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RELATION BETWEEN PHOTOSYNTHETIC AND PHENOLASE ACTIVITIES IN SPINACH CHLOROPLASTS

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Key Word Index—*Spinacia oleracea*; Chenopodiaceae; spinach; activation; chloroplast; latency; osmotic swelling; phenolase; photosynthesis; photo-oxidation.

Abstract—During 0°-storage of class I chloroplasts from spinach leaves, activation of phenolase strongly correlates with the inactivation of photosynthetic reduction of 3-phosphoglyceric acid, indicating that intact chloroplasts do not contain active phenolase, but that latent phenolase is activated irreversibly by ageing. Light activation of spinach phenolase cannot be studied in photosynthetically active chloroplasts because it is superimposed by photoreactions. Phenolase activity in functionally intact thylakoid preparations, measured in the dark immediately after light-induced reversible volume changes, did not indicate any reversible activation of phenolase. In contrast, light increased irreversible activation.

INTRODUCTION

The large amount of latent phenolase in chloroplasts from several plants [1] and the low phenolase activity in freshly prepared chloroplasts [1, 2] imply that chloroplast phenolase may be inactive *in vivo*. Tolbert [1] found that in chloroplast preparations of some plants (e.g. wheat) phenolase is inactive in the dark, but is activated (partially reversible) by light, independent of photosynthetic reactions. With spinach chloroplast preparations, in contrast, Tolbert found that light increases pre-existing phenolase activity.

In contrast to some other plants (e.g. wheat [1]), spinach chloroplast phenolase is spontaneously activated by its release from thylakoid membranes during homogenization and storage [2]. During storage, thylakoid membranes swell and lose their photosynthetic activity [3].

Photosynthetically active, broken chloroplasts undergo reversible volume changes in the light or in the presence of several osmotically active substances (e.g. [4]). Possibly, phenolase may be activated *in vivo* reversibly in this way. Irreversible activation of chloroplast phenolase by light is known from results of Siegenthaler [3, 5].

We have used class I chloroplasts from spinach leaves to prove earlier findings indicating zero activity of phenolase *in vivo* and have found no reversible but only irreversible activation of phenolase in broken chloroplasts under conditions of light/dark reversible volume changes. These experiments are reported here.

RESULTS AND DISCUSSION

Phenolase activity in isolated class I *chloroplasts*

A suspension of isolated class I chloroplasts was prepared following the methods of ref. [6]. According to phase contrast microscopy *ca* 80% of the chloroplasts have been in a class I status. The loss of the chloroplast envelopes during storage is indicated by their decreasing photosynthetic activity, measured with 3-phosphoglyceric acid (3PGA) [7]. Upon storage of the chloroplast suspension at 0° in the dark, photosynthetic activity decreases, whereas phenolase activity increases (Fig. 1). The strong negative linear correlation between these activities indicates that phenolase activity in intact chloroplasts is zero and that activation of latent enzyme occurs upon rupture of the chloroplast envelope.

Interference between phenolase activity and photosynthetic activity in illuminated chloroplasts

Reversible volume changes of thylakoids require photosynthetically active broken chloroplasts (e.g. [4]). Tolbert's [1] experiments with spinach chloroplasts did not indicate any photosynthetic reaction when phenolase activity was followed in the light after a dark period. During short exposure to light (Fig. 2b) O_2 consumption by phenolase-catalysed 4-methylcatechol (4MC) oxidation is superimposed by photosynthetic O_2 evolution. This makes measurement of phenolase activity in the light difficult. Phenols or quinones may react as photosynthetic co-factors, donors or acceptors [8] in the light. Therefore light activation of phenolase was tested in the dark immediately following a light period. O_2 consumption by phenolase-mediated oxidation of 4MC in the dark is superimposed by photosynthetic O_2 evolution in the light although no Hill reagent has been added (Fig. 2b, c). However, quinones formed in the dark act as Hill reagents in the light. This can be concluded from the fact that O_2 evolution is higher with an aged soln of 4MC which contains more quinones due to autoxidation (Fig. 2a, c). Inhibition by (3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) (Fig. 2d) indicates that O_2 evolution is due to the photosynthetic light reaction, and not to light-induced dismutation of H_2O_2. With chloroplasts aged at 0° (Fig. 2e, light), after switching on the light only a minimal O_2 evolution is found. Concomitant loss of photosynthetic activity and activation of phenolase is rapid (Fig. 1, Fig.

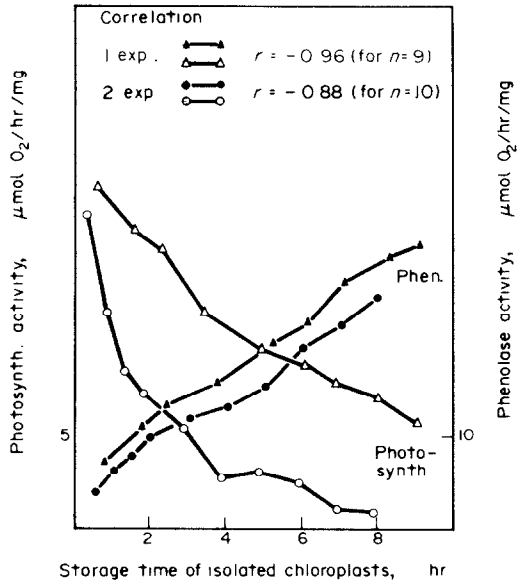

Fig. 1. Photosynthetic and phenolase activities of isolated class I chloroplasts during *in vitro* storage. Freshly prepared intact chloroplasts were kept in the dark in an ice-bath in the medium of ref. [6] with 0.6% BSA added. In the same medium O_2 evolution was measured polarographically at pH 6.8 and 25° under saturating white light. As a Hill reagent, 3PGA at a final concn of 5 mM was used. O_2 consumption by phenolase was followed in the same assay system in the dark using a final concn of 7.5 mM 4MC. Chloroplasts equivalent to 100–200 μg chlorophyll were taken for each measurement and activities were calculated on a chlorophyll base. Correlation coefficients (r) were calculated by means of linear regression analysis. Two representative experiments are given in the figure. Triangles and circles represent two expts with chloroplasts from different plots of spinach leaves. Open symbols: photosynthetic activity, closed symbols: phenolase activity.

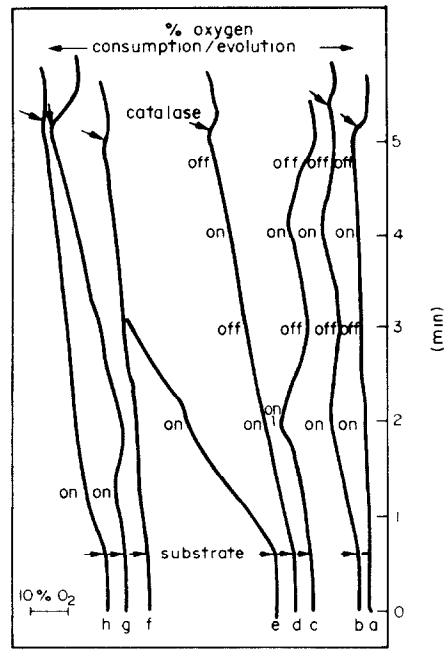

Fig. 2. Phenolase activity in darkened and illuminated broken chloroplasts. Class II (broken) chloroplasts were prepared according to ref. [12] in a medium containing 350 mM NaCl. The resultant sediment was resuspended in a medium containing 150 mM sucrose and 50 mM tricine NaOH buffer, pH 6.8. This stock suspension was kept in an ice-bath in the dark. Phenolase activity was determined in the same buffer in the dark or in white light at 25°. In some experiments 10 μl DCMU dissolved in 40% EtOH (final concn 50 mM) or 10 μl catalase (commercial prepn, 1300 units) were added to the reaction mixture. In each expt chloroplasts equivalent to 100 μg chlorophyll (2 b–e) or 500 μg (2 f–h) were used. (a) Autoxidation of 7.5 mM 4-methylcatechol (4MC) (values are identical in the absence or presence of DCMU). (b–e) Enzymatic oxidation of 7.5 mM 4MC. (b) Freshly prepared chloroplast suspension plus freshly prepared substrate soln. (c) Freshly prepared chloroplast suspension plus substrate soln, the latter stored 24 hr at room temp. (d) As in (b) but 50 μM DCMU added. (e) Chloroplast suspension stored 24 hr at 0° in the dark plus substrate soln as in (b). (f–h) Enzymatic oxidation of 10 mM DL-tyrosine (the rate of autoxidation is negligible). (f) Freshly prepared chloroplasts, darkened reaction cuvettes. (g) Freshly prepared chloroplasts in white light. (h) As in (g) but in the presence of 50 μM DCMU: on/off: light on/off; catalase: addition of 10 μl catalase suspension; substrate: addition of 100 μl substrate soln.

2b, e), especially at room temperature, and does not allow measurements in functionally intact thylakoids over a period longer than a few mins. (Tolbert followed light activation of phenolase in 30–50-min incubation at 26° [1].) During prolonged incubation in white light at room temperature, an irreversible activation of latent phenolase will occur which is due to increased damage of thylakoid membranes [3, 5].

In contrast to *o*-diphenols, monophenols are not oxidized by isolated spinach chloroplast phenolase [9]. Only in crude leaf extracts are monophenols oxidized at a very slow rate. However, by light, the tyrosine oxidation is stimulated nearly two-fold (Fig. 2f, g). This stimulation depends on photosynthetic electron transport, because it is inhibited by DCMU (Fig. 2h). But this enhanced oxidation leads to the formation of H_2O_2 as is shown by the addition of catalase, whereas the final product of the enzymatic oxidation of *o*-diphenols is H_2O (Fig. 2b). Nonenzymatic autoxidation of phenols always yields H_2O_2 (Fig. 2a). Such a type of photo-oxidation has been described earlier by Trebst and Eck [10] and is not mediated by phenolase. Reactions yielding H_2O_2 with *o*-diphenols as substrates in the presence of SH-compounds have been found with pea chloroplasts [11], this type of photo-oxidation not being sensitive to DCMU.

Activation of latent phenolase during osmotic swelling or shrinking of chloroplasts

The irreversible swelling of thylakoid membranes during storage of isolated chloroplasts or washings with hypotonic buffers results in a strong increase of phenolase activity [2, 3, 9]. Light causes freshly prepared chloroplasts and thylakoids to undergo reversible volume changes in the absence of inhibitors of non-cyclic electron flow such as DCMU (e.g. [4]). These changes are reversed in subsequent darkness. Experiments were run to find out if reversible conformational changes of the membranes are accompanied by reversible changes in the phenolase activity. In these experiments phenolase activities were always determined in the dark immediately after the

Table 1. Chloroplast volume and phenolase activity after 5 min incubation of broken chloroplasts in the dark or in white light

Medium		% Vol. after 5 min incubation (the volume in 300 mM sucrose in the dark is equal to 100%)	% Increase in phenolase activity after 5 min incubation (100% = activity before incubation)
300 mM sucrose	D	100	114
	L	86	122
300 mM sucrose + DCMU	D	96	116
	L	100	119
150 mM NaCl	D	74	128
	L	78	165
1.5 mM NaCl	D	160	139
	L	148	229
150 mM NH_4Cl	D	56	126
	L	84	154
150 mM NH_4OAc	D	108	143
	L	84	147
150 mM NaOAc	D	70	122
	L	56	128
150 mM Methylamine	D	72	131
	L	92	144
150 mM NaCl + DCMU	D	73	151
	L	73	172
150 mM NaCl + atebrine	D	112	125
	L	124	145

Class II chloroplasts were prepared and stored as described in Fig. 2. Aliquots from the stock suspension were incubated in the dark (D) or in white light (L) at room temp. for 5 min in the presence of additives as shown in the table. The incubation media contained (final concn) 10% chloroplast stock suspension, 50 mM tricine–NaOH buffer, pH 6.8, and 0.8 mM $K_3[Fe(CN)_6]$ modified from [12] and additives as indicated in the table (pH adjusted to 6.8). With 0.25-ml aliquots in a total vol. of 3 ml containing 150 mM sucrose and 50 mM tricine–NaOH buffer, pH 6.8, phenolase activity was determined in the dark before and after incubation. Initial phenolase activities of each sample before incubation were set to be 100%. Additionally, immediately after incubations aliquots were fixed with glutaraldehyde (see Experimental) and the packed vol. determined. The packed vol. of the sample incubated 5 min in the dark in sucrose medium was taken as 100%.

exposure to light for 5 min of chloroplast suspensions incubated in the presence of acceptors of non-cyclic electron flow. Control samples were stored in total darkness.

The incubation media contained a series of buffers and uncouplers which have been reported to cause different rates of reversible swelling or shrinking of chloroplasts in the dark or in the light (e.g. [4, 12]). Several experiments with each of the media containing components as shown in Table 1 revealed great variations in phenolase activity and in thylakoid volume changes, depending on the varying photosynthetic activities and on the age of the leaves. However, the fundamental pattern of the light–dark differences of phenolase activation and of the tendencies of swelling or shrinking respectively were the same as shown in a representative series of experiments presented in Table 1. Separate experiments with isolated membrane-free preparations of spinach leaf phenolase showed that phenolase activity was not inhibited by the incubation media (data not shown). In these experiments, which were reproduced with several chloroplast preparations, thylakoid-packed volume changes were found to be the same in principle as in published data, except for media containing atebrine, which was known to cause light shrinking [13].

As the main result of these experiments, it must be concluded that osmotically or light-induced reversible volume changes do not cause reversible phenolase activation but in contrast are accompanied by irreversible activation of phenolase. Although the volume changes were reported to be reversible (e.g. [4]), the increase of phenolase activity was irreversible, i.e. there was no decline of activity following light or dark incubation. Repeated light–dark changes or prolonged incubation resulted in still higher phenolase activities (data not shown). Further, phenolase is also activated in the presence of DCMU, despite the fact that conformational changes of thylakoid membranes are totally suppressed ([7] and Table 1), and in the presence of uncouplers, which inhibit the establishment of the pH gradient in the light. Thus this activation is independent of reversible volume change. This is further supported by the fact that there is no correlation between either osmotic volume change of chloroplasts in the dark- or light-induced volume changes and the degree of phenolase activation. Comparing the dark incubations only it is obvious that the increase of phenolase activity depends on the composition of the medium rather than on reversible volume changes.

Independent of the osmotics and additives used, light activation was higher than dark activation in all experiments (Table 1). Since there is no correlation of light activation and shrinking or swelling respectively and because phenolase activation by light was irreversible in all cases, the increase of phenolase activity must be due to other changes in the thylakoid membranes which are irreversible. Siegenthaler [3, 5] found irreversible activation of chloroplast phenolase by light to depend on

the release of fatty acids from the membranes. This is supported by a recent abstract of Golbeck and Cammarata [14] reporting activation of latent spinach phenolase by C_{18} fatty acids. These irreversible changes depend on *in vitro* conditions. They cannot be expected to occur *in vivo*. Figure 2b, c shows that very short (1 min) exposure to light does not increase phenolase activity significantly. Thus intact tissues lack active phenolase even during light-induced thylakoid volume changes. The activation is a post-mortem process, as has also been discussed for some other plants (see ref. [15]).

EXPERIMENTAL

Spinacia oleracea L. cv Matador was purchased locally or field-grown in early spring. Only non-senescent and turgescent leaves of young plants were used. Leaves were washed 2× with H_2O, midribs removed and intact or broken chloroplasts were prepared according to the methods of refs. [6] and [12] respectively. Polarographic measurements were performed using a Clark-type electrode and magnetically stirred cuvettes. The reaction mixture contained 0.1 ml substrate soln, 1.0–0.5 ml chloroplast suspension from the stock soln and air-satd buffer (see Figs. 1 and 2), yielding 3 ml final vol. White light was provided using a quartz iodine lamp (Zeutschel/systematic M 3). Light intensity in front of the H_2O bath surrounding the reaction cuvette was 30000 lx. Vols of chloroplasts were determined as packed vols according to [12]. Samples containing the same amount of chlorophyll were fixed by addition of glutaraldehyde (final concn 5%) and chloroplasts were spun down in hematocrit tubes for 20 min at 2500 *g* to give constant readings (light-treated samples were fixed during illumination).

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