

# 3-Phosphoglycerate Phosphatase Activity in Chloroplast Preparations as a Result of Contamination by Acid Phosphatase<sup>1</sup>

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## ABSTRACT

The presence of a nonspecific acid phosphatase which had high activity with 3-phosphoglycerate as substrate has recently been reported in *Spinacia oleracea* L. chloroplasts (Mulligan, Tolbert 1980 Plant Physiol 66: 1169-1173). The subcellular localization of this activity has been reinvestigated by differential centrifugation of spinach leaf homogenates. The fraction sedimenting at 1,200g comprised mostly intact chloroplasts and contained more than half the chlorophyll but only 5% of the 3-phosphoglycerate phosphatase activity present in the homogenate. The fraction of the homogenate pelleting at 5,000g contained broken chloroplasts and had considerable 3-phosphoglycerate phosphatase activity. Further purification of the 1,200g pellet fraction on a Percoll step gradient yielded greater than 95% intact chloroplasts, yet the phosphatase activity was reduced more than 15-fold on a chlorophyll basis by this purification.

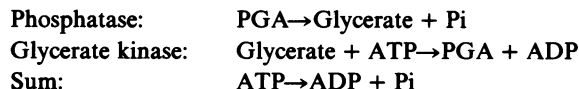
When the intact chloroplast and cytoplasmic fractions of mesophyll protoplasts were separated by silicone oil filtering centrifugation, the chloroplast fraction contained more than 90% of the chlorophyll but had less than 12% of the 3-phosphoglycerate phosphatase activity. By contrast, more than 60% of the 2-phosphoglycolate phosphatase was recovered in this chloroplast fraction supporting previous evidence that this phosphatase is localized in the chloroplast stroma.

It is concluded that 3-phosphoglycerate phosphatase activity is not localized in the chloroplast but that the activity present in chloroplast preparations results from contamination by acid phosphatase, which either binds to the thylakoid membranes during preparation or is present as some other contaminant in the preparation. Inasmuch as the enzyme acts on a broad range of substrates its presence in chloroplast preparations, particularly when the percentage of intact chloroplasts is low, could produce artifacts in metabolic studies such as measurement of phosphorylation.

Randall and Tolbert (9) first reported a PGA<sup>2</sup> phosphatase in plants and characterized the enzyme from sugar cane leaves. In spinach leaves, most of this activity was soluble (11) although about 20% of the enzyme was bound to starch grains (10) which were presumably derived from chloroplasts. Subsequently, Mulligan and Tolbert (8) reported that about 25% of the PGA phosphatase activity in a spinach leaf homogenate was firmly bound to the chloroplast thylakoids. The enzyme was by no means specific for PGA and, as suggested by Mulligan and Tolbert, had the characteristics of an acid phosphatase. The presence of a thylakoid-bound acid phosphatase has also been reported in *Sorghum* (12). Other phosphatases localized in the chloroplast

include P-glycolate phosphatase (13), inorganic pyrophosphatase (18), fructose-1,6-bisphosphatase (2) and sedoheptulose-1,7-bisphosphatase (21) but all of these are virtually absolutely specific for their substrates and show negligible activity with other phosphate esters.

The presence of nonspecific phosphatases in chloroplasts has serious implications for the regulation of photosynthetic carbon metabolism. An uncontrolled hydrolysis of PGA concurrent with its formation by glycerate kinase (which is located in the chloroplast [20]) would constitute a futile cycle which could effectively deplete the chloroplast of ATP:



Because the level of PGA also regulates other reactions in the chloroplast, including enzymes of the photosynthetic carbon reduction cycle and of starch synthesis (15), its uncontrolled hydrolysis would have far reaching effects on carbohydrate metabolism. For other phosphate esters of the carbon reduction cycle, which have no specific kinase for their regeneration, their hydrolysis would result in depletion of the levels of sugar phosphate intermediates and inhibition of CO<sub>2</sub> fixation (15). The PGA phosphatase activity of spinach thylakoids reported by Mulligan and Tolbert (8) was greater than 100 μmol mg<sup>-1</sup> Chl h<sup>-1</sup> compared with a rate of PGA synthesis (from ribulose-1,5-bisphosphate) of about 200 μmol mg<sup>-1</sup> Chl h<sup>-1</sup> at air levels of CO<sub>2</sub> (15). The rates of PGA phosphatase are thus high enough to inhibit photosynthesis unless this activity is regulated or is compartmentalized within the chloroplast.

It is important to establish whether such a nonspecific phosphatase is located in the chloroplast. The results presented in this paper suggest that PGA phosphatase activity does not occur in chloroplasts *in vivo* but that the activity in chloroplast preparations results from contamination by an acid phosphatase, either adsorbed onto the thylakoid membranes during preparation or present on some other membrane.

## MATERIALS AND METHODS

**Plant Material.** Spinach (*Spinacia oleracea*, L. cv. Hybrid 102) was grown in a growth cabinet with 14 h day and day/night temperatures of 25°C/20°C. Light was supplied by a mixture of fluorescent and incandescent lights giving 350 μE m<sup>-2</sup> s<sup>-1</sup> (400-700 nm). The plants were grown in pots filled with gravel and drip irrigated with half strength Hoagland nutrient solution. Leaves were harvested after 3 to 4 weeks.

**Fractionation of Leaf Homogenate.** All procedures were carried out at 0°C using chilled glassware and apparatus. Leaves (30-40 g) were disrupted for 3 s in a Polytron blender with 200 ml of a semifrozen slurry of 330 mM sorbitol, 5 mM MgCl<sub>2</sub>, 30 mM Mes-

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<sup>2</sup> Abbreviations: PGA, 3-phosphoglycerate; P-glycolate, 2-phosphoglycolate.

KOH, 2 mM isoascorbate, and 0.1% BSA (pH 6.5). The brei was squeezed through two layers of Miracloth containing a layer of cotton wool and the filtrate was centrifuged at 1,200g for 1 min in a swingout rotor. The pellet was resuspended in 330 mM sorbitol, 2 mM EDTA, 1 mM MgCl<sub>2</sub>, 1 mM MnCl<sub>2</sub>, 50 mM Hepes-KOH, and 0.2% BSA (pH 7.6). The supernatant was centrifuged at 5,000g for 5 min and then at 50,000g for 30 min in a Sorvall RC2B centrifuge (SS34 rotor). The two resulting pellets were resuspended in 330 mM sorbitol, 50 mM Mes-KOH (pH 6.0).

**Purification of Chloroplasts.** The 1,200g pellet fraction (about 6 ml) was placed into two centrifuge tubes and each was under-layered with 4 ml 330 mM sorbitol, 2 mM EDTA, 1 mM MgCl<sub>2</sub>, 1 mM MnCl<sub>2</sub>, 50 mM Hepes-KOH, 0.2% BSA (pH 7.6) plus 40% (v/v) Percoll. After centrifuging at 1,200g for 1 min, the broken chloroplasts formed a sharp band at the interface of the Percoll pad, whereas the intact chloroplasts were pelleted. The material at the interface was removed with a Pasteur pipette and the remainder of the supernatant was withdrawn and discarded. The pellet was resuspended in the above medium minus Percoll.

**Fractionation of Protoplasts.** Spinach mesophyll protoplasts were prepared by digestion of leaf slices with Cellulase and Macerozyme and purified on a sucrose-sorbitol step gradient as described previously (14). The protoplasts were ruptured and the chloroplast and cytoplasmic fractions separated by silicone oil filtering centrifugation as described previously (14).

**Phosphatase Activity.** Hydrolysis of PGA was measured with 25 mM Mes-KOH (pH 6.0) plus 10 mM PGA in a total volume of 0.5 ml. The reaction was started by adding PGA and the samples were incubated for 10 min at 22°C before stopping the reaction by adding 0.5 ml 25% TCA. Protein was removed by centrifugation and Pi in the supernatant was estimated with molybdate-FeSO<sub>4</sub> according to the method of Taussky and Shorr (19). For each sample, a blank was run under identical conditions but with PGA added after TCA to correct for Pi present in the sample. For each fraction it was established that the phosphatase activity increased linearly with the amount of enzyme added. The fractions were sufficiently diluted with Mes buffer to ensure rupture of any intact organelle. P-Glycolate phosphatase was measured as for PGA phosphatase except that the assay contained 2 mM P-glycolate and 1 mM MgCl<sub>2</sub> instead of PGA. Chl was determined by the method of Arnon (1). The percentage of intact chloroplasts in each fraction was determined by measuring ferricyanide-dependent O<sub>2</sub> evolution before and after osmotic shock as described by Lilley *et al.* (4).

## RESULTS

**Fractionation of Spinach Leaf Homogenate.** The filtered homogenate of spinach leaves contained considerable PGA phosphatase activity (150–400 μmol mg<sup>-1</sup> Chl h<sup>-1</sup>). When the filtrate was

examined with a light microscope, no whole cells or cell wall fragments were observed. More than half of the PGA phosphatase activity was sedimented by centrifugation at 50,000g for 30 min (Table I), indicating that this proportion of the phosphatase was membrane-bound or contained inside membrane vesicles. Table I shows the distribution of Chl and PGA phosphatase activity in the various fractions obtained by differential centrifugation of the homogenate. The 1,200g pellet contained more than half of the Chl and consisted mostly of intact chloroplasts yet only 5% of the PGA phosphatase activity was recovered in this fraction. The 5,000g pellet contained slightly less than half the Chl and consisted of broken chloroplasts, probably contaminated by other membrane fragments. This fraction contained considerable PGA phosphatase activity, amounting to almost half of the total pelletable activity. The 50,000g pellet contained only a small amount of Chl but was also high in PGA phosphatase activity. The activity in each of the three pellet fractions was only slightly decreased if the pellets were resuspended and washed indicating that the activity was membrane bound. Inasmuch as no obvious starch ring was observed by eye in any of the pellets, it seems unlikely that the pelleted activity was bound to starch grains as found by Randall and Tolbert (10). On a Chl basis, the specific activity of PGA phosphatase was greatest in the 50,000g pellet and least in the 1,200g pellet. The specific activity of the thylakoid fraction (5,000g pellet) was similar to that reported by Mulligan and Tolbert (8), whereas that in the intact chloroplasts (1,200g pellet) was much less.

**Purification of Chloroplasts.** Because the distribution of PGA phosphatase activity did not parallel that of Chl (Table I) it seemed that at least some of the membrane-bound enzyme was not associated with the chloroplasts. It was therefore difficult to distinguish whether the activity in the intact chloroplast fraction resulted from a small part of the total activity which was genuinely associated with chloroplast membranes or from contamination of this fraction by other membranes. Intact chloroplasts can be separated from thylakoid membranes by centrifugation through Percoll, a procedure which also greatly reduces the contamination by mitochondria, peroxisomes, and other membranes (6, 7). When the 1,200g pellet fraction was layered over Percoll and recentrifuged, the resulting pellet contained chloroplasts which were greater than 95% intact yet only 2% of the PGA phosphatase was pelleted by this procedure (Table II). In a number of experiments, the specific activity of PGA phosphatase in intact chloroplasts purified by this method was less than 3 μmol mg<sup>-1</sup> Chl h<sup>-1</sup>. Most of the PGA phosphatase activity was recovered with the band of thylakoid membranes at the interface of the Percoll pad (Table II).

**Fractionation of Protoplasts.** The results of Tables I and II

Table I. Distribution of Chl and Phosphatase Activity following Differential Centrifugation of a Spinach Leaf Homogenate

The various fractions were prepared as described in "Materials and Methods." The per cent chloroplasts intact was determined from ferricyanide-dependent oxygen evolution. Phosphatase activity was determined with PGA as substrate.

Fraction	Chloroplasts Intact %	Chl mg	Phosphatase Activity		Total	
			μmol h <sup>-1</sup>	μmol mg <sup>-1</sup> Chl h <sup>-1</sup>	Chl %	Phosphatase %
Homogenate		20.1	7,287	362	100	100
1,200g pellet	62	10.8	372	34	53.7	5.1
5,000g pellet	3	8.7	1,802	207	43.2	24.7
50,000g pellet	0	0.7	1,815	2,593	3.4	24.9
50,000g supernatant		0	2,959		0	40.6
Total recovered		20.2	6,948		100.3	95.3

Table II. *Distribution of Chl and Phosphatase Activity following Purification of the 1,200g Pellet Fraction.*

The 1,200g pellet fraction (Table I) was purified on a Percoll step gradient as described in "Materials and Methods." Phosphatase activity was determined with PGA as substrate.

Fraction	Chloroplasts Intact %	Chl mg	Phosphatase Activity		Total	
			$\mu\text{mol h}^{-1}$	$\mu\text{mol mg}^{-1}$ <i>Chl h}^{-1}</i>	Chl	Phosphatase %
1,200g pellet	62	7.2	248	34	100	100
Percoll interface	14	4.4	234	53	61.1	94.4
Percoll pellet	97	2.5	6	2	34.7	2.4
Total recovered		6.9	240		95.8	96.8

Table III. *Distribution of 3-Phosphoglycerate Phosphatase and Phosphoglycolate Phosphatase Activities following Fractionation of Spinach Leaf Protoplasts*

Phosphatase activities were determined in an unfractionated protoplast extract and in the separated cytoplasmic and chloroplast fractions as described in "Materials and Methods." Specific activities ( $\mu\text{mol mg}^{-1}$  Chl  $\text{h}^{-1}$ ) in the protoplast extract were: PGA phosphatase, 41; P-glycolate phosphatase, 217. More than 90% of the Chl was recovered in the chloroplast fraction.

Fraction	PGA Phosphatase	P-Glycolate Phosphatase	P-Glycolate Phosphatase
			PGA Phosphatase
	%		ratio
Protoplast extract	100.0	100.0	5.3
Cytoplasmic fraction	82.7	36.9	2.4
Chloroplast fraction	11.0	63.1	30.4
Per cent recovered	93.7	100.0	

clearly indicate that PGA phosphatase is not a chloroplast enzyme, contradicting previous findings (8, 10). For confirmation, spinach leaf protoplasts were prepared to check the localization of this enzyme and of P-glycolate phosphatase, which has been shown to be located in the stroma of chloroplasts (17). On a Chl basis the PGA phosphatase activity of purified protoplasts was less than half that in a mechanically prepared homogenate from the same leaves, suggesting that some of the phosphatase in the homogenate was derived from cells which do not form protoplasts during digestion of the leaf tissue.

The chloroplast and cytoplasmic fractions were prepared from protoplasts as described before (14). The protoplasts are ruptured by rapid centrifugation through a narrow aperture nylon mesh and the chloroplasts, which mostly remain intact, are then separated from the cytoplasm by sedimentation through a layer of silicone oil below the mesh. Within 6 to 8 s of starting the centrifuge, 90% of the chloroplasts are separated into the pellet fraction which contains only 10% to 15% contamination by mitochondria and peroxisomes and less than 5% by soluble cytoplasm as judged by the distribution of marker enzymes (14). Less than 12% of the PGA phosphatase activity was recovered in the chloroplast fraction (Table III) and because this is below the known levels of contamination by mitochondria and peroxisomes, even this low level of activity is probably not in the chloroplasts. By contrast, the chloroplast fraction contained more than 60% of the P-glycolate phosphatase activity (Table III). This does not suggest that some of the P-glycolate phosphatase is outside the chloroplast since nonspecific phosphatases in the cytoplasmic fraction would also hydrolyze P-glycolate under these assay conditions, leading to artificially high P-glycolate phosphatase activities in the protoplast and cytosolic fractions. The ratio of P-glycolate phosphatase to PGA phosphatase in the protoplasts was similar to that reported previously for spinach leaves (11) but was increased almost 6-fold in the chloroplast fraction, indicating that the two enzymes were not both located in the chloroplast. Similar results to those in Table III were obtained when protoplasts from barley or wheat leaves were used.

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**Binding of PGA Phosphatase to Thylakoids.** Since PGA phosphatase is not localized in intact chloroplasts (Tables II and III) there are two possible explanations for the activity in the 5,000g pellet fraction, which contains chloroplast thylakoids. Either the phosphatase is a soluble enzyme which becomes adsorbed onto the thylakoids when the tissue is disrupted or the activity results from contamination of this fraction by some other membrane which has bound phosphatase. To test the possibility that the enzyme binds to thylakoid membranes during preparation, thylakoids free of phosphatase were prepared from the Percoll-purified intact chloroplasts by brief osmotic shock. When these thylakoids were washed through the 50,000g supernatant, the PGA phosphatase activity was only increased from 0.9 to 4.5  $\mu\text{mol mg}^{-1}$  Chl  $\text{h}^{-1}$ , compared to specific activities in excess of 200  $\mu\text{mol mg}^{-1}$  Chl  $\text{h}^{-1}$  for the 5,000g pellet fraction (Table I). Even if the intact chloroplasts were disrupted into the 50,000g supernatant with the Polytron blender, the pelletable activity did not exceed 10  $\mu\text{mol mg}^{-1}$  Chl  $\text{h}^{-1}$ . All other attempts to bind the soluble PGA phosphatase to thylakoids were unsuccessful.

## DISCUSSION

The results clearly demonstrate that PGA phosphatase is not a chloroplast enzyme nor is it associated with intact chloroplasts even though the activity in crude chloroplast preparations can be quite high. Intact chloroplasts purified from a leaf homogenate (Table II) or from protoplasts (Table III) had PGA phosphatase activity of less than 5  $\mu\text{mol mg}^{-1}$  Chl  $\text{h}^{-1}$  and even this low level probably results from contamination. The activity present in crude chloroplast preparations (Table I) could result from adsorption of a soluble enzyme onto the thylakoid membranes during disruption of the tissue, but attempts to bind soluble phosphatase onto thylakoids prepared from intact chloroplasts were not successful. When the enzyme is present in thylakoid preparations it is tightly membrane bound (8, 12), which would not be expected if a soluble enzyme was adsorbed onto membranes. It seems more likely that the phosphatase activity in chloroplast preparations results from contamination by a phosphatase on some other membrane. Mulligan and Tolbert (8) demonstrated that the enzyme in chloroplast preparations is not specific for PGA. With *p*-nitrophenyl phosphate, the pH optimum was below 5.5 and showed no cation requirement which suggests that the enzyme was a general acid phosphatase. Although the localization of acid phosphatase in plant cells has not been completely resolved, it appears that the major proportion of the enzyme is in the vacuole (3, 5). It is possible that chloroplast preparations are contaminated by vacuolar material containing acid phosphatase.

The chloroplasts used by Mulligan and Tolbert (8) were pre-

pared by centrifuging for 5 min at 5,000g or for 10 min at 12,000g and would undoubtedly also have been contaminated by other membranes. However, the PGA phosphatase activity remained associated with the thylakoid fraction even after sucrose density gradient centrifugation (8). This highlights the care which must be taken in determining the localization of enzymes in plant cells and the dangers in using Chl as a marker for chloroplasts. In addition, it is clear that thylakoids prepared directly from leaf homogenates are likely to be contaminated by hydrolytic enzymes. Studies involving the formation or hydrolysis of phosphate esters would be greatly influenced by the presence of acid phosphatase. For example, even a relatively low rate of hydrolysis of ATP can significantly alter the apparent stoichiometry of phosphorylation in chloroplasts (16). Even for studies which require only thylakoids, it would be preferable to isolate and purify intact chloroplasts and then prepare thylakoids from them by osmotic shock.

Compartmentation of synthetic and degradative pathways is an important mechanism of metabolic regulation. The effects of an unregulated phosphatase in the chloroplast would be far reaching and it is hardly surprising that all of the phosphatases so far found in chloroplasts are highly substrate-specific. A similar argument against the existence of unregulated phosphatases might also be proposed for the cytosol. The existence of a PGA phosphatase was first proposed to account for the apparent flow of carbon from PGA to glycerate during short duration labeling studies with  $^{14}\text{CO}_2$  (8). If this conversion is achieved by a phosphatase, it is now clear that the hydrolysis must occur outside of the chloroplast. Further studies are required to assess the magnitude of the flux of carbon in this pathway from PGA to glycerate and serine and the steps involved as this would influence carbon flow through the photorespiratory pathway which is in the opposite direction (serine to PGA) and hence the regeneration of PGA for the photosynthetic carbon reduction cycle.

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