Resistance to Phosphinothricin (Glufosinate) and Its Utilization as a Nitrogen Source by *Chlamydomonas reinhardtii*

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Wild-type strain 21gr of the green alga *Chlamydomonas reinhardtii* was resistant to the ammonium salt of L-phosphinothricin (PPT, also called glufosinate), an irreversible inhibitor of glutamine synthetase activity and the main active component of the herbicide BASTA (AgrEvo, Frankfurt am Main, Germany). Under the same conditions, however, this strain was highly sensitive to L-methionine-S-sulfoximine, a structural analog of PPT which has been reported to be 5 to 10 times less effective than PPT as an inhibitor in plants. Moreover, this alga was able to grow with PPT as the sole nitrogen source when this compound was provided at low concentrations. This utilization of PPT was dependent upon the addition of acetate and light and did not take place in the presence of ammonium. Resistance was due neither to the presence of N-acetyltransferase or transaminase activity nor to the presence of glutamine synthetase isoforms resistant to PPT. By using L-[methyl-¹⁴C]PPT, we demonstrated that resistance is due to lack of PPT transport into the cells. This strongly suggests that PPT and L-methionine-S-sulfoximine enter the cells through different systems. Growth with PPT is supported by its deamination by an L-amino acid oxidase activity which has been previously described to be located at the periplasm.

L-Phosphinothricin [PPT; L-homoalanine-4-yl-(methyl)phosphinic acid] is the active ingredient of the nonselective herbicide BASTA (AgrEvo, Frankfurt am Main, Germany), an inhibitor of glutamine synthetase (GS) activity from many different sources, including bacteria and plants (2, 13, 14). It was first discovered as part of bialaphos, a dialanyl tripeptide antibiotic produced by some *Streptomyces* strains (3, 18). L-Methionine-S-sulfoximine (MSX) is a structural analog of PPT which also inhibits GS from different sources (11, 14, 21). Both compounds act as competitive inhibitors, but they eventually inactivate GS irreversibly after phosphorylation at the active site of the enzyme (14, 21). In plants, PPT has been reported to inhibit at least 5 to 10 times more effectively than MSX (13).

The metabolism of PPT and the enzyme activities leading to the resistance to this inhibitor have been studied mainly in bacteria (Fig. 1). So, in the natural PPT-producing strains of *Streptomyces* and some other, unrelated, resistant bacteria, a specific PPT N-acetyltransferase activity is present (2, 24) which catalyzes the conversion of PPT to N-acetyl-PPT. Gene cloning of this PPT-acetylating enzyme has allowed the isolation of transgenic plants highly resistant to this herbicide (6, 8). Besides this acetylating activity, PPT can be deaminated to its corresponding keto acid, 4-methylphosphinico-2-oxobutanoic acid (PPO), by enzymatic transamination (2, 22, 25). Sometimes, this transamination seems to confer resistance to PPT in the absence of the acetylating enzyme, although other bacteria expressing this activity are still sensitive (2). Besides transamination, PPO can also be formed through oxidative deamination by an L-amino acid oxidase, which allows some bacteria to grow by utilizing PPT as a nitrogen source (2). However, no evidence that the activity of L-amino acid oxidase can confer resistance to PPT has been found. Eventually, PPO can be

decarboxylated by a noncatalyzed reaction to 3-methylphosphinopropionic acid (2, 25).

Growth of *Chlamydomonas reinhardtii* cells is blocked upon addition of low concentrations of MSX (11). However, the effects of PPT on this alga have never been characterized. In this study, we investigated the *Chlamydomonas* enzyme activities acting on PPT and the reasons why the wild-type strain of *C. reinhardtii* is selectively resistant to PPT and not to MSX. In this report, we describe how these cells can utilize PPT as a nitrogen source for growth.

MATERIALS AND METHODS

Chemicals. The ammonium- and acid-free forms of PPT, L-[methyl-¹⁴C]PPT, and their derivatives PPO, 3-methylphosphinopropionic acid, and N-acetyl-PPT were provided by H. Köcher (AgrEvo). MSX was purchased from Sigma Chemical Co. Only the active form of PPT was used throughout this work. When needed, the acid-free form of PPT was neutralized with potassium hydroxide before use.

Cell culture and growth conditions. Haploid wild-type cells of *C. reinhardtii* 21gr were provided by Emilio Fernández (University of Córdoba, Córdoba, Spain). Cells were grown at 25°C under continuous illumination (10 to 20 W/m²) in either minimal medium with air enriched with 2 to 5% (vol/vol) CO₂ or in TAP (Tris-acetate-phosphate) medium (12) containing the nitrogen source indicated in Results. Solid media were prepared with 1.7% (wt/vol) agar (Difco, Detroit, Mich.). PPT and MSX were filter sterilized and added to autoclaved media after cooling to 48°C to prevent hydrolysis. To determine growth in liquid media, we measured the chlorophyll content at 652 nm after extraction with acetone (1). Growth on solid media was determined by the modified drop method (11), in which 1 × 10³ to 3 × 10³ cells, contained in 20-μl drops, were grown on solid plates and then collected and extracted for determination of their chlorophyll content. Purity of cultures was always checked before and after each experiment.

Enzyme activities and assay conditions. When activities were assayed in vitro, cell extracts were previously dialyzed through a Sephadex G-25 column (PD-10; Pharmacia) equilibrated with the corresponding assay buffer in accordance with the manufacturer's recommendations. GS activity was measured by the reverse γ-glutamyltransferase reaction (23) either in situ in toluene-permeabilized cells or in vitro in extracts obtained after freezing and thawing of cell pellets (9). To determine the sensitivity of GS to PPT in vivo, an in situ assay was carried out with toluenized cells as described by Florencio and Vega (9). The in vitro sensitivity of GS to PPT was assessed in cell extracts by adding 50 μM PPT plus 50 μM ATP in 50 mM morpholine propanesulfonic acid (MOPS)-KOH buffer (10 mM dithioerythritol, 10 mM MgCl₂), pH 7.0. PPT N-acetyltransferase activity was assayed by incubating cell extracts in a 50 mM Tris-HCl buffer (pH 8.0) in the presence of 5 mM [¹⁴C]PPT (0.5 mCi/mol) and 2 mM acetyl coenzyme A

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FIG. 1. Metabolism of PPT in bacteria according to references 2 and 25. The steps involved are catalyzed by the following enzymes: step 1, PPT *N*-acetyltransferase (*N*-ac-PPT); step 2, deaminases, including L-amino acid oxidase; step 3, transaminase. Step 4 presumably involves nonenzymatic decarboxylation. MPP, 3-methylphosphinopropionic acid.

and then monitoring the appearance of *N*-acetyl-PPT by thin-layer chromatography (TLC) as described below. L-Amino acid oxidase activity was determined with L-alanine as the substrate by measuring the production of hydrogen peroxide with *o*-dianisidine and horseradish peroxidase in extracts obtained after sonication of cell suspensions at 40 W for 30 s (19). Total deaminase activity was assayed either by monitoring the appearance of ammonium in extracts incubated with 1 mM PPT in Tris-HCl buffer (100 mM, pH 8.0), or in cell suspensions by measuring the appearance of labeled PPO in the supernatant after adding [^{14} C]PPT as a substrate. Transaminases were assayed in 20 mM Tris-HCl buffer–2 mM PPT–2 mM α -ketoglutarate–10 μ M pyridoxal phosphate at pH 8.0 by monitoring the appearance of PPO by TLC or the disappearance of PPT by high-performance liquid chromatography (HPLC) as described below. To quantitate activity, cellulose from the TLC sheets containing the label was scraped off and radioactivity was measured in a liquid scintillation counter (Beckman LS 3801). To distinguish the production of PPO by either transamination or oxidative deamination by amino acid oxidase, a parallel assay was carried out in the presence or absence of added substrates other than PPT (2). This was also corroborated by running assays with extracts obtained after thawing the cells, under which conditions the L-amino acid oxidase activity is not extracted (19). A unit of enzymatic activity is the amount of enzyme which catalyzes the transformation of 1 μ mol of substrate per min under optimal assay conditions.

Analytical methods and transport assays. Ammonium was determined with glutamate dehydrogenase as described by Romero et al. (20). TLC was done on Cellulose F-coated sheets (E. Merck AG, Darmstadt, Germany) by using *n*-propanol–25% NH_4OH (3:2, vol/vol) as the solvent. Cold PPO, 3-methylphosphinopropionic acid, and *N*-acetyl-PPT were cochromatographed and stained with bromocresol green (27) and used as a control to determine the corresponding R_f s. The concentrations of PPT and amino acids derived from the transaminase assays were determined by HPLC (Spherisorb ODS2 [Teknochroma, Madrid, Spain], 15 by 0.46 cm, 5 μ m), after derivatization at 30°C for 1 h with dansyl chloride in the dark, by monitoring the UV absorption at 250 ± 6 nm in a diode array (System Gold; Beckman) under conditions described elsewhere (15). Keto acids were determined from liquid media with 2,4-phenylhydrazine as described by Piedras et al. (19). Protein was estimated as described by Bradford (4), with ovalbumin as the standard.

The metabolism and transport of L-[methyl- ^{14} C]PPT in whole cells were analyzed by centrifuging 300 μ l of cell suspensions for 10 to 15 s in a Beckman microcentrifuge E in a 0.4-ml Eppendorf tube containing at the bottom 20 μ l of a perchloric acid–water mixture (15:85, vol/vol) and 80 μ l of a dinonylphthalate–silicone DC-550 mixture (40:60, vol/vol) (Fluka Chemica AG, Buchs, Switzerland). Under these conditions, cells are washed on their way through the silicone phase and drop to the bottom phase, where they are immediately lysed. Internal metabolites are collected from the bottom phase, whereas the supernatant remains in the top phase.

RESULTS

Resistance of *C. reinhardtii* to PPT. Wild-type strain 21gr of the green alga *C. reinhardtii* was resistant to the ammonium salt of PPT, the main active component of the herbicide

FIG. 2. Growth of *Chlamydomonas* cells with ammonium-PPT. Cells were incubated for 7 days in plates with TAP medium supplemented with the indicated concentrations of ammonium-PPT, and then growth was determined by the modified drop method (11). AMM, plate control, to which 2.5 mM NH_4Cl was added as the only nitrogen source.

BASTA (Fig. 2). Total growth increased with increasing concentrations of ammonium-PPT (up to 2.5 mM [500 μ g/ml]). Growth took place with ammonium as the nitrogen source, and no consumption of PPT was observed (Fig. 3). In fact, growth halted when ammonium was exhausted from the medium. Under these conditions, GS activity levels did not change in the presence of PPT compared with that of control, untreated cells (Fig. 4). These cells were, however, extremely sensitive to MSX

FIG. 3. Utilization of ammonium-PPT by *C. reinhardtii* cells. Cells were grown in liquid medium with 1.5 mM (300 μ g/ml) ammonium-PPT. The concentrations of ammonium and chlorophyll were determined as described in Materials and Methods. PPT concentrations were determined by HPLC.

FIG. 4. Effects of PPT and MSX on GS activity and ammonium consumption of *C. reinhardtii*. Cells were placed in liquid TAP medium–4 mM NH_4Cl . GS activity was then determined in situ in control cells without any addition ($\circ$) and with addition of 1.5 mM K-PPT ($\blacksquare$) or of 0.27 mM MSX ($\blacktriangle$) at the time indicated by the arrow.

at concentrations as low as 0.27 mM (50 $\mu\text{g/ml}$), at which both GS activity and ammonium utilization were completely abolished (Fig. 4). To determine whether resistance was due to the presence of a GS activity resistant to PPT, an in vitro assay was carried out. Neither of the GS isoenzymes of *C. reinhardtii*, however, was resistant to PPT, since their activities were completely inhibited when extracts were incubated with 50 μM PPT and ATP for only 10 min (results not shown).

When cells were incubated for 10 h in media with ammonium and 1 mM [^{14}C]PPT (0.5 mCi/mol), an insignificant amount of label entered the cells (less than 80 dpm/300 μl of cell suspension collected), thus proving that the resistance to PPT was due to the inability of this strain to transport PPT into the cells.

***Chlamydomonas* growth with PPT.** Significant growth was observed when *Chlamydomonas* cells were incubated in TAP medium to which only the potassium salt of PPT was added (Fig. 5). No growth was observed in plates lacking PPT, and they were used as a control. This growth depended upon the presence of acetate and light, since it was not observed in minimal medium or in the dark (results not shown), and increased when the concentration of K-PPT was raised. Strong inhibition took place, however, when the concentration of K-PPT was over 1.5 mM. Again, when cells grew with 1.5 mM K-PPT, a level of labeled PPT that is very low, yet similar to

FIG. 5. Growth of *C. reinhardtii* cells with the potassium salt of PPT. (A) Cells were grown on solid TAP plates with the indicated concentrations of K-PPT as the sole nitrogen source, and then growth was determined by the modified drop method (11). (B) Cells were incubated in liquid TAP medium supplemented with L-[methyl- ^{14}C]PPT (50 mCi/mol) for 10 h, and then they were centrifuged through a dinonylphthalate-silicone layer as described in Materials and Methods. From the bottom layer, 10- μl aliquots were collected, and their radioactivity was measured in a liquid scintillation counter.

that found in media with ammonium, was found within the cells; this increased linearly with increasing concentrations of external PPT (Fig. 5). By HPLC and TLC analyses with [^{14}C]PPT, we demonstrated that *C. reinhardtii* cells grew by utilizing PPT as a source of nitrogen, by deaminating this compound to the corresponding keto acid, PPO, which appeared stoichiometrically in the supernatant (Fig. 6). The appearance of this keto acid was also corroborated by determining its presence with 2,4-phenylhydrazine (results not shown). No appearance of other PPT derivatives, such as *N*-acetyl-PPT or 3-methylphosphinopropionic acid, was detected in the supernatant by TLC analyses. Although we had clear evidence that PPT was being deaminated, no accumulation of ammonium in the media was observed.

Figure 7 shows the time course of the appearance of L-amino acid oxidase activity in nitrogen-starved cells compared with cells incubated in the presence of K-PPT. In both cases, similar lag phases and levels of amino acid oxidase were observed, although the enzyme levels diminished shortly after growth started in the presence of K-PPT. Amino acid oxidase activity was lacking in cells growing in media with ammonium.

Formation of PPT metabolites in cell extracts. When assays were carried out in vitro (Fig. 8), no *N*-acetyltransferase activity was detected. However, *C. reinhardtii* extracts showed a PPT- α -ketoglutarate aminotransferase activity which rendered PPO as the end product. When PPT was used as the substrate, L-amino acid oxidase was detected in sonicated ex-

FIG. 6. Consumption of PPT by *C. reinhardtii* cells. (A) Cells grown in minimal medium with ammonium were washed by centrifugation and resuspended in TAP medium lacking nitrogen for 8 h. Then, 1.5 mM [^{14} C]PPT was added (50 mCi/mol) and the concentrations of PPT (■), PPO (▲), and ammonium (○) were determined. □, concentration of total labeled compounds remaining in supernatant. (B) TLC analysis of supernatant performed at time zero (left) and after 24 h of growth (right).

tracts but not in those that were obtained after thawing of the cell pellets.

DISCUSSION

The unicellular green alga *C. reinhardtii* is extremely sensitive to GS inhibitors such as MSX, which causes rapid and complete inhibition of their GS isoforms both in vivo and in vitro (11). Another known inhibitor of GS is PPT, the main active component of the herbicide BASTA and a structural analog of MSX which has been reported to be 5 to 10 times more effective in plants (13).

Surprisingly, however, *C. reinhardtii* was resistant to the ammonium salt of PPT at concentrations up to 10 times higher than those of MSX (0.25 versus 2.5 mM). Under these conditions, growth was supported by consumption of only ammonium as a nitrogen source, whereas the concentrations of PPT remained unaltered (Fig. 3). PPT was unable to inactivate GS in vivo, since no alteration of this activity was detected in our in situ assays (Fig. 4). The fact that GS was completely functional under these conditions strongly suggests that ammonium is incorporated through the GS-glutamate synthase cycle, which is the usual pathway utilized by *C. reinhardtii* (5). However, neither of the two GS isoenzymes present in *C. reinhardtii* (11) is resistant to PPT, since they were completely inhibited in assays performed in cell extracts.

FIG. 7. Induction of L-amino acid oxidase in *C. reinhardtii*. Cells grown in media with ammonium were washed by centrifugation and transferred to TAP ammonium liquid medium lacking nitrogen (○) or containing 1.5 mM K-PPT (■). L-Amino acid oxidase activity was determined in cell extracts at the indicated times. Chl, *C. reinhardtii*.

Next, we became interested in investigating the presence of putative enzyme activities capable of inactivating PPT. Two of the three major different PPT-metabolizing enzymes are capable of conferring resistance to PPT: a PPT *N*-acetyltransferase (2, 6, 8, 24) and an α -ketoglutarate-PPT aminotransferase (2). The acetylating enzyme was discovered in some bialaphos-producing *Streptomyces* strains which synthesize it as a way to protect themselves against the toxin. The transaminase activity has been found in some resistant bacteria lacking acetylating activity. However, the mechanism by which this transaminase activity confers resistance to PPT remains unclear, since some bacteria expressing this activity are sensitive (2). A third PPT-metabolizing activity, namely, an L-amino acid oxidase, does not seem to confer resistance but is essential for the utilization of PPT as a nitrogen source for growth (2).

The facts that the concentration of PPT remained unaltered in the supernatant when cells grew in the presence of ammo-

FIG. 8. PPT-metabolizing activities of *C. reinhardtii* assayed in cell extracts by TLC. Cells were incubated in TAP medium lacking nitrogen for 10 h and then collected by centrifugation. Activity was then assayed in extracts. A, PPT acetyltransferase in thawed extracts; B, PPT- α -ketoglutarate aminotransferase in thawed extracts; C, L-amino acid oxidase assayed in thawed extracts; D, L-amino acid oxidase assayed in extracts obtained after sonication as detailed in Materials and Methods. In each panel, the left lane corresponds to the reaction mixture at time zero and the right lane corresponds to the same extract assayed after incubation for 1.5 h at 30°C.

nium and that no derivatives of PPT were detected (Fig. 3) strongly suggest that the cause of the resistance is lack of PPT transport into the cells. This was confirmed by using [^{14}C]PPT, as no significant amount of label entered the cells after various periods of incubation. Thus, we could not perform transport experiments with this compound. Alternatively, PPT could enter the cells but be immediately pumped out by the cells. This possibility is, however, highly improbable for several reasons. (i) *C. reinhardtii* lacks an active system able to transport any amino acid except arginine (12, 17), which explains why the only autotrophic mutants ever isolated are those affected in the synthesis of this amino acid (12). (ii) If PPT were excreted, it would be expected that other structurally related amino acids could also be pumped out by *C. reinhardtii*. Excretion of amino acids by this alga never has been observed and would surely have deleterious effects on growth. (iii) Once accumulated, labeled PPT was never observed to be excreted to the medium (results not shown). Under these same conditions, however, MSX did enter the cells, since both GS isoforms were completely inhibited after only 90 min of treatment. This strongly indicates that the two compounds, although structurally related, are transported through different systems. It should be expected if we consider that under the conditions we used to run the experiments (pH 7.5), PPT is negatively charged (7), whereas most of the MSX is in its isoelectric, neutral form. Besides, by using [^{14}C]-labeled amino acids, it has been demonstrated that *Chlamydomonas* cells transport only arginine (12, 17). However, growth was inhibited when the concentration of K-PPT was over 1.5 mM (Fig. 5A) and no growth at all was observed with 5 mM K-PPT (11). This inhibition correlates with the increased amount of labeled PPT that entered the cells (Fig. 5B), which could partially inhibit GS activity. The fact that this inhibition was not observed when cells were grown with ammonium-PPT can be explained if we assume that with an excess of ammonium, a higher internal concentration of glutamate is present (16), which should protect against competitive inactivation of GS by PPT (14). The mechanism by which PPT enters cells remains unknown, but the time course in Fig. 5 strongly suggests that PPT enters cells through diffusion, which explains the fact that the same level of labeled PPT was found in the presence and in the absence of ammonium. Thus, *C. reinhardtii* is resistant only to low concentrations of PPT, when the amount of internal PPT is below a threshold level. In any case, resistance was not due to the presence of an acetylating enzyme, since we could not detect it in assays carried out both *in vivo* and *in vitro*. However, a PPT- α -ketoglutarate aminotransferase has been found in *C. reinhardtii* (Fig. 7), whose participation in the resistance must be excluded, since at low concentrations of K-PPT, no significant levels of label entered the cell, and this activity did not protect the cells when the concentration of K-PPT increased.

When PPT was added as the potassium salt, *C. reinhardtii* utilized PPT as the sole nitrogen source for growth (Fig. 5). No growth in TAP medium was detected in the absence of an added nitrogen source, and this condition was used as a control. We demonstrated by TLC and HPLC analyses that PPT was specifically consumed under these conditions. Besides, this growth was dependent upon the presence of acetate and did not take place in minimal medium. PPO was found to be present in supernatants of cells growing with 1 mM [^{14}C]PPT (Fig. 6). This proves that PPT was being deaminated and strongly supports the involvement of L-amino acid oxidase activity in its utilization. In fact, *Chlamydomonas* cells can grow in the presence of acetate at the expense of the ammonium supplied after enzymatic deamination of at least 12 L-amino acids with an L-amino acid oxidase (17). By using [^{14}C]-labeled

amino acids, Muñoz-Blanco et al. (17) demonstrated that the corresponding keto acid was left in the supernatant and that no significant label entered the cells. The same happens with PPT. Induction of L-amino acid oxidase activity required acetate and was sensitive to ammonium and light deprivation, which explains why PPT was not used in the presence of ammonium or in the dark. This amino acid oxidase recognizes PPT when assayed *in vitro* (Fig. 8), and it has been reported to be at the periplasmic space (17, 19, 26), which explains the ability of *C. reinhardtii* to use this compound as a nitrogen source even though its cells lack the ability to transport it within themselves. When K-PPT was used, no accumulation of ammonium was observed, which can be explained if we take into account the fact that a high-affinity ammonium-specific permease is induced in *Chlamydomonas* cells under these nitrogen-limiting conditions (10).

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