Plant Physiol. (1983) 71, 187–189
0032-0889/83/71/0187/04/$00.50/0

Reduced Glutathione as an Effector of Phosphoenolpyruvate Carboxylase of the Crassulacean Acid Metabolism Plant *Sedum praealtum* D.C.

Received for publication July 9, 1982 and in revised form September 30, 1982

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ABSTRACT

Reduced glutathione, but not mercaptoethanol or dithiothreitol, inhibits phosphoenolpyruvate carboxylase (PEPC) in desalted leaf extracts from *Sedum praealtum* D.C. The inhibition is more evident at low pH values (< 7.2) and becomes increasingly smaller at higher pH. In the presence of the inhibitor, the hyperbolic rate curve of night PEPC is transformed to sigmoid and the $S_{0.5}$ is increased. When the enzyme is extracted during the day, the rate curve is sigmoid and it is not changed by the inhibitor, though the $S_{0.5}$ is further increased. Oxidized glutathione is completely inactive. Levels of reduced glutathione in leaf tissue are distinctly higher in the light. A role of photosynthetically reduced glutathione in the regulation of PEPC in Crassulacean acid metabolism species appears probable.

In CAM, the activity of PEPC[1] should be strictly regulated *in vivo*, since the biochemical shift from dark CO_2 fixation to reductive assimilation in the light poses opposite requirements on this enzyme; high activity in the dark should alternate with quiescence in the light, if futile cycles of CO_2 refixation on PEP were to be avoided (7, 9, 12, 17).

Although the details of the regulatory mechanism remain obscure, an overall outline emerges from the results of recent investigations; the onset of light brings about some distinct changes on PEPC properties, which, in unison with higher malate concentration and lower pH, can effectively turn the enzyme off *in vivo* (6, 7, 9, 15–17). An alleged conformational change (5, 9, 17) confers higher sensitivity towards malate (6, 7, 9, 15, 17), suppresses activity at lower pH (9, 15, 17), and increases $S_{0.5}$ for PEP, due to the induction of positive cooperativity (9).

In the course of a previous work in our laboratory, we noticed that GSH inhibited the activity of the day form of PEPC from the CAM plant *Sedum praealtum* D.C., though it did not affect the rate of the fast *in vitro* reversion to the more active night form in the absence of glycerol (9). Since sulfhydryl groups have been implicated in the activity of PEPC from C_4 plants (3, 10), we studied in more detail the effects of glutathione on this enzyme from *S. praealtum* D.C. in the hope of gaining some insight concerning the chemical signals affecting the night/day changes in the behavior of PEPC in CAM.

MATERIALS AND METHODS

Plants were grown in soil pots in a growth chamber with temperature, RH, and light/dark cycles of 28/18°C, 40/70%, and 10.5/13.5 h, respectively. Irradiance at plant level was around 250 $\mu E/m^2 \cdot s$ PAR, given by a mixture of fluorescent and incandescent lamps. Under these conditions, CAM plants exhibited typical CAM, as judged by nocturnal CO_2 absorption and malate accumulation (9).

For enzyme extraction from *Sedum praealtum*, three mature leaves of about the same age (length, 4 cm; weight, 2.5 g) were ground in a prechilled mortar with a small amount of purified sea sand and 5 ml of extraction medium containing 0.2 M Tris-HCl (pH 7.6), 1 mM EDTA, 10 mM $MgCl_2$, 5% w/v polyethylenglycol, 1% v/v Triton X-100, and 20% v/v glycerol. The extract was centrifuged and the clear supernatant was desalted through a 12 × 1 cm Sephadex G-25 column equilibrated with 0.1 M Tris-HCl (pH 7.6) in 20% v/v glycerol. Triton X-100 was used to maximize enzyme extractability, while glycerol has been shown to stabilize the enzyme against fast activity changes after extraction (9). 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 of the indicated pH, 0.14 mM NADH, 1 mM $NaHCO_3$, 5 mM $MgCl_2$, 4.5 units of malate dehydrogenase (pig heart; Sigma), and PEP and GSH as specified. The reaction was started with 100 μl of desalted extract and its initial rate was measured by the decrease in *A* at 340 nm (oxidation of NADH).

For the estimation of GSH, the tissue (3 g) was extracted in a prechilled mortar with 10 ml of 0.25 M phosphate buffer (pH 6.8), and the homogenate was centrifuged at 30,000*g* for 10 min. All above steps were carried out at 0 to 4°C. The supernatant was immediately deproteinized by incubation at 100°C for 3 min and centrifugation in the cold (4°C) at 15,000*g* for 5 min. Sulfhydryl and GSH content in the supernatant were measured as described by De Kok *et al.* (2)

All experiments were performed at least three times; results from representative experiments are shown in figures and tables.

RESULTS

GSH inhibits the activity of PEPC from *S. praealtum* D.C. at pH 7.1 in a concentration-dependent manner, whereas at pH 8.1 the extent of inhibition is practically nil (Fig. 1). As it is usual with PEPC, the absolute values of extracted activities vary considerably from experiment to experiment and the percent inhibition also fluctuates, but the general picture is repeatedly the same. GSH affects both the day and night form of the enzyme, though the inhibition is always stronger on the former.

The observed inhibition could not be attributed to an artifact of NAD reduction by GSH through a reversed activity of glutathione reductase; such an activity was absent in our preparations under the conditions of the assay. The pH/activity and inhibition profiles of the two forms of PEPC are shown in Figure 2. The small difference in the extent of inhibition on the two forms of the

[1] Abbreviation: PEPC, phosphoenolpyruvate carboxylase.

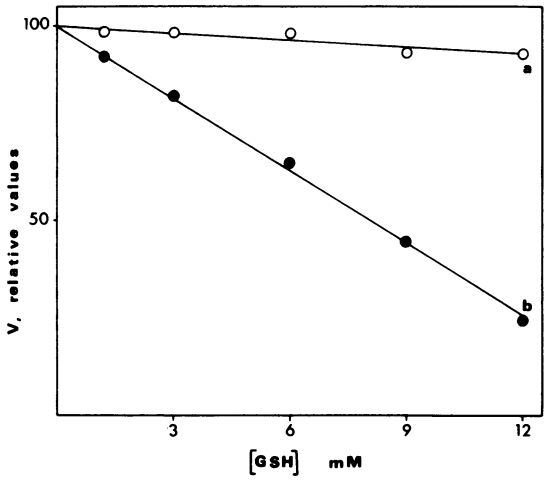

FIG. 1. Inhibition of PEPC from *S. praealtum* by GSH at pH 7.1 (●) or 8.1 (○). Enzyme extracted during early light period (1–2 h after the lights are on). Assay started with the addition of desalted enzyme. PEP at 1.82 mM.

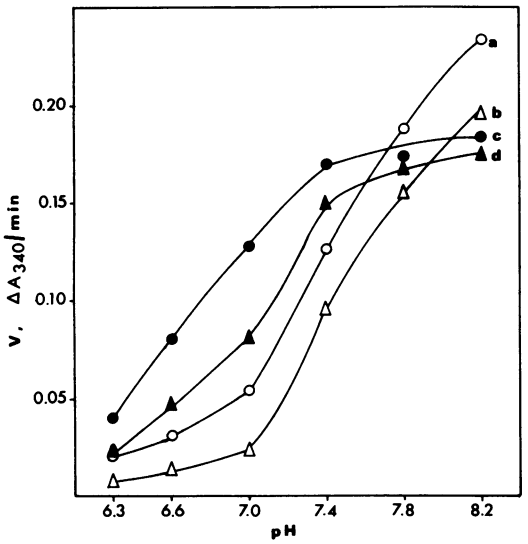

FIG. 2. Rate/pH and inhibition profiles for PEPC from *S. praealtum*. Enzyme extracted during early light (a,b) or late dark (c,d) period. Assay with (b,c) or without (a,c) 6 mM GSH. Assay started with the addition of desalted enzyme. PEP at 1.82 mM; pH 6.3 to 6.6, Mes-KOH buffer; pH 7.0 to 8.2, Tris-HCl buffer. Late dark and early light denote 1 to 2 h before and after the lights are on, respectively.

enzyme does not distort their distinct pH dependence (9). Unexpectedly, this inhibitory effect of GSH is specific, *i.e.* it is not observed with other thiols. DTT up to 5 mM and mercaptoethanol up to 30 mM do not affect the enzymic activity. Similarly, the effect seems to be specific for PEPC extracted from certain CAM and C_3 plants, while activities from C_4 plants are practically unaffected by GSH in the assay medium (Table I). In addition, the oxidized forms of glutathione and DTT do not affect the enzymic activity from *S. praealtum* D.C.

Activity *versus* PEP concentration curves of the two forms of PEPC from *S. praealtum* D.C. (Fig. 3) show some details of the GSH inhibition. The data shown in Figure 3 have been processed by Hill plots and the numerical results are shown in Table II. In both the night and day forms of the enzyme, GSH decreases the affinity for PEP (higher $S_{0.5}$), so that the inhibition becomes much stronger at low substrate concentrations, which are supposed to prevail *in vivo* at the carboxylation sites. Maximum activities are

Table I. *Percent Inhibition by GSH of PEPC Activity from Various CAM, C_4, and C_3 Species*

GSH at 6 mM; PEP at 1.82 mM. At pH 8.1, the activity was not inhibited in the CAM and C_4 species.

Plant Species	Inhibition	
	pH 7.1	pH 8.1
	%	
CAM plants		
Sedum praealtum D.C.	43	
Hoya carnosa (L.) R.Br.	58	
Aloe sp.	36	
Echeveria sp.	35	
Kalanchoë tubiflora (Harvey) Hamet	15	
Kalanchoë daigremontiana Hamet et Perr.	0	
Sedum sediforme (Jacq.) C. Pau[a]	9	
C_4 plants		
Digitaria sanguinalis (L.) Scop.	0	
Sorghum bicolor (L.) Moench	20	
Atriplex halimus L.	8	
Cyperus rotundus L.	0	
C_3 plants (field grown)		
Capparis spinosa L.	32	7
Robinia pseudacacia L.	72	50
Inula viscosa (L.) Aiton	82	56
Tradescantia sp.	46	8

[a] Not previously reported as CAM, but found to exhibit diurnal malate fluctuations (unpublished results).

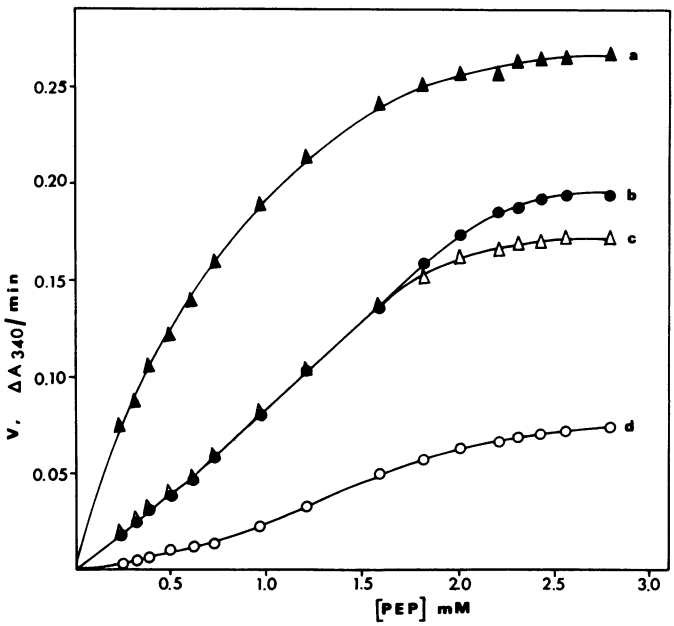

FIG. 3. The effect of 6 mM GSH on the rate curves of PEPC from *S. praealtum* extracted during late dark and early light period. a, Night enzyme; B, night enzyme plus GSH; c, day enzyme; d, day enzyme plus GSH. Assay started with the addition of desalted enzyme; pH = 7.1.

also decreased, a fact that may not have physiological significance, but reveals a complex mode of inhibition. Most interesting is the transformation of the hyperbolic Michaelis-Menten behavior of the night form of PEPC to sigmoid with a *n* around 2, an effect similar to that of light (9). The signoidicity obtained with the day form of PEPC is not accentuated further by GSH, though the activity at low PEP levels is much more depressed.

Determinations of GSH content in whole leaf tissues of *S.*

Table II. *Kinetic Properties of PEPC Extracted during Late Dark and Early Light Period*

		V_{max}	$S_{0.5}$	n
		$\Delta A/min$	mM	
Dark	− GSH	0.344	0.87	1
	+ GSH	0.202	1.21	2.2
Light	− GSH	0.243	1.42	2.0
	+ GSH	0.135	2.35	1.9

Table III. *GSH Content of the Leaves at Late Dark and Early Light Period*

Means from five separate determinations.

	GSH
	$\mu g/g$ *fresh wt*
Dark	2.87 ± 0.16
Light	3.99 ± 0.12
Increase	39%

praealtum D.C. showed that the levels of this metabolite are increased significally soon after the onset of light (Table III). Though the compartmentation of GSH and its concentration at the carboxylation sites remain unknown, the results imply that GSH could be a physiological modulator of PEPC, suppressing the day form to a minimum activity.

DISCUSSION

The main question arising from our results concerns the physiological relevance of the observed GSH effect. The smooth functioning of CAM calls for a suppression of PEPC activity after the onset of light, and such a partial inactivation is apparently brought about by a combination of a light-induced change in enzymic properties, low pH, and high malate concentrations (6, 7, 9, 15–17). The existence of additional controls, however, cannot be excluded and glutathione, a sulfhydryl-disulphide component of most, if not all, living cells, could play such a role. This interpretation is supported by the specifity of the PEPC inhibition by GSH, which could not be substituted in this effect by other thiols. The fact that PEPC from CAM species (and not C_4-PEPC) is affected also suggests a physiological role, since only in CAM should the activity of PEPC be turned off in the light. It is not, however, equally easy to assign an *in vivo* role to the observed similar effect of GSH on C_3-PEPC. In addition, a large number of C_3-species should be examined, inasmuch as GSH was reported in earlier works (1, 11) as a necessary component of the extraction and assay media for stabilization of the enzymic activity from C_3 plants.

The measurements of GSH in the leaves of *S. praealtum* show a significant increase in the light, though the concentrations on a whole leaf basis are generally low. However, in view of the usual compartmentation of metabolites, one can visualize easily that GSH may reach the effective concentrations at the carboxylation sites; *e.g.* high concentrations of glutathione (up to 3.5 mM), predominantly in the reduced form, have been reported in chloroplasts (4). Critical in this respect would be the site of the light-induced GSH increase, in relation to the location of PEPC in CAM cells. Though the latter is still considered uncertain (13), recent evidence points towards a cytoplasmic PEPC (14, 18).

Whether cytoplasmic or chloroplastic, it can be estimated that the light-generated amount of GSH (≈ 1.1 $\mu g/g$ fresh weight) should be restricted to the 1/1,000 of the total volume of the leaf tissue, if it were to reach the *in vitro* effective concentration (3–4 mM). Such an 'active volume' for PEPC is not unreasonable, if we take into account the extreme vacuolation of the CAM cell (8) and the existence of nonphotosynthetic cells in the tissue used.

In addition to the increase of the total amount of GSH, there is also the possibility for a light-induced transport towards the active site; in that case, either the active volume or the final concentration of GSH could be much higher.

Concerning the effect of light in transforming PEPC to a less active form (9), it is not possible at present to decide whether it is mediated by GSH in *S. praealtum*. Both light and GSH induce a sigmoidicity in the rate curves, but GSH does not affect the reversion of the less active day-form to the active night-form after extraction (9).

Several additional questions remain open for future work. Because sulfhydryl group(s) have been implicated in the activity of PEPC (3, 10), a sulfhydryl-disulphide exchange seems the most probable mechanism of GSH action. Such an effort could be either direct or enzyme-mediated and its elucidation is the object of our current effort. Also, it remains to be seen whether there are real inherent differences among CAM species concerning the effect of GSH on PEPC or whether the lack of response in some of them is an artifact of extraction.

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