Phosphoenolpyruvate carboxylase from pennywort (*Umbilicus rupestris*)

Changes in properties after exposure to water stress

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(Received 8 August 1983/Accepted 31 October 1983)

Umbilicus rupestris (pennywort) switches from C_3 photosynthesis to an incomplete form of crassulacean acid metabolism (referred to as 'CAM-idling') when exposed to water stress (drought). This switch is accompanied by an increase in the activity of phosphoenolpyruvate carboxylase. This enzyme also shows several changes in properties, including a marked decrease in sensitivity to acid pH, a lower K_m for phosphoenolpyruvate, very much decreased sensitivity to the allosteric inhibitor malate, and increased responsiveness to the allosteric effector glucose 6-phosphate. The M_r of the enzyme remains unchanged, at approx. 185 000. These changes in properties of phosphoenolpyruvate carboxylase are discussed in relation to the roles of the enzyme in C_3 and in CAM plants.

CAM is a common metabolic adaptation to water stress in succulent plants. In CAM, net fixation of CO_2 occurs at night, through the activity of phosphoenolpyruvate carboxylase (EC 4.1.1.31). The oxaloacetate thus formed is converted into malate. During daylight, the malate is decarboxylated, and the CO_2 is re-fixed via the Calvin cycle. Several plant species have been shown to exhibit conventional C_3 photosynthesis under normal conditions, but to switch to CAM when exposed to water stress (reviewed by Ting & Rayder, 1982). In such plants, the switch from C_3 photosynthesis to CAM is accompanied by changes in the properties of phosphoenolpyruvate carboxylase (Greenway *et al.*, 1978; Kluge *et al.*, 1981; von Willert *et al.*, 1976a,b; Winter, 1974, 1979, 1980). In particular, the enzyme becomes more tolerant of acid pH, less sensitive to malate as an allosteric inhibitor and more responsive to glucose 6-phosphate as an allosteric effector. In addition to the change in properties, there is also an increase in the activity of phosphoenolpyruvate carboxylase (von Willert *et al.*, 1976a,b).

More recently, it has been shown that plants in the genera *Peperomia* and *Pereskia* switch from C_3 photosynthesis to an incomplete form of CAM when exposed to water stress. In these plants, there is no nocturnal net fixation of CO_2 . There is, how-

ever, nocturnal accumulation of acid, which arises from fixation of CO_2 generated in respiration (Hanscom & Ting, 1978; Rayder & Ting, 1981; Ting & Rayder, 1982). This incomplete form of CAM has been termed 'CAM-idling' (see, e.g., Ting & Rayder, 1982).

We have been investigating responses to water stress in *Umbilicus rupestris* (pennywort or navelwort). This is a succulent plant of western Europe which, by virtue of its habitat (walls, cliffs and rock faces) is exposed from time to time to water stress. We have shown that when *U. rupestris* plants are exposed to water stress, they switch from C_3 photosynthesis to CAM-idling (Daniel, 1982). *U. rupestris* is therefore a good subject for a study of the properties of phosphoenolpyruvate carboxylase in relation to the switch from C_3 photosynthesis to CAM-idling, particularly in order to determine whether the switch to CAM-idling is accompanied by dramatic changes in enzyme properties (such as occur in the switch from C_3 to full CAM in *Mesembryanthemum*).

Materials and methods

Plants

Umbilicus rupestris (Salis) Dandy (pennywort, navelwort) was collected from sites in South Glamorgan and Dyfed, Wales, and propagated in John Innes no. 2 compost at the University College Field Station, Leckwith, Cardiff. When

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Abbreviation used: CAM, crassulacean acid metabolism.

the larger leaves were approx. 40 mm in diameter, the plants were moved to a growth chamber. The temperature in the growth chamber was controlled to give a sine-wave diurnal fluctuation, with a peak of 25°C at mid-day and a low of 10°C at midnight. The lighting regime gave 8 h light ($192 \text{ W} \cdot \text{m}^{-2}$) and 16 h dark. Plants were kept under these conditions for 2 weeks, during which time they were watered daily, from below. After 2 weeks, the experimental plants were exposed to water stress simply by cessation of watering. Control plants were watered as usual. The CAM-idling pattern of metabolism (and associated stomatal behaviour) became established on day 4–5 of the drought regime. Phosphoenolpyruvate carboxylase was prepared on day 6 or 7.

Preparation of phosphoenolpyruvate carboxylase

Leaves were harvested in the middle of the light period. Leaf tissue (20 g) was homogenized with a pestle and mortar in 20 ml of 200 mM-Tris/HCl buffer, pH 8.0, containing 10 mM-MgCl₂ and 2 mM-Na₂EDTA. Solid polyvinylpyrrolidone was added (0.2 g/20 ml) before homogenization. The homogenate was filtered through four layers of coarse nylon gauze and then centrifuged at 36000g for 30 min at 4°C. The supernatant was applied to a DEAE-cellulose column (15 mm × 50 mm) ('DEAE-C I'); the column was washed with homogenization buffer to remove unbound proteins (which did not contain phosphoenolpyruvate carboxylase activity). Phosphoenolpyruvate carboxylase was eluted from the column with 300 mM-KCl in 200 mM-Tris/HCl (pH 8.0). Fractions containing enzyme activity were pooled; glycerol was added (final concn. 25%, v/v) to stabilize the enzyme.

The general characterization of the enzyme and all determinations of the effects of regulatory metabolites were done at this stage of purification.

Assay of phosphoenolpyruvate carboxylase

(i) *Coupled assay.* Spectrophotometric assays were performed by coupling the phosphoenolpyruvate carboxylase activity with malate dehydrogenase (EC 1.1.1.37) and monitoring the oxidation of NADH by the decrease in A_{340} . At the DEAE-C I stage of purification (see above), enough malate dehydrogenase was present to sustain the coupled reaction without any restriction. At later stages of purification, malate dehydrogenase (Sigma Chemical Co.) was added at 10 units/assay. The standard assay was done in a 1.0 ml spectrophotometer cuvette, containing 50 mM-Tris/HCl buffer (pH 8.0), MgCl₂ (15 mM), NaHCO₃ (10 mM), phosphoenolpyruvate (2 mM) and NADH (0.15 mM). The reaction was initiated by addition, with rapid mixing, of 35 µl of enzyme. Under standard condi-

tions, the reaction was linear with respect to time for 7–8 min.

(ii) *Radiochemical assay.* This was done as described by Goatly & Smith (1974).

Further purification

The enzyme eluted from DEAE-C I was precipitated with 30–60% satd. (NH₄)₂SO₄. The precipitate was dissolved in 200 mM-Tris/HCl, pH 8.0, containing 10 mM-MgCl₂ and 2 mM-Na₂EDTA, and then passed through a column (25 mm × 60 mm) of Sephadex G-50 equilibrated against the same buffer. The enzyme was then applied to a column of DEAE-cellulose (12 mm × 120 mm) ('DEAE-C II'). The column was washed with 200 mM-Tris/HCl buffer, pH 8.0, and then phosphoenolpyruvate carboxylase was eluted from the column in a 72 ml linear gradient of KCl (0–600 mM) in 200 mM-Tris/HCl buffer; 4.0 ml fractions were collected automatically.

Gel filtration

The M_r of the purified phosphoenolpyruvate carboxylase (from DEAE-C II) was estimated by gel filtration through a column of Sepharose CL 4B (30 mm × 320 mm), with a series of proteins of known M_r as standards.

Electrophoresis

Purified phosphoenolpyruvate carboxylase activity (from DEAE-C II) was applied to 6%-polyacrylamide gels; electrophoresis was performed under non-denaturing conditions as described by Karn *et al.* (1973). After electrophoresis, phosphoenolpyruvate carboxylase activity was detected as described by von Willert *et al.* (1976b).

Protein determination

This was done by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Results

Reproducibility of results

Unless otherwise stated, all numerical results are means ± S.E.M. for six determinations.

Detection of phosphoenolpyruvate carboxylase activity

At no time were we able to detect phosphoenolpyruvate carboxylase activity in crude homogenates. Passage of crude homogenates or of 36000g supernatants through Sephadex G-50 failed to yield activity, as did (NH₄)₂SO₄ fractionation of 36000g supernatants. Of all methods tried, only passage of 36000g supernatants through DEAE-cellulose (as described above) routinely yielded active enzyme preparations.

Comparison of enzyme properties in control and water-stressed plants

The pH optimum for phosphoenolpyruvate carboxylase was 8.0–8.2 in both sets of plants. However, the enzyme from stressed plants was more tolerant of acid pH than was the enzyme from control plants. At the pH optimum, maximum rates (in the presence of saturating concentration of phosphoenolpyruvate) were obtained with 15mM-MgCl₂, 10mM-NaHCO₃ and 0.15mM-NADH. Under all conditions, the enzyme from both control and stressed plants showed an absolute requirement for phosphoenolpyruvate. In the absence of specific regulatory metabolites, there was no evidence for co-operativity with respect to phosphoenolpyruvate (Fig. 1). With the enzyme from control plants, the K_m for phosphoenolpyruvate was 0.23 ± 0.016 mM at pH 8.2 and 1.25 ± 0.21 mM at pH 7.2. In stressed plants, the K_m for phosphoenolpyruvate was 0.20 ± 0.018 mM at pH 8.2 and 0.86 ± 0.20 mM at pH 7.2. Thus, in stressed plants, the enzyme had a slightly lower K_m for phosphoenolpyruvate, particularly at sub-optimal pH values (for data at pH 8.2, $P = 0.15$; for data at pH 7.2, $P = 0.10$).

Phosphoenolpyruvate carboxylase from both control and stressed plants was activated by glucose 6-phosphate; the activation reached a plateau at 2mM-glucose 6-phosphate. In the presence of glucose 6-phosphate there was again no evidence for co-operativity with respect to phosphoenolpyruvate (Fig. 2), but the K_m for phosphoenolpyruvate was markedly decreased: in control plants, 0.125mM at pH 8.2 and 0.39mM at pH 7.2; in stressed plants, 0.056mM at pH 8.2 and 0.097mM at pH 7.2. The activatory effect of glucose 6-phosphate was thus more marked at pH 7.2 than at

pH 8.2, and was very much more marked with the enzyme from stressed plants than with the enzyme from control plants.

L-Malate was a potent inhibitor of phosphoenolpyruvate carboxylase at all concentrations tested (1–32mM). Surprisingly, in the presence of malate, there was evidence for co-operativity with respect to phosphoenolpyruvate, as shown by the non-linear Lineweaver–Burk plot (Fig. 3). The inhibitory effect of malate was more marked at pH 7.2 than at pH 8.2. The enzyme from stressed plants showed a very much decreased sensitivity to malate, and this decrease was particularly marked at pH 7.2 (Fig. 3).

Other metabolites, including citrate, L-aspartate, ADP and ATP, also inhibited phosphoenolpyruvate carboxylase, but there were no differences with these inhibitors between the enzymes from control and stressed plants.

Enzyme activity in control and stressed plants

The specific activities of phosphoenolpyruvate carboxylase measured at the earliest possible stage of purification (i.e. after DEAE-C I) are shown in Table 1. It is clear that exposure of plants to water stress led to a relatively small, but nonetheless significant, increase in enzyme activity, particularly when measured at pH 7.2.

Absence of multiple forms of phosphoenolpyruvate

(i) When applied to DEAE-C II, and then eluted in a linear gradient of KCl, the enzyme from both control and stressed plants appeared as a single peak of activity at 300mM-KCl (Fig. 4). Modifying the elution procedure by eluting with a gradient of Tris/HCl (50–200mM) before the KCl gradient did not in any way alter the elution profile. Thus there

Fig. 1. Kinetics of *U. rupestris* phosphoenolpyruvate carboxylase

(a) Enzyme from control plants; (b) enzyme from water-stressed plants. ●, Enzyme assayed at pH 8.2; ○, enzyme assayed at pH 7.2. Velocity (v) was measured as change in A_{340}/min .

Fig. 2. Effect of glucose 6-phosphate on phosphoenolpyruvate carboxylase activity (a) Enzyme from control plants assayed at pH 8.2; (b) enzyme from control plants assayed at pH 7.2; (c) enzyme from water-stressed plants assayed at pH 8.2; (d) enzyme from water-stressed plants assayed at pH 7.2. ●, Normal assay; ○, assay mixture containing 2 mM-glucose 6-phosphate. Velocity (v) was measured as in Fig. 1.

Table 1. Activity of phosphoenolpyruvate carboxylase in control and water-stressed plants

Results are means $\pm$ S.E.M. ($n = 6$). For details see the text.

Assay pH	Enzyme activity ($\mu\text{mol/min per mg of protein}$)		
	Control plants	Water-stressed plants	
8.2	0.075 ± 0.018	0.098 ± 0.004	$P = 0.15$
7.2	0.065 ± 0.019	0.095 ± 0.004	$P = 0.10$

was no evidence for different forms of the enzyme differing in charge density.

(ii) Gel filtration on Sepharose CL 4B of the enzyme population eluted from DEAE-C II again gave rise to a single peak of enzyme activity. For

both control and stressed plants, the M_r was estimated as $185\,300 \pm 24\,000$.

(iii) Although ion-exchange chromatography and gel filtration did not provide any evidence for enzyme heterogeneity, there was some indication

Fig. 3. *Effect of malate on phosphoenolpyruvate carboxylase activity*

(a) Enzyme from control plants assayed at pH 8.2; (b) enzyme from control plants assayed at pH 7.2; (c) enzyme from water-stressed plants assayed at pH 8.2; (d) enzyme from water-stressed plants assayed at pH 7.2. ●, Normal assay; ○, assay containing 2 mM-malate; □, assay containing 8 mM-malate. Velocity was measured as in Fig. 1.

Fig. 4. *Ion-exchange chromatography of phosphoenolpyruvate carboxylase on DEAE-cellulose (DEAE-C II)*
For details, see the text.

of heterogeneity when the enzyme population from DEAE-C II was fractionated by electrophoresis in polyacrylamide gels, under non-denaturing conditions. With enzyme extracted from either control or stressed plants, most of the activity appeared as a large deeply stained band (Fig. 5). In addition, there was a very minor weakly stained band with a slightly lower mobility than the major band. This was present in enzymes extracted from control and from stressed plants.

Discussion

In this paper, we have presented a characteriza-

Fig. 5. Disc polyacrylamide-gel electrophoresis under non-denaturing conditions of phosphoenolpyruvate carboxylase eluted from DEAE-cellulose (DEAE-C II)

The three lanes represent three different samples of the same enzyme preparation.

tion of phosphoenolpyruvate carboxylase from a plant in which CAM-idling is inducible. This is the first characterization of the enzyme in a plant showing this particular metabolic switch. Detection of enzyme activity depends on close adherence to the described extraction procedure, which involves passage of the crude enzyme preparation through DEAE-cellulose, and it is likely that passage through the ion-exchange column removes an inhibitor or inactivator of the enzyme.

The general properties of phosphoenolpyruvate carboxylase from control plants are very similar to those of this enzyme from a range of plant species. In particular, the alkaline pH optimum, the inhibition by malate (and aspartate), and the stimulation by glucose 6-phosphate, coupled with the enhanced effects of these regulatory metabolites at suboptimal pH, very closely parallel the properties described for phosphoenolpyruvate carboxylase from dark-grown maize, *Zea mays* (Wong & Davies, 1973), and from potato, *Solanum tuberosum* (Bonugli & Davies, 1977).

On exposure of plants of *U. rupestris* to water stress, i.e. to conditions under which a switch from C_3 photosynthesis to CAM-idling occurs, there is a marked change in enzyme properties. Firstly, there is an increase in activity, as expressed either per unit of protein or per leaf (latter results not shown). The increase is not as large as occurs, for example, when *Mesembryanthemum crystallinum* switches from C_3 photosynthesis to full CAM (Winter, 1974, 1979), but is nevertheless significant. Secondly, the response to inhibitors changes: the enzyme becomes much less sensitive to acid pH and to malate, and much more responsive to glucose 6-phosphate. Similar changes occur in the phosphoenolpyruvate carboxylase of *Mesembryanthemum* during the switch from C_3 photosynthesis to CAM (see the introduction). These changes were not caused by changes in properties of the endogenous malate dehydrogenase used in the linked assay, since addition of excess exogenous malate dehydrogenase did not abolish the changes.

The properties of phosphoenolpyruvate carboxylase from water-stressed *U. rupestris* are in fact very similar to the properties of phosphoenolpyruvate carboxylase from various constitutive CAM plants (Nott & Osmond, 1982). It is concluded that the switch from C_3 photosynthesis to CAM-idling in *U. rupestris* is accompanied by a change in the properties of phosphoenolpyruvate carboxylase, which thus comes to resemble the enzyme present in CAM plants. This indicates that the plant's inability to perform full CAM under water stress does not lie in the properties of the carboxylating enzyme. It is likely that the failure of stressed *U. rupestris* to carry out net fixation of CO_2 in the dark is a result of stomatal behaviour (Daniel, 1982). This suggestion receives support from our recent findings that leaves from which the lower epidermis is removed are able to fix $^{14}CO_2$ in the dark, and that this ability is very much greater in stressed than in unstressed plants (J. A. Bryant & D. Bartholomeusz, unpublished work).

In *U. rupestris*, we did not detect any new separable form of phosphoenolpyruvate carboxylase during the induction of CAM-idling. In this respect, the data are comparable with those for *Mesembryanthemum*, in which, contrary to earlier suggestions, there is again no appearance of a separable new form of the enzyme when the plants switch from C_3 to CAM (Winter *et al.*, 1982). Further, in a wide variety of CAM plants, there is no evidence for the existence of multiple forms of phosphoenolpyruvate carboxylase (Nott & Osmond, 1982), and the single enzyme species observed is assumed to fulfil all of the different needs for carboxylation of phosphoenolpyruvate. This contrasts with the situation in sugar cane, where

exposure of dark-grown plants to light causes the appearance of a second form of phosphoenolpyruvate carboxylase, which is associated with C_4 photosynthesis (Goatly & Smith, 1974).

The M_r for the phosphoenolpyruvate carboxylase of *U. rupestris* in both control and water-stressed plants is of the same order as reported for a wide range of normal CAM plants (Kluge & Ting, 1978; Nott & Osmond, 1982). In comparison with the data for *Bryophyllum*, *Sedum* and *Kalanchoë*, it is likely that the M_r of approx. 185 000 observed in our experiments represents a dimer of two identical subunits, and that, as in *Kalanchoë*, the very minor sub-population observed in polyacrylamide gels is a tetramer. We were, however, unable to confirm this by electrophoresis under denaturing conditions, because of difficulties in obtaining enzyme activity after exposure to sodium dodecyl sulphate.

In *U. rupestris*, the switch from C_3 to CAM-iddling and the changes in the properties of phosphoenolpyruvate carboxylase are very clear responses to water stress. However, the exact nature of the signal that actually causes the metabolic switch is unknown. The leaves are succulent, and, although water stress causes a fall in water potential, the metabolic switch occurs before the decrease in leaf water potential can be detected.

We are grateful to Professor H. Woolhouse, Dr. Alison Smith, Dr. W. Cockburn and Dr. R. A. John for helpful discussion. P. P. D. thanks the British Council and University College, Cardiff, for financial support.

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