Molecular and Biophysical Analysis of Herbicide-Resistant Mutants of *Chlamydomonas reinhardtii*: Structure-Function Relationship of the Photosystem II D1 Polypeptide

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Plants and green algae can develop resistance to herbicides that block photosynthesis by competing with quinones in binding to the chloroplast photosystem II (PSII) D1 polypeptide. Because numerous herbicide-resistant mutants of *Chlamydomonas reinhardtii* with different patterns of resistance to such herbicides are readily isolated, this system provides a powerful tool for examining the interactions of herbicides and endogenous quinones with the photosynthetic membrane, and for studying the structure-function relationship of the D1 protein with respect to PSII electron transfer. Here we report the results of DNA sequence analysis of the D1 gene from four mutants not previously characterized at the molecular level, the correlation of changes in specific amino acid residues of the D1 protein with levels of resistance to the herbicides atrazine, diuron, and bromacil, and the kinetics of fluorescence decay for each mutant, which show that changes at two different amino acid residues dramatically slow PSII electron transfer. Our analyses, which identify a region of 57 amino acids of the D1 polypeptide involved in herbicide binding and which define a D1 binding niche for the second quinone acceptor, Qe of PSII, provide a strong basis of support for structural and functional models of the PSII reaction center.

INTRODUCTION

Photosynthesis, the chemical process by which light energy is converted into chemical energy, takes place in the chloroplast of plants and algae. Photosystem II (PSII) is the thylakoid membrane protein-pigment complex that mediates the initial light-driven reactions in which electrons are removed from water, yielding oxygen and hydrogen ions (for general review, see Clayton, 1980; Dismukes, 1988). The electrons are transferred through a series of intermediate acceptors to the first and second stable quinone acceptors of PSII, Qa and Qb, respectively (Velthuys, 1981). Much recent work has focused on describing the structural composition of the PSII complex, and, in particular, of the PSII reaction center. Our interest lies in elucidating how the structure of the reaction center relates to its function in mediating the transfer of electrons from water to Qa.

A number of chemically different classes of herbicides block the photosynthetic electron transport chain on the reducing side of PSII. These include the s-triazines, substituted ureas, and phenolic derivatives, all of which can be used as probes to study the function of the thylakoid membrane (for reviews, see Trebst, 1980; Arntzen et al., 1982). The different herbicides compete with each other and with quinone for binding to thylakoid membranes (Tischer and Strotmann, 1977), and they appear to disrupt electron transfer by displacing the bound plastoquinine, Qa (Vermaas et al., 1983). Photoaffinity labeling of thylakoid membranes with 14C-azidoatrazine indicates that the herbicide analog binds, in herbicide-sensitive plants and algae, and with greatly reduced affinity in herbicide-resistant strains, to a PSII polypeptide of 32,000 apparent molecular weight (Pfister et al., 1981; Steinback et al., 1981; Tellensbach et al., 1983; Erickson et al., 1984a). This polypeptide, called D1 in *Chlamydomonas reinhardtii* (Chua and Gilham, 1977), is encoded by the chloroplast psbA gene (Zurawski et al., 1982), is highly conserved in all species examined to date (see Erickson et al., 1985b, for review), and is an integral part of the active PSII reaction center core (Barber...
The role of the D1 polypeptide in determining resistance to triazine herbicides was demonstrated by the isolation and characterization of psbA from plants and algae resistant to high levels of the herbicide atrazine. DNA sequence analysis of these mutant genes showed that a single base pair change in psbA, compared with the sequence of psbA from herbicide-sensitive strains, resulted in a single amino acid substitution at residue 264 which changed the wild-type serine residue to alanine in Chlamydomonas (Erickson et al., 1984a) and to glycine in Amaranthus hybridus and Solanum nigrum (Hirschberg and McIntosh, 1983; Hirschberg et al., 1984; Goloubinoff et al., 1984). Transformation of cyanobacteria with a mutant cyanobacterial psbA gene coding for alanine at position 264 produced herbicide-resistant cyanobacteria (Golden and Haselkorn, 1985) and provided definitive confirmation that such mutations result in the herbicide-resistant phenotype. These molecular genetic results were consistent with classical genetic evidence that the herbicide-resistance trait is maternally inherited (Souza-Machado et al., 1978; Galloway and Mets, 1982, 1984; Erickson et al., 1984a) and, coupled with the physical characterization of the herbicide-resistant and sensitive strains, strongly suggested that the psbA gene product was the "herbicide-binding" protein and served as the Qa-binding protein of PSII.

Our previous work in characterization of herbicide-resistant mutants of C. reinhardtii revealed additional mutation sites in psbA that alter D1 amino acid residues 219 and 255 and result in unique patterns of resistance to the herbicides atrazine and diuron (Erickson et al., 1985a). In contrast to the changes described at residue 264 which dramatically slow electron transfer in both plants and algae (Bowes et al., 1980; Erickson et al., 1984a), the amino acid substitutions at residues 219 and 255 do not appear to affect the rate of Qa to Qb electron transfer (Galloway and Mets, 1984). Hence, herbicide-resistant mutants that have single amino acid substitutions in the D1 protein are important tools for studying PSII electron transfer and, indirectly, quinone binding. Unlike the photosynthetic reaction centers, which have been successfully crystallized and analyzed by x-ray diffraction (Deisenhofer et al., 1985; Allen et al., 1987), the chloroplast PSII reaction center has resisted a similar type of analysis, and PSII crystals that can be effectively analyzed by x-ray diffraction have not been obtained. Thus, the combined biochemical, molecular, and genetic approach provides a powerful means of dissecting structure-function relationships in the PSII reaction center.

To further explore the functional regions of the D1 protein and to delineate the extent of the Qa-binding site more precisely, we have characterized four additional uniparental mutants of Chlamydomonas. Here we report the results of sequence analysis of the D1 gene, psbA, from four mutants, Ar204, Br24, Br202, and Dr18, and the identification of two new mutation sites which result in amino acid substitutions at residues 256 and 275 of the D1 protein. In addition, we have studied the rates of electron transfer between Qa and Qb in these four mutants and in the three we previously characterized, by observing fluorescence induction kinetics and fluorescence decay kinetics. (In the strict sense we mean decay in the fluorescence yield, but we use the term fluorescence decay for simplicity.) We have also compared the relative resistance of all seven mutants to the herbicides atrazine, bromacil, and diuron. These data allow us to correlate the presence of specific amino acid substitutions in D1 with herbicide resistance patterns and with the rate of Qa to Qb electron transfer.

RESULTS

Isolation and Genetic Characterization of Herbicide-Resistant Mutants

Dr18 was obtained by mutagenizing C. reinhardtii wild-type (wt) strain 137c(+) with N-methyl-N′-nitro-N-nitroso-guanidine (Gilham, 1965) and selecting on diuron (DCMU) gradient plates of solid minimal medium as previously described by McBride et al. (1977). Br24 was obtained following a similar procedure, except that bromacil gradient plates were substituted for the DCMU gradient plates. In both cases, no attempt was made to reduce the copy number of the chloroplast genome by treatment with 5-fluoro-2'-deoxyuridine before mutagenesis. Analysis of 17 tetrads from matings of Dr18 with wt cells and eight tetrads from matings of Br24 with wt cells showed a uniparental mode of inheritance for both herbicide-resistant phenotypes. In addition, both mutants showed resistance to herbicide in vitro, monitored by Hill activity of chloroplast membrane preparations (Lien et al., 1977). The isolation and phenotypic characterization of uniparental herbicide-resistant mutants of C. reinhardtii selected for their resistance to atrazine (mutants Ar204, Ar207, Galloway and Mets, 1984), diuron (mutant DCMU4, Erickson et al., 1984a; mutant Dr2, Galloway and Mets, 1984), and bromacil (mutant Br202, Galloway and Mets, 1984) have been previously reported as noted.

Molecular Characterization of psbA from Four Herbicide-Resistant Mutants

psbA, the chloroplast gene coding for D1, contains five exons spanning approximately 7 kb and is present in two copies per circular chloroplast molecule of C. reinhardtii (Erickson et al., 1984b). Total DNA from the mutants was
isolated and digested with BamHI and BgII, generating BamHI-BgII restriction fragments of approximately 10 kb and 12 kb which each contain one copy of psbA. Digested DNA from Ar204, Br202, Br24, and Dr18 was cloned in Escherichia coli using plasmid vectors as described in "Methods," and recombinant clones containing psbA were identified by colony hybridization. Because all three of the herbicide-resistant mutation sites previously characterized (Erickson et al., 1984a, 1985a) were localized in exons 4 and 5 of psbA, only these two exons were sequenced for each of the four new mutants listed above, using the Maxam-Gilbert sequence strategy diagrammed in Figure 1 and described in "Methods." We find that an adenine to cytosine transversion changes the wild-type leucine codon 275 to phenylalanine in mutant Br202. Mutants Ar204 and Br24 both have an identical mutation in which a guanine to adenine transition changes the wild-type glycine codon 256 to aspartic acid. These latter two mutants were isolated in different laboratories and selected for resistance to different herbicides (atrazine in the case of Ar204, bromacil in the case of Br24). Sequence analysis of psbA from mutant Dr18 reveals a guanine to adenine transition which changes valine codon 219 to isoleucine in the mutant. This same change was reported previously in the independently isolated diuron-resistant mutant Dr2 (Erickson et al., 1985a). Table 1 shows the results of psbA sequence analysis of the four new mutants, as well as the three previously characterized, and summarizes the change deduced in the D1 protein for each mutant. Except for the single mutation observed in each mutant as indicated in Table 1, no other changes were found in exons 4 and 5 in any of these mutants. In contrast to the extensive molecular analysis of our previous work, no attempt was made to sequence both copies of psbA from these four new mutants. When a mutation eliminates or creates a restriction enzyme site, the presence of that mutation can be verified for a large population of cells by performing the diagnostic restriction digestion followed by a genomic DNA gel blot, as was done for DCMU4 (Erickson et al., 1985a) and for Br202, Br24, Dr2, and Dr18 (J.M. Erickson, unpublished). In all cases, overexposure of the blot showed that all detectable copies of psbA contained the mutation. Genetic evidence in cyanobacteria strongly suggests that herbicide resistance is a recessive trait, i.e. that a mixture of wild-type and mutant gene products results in a herbicide-sensitive phenotype (Pecker et al., 1987). Hence, we find, as expected, only mutant copies of psbA in our strains that were selected for herbicide resistance.

### Table 1. psbA Mutations in Herbicide-Resistant Strains of C. reinhardtii

<table>
<thead>
<tr>
<th>Strain</th>
<th>psbA Sequence</th>
<th>Codon Change</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>tta GTT act</td>
<td>Val → ile</td>
<td>219</td>
</tr>
<tr>
<td>Dr2, DR18</td>
<td>tta ATT act</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>tac TTT ggt</td>
<td>Phe → Tyr</td>
<td>255</td>
</tr>
<tr>
<td>Ar207</td>
<td>tac TAT ggt</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>ttt GGT gtt</td>
<td>Gly → Asp</td>
<td>256</td>
</tr>
<tr>
<td>Ar204, Br24</td>
<td>ttt GAT gtt</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>gct TCT ttc</td>
<td>Ser → Ala</td>
<td>264</td>
</tr>
<tr>
<td>DCMU4</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>ttc TTA gct</td>
<td>Leu → Phe</td>
<td>275</td>
</tr>
<tr>
<td>Br202</td>
<td>ttc TTC gct</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Relative Resistance of Mutants to Herbicides

All seven herbicide-resistant mutants were tested in vivo for their relative levels of resistance to atrazine, bromacil, and diuron by plating comparable numbers of cells on minimal agar plates containing varying amounts of a given herbicide, and comparing their growth to that of wild-type 137c(+) cells. Bromacil and atrazine were added to a series of plates at concentrations of 1 μM, 2 μM, 5 μM, 10 μM, 20 μM, 50 μM, 100 μM, and 500 μM. Diuron was added to plates at final concentrations of 0.1 μM, 0.2 μM, 0.5 μM, 1 μM, 2 μM, 5 μM, 10 μM, 20 μM, 50 μM, 100 μM, and 250 μM. Growth of wild-type cells was inhibited by 1 μM diuron, 2 μM bromacil, and 5 μM atrazine. The resistance levels of all mutants are summarized in Table 2.

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**Figure 1. Restriction Map of psbA from C. reinhardtii.**

The map shows the five exons (I to V, black boxes) and the Maxam-Gilbert DNA sequencing strategy for exons 4 and 5 contained in the 1300-bp XbaI-EcoRI fragment and the 1100-bp HindIII-KpnI fragment, respectively. The sizes of the four small DNA fragments sequenced are indicated in base pairs. Arrows show the approximate extent of sequencing from each labeled end. Each fragment was labeled separately at both the 5' and 3' end, so that both strands corresponding to the entire exon 4 and 5 regions were sequenced for each mutant. Restriction sites are as follows: D, Ddel; H, HindIII; Hf, HinfI; K, KpnI; R, EcoRI; X, XbaI.
Table 2. Summary of Properties of Herbicide-Resistant Mutants of C. reinhardtii

<table>
<thead>
<tr>
<th>Codons</th>
<th>Resistance</th>
<th>Fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>WT</td>
<td>MT</td>
</tr>
<tr>
<td>219</td>
<td>Val</td>
<td>Ile</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>255</td>
<td>Phe</td>
<td>Tyr</td>
</tr>
<tr>
<td>256</td>
<td>Gly</td>
<td>Asp</td>
</tr>
<tr>
<td>264</td>
<td>Ser</td>
<td>Ala</td>
</tr>
<tr>
<td>275</td>
<td>Leu</td>
<td>Phe</td>
</tr>
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<td></td>
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</tr>
</tbody>
</table>

Resistance of C. reinhardtii mutant strains to atrazine (A), bromacil (B), and diuron (D) is indicated relative to the wild-type strain as described in “Results.” Codon numbers are for the D1 polypeptide. Amino acid residues in wild-type (WT) and mutant (MT) strains are indicated. Fluorescence induction and decay measurements are as described in “Methods” and in the legends to Figures 2 to 4. T(1/2) is the half-time of fluorescence decay (see Figure 3 and text). The number of independent measurements of decay half-time is indicated in parentheses for each mutant.

Electron Transfer from Qa to Qb in Herbicide-Resistant Mutants

Fluorescence Induction Kinetics

Electron transfer on the reducing side of photosystem II was monitored in the seven herbicide-resistant mutants whose properties are described in Table 2 by analyzing the kinetics of fluorescence induction as described in “Methods.” Figure 2 depicts the fluorescence induction kinetics for Br24 and wild-type cells. The inset in Figure 2 shows the initial fluorescence rise on an expanded time scale, which clearly depicts the difference between the two cell lines. Our measurements for mutants Ar204 and DCMU4 show a more rapid rise in the fluorescence induction curve when compared with wild-type cells (data not shown), which is consistent with previous results (Erickson et al., 1984a; Galloway and Mets, 1984). Fluorescent rise curves of this type have also been found for the atrazine-resistant biotypes of Amaranthus and have been interpreted as a slowing of electron transfer between Qa and Qb (Bowes et al., 1980; Arntzen et al., 1982). In contrast, our measurement of fluorescence induction transients for the herbicide-resistant mutants Ar207, Br202, Dr2, and Dr18 were not significantly different from those of wild-type cells (data not shown).

Fluorescence Decay Curves

To determine the rate of electron transfer from Qa to Qb more precisely, the kinetics of reoxidation of Qa by Qb was measured with a pulse-modulated fluorimeter (Schreiber, 1986). Analysis of single, isolated colonies on agar plates or liquid cells in suspension gave similar results. Fluorescence decay curves following an actinic saturating flash were determined using a pulsed low-intensity measuring beam (see “Methods”). Plotting the decay of fluorescence on a logarithmic scale allows one to determine, by linear extrapolation of the initial part of the curve In(a-x) versus time, the decay half-time, T(1/2), where a represents the maximum fluorescence and a-x the fluorescence as a function of time. Such a plot is shown in the bottom half of Figure 3. Only the initial part of the curve can be linearized in a first-order plot. This value is taken as a specific indicator of the reoxidation of Qa by Qb. The latter part of the curve (>300 μsec), however, is also Qa:Qb dependent. This was demonstrated by the complete inhibition of the fluorescence decay in the presence of a PSI inhibitor (data not shown). Figures 3 and 4 show the fluorescence decay curves obtained for wild-type cells and the seven mutants Dr18, Dr2, Br202, Ar207, DCMU4, Br24, and Ar204. The corresponding values for the decay half-times are indicated in Table 2. The decay curves and T(1/2) values obtained for Dr18 and Dr2 are very similar (Figure 3), as expected, since the two mutants carry the same chloroplast mutation (Table 1). Mutants Ar204 and Br24, which were isolated independently and which contain identical mutations, both show an identical and significant decrease in the rate of Qa reoxidation relative to wild-type cells (Figure 4, Table 2), indicating a slowing of electron transfer between Qa and Qb. The same is true for the DCMU4 mutant (Figure 4). The fluorescence decay curves obtained with all mutants were highly reproducible. The estimated T(1/2) values for Ar207, Br202, Dr2, and Dr18 are slightly but consistently higher than that seen for wild-type cells (Figures 3 and 4, Table 2).

DISCUSSION

Analysis of the psbA genes from this characterization of herbicide-resistant mutants and from our previous studies...
Wild-type strain 137c (solid line); herbicide-resistant mutant Br24 (dashed line). Relative fluorescence was measured as indicated in "Methods." The inset shows the initial fluorescence rise on an expanded time scale. Bars indicate the different time scales.

(Erickson et al., 1984a, Erickson et al., 1985a) have together identified five different mutation sites in psbA that result in amino acid substitutions at five distinct amino acid residues of the D1 reaction center polypeptide of PSII. Each of these mutations can be correlated with a specific pattern of resistance to the PSII inhibitors atrazine, bromacil, and diuron, and with specific rates of electron transfer from QA to QB (Table 2) in the absence of herbicide. An additional psbA mutation affecting alanine codon 251 has recently been identified in a metribuzin-resistant mutant of Chlamydomonas (Johanningmeier et al., 1987), bringing the total number of mutant sites found in the Chlamydomonas D1 polypeptide to six. These mutations serve as probes for assessing the role of specific amino acid residues of D1 in quinone-binding and electron transfer on the reducing side of PSII.

Figure 5 shows the locations of these six sites in the D1 polypeptide of C. reinhardtii. This particular membrane-spanning model, with five transmembrane domains (after Trebst, 1987), is based on information obtained from a variety of molecular, biochemical, and physical studies of chloroplast and bacterial reaction centers. Active PSII core complexes capable of efficient primary charge separation have recently been isolated from thylakoid membranes of spinach and pea (Barber et al., 1987; Danielius et al., 1987; Marder et al., 1987; Nanba and Satoh, 1987). These PSII cores contain the D1 and D2 polypeptides, cytochrome b559, chlorophyll a, pheophytin, and β-carotene. The chloroplast PSII reaction center is related both structurally and functionally to the reaction centers of the purple photosynthetic bacteria (for review, see Barber, 1987; Rochaix and Erickson, 1988). In each case, pheophytin acts as an intermediate electron acceptor and a very similar quinone-iron complex exists that contains the primary and secondary stable quinone electron acceptors QA and QB. In the bacterial reaction centers, QA and QB are bound to the M and L reaction center polypeptides, respectively. The L subunit has also been shown to bind PSII herbicides (Brown et al., 1984; DeVitry and Diner, 1984; Okamura, 1984). The molecular structures of the reaction center of Rhodopsseudomonas viridis (Deisenhofer et al., 1985) and Rhodobacter sphaeroides (Allen et al., 1987) have recently been determined by x-ray crystallographic analysis. In both of these organisms, the L and M subunits form a pair,
Figure 4. Fluorescence Decay Curves for Dark-Adapted Cells of C. reinhardtii.

Wild-type strain 137c compared with herbicide-resistant mutants Ar207 and Br202 (upper panel) and DCMU4, Br24, and Ar204 (lower panel), following an actinic flash as described in "Methods." Curves are normalized to the same maximum fluorescence. Bar represents time scale.

Immuno logic probing of thylakoid membranes with antibodies specific for proposed stromal and luminal residues of the D1 polypeptide (Sayre et al., 1986) gives results that strongly support the general model for D1 shown in Figure 5. This model, however, is in conflict with previous studies that suggest that the COOH terminus of D1 is in the stroma and not the lumen (Marder et al., 1984), and with our previously published model (Erickson et al., 1985a), based on Rao et al. (1983), which predicted seven transmembrane helices for D1. Although more work on the structure of D1 is required, the current model is most consistent with available data. Given this model, we see that four of the D1 residues that are changed in the herbicide-resistant mutants (Ala-251, Phe-255, Gly-256, and Ser-264) are localized on the stromal side of the membrane in a 14-amino acid region of D1 between the fourth and fifth transmembrane domains. Although the remaining two residues, Val-219 and Leu-275 are further away in the primary amino acid sequence of D1, the membrane-folding model places them in close proximity to the other altered residues on the stromal side of the thylakoid membrane. Recent analysis of herbicide-resistant mutants in cyanobacteria have identified phenylalanine codon 211 as another site at which a change confers resistance to atrazine and diuron (Gingrich et al., 1988). Thus, all these mutation sites fall in one localized region of the protein and define the QA-binding pocket. X-ray diffraction analysis of the bacterial reaction center crystals suggests that amino acid residues His-217, Trp-250, and Ala-258 of the M subunit are contacts for binding QA (Deisenhofer et al., 1985; Trebst, 1987). Based on similarities of M with D2, and D2 with D1, the corresponding residues of the D1 protein are His-215, Phe-255, and Ser-264. These three residues are close to or coincide exactly with residues that are altered in our herbicide-resistant mutants.

Although several herbicide-resistant mutants have been examined at the level of psbA sequence in higher plants, algae, and cyanobacteria as previously noted, as well as in Euglena (Johanningmeier and Hallick, 1987), no changes in other parts of D1 or in other PSII core polypeptides have been reported in conjunction with herbicide resistance. This raises the possibility that the conformation of the herbicide and/or the QA-binding site is not significantly influenced by other regions of the D1 polypeptide or other peptides. PSII inhibitors, however, can have effects on the donor side of PSII which appear to be mediated through the transmembrane helices (Renger, 1973; Carpentier et al., 1985; Trebst, 1987). It has also been reported that the metribuzin-resistant mutant of C. reinhardtii, which has alanine codon 251 changed to valine, is affected on the donor side of PSII (Johanningmeier et al., 1987).

In this study we have used measurements of chlorophyll fluorescence decay curves to explore the rate of QA reoxidation in seven herbicide-resistant mutants. In some of these mutants, the rate of electron transfer from QA to Qb is strongly reduced, whereas in others it is barely affected.

each folded through the membrane with five transmembrane domains that put the two quinone binding sites in close proximity. DNA sequence analysis of the genes coding for the chloroplast D1 and D2 polypeptides of Chlamydomonas showed that these two proteins are of similar size (352 amino acid residues each), have several highly homologous regions, and have nearly identical hydrophatic profiles (Rochaix et al., 1984). Moreover, a local sequence homology is apparent between the D1, D2, and the L and M subunits of the bacterial reaction center (Rochaix et al., 1984; Youvan et al., 1984; Erickson et al., 1985b; Williams et al., 1986). Based on the above information, as well as the structural and functional homology between the two bacterial and PSII subunits, it has been proposed that D2 and D1 are the apoproteins that bind QA and Qb, respectively, and that these two proteins can be folded with five transmembrane domains each to form a PSII core with chlorophyll, pheophytin, and quinone that resembles the bacterial reaction center (Trebst, 1987).
Figure 5. Model of the *C. reinhardtii* D1 Protein with Five Transmembrane Helices (I to V, Boxed Regions) and a Helix Parallel to the Membrane which Contains Residues 251 to 261 (Boxed), after Trebst (1987).

The amino-terminal end (NH$_2$) is in the stroma, whereas the carboxyl terminus (COOH) is in the lumen. Circles represent amino acid residues, as indicated by the single letter code. Numbers correspond to the amino acid residue. The six residues that are altered in herbicide-resistant mutants as described in the text are marked with dark circles, and are all localized toward the stromal side of the thylakoid membrane. Histidine residues 215 and 272 (circles with dark edges) are involved in iron binding (Fe$^{2+}$). The azido-atrazine binding area between residues 214 and 224 (Wolber et al., 1986) is indicated by a black curving line.

Mutants of the latter group include Dr2, Dr18, Ar207, and Br202 (see Table 2). In these mutants, the fluorescence decay half-time is consistently longer than in wild-type cells, indicating a small decrease in the rate of electron transfer. The consistent difference between the fluorescence decay curves of these mutants and wild-type cells observed at later times, e.g. 1 to 2 msec (Figures 3 and 4) is difficult to interpret. This may be because the measurements in this time frame are affected by properties or components of PSII, such as structural and functional heterogeneities, other than the Q$_A$:Q$_B$ interaction. Our results on Dr2 are at variance with those of Haworth and Steinback (1987), who concluded from fluorescence induction transients that the rate of electron transfer from Q$_A$ to Q$_B$ is strongly reduced in this mutant. We do not have an explanation for this discrepancy, but note that chlorophyll fluorescence decay provides a more direct measurement for rates of electron transfer between the two quinones.

Although Dr2 is moderately resistant to diuron and other PSII inhibitors except for s-triazines, and shows an elevated $I_{50}$ value for diuron inhibitors of the in vitro Hill reaction (Galloway and Mets, 1984), surprisingly, no difference in binding of radioactive diuron to the thylakoids of Dr2 and wild-type cells could be found (Haworth and Steinback, 1987).

Fluorescence decay measurements in the mutants DCMU4, Ar204, and Br24 indicate that the rate of Q$_B$ to Q$_A$ electron transfer is considerably slower in these mutants than in wild-type cells. It has also been shown previously that the decay of fluorescence in chloroplasts from resistant biotypes of *Amaranthus* is more than 10-fold slower than in sensitive strains (Bowes et al., 1980). The mutation in DCMU4 changes serine 264 of the D1 protein to alanine, and the mutant strain is resistant to high levels of atrazine and low levels of diuron. In both Ar204 and Br24, glycine residue 256 is changed to aspartic
acid as the result of a point mutation in psbA. These two mutants are resistant to moderate levels of atrazine and bromacil (Table 2). The metribuzin-resistant mutant M22 has a change of alanine to valine at codon 251, is also resistant to atrazine and diuron, and has a slowed $Q_x$ to $Q_o$ electron transfer (Johanningmeier et al., 1987). Hence, in C. reinhardtii, amino acid substitutions have been found at D1 residues 251, 256, and 264 that significantly reduce the rate of electron transfer on the reducing side of PSII. The drastic effect of the glycine 256 change on electron transfer is understandable since the mutation introduces a negative charge and thereby seriously disrupts the ionic environment of this region. In the model shown in Figure 5, alanine 251, phenylalanine 255, and glycine 256 are part of the surface parallel helix formed between the fourth and fifth transmembrane helices.

Although herbicides that compete with quinone for binding to the thylakoid membrane have been useful probes for exploring structure-function relationships on the reducing side of PSII, studies with herbicide-resistant mutants have obviously been limited to the specific regions of D1 involved in herbicide binding. Because compounds that inhibit electron transfer on the oxidizing side of PSII are often highly toxic to cells (Carpentier et al., 1985), a similar extension of this approach to studying structure-function relationships in other regions of the D1 protein has not been possible. However, a recent report has demonstrated that the chloroplast genome of C. reinhardtii can be transformed with exogenous DNA (Boynton et al., 1988). Using this technology, we have transformed a mutant strain of C. reinhardtii, which lacks the chloroplast gene coding for the D1 polypeptide, with the cloned D1 gene from our Br202 herbicide-resistant mutant and obtained transformants that have the herbicide-resistant phenotype of the original Br202 mutant (J. M. Erickson and M. C. Yee, unpublished results). Boynton et al. (1988) also report the recovery of herbicide-resistant transformants after transformation of the same strain with the cloned psbA gene from our DCMU4 mutant. These results not only confirm that the chloroplast herbicide resistance trait is a direct result of a single amino acid substitution in the D1 polypeptide, but also show that psbA can be successfully transformed into the chloroplast genome of a psbA deletion mutant. Thus it is now possible, using site-directed mutagenesis and subsequent transformation, to extend structure-function analyses of the D1 polypeptide to any region of interest in this important PSII reaction center component.

**METHODS**

**Algal and Bacterial Strains**

Chlamydomonas reinhardtii wild-type strain 1737c(+) and the herbicide-resistant mutant strains were maintained in dim light on Tris-acetate-phosphate (TAP) agar plates (Gorman and Levine, 1965). Br24 and Dr18 were isolated as bromacil- and diuron-resistant mutants, respectively, as described in "Results." The isolation of mutants DCMU4, Ar204, Ar207, Br202, and Dr2 has been previously described (Erickson et al., 1984a; Galloway and Mets, 1984). Escherichia coli strains JM83 or JM101 were used for the propagation of plasmid vectors and recombinant plasmids.

**Isolation of DNA and Cloning of psbA**

Total DNA was prepared from 40-ml TAP cultures of the different C. reinhardtii mutants using a modification of the procedure of Weeks et al. (1986) as previously described (Erickson et al., 1986). The DNAs were double-digested with BamHI and BglII and ligated with DNA from the plasmid pUC19 vector or the Bluescript KS+ vector (Stratagene), after linearization of the vector DNA with BamHI, treatment with calf intestine alkaline phosphatase, and phenol extraction. Calcium-shocked E. coli cells were transformed with the ligated DNA and plated on L agar plates containing 100 μg/ml of ampicillin. Colonies appearing after incubation at 37°C were transferred to nitrocellulose filters (Grinstein and Hoggness, 1975) and hybridized with a $^{32}$P nick-translated DNA fragment from the wild-type psbA gene (gel-isolated 1.3-kb XbaI/EcoRI fragment, Erickson et al., 1984b). Hybridizing colonies were isolated and rapid preparations of plasmid DNA from 1.5-ml cultures were performed by a modification of the Holmes and Quigley (1981) procedure as previously described (Erickson et al., 1984a).

**DNA Sequence Analysis**

The 1.3-kb XbaI-EcoRI and the 1.1-kb HindIII-KpnI fragments of psbA (Erickson et al., 1984b) were isolated by digesting the cloned psbA gene of each mutant with these restriction enzymes and electrophoretically separating the fragments from preparative polyacrylamide gels. After digestion of these fragments with Ddel and Hinfl, as shown in Figure 1, the digested fragments were labeled at either the 3’ or 5’ ends as previously described (Erickson et al., 1984b), strand-separated by electrophoresis on a polyacrylamide gel, and sequenced by the chemical cleavage method of Maxam and Gilbert (1980). Reaction products were fractionated by electrophoresis on 6% and 20% polyaerylamide gels containing 6 μm urea and visualized by autoradiography with Kodak XAR5 x-ray film.

**Fluorescence Measurements**

Kinetic studies of chlorophyll fluorescence decay were performed with a pulse-modulated fluorimeter (Walz Co., Effeltrich, Federal Republic of Germany), which has been described in detail elsewhere (Schreiber, 1986). During the measuring period of 2 msec, the weak noninductive monitoring beam ($\lambda = 660$ nm) was modulated at a frequency of 100 kHz. The common end of the 4-branched fiber optic system was positioned about 1 mm above the surface of agar plates containing the algal strains. The total area analyzed on the plates was 113 mm².

Chlorophyll fluorescence decay curves representing the $Q_x$ reoxidation were recorded following an actinic, saturating xenon flash of 8 μsec width ($T_{1/2}$) obtained from an FX-800 flash tube (EG+G, Salem, MA) operated at an energy of 2 joules. The actinic
light passed through a Calflex Cyanll (Balzers, Liechtenstein) and a BG 28, Schott (Duryea, PA) glass filter before it was coupled into the light guide system. Due to internal gating of the pulse-modulated fluorimeter detector, the first signal point was recorded at 110 \, \mu s after triggering of the actinic flash. The fluorescence signal was stored in a digital oscilloscope (Explorer III, Nicolet, Madison, WI) using 4096 samples at 12-bit resolution and then subsequently transferred on-line to a Hewlett Packard 9920 computer for further analysis. For each decay curve, 10 to 20 transients were averaged by flashing the sample repetitively every 8 sec. After reploting the data in a first-order analysis, a linear regression line was fitted to the first 150 \, \mu s of the data for calculation of the decay half-time.

Fluorescence induction transients were measured in continuous actinic blue light (excitation filter: Corning 4-96) derived from a Schott KL 500 fiberoptic light source. The actinic light passed through a mechanical shutter (opening time, 1 msec) and was directed by fiber optics to the surface of the agar plate. Fluorescence was isolated from scattered actinic light by a Schott RG 665 red glass filter and detected by a UDT 500 photodiode/amplifier combination (United Detector Technology, Hawthorne, CA). Storage and processing of the transients were as described above.

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