Effects of pH on the induction of phosphoenolpyruvate carboxylase kinase in Kalanchoë fedtschenkoi

Katrina M. Paterson, Hugh G. Nimmo *

Plant Science 154 (2000) 135–141

Plant Molecular Science Group, Division of Biochemistry and Molecular Biology, Bower Building, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow G12 8QQ, UK

Received 27 September 1999; received in revised form 22 November 1999; accepted 22 November 1999

Abstract

Previous work has shown that an increase in cytosolic pH plays an important role in the induction of phosphoenolpyruvate carboxylase (PEPc) kinase by light in C4 plants. The potential involvement of a similar effect in the induction of PEPc kinase in the Crassulacean acid metabolism (CAM) plant Kalanchoë fedtschenkoi was assessed using leaf disks. Treatment of disks with the weak base NH4Cl did not affect induction of the kinase. Prolonged treatment of disks with weak acids prevented both decarboxylation of malate during the day and induction of the kinase in the following night, but short treatments had no effect. The data are consistent with the view that a high cytosolic malate content can prevent induction of phosphoenolpyruvate carboxylase kinase. Changes in cytosolic pH may affect induction of the kinase in CAM plants by a secondary effect rather than as part of a primary signalling pathway. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Crassulacean acid metabolism; Kalanchoë fedtschenkoi; Malate; pH; Phosphoenolpyruvate carboxylase kinase; Protein phosphorylation

1. Introduction

Phosphoenolpyruvate carboxylase (PEPc) catalyses the primary assimilation of CO2 in both Crassulacean acid metabolism (CAM) and C4 plants. It is an allosteric enzyme, inhibited by malate and activated by glucose 6-phosphate [1]. Superimposed on this, PEPc is regulated by reversible phosphorylation, which plays a central role in controlling flux through the enzyme in vivo. PEPc is phosphorylated by a specific Ca2+ independent PEPc kinase, which significantly reduces the sensitivity of PEPc to inhibition by malate, and is dephosphorylated by protein phosphatase 2A [2–5]. PEPc becomes phosphorylated during the night in CAM plants and in the light in C4 plants [4,5]. It is now accepted that the function of this phosphorylation in C4 plants is to allow PEPc to fix CO2 during the day in the face of the high cytosolic malate content of mesophyll cells that is needed to sustain malate diffusion to bundle sheath cells in the C4 pathway [4,6]. Correspondingly, in CAM plants the function of the phosphorylation is presumed to be to allow CO2 fixation to continue during the nocturnal period of malate accumulation [5].

Much attention has been focussed on the events that lead to phosphorylation of PEPc in the light in C4 plants. This involves increases in PEPc kinase activity [7,8] resulting from induction of PEPc kinase gene expression in the light [9,10]. Changes in protein phosphatase 2A activity have not been detected [8]. The signal transduction pathway involves both a light-dependent increase in cytosolic pH and an increase in cytosolic Ca2+ ion concentration, which may itself be triggered by the change in pH [4,11]. The effects of light can be blocked by the calmodulin antagonist W7, leading to the suggestion that activation of a calcium-dependent protein kinase may be required for the induction of PEPc kinase [11].

* Corresponding author. Tel.: + 44-141-330-4721; fax: + 44-141-330-4447.
E-mail address: h.g.nimmo@bio.gla.ac.uk (H.G. Nimmo)

0168-9452/00 $ - see front matter © 2000 Elsevier Science Ireland Ltd. All rights reserved.
PH: S0168-9452(99)00249-6
In contrast to the response to light in C₄ plants, in CAM plants the phosphorylation state of PEPc is controlled by a circadian oscillator such that the enzyme is phosphorylated during the dark period in a normal diurnal cycle [12,13]. This is caused by induction of PEPc kinase gene expression rather than by changes in protein phosphatase 2A activity [9,14,15]. Little is known about the signal transduction pathway that connects the circadian oscillator to the expression of PEPc kinase. The objective of this work was to investigate whether changes in cytosolic pH are involved in the signalling process. No evidence to suggest a role for increases in cytosolic pH was obtained. While agents that promote cytosolic acidification do block induction of PEPc kinase, the underlying mechanism is indirect and may be secondary to a reduction in the decarboxylation of malate.

2. Methods

2.1. Plant material

*Kalanchoë* (*Bryophyllum*) *fedtschenkoi* Hamet et Perrier plants were maintained and harvested as described previously [12]. The photoperiod was 8 h, from 08:00 until 16:00 h. The day and night temperatures were 27 and 15°C, respectively. Unless stated otherwise, leaf disks (1.5 cm diameter) were cut at the start of the photoperiod and washed in the stated incubation medium for 2 × 60 min periods on a shaking incubator. They were then incubated further as shown in the figures in growth room conditions.

2.2. Preparation of extracts

Leaf disks (approximately 1 g) were ground in a mortar and pestle with some sand, sodium bicarbonate (50 mg g⁻¹ tissue) and a few drops of octanol in 100 mM Tris/HCl pH 8.0 containing 1 mM benzamidine HCl, 1 mM dithiothreitol, 2 mM EDTA and 10 mM L-malate (1 ml g⁻¹ tissue). Extracts were centrifuged at 12 000 × g for 5 min and desalted as described previously [14].

2.3. Assay procedures

The activity of PEPc and its apparent $K_i$ for L-malate were measured as described previously [12], from duplicate assays that agreed to within 15%. PEPc kinase was assayed by monitoring the incorporation of $^{32}$P from [$\gamma$-$^{32}$P]ATP into purified, dephosphorylated PEPc [14] by phosphorimaging of the PEPc band following SDS gel electrophoresis [9]. Assays were carried out in duplicate and representative results are shown. PEPc kinase translatable mRNA was quantified as described previously [9]. Briefly, total RNA was isolated and translated in a rabbit reticulocyte lysate. The PEPc kinase activity of the translation products was quantified as above. The $^{32}$P incorporation data were corrected for variations in the efficiency of translation between different samples of RNA as revealed by incorporation of $^{35}$S from $^{[35}$S] methionine into protein. The data are thus equivalent to those obtained from Northern analysis corrected for any differences in the loading of RNA.

The L-malate content of leaf disk cell sap was measured as in [13].

3. Results

3.1. Induction of PEPc kinase in leaf disks

To define a system in which possible elements of a signal transduction pathway could be manipulated, the induction of PEPc kinase was studied in leaf disks. Disks were cut from mature *K. fedtschenkoi* leaves in the middle of the photoperiod and shaken gently in distilled water in petri dishes, for the remainder of the photoperiod (4 h) and the following dark period. The water was replaced after 1 and 2 h; control experiments revealed that the leakage of malate from damaged tissue had ceased after this time (data not shown). The PEPc kinase activity and the malate sensitivity of PEPc were measured in disk extracts prepared after different times of incubation, and compared to those in extracts of intact leaves. Fig. 1 shows that PEPc kinase activity was induced during the dark period in both intact leaves and leaf disks. Moreover, the apparent $K_i$ of PEPc for malate rose from 0.5 mM at the end of the photoperiod to 2.2 mM in disks and 2.6 mM in detached leaves in the middle of the dark period (not shown). This suggests that incubation of leaf disks could be used as a model system for studies of signal transduction in the induction of PEPc kinase in *K.
fedtschenkoi. Disks were therefore cut in the middle of the photoperiod, washed for 2 × 1 h periods, and then transferred to solutions of NH₄Cl (10–50 mM) at the start of the dark period. NH₄Cl has been shown to cause an increase in cytosolic pH that is required for the light-induced phosphorylation of PEPc in C₄ species [11,16,17]. However, treatment of disks with NH₄Cl had no effect on the phosphorylation of PEPc as judged by the decrease in its sensitivity to inhibition by malate (not shown).

3.2. Agents which reduce cytosolic pH prevent induction of PEPc kinase and decarboxylation of malate

Disks were cut and transferred to either water, 3 mM acetic acid, 3 mM propionic acid or 1 mM HCl at the start of the photoperiod. The pH of the incubation medium was approximately 3.7 for each acid. The disks were incubated for 16 h, until the middle of the following dark period. At this time, the apparent Kᵢ values of the PEPc for malate were 0.5 mM in the acetic acid- and propionic acid-treated disks, 1.1 mM in water-treated disks and 1.2 mM in HCl-treated disks. In this experiment, the increase in the apparent Kᵢ of PEPc for malate in control disks in the dark was modest. However, it is clear that treatment with the two membrane-permeable weak acids prevented the phosphorylation of PEPc, whereas treatment with the impermeable HCl did not. Since the metabolic consequences of the uptake of acetate and propionate ions must be different, these data suggest that the lack of phosphorylation of PEPc must result from an effect common to both acids, such as cytosolic acidification. In experiments to define the lowest effective concentration of acetic acid, it was found that 1 mM acetic acid partially prevented the nocturnal phosphorylation of PEPc but that 0.1 mM acetic acid had little effect (not shown).

The duration of treatment with acetic acid required to prevent the nocturnal phosphorylation of PEPc was investigated in a time-course experiment. Disks were cut at 08:00 h, the start of the photoperiod, and transferred to 3 mM acetic acid either immediately or at 12:00, 15:00 or 18:00 h. Disk extracts were prepared at midnight and assayed for PEPc kinase activity, PEPc kinase translatable mRNA, the malate sensitivity of PEPc and total malate content. The results in Fig. 2 show that treatment with acetic acid from 08:00 h prevents the nocturnal increases in PEPc kinase translatable mRNA, PEPc kinase activity and the apparent Kᵢ of PEPc for malate. However, treatments for shorter periods, even from 12:00 h onwards, have markedly less effect. It seems that disks have to be treated with acetic acid for at least 8–12 h for the effect on phosphorylation of PEPc to be detectable.

The total malate content of disks over the time-course is shown in Fig. 3. These results show that the malate content of disks is, as expected, high at the start of the experiment as a result of nocturnal CO₂ fixation. In control disks incubated in water, the malate content falls during the photoperiod and rises during the following dark period. Hence the disks qualitatively reproduce the changes in malate content observed in intact leaves [12].
Fig. 2. Acetic acid treatment of leaf disks blocks the nocturnal induction of PEPc kinase and the phosphorylation of PEPc. Leaf disks were cut at the start of the photoperiod and incubated in either water (lane 1) or 3 mM acetic acid. Disks were transferred from water to 3 mM acetic acid at 08:00 h (lane 2), 12:00 h (lane 3), 16:00 h (lane 4) or 18:00 h (lane 5). All disks were incubated until the middle of the following dark period. (A) PEPc kinase activity. The panel shows phosphorimages of 32P-labelled PEPc resolved by SDS gel electrophoresis. The numbers under the lanes show the incorporation of 32P into PEPc expressed relative to the maximum values; (B) PEPc kinase translatable mRNA. The panel shows phosphorimages of immunoprecipitated 32P-labelled PEPc in assays of the PEPc kinase activity in translation products. The numbers under the lanes show the incorporation of 32P into PEPc expressed relative to the maximum values, corrected for differences in the efficiency of translation of different RNA samples [9]; (C) the apparent $K_i$ for malate (mM).

However, treatment with acetic acid from 08:00 h onwards markedly reduces the decline in malate, and prevents any subsequent increase in malate in the following dark period. This observation is consistent with the suggestion that, in both CAM [18] and C₄ [10] plants, a metabolite signal (possibly a high cytosolic level of malate) can inhibit expression of PEPc kinase.

To test this hypothesis further, detached leaves were maintained in CO₂-free air overnight to reduce the nocturnal accumulation of malate. Disks were cut from these leaves and control leaves at 08:00 h and incubated in either water or 3 mM acetic acid. As shown in Fig. 4, the treatment in CO₂-free air reduced but did not eliminate the accumulation of malate during the night; the residual accumulation is probably due to the re-fixation of respiratory CO₂. Incubation of the disks from leaves that had been maintained in CO₂-free air in 3 mM acetic acid slightly reduced the decline in malate content observed during the day, but did not prevent some malate accumulation during the following dark period. However, it did prevent the nocturnal phosphorylation of PEPc, as judged by its apparent $K_i$ for malate in the middle of the dark period. Attempts to reduce nocturnal CO₂ fixation still further by allowing leaves to take up the PEPc inhibitor 3,3-dichloro-2-dihydroxypophinoylmethyl-2-propenoate (e.g. Ref [19]) were unsuccessful.

4. Discussion

An increase in cytosolic pH is thought to play an important signal transduction role in the induction of PEPc kinase expression by light in C₄ plants [4,11,16,17]. In CAM plants, PEPc kinase expression is controlled by a circadian oscillator rather than by light [5,9,14]. The experiments reported here were undertaken to investigate the possibility that cytosolic pH changes play a role in this circadian control of PEPc kinase in CAM plants. The data provide no support for this suggestion. Incubation of K. fedtschenkoi leaf disks with NH₄Cl to increase cytosolic pH neither increased nor accelerated the nocturnal phosphorylation of PEPc. Incubation of disks for up to 12 h in the presence of acetic acid to reduce cytosolic pH did not prevent the phosphorylation of PEPc. However, the extent to which cytosolic pH was affected by these treatments is not known. It remains possible that the signalling pathway between the circadian oscillator and the induction of PEPc kinase does involve an increase in cytosolic pH but that the treatments used did not affect this signal. Prolonged incubation of disks in the presence of acetic or propionic acids to lower cytosolic pH did block the induction of PEPc kinase, but this may be due to the impaired decarboxylation of malate under these conditions rather than to inhibition of a signalling process as such.

Recent work has suggested that a high level of cytosolic malate may repress the expression of PEPc kinase in CAM plants [18]. Although reliable estimation of the subcellular distribution of
malate in CAM plants has not yet been achieved, our data are consistent with this view. Treatment of disks with acetic acid from 08:00 h onwards clearly inhibits the decarboxylation of malate. The mechanism underlying this effect is not clear, it may represent conversion of NAD-malic enzyme to a low-activity form (e.g. Ref. [20]). Disks cut from control leaves and treated with acetic acid from 08:00 h failed to accumulate malate in the following dark period. In contrast, similarly treated disks from leaves maintained in CO2-free air to reduce their malate content did accumulate malate during the next dark period. This demonstrates that acetic acid treatment does not damage the tissue or inactivate PEPc in such a way as to prevent CO2 uptake. Since PEPc is cytosolic, it suggests that the failure to accumulate malate in acetic acid-treated disks seen in Fig. 3 caused by a failure to transport malate into the vacuole. This could be due to a decrease in the pH gradient between the cytosol and the vacuole, or just to inhibition of malate uptake across the tonoplast. The elevated cytosolic malate would then prevent expression of PEPc kinase, as seen in Fig. 2. A similar failure to transport malate into the vacuole would occur in the acetic acid-treated disks from leaves deprived of CO2 overnight. This could account for the lack of phosphorylation of PEPc (as judged by its apparent $K_i$ for malate) shown in Fig. 4. However, the cytosolic malate in these disks would be lower than that in the acetic acid-treated disks from control leaves. This presumably allows the dephosphorylated form of PEPc to fix some CO2 into malate, as observed in Fig. 4. The data in Figs. 3 and 4 suggest that there is relatively little difference in the total malate content of these

### Fig. 3

Acetic acid treatment of leaf disks blocks the destruction and re-synthesis of malate. Leaf disks were incubated in water (control) or transferred to 3 mM acetic acid at the indicated times as in Fig. 2. The malate content of cell sap from four disks was measured at the start of the experiment (08:00 h, filled), at 16:00 h (open), midnight (horizontal hatch) and 07:00 h (diagonal hatch).

### Fig. 4

Acetic acid treatment of disks from leaves treated with CO2-free air overnight. Detached leaves were kept in a stream of CO2-free air overnight. Disks were cut from these and control leaves at the start of the photoperiod and incubated in either water or 3 mM acetic acid. Treatment 1, normal air, water; treatment 2, CO2-free air, water; treatment 3, normal air, acetic acid; treatment 4, CO2-free air, acetic acid. The malate content of cell sap from four disks was measured at the start of the experiment (08:00 h, filled), at the end of the photoperiod (16:00 h, open) and in the middle of the following dark period (horizontal hatch). The apparent $K_i$ of PEPc for malate was measured in the middle of the following dark period.
acetic acid-treated disks. However, it should be noted that a significant part of the total malate at the end of the photoperiod in *K. fedtschenkoi* leaves seems metabolically inert, in that it is never decarboxylated even in prolonged light at high temperature [21], both of which promote breakdown of malate. Hence, there is likely to be a significant difference in the compartmentalisation of malate between the two conditions, sufficient to allow flux through PEPc in one case but not in the other.

In conclusion, the data do not suggest that cytosolic pH changes are involved in signal transduction affecting the expression of PEPc kinase in CAM plants. While acid treatment of disks does prevent induction of the kinase, this may be secondary to an inhibition of malate breakdown during the day and nocturnal uptake of malate into the vacuole.

**Acknowledgements**

K.M. Paterson thanks BBSRC for a studentship.

**References**


