Volume 213, number 1, 18–22 FEB 04477 March 1987

Changes in the kinetic properties and phosphorylation state of phospho*enol*pyruvate carboxylase in *Zea mays* leaves in reponse to light and dark

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Received 12 December 1986

In plants such as *Zea mays* that carry out C_4 metabolism, phospho*enol*pyruvate carboxylase catalyses the primary fixation of atmospheric CO_2. The properties of this enzyme from *Z. mays* leaves kept in light and in darkness are different. In brightly illuminated leaves, which are actively fixing CO_2, the enzyme is less sensitive to feedback inhibition by malate and is phosphorylated on one or more serine residues. In darkened leaves, which are not photosynthesising, the enzyme is more sensitive to inhibition by malate and is much less phosphorylated. This indicates that the activity of the enzyme is controlled by a reversible phosphorylation.

C_4 photosynthesis; Light/dark regulation; Phosphorylation; Phospho*enol*pyruvate carboxylase; Malate inhibition; *(Zea mays)*

1. INTRODUCTION

Phospho*enol*pyruvate carboxylase (EC 4.1.1.31, PEPc) catalyses the key step in the fixation of atmospheric CO_2 into malate in the mesophyll cells of plants which carry out C_4 photosynthesis. The malate is transported to the bundle sheath cells and decarboxylated. The CO_2 is then refixed in the photosynthetic carbon reduction cycle. For C_4 plants, this enzyme is in a more active form in extracts from illuminated leaves than from leaves in darkness, as indicated by changes in V_{max}, sensitivity to feedback inhibition by malate or activation by glucose 6-phosphate [1–4]. This activation was distinct from the light stimulated de novo synthesis of PEPc which has been observed in many C_4 plants [4].

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Crassulacean acid metabolism (CAM) plants use PEPc to fix atmospheric CO_2 into malate at night. They then release and refix the CO_2 photosynthetically during the day. In several CAM plants it has been shown that PEPc occurs in a more active, phosphorylated form at night and as a less active, unphosphorylated form during the day [5–7]. Here, we demonstrate that the enzyme in a C_4 plant is also regulated by phosphorylation.

2. EXPERIMENTAL

2.1. *Materials*

Sorbitol was obtained from BDH. Control rabbit serum and rabbit antiserum raised against purified PEPc from *Bryophyllum fedtschenkoi* were obtained as described in [6]. PEPc was purified to homogeneity from *Zea mays* leaves by chromatography on Bio-Gel HTP and Mono Q. The sources of other materials were as given in [5,6,8].

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/87/$3.50

2.2. *Plants*

Z. mays var. Goal plants were grown in a greenhouse under long-day conditions for 4–6 weeks and then transferred to a short-day growth room for 1–3 weeks before use. The 8 h light period was at 27°C and 80 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (400–700 nm) and the dark period was at 15°C.

Detached leaves were illuminated by tungsten halogen headlamps (12 V, 55 W, H3 bulbs). The light was passed through 15 cm water to reduce heating effects.

2.3. *Assays of PEPc*

PEPc activity was assayed spectrophotometrically at 25°C. The standard assay mixture contained, in 1 ml, 50 mM Hepes-NaOH (pH 7.0), 10 mM $MgCl_2$, 10 mM bicarbonate, 0.2 mM NADH, 5 U malate dehydrogenase, 0.5 mM phospho*enol*pyruvate, 5 mM glucose 6-phosphate and 0.05 ml extract (added last). One unit is the amount of enzyme required to catalyse the production of 1 μmol oxaloacetate per min.

The enzyme gave nonlinear progress curves; the rate accelerated for the first few minutes, particularly for extracts of illuminated leaves, aged extracts and extracts prepared with liquid N_2. In all cases the final linear rate was measured. Addition of lactate dehydrogenase (5 U) to some assays showed that the non-enzymic decarboxylation of oxaloacetate [9] was not significant.

The apparent K_i for malate was estimated as in [5].

2.4. *Labelling with ^{32}P*

Detached portions of leaf (0.4 g) were allowed to take up carrier-free ^{32}P (40 μCi). They were then left resting in H_2O for 24 or 48 h in the short-day growth room before any light treatment and extraction.

2.5. *Preparation of leaf extracts*

The extraction procedure was based upon that of Huber and Sugiyama [4]. For most experiments the leaves were chopped up with scissors and homogenised at 5°C in 4 vols 0.1 M Tris-HCl, pH 7.0 (at 25°C), 1 mM EDTA, 10 mM $MgCl_2$, 20% (w/v) sorbitol, 0.05% (v/v) 2-mercaptoethanol ('extraction buffer') containing Polyclar AT (10% of leaf weight) in a 100 ml capacity Waring blender for 20 s at low speed. The extract was rapidly centrifuged for 2 min at 11600 × *g*. The supernatant was used immediately or after rapid gel filtration (Sephadex G-25M) into extraction buffer. As indicated in the text, in some experiments (particularly those involving ^{32}P), 0.4 g portions of leaf were frozen in liquid N_2, pulverised with 40 mg Polyclar AT, acid-washed sand and liquid N_2 in a chilled mortar and then ground with 2 ml extraction buffer. The extract was then centrifuged as above.

2.6. *Immunoprecipitation*

Undesalted extract containing 0.12 U PEPc was mixed with 0.3 ml antiserum and left at 0°C for 60 min. The mixture was centrifuged for 2 min at 11600 × *g* and the pellet was washed with 1 ml of 1.5 M NaCl, 2 mM EDTA, pH 7. The pellet was resuspended in 0.18 ml SDS sample buffer, boiled for 3 min and analysed by SDS-polyacrylamide gel electrophoresis. This provided enough material for 6 gel tracks. In controls the antiserum was replaced by an equal volume of preimmune serum.

2.7. *Polyacrylamide gel electrophoresis*

SDS-polyacrylamide (8%) slab gels were run as in [10]. Autoradiography was carried out as in [5]. 'Cleveland mapping' was carried out as in [11] using chymotrypsin and V8 protease.

2.8. *Isolation and analysis of ^{32}P-labelled PEPc*

Z. mays leaves (1 g) were labelled with $^{32}P_i$, illuminated for 1 h at 800 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and extracted with liquid N_2. PEPc was immunoprecipitated and purified by SDS-polyacrylamide gel electrophoresis as described above. The enzyme band was extracted, hydrolysed in 5 M HCl for 2 h at 110°C and analysed for ^{32}P-labelled amino acids by two-dimensional electrophoresis on silica gel thin layers and autoradiography as in [12]. The standard markers (P-Ser, P-Thr, P-Tyr, P_i) were detected by staining for phosphate and amino acids as in [12].

3. RESULTS

3.1. *Changes in the sensitivity of PEPc to inhibition by malate in response to light and dark*

The kinetic properties of PEPc were examined in freshly prepared, desalted extracts of *Z. mays*

leaves. The apparent K_i of the enzyme for malate was found to be 2–3-times higher in extracts of leaves which had been illuminated than in extracts of leaves which had been kept in darkness. After illumination the enzyme showed sigmoidal inhibition by malate with an apparent K_i of 0.9–1.2 mM and after a period of darkness the enzyme showed hyperbolic inhibition by malate with an apparent K_i of 0.3–0.6 mM (fig.1). A similar difference was observed in undesalted extracts and in extracts prepared by both the methods described in section 2. The apparent K_i for malate increased as the extracts aged.

At 27°C and 800 μmol·m^{-2}·s^{-1} the conversion of the enzyme from the dark, more malate-sensitive form to the light, less malate-sensitive form took ~1 h to reach completion after the illumination began (fig.2a). Similarly, the conversion of the light form of the enzyme to the dark form took ~1 h to reach completion after transfer to darkness (fig.2b).

When previously darkened leaves were exposed to light intensities of <300 μmol·m^{-2}·s^{-1} for 1 h there was little or no increase in the apparent K_i for malate of PEPc. Above ~500 μmol·m^{-2}·s^{-1} the increase in the apparent K_i for malate was completed within 1 h and the extent of the increase was independent of the photon fluence rate.

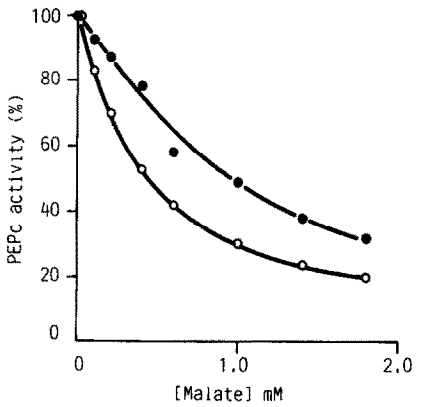

Fig.1. Inhibition by malate of PEPc extracted from illuminated and non-illuminated leaves. Enzyme activity is expressed as a percentage of the activity in the absence of malate. 100% represents 0.005 U/ml. Extracts prepared from leaves maintained in darkness for 10 h at 15°C (○) and illuminated for 1 h at 27°C (800 μmol·m^{-2}·s^{-1}) (●).

Identical immunotitration curves were obtained for the precipitation of PEPc activity from extracts of *Z. mays* leaves after exposure to darkness (10 h, 16°C) and after illumination (1 h, 800 μmol·m^{-2}·s^{-1}, 27°C) suggesting that the total amount of PEPc protein does not change during the short-term (1 h) response to light.

3.2. *Changes in the ^{32}P content of PEPc in response to light and dark*

Portions of *Z. mays* leaves were prelabelled with $^{32}P_i$, subjected to periods of light and darkness and extracted as described in section 2 and fig.3. Very similar amounts of a protein of subunit M_r 109000 were immunoprecipitated from each of the extracts as judged by staining with Coomassie brilliant blue (fig.3a, tracks 11–15). This protein band was shown to be PEPc by comparing the pattern of proteolytic fragments obtained from the subunit 109 kDa band with that obtained from purified *Z. mays* PEPc by Cleveland mapping. No subunit 109 kDa band was observed in control immunoprecipitates (fig.3a,b, tracks 6–10).

The apparent K_i for malate of the PEPc in each extract shown in fig.3 is also shown in fig.2 (▲). The PEPc samples with the lowest apparent K_i values for malate (0.5–0.6 mM) extracted after 10 h darkness (fig.3b, track 11) and after 1 h light followed by 1.5 h darkness (fig.3b, track 15) appeared to contain the least ^{32}P. The sample with the highest apparent K_i for malate (1.2 mM) extracted after illumination for 1 h (fig.3b, track 13) appeared to contain the most ^{32}P. The differences in the extent of labelling of PEPc with ^{32}P are unlikely to have arisen as a result of changes in the specific radioactivity of a precursor pool of [^{32}P]ATP. Leaves which had been prelabelled for 48 h under short-day conditions gave results similar to those shown in fig.3. The labelling of most proteins in the five extracts appeared to be similar (fig.3b, tracks 1–5). Furthermore, it is unlikely that changes in the specific radioactivity of the precursor pool could account for the decrease in the ^{32}P labelling of PEPc after transfer to darkness (fig.3b). The ^{32}P in PEPc was not increased by low illumination (<300 μmol·m^{-2}·s^{-1}) for 1 h which was insufficient to increase the apparent K_i for malate (not shown).

The ^{32}P present in PEPc did not represent a covalent intermediate in the catalytic mechanism,

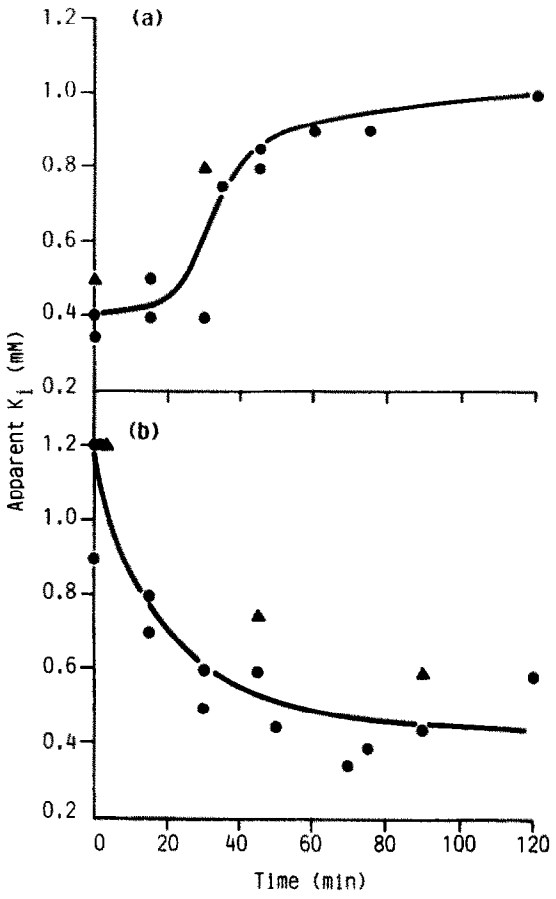

Fig.2. Time courses of the changes in the malate sensitivity of PEPc. Leaves were illuminated, extracted and assayed for PEPc as described in section 2. (a) Leaves were kept in darkness for 10–14 h at 15°C and then detached and transferred to light (800 μmol·m^{-2}·s^{-1}) at 27°C at zero time. (b) Detached leaves were illuminated (800 μmol·m^{-2}·s^{-1}) for 1 h at 27°C and then transferred to darkness at 27°C at zero time. (●) Leaves extracted in a Waring blender. (▲) Leaves extracted in liquid N_2; the PEPc in these leaves was analysed for ^{32}P as shown in fig.3.

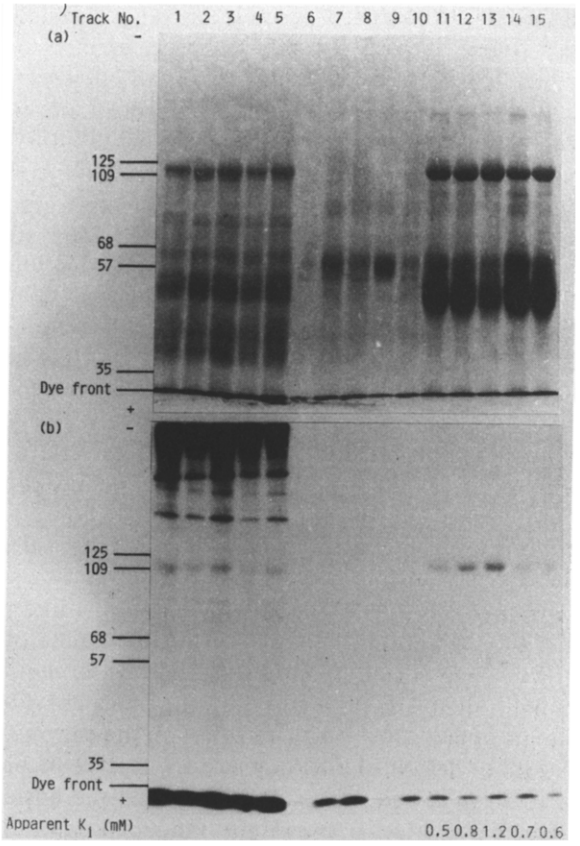

Fig.3. SDS-polyacrylamide gel electrophoresis of extracts from ^{32}P-labelled leaves. Portions of leaf were labelled with ^{32}P for 24 h and then maintained in darkness for 10–12 h. They were then extracted immediately (tracks 1,6,11), after 30 min illumination (tracks 2,7,12), after 60 min illumination (tracks 3,8,13), after 60 min illumination followed by 45 min darkness (tracks 4,9,14) and after 60 min illumination followed by 90 min darkness (tracks 5,10,15) as described in fig.2. Tracks 1–5, leaf extract containing 0.01 U PEPc. Tracks 11–15, immunoprecipitate containing 0.02 U PEPc. Tracks 6–10, as tracks 11–15 but substituting preimmune serum for antiserum. (a) Coomassie brilliant blue-stained gel. (b) Autoradiograph. The numbers on the left indicate M_r values × 10^{-3} of the marker proteins. The apparent K_i for malate of PEPc was measured in each extract and is indicated beneath the appropriate immunoprecipitate.

since preincubation of ^{32}P-labelled extracts with unlabelled substrates for 1 h at 25°C before immunoprecipitation did not remove any ^{32}P from PEPc.

The ^{32}P-labelled PEPc was analysed for ^{32}P-labelled amino acids as described in section 2. Three radioactive spots were observed, corresponding to P_i, phosphoserine and an unknown. Longer hydrolysis (4 h) increased the ^{32}P in P_i and reduced the ^{32}P in the unknown. It seems likely, therefore, that the unknown spot represents a phosphopeptide and that *Z. mays* PEPc is phosphorylated on one or more serine residues.

4. CONCLUSIONS

The difference in the malate sensitivity of PEPc in illuminated and non-illuminated leaves of *Z. mays* shown in fig.1 is very similar to that observed previously [4]. Immunodiffusion experiments [4] and the immunoprecipitation experiments described here suggest that de novo synthesis of PEPc is not involved in this effect. The reduction in the malate sensitivity of PEPc requires high light intensities and was relatively slow to reach completion (~1 h at 27°C). The reversal of this effect in darkness was similarly slow. These results are very similar to those obtained for *Salsola soda* [2]. A reduction in the sensitivity of PEPc to feedback inhibition by malate would be expected to make the enzyme more active in vivo, since malate concentrations are thought to be high in mesophyll cells (see e.g. [13,14]).

From the results shown in fig.3 it seems likely that the increase in the apparent K_i for malate of PEPc which occurs upon illumination of *Z. mays* leaves ([4]; figs 1,2) is caused by the phosphorylation of one or more serine residues on the enzyme. Phosphorylation of PEPc in vitro will have to be carried out in order to establish whether there is a direct correlation between the kinetic properties and phosphorylation state of the enzyme and to determine the stoicheiometry of phosphorylation.

PEP carboxylases from C_4 and CAM plants are distinct enzymes but appear to fulfill similar functions. The proposed activation of *Z. mays* PEPc by phosphorylation rendering the enzyme less sensitive to feedback inhibition by malate is very similar to that proposed for PEP carboxylases from several CAM plants [5–7], although in C_4 plants PEPc is normally active during the day, whereas in CAM plants PEPc is normally active at night.

ACKNOWLEDGEMENTS

This work was supported by grants AG17/151 and AG17/178 from the Agriculture and Food Research Council.

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