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Persistent circadian rhythms in the phosphorylation state of phosphoenolpyruvate carboxylase from *Bryophyllum fedtschenkoi* leaves and in its sensitivity to inhibition by malate

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Abstract. Phosphoenolpyruvate carboxylase (EC 4.1.1.31; PEPCase) from *Bryophyllum fedtschenkoi* leaves has previously been shown to exist in two forms in vivo. During the night the enzyme is phosphorylated and relatively insensitive to feedback inhibition by malate whereas during the day the enzyme is dephosphorylated and more sensitive to inhibition by malate. These properties of PEPCase have now been investigated in leaves maintained under constant conditions of temperature and lighting. When leaves were maintained in continuous darkness and CO_2-free air at 15 °C, PEPCase exhibited a persistent circadian rhythm of interconversion between the two forms. There was a good correlation between periods during which the leaves were fixing respiratory CO_2 and periods during which PEPCase was in the form normally observed at night. When leaves were maintained in continuous light and normal air at 15 °C, starting at the end of a night or the end of a day, a circadian rhythm of net uptake of CO_2 was observed. Only when these constant conditions were applied at the end of a day was a circadian rhythm of interconversions between the two forms of PEPCase observed and the rhythms of enzyme interconversion and CO_2 uptake did not correlate in phase or period.

Key words: *Bryophyllum* – Circadian rhythm – Crassulacean acid metabolism – Malate inhibition – Phosphoenolpyruvate carboxylase – Phosphorylation (reversible).

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Abbreviations: CAM = Crassulacean acid metabolism; FW = fresh weight; PEPCase = phosphoenolpyruvate carboxylase; RuBPCase = ribulose-1,5-bisphosphate carboxylase

Introduction

Phosphoenolpyruvate carboxylase (PEPCase; EC 4.1.1.31) plays an important regulatory role in Crassulacean acid metabolism (CAM). At night the enzyme is responsible for the fixation of atmospheric CO_2 into malate. This malate is stored in the vacuole. In order to avoid futile cycling the enzyme must be inactive during the day, when CO_2 is released from malate intracellularly and refixed photosynthetically using ribulose-1,5-bisphosphate carboxylase (RuBPCase; EC 4.1.1.39). No diurnal variation in the amount of PEPCase protein was found immunologically (Brulfert et al. 1982; Nimmo et al. 1984). The enzyme is subject to feedback inhibition by malate and it is widely thought to be regulated by the periodic accumulation of malate in the cytoplasm (for reviews, see Kluge and Ting 1978; Osmond and Holtum 1981). There is considerable evidence that the kinetic properties of PEPCase from CAM plants change reversibly during the diurnal cycle (Greenway et al. 1978; Von Willert et al. 1979; Kluge et al. 1980, 1981a; Manetas 1982; Winter 1980a, 1982; Brulfert and Queiroz 1982; Buchanan-Bollig and Smith 1984). When PEPCase was assayed in rapidly prepared extracts from *Bryophyllum fedtschenkoi* leaves, the enzyme was found to be more sensitive to inhibition by malate during the day than at night. The periods of interconversion between the two forms of the enzyme both occured during the dark period (Nimmo et al. 1984). The interconversions have also been shown to occur sequentially, without an intervening change in the lighting or temperature conditions, in several other CAM plants (Kluge et al. 1981a; Winter 1982; Buchanan-Bollig and Smith 1984). The enzyme from *B. fedtschenkoi*

leaves was shown to be phosphorylated on a serine residue at night and to be dephosphorylated during the day (Nimmo et al. 1984, 1986). Phosphorylation of PEPCase at night has also been detected in three species of *Kalanchoë* (Brulfert et al. 1986).

In day/night conditions CAM plants exhibit a diurnal rhythm of net uptake of CO_2 during the night and net output of respiratory CO_2 during the day. In many cases this periodic uptake of CO_2 has been shown to persist under constant conditions of lighting and temperature. Such persistent circadian rhythms have been studied extensively for *B. fedtschenkoi* in continuous darkness and CO_2-free air (Wilkins 1959, 1960, 1962, 1967, 1973, 1983; Warren and Wilkins 1961) and, more recently, in continuous light and normal air (Wilkins 1984). In *Kalanchoë daigremontiana* in continuous light and normal air a persistent rhythm in the activity of PEPCase, measured in freshly prepared extracts, has been observed (Buchanan-Bollig and Smith 1984) and in *B. fedtschenkoi* in continuous darkness and CO_2-free air a similar persistent rhythm in the activity of PEPCase was observed (Wilkinson and Smith 1976).

In this paper we have examined the kinetic properties of PEPCase in *B. fedtschenkoi* leaves maintained under constant conditions of temperature and either light or darkness. In those cases where the enzyme was found to exhibit a persistent rhythm of interconversion between two forms which differed in their sensitivity to inhibition by malate, we have also examined the phosphorylation state of PEPCase.

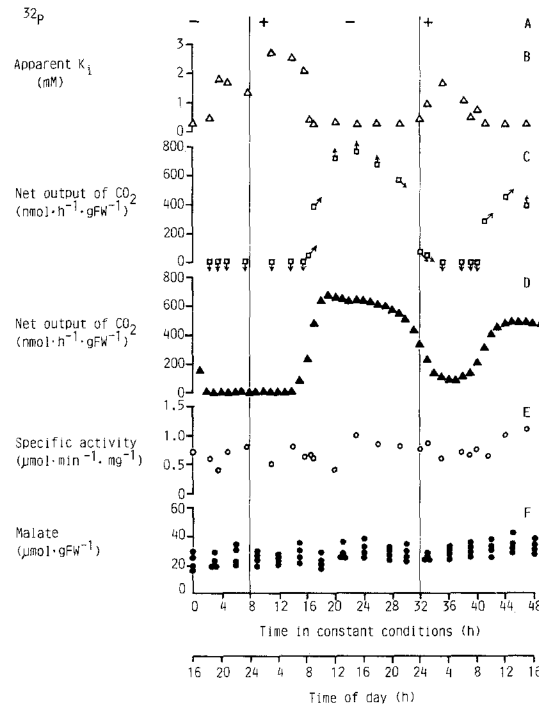

Fig. 1. The malate sensitivity and phosphorylation state of PEPCase in *Bryophyllum fedtschenkoi* leaves transferred to continuous darkness and CO_2-free air at 15 °C after a normal day. The leaves were transferred from the growth room to constant conditions at 16.00 h. Each point represents the measurement for a single leaf. *A.* Phosphorylation state of PEPCase (see **Fig. 2**) phosphorylated (+) dephosphorylated (−). *B.* Apparent K_i for malate of PEPCase (△). *C.* Net output of CO_2 of leaves measured shortly before they were extracted for the PEPCase assays shown in *A*, *B* and *E* (□). The position of the leaves in the rhythm of CO_2 output is shown: no output (↓), increasing output (↗), decreasing output (↘), maximum of a peak of output (↑). *D.* Typical rhythm of net output of CO_2 measured for a single leaf (▲). *E.* Specific activity of PEPCase in the leaves used for the measurements shown in *B* and *C* (○). *F.* Malate content of leaves (●)

Material and methods

Plant material. Plants of *Bryophyllum* (*syn. Kalanchoë*) *fedtschenkoi* Hamet et Perrier were grown and harvested as described by Nimmo et al. (1984). The day in the growth room was from 8.00 to 16.00 h at 27 °C with an average photon fluence rate (400–700 nm) of 80 $\mu mol \cdot m^{-2} \cdot s^{-1}$ provided by a bank of white and warm-white flourescent tubes 75/85 W supplemented with 40 W tungsten lamps. The night temperature was 15 °C.

Maintenance of leaves under constant conditions and measurement of their net rate of output or uptake of CO_2. Detached leaves were removed from the growth room at 8.00 h or 16.00 h as indicated in the text and placed in gas-tight brass containers surrounded by a water jacket maintained at 15 °C as described by Wilkins (1983). The leaves were maintained in continuous light with an average photon fluence rate (400–700 nm) of 15 $\mu mol \cdot m^{-2} \cdot s^{-1}$ provided by white fluorescent tubes or in continuous darkness as indicated. A stream of normal air or CO_2-free air was passed over the leaves at a rate of 1.55 $l \cdot h^{-1}$ and the CO_2 content of the emergent gas was analysed using an infra-red gas analyser as described by Wilkins (1973). Net uptake of CO_2 is represented in the figures as negative net output of CO_2.

Assay of PEPCase. Extracts of leaves were rapidly prepared, desalted (≤10 min) and assayed for PEPCase in the presence and absence of malate (Nimmo et al. 1984). The apparent K_i for malate of PEPCase was estimated as described by Nimmo et al. (1984). The standard assay mixture contained, in 1 ml, 50 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris)-HCl, pH 7.8, 5 mM $MgCl_2$, 2 mM phosphoenolpyruvate, 0.2 mM NADH,10 mM $NaHCO_3$, 10 µg malate dehydrogenase, 20 µl extract. The oxidation of NADH was monitored spectrophotometrically at 25 °C. Specific activity is defined as total enzyme activity ($\mu mol \cdot min^{-1}$) divided by total protein (mg).

Assay of protein. Protein was measured by the method of Bradford (1976).

Assay of malate. Leaves were rapidly pressed through a garlic press and the sap was centrifuged at 11600 *g* for 1 min and assayed for malate by the method of Möllering (1974). This method of extraction gave results identical to those obtained using extraction into perchloric acid (Nimmo et al. 1984).

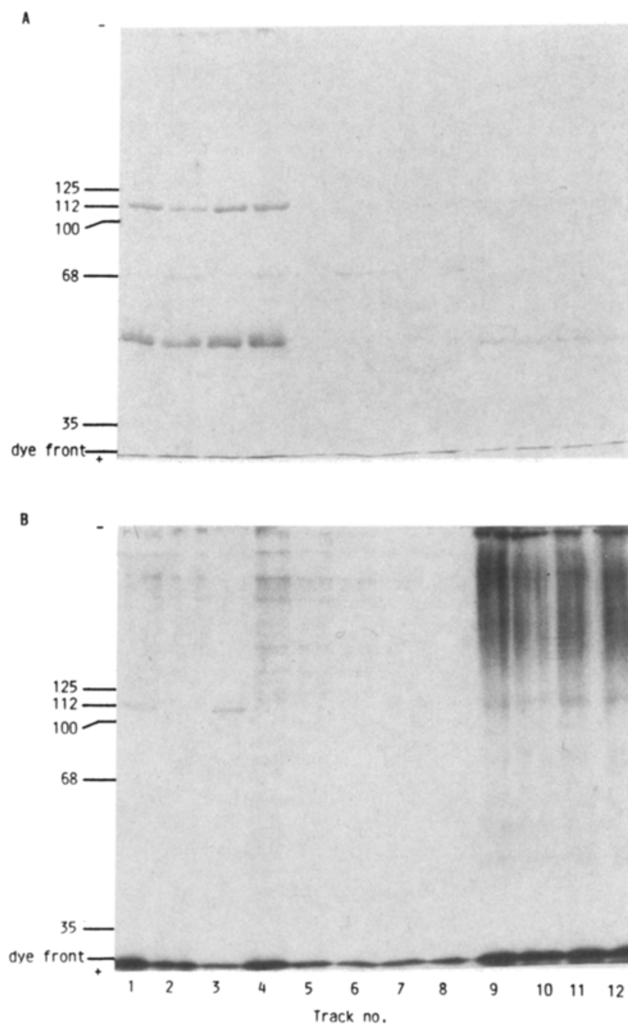

Fig. 2 A, B. The incorporation of ^{32}P into PEPCase in *Bryophyllum fedtschenkoi* leaves maintained at 15 °C in continuous darkness and CO_2-free air. The leaves were labelled with ^{32}P-inorganic phosphate and PEPCase was extracted, immunoprecipitated and analysed for covalently-bound ^{32}P by electrophoresis on a sodium dodecyl sulfate polyacrylamide (8%, w/v) gel as described in methods. **A.** Gel stained for protein with Coomassie Billiant Blue. **B.** Autoradiograph. *Tracks 1–4:* immunoprecipitate from leaf extract containing PEPCase (0.07 μmol·min^{-1}) using anti-PEPCase antiserum. *Tracks 5–8* as *tracks 1–4* but substituting normal rabbit serum for anti-PEPCase antiserum. *Tracks 9–12:* leaf extract containing PEPCase (0.01 μmol·min^{-1}). Extracts and immunoprecipitates were prepared at intervals after the leaves were transferred to constant conditions of darkness and CO_2-free air; *tracks 1, 5, 9:* 33 h; *tracks 2, 6, 10:* 22 h; *tracks 3, 7,11:* 10 h; *tracks 4, 8, 12:* 0 h. The numbers on the left indicate M_r values·10^{-3} of marker proteins; PEPCase has a subunit M_r of 112000 (Nimmo et al. 1986)

Labelling of leaves with ^{32}P and subsequent immunoprecipitation of PEPCase from the leaves. Detached leaves, weighing ~3 g, were each allowed to take up 1.885·10^7 Bq of carrier-free ^{32}P-inorganic phosphate for 48 h whilst they were maintained under normal short-day conditions (Nimmo et al. 1984). They were then transferred to individual leaf chambers and maintained under constant conditions for the times indicated. The radioactive leaves were rapidly extracted as described by Nimmo et al. (1984). Phosphoenolpyruvate carboxylase was immunoprecipitated as described by Nimmo et al. (1986) and the precipitates were analysed for ^{32}P by autoradiography after polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS; Nimmo et al. 1984, 1986).

Results and discussion

Leaves maintained in continuous darkness and CO_2-free air. When detached leaves of *B. fedtschenkoi* were transferred to constant conditions at 16.00 h, at the end of a normal day, and maintained at 15 °C in continuous darkness and a stream of CO_2-free air, they exhibited a persistent periodic net output of CO_2 as described previously (Wilkins 1959). The amplitude of the rhythm was variable but the period and the times of the peaks of output of CO_2 were reproducible from one leaf to another (Fig. 1C, D). The specific activity of PEPCase, measured under the standard assay conditions, was variable from one leaf to another but did not change in a periodic manner (Fig. 1E). Under these conditions the apparent K_i for malate of PEPCase changed in a periodic manner which was in phase with the rhythm of CO_2 output. When CO_2 was being released maximally from the leaves, the apparent K_i for malate of PEPCase was low (0.3 mM) and, when there was a reduced net release of CO_2, PEPCase had a higher apparent K_i for malate (up to 2.7 mM; Fig. 1B). The periods during which PEPCase had a higher apparent K_i for malate and the periods in which the net output of CO_2 was reduced declined in a similar manner. This suggests that the less malate-sensitive form of PEPCase is the physiologically active form and is consistent with the proposal that PEPCase is solely responsible for the circadian rhythm of fixation of CO_2 under these conditions (Warren and Wilkins 1961).

Leaves were labelled with ^{32}P and then maintained in continuous darkness and CO_2-free air at 15 °C as described above. At intervals PEPCase was extracted, immunoprecipitated and examined for covalently-bound ^{32}P. The enzyme was found to contain covalently-bound ^{32}P, presumably in the form of phosphoserine (Nimmo et al. 1986), when its apparent K_i for malate was high and no ^{32}P was detected in PEPCase when its apparent K_i was low (Figs. 1A, 2).

There is a ^{32}P-containing component in the leaf extracts which appears to comigrate with PEPCase on polyacrylamide gel electrophoresis (Fig. 2B, tracks 9–12). This component is clearly not PEP-

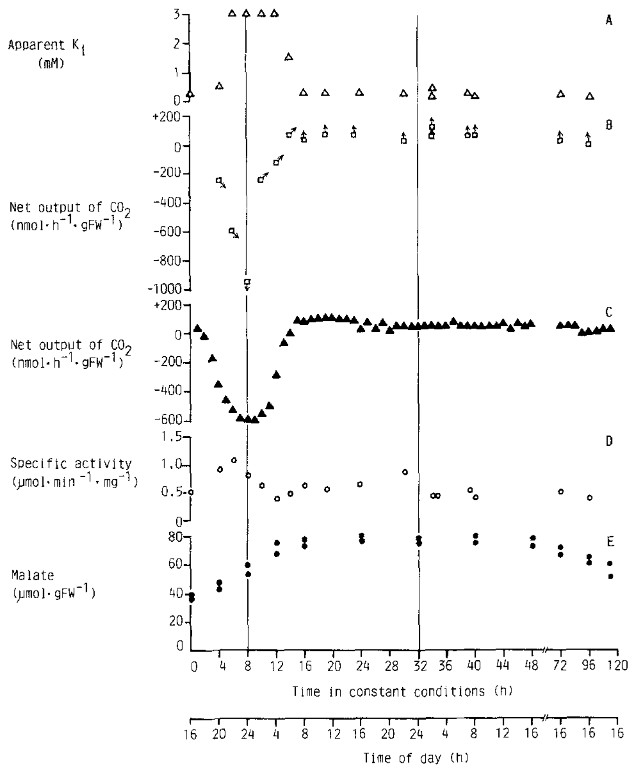

Fig. 3. The malate sensitivity of PEPCase in *Bryophyllum fedtschenkoi* leaves transferred to continuous darkness and normal air at 15 °C after a normal day. The leaves were transferred from the growth room to constant conditions at 16.00 h. Each point represents the measurement for a single leaf. *A*. Apparent K_i for malate of PEPCase (△). *B*. Net output of CO_2 of leaves measured shortly before they were extracted for the PEPCase assays shown in A and D (□). The uptake or output of CO_2 by the leaves is shown: maximum uptake (↓), increasing uptake (↘) decreasing uptake (↗), maximum output (↑). *C*. Typical net uptake and output of CO_2 measured for a single leaf (▲). *D*. Specific activity of PEPCase in the leaves used for the measurements shown in A and B (○). *E*. Malate content of leaves (●)

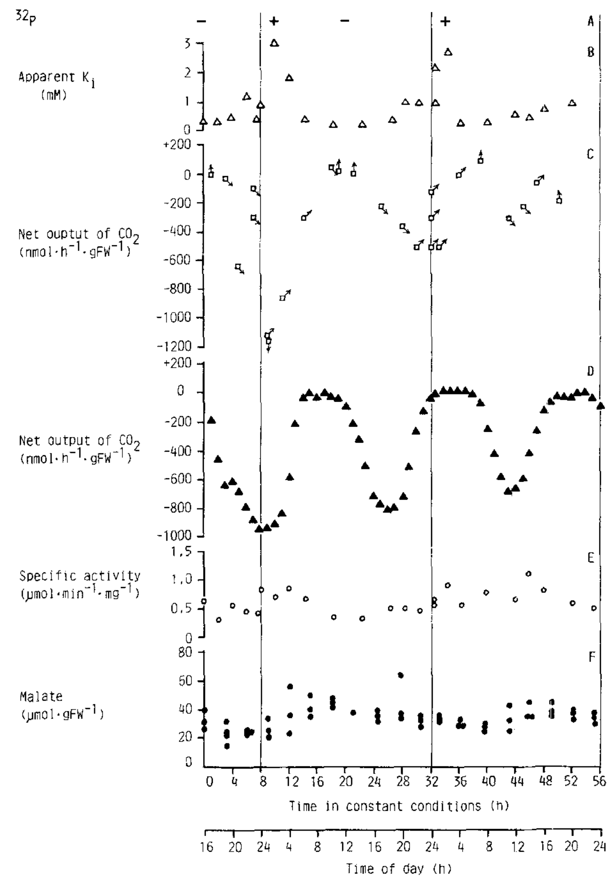

Fig. 4. The malate sensitivity and phosphorylation state of PEPCase in *Bryophyllum fedtschenkoi* leaves transferred to continuous light and normal air at 15 °C after a normal day. The leaves were transferred from the growth room to constant conditions at 16.00 h. Each point represents the measurement for a single leaf. *A*. Phosphorylation state of PEPCase (see **Fig. 5**) phosphorylated (+), dephosphorylated (−). *B*. Apparent K_i for malate of PEPCase (△). *C*. Net output of CO_2 of leaves measured shortly before they were extracted for the PEPCase assays shown in *A*, *B* and *E* (□). The position of the leaves in the rhythm of CO_2 uptake is shown: maximum uptake (↓), decreasing uptake (↗), increasing uptake (↘), minimum uptake (↑). *D*. Typical rhythm of net uptake of CO_2 measured for a single leaf (▲). *E*. Specific activity of PEPCase in the leaves used for the measurements shown in *B* and *C* (○). *F*. Malate content of leaves (●)

Case, since it is much more heavily labelled with ^{32}P and the labelling does not vary during the circadian rhythm. As can be seen (Fig. 2B, tracks 1–4) other ^{32}P-labelled material was immuno-precipitated with PEPCase. Most of this material was probably trapped non-specifically in the precipitates (see Fig. 2B, tracks 5–8). In any case, we have shown by Cleveland mapping that the band of protein in the immunoprecipitate with a subunit M_r of 112000 is identical to purified PEPCase (Nimmo et al. 1986).

The leaves used in these experiments contained ~80 µmol malate·gFW^{-1} at the end of the night (8.00 h) and ~25 µmol malate·gFW^{-1} at the end of the day (16.00 h), when maintained under normal short-day conditions. When the leaves were maintained under constant conditions as described above they accumulated much less malate (≤40 µmol·gFW^{-1}; Fig. 1F) than that accumulated in a normal night. Similar results have been obtained previously (Bollig and Wilkins 1979; Kluge et al. 1981a). The variability of the malate content of *B. fedtschenkoi* leaves (Fig. 1F) made it impossible to detect any small periodic changes in the malate content of the leaves.

When leaves were maintained in darkness and CO_2-free air at 15 °C for several days, the amount of malate in the leaves remained relatively low

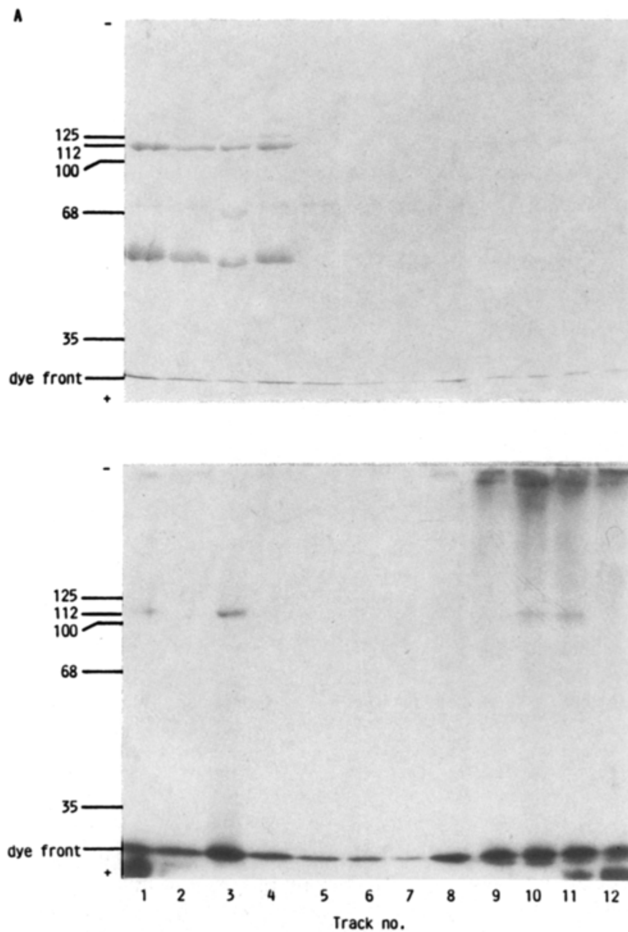

Fig. 5 A, B. The incorporation of ^{32}P into PEPCase in *Bryophyllum fedtschenkoi* leaves maintained at 15 °C in continuous light and normal air. The leaves were labelled with ^{32}P-inorganic phosphate and PEPCase was extracted, immunoprecipitated and analysed for covalently-bound ^{32}P by electrophoresis on an SDS polyacrylamide (8% w/v) gel as described in the *methods*. **A.** Gel stained for protein with Coomassie Brilliant Blue. **B.** Autoradiograph. *Tracks 1–4,* immunoprecipitate from leaf extract containing 0.07 $\mu mol \cdot min^{-1}$ PEPCase using anti-PEPCase antiserum. *Tracks 5–8,* as *tracks 1–4* but substituting normal rabbit serum for anti-PEPCase antiserum. *Tracks 9–12,* leaf extract containing 0.01 $\mu mol \cdot min^{-1}$ PEPCase. Extracts and immunoprecipitates were prepared at intervals after the leaves were transferred to constant conditions; *tracks 1, 5,* **9**: 34 h; *tracks 2, 5, 10:* 20 h; *tracks 3, 6, 11:* 10 h; *tracks 4, 7, 12:* 0 h. The numbers on the left indicate M_r values $\times 10^{-3}$ of marker proteins and PEPCase

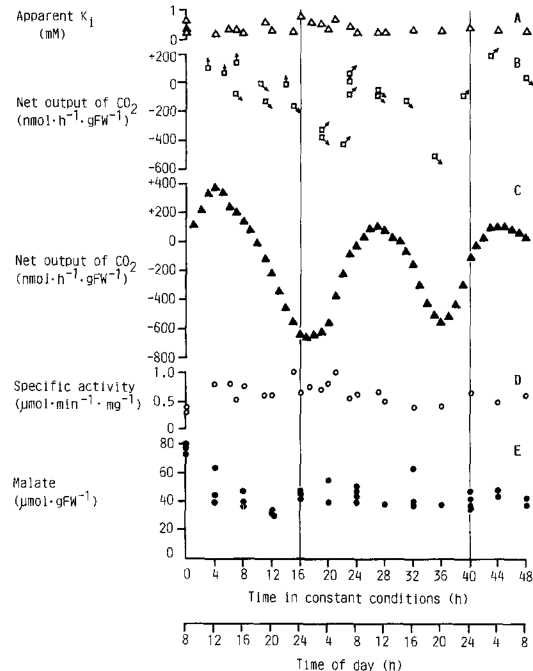

Fig. 6. The malate sensitivity of PEPCase in *Bryophyllum fedtschenkoi* leaves maintained at 15 °C in continuous light and normal air after a normal night. The leaves were transferred from the growth room to constant conditions at 8.00 h. Each point represents the measurement for a single leaf. *A.* Apparent K_i for malate of PEPCase (△). *B.* Net output of CO_2 of leaves measured shortly before they were extracted for the PEPCase assays shown in *A* and *D* (□). The position of the leaves in the rhythm of CO_2 uptake is shown minimum uptake (↑), increasing uptake (↘), decreasing uptake (↗) *C.* Typical rhythm of net uptake of CO_2 measured for a single leaf (▲). *D.* Specific activity of PEPCase in the leaves used for the measurements shown in *A* and *B* (○). *E.* Malate content of leaves (●)

(~30 $\mu mol \cdot gFW^{-1}$), the maximum net rate of output of CO_2 decreased very slowly and the periods in which the CO_2 output was reduced and PEPCase had an increased apparent K_i for malate gradually disappeared. It seems likely that the circadian rhythm of CO_2 uptake dies out because the energy reserves of the leaves have been used up (Osmond 1978) rather than because the vacuoles are completely filled with malate (Wilkins 1984). However, one of the consequences of using up all the energy reserves may be to reduce the transport of malate from the cytoplasm to the vacuole and thus the rhythm may die out because the cytoplasmic malate concentration has risen (Wilkins 1984).

For comparison leaves were also maintained in normal air and continuous darkness at 15 °C starting at 16.00 h. The first 16 h corresponded to a normal night. During the night there was a period during which malate was accumulated, PEPCase was phosphorylated and less sensitive to malate (Nimmo et al. 1984) and there was net uptake of CO_2 (Fig. 3B, C). Thereafter the rate of net output of CO_2 and the amount of malate in the leaves slowly declined over several days of continuous darkness and PEPCase remained in the form with a lower apparent K_i for malate (Fig. 3). Similarly, when leaves were transferred to CO_2-free air and continuous darkness at 15 °C at 8.00 h, at the end of a normal night when the leaves contained a large amount of malate (Nimmo et al. 1984), a steady output of CO_2 was observed which slowly

declined over several days. The maximum net rate of CO_2 output was lower in normal air than in CO_2-free air (Figs. 1, 3).

Leaves maintained in continuous light and normal air. When detached leaves of *B. fedtschenkoi* were transferred to constant conditions at 16.00 h and then maintained at 15 °C in continuous light and a stream of normal air, they exhibited a persistent periodic net uptake of CO_2 (Fig. 4C, D) as described previously (Wilkins 1984). The times of maximum net uptake and the amplitude and period of the rhythm were variable from one leaf to another (Fig. 4C, D). The apparent K_i for malate of PEPCase changed in a periodic manner which was slightly out of phase with the rhythm of net CO_2 uptake (Fig. 4B). The times during which PEPCase had a higher apparent K_i for malate occurred slightly later than the periods of maximum CO_2 uptake (Fig. 4B, C, D) and the period of the rhythm in the apparent K_i of PEPCase (19–24 h) was slightly longer than the period of the rhythm in net CO_2 uptake (16–19 h).

Leaves were labelled with ^{32}P and then maintained at 15 °C in continuous light and normal air as described above and at intervals PEPCase was extracted, immunoprecipitated and examined for covalently-bound ^{32}P. The enzyme PEPCase was found to contain covalently-bound ^{32}P when its apparent K_i for malate was high and no ^{32}P was detected in PEPCase when its apparent K_i was low (Figs. 4A, 5).

When leaves were maintained under the constant conditions described above, they accumulated much less malate ($\leq 50\ \mu mol \cdot gFW^{-1}$; Fig. 4F) than that accumulated in a normal night ($\sim 80\ \mu mol \cdot gFW^{-1}$). Similar results have been obtained with other CAM plants (Kluge and Ting 1978; Winter 1982; Buchanan-Bolling 1984; Buchanan-Bolling and Smith 1984); however, Kluge et al. (1981b) observed that *K. daigremontiana* leaves accumulated similar amounts of malate during a normal night and when maintained in constant light. As described earlier, the malate content of the *B. fedtschenkoi* leaves was very variable and this made it impossible to detect any small periodic changes in the malate content of the leaves (Fig. 4F). Such periodic changes have been observed in other CAM plants maintained in normal air and continuous light (Kluge and Ting 1978; Buchanan-Bollig and Smith 1984). The lighting fluence rate used in these experiments ($15\ \mu mol \cdot m^{-2} \cdot s^{-1}$) may have been sufficiently high to damp the oscillations in the malate concentrations (Buchanan-Bollig 1984).

When leaves were maintained in continuous light and normal air at 15 °C for many days the amount of malate in the leaves remained relatively low ($\leq 50\ \mu mol \cdot gFW^{-1}$), the periods of net uptake of CO_2 slowly declined and the periods in which PEPCase had an increased apparent K_i for malate declined fairly rapidly.

When leaves were transferred to normal air and continuous light at 15 °C at 8.00 h, at the end of a normal night, instead of at 16.00 h, a similar persistent rhythm of uptake of CO_2 was observed (Fig. 6B, C). For the first 8 h under these conditions, the amount of malate in the leaves declined, there was a low rate of net output of CO_2 and the PEPCase remained in the form with a lower apparent K_i for malate (Fig. 6). This behaviour was similar to that observed during a normal day when the temperature was higher (27 °C; Nimmo et al. 1984). The rhythm of CO_2 uptake for leaves transferred to continuous light at 8.00 h had the same period as that observed for leaves transferred at 16.00 h (Figs. 4, 6). The amplitudes of both rhythms varied greatly but on average the rhythms started at 8.00 h had about half the amplitude of rhythms started at 16.00 h (Figs. 4, 6). The peaks of maximum net CO_2 uptake occurred 1-2 h later for the rhythm started at 8.00 h than for the rhythm started at 16.00 h (Figs. 4, 6). Both rhythms of CO_2 uptake persisted for many days. In contrast to the results observed for leaves transferred to continuous light at 16.00 h, little if any change in the apparent K_i for malate of PEPCase was observed in leaves transferred to continuous light at 8.00 h (Fig. 6A). For both rhythms of CO_2 uptake in continuous light, the specific activity of PEPCase, measured under the standard assay conditions, was variable from leaf to leaf but showed no consistent periodic variation (Figs. 4E, 6D). After the first 8 h under constant conditions the concentration of malate in the leaves remained relatively low ($\leq 50\ \mu mol \cdot gFW^{-1}$; Fig. 6E).

In normal air and continuous light at 15 °C the circadian rhythms of net uptake of CO_2 and of conversion of PEPCase to the less malate-sensitive form did not correlate well in phase, period or persistence of the rhythms (Figs. 4, 6). Similarly it has been shown that the rhythm of periodic accumulation of malate in leaves of *Bryophyllum calcynum* maintained in normal air and continuous light declined much more rapidly than the rhythm of periodic net uptake of CO_2 (Kluge and Ting 1978; Nungesser et al. 1984). Under these conditions the leaves have the potential to fix atmospheric CO_2 by the C_3 pathway using RuBPCase and by the CAM pathway using PEPCase pro-

vided that the stomata are open. Several attempts have been made to assess the relative amounts of CO_2 fixed by the C_3 and CAM pathways in the light. In continuous light *Kalanchoë pinnata* leaves fixed atmospheric CO_2 using both pathways as judged by the light-dependence and O_2-sensitivity of CO_2 fixation and the CO_2 compensation point (Winter 1980b). The pattern of incorporation of $^{13}CO_2$ into malate in *Kalanchoë tubiflora* leaves in continuous light at 15 °C was studied and the results indicated that both carboxylases were fixing atmospheric CO_2 directly and that at higher irradiances a greater proportion of the CO_2 was fixed by RuPBCase (Ritz et al. 1986). Similarly, studies on the uptake of $^{14}CO_2$ into malate in *K. daigremontiana* leaves in continuous light indicated that both enzymes were fixing atmospheric CO_2 (Buchanan-Bollig and Smith 1984; Buchanan-Bollig et al. 1984). Several studies have indicated that both enzymes fix atmospheric CO_2 during phase II of the normal day in CAM plants (Kenyon et al. 1981; Fischer and Kluge 1984; Littlejohn and Ku 1984). Kluge and Ting (1978) concluded that low day temperatures favour the activity of both enzymes in phase II. It seems probable, therefore, that the CO_2 fixation rhythms in continuous light shown in Figs. 4 and 6 reflect the activity of RuBPCase and PEPCase.

Conclusions

The results shown in Figs 1, 2, 4, 5 demonstrate that persistent, circadian rhythms in the properties of PEPCase occur in *B. fedtschenkoi* leaves maintained under some but not all constant conditions. Rhythms were observed in the reversible phosphorylation of PEPCase and in the interconversion of PEPCase between two forms which differ in their sensitivity to inhibition by malate. This is in agreement with earlier studies which showed that a light/dark transition was not necessary for the interconversion of the two kinetically distinct forms of PEPCase (Kluge et al. 1981a; Winter 1982; Wilkinson and Smith 1976; Buchanan-Bollig and Smith 1984; Nimmo et al. 1984).

Under constant conditions in continuous light when *B. fedtschenkoi* leaves were exhibiting persistent circadian rhythms of uptake of CO_2, there was no direct correlation between the circadian rhythms of CO_2 uptake and of interconversion of PEPCase between two forms (Figs. 4, 6). Indeed, when the constant conditions were started at the end of the night, no interconversion of PEPCase was observed, whereas a persistent rhythm of CO_2 uptake was clearly apparent (Fig. 6).

In all cases where the phosphorylation state of PEPCase has been examined, the form of the enzyme which was more sensitive to inhibition by malate was dephosphorylated and the form which was less sensitive to inhibition by malate was phosphorylated (Figs. 1, 2, 4, 5; see also Nimmo et al. 1984). These results suggest that the phosphorylation of the enzyme causes a reduction in its sensitivity to inhibition by malate. Direct confirmation of this will require the interconversion of the two forms of the enzyme in vitro using isolated PEPCase kinase and PEPCase phosphatase. We have shown that purified phosphorylated PEPCase from *B. fedtschenkoi* can be dephosphorylated by alkaline phosphatase in vitro and that this dephosphorylation is accompanied by a decrease in the apparent K_i of PEPCase for malate (Nimmo et al. 1986). A similar result was obtained using acid phosphatase and PEPCase from *Kalanchoë blossfeldiana* (Brulfert et al. 1986).

Both forms of PEPCase had similar specific activities when extracts were assayed in the absence of malate (Figs. 1, 3, 4; see also Nimmo et al. 1984). However, it seems probable that in vivo the concentration of malate in the environment of PEPCase is normally sufficiently high that the less malate-sensitive form of PEPCase is considerably more active than is the more malate-sensitive form. A similar proposal was made for PEPCase from *Mesembryanthemum crystallinum* (Winter 1982). Both forms of the enzyme may also be regulated by changes in the concentration of malate in their immediate environment. The concentration range which inhibited the less sensitive form was about 10 times higher than that which inhibited the more sensitive form (Figs. 1, 3, 4; see also Nimmo et al. 1984). In order to assess the relative contribution of feedback inhibition and phosphorylation to the regulation of PEPCase under any given set of circumstances, it will be necessary to measure the concentrations of malate which are experienced by PEPCase in vivo.

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References

Bollig, I.C., Wilkins, M.B. (1979) Inhibition of the circadian rhythm of CO_2 metabolism in *Bryophyllum* leaves by cycloheximide and dinitrophenol. Planta **145**, 105–112

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. Anal. Biochem. **72**, 248–254

Brulfert, J., Queiroz, O. (1982) Photoperiodism and Crassulacean acid metabolism. III. Different characteristics of the photoperiod-sensitive and non-sensitive isoforms of phos-

phoenolpyruvate carboxylase and Crassulacean acid metabolism operation. Planta **184**, 339–343

Brulfert, J., Vidal, J., Gadal, P., Queiroz, O. (1982) Daily rhythm of phosphoenolpyruvate carboxylase in Crassulacean acid metabolism plants. Immunological evidence for the absence of a rhythm in protein synthesis. Planta **156**, 92–94

Brulfert, J., Vidal, J., Le Marechal, P., Gadal, P., Queiroz, O., Kluge, M., Kruger, I. (1986) Phosphorylation-dephosphorylation process as a probable mechanism for the diurnal regulatory changes of phosphoenolpyruvate carboxylase in CAM plants. Biochem. Biophys. Res. Commun. **136**, 151–159

Buchanan-Bollig, I.C. (1984) Circadian rhythm in *Kalanchoë:* effects ot irradiance and temperature on gas exchange and carbon metabolism. Planta **160**, 264–271

Buchanan-Bollig, I.C., Fischer, A., Kluge, M. (1984) Circadian rhythms in *Kalanchoë:* the pathway of $^{14}CO_2$ fixation during prolonged light. Planta **161**, 71–80

Buchanan-Bollig, I.C., Smith, J.A.C. (1984) Circadian rhythms in Crassulacean acid metabolism: phase relationships between gas exchange, leaf water relations and malate metabolism in *Kalanchoë daigremontiana.* Planta **161**, 314–319

Fischer, A., Kluge, M. (1984) Studies on carbon flow in Crassulacean acid metabolism during the initial light period. Planta **160**, 121–128

Greenway, H., Winter, K., Lüttge, U. (1978) Phosphoenolpyruvate carboxylase during development of Crassulacean acid metabolism and during a diurnal cycle in *Mesembryanthemum crystallinum.* J. Exp. Bot. **29**, 547–559

Kenyon, W.H., Holaday, A.S., Black, C.C. (1981) Diurnal changes in metabolite levels and Crassulacean acid metabolism in *Kalanchoë daigremontiana* leaves. Plant Physiol. **68**, 1002–1007

Kluge, M., Böcher, M., Jungnickel, G. (1980) Metabolic control of Crassulacean acid metabolism: evidence for diurnally changing sensitivity against inhibition by malate of PEP-carboxylase in *Kalanchoë tubiflora* Hamet. Z. Pflanzenphysiol. **97**, 197–204

Kluge, M., Böhlke, C., Queiroz, O. (1981b) Crassulacean acid metabolism (CAM) in *Kalanchoë:* changes in intercellular CO_2 concentration during a normal CAM cycle and during cycles in continuous light or darkness. Planta **152**, 87–92

Kluge, M., Brulfert, J., Queiroz, O. (1981a) Diurnal changes in the regulatory properties of PEP-carboxylase in Crassulacean acid metabolism (CAM). Plant Cell Environ. **4**, 251–256

Kluge, M., Ting, I.P. (1978) Crassulacean acid metabolism. Analysis of an ecological adaptation. Springer, Berlin Heidelberg New York

Littlejohn, R.O., Ku, M.S.B. (1984) Characterisation of early morning Crassulacean acid metabolism in *Opuntia erinacea* var. *Columbiana* (Griffiths) L. Benson. Plant Physiol. **74**, 1050–1054

Manetas, Y. (1982) Changes in properties of PEP-carboxylase from the CAM plant *Sedum prealtum* DC upon dark/light transition and their stabilisation by glycerol. Photosynth. Res. **3**, 321–333

Möllering, H. (1974) L-(−)-Malate. Determination with MDH and GOT. In: Methods of enzymatic analysis, 2nd edn., vol. 3, pp. 1589–1593, Bergmeyer, H.U., ed. Academic Press, New York

Nimmo, G.A., Nimmo, H.G., Fewson, C.A., Wilkins, M.B. (1984) Diurnal changes in the properties of phosphoenolpyruvate carboxylase in *Bryophyllum* leaves: a possible covalent modification. FEBS. Lett. **178**, 199–203

Nimmo, G.A., Nimmo, H.G., Hamilton, I.D., Fewson, C.A., Wilkins, M.B. (1986) Purification of the phosphorylated night form and dephosphorylated day form of phosphoenolpyruvate carboxylase from *Bryophyllum fedtschenkoi.* Biochem. J. **239**, 213–220

Nungesser, D., Kluge, M., Tolle, H., Oppelt, W. (1984) A dynamic computer model of the metabolic and regulatory processes in Crassulacean acid metabolism. Planta **162**, 204–214

Osmond, C.B. (1978) Crassulacean acid metabolism: a curiosity in context. Annu. Rev. Plant Physiol **29**, 379–414

Osmond, C.B., Holtum, J.A.M. (1981) Crassulacean acid metabolism. In: The biochemistry of plants, vol. 8, pp. 283–328, Hatch, M.D., Boardman, N.K., eds. Academic Press, London New York

Ritz, D., Kluge, M., Veith, H.J. (1986) Mass-spectrometric evidence for the double-carboxylation pathway of malate synthesis by Crassulacean acid metabolism plants in light. Planta **167**, 284–291

von Willert, D.J., Brinckmann, E., Scheitler, B., Thomas, D.A., Treichel, S. (1979) The activity and malate inhibition/stimulation of phosphoenolpyruvate carboxylase in Crassulacean acid metabolism plants in their natural environment. Planta **147**, 31–36

Warren, D.M., Wilkins, M.B. (1961) An endogenous rhythm in the rate of dark-fixation of carbon dioxide in leaves of *Bryophyllum fedtschenkoi.* Nature **191**, 686–688

Wilkins, M.B. (1959) An endogeneous rhythm in the rate of carbon dioxide output of *Bryophyllum.* I. Some preliminary experiments. J. Exp. Bot. **10**, 377–390

Wilkins, M.B. (1960) An endogeneous rhythm in the rate of carbon dioxide output of *Bryophyllum.* II. The effects of light and darkness on the phase and period of the rhythm. J. Exp. Bot. **11**, 269–288

Wilkins, M.B. (1962) An endogenous rhythm in the rate of carbon dioxide output of *Bryophyllum.* III. The effects of temperature on the phase and period of the rhythm. Proc. R. Soc. London B **156**, 220–241

Wilkins, M.B. (1967) An endogenous rhythm in the rate of carbon dioxide output of *Bryophyllum.* IV. The dependence of rhythmicity on aerobic metabolism. Planta **72**, 66–77

Wilkins, M.B. (1973) An endogenous cicadian rhythm in the rate of carbon dioxide output of *Bryophyllum.* VI. Action spectrum for the induction of phase shifts by visible radiation. J. Exp. Bot. **24**, 488–496

Wilkins, M.B. (1983) The circadian rhythm of carbon-dioxide metabolism in *Bryophyllum:* the mechanism of phase-shift induction by thermal stimuli. Planta **157**, 471–480

Wilkins, M.B. (1984) A rapid circadian rhythm of carbon-dioxide metabolism in *Bryophyllum fedtschenkoi.* Planta **161**, 381–384

Wilkinson, M.J., Smith, H. (1976) Properties of phosphoenolpyruvate carboxylase from *Bryophyllum fedtschenkoi* leaves and fluctations in carboxylase activity during the endogenous rhythm of carbon dioxide output. Plant Sci. Lett. **6**, 319–324

Winter, K. (1980a) Day/night changes in the sensitivity of phosphoenolpyruvate carboxylase to malate during Crassulacean acid metabolism. Plant Physiol. **65**, 792–796

Winter, K. (1980b) Carbon dioxide and water vapour exchange in the Crassulacean acid metabolism plant *Kalanchoë pinnata* during a prolonged light period. Metabolic and stomatal control of carbon metabolism. Plant Physiol. **66**, 917–921

Winter, K. (1982) Properties of phosphoenolpyruvate carboxylase in rapidly prepared, desalted leaf extracts of the Crassulacean acid metabolism plant *Mesembryanthemum crystallinum* L. Planta **154**, 298–308

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