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Changes in properties of phosphoenolpyruvate carboxylase from the CAM plant *Sedum praealtum* D.C. upon dark/light transition and their stabilization by glycerol

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Abstract. A pronounced decrease in phosphoenolpyruvate carboxylase (PEPC) activity is observed upon dark/light transition in *Sedum praealtum* D.C., only when glycerol is included in the extraction medium. If glycerol is omitted, the activity extracted in light is initially low, but soon reaches night levels. The stabilization of the light-induced form of the enzyme by glycerol, in crude or desalted extracts, made it possible to study its kinetic properties in comparison to those of the dark form. The behaviour towards substrate (PEP) changes from hyperbolic (dark) to sigmoid (light), $S_{0.5}$ is increased and the enzymic activity becomes more sensitive to malate inhibition. Quite different activity/pH profiles are also obtained for the two forms of PEPC.

It is inferred that the *in vivo* regulation of PEPC in CAM is effected by a concerted action of light, malate and pH shifting.

Introduction

In Crassulacean acid metabolism (CAM) plants, malic acid is accumulated in the dark and decarboxylated during the following light period, providing the CO_2 for photosynthetic carbon reduction [12]. Phosphoenolpyruvate carboxylase (PEPC), the enzyme responsible for dark fixation of CO_2, should be inoperative in the light, if futile cycles of CO_2 refixation on phosphoenolpyruvate (PEP) were to be avoided. This theoretical prediction is supported by experimental evidence showing that $^{14}CO_2$, given during the period of malate decarboxylation, is not incorporated into malate [17]. The need for a strict control of PEPC activity is evident and it has been proposed that CAM regulation could be understood on the basis of malate compartmentation and its inhibitory action on PEPC. According to this scheme, the onset of light triggers a massive exit of malate from the vacuole to the cytoplasm, resulting in PEPC inhibition [12, 16].

There are, however, reports concerning diurnal changes in PEPC activity, which point towards a more complex mode of PEPC regulation. Some investigators have found PEPC activity extractable during the day to be only a fraction of that extracted at night [5, 19, 21, 22, 23, 24]. It has also been shown that the enzyme is more sensitive to malate inhibition when extracted during the day [9, 10, 21, 24]. In other studies, however, such diurnal

fluctuations in activity were not observed [2, 3, 7]; furthermore, opposite changes have been reported in at least one case [9]. Winter [24] found that the enzyme extracted during the day is quickly activated, so that very soon after extraction, night activity values are obtained. Concurrently, the sensitivity towards malate decreases to the night levels. In such a situation, one can easily fail to detect dark/light fluctuations in PEPC activity, due to the inevitable delay in assaying activity after extraction. It was, therefore, proposed [5, 24] that the enzyme can exist in two different conformational states: one of them, prevailing in darkness, has properties commensurate with efficient CO_2 fixation (high activity, low sensitivity to malate); the other, with low activity and high sensitivity to malate, is dominant in the light.

Kinetic and regulatory properties of PEPC in CAM plants have been studied mainly in partially purified preparations [11, 13]; in one case the enzyme was purified to homogeneity [8] and kinetic parameters for substrate and malate were studied [18]. Apparently, all the above studies refer to the active state of the enzyme, which might differ substantially from the alleged *in vivo* state in the light. Therefore, the need for a stabilization of the latter as a prerequisite for the study of its kinetic properties is evident. In the present paper, conditions for enzyme extraction from *Sedum praealtum* D.C. are described, which result in wide dark/light fluctuations in activity. In addition, the form prevailing in light is stabilized, allowing the comparative study of such properties as pH dependence, kinetic behaviour and sensitivity to malate inhibition.

Material and methods

Cuttings of *Sedum praealtum* D.C. were grown in soil pots in a growth chamber with temperature, relative humidity and light/dark cycles of 28 °/18 °C, 40/70% and 10.5/13.5 hours respectively. Irradiance at plant level was around 250 $\mu E/m^2 \cdot sec$ photosynthetically active radiation (PAR), given by a mixture of fluorescent and incandescent lamps.

For enzyme extraction, 3 mature leaves of about the same age (length 4 cm, weight 2.5 g) were ground for 90 sec in a prechilled mortar with a small amount of purified sea sand and 5 ml of extraction medium (0.2 M Tris-HCl, pH 7.6, 1 mM EDTA, 10 mM $MgCl_2$ and 5% w/v polyethylene glycol). Triton X-100 at 1% v/v and glycerol 20% v/v were also included in the extraction medium as specified in the text. The extraction was done immediately after removal of the leaves from the plants. The extract was centrifuged for 90 sec and the clear supernatant was either used immediately (crude extract) or desalted through a 12 x 1 cm Sephadex G25 column equilibrated with 0.1 M Tris-HCl, pH 7.5 in 20% v/v glycerol (desalted extract). All above steps were carried out at 4 °C.

Assays of PEPC were run at 30 °C in 3 ml final volume of 0.1 M Tris-HCl, (pH 7.1 or 8.1), 0.14 mM NADH, 1 mM $NaHCO_3$ 5 mM $MgCl_2$, 4.5 units of

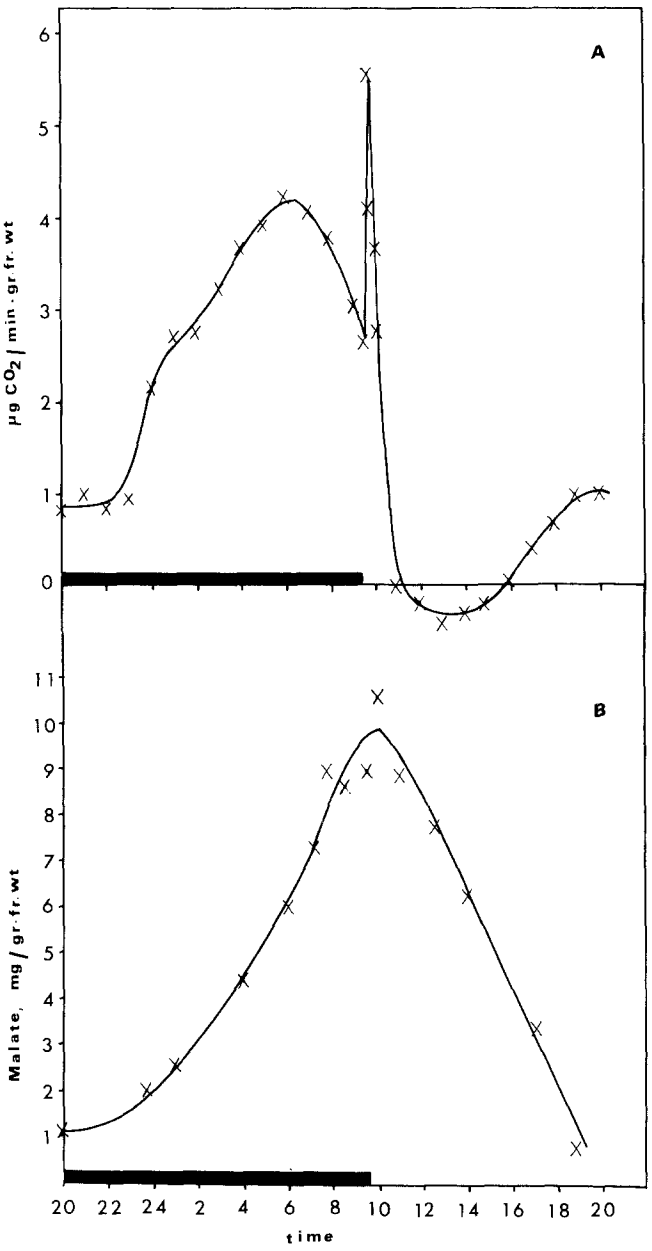

Figure 1. CAM activities during a diurnal cycle in *Sedum praealtum*. Light was given from 9.30 h in the morning until 20.00 h in the evening. A: net CO_2 absorption and B: malic acid content of the leaves

malate dehydrogenase (pig heart, Sigma) and PEP as specified. The reaction was started with 100 µl of crude or desalted extract and its rate was measured by the decrease in absorbance at 340 nm (oxidation of NADH).

The malate content of the tissue was measured enzymatically [6] and CO_2 exchanges were monitored with an infrared gas analyzer, connected in an open system with a cuvette containing a leafed twig attached to the plant.

All experiments in the figures and tables were performed at least three times and results from a representative experiment are given.

Results

As shown in Figure 1, *Sedum praealtum* exhibits net CO_2 assimilation and malate accumulation during the dark period. Malate is consumed in the light and, limited CO_2 exchanges occur between the plant and the atmosphere. These data constitute unequivocal evidence that our experimental plants performed typical CAM under the growth conditions employed.

In preliminary attempts to extract PEPC, it was found that Triton X-100, at 1% v/v in the extraction medium, greatly increased the extractability of the enzyme. This was true not only for *Sedum praealtum* but for other CAM plants as well (Table 1). Subsequently, this detergent was included as a routine component of the extraction medium, unless otherwise specified.

Changes in PEPC activity upon dark/light transition

The enzyme activities (pH 7.1) obtained at various times before and after dark/light transition were dependent on the composition of the extraction medium (Fig. 2B). In the absence of Triton X-100 and glycerol, low values were found and only a 50% decrease in activity was observed during the first 1–2 hours in light. Triton X-100 gave higher values in the light than in the dark, whereas in the presence of both Triton X-100 and glycerol (20% v/v) a very pronounced decrease in activity was obtained upon the onset of light. The low values persisted for about 2 hours and a gradual increase was evident thereafter. By comparison of Figures 1A and 2B, it can be seen that the pattern of CO_2 exchanges during the day correlates well with PEPC activity values found with glycerol in the extraction medium.

Table 1. The effect of Triton-X100 on the extractable activity of PEPC. Assay with PEP = 1.82 mM at pH = 8.1. Enzyme extracted during early light period. —: extraction without Triton. +: extraction with 1% Triton

Species	µMoles CO_2/min.g.fr.wt.		
	—	+	% increase
Kalanchoe daigremontiana	2.2	5.0	127
Aloe sp	14.6	33.3	128
Hoya carnosa	17.5	27.2	55
Sedum praealtum	12.5	21.8	71

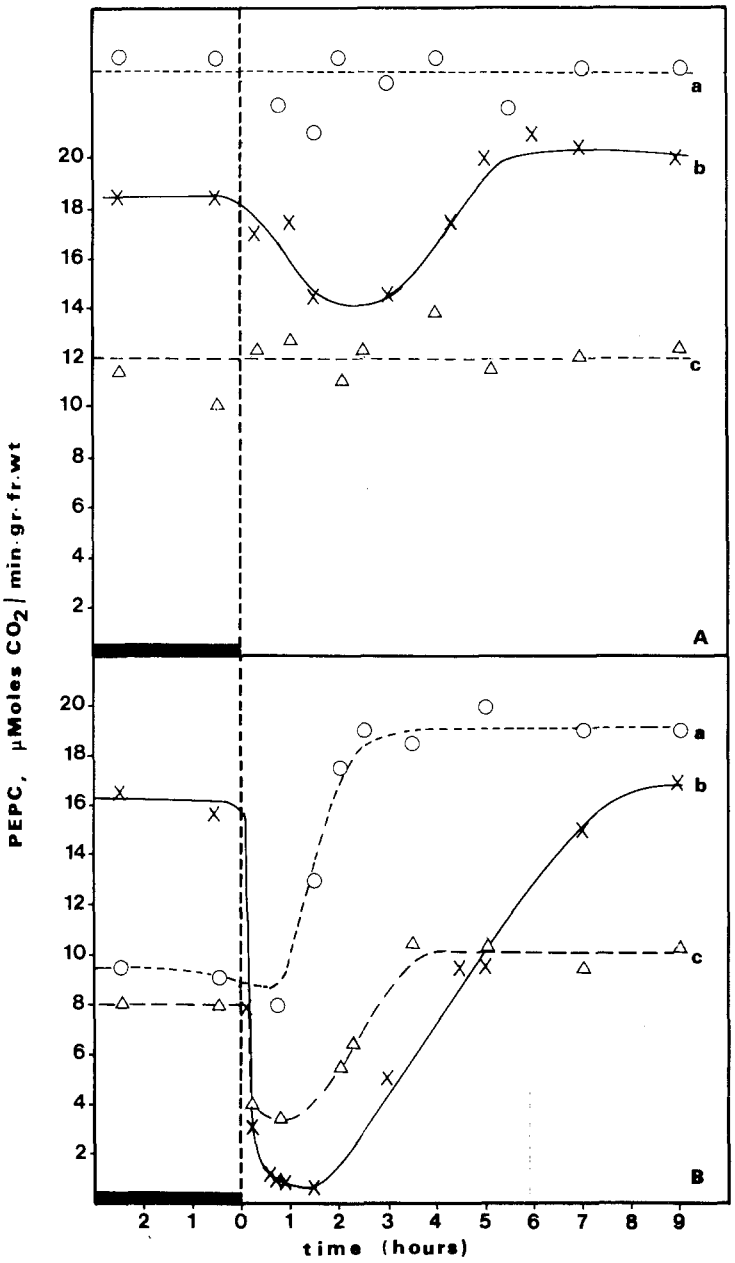

Figure 2. PEPC activity in crude leaf extracts around the night/day transition, as affected by the composition of the extraction medium. Enzymic source: *Sedum praealtum*. Assay run 5 min after removing the leaves from the plant. Extraction buffer with 1% Triton-X100 (a), 1% Triton-X100 plus 20% glycerol (b), or with no addition (c). PEP = 1.82 mM. A: pH = 8.1; B: pH = 7.1

All the above assays were run on crude extracts, 5 minutes after removing the leaves from the plants and at pH 7.1. When the assay was run at pH 8.1 (Fig. 2A) only minor changes in PEPC activity were found, even in the presence of glycerol in the extraction medium. Upon desalting through Sephadex G25 the differences between dark and light activities became smaller but not abolished.

Stability of the enzymic activity in crude extracts

The enzymic activity extracted at darkness was stable at 0 °C and for at least 3 hours, both in the presence and absence of glycerol. In contrast, enzyme extracted without glycerol during the light period showed a gradual activation on storage at 0 °C, whereas the addition of glycerol in the extraction medium prevented this apparent activation (Fig. 3). The same was true after desalting through Sephadex G25 equilibrated with or without glycerol respectively.

The gradual increase in PEPC activity in the absence of glycerol could not be prevented by incubation of the enzymic extract at 0 °C with 5 mM reduced

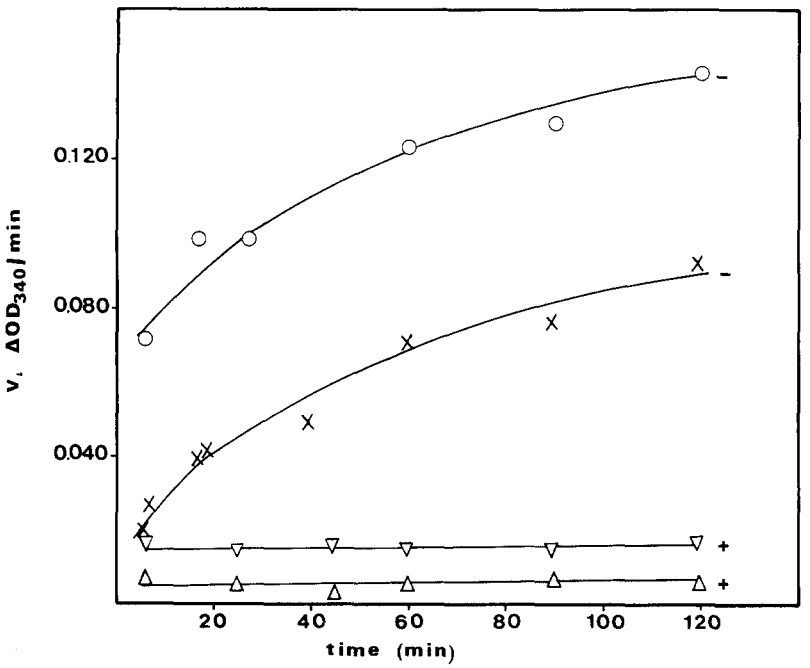

Figure 3. Stability of PEP activity extracted during the early light period, upon storage of the crude extract at 0°C. Enzymic source as in Fig. 2. Assay started at the indicated times by the addition of enzyme. Extraction with (+) or without (—) glycerol. PEP at 0.97 mM (△——△, X——X) or at 1.82 mM (▽——▽, ○——○). Assay pH = 7.1. Desalted extracts gave similar results

or oxidized glutathione, mercaptoethanol, dithiothreitol, ATP, P_i or 5 mM ATP + 1 mM cAMP.

Apparently, when glycerol is included in the extraction medium, the PEPC activities obtained not only fluctuate in accordance with the physiological demands for proper CAM function, but are also stabilized against activation in storage.

The stability offered by glycerol made it possible to study differences in enzymic properties between desalted preparations extracted before or after the dark/light transition.

Dark/light changes in pH dependence of activity

Activity/pH profiles were determined in desalted preparations, extracted at late dark or early light period, i.e. 1–2 hours before and after the onset of light. In this and subsequent experiments care was taken to compare equivalent amounts of enzyme, by using the same amount of enzymic extracts, taken from leaf samples of equal weight and comparable age.

As shown in Fig. 4, the pH profiles of the alleged two forms of PEPC were found to be quite different. A broad maximum occurs between pH 7.4–8.3 in the dark-form, whereas the light-form of the enzyme does not

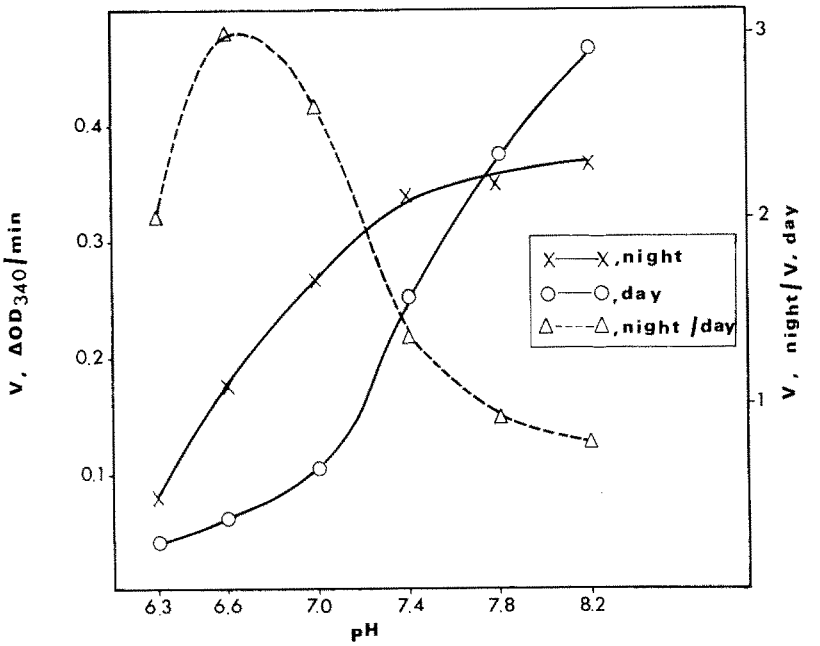

Figure 4. Rate/pH and night/dark activity/pH curves for PEPC from *Sedum praealtum*. Enzyme extracted during late dark or early light period. Assay started with the addition of desalted enzyme. PEP at 1.82 mM. pH 6.3 – 6.6, MES-KOH buffer. pH 7.0–8.2, Tris-HCl buffer

reach its optimum even at pH 8.2. Thus, in the pH region of 6.3–7.6 the former is more active, but above pH 7.6 the activities of the two forms are almost equal. This is shown in Fig. 4 as the ratio of dark/light activities in the pH ranges examined.

Dark/light changes in kinetic behaviour and sensitivity to malate inhibition

In agreement with earlier reports [9, 10, 21, 24] the light form of PEPC was found to be more sensitive to malate inhibition at both the substrate (PEP) concentrations used (Fig. 5). Since the extent of inhibition appeared to depend on PEP concentration, a study of the kinetic properties of the enzyme in the presence and absence of the inhibitor was carried out, at pH 7.1.

This particular pH was selected in an attempt to simulate the pH prevailing at the site of PEPC action during the first hours of light, when malate comes out of the vacuole, or at late dark period, when malate surpasses the storage capacity of the vacuole and starts accumulating in the cytoplasm.

As shown in Fig. 6A, the decrease of enzymic activity in the light is much more evident at low than at high PEP concentrations, e.g. the ratio of the light/dark activities, which is 0.12 at 0.5 mM PEP, becomes 0.47 at infinite (PEP) (Vmax). This is mainly due to the change of the rate vs substrate curves from hyperbolic in the dark-form to sigmoid in the light-form. Accordingly, double reciprocal plots give lines broken upwards for the light-form

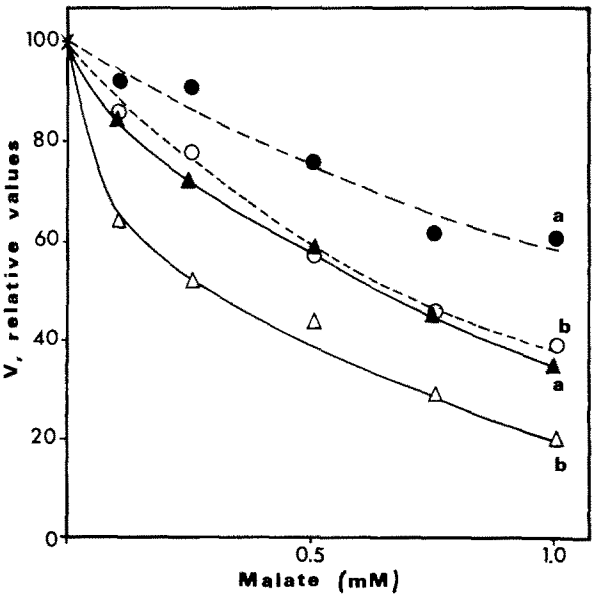

Figure 5. Inhibition of PEPC from *Sedum praealtum* by malate at pH 7.1. Enzyme extracted during late dark (a) or early light (b) period. Assay started with the addition of desalted enzyme. PEP at 1.46 mM (▲——▲, △——△) or at 2.92 mM (●——●, ○——○)

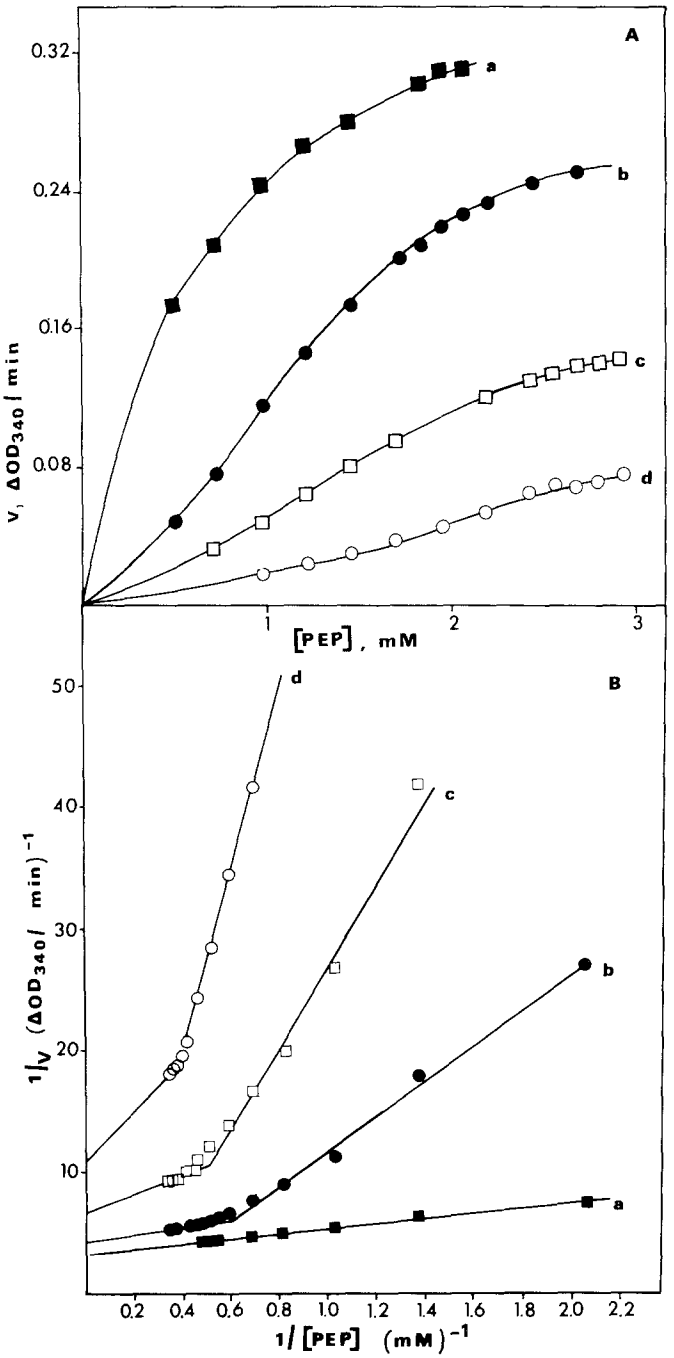

Figure 6. A: The effect of 0.5 mM malate on the rate curves of PEPC from *Sedum praealtum*, extracted during late dark and early light periods. a: night enzyme, b: night enzyme plus malate, c: day enzyme, d: day enzyme plus malate. Assay started with the addition of desalted enzyme. pH = 7.1. B: Double reciprocal plots of the data

Table 2. Kinetic properties of PEPC extracted during late dark and early light period

		Vmax (ΔOD/min)	$S_{0.5}$ (mM)	n
Night	– malate	0.411	0.68	1
	+ malate	0.344	1.46	1.51
Day	– malate	0.193	1.75	1.47
	+ malate	0.123	2.35	2.40

of enzyme (Fig. 6B) and a Hill analysis of the data reveals higher $S_{0.5}$ and n values (Table 2).

The effect of 0.5 mM malate on the two forms of the enzyme is also shown in Figs. 6A, B. The hyperbolic rate curve of the dark form is transformed to sigmoid by malate with a corresponding increase in $S_{0.5}$ and n. (Figs. 6A, B and Table 2). Similar is the effect of malate on the light form of PEPC; $S_{0.5}$ and n are increased even further than in the uninhibited enzyme.

Discussion

It is reasonable to propose that the regulation of CAM may involve a diurnal fluctuation of PEPC activity. The experimental search for such a difference is often based on the tacit assumption that extractable activity can serve as a relevant measure of the situation *in vivo*. The conflicting results (see Introduction) however, show that this assumption is not always valid. As shown recently [5, 24], activity differences wide enough to be physiologically meaningful can be obtained in *Mesenbryanthemum crystallinum* only when the assay is performed within a few minutes after extraction. Though activity extracted at night was high and stable, that extracted at day was initially low but increased with time, reaching the night values in 20 minutes after extraction.

It has been proposed, therefore, that PEPC in CAM plants can take two different conformations [24]; the less active day conformation is unstable under the usual extraction and storage conditions, changing rapidly to the more active and stable night conformation.

We found the same behaviour in PEPC from *Sedum praealtum* D.C. and in addition we stabilized the less active day-form with high concentrations of glycerol (Figs. 2 and 3), a component of the extraction medium used by von Willert's group [21, 22]. Apparently, it was this additive that enabled them to find a pronounced drop in extractable PEPC activity soon after the onset of light, without the precaution of assaying immediately after extraction. Glycerol has also been used as a stabilizer of PEPC from C_4 plants [4, 15, 26]. An attempt to stabilize the malate sensitive day-form of this enzyme in a CAM plant, by extraction and storage at low pH (7.0) and 10 mM malate, has been reported [25].

The stabilization of the day-form offered the opportunity for a com-

parative investigation of the kinetic behaviour and pH profiles of the two conformational states of the enzyme. The data obtained for the less active day-form are quite interesting and novel, because the information available so far for PEPC from CAM plants [8, 10, 11, 13, 20] is appparently related to the stable activated state of the enzyme.

The differences in kinetic behaviour, pH profiles and sensitivity to malate are not only pronounced but physiologically meaningful as well. The main difference between the two forms of PEPC in pH dependence is the much stronger depression of the day activity at low pH, particularly in the region 6.5–7.0, where the ratio of the activities (night/day) becomes almost 3 (Fig. 4). Thus, the lowering of the cytoplasmic pH, which is assumed to happen with the onset of light and the consequent exit of malate from the vacuole, is more suppressive for the activity of the day-form, than it would have been for the unaltered night-form of the enzyme. An extension of our observations to other CAM species appears to be necessary, however, before a generalization can be drawn, since the pH profiles of PEPC of CAM plants seem to be quite variable [5, 8, 11, 13]. Our data, obtained with desalted extracts, are in agreement with earlier results [21, 24] based on crude extracts; in these cases interference from the coextracted malate could not be avoided and so the validity of the results was rather questionable.

The change of the rate curves from hyperbolic (night-form) to sigmoid (day-form) may be physiologically relevant, since the suppression of PEPC activity in the light becomes increasingly strong at lower PEP concentrations, which are expected to prevail *in vivo* at the carboxylation sites. The hyperbolic rate curves reported in the past for PEPC from CAM species [10, 11, 20] are not in disagreement with our results, since no precautions had been taken to stabilize the day-form of the enzyme and a reversion to the night-form could have occurred before the assays.

Equally important is the increased sensitivity of the day-form towards malate, a finding that confirms earlier results [9, 10, 21, 24]. The effect of malate in transforming the rate curves to sigmoid enhances further its inhibitory action at the physiological levels of PEP concentration. It appears that the regulation of the PEPC activity in CAM may be effected by a concerted action of light, pH shifting and malate.

It is now clear that light brings about a transformation of PEPC from an active to an inactive (or less active) form, but the mechanism of this effect is still unclear. The results obtained with reduced or oxidized thiols on the *in vitro* rate of activation suggest that this change does not involve sulfhydryl-bisulphide interconversions, as it has been inferred for the C_4-enzyme [14]. Similarly, phosphorylation by a protein kinase [1] does not seem to be responsible for the inactivation of the enzyme, since the addition of ATP or ATP + cAMP or P_i does not affect the rate of reactivation of the day-form *in vitro;* if the reactivation were due to a phosphatase, the inorganic phosphate would have been expected to inhibit it.

Our results raise several additional questions, which remain to be answered in the future. The differential effect of Triton X-100 on the extractability of PEPC activity during the dark or the light phase of the diurnal cycle might be related to a reversible binding of the enzyme on lipophilic components of the cell. The mechanism of the glycerol effect in the stabilization of the inactive day-form of the enzyme is still unexplored, though it may simply be related to the increased viscosity of the medium. Finally, if, as seems probable, the activation of the day-form after grinding is due to the destruction of the internal compartmentation of metabolites, the extent of activation and its avoidance may depend on the species used for experimentation. It is indeed our experience that the behaviour of PEPC in crude or desalted extracts from different species (CAM or C_4) is variable, depending not only on the species used, but on growth conditions as well. Whether this variability is inherent or imposed by coextracted metabolites or macromolecules remains an open question.

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