Isolation and Distribution of Phosphoglycolate Phosphatase [1, 2]

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A phosphatase specific for P-glycolate[5] was purified from extracts of tobacco leaves until no other phosphatase activity could be demonstrated in the preparation (10). Other plants have now been examined for the presence of this phosphatase. A new isolation procedure for the enzyme was developed because the $(NH_4)_2SO_4$ fractionation procedure, which was used with tobacco leaf extracts, inactivated the enzyme when applied with most other tissue. The enzyme has been found in green leaves but not in roots or etiolated leaves.

P-glycolate phosphatase is of interest because P-glycolate and glycolate are labeled rapidly during photosynthesis with $C^{14}O_2$ (1, 2, 3, 11). Also glycolate rapidly appears outside of isolated chloroplasts during photosynthesis (3), and thus the phosphatase may be a part of a chloroplast permease system. If this were possible then the phosphatase should be either a part of the chloroplast or closely associated with the chloroplast in vivo. Data will be presented with isolated chloroplast which support these suggestions.

Materials and Methods

Enzyme Assays. Substrates and enzymic assays were the same as those used previously (10). Barium salts of the substrates were dissolved by suspension with Dowex-50 (Na^+). Solutions of P-glycolate and 3-P-glycerate were stable for months, but fresh phenolphthalein-diP was prepared weekly.

All enzyme assays were run at 30° in 3 ml volume containing 3 μmole $CoSO_4$ or $MgSO_4$, 33 μmole Tris-acetate buffer at pH 6.3, 1 to 5 μmole substrate, and enzyme. The reactions were stopped by addition of 1 ml of 10% trichloroacetic acid, the precipitate removed by centrifugation, and the orthophosphate determined in the supernatant fluid. Protein concentration on another sample was determined by the 280/260 ratio (15) when crude sap or acetone precipitates were used and by Lowry's modified Folin-Ciocalteu method with bovine serum albumin as a standard (5) when purer enzyme preparations were assayed. Protein determination by absorbancy with the partially purified enzyme could not be used because of an associated material with high absorbancy at 260 mμ. A unit of enzyme activity was defined as the release of 1 μg of phosphorus in 10 minutes and specific activity as units of activity per milligram protein.

Plant Material. Tobacco leaves were either grown in the field or greenhouse. Spinach leaves were purchased from local markets. Alfalfa was field grown. Thatcher wheat was grown in the greenhouse and etiolated wheat was grown in dark boxes placed on the floor of a commercial plant growth chamber with temperature inside the boxes maintained between 13 to 16°.

Criteria of Enzyme Specificity. Measurements of the specific P-glycolate phosphatase activity were complicated by action of nonspecific phosphatases. Consequently quantitative parallel measurement of the rate of hydrolysis of other phosphate esters was necessary in order to indicate the degree of purification at each step of the enzyme isolation procedure. Phenolphthalein-diP and 3-P-glycerate were used for this purpose because phosphatases capable of hydrolyzing them were active in leaf extracts, and these phosphatases remained most tenaciously with the P-glycolate phosphatase during the isolation procedure (10). The 2 indexes of P-glycolate phosphatase purification were therefore the increase in specific activity of the preparation and an increasing ratio of activity which favored the hydrolysis of P-glycolate with respect to the hydrolysis of 3-P-glycerate and phenolphthalein-diP. The data are expressed as a ratio of activity with phosphoglycolate phosphatase activity equal to one.

Results

Enzyme Isolation. The enzyme isolation procedure developed by Richardson and Tolbert (10) involved $(NH_4)_2SO_4$ fractionation, calcium phosphate gel adsorption, and then DEAE cellulose column fractionation. This procedure was effective when starting with tobacco leaves, but little activity was obtained with alfalfa, wheat, or barley leaves. During am-

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[5] Abbreviations: phosphoglycolate, P-glycolate; 3-phospho-D-glycerate, 3-P-glycerate; phenolphthalein diphosphate, phenolphthalein-diP; D-fructose-1,6-diphosphate, fructose-diP.

monium sulfate fractionation of enzyme preparations most of the activity was lost. In addition, after removal of P-glycolate phosphatase from calcium phosphate gel by elution with phosphate buffer, substantial amounts of P_i remained associated with the enzyme. Exhaustive dialysis would remove enough of the P_i to permit running the assay. However, in a subsequent section it will be shown that dialysis also inactivated the enzyme. Consequently, a more rapid isolation procedure which gave better recoveries of P-glycolate phosphatase was developed so that physiological studies could be undertaken.

Wheat leaves (50 g) were ground with 100 ml of cold 0.05 M Tris-acetate buffer at pH 6.3 for 1 minute in a Waring blendor. The homogenate was squeezed through cheesecloth and the sap clarified by centrifugation at 8000 × *g* for 10 minutes. The activity of the sap is shown in the first line of table I. The sap was added to 3 volumes of acetone prechilled to −30 to −40°. After standing for 10 minutes the precipitate was removed by centrifugation and redissolved in 25 ml of 0.02 M Tris-acetate buffer at pH 7.2. Insoluble protein was removed by centrifugation. This preparation is designated as acetone precipitate in table I. Generally nearly complete recovery of P-glycolate phosphatase was achieved and the preparations were stable for at least several weeks when stored at 0 to 4°. Active preparations have been maintained for over a half year at −20°.

The third step in the isolation was separation on a DEAE-cellulose column. Dry DEAE-cellulose (2 g), prepared as described in Methods in Enzymology (8), were suspended in 200 ml of 0.02 M Tris-acetate buffer, pH 7.2, to make a column 12 × 120 mm. Columns were packed by gravity alone, washed with buffer, and allowed to stand in a cold room at least 2 hours before introducing a sample of the acetone precipitated enzyme containing about 200 mg of protein. The column was then washed with 600 ml of the Tris-acetate buffer, pH 7.2, and developed with a nonlinear gradient of NaCl in 0.02 M Tris-acetate buffer. The enzyme eluted from the column in fractions corresponding to about 0.12 to 0.135 M NaCl.

A fourth step in the purification scheme which was sometimes employed with the experiments in this report was passage of the enzyme fractions after DEAE-cellulose fractionation through Sephadex G-50 to remove salt and low molecular weight components. This step did not change the degree of purification of the enzyme. However, the enzyme after treatment with Sephadex G-50 or dialysis was very heat labile at 30°. Even at 0°, enzyme activity was lost after 1 or 2 days.

Cofactors. P-glycolate phosphatase as prepared by ammonium sulfate fractionation and calcium phosphate gel adsorption from tobacco leaves required cobalt for activity and magnesium was less effective (10). With the phosphatase from wheat leaves isolated by acetone precipitation a metal cation was also essential for enzyme activity after separation on DEAE-cellulose or Sephadex G-50 treatment. At cation concentrations of 10^{-3} M, enzyme activity was greatest with magnesium; manganese was also effective, but cobalt was less effective (table 2).

pH Optimum. When starting with wheat, spinach, or tobacco leaves, the pH optimum of the enzyme in the crude sap was 6.3. The pH optimum did not

Table II. *Metal Activation of P-glycolate Phosphatase*

The enzyme was isolated from green Thatcher wheat to the stage of purification indicated. All final concentrations of metal cations were at 1 × 10^{-3} M.

Metal	Units activity/ml	
	DEAE cellulose preparation	Sephadex G-50 treated enzyme
none	0	0
Co++	1.4	0.5
Zn++	1.6	1.5
Mg++	2.6	2.5
Mn++	2.2	1.0
Cu++	...	0
Fe++	...	0.3

Table I. *Comparative Enzyme Activity of Green Leaves, Illuminated and Nonilluminated Etiolated Leaves, and Roots of Wheat Plants*

Tissue	Crude sap			Acetone precipitate enzyme			DEAE-cellulose enzyme	
	ratio*	unit per g**	specific activity***	ratio*	unit per g**	specific activity***	ratio*	specific activity***
green leaves	1 : 0.2 : 0.4	900–1,500	25	1 : 0.3 : 0.3	800	160	1 : 0.1 : 0.3	400
etiolated leaves plus 24 hr light	1 : 0.7 : 7	300–400	6	1 : 0.6 : 1	230–300	30–40	1 : 0.6 : 1	300–400
etiolated leaves plus 12 hr light	1 : 0.6 : 9	300–330	5	1 : 0.8 : 3	190–200	20	1 : 0.5 : 1.2	60–140
etiolated leaves	1 : 1 : 9	110–150	2–3	1 : 1 : 3	100–150	14	1 : 0.6 : 1.7	60–95
roots	1 : 3 : 6	25	2.4	1 : 2 : 3	16	9	1 : 1 : 1.7	66–88

* Ratio of rate of hydrolysis of P-glycolic acid, 3-P-glyceric acid, and phenolphthalein-diP.
** Units of activity for hydrolysis of P-glycolic acid per gram wet weight.
*** Specific activity of P-glycolic acid phosphatase; units of enzyme per milligram protein.

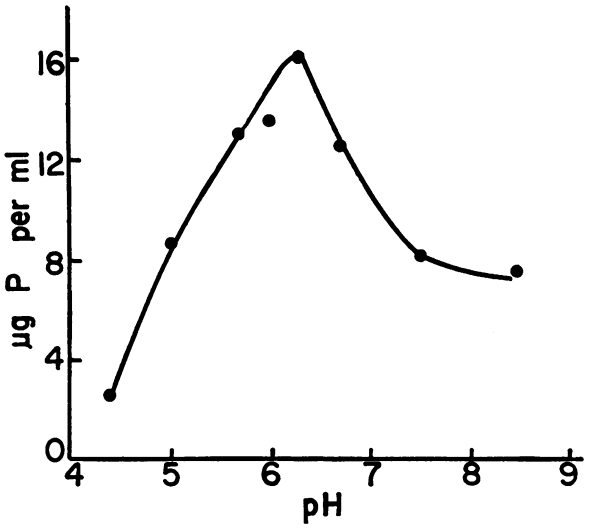

FIG. 1. pH optimum of P-glycolate phosphatase. Reaction mixture contained the following in a total volume of 3.0 ml: 1 μmole of P-glycolate, 100 μmole of Tris-acetate buffer at the pH indicated, 3 μmoles of $MgSO_4$, and 1 ml of enzyme preparation from a DEAE-cellulose column. The enzyme had passed through Sephadex G-50 before use.

change after purification by acetone precipitation, by DEAE-cellulose chromatography (fig 1), and by Sephadex treatment of the enzyme. These results are in contrast to a change in the pH optimum to 5.0 for P-glycolate phosphatase from tobacco leaves after $(NH_4)_2SO_4$ fractionation (10). It appears that the $(NH_4)_2SO_4$ precipitation not only partially inactivated the enzyme but also resulted in a shift in the pH optimum. When the tobacco enzyme was purified without $(NH_4)_2SO_4$ fractionation, the pH optimum remained at pH 6.3.

Formation of P-glycolate Phosphatase during Greening. Root tissue from Thatcher wheat plants (table I, last line) contained only a small amount of active P-glycolate phosphatase in the crude sap when compared to green leaves. Furthermore, at each step of the purification procedure for the P-glycolate phosphatase the hydrolysis of P-glycolate was slow as compared to the rates of hydrolysis of 3-P-glycerate and phenolphthalein-diP. From comparing 1,500 units of extractable activity per gram green leaf tissue with 25 units per gram root tissue, it is suggested that root tissues do not contain a significant amount of P-glycolate phosphatase. Since no phosphatase with specificity for P-glycolate was obtained from root extracts by the isolation procedure, the small hydrolysis which was observed by root extracts could have been catalyzed by nonspecific phosphatases.

The phosphatase activity in etiolated wheat leaves (0 hr of light in table I) was similar to that in the wheat roots. Green leaves contained 10 times as many units of P-glycolate phosphatase activity per gram of leaf as did etiolated leaves. In addition, the phosphatase from etiolated leaves when partially purified still did not show a specificity for P-glycolate. The immense increase in P-glycolate phosphatase activity in fully green wheat grown in the light and the specificity of the enzyme from green leaves for P-glycolate is emphasized by data in table I. During the first hours of greening in the light, P-glycolate phosphatase activity increased slowly. After 12 hours the rate of P-glycolate hydrolysis had doubled; after 24 hours the enzyme was not as active as in plants grown in the light for 4 to 5 days. In crude sap prepared from normal green leaves the rate of hydrolysis of P-glycolate at pH 6.3 was 5 times greater than the rate of hydrolysis of 3-P-glycerate. These data indicate a rapid rate of P-glycolate hydrolysis in green tissue, in comparison with other phosphate esters.

Distribution of Phosphatase between Chloroplasts and Cytoplasm. In previous work the phosphatase was isolated from aqueous plant extracts which did not indicate definitely where the phosphatase might be located in the cell. Since P-glycolate is a product of photosynthesis, the association of the phosphatase with chloroplasts was investigated. Spinach leaves, (200 g) without midribs, were ground with 400 ml of 0.5 M NaCl in 0.05 M Tris-acetate buffer at pH 7.2 in a Waring blendor. After adjusting the pH to 7.0, cell debris was removed by centrifugation at 200 × *g* for 2 minutes. Chloroplasts were removed by centrifuging at 1000 × *g* for 10 minutes. The units of P-glycolate phosphatase activity in the supernatant fluid represented 91 % of the total activity in the extract (table III). Similarly, about 91 % of the total P-glycolate phosphatase activity was found in the soluble fraction when chloroplasts were isolated from wheat, tobacco, and alfalfa leaves. The chloroplasts were washed with 400 ml of the Tris-acetate and NaCl solution and again removed by centrifugation. 6.2 % of the phosphatase activity was recovered in the washings. The washed chloroplasts were suspended in 133 ml of 0.001 M Tris-acetate buffer, pH 7.3, for 30 minutes. After this hypotonic treatment, the chloroplasts were removed by centrifugation at 8000 × *g* for 10 minutes. Both the aqueous chloroplast washings and the final chloroplast fragments resuspended in 0.01 M Tris buffer at pH 6.3 were assayed for phosphatase activity. From the aqueous washings of the isolated chloroplast fragments 1.3 % of the total P-glycolate phosphatase activity was obtained. Although this amount of activity was a small portion of the total activity in the cell it was highly specific for the P-glycolate substrate. In some experiments the aqueous chloroplast washings would catalyze the hydrolysis of only P-glycolate and would not attack the other phosphate esters which were used. In most experiments little or no P-glycolate phosphatase activity remained with the water washed chloroplasts. On the other hand, other phosphatases remained with the chloroplast fragments or in the first supernatant fluid from which the chloroplasts were isolated. Fructose-diP hydrolysis was also tested

Table III. *Distribution of P-glycolate Phosphatase between Chloroplasts and Cytoplasm*

Fraction	Experiment and plant	Total units of activity	% of total activity	Specific activity units/mg protein	Ratio of P-glyc: 3-PGA: PdiP: FdiP*
Supernatant fluid	A. Wheat	32,500	91.5	...	1: 0.4: 1.4
	B. Spinach	78,300	91.5	15.1	1: 0.3: 0.3
	C. Spinach	22,600	91.0	...	1: 0.8: 1.7: 0.8
NaCl washing from chloroplasts	A. Wheat	2,312	6.5	...	1: 0.5: 1.5
	B. Spinach	4,250	7.0	10.0	...
	C. Spinach	1,550	6.2	...	1: 1.4: 2.6: 1.1
H_2O washing from chloroplasts	A. Wheat	297	0.9	...	1: 0: 0
	B. Spinach	1,625	1.5	46.0	1: 0: 0
	C. Spinach	330	1.3	...	1: 0: 0.4: 0.1
Washed chloroplasts	A. Wheat	25	0	...	...
	B. Spinach	625	0.5	0.8	1: 0: 8.0
	C. Spinach	310	1.3	...	1: 1.2: 2.1: 1.8

* P-glyc, phosphoglycolate; 3-PGA, 3-phosphoglycerate; PdiP, phenolphthalein diphosphate; FdiP, fructose-1,6-diphosphate.

in these experiments. The phosphatase for fructose-diP should be present in the chloroplasts where it is necessary for fructose-6-P formation after aldolase catalyzed synthesis of fructose-diP during photosynthesis. Phosphatase activity for hydrolysis of fructose-diP remained with the washed chloroplast fragments.

Discussion

The activity of the phosphatase specific for P-glycolate is at least equal to or greater than the rate of CO_2 fixation during photosynthesis. A rate of 1,500 μg P_i formation from P-glycolate per 10 minutes is equal to 280 μmoles per hour per gram wet weight. Peterkofsky and Racker (7) determined CO_2 fixation rates of 32 μmoles per hour per milligram of chlorophyll, for spinach leaves and 1 g wet weight of wheat leaves contains 0.5 to 1 mg chlorophyll. Although no exact quantitative comparison can made between P-glycolate phosphatase and other leaf phosphatases, sap from green wheat leaves at pH 6.3 hydrolyzed P-glycolate 5 times faster than 3-P-glycerate. The pH optimum of 6.3 for P-glycolate hydrolysis is not characteristic of the usual acid or alkaline phosphatases with optimum around 5.0 and 9.5 respectively. Erythrocyte phosphatase, with a pH optimum of 6.3 (6), might be compared to P-glycolate phosphatase in this respect.

Kearney and Tolbert (3) indicated that P-glycolate may be an intermediate in the glycolate pathway of metabolism (9) which is associated with the photosynthetic carbon cycle. The presence of a specific phosphatase for P-glycolate in green leaves but not in etiolated leaves or roots also supports the concept that this system is closely associated with photosynthesis. The next 2 enzymes of the glycolate pathway (9) after the P-glycolate phosphatase are also inactive or absent in etiolated plants. Part of the glycolate oxidase is present as a proenzyme and part is not detectable (4). A glyoxylate-serine transaminase is also absent in etiolated plants (E. R. Waygood, personal communication). Thus, three consecutive enzymes of the glycolate pathway are inactive or not present in the etiolated tissue.

The mechanism for formation of the phosphatase during greening of the etiolated plant has been considered. Glycolate oxidase from etiolated leaves was activated in vivo and in vitro by excess substrate (4, 13). Thus the substrate P-glycolate might likewise activate a specific phosphatase. However, when etiolated wheat leaves were homogenized in the presence of P-glycolate or glycolate, no increase in phosphatase activity was observed. In vivo tests were not run because of insufficient substrate.

The studies on formation of the enzyme during greening were qualitative, but they suggested that the rate of formation of this phosphatase was much slower than the formation of chlorophyll (12) and also slower than the development of the photosynthetic carbon cycle (14). In the latter case, green plants did not acquire the ability to synthesize much glycine and serine from $C^{14}O_2$ during photosynthesis until after 24 hours of light. This delay in glycine and serine synthesis during photosynthesis is consistent with the slow development of P-glycolate phosphatase which catalyzes a prior step in the glycolate pathway (9).

The association of P-glycolate phosphatase with the chloroplasts is suggested by the fact that about 9% of the activity remained with the unwashed chloroplast fraction. The formation of the enzyme during greening of the etiolated leaf is consistent with a relationship to photosynthesis and the chloroplasts. However, the phosphatase was readily removed from the chloroplast particles by washing and the phosphatase activity was highly specific for P-glycolate. This fact suggests a loose but perhaps special association of P-glycolate phosphatase with the chloroplast surface. Our observations on the specific appearance of C^{14}-labeled glycolate outside

of isolated chloroplasts after $C^{14}O_2$ fixation in the light has also suggested that P-glycolate phosphatase should be associated with movement of this product of photosynthesis out of the chloroplasts. The appearance of some P-glycolate outside of isolated chloroplasts probably reflects in part the loss of the phosphatase activity from the chloroplasts during their isolation.

Summary

A new procedure involving acetone precipitation and DEAE-cellulose fractionation was developed for partial purification of phosphoglycolate phosphatase from tobacco, wheat, spinach, or alfalfa plants. This specific phosphatase was present in large amounts in green leaves, but little activity was detected in etiolated leaves or roots of Thatcher wheat plants. During greening of etiolated tissue in the light the phosphate activity developed slowly during several days. The pH optimum of the enzyme was 6.3, and Mg^{++} or Mn^{++} ions were necessary for activity. About 91 % of the total enzyme activity was recovered in the supernatant fluid after removal of chloroplasts. The remaining activity could be removed from the chloroplasts by washing with 0.35 M NaCl and then by water. The phosphatase activity recovered from chloroplast fragments by water washing was highly specific for phosphoglycolate.

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