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The roles and regulation of phosphoenolpyruvate carboxykinase in C_4 and CAM plants

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Introduction

The photosynthetic CO_2-concentrating mechanisms found in plants with C_4 photosynthesis, Crassulacean Acid Metabolism and some aquatic organisms are characterised by a variety of decarboxylases, including the malic enzymes and PCK (Leegood et al., 1996; Reiskind and Bowes, 1991; Reinfelder et al., 2000). PCK catalyses the ATP-dependent decarboxylation of oxaloacetate (OAA) to phosphoenolpyruvate (PEP). C_4 plants are classified into three sub-types, depending upon the principal decarboxylase present in the bundle sheath. In PCK-types, such as *Panicum maximum*, PCK acts in tandem with NAD-malic enzyme to decarboxylate C_4 acids (Burnell and Hatch, 1988), while in some NADP-malic enzyme types, such as maize, PCK appears to act as an auxiliary decarboxylase involved in the decarboxylation of aspartate (Walker et al., 1997; Wingler et al., 1999).

In the leaves of C_4 plants, PCK activity must be regulated so as to prevent depletion of OAA and/or ATP in the cytosol in the dark, but no mechanism of regulation has been elucidated (Carnal et al., 1993). Diurnal changes in PCK activity have previously been reported for leaves of the CAM plants, *Aloe vera* (Lin et al., 1991) and pineapple (Lin et al., 1994), with a two- to four-fold increase in activity recorded during the daytime deacidification (decarboxylation) phase, but the reasons for this change were not elucidated. PCK is subject to reversible protein phosphorylation in many plant tissues (Walker and Leegood, 1997). It is phosphorylated in darkened cotyledons of gluconeogenic seedlings, such as cucumber, and in darkened leaves of PCK-type CAM plants, such as *Tillandsia* spp. (Walker and Leegood, 1997). Although PCK is phosphorylated during darkness in the leaves of some C_4 plants, such as *P. maximum*, it is not phosphorylated in many others, such as *Urochloa panicoides*, that have smaller molecular mass forms of PCK (Walker et al., 1997, Walker and Leegood, 1996). Like a number of other enzymes regulated by phosphorylation, PCK possesses a readily proteolysed N-terminal sequence containing a phosphorylation site(s) that is absent from enzymes with otherwise very similar sequences, for example, in bacteria or fungi (Walker et al., 1997). Although PCK from *P. maximum* has not been sequenced, the available PCK sequences indicate that the N-terminal region may contain up to two potential phosphorylation sites. One is a consensus sequence for recognition by cAMP-dependent protein kinases and the other is a consensus sequence for the SNF-1 related protein kinases (Leegood and Walker, 1999). The latter are thought to be global regulators of carbon metabolism in plants, implicated in the regulation of sucrose-P synthase, nitrate reductase and

HMG-CoA reductase (Halford and Hardy, 1998). Both these potential sites are absent from *U. panicoides* PCK, that is not phosphorylated (Finnegan and Burnell, 1995).

Results and Discussion

We have purified PCK from the C_4 grass, *P. maximum*, and improved upon earlier purification methods by developing a procedure that results in minimal proteolysis of PCK, thus the N-terminal region, together with its phosphorylation site, is retained. To prevent proteolysis, all purification procedures were done at pH 9.8 (Walker et al., 1995). PCK was separately purified from ^{32}P-labelled darkened and illuminated leaves of *P. maximum* to enrich the phosphorylated and dephosphorylated forms.

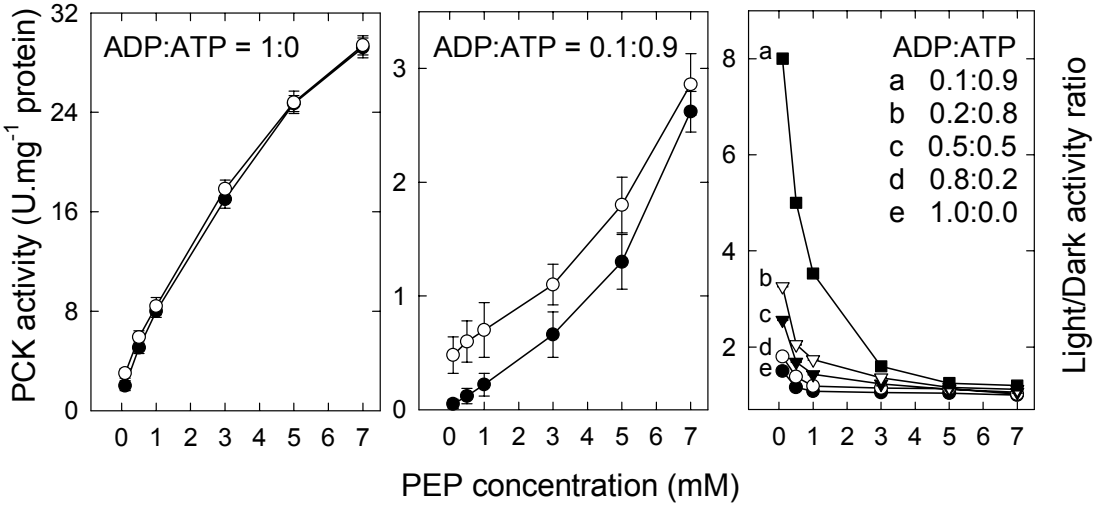

Fig. 1. Effect of phosphorylation on the carboxylase activity of PCK. The affinity of the phosphorylated and non-phosphorylated forms of PCK for PEP was determined at different ratios of ATP:ADP. The ratio of activities at different ATP:ADP ratios is shown on the right-hand side. The total concentration of adenylates was 1 mM.

To determine how phosphorylation of PCK affects its activity, the properties of the phosphorylated and largely non-phosphorylated forms of the enzyme were compared. In the carboxylation direction both the concentration of ATP and ADP and the ratio of ADP:ATP altered the affinity of the enzyme for ADP and PEP and these interactions were modulated by phosphorylation (Fig. 1). The activities of non-phosphorylated and phosphorylated PCK were the same in the absence of ATP. The difference in activity between the two forms was greatest at lower concentrations of PEP and higher ratios of ATP:ADP. Assayed in the carboxylation direction in the absence of ATP, the response to increasing PEP was hyperbolic, but increasing the amount of ATP strongly inhibited the rate and induced sigmoidal behaviour. Similar results were obtained for assay in the direction of decarboxylation (Fig. 2).

PCK purified from illuminated leaves was incubated *in vitro* with ^{32}P-ATP and the catalytic subunit of cAMP-dependent protein kinase (Fig. 2). The stoichiometry of phosphorylation was about 0.7 mol Pi per mol PCK subunit. PCK phosphorylated *in vivo*, when assayed as a decarboxylase, showed a decrease in activity over a range of OAA concentrations, showing similar properties to the phosphorylated enzyme purified from darkened leaves.

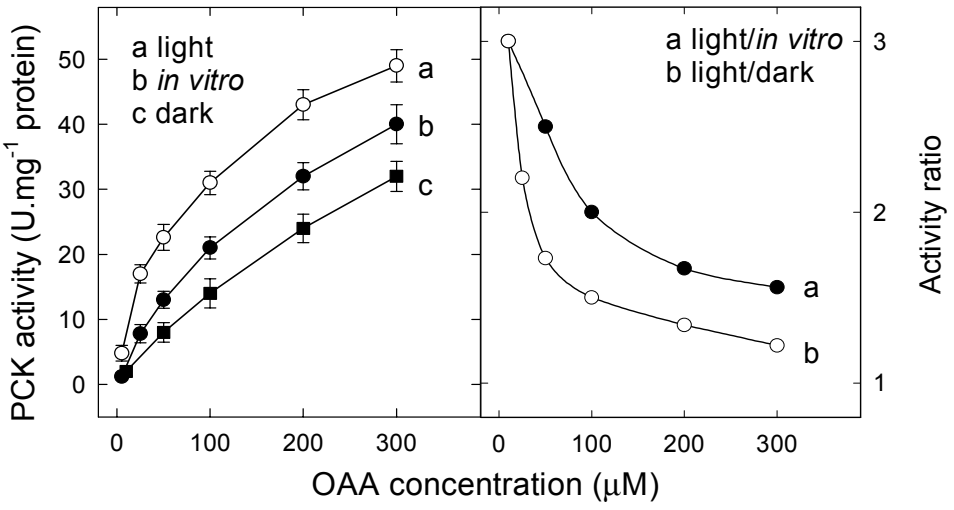

Fig. 2. The effect on PCK activity of phosphorylation *in vivo* by cyclic AMP-dependent protein kinase. Left: the response to OAA concentration of PCK phosphorylated *in vivo* (the dark form of the enzyme) or *in vitro* (the dark form incubated with cAMP-dependent protein kinase) and the non-phosphorylated (light) form of the enzyme. Assays were done under selective conditions (0.8 mM ATP and 0.2 mM ADP). Right: the ratio of the activity of non-phosphorylated enzyme to the enzyme phosphorylated either *in vivo* or *in vitro*.

We investigated whether or not changes in the properties of PCK activity were correlated with changes in phosphorylation state in leaves of *P. maximum* and pineapple during a light-dark cycle. There was no change in the abundance of PCK protein, but PCK was much more phosphorylated in darkened leaves than illuminated leaves (data not shown). The carboxylase activity of PCK was measured in crude extracts of leaves using (i) assay conditions that allowed measurement of maximum activity (Vmax) or (ii) under assay conditions that revealed a large difference in activity between phosphorylated and non-phosphorylated enzyme by the inclusion of ATP (selective). When activity was measured under conditions that discriminate between phosphorylated and non-phosphorylated forms of the enzyme, much less activity was present in extracts of darkened leaves (Fig. 3). In contrast, similar measurements made on PCK extracted from leaves of maize or *U. panicoides* showed no change in activation state (data not shown). PCK in these plants is not phosphorylated (Walker et al., 1997).

Our results are consistent with a phosphorylation-based mechanism for the light-dark regulation of PCK in the leaves of C_4 plants. The diurnal changes in the properties of PCK are the combined result of differences in substrate affinities and in sensitivity to adenylates of the phosphorylated and dephosphorylated enzyme. This is the first evidence from any organism for changes in the regulatory properties as a result of covalent modification of PCK.

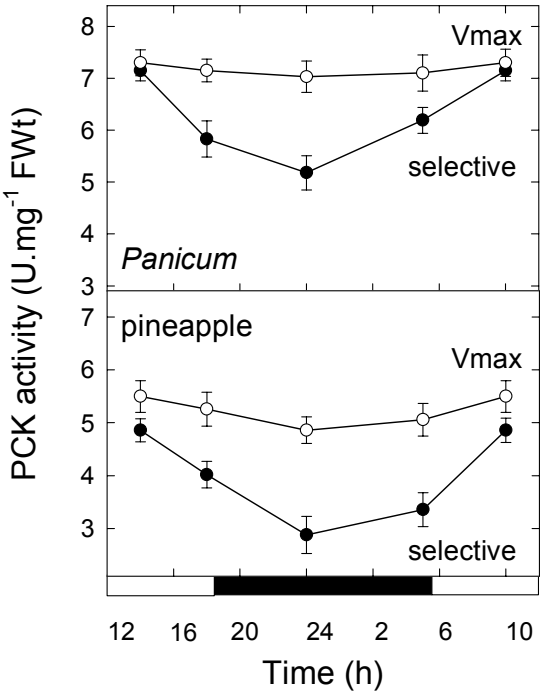

Fig. 3. Diurnal changes in the activity of PCK in leaves of *P. maximum* and pineapple. PCK was measured either under conditions that estimate maximum activity (Vmax; 5 mM PEP, 5mM $MnCl_2$, 0.5 mM ADP) or in a selective assay (5 mM PEP, 10 µM $MnCl_2$, 4 mM $MgCl_2$, 0.8 mM ATP, 0.2 mM ADP) that discriminates between the phosphorylated and non-phosphorylated forms of the enzyme.

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