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Detecting Photoactivation of Phosphoenolpyruvate Carboxylase in C_4 Plants[1]

AN EFFECT OF pH

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ABSTRACT

Photoactivation of phosphoenolpyruvate carboxylase in C_4 plants is detected more efficiently when activity is assayed at suboptimum pH (*e.g.* 7.2); the magnitude of the light effect is often larger at low phosphoenolpyruvate concentration.

Darkness and low assay pH induce an allosteric behavior (positive cooperativity with phosphoenolpyruvate) which is relieved in light or by higher pH; thus, normal Michaelis-Menten kinetics are exhibited only when the enzyme is extracted during the day and assayed at pH 8.2.

Light activation, pH, and substrate level appear to be components of a regulatory device suppressing the activity in darkness and enhancing it under light.

Light activation of PEPCase[2] in *Salsola soda* L. and other C_4 plants was recently reported from our laboratory (8). The magnitude, however, of the detectable light effect on different species was variable and, further, no activation could be observed in several C_4 plants. This variability could be interpreted in two ways; either photoactivation of PEPCase occurred indeed in some, but not all, C_4 species or the failure to observe the light effect was due to unsuitable extraction or/and assay conditions. The latter alternative seemed more reasonable, not only out of respect for Occam's razor but mainly because pH-dependent failures in detecting effects of light on enzymic properties are already known. The photoactivation of chloroplastic F-1,6-P_2 phosphatase could not be detected at pH 8.8 (optimum for activity), but only at pH 7.9 (9) or 8.1 (3). Similarly, a more pronounced activation of the purified enzyme by DTT at pH 7.9 than 8.8 (1) and by reduced Fd at pH values lower than 8.0 (2) have also been reported. The low activity (7, 14) and high sensitivity to malate (14) of the day form of PEPCase from *Mesembryanthenum crystallinum* (performing CAM) were much more evident at a lower pH than at 8.0. In *Sedum praealtum* this low activity of PEPCase after the onset of light could only be shown by stabilization with glycerol in the extraction medium (10).

It was tempting, therefore, to investigate the effect of extraction and assay pH on the detection of PEPCase's photoactivation, selecting species in which our previous attempt (8) to show activation by light was unsuccessful.

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[2] Abbreviations: PEPCase, phosphoenolpyruvate carboxylase; F-1,6-P_2, fructose-1,6-bisphosphate; G-6-P, glucose-6-phosphate.

MATERIALS AND METHODS

Plants were grown in soil pots in the open during September and October 1983. *Saccharum ravenae* (L.) Murray, *Amaranthus* sp, and *Cynodon dactylon* (L.) Pers. were chosen as experimental plants because no light activation of their PEPCase had been found in a previous survey (8) using routine extraction (pH 7.7) and assay (pH 8.2, PEP 1.82 mM) media. *Setaria verticillata* (L.) Beauv. was also used, since only a 20% activation had been determined in the same survey.

For enzyme extraction, 500 mg of mature leaves were ground in a prechilled mortar with purified sea sand and 5 ml of extraction medium (0.1 M Tris-HCl, pH 7.2 or 8.2, 1 mM EDTA, 10 mM $MgCl_2$, 25% v/v glycerol, 3% w/v PVP plus a small amount of insoluble polyvinylpolypyrrolidone). The extract was centrifuged for 1 min and the clear supernatant desalted through a 12 × 1 cm Sephadex G25 column equilibrated with 0.1 M Tris-HCl (pH 7.2 or 8.2) in 25% v/v glycerol. The pH of equilibration was the same as that of extraction buffer. All above steps were carried out at 4°C, and lasted for 15 min.

Assays of PEPCase activity were run at 30°C in 3 ml final volume of 1 mM $NaHCO_3$, 5 mM $MgCl_2$, 0.14 mM NADH, 4.5 units of malate dehydrogenase (pig heart, Sigma), PEP as specified, and either 0.1 M Tris-HCl (pH 7.2–8.2) or 0.1 M Hepes-KOH (pH 6.9). Enzyme activity at pH 7.6 was the same in Tris or Hepes buffer. The reaction was started with the addition of enzyme and its rate was measured by the decrease in absorbance at 340 nm (oxidation of NADH). The first absorbance difference was taken between 60 and 105 se after the addition of the enzyme; three to four additional measurements were taken subsequently, to assess the rate of the decline in activity (6); true initial activities were calculated by mathematical extrapolation to zero time. The reasoning for this procedure was discussed previously (6).

RESULTS AND DISCUSSION

A series of pilot experiments was initially carried out to assess the effect of several factors on PEPCase activity extracted at day or night. Extractions and assays were done at pH 7.2 and 8.2, using a low (0.49 mM) and a high (1.82 mM) PEP level in the assay. Leaves were extracted at the end of the dark period and about 2 h after the onset of light. All 16 possible combinations of the above variables were examined on each of the plant species used (Table I). Only the data obtained with high PEP in the assay are presented in detail, since those with low PEP showed the same general trends. Only in *C. dactylon*, and less in *Amaranthus*, the day/night ratios were higher when activities were assayed at pH 7.2 and low (0.49 mM) PEP.

It is obvious that a considerable photoactivation of PEPCase (2- to 3-fold) can be detected in the four species examined if the proper combination of extraction and assay pH values is used.

Table I. *Effects of Extraction and Assay pH on the Detection of PEPCase Photoactivation in C_4 Plants*

The magnitude of photoactivation is given (in parentheses) as day/night activity ratio. PEP in the assay at 1.82 mM; light at about 2000 μE/m²·s PAR from sunlight

Treatment	Enzymic Activity (Activity Ratio)							
	Assay at pH 7.2				Assay at pH 8.2			
	Extraction pH 7.2		Extraction pH 8.2		Extraction pH 7.2		Extraction pH 8.2	
A. *S. ravenae*[a]								
Light	9.8	(3.5)	11.9	(1.1)	23.0	(2.6)	23.4	(0.8)
Dark	2.8		10.6		9.0		28.5	
B. *Amaranthus* sp[a]								
Light	8.4	(1.8)	8.0	(1.9)	8.8	(1.2)	7.8	(1.6)
Dark	4.7		4.3		7.3		4.8	
C. *S. verticillata*								
Light	9.8	(2.3)	16.7	(3.0)	18.4	(0.8)	25.3	(1.3)
Dark	4.2		5.5		22.4		18.9	
D. *C. dactylon*								
Light	34.6	(2.0)	30.4	(1.1)	36.5	(0.8)	23.8	(1.0)
Dark	17.7		27.0		44.6		22.8	

[a] Means of two replications.

Table II. *Effect of Assay pH on Kinetic Parameters and Calculated Allostericity (Hill Coefficient) of PEPCase from S. verticillata in Light and Darkness*

Light was at about 2000 μE/m²·s PAR from sunlight. Extraction was at pH 8.2.

pH	Day			Night		
	V_{max}	$S(PEP)_{0.5}$	n	V_{max}	$S(PEP)_{0.5}$	n
	μmol CO_2/min·g fresh wt	mM		μmol CO_2/min·g fresh wt	mM	
7.2	30.0	3.38	1.9	9.8	2.35	3.3
7.5	24.7	1.55	1.8	15.5	1.78	2.4
7.8	24.7	1.43	1.2	15.5	1.40	1.5
8.2	24.7	0.95	1.0	15.5	0.90	1.4

A low pH in the assay appears to be the decisive factor in obtaining a large day/night activity ratio, whereas the response to pH of extraction depends on species. In *Saccharum ravenae*, extraction at high pH suppresses the day/night activity differences. This effect could be interpreted either as an activation or as an inactivation of the night form of the enzyme by high or low pH, respectively. We are inclined to accept the former alternative, since a pH of 7.2 seems much more probable for the *in vivo* environment of a cytoplasmic enzyme. In addition, photoactivation of PEPCase is a common phenomenon among C_4 species (8 and this study) and the latter alternative would make *S. ravenae* the only exception. The same effect of high extraction pH is observed in *C. dactylon*, but only when the assay is run at pH 7.2 and high PEP. In contrast, the extraction pH does not interfere with the detection of the light effect in *Amaranthus*, whereas in *S. verticillata* extraction at pH 8.2 results in somewhat higher day/night activity ratios, mainly due to an increase of the day activity. Higher day/night ratios are determined with low PEP in the assay (pH 7.2) in *Amaranthus* and *C. dactylon*, suggesting a more effective suppression of night PEPCase activity under rate-limiting PEP concentrations.

Detailed kinetic analyses, aiming at an understanding of the light-dark modulation of enzymic behavior at different pH values, were subsequently carried out. PEPCase activity, extracted (pH 8.2) from *S. verticillata* at night and day, was assayed at 9 to 11 different PEP levels (0.49–4.1 mM) and four pH values, spanning the range 7.2 to 8.2. The kinetic parameters obtained after a Hill analysis of the data are given in Table II.

The allosteric behavior of the enzyme becomes more pronounced in darkness and low pH, normal Michaelis-Menten kinetics being obtained only in light and high pH. The effect of suboptimum pH in inducing positive cooperativity with the substrate (PEP), in purified PEPCase from corn, has already been reported (12, 13). The modulation of this allostericity by light and darkness has also been found in *Salsola soda* (8). Both observations are now verified on PEPCase from *S. verticillata* and a suggestion on their apparent physiological significance can be made (see "Concluding Remarks").

Maximum activity is increased by light and remains constant in the pH range 7.5 to 8.0 both under light or darkness. An independence of V_{max} from pH has been proposed by O'Leary (11) after a detailed kinetic study with purified PEPCase from corn. Though O'Leary claims no effect on V_{max} down to pH 6.5, this insensitivity seems to break at pH 7.2 in our preparations. The physiological significance of V_{max}, however, is rather doubtful, since enzymes *in vivo* are not functioning at full capacity, as a rule, and there is no evidence that PEPCase is an exception. The pH activity profiles of the day and night form of *Setaria* PEPCase, at a relatively low PEP level, are shown in Figure 1; the two forms show a comparable pH dependence but the activity of the night form is increased to a larger extent when assayed at alkaline pH values. Thus, the photoactivation (day/night activity ratio) is much more evident in the pH range 7.2 to 7.5.

Similar results were obtained from *C. dactylon*, with PEPCase activities extracted either at pH 7.2 or at 8.2 and assayed at low (0.36 mM) and high (2.43 mM) PEP levels (Fig. 2). With low PEP, the higher day/night activity ratio is obtained at pH 7.2, whereas the optimum pH for detecting photoactivation becomes even lower when activities are assayed at high PEP concentration. Though detailed kinetic analyses at various pH values were not attempted, it is apparent that the response of *Cynodon* PEPCase to pH and light follows the basic pattern obtained with *S. verticillata, i.e.* enhancement of allosteric behavior by lower pH and darkness.

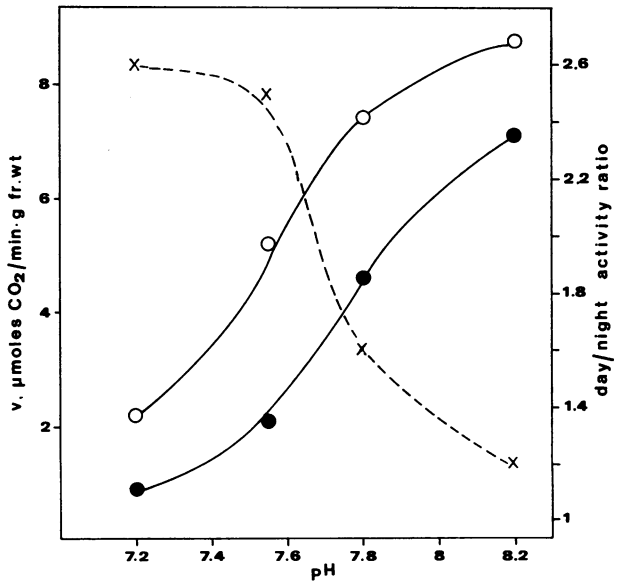

FIG. 1. Day- (O——O) and night-extracted (●——●) activity of PEPCase, assayed at different pH values and the respective day/night activity ratios (×——×) as a measure of the detected photoactivation. Extraction at pH 8.2; PEP at 0.73 mM; light at about 2000 μe/m²·s PAR from sunlight; enzymic source: leaves of *S. verticillata*.

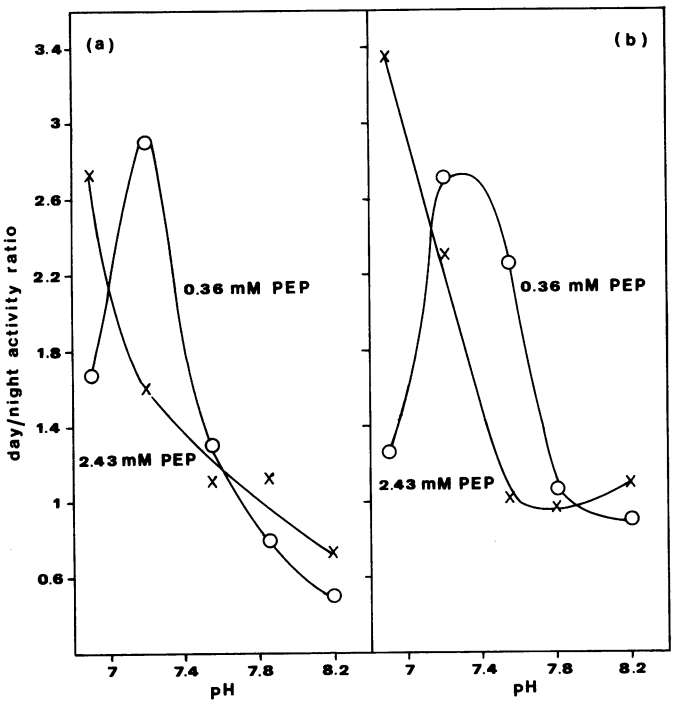

FIG. 2. Magnitude of detected PEPCase photoactivation in *C. dactylon*, at different assay pH values and two PEP levels. (a), Extraction at pH 8.2; (b), extraction at pH 7.2; light at about 2000 $\mu E/m^2 \cdot s$ from sunlight.

CONCLUDING REMARKS

The failure to detect the photoactivation of PEPCase in earlier works has been attributed to the presence of thiols in the extraction media (5, 8). It is now evident that unsuitable assay pH was an additional factor, since the highest day/night activity ratio is obtained when the assay is run at a pH (7.2–7.4), much lower than the optimum for PEPCase activity. In some cases, a low substrate (PEP) concentration (*e.g.* 0.5 mM) also helps in assaying for photoactivation, provided that PEPCase activity is detectable under both the above limitations. A high pH should also be avoided in extraction, since it may obscure the picture of photoactivation (*e.g.* in *S. ravenae*); the intermediate pH 7.7 could serve as a compromise between the two extremes tested in extraction.

Most interesting is the emerging picture of a cooperative action of light and other factors in PEPCase modulation. The strong sigmoidicity of the rate curves in darkness and low pH suggests that PEPCase activity *in vivo* could be suppressed to very low values during the night, if pH and PEP levels were kept low in the carboxylation site. On the other hand, only moderate increase of PEP and/or pH after the onset of light, in concert with the light-induced changes in the properties of the enzyme, could support a high activity during the day. Though substantial data on cytoplasmic pH and PEP levels, in light or darkness, are not available, the above postulates do not seem unreasonable, in view of the fact that variations in the levels of metabolites and in the pH are not rare in the biochemical system *in vivo*.

It is noteworthy that the enzymic capacity for PEP carboxylation in mesophyll cells of C_4 plants is estimated to be 4-fold higher than the observed photosynthetic rates (Table 10.1 in Ref. 4). Therefore, there is ample margin for a loss of PEPCase capacity (*i.e.* by functioning at suboptimum pH and low PEP levels), if the regulation of the activity by light and other modulators is more efficient at lower pH.

It is not yet evident why photoactivation is routinely observed in some C_4 species (8), but only under special precautions in others. Apparently, the two forms of the enzyme are easily interconvertible and their stability during extraction and assay varies considerably, depending on pH and/or coextracted compounds. We have used glycerol at high concentration (8, 10, and this work) for stabilization of the prevailing *in vivo* enzymic form, but a more efficient method is needed for future work.

In the light of our results, it is also obvious that the two forms of PEPCase may respond differentially to the known metabolic effectors (G-6-P, malate, etc.) and a reevaluation of their action at a suboptimal pH seems necessary.

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