by incubating extract containing 0.002 units of PEPc activity in 50 mM Tris–HCl, pH 7.8, containing 5 mM $MgCl_2$, 0.01 mM [γ-^{32}P]ATP (1 μCi), 1 mM benzamidine hydrochloride, 10 μg antipain/ml, 10 μg leupeptin/ml, 5 nM okadaic acid, either with or without 0.03 units of purified dephosphorylated PEPc in a total volume of 25 μl. Incubations (10 min at 30°C) were terminated by the addition of SDS sample buffer and heating to 100°C. Reaction mixtures were analysed by SDS–PAGE (Laemmli, 1970) (8% gels) and autoradiography.

Partially purified PEPc kinase activity was assayed in conditions based on those of Jiao and Chollet (1989). The kinase (17 μl) was incubated with 0.03 units of purified dephosphorylated PEPc in Buffer B containing 10 μg antipain/ml, 10 μg leupeptin/ml, 5 mM $MgCl_2$ and 0.1 mM [γ-^{32}P]ATP (1 μCi) in a total volume of 25 μl at 30°C for 10 min unless stated otherwise. Incorporation of ^{32}P into PEPc was monitored either by SDS–PAGE, autoradiography and scintillation counting of the PEPc band or after precipitation and washing of the protein with trichloracetic acid (Corbin and Reimann, 1974). Time course experiments were carried out using these conditions on a larger scale. ^{32}P incorporation was measured using 25 μl samples and the K_i of PEPc for malate was measured by assaying four 2.5 μl samples at different concentrations of malate.

The activity of PEPc was assayed, and its K_i for malate estimated using different malate concentrations, as described in Nimmo *et al.* (1984). PP2A activity was assayed using [^{32}P]casein (Cohen *et al.*, 1988). One unit of enzyme activity is the amount required to catalyse the formation of 1 μmol of product/min. Protein was assayed as described by Bradford (1976).

Peptide mapping

Phosphorylated PEPc labelled with ^{32}P in intact leaves was isolated by immunoprecipitation from $^{32}P_i$-labelled leaves as described by Nimmo *et al.* (1984). PEPc was ^{32}P-phosphorylated *in vitro* by the partially purified PEPc kinase as described above. Samples were resolved by SDS–PAGE on 8% gels. The PEPc bands (M_r 112 000 and 123 000) were excised and run on a 15% gel with or without digestion by *Staphylococcus aureus* V8 protease (Cleveland *et al.*, 1977).

Measurement of CO_2 output in continuous darkness

Detached leaves were removed from the growth room at 16.00 h and placed in a gas-tight brass container surrounded by a water jacket maintained at 15°C (Wilkins, 1983) in continuous darkness unless stated otherwise. A stream of CO_2-free air was passed over the leaves at a rate of 1.55 l/h and the CO_2 content of the emergent gas was determined using an infra-red gas analyser (Wilkins, 1973). Leaves were allowed to take up protein synthesis inhibitors or solvent through the transpiration stream. In some experiments, leaves were illuminated for 6 h at an average photon fluence rate (400 –700 nm) of 15 μmol/m^2/s provided by white fluorescent tubes.

Treatment of leaves with protein synthesis inhibitors

A stock solution of cycloheximide (5 mM) was made up in 1% (v/v) ethanol and diluted with H_2O. Puromycin was dissolved in H_2O. For experiments on PEPc kinase activity, leaves were detached at 09.00 h and allowed to take up H_2O, appropriately diluted ethanol or protein synthesis inhibitors in the growth room. Extracts were prepared during the following night at 02.00 h. For experiments on CO_2 output, leaves were allowed to take up inhibitors when they were placed in constant conditions (see above).

Acknowledgements

This work was supported by the Agricultural and Food Research Council, London.

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Received on March 27, 1991