Effects of Photosystem II Herbicides on the Photosynthetic Membranes of the Cyanobacterium *Aphanocapsa 6308*¹

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**ABSTRACT**

The effects of the photosystem II herbicides diuron (3-(3,4-dichlorophenyl)-1,1-dimethylurea) and atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine) on the photosynthetic membranes of a cyanobacterium, *Aphanocapsa 6308*, were compared to the effects on a higher plant, *Spinacia oleracea*. The inhibition of photosystem II electron transport by these herbicides was investigated by measuring the photoreduction of the dye 2,6-dichlorophenol-indophenol. A labeled herbicide was used to investigate herbicide binding with calculated binding constants (K) being 8.2 × 10⁻⁸ molar for atrazine and 1.7 × 10⁻⁹ molar for diuron. Competitive binding studies carried out on *Aphanocapsa* membranes using radiolabeled [³¹C]atrazine and unlabeled diuron revealed that diuron competed with atrazine for the herbicide-binding site. Experiments involving the photoaffinity label [³¹C]azidoatrazine (2-azido-4-ethylamino-6-isopropylamino-2-triazine) and autoradiography of polyacrylamide gels indicated that the herbicide atrazine binds to a 32-kilodalton protein in *Aphanocapsa 6308* cell extracts.

A number of commercially important herbicides have been shown to inhibit PSII electron transport in higher plants and algae. Diuron was shown to inhibit O₂ evolution in *Scenedesmus* (5) and s-triazines were shown to act at the same place in *Ankistrodesmus* (16). More recently, investigations have demonstrated that there is a direct correlation between the binding of various herbicides, including atrazine¹ and diuron, and the inhibition of electron transport in *Spinacia oleracea* (spinach) (22) and *Senecio vulgaris* L. (13). In addition, these investigations showed that diuron competes with atrazine for the herbicide-binding site in both plants (13, 22). Fluorescence induction experiments (17) and trypsin treatment of chloroplast thylakoids (20) and PSII particles (11) have suggested that PSII herbicides inhibit electron transport at the level of the secondary electron acceptor B, by causing a decrease in the oxidation/reduction potential of B with respect to the primary acceptor Q, thus making it inaccessible to reduction by Q (12). Trypsin treatment of chloroplast thylakoids and PSII particles (7, 11) has resulted in the proteolytic modification of a 32-kD protein and the concurrent loss of herbicide sensitivity, thus implicating a 32-kD protein as the herbicide receptor (11). In addition, a photoaffinity label, [³¹C]azidoatrazine, has been shown to bind specifically to a 32 to 34-kD protein in chloroplast thyalkloid preparations (15).

Most photosynthetic cyanobacteria, or blue-green algae, are sensitive to PSII inhibitors. Several investigations of the effect of herbicides on electron transport in cyanobacteria concluded that the herbicide diuron acts on the oxidizing side of PSII, rather than on the reducing side as in higher plants (6, 19). More recent studies of the inhibition of photosynthetic electron transport by diuron in the photoheterotrophic cyanobacterium *Aphanocapsa 6714* have concluded that diuron acts at the same step in cyanobacterial electron transport as in that of higher plants (4). In addition, a correlation was found between diuron sensitivity and the presence of a 33-kD polypeptide in *Aphanocapsa 6714* cell extracts (3).

In the present study, the herbicide receptor site of the photosynthetic membranes of the photoautotrophic *Aphanocapsa 6308* was investigated, utilizing many of the techniques employed in the characterization of inhibitor binding to higher plants. The results were then compared to those obtained for spinach stroma-free thylakoids. The covalent binding of the radiolabeled photoaffinity azido derivative of atrazine to a 32-kD protein in *Aphanocapsa* membranes identified an herbicide receptor.

**MATERIALS AND METHODS**

**Biological Material.** The unicellular cyanobacterium *Aphanocapsa 6308* (ATCC 27150) was grown in medium 11 (1) supplemented with 2.4 g/l Na₂CO₃ at 35°C under cool fluorescent bulbs at 10,800 lux in Roux flasks bubbled with 5% CO₂ in air. Fresh *Spinacia oleracea* leaves were obtained from a local supermarket.

**Spinach Thylakoid Preparation.** All procedures were carried out at 4°C. Fresh spinach leaves were ground in 100 mm Tricine-NaOH buffer, pH 7.8, containing 400 mm sorbitol and 5 mm MgCl₂ in a Waring blender at low speed. The resulting suspension was strained through cheesecloth and then centrifuged at 2,000g for 5 min. The pelleted chloroplasts were resuspended in 20 mm Tricine-NaOH buffer, pH 7.8, containing 5 mm MgCl₂ and left on ice for 5 min. After centrifugation at 2,000g for 5 min, the pelleted stroma-free thylakoids were resuspended in 50 mm sorbitol. The Chl concentration was determined using its extinction coefficient and the A₆₆₅ nm of 80% (v/v) acetone extracts (2).

**Aphanocapsa Membrane Preparation.** All procedures were carried out at 4°C. Cells were harvested by centrifugation at 5,000g for 15 min and washed in 50 mm Tricine-NaOH buffer, pH 7.5, containing 5 mm MgCl₂. After further centrifugation at 5,000g for 10 min, the cells were resuspended in Tricine buffer and broken with a Yeda press at 1,750 p.s.i. The broken cell suspension was diluted in buffer, pH 7.5, containing 10 mm Tricine-NaOH, 10

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³ Abbreviations: atrazine, 2-chloro-4-ethylamino-6-isopropylamino-s-triazine; DCPIP, 2,6-dichlorophenol-indophenol; azidoatrazine, 2-azido-4-ethylamino-6-isopropylamino-s-triazine; B, secondary electron acceptor in PSII; Q, primary electron acceptor in PS II.
mm NaCl, 10 mm MgCl₂, and 100 mm sorbitol. Cell debris was removed by centrifugation at 1,000 g and the remaining supernatant was centrifuged at 27,000 g for 10 min to pellet the photosynthetic membranes. The cell debris was washed in the second buffer and the centrifugations at 1,000 g and 27,000 g were repeated several times to increase the yield of the photosynthetic membranes. The membranes were resuspended in the 10 mm buffer for further assays. The Chl concentration was determined by reading the A₆₆₅nm in 80% (v/v) acetone (10).

Electron Transport Assays. Electron transport assays were carried out by measuring photoinduced absorbance changes (A₅₉₀ nm) in the dye DCPIP with time with a Gilford 240 spectrophotometer. Reaction mixtures contained 5 µg Chl/ml, 5 mM NH₄Cl, 10 mM MgCl₂, 50 mM Na,K-phosphate (pH 6.8), 100 mM sorbitol, and a DCPIP concentration giving a final A₅₉₀nm of 0.4 to 0.5. Photochemical assays of spinach electron transport activity were carried out at a saturating light intensity (Sylvania 150-w projector flood light) of 2400 µE/m²·s, while Aphanocapsa 6308 assays were carried out at a saturating light intensity of 3000 µE/m²·s. Assays were conducted at 25°C for 90 s.

Herbicide-Binding Assays. Reaction mixtures for herbicide-binding assays and competition studies contained 20 mm Tricine-NaOH, 5 mM MgCl₂, 100 mM sorbitol, and 25 to 50 µg Chl/ml. For herbicide-binding assays, small amounts of either uniformly ring-labeled [¹⁴C]atrazine (9.5 Ci/mmol) or [¹⁴C]diuron (93.9 Ci/mmol) were added to the reaction mixture. For competition studies, varying amounts of unlabeled diuron were added to samples containing a constant amount (0.482 µM) of labeled atrazine. Samples were centrifuged at 4°C for 10 min at 27,000 g. Aliquots of 0.8 ml of clear supernatant were removed and added to 10 ml of Aquasol-2 scintillation cocktail (New England Nuclear). Radioactivity of samples was measured by liquid scintillation spectrometry. The amount of bound inhibitor was calculated from the difference between the total radioactivity added to the membrane suspension and the amount of free inhibitor found in the supernatant after centrifugation. Assays were conducted at 25°C, with herbicide solutions prepared in ethanol. Binding constants were calculated by linear regression analysis of double reciprocal data plots as described by Tischer and Strotmann (22).

Sucrose Density Gradient Centrifugation. Continuous gradients were prepared with solutions of 0.2 to 1 M sucrose in a linear gradient maker. Approximately 1 ml of Aphanocapsa 6308 crude extract or resuspended membranes was layered on top. The gradients were centrifuged at 23,000 rpm for 14 h at a temperature of 4°C in an SW 41 swing bucket rotor of a Sorvall OTD-65 ultracentrifuge.

Polyacrylamide Gel Electrophoresis. SDS-polyacrylamide gel electrophoresis was carried out using the discontinuous buffer system of Laemmli (8) and a slab gel apparatus modeled after that of Studier (21). Samples were solubilized in sample buffer for 20 min at room temperature and applied to a 10 to 17% (w/v) linear acrylamide gradient gel with a 5% (w/v) stacking gel. Electrophoresis was carried out in a cold room (4°C) and the gels were stained with Coomassie blue for the detection of proteins.

Photoaffinity Labeling. Ultraviolet irradiation of sample exposed to the photoaffinity label [¹⁴C]azidoatrazine (37.1 µCi/mg) was carried out for 10 min at a distance of approximately 5 cm from the UV source. Polyacrylamide gels were analyzed for covalently bound [¹⁴C]azidoatrazine by x-ray fluorography (9). Gels were soaked in 22% (w/v) PPO in dimethyl sulfoxide, followed by drying onto filter paper (9). The gel and x-ray film were wrapped in aluminum foil and kept at -80°C for 2 to 4 weeks before the autoradiogram was developed. Mol wt were determined using standards on each gel followed by graphing of the log of the mol wt of the standard versus the distance traveled on the gel.

RESULTS

Electron Transport Studies. The effect of the PSII herbicides diuron and atrazine on the photosynthetic electron transport activity of isolated Aphanocapsa 6308 membranes and spinach stroma-free thylakoids was determined by measuring the decrease in electron transport activity as a function of increasing concentration of herbicide added to the reaction mixture. Addition of diuron to both Aphanocapsa 6308 membranes and spinach thylakoids decreased the rate of DCPIP reduction, indicating a decrease in the rate of electron flow through the photosynthetic electron transport chain (Fig. 1). The I₅₀ concentration, defined as the herbicide concentration resulting in a 50% inhibition of photosynthetic electron transport, was calculated from these data.

Table I shows that the diuron I₅₀ concentrations of 5.0 x 10⁻⁹ M for spinach thylakoids and 6.8 x 10⁻⁸ M for Aphanocapsa 6308 membranes are very similar. The atrazine I₅₀ concentration of 8.8 x 10⁻⁸ M for Aphanocapsa membranes is significantly higher than the diuron I₅₀ concentration.

Herbicide-Binding Studies. Binding studies using radioactively labeled herbicides can be used to characterize directly the amount of bound inhibitor, the binding constant of each inhibitor, and the number of inhibitor-binding sites for each inhibitor at equilibrium (13). The amount of diuron or atrazine bound to isolated Aphanocapsa membranes increased as the amount of free diuron or atrazine added to the reaction mixture increased (Fig. 2). Binding constants and the maximum number of available binding sites can be calculated from double reciprocal plots of binding data. Double reciprocal plots of the data in Figure 2 are shown in Figure 3. The y intercept is a measure of the number of inhibitor binding sites on a Chl basis, the number of binding sites per 'photosynthetic unit' (13). For Aphanocapsa 6308 membranes, the number of atrazine-binding sites was calculated to be 375 nmol Chl/nmol atrazine bound, while the number of diuron-binding sites was found to be 295 nmol Chl/mol diuron bound (Table I). The binding constants, K, as calculated from the x intercept, were 8.2 x 10⁻⁹ M for atrazine and 1.7 x 10⁻⁷ M for diuron (Table I). The binding of atrazine or diuron to spinach stroma-free thylakoids was analyzed in the same way for Aphanocapsa 6308 (Table I).

Competition experiments between labeled atrazine and unlabeled diuron indicate whether the two herbicides share a binding site in the photosynthetic membranes of Aphanocapsa 6308. After exposing isolated Aphanocapsa membranes to radioactively labeled atrazine, increasing concentrations of unlabeled diuron were added to the assay mixture. The results of these experiments are shown in Figure 4. The results indicate that atrazine and diuron do not compete for any common binding sites on the membranes.


Table 1. Comparison between \( I_{50} \) Values, Calculated Binding Constants (\( K \)), and Number of Binding Sites for Diuron and Atrazine in Aphanocapsa 6308 Membranes and Spinach Stroma-Free Thylakoids

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Aphanocapsa</th>
<th>Spinach</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( I_{50} )</td>
<td>( K )</td>
</tr>
<tr>
<td>Diuron</td>
<td>( 6.8 \times 10^{-9} )</td>
<td>( 1.7 \times 10^{-7} )</td>
</tr>
<tr>
<td>Atrazine</td>
<td>( 8.8 \times 10^{-8} )</td>
<td>( 8.2 \times 10^{-8} )</td>
</tr>
</tbody>
</table>

*Values for \( K \) and nmol Chl/nmol inhibitor were determined from linear regression analysis of reciprocal plots such as shown in Figure 3 and values for \( I_{50} \) were determined from data shown in Figure 1. ND, not determined.

**Figure 2.** Binding of \( ^{14} \text{C} \) diuron and \( ^{14} \text{C} \) atrazine to Aphanocapsa 6308 membranes. The amount of bound inhibitor, expressed as a function of Chl concentration, was calculated from the difference between the radioactivity added and the free inhibitor left in the supernatant. (C) Atrazine; (O) diuron.

**Figure 3.** Double reciprocal plots of \( ^{14} \text{C} \) diuron and \( ^{14} \text{C} \) atrazine binding to Aphanocapsa 6308 membranes. (C) Atrazine; (O) diuron.

**Figure 4.** Competition between \( ^{14} \text{C} \) atrazine and unlabelled diuron in Aphanocapsa 6308 membranes. The amount of \( ^{14} \text{C} \) atrazine bound, expressed as a function of Chl concentration, was calculated from the difference between the total \( ^{14} \text{C} \) atrazine added and the free atrazine left in the supernatant. The amount of bound \( ^{14} \text{C} \) atrazine was plotted as a function of unlabelled diuron concentration. Concentration of \( ^{14} \text{C} \) atrazine was \( 0.482 \mu \text{M} \).

Staining bands from Aphanocapsa are those at mol wt between 16 and 18 kD associated with the phycobiliproteins; other heavy bands are noted at 29 kD, the 32 to 34-kD region, 45 kD, and in the 65-kD area. Heavily stained bands in the spinach membrane preparation are observed at 25, 32, 68, and 75 kD. A number of proteins in Aphanocapsa migrate the same distance as those of spinach, indicating comparable mol wt.

Purification of cyanobacterial membranes on a continuous sucrose gradient shows that many soluble proteins can be removed or reduced in concentration in the membrane fractions (Fig. 6). In particular, the phycobiliproteins are greatly decreased in concentration. The 32-kD area becomes more clearly resolved in the most purified membrane fraction (Fig. 6a). Figure 7 shows the results of labeling the sucrose gradient-purified membrane proteins of Aphanocapsa with the photoaffinity label \( ^{14} \text{C} \) azidoatrazine. The figure compares the Coomassie blue-stained gel of membranes with the corresponding fluorogram, developed on x-ray film. Radioactive label was detected only in a polypeptide at an apparent mol wt of 32 kD.

**DISCUSSION**

The \( I_{50} \) concentration of an inhibitor is a useful means of comparing the effect of the inhibitor on different systems. The spinach \( I_{50} \) concentration of diuron \( (5.0 \times 10^{-9} \text{ M}, \text{ Table I}) \) is significantly lower than the reported value of \( 4.0 \times 10^{-8} \text{ M} \) (22);
however, the conditions of the experiment were different and a different electron acceptor was used. Similarly, the *Aphanocapsa*
6308 $I_0$ concentration of diuron (6.8 × 10⁻⁷ M, Table I) is also
significantly lower than the value reported for *Aphanocapsa* 6714
thylakoids of 1.5 × 10⁻⁷ M (4). In addition to the fact that the
experimental conditions were again different, the variation be-
tween the $I_0$ values may be due to the fact that 6714 is a
photoheterotroph (18) while 6308 is photoautotrophic and there-
fore likely to have different biochemical properties. That the
diuron $I_0$ concentrations for *Aphanocapsa* 6308 and for spinach
thylakoids were similar when determined under the same ex-
perimental conditions is evidence that the affinity of diuron for the
herbicide-binding site of these two organisms is comparable.

The atrazine-binding constant for spinach (4.0 × 10⁻⁸ M, Table
I) is different from the value of 1.4 × 10⁻⁷ M reported (22), but the
same as the value of 4.0 × 10⁻⁸ M reported for *S. vulgaris* L. (13).

The similarity of binding constants for diuron measured using
*Aphanocapsa* 6308 and spinach in experiments performed under
similar conditions as reported in Table I and also the similarity of
atrazine constants are further evidence suggesting that the mech-
anism of herbicide action in the two organisms may be the same.

Tischer and Strotmann (22) showed that inhibitor binding is
directly related to inhibition of electron transport in spinach
chloroplasts so that binding can be used for studies of inhibitor-
receptor interaction. If two inhibitors react with the same electron
carrier by the same mechanism, binding of one should be com-
petitively affected by the other. Tischer and Strotmann (22)
showed that diuron and atrazine act at the same site in spinach
chloroplasts by analysis of competition between the herbicides.
Figure 4 shows that the same competition takes place in *Aphano-
capsa* membranes, suggesting that the herbicides share a similar
mode of action in the two systems. Astier and Joset-Espardellier

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Figs. 5, 6, and 7. Arrow at left indicates 32-kD polypeptide. Chl (7–15 mg) was applied to the sample wells. Fig. 5 (left), Coomassie blue-stained gradient SDS gel of membranes from *Aphanocapsa* (a) and spinach (b), not purified by sucrose gradient. Membranes were prepared as for measurement of PSII activity and then washed three times with breaking buffer. Standards are BSA, 68 kD; ovalbumin, 48 kD; pepsin, 34.7 kD; trypsinogen, 24 kD; β-lactoglobulin, 18.4 kD; and lysozyme, 14.3 kD. Fig. 6 (center), Coomassie blue-stained gradient SDS gel of membranes of *Aphanocapsa* purified by continuous sucrose gradient (a), membrane fraction put on the sucrose gradient (b), and crude extract (c). Fig. 7 (right), comparison of Coomassie blue-stained gradient SDS gels of [¹⁴C]azidoatrazine-labeled membranes of *Aphanocapsa* purified by continuous sucrose gradient (a) and fluorogram of the gel (b).
(3) demonstrated the simultaneous resistance to diuron and atrazine after a single mutational event. This also suggests that the sites of action of both inhibitors are on the same protein.

Polyacrylamide gel electrophoresis of cyanobacterial membrane preparations revealed a large number of proteins, some of which appear to co-migrate with spinach membrane proteins. In Figure 5, a group of *Aphanocapsa* membrane proteins is shown to travel the same distance on the gel as a heavily staining 32-kD polypeptide in the spinach preparation. Pfister and co-workers (14, 15), using the photoaffinity label azidoatrazine, have identified a 32-kD polypeptide as the herbicide-binding protein in chloroplast membranes. They suggest that this 32-kD protein is the binding protein for all PSII inhibitors and that it is associated with the secondary electron acceptor B.

Astier and Joset-Espardellier (3) have shown that a 33-kD protein may be involved in herbicide binding in *Aphanocapsa* 6714, since this protein is missing in diuron-resistant mutants of the strain. Figure 7 shows that a single protein in *Aphanocapsa* 6308 membranes binds the photoaffinity label azidoatrazine, suggesting that this protein, with an apparent mol wt of 32 kD, is the herbicide-binding protein.

**CONCLUSIONS**

The results of these investigations indicate that the binding properties of the herbicides diuron and atrazine to *Aphanocapsa* 6308 membranes and spinach thylakoids are comparable. In addition, it appears that diuron and atrazine bind competitively to the same site in *Aphanocapsa* 6308 membranes, as has been shown to be true in spinach thylakoids and those of other higher plants.

*Aphanocapsa* 6308 membrane proteins were characterized by means of polyacrylamide gel electrophoresis and directly compared to spinach thylakoid proteins. The binding of the photoaffinity label [14C]azidoatrazine specifically to a membrane polypeptide of 32 kD is significant since a protein of this mol wt has been shown to specifically bind azidoatrazine in higher plants. Continued investigations of the interaction of herbicides with *Aphanocapsa* 6308 membrane proteins would further reveal the similarities and differences between *Aphanocapsa* 6308 and higher plant herbicide-binding and photosynthetic function.

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