Diurnal changes in the properties of phosphoenolpyruvate carboxylase in *Bryophyllum* leaves: a possible covalent modification

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In plants that show Crassulacean acid metabolism, phosphoenolpyruvate carboxylase catalyses the key step of CO_2 fixation at night. We show here that the properties of this enzyme from *Bryophyllum fedtschenkoi* undergo marked changes between night and day; the night form is much less sensitive to feedback inhibition by malate than is the day form. Incubation of leaves with $^{32}P_i$ followed by extraction and immunoprecipitation of phosphoenolpyruvate carboxylase showed that only the night form contained ^{32}P. This suggests that the activity of the enzyme is controlled by a covalent modification mechanism.

Crassulacean acid metabolism Bryophyllum (Kalanchoë) fedtschenkoi *Diurnal rhythm*
Phosphoenolpyruvate carboxylase *Covalent modification* *Malate inhibition*

1. INTRODUCTION

Plants which have Crassulacean acid metabolism show diurnal rhythms of CO_2 fixation. At night they fix CO_2 into malate via phosphoenolpyruvate carboxylase (EC 4.1.1.31, PEPc) and during the day the CO_2 is released from malate and refixed photosynthetically. To avoid futile cycling, PEPc must be inactivated during the day. There is considerable evidence that the enzyme can be inhibited by malate and the periodic accumulation of malate in the cytoplasm may regulate the enzyme activity [1–4]. No periodic change in total enzyme protein has been found immunologically [5]. There have been reports of diurnal changes in the specific activity of the enzyme and in its sensitivity to inhibition by malate when assayed in crude extracts of several Crassulacean acid metabolism plants [6–8]. Some Crassulacean acid metabolism plants exhibit circadian rhythms of CO_2 fixation when maintained under constant environmental conditions and in some of these cases circadian changes in the specific activity of PEPc have been observed [8,9]. The mechanisms responsible for these diurnal and circadian changes in activity are not known.

Bryophyllum fedtschenkoi shows both diurnal and circadian rhythms of fixation of CO_2 into malate via PEPc [10–12]. In a previous paper from this laboratory it was reported that no diurnal variation in the specific activity of PEPc in *B. fedtschenkoi* was observed [13]. In contrast, circadian variations in specific activity of the same enzyme in constant darkness and CO_2-free air have been reported [9]. This paper reports diurnal variations in the malate sensitivity of PEPc from *B. fedtschenkoi* and provides preliminary evidence that reversible covalent modification may be responsible for the variation.

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Abbreviation: PEPc, phosphoenolpyruvate carboxylase

2. EXPERIMENTAL

2.1. *Materials*

Bovine serum albumin (fraction V) was obtained

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from Sigma, β-galactosidase (*Escherichia coli*) and lactate dehydrogenase (rabbit muscle) from Boehringer, V8 protease from Miles and chymotrypsin from Worthington. Fatty acid synthase was purified from lactating rabbit mammary gland [14] and PEPc was purified from *B. fedtschenkoi* [15]. An ammonium sulphate precipitate of rabbit antiserum raised against purified PEPc from *Sorghum* [16] was a gift from Professor P. Gadal and Dr J. Vidal. Donkey anti-rabbit IgG antiserum and normal rabbit serum were obtained from the Scottish Antibody Production Unit, Carluke, Lanarkshire. $^{32}P_i$ (carrier free) was obtained from Amersham International. The sources of other materials were as given in [13,15].

2.2. *Plants*

B. (Kalanchoë) fedtschenkoi Hamet et Perrier plants were grown under long day conditions and then transferred to a short day growth room [15]. The light period was from 08.00 to 16.00 h and the temperature was 28°C during the day and 16°C at night. Plants spent 6–12 weeks under short day conditions before use. Similar sized and aged leaves, 6–8 pairs from the apex of the shoot, were used.

2.3. *Assays*

PEPc activity was assayed spectrophotometrically [15]. The standard assay mixture contained, in 1 ml, 50 mM Tris–HCl, 5 mM $MgCl_2$, 2 mM phosphoenolpyruvate, 0.2 mM NADH, 10 mM sodium bicarbonate, 5 units malate dehydrogenase (pH 7.8) and 0.02 ml extract (added last). Under these conditions the enzyme rate was linear for at least 30 min. One unit is the amount of enzyme required to catalyse the production of 1 μmol oxaloacetate per min.

For malate determinations, leaves were chopped up with scissors and homogenised for 90 s in 4 vols 6% (v/v) ice-cold perchloric acid with 2–3 drops octan-1-ol in a 25 ml capacity MSE Atomix blender. Leaf debris was removed by centrifugation at 11600 × *g* for 2 min. The perchlorate was precipitated and malate was measured as in [17].

Protein was measured as in [18].

2.4. *Estimation of inhibition of PEPc by malate during the diurnal cycle*

Eight assays were carried out almost simultaneously using concentrations of malate of 0, 0.2, 0.5, 0.7, 1.0, 2.0, 3.0 and 5.0 mM. The rates were linear 5 min after the addition of the extract [15] and the linear part of the rate was measured. The concentration of malate required for 50% inhibition of PEPc was estimated from a plot of rate *vs* [malate].

2.5. *Labelling with ^{32}P*

For each experiment one leaf was removed from a plant at 03.00 h and another at 11.00 h and each was placed with its petiole resting in a separate container with 0.05–0.10 ml (0.5 mCi) carrier-free $^{32}P_i$. Each leaf was left in the growth room for 24 h by which time it had taken up all the phosphate. It was then given 0.2 ml H_2O and left for a further 24 or 48 h before extraction.

2.6. *Preparation of leaf extracts*

Leaves were chopped up with scissors and homogenised in 5 vols (^{32}P and antibody experiments) or 10 vols (all other experiments) of 100 mM Tris–HCl, 2 mM EDTA, 2% (w/v) polyethylene glycol 20000, 1 mM dithiothreitol (pH 7.5), ('extraction buffer') with 2–3 drops octan-1-ol in a 100 ml capacity MSE Atomix blender for 1 min at full speed (^{32}P experiments) or in a 100 ml capacity Waring blender for 20 s at low speed (all other experiments). The homogenate was filtered through muslin and centrifuged for 2 min at 11600 × *g*. The supernatant was used immediately or after gel filtration into extraction buffer using Sephadex G-25M. Preparation of extracts took no more than 4 min and of desalted extracts 10 min.

2.7. *Immunoprecipitation*

Samples of undesalted extract were mixed with 5 M NaCl and 10% (v/v) Triton X-100 to give final concentrations of 1.5 M and 1%, respectively. They were then mixed with rabbit anti-PEPc antiserum and left in ice for 15 min, incubated for a further 30 min in ice with donkey anti-rabbit IgG antiserum and then centrifuged for 2 min at 11600 × *g*. The precipitates were washed with 1 ml of 1.5 M NaCl, 2 mM EDTA (pH 7.0). Controls were carried out using normal rabbit serum instead of the rabbit anti-PEPc antiserum.

2.8. *Polyacrylamide gel electrophoresis*

8% polyacrylamide slab gels were run in the presence of SDS as in [19]. Autoradiography was carried out using Kodak X-OMAT AR film with an intensifying screen. Antibody precipitates were redissolved in 8 M urea, 1% (v/v) 2-mercaptoethanol overnight at room temperature before SDS sample buffer was added. The molecular mass marker proteins were: fatty acid synthase (250 kDa), β-galactosidase (125 kDa), serum albumin (68 kDa) and lactate dehydrogenase (35 kDa). Limited proteolysis and peptide mapping were carried out as in [20] using chymotrypsin and V8 protease.

3. RESULTS AND DISCUSSION

3.1. *Sensitivity of PEPc to inhibition by malate during the diurnal cycle of light and darkness*

The sensitivity of PEPc to inhibition by malate was examined in freshly prepared and desalted extracts of *B. fedtschenkoi* leaves. There was a marked difference in the sensitivity of the enzyme extracted between 07.00 and 20.00 h and that extracted between 22.00 and 04.00 h (fig.1,2). At 07.00–20.00 h the enzyme showed hyperbolic inhibition by malate with an apparent K_i of 0.3 mM under the assay conditions. In contrast, at 21.00–04.00 h the enzyme was much less sensitive

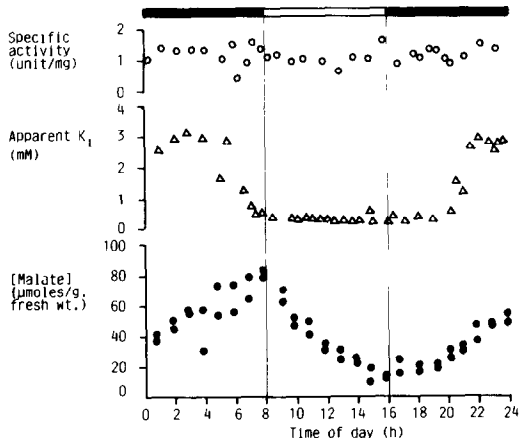

Fig.1. Diurnal rhythm in the malate sensitivity of PEPc in *B. fedtschenkoi*. The specific activity (○) and apparent K_i for malate (△) of PEPc and the malate content (●) were measured in leaves extracted throughout the day as described in section 2. Each point represents a single leaf. PEPc assays were carried out on desalted extracts. Dark period (▬), light period (▭).

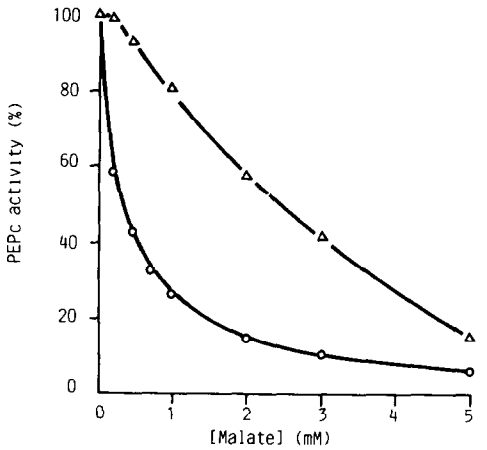

Fig.2. Inhibition by malate of PEPc extracted during the day and during the night. Enzyme activity is expressed as a percentage of the activity in the absence of malate. 100% represents 0.002 unit/ml. Extracts prepared at 11.00 h (○) and 23.00 h (△).

to malate inhibition, the inhibition was sigmoid and the concentration of malate required for 50% inhibition was 2.5–3.0 mM (fig.2). The total PEPc activity, measured with saturating substrates and in the absence of malate, was unchanged during the diurnal cycle (fig.1), in agreement with previous results for *B. fedtschenkoi* [13]. The results shown in fig.1 are in general agreement with those obtained for *M. crystallinum* [7].

The period of conversion of the enzyme from the less sensitive, night form to the more sensitive (and presumably less active in vivo), day form coincided with the cessation of malate accumulation and the period of conversion from the day form to the night form coincided with the onset of malate accumulation by the leaves. Both conversions occurred during the dark period. This presumably reflects the fact that under constant conditions of darkness and temperature, in CO_2-free air, *B. fedtschenkoi* has an endogenous circadian rhythm of CO_2 fixation via its Crassulacean acid metabolic pathway and PEPc [10–12]. The results shown in fig.1 suggest that the interconversion of the enzyme between the two kinetically distinct forms is controlled by the endogenous rhythm rather than directly by light.

These results (fig.1,2) appear to contrast with an earlier report that there was no difference in the malate sensitivity of PEPc extracted from *B. fedtschenkoi* at night and during the day [13].

However, in the earlier work, the malate sensitivity was measured only at 12.00 and 18.00 h and fig.1 shows that the enzyme is not converted to the night form until 20.00 h. A similar delay in conversion from the day to night form after the light has been switched off was observed for *M. crystallinum* [7]. For *Kalanchoe diagremontiana* a difference in the specific activities of the day and night forms of PEPc has been reported. Both interconversions, from day to night form and from night to day form, occurred during the light period [8].

The malate sensitivity of PEPc declined slowly as the extract aged but this decline was not observed in the presence of the assay mixture, in agreement with the results for *M. crystallinum* [21]. This effect of ageing appears to be less rapid in *B. fedtschenkoi* than in *M. crystallinum*. No significant differences in malate sensitivity or specific activity of the enzyme were observed between fresh extracts and desalted extracts, assayed 4 and 10 min after extraction, respectively. It is therefore unlikely that the lower sensitivity of the enzyme extracted at night is due to much more rapid ageing of night extracts compared with day extracts. At 02.00 h PEPc is in the night form and at 11.00 h it is in the day form, yet the concentration of malate is the same at these times (fig.1). Mixing a night extract and a day extract did not cause interconversion of either form of the enzyme. Clearly, the two forms of PEPc do not arise after extraction as a consequence of the considerable variation in the concentration of malate.

3.2. *Immunoprecipitation of PEPc and labelling with ^{32}P*

Previous studies in *K. blossfeldiana* using immunoprecipitation of PEPc showed that there was no change in the total enzyme protein during the diurnal cycle [5]. Similarly, the precipitation of PEPc activity from day and night extracts of *B. fedtschenkoi* leaves, using antiserum raised against *Sorghum* PEPc, gave identical immunotitration curves (not shown).

PEPc was precipitated from extracts of two leaves prelabelled with $^{32}P_i$. One extract was prepared during the day and the enzyme was shown to have an apparent K_i of 0.3 mM and the other was prepared at night when the enzyme had an apparent K_i of 3 mM. The precipitates were analysed by SDS slab gel electrophoresis. Very similar amounts of a protein of subunit M_r 117000 were precipitated in both extracts, as judged by staining with Coomassie brilliant blue (fig.3a, tracks 4 and 5). This protein band was shown to be PEPc by comparing the patterns of proteolytic fragments obtained for the 117-kDa protein band and for purified PEPc by the method in [20] (not shown).

Only the precipitated enzyme from the night extract contained ^{32}P (fig.3b, tracks 4 and 5). No ^{32}P or protein appeared in this position on the gel from

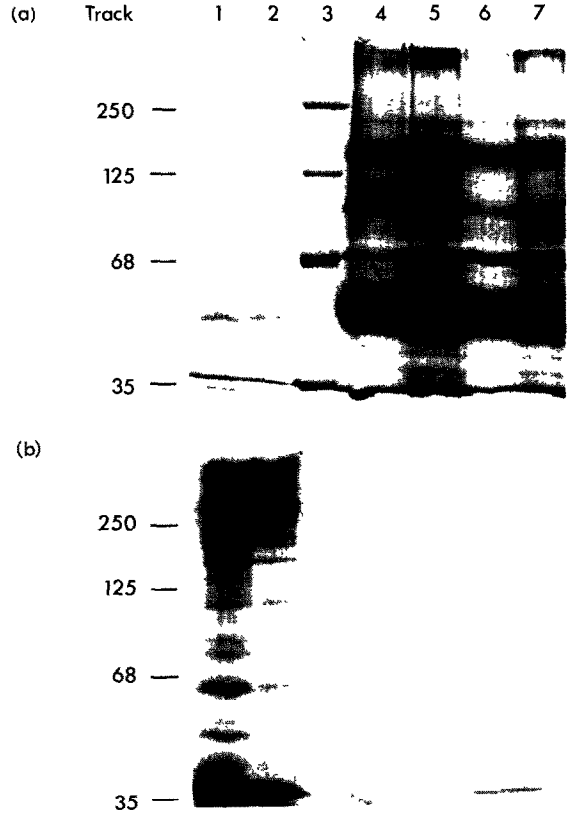

Fig.3. SDS-polyacrylamide gel electrophoresis of extracts from ^{32}P-labelled leaves. The leaves were incubated for 72 h with ^{32}P before extraction. The day extract was prepared at 13.00 h and the night extract at 02.45 h. (a) Coomassie brilliant blue stain, (b) autoradiograph. Track 1, day extract containing 0.01 unit PEPc. Track 2, night extract containing 0.01 unit PEPc. Track 3, marker proteins. Track 4, immunoprecipitate from day extract containing 0.07 unit PEPc using anti-PEPc antiserum. Track 5, immunoprecipitate from night extract containing 0.07 unit PEPc using anti-PEPc antiserum. Tracks 6 and 7, as tracks 4 and 5 but substituting normal rabbit serum for anti-PEPc antiserum. The numbers on the left indicate M_r values $\times 10^{-3}$ of the marker proteins.

control precipitates using normal rabbit serum (fig.3a,b, tracks 6 and 7). The total time of incubation with ^{32}P and the time spent in the dark and in the light were very similar for the two leaves. The general distribution of labelling in the two extracts (fig.3b, tracks 1 and 2) was similar. The day extract was slightly more heavily labelled than the night extract; since, in contrast, PEPc was only labelled in the night extract, this emphasises that the difference in the labelling of PEPc was not due to the different order of light and dark periods experienced by the two leaves whilst they were incubated with ^{32}P. The difference in the labelling of the PEPc between the night and day extracts could have arisen without any change in the amount of the phosphorus-containing group attached to the enzyme, if the specific radioactivity of the precursor was much higher at night. This parameter cannot yet be measured because the nature of the precursor is unknown. However, experiments have been carried out in which leaves were incubated with ^{32}P for 2 and 3 days and the labelling patterns were the same in both cases. This suggests that the precursor had reached equilibrium with the ^{32}P within 2 days. It could be argued that, if the catalytic mechanism of PEPc were to involve a phosphoenzyme intermediate, then the presence of ^{32}P in the night form of the enzyme might merely indicate that this form, being less sensitive to malate inhibition, is active in vivo whereas the day form is not. However, current views on the mechanism of the enzyme suggest that it does not involve a phosphoenzyme intermediate (review [22]).

It seems likely, therefore, that the differences in the malate sensitivity of PEPc extracted during the day and night are due to a reversible covalent modification of the enzyme, involving the incorporation of a phosphorus-containing group to form the less sensitive, night form of the enzyme. Confirmation that covalent modification can change the activity of PEPc will require purification of the modified form of the enzyme or labelling of purified enzyme in vitro. An endogenous circadian rhythm of fixation of respiratory CO_2 into malate via PEPc occurs when leaves of *B. fedtschenkoi* are kept in constant darkness in CO_2-free air [10–12]. A preliminary experiment indicates that a reversible covalent incorporation of ^{32}P into PEPc also occurs during this circadian rhythm.

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