Site-Specific Mutagenesis of the D1 Subunit of Photosystem II in Wild-Type Chlamydomonas

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The structure and functional mode of photosystem II reaction center protein D1 can be studied by analyzing the effects of amino acid substitutions within the binding niche for Qa, the second stable electron acceptor of photosystem II, on herbicide binding. Here we report on site-directed mutagenesis of the psbA gene coding for the D1 protein in the unicellular alga Chlamydomonas reinhardtii. The chloroplasts of wild-type cells were transformed using the particle gun. The plasmids introduced carried an in vitro mutated fragment of the psbA gene. We obtained a double mutant with replacements of amino acids 264 and 266 and a triple mutant having an additional substitution in position 259. The sensitivities of both mutants toward several types of herbicides are given and compared with those of a mutant having only a substitution at position 264.

INTRODUCTION

In photosynthesis of cyanobacteria, algae, and higher plants, electrons are transferred from water to plastoquinone by photosystem II. The reaction center core of this pigment-protein complex is formed by the polypeptides D1, D2, and cytochrome b559 in eukaryotes encoded on the chloroplast genome. The first stable acceptor of electrons, QA, is firmly bound to the D2 protein. It transfers one electron at a time to the two-electron carrier QB. QB is bound to the D1 protein and is released once it is fully reduced (for review, see Barber, 1987).

Many herbicides inhibit photosynthesis by preventing electron transfer from QA to QB. They compete with QB for its binding niche at the D1 protein (for review, see Kyle, 1985). Herbicide-tolerant biotypes of higher plants, algae, and cyanobacteria have amino acid substitutions in a special region of the D1 protein (Hirschberg and McIntosh, 1983; Erickson et al., 1984b, 1985; Golden and Haselkorn, 1985). These amino acid substitutions are instrumental for predicting the three-dimensional folding of this polypeptide and especially of the QB binding niche (Trebst, 1986, 1987). The information from further herbicide-tolerant mutants on cross-resistance and their functional properties in electron flow through the plastoquinones refine the understanding of the structure and function of the acceptor side of photosystem II. Recently, more herbicide-tolerant mutants have been isolated and their DNA sequence (reviewed in Trebst, 1990). It is possible in cyanobacteria to design site-directed changes of amino acids of the D1 protein (Debus et al., 1988; Ohad and Hirschberg, 1990). It seemed desirable to have this method also available for eukaryotic organisms because it not only allows one to confirm predictions of the role of certain amino acids in the D1 protein but also to generate mutants with changes of amino acids that one cannot select for directly. The particle gun (Klein et al., 1987) has made it possible to transform chloroplasts of the alga Chlamydomonas reinhardtii (Boynton et al., 1988). We used this method to obtain two new mutants of the D1 protein.

RESULTS

Site-Specific Mutagenesis of Wild-Type C. reinhardtii Chloroplasts

The chloroplast psbA gene of C. reinhardtii is interrupted by four introns. The carboxyl-terminal part of the D1 protein, as of amino acid 253, is encoded in exon 5 (Erickson et al., 1984a). This exon, together with some adjacent sequence, was subcloned into pBSK-., as shown in Figure 1. Changes of codons for asparagine 266 to threonine (AAC to ACC) and isoleucine 259 to serine (ATC to AGC) were introduced in vitro. To allow selection of mutated C. reinhardtii cells, the codon for serine 264 was also changed to alanine (TCT to GCT). This mutation is well known in algae (Erickson et al., 1984b) and cyanobacteria (Golden and Haselkorn, 1985) and confers resistance to several herbicides.

1 To whom correspondence should be addressed.
In Vitro Mutagenesis of psbA of C. reinhardtii.

The EcoRI fragment R24 (Rochaix and Malnoe, 1978) was used to subclone the HindIII-KpnI 1100-bp fragment containing exon 5 of the psbA gene into vector pBS. Single-stranded DNA was prepared from the resulting plasmid pHK1100 and used for site-specific mutagenesis. The wild-type sequence is given, and introduced changes of nucleotides are indicated by arrows. A portion of the amino acid sequence encoded by the DNA of wild-type and mutated constructs is shown. The numbers of replaced amino acids are given above the wild-type sequence. Restriction sites are as follows: E, EcoRI; B, BamHI; H, HindIII; K, KpnI.

The plasmids carrying the mutated clones were used to transform C. reinhardtii wild-type cells. It was possible to select photoautotrophically growing cells on plates containing $10^{-5}$ M metribuzin in the medium; control cells that had been bombarded with plasmids carrying an unmodified exon 5 did not give colonies in the presence of metribuzin. Four colonies each obtained with plasmid pcrD1-264A-266T and plasmid pcrD1-259S-264A-266T were picked for further examination.

Total RNA was isolated from 250-mL suspension cultures. The region of interest was determined by sequencing the psbA mRNA with a psbA-specific oligonucleotide, as described in Methods. This technique gives some background, but in each case it was possible to determine the sequence unambiguously. A comparison of wild-type and mutant sequences is given in Figure 2. All of the strains obtained from transformation with pcrD1-264A-266T had the 266-threonine mutation not selected for in addition to the 264-alanine mutation that was selected for. All of the strains obtained from transformation with pcrD1-259S-264A-266T also contained the 259-serine mutation. Thus, a C. reinhardtii strain with a double mutation and another with a triple mutation in the psbA gene were obtained and have been designated A264T266 and S259A264T266, respectively.

To determine whether the strains were homoplasmic or heteroplasmic, we analyzed total chloroplast DNA. The base pair change in strain S259A264T266 at amino acid 259 not only alters the isoleucine codon to serine but also creates a new HindIII restriction site (AATCTT-AAGCTT, see Figure 1). The wild-type gene 4.0-kb HindIII fragment containing exon 5 is cut in the mutant gene to give a 3.6-kb and a 0.4-kb fragment. Figure 3 shows the expected change in a DNA gel blot hybridization pattern. The 0.4-kb fragment cannot be seen in the autoradiogram because the oligonucleotide we used as probe hybridized downstream of the newly generated restriction site. There was no wild-type signal at 4.0 kb detectable in the DNA isolated from mutant S259A264T266 either when we loaded five times the amount of DNA given in Figure 3 or when the film was overexposed.

Stability of the Mutants

In general, strains A264T266 and S259A264T266 have been maintained under autotrophic conditions on HS agar.
Figure 3. DNA Gel Blot Analysis of Chloroplast DNA from Mutant and Wild-Type Cells.

Chloroplast DNA was purified from wild-type strain and from mutant S259A264T266, and 1 μg of each DNA was digested with HindIII and electrophoresed in an 0.8% agarose gel. The DNA was transferred to a nylon membrane and probed with a 32P-labeled oligonucleotide hybridizing to a part of the exon 5 sequence downstream from the mutagenized site. In the wild-type psbA gene, exon 5 is part of a HindIII restriction fragment about 4 kb in size (lane 1). In the mutant gene, the fragment is cut at a new HindIII site to produce a fragment 0.4 kb smaller (lane 2). The autoradiogram is shown. Migration of BstEII-digested λ DNA size marker is indicated in the left margin.

containing 10^-5 M metribuzin. The cultures were kept in parallel without any selection either on HS plates or in suspension by diluting them about 10-fold every week for 6 months. Even under these conditions, the transformants proved to be stable and maintained their ability to grow on metribuzin.

Herbicide Cross-Resistance

The photosynthetic rates [H2O to 2,6-dichlorophenol-indophenol (DCPIP)] in thylakoids isolated from mutant cells were similar to those measured in thylakoids isolated from the wild-type cells. Rates varied between 80 μmol and 130 μmol of DCPIP per milligram of chlorophyll/hr. The p50 values were determined for several types of herbicides in isolated thylakoids. The mutants were compared with each other and to mutant MZ1 (Pucheu et al., 1984), which has a single substitution of serine 264 to alanine (Johanningmeier et al., 1988). MZ1 is descended from wild-type 2137 mt+. The R/S values summarized in Table 1 show the relative sensitivity of mutants versus their respective wild-type strains. The values of several of the originally picked colonies of each mutant have been determined separately. They did not differ from each other by more than 10% for any of the tested inhibitors; the average values are given in Table 1.

For metribuzin, atrazine, and urea derivatives, the replacement of asparagine 266 by threonine in addition to the mutation 264-alanine led to a slightly increased sensitivity, i.e., to a partial reversal of the tolerance induced by the substitution of 264 alone, which was 5000-fold, 160-fold, and 50-fold to 80-fold more resistant to metribuzin, atrazine, and urea derivatives, respectively. The OH group introduced at position 259 led to an additional increase of sensitivity toward both urea derivatives and atrazine.

On the other hand, the resistance was increased in the double mutant A264T266 for two other of the tested substances, phenmedipham (fivefold) and the cyanoacrylate (fourfold). For cyanoacrylate, tolerance was reduced in the triple mutant. The 264 serine-to-alanine mutation did not lead to tolerance for phenolic herbicides, and there was even a small increase in sensitivity (Janatkova and Wildner, 1982). The additional mutations at amino acids 259 and 266 gave only minor changes; the mutant A264T266 was 3.2 times more resistant to the hydroxyquinoline tested and 1.6 times more resistant to ioxynil as compared with the mutant MZ1. In the triple mutant, the sensitivity was increased (sixfold for ioxynil compared with the double mutant).

DISCUSSION

One approach for gaining information to better understand primary photosynthetic processes is to change amino acids of reaction center proteins. This kind of manipulation has been restricted to photosynthetic bacteria and cyanobacteria, and it has not been possible to transform chloroplasts of algae or higher plants.

The particle gun invented by Sanford (Klein et al., 1987) now allows the delivery of DNA into the chloroplast of C. reinhardtii cells (Boynton et al., 1988; Blowers et al., 1989). In most cases, deletion mutants are used for this kind of experiment. The complementation of the missing gene by the introduced gene allows easy selection of transformants. However, there is a disadvantage to using a deletion mutant in the case of the psbA gene. In C. reinhardtii, this gene contains several introns and spans a region of about 7 kb (Erickson et al., 1984a). The deletion mutant (FuD7) available is missing exon 2 to 5, a total of 8 kb (Bennoun et al., 1986). Mutagenesis of this large piece of DNA poses several problems. The mutant oligonucleotide might hybridize to part of the sequence not supposed to change (D. Naber and S. Heiss, unpublished data), or several additional cloning steps would be needed. It would be difficult to detect unwanted changes of the gene because of its large size. In addition, the complementation of FuD7 by the wild-type gene might lead to heteroplasmicity.

Therefore, it seemed worthwhile to take another approach to circumvent these problems. Instead of using a deletion mutant, we transformed C. reinhardtii wild-type
Table 1. Relative Resistance (R/S) of Mutants to Various Herbicides

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>264 Ser-Ala</th>
<th>264 Asn-Thr</th>
<th>259 Ile-Ser</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metribuzin</td>
<td>5000</td>
<td>1580</td>
<td>1550</td>
</tr>
<tr>
<td>Metamitron</td>
<td>30</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Atrazine</td>
<td>160</td>
<td>40</td>
<td>20</td>
</tr>
<tr>
<td>DCMU</td>
<td>200</td>
<td>80</td>
<td>13</td>
</tr>
<tr>
<td>Benzthiazuron</td>
<td>80</td>
<td>50</td>
<td>20</td>
</tr>
<tr>
<td>Phenmedipham</td>
<td>200</td>
<td>1000</td>
<td>1260</td>
</tr>
<tr>
<td>Cyanacrylate</td>
<td>30</td>
<td>130</td>
<td>30</td>
</tr>
<tr>
<td>Hydroxyquinoline</td>
<td>0.5</td>
<td>1.6</td>
<td>0.6</td>
</tr>
<tr>
<td>Ioxynil</td>
<td>0.5</td>
<td>0.8</td>
<td>0.13</td>
</tr>
<tr>
<td>Ketonitirile</td>
<td>0.6</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td>Bromonitrothylurea</td>
<td>0.4</td>
<td>0.16</td>
<td>0.1</td>
</tr>
</tbody>
</table>

The pI50 values were determined in isolated thylakoids as the inhibitor concentration which blocks half of the Hill activity. R/S is the ratio of the pI50 of the mutant to that of the wild type from which it descends. Cyanoacrylate, 2-cyano-3-ethyl-3-(4-chlorbenzyl)-aminoacrylate (Phillips and Huppatz, 1984); hydroxyquinoline, 3-bromo-2,6-di-trifluoromethylhydroxyquinoline (Trebst et al., 1989); ketonitirile, 2-phenylthiazolyC3-hydroxy-4-phenyl-butenonitrile (Buehmann et al., 1987); DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

Figure 3. The A264T266 double mutant described here reversed the tolerance toward metribuzin, atrazine, and urea derivatives obtained in the mutant MZ1, which has only the substitution of amino acid 264. Four phenolic herbicides were tested, two of which inhibited electron flow more and two less than they do in MZ1. This result seems to conflict with the results of Astier and coworkers (Ajlani et al., 1989a) because their mutant is 10-fold more resistant to the phenol-type herbicide ioxynil than its wild-type and sevenfold more resistant to metribuzin. It is not possible to decide whether the different effects observed are due to a different structure of the herbicide binding niche of C. reinhardtii and Synechocystis or whether the additional change in position 264 contributes to this result. A single mutation at position 266 in C. reinhardtii is being prepared to find out whether the amino acids at position 264 and 266 interact in binding of herbicides or whether they contribute independently to herbicide binding (Horovitz et al., 1989).

Until now, a mutant of amino acid 259 has not been described. We substituted isoleucine with serine. Because of this additional OH group, the sensitivity against some of the inhibitors was increased up to sixfold for DCMU and ioxynil. For other compounds, no change in sensitivity was observed. The properties of herbicide binding on the D1 protein can be translated directly into the properties and the dimensions of the Qo binding site. However, more data, especially from different mutants, are necessary to obtain a final picture of this site. The approach we have described here will allow us to produce more site-directed mutants of the D1 protein in a eukaryotic system.

METHODS

Algal and Bacterial Strains

Chlamydomonas reinhardtii wild-type strain 11/32 b (Sammlung von Algenkulturen, Göttingen, Federal Republic of Germany) was maintained in dim light on HSA agar (Sueoka, 1960). Escherichia coli strains JM101 and TG1 (Amersham, oligonucleotide-directed in vitro mutagenesis system) were used for propagation of plasmid vectors.

Plasmids and in Vitro Mutagenesis

Plasmid pcp58 containing C. reinhardtii chloroplast DNA EcoRI fragment R24 was obtained from Dr. Jean-David Rochaix. After double digestion, the 3.6-kb EcoRI-BamHI fragment was isolated and inserted into cloning vector pBSsK+ (Stratagene). The resulting plasmid was cut with HindIII and religated. Cutting with Kpnl and insertion of the resulting 1.1-kb fragment into the Kpnl site of vector pBSsK+ gave pHK1100. This plasmid contains exon 5 of the C. reinhardtii psbA gene and about 450 bp upstream and 250 bp downstream from it.
Plasmids pcdD1-264A-266T and pcdD1-259S-264A-266T were produced by site-directed mutagenesis of pHK1100 using the method of Nakamaye and Eckstein (1986). All system components were purchased from Amersham and used according to their recommendations.

For single-stranded DNA preparation, the Bluescript system (Stratagene) was used instead of the M13 phage. Three micrograms of single-stranded DNA were annealed with 5 pmol of phosphorylated oligonucleotide designed to replace the codon for serine 264 with alanine and the codon for asparagine 266 with threonine. After the in vitro mutagenesis reaction plasmids were transferred into E. coli strain TG1, plasmid clones were screened by dieoxy sequencing. As primer, an oligonucleotide (opsbA9) was used that comprised nucleotides 874 to 897 of the C. reinhardtii psbA gene (Erickson et al., 1984a). At month that no other nucleotides within exon 5 were changed during the reaction, the selected clones were also sequenced using a primer corresponding to nucleotides 1053 to 1087.

In one clone, we found that an additional nucleotide substitution had occurred close to the 3' end of our mutant oligonucleotide. It changed the codon for isoleucine 259 to serine.

Transformation of C. reinhardtii Chloroplasts

C. reinhardtii wild-type cells were grown at 25°C in liquid HSHA medium (Sueoka, 1960) in light (7000 lux), harvested at mid-log phase, and resuspended in HSHA media, and aliquots containing 1 to 2 x 10^7 cells were spread onto HSHA media in 9 x 50 mm Petri dishes. Cells were kept in darkness overnight and transformed by bombarding them with tungsten microprojectiles coated with DNA (Klein et al., 1987; Boynton et al., 1988).

After incubation for 18 hr to 24 hr in the dark, cells were transferred to 82-mm Petri dishes of HS liquid medium (Sueoka, 1960) containing metribuzin (10^-5 M, Pucheu et al., 1984) and incubated at 25°C in dim light (300 lux). Metribuzin-resistant colonies appeared after 3 weeks to 4 weeks. They were maintained on HS plates and in HS liquid medium with and in parallel without further selection.

RNA Isolation, Preparation of cDNA, and Sequencing

C. reinhardtii cultures were grown in 250 mL of HSHA medium and harvested at end-log phase, and total RNA was prepared essentially as described by Johannimier et al. (1987). 5'-32P-labeled oligonucleotide (7 x 10^6 cpmm) opsbaA9 was annealed to approximately 70 µg of RNA. Reverse transcriptase (Promega Biotec) was used to produce cDNA fragments in the presence of dieoxyxynucleotide triphosphates (Hamly et al., 1976; Zimmern and Kaesberg, 1978; Johannimier et al., 1987). Samples were separated on 8% polyacrylamide-urea gels.

Preparation of Chloroplast DNA and DNA Gel Blot Analysis

C. reinhardtii cells were grown in 5 L of HSHA medium and harvested at mid-log phase, and their chloroplast DNA was isolated in sodium iodide gradients according to the procedure described by Harris (1986). One microgram of wild-type and mutant S259A264T266 DNA each was digested with the restriction enzyme HindIII and electrophoresed on an 0.8% agarose gel. DNA was transferred to GeneScreen nylon filters (DuPont-New England Nuclear). Filters were probed with the 5'-32P-labeled oligonucleotide opsba3, comprising nucleotides 844 to 859 of the C. reinhardtii psbA gene. Prehybridization and hybridization were performed at 31°C in 5 x SSC, 5 x Denhardt's solution, 0.1% SDS, and 100 µg/mL herring sperm DNA. For hybridization, labeled oligonucleotides were added to the buffer at a concentration of 0.5 x 10^6 cpmm/mL. Filters were washed in 4 x SSC at room temperature and exposed to Kodak XAR-5 film with two intensifying screens at -80°C for 2 days.

Measurements of Herbicide Cross-Resistance

End-log phase cultures (250 mL of HSHA medium) were harvested and cells were broken by Yedapress (Scientific Instruments, Rehovot, Israel) treatment in 20 mM Hepes buffer, pH 7.5; with 0.3 M sorbitol, 5 mM MgCl2, 1 mM MnCl2, and 2 mM KCl at a concentration of 0.3 mg of chlorophyll/mL. Thylakoids were pelleted, resuspended in the above-mentioned buffer at 1 mg of chlorophyll/mL, and preincubated with the inhibitors for 5 min in the dark. Photosynthetic electron transport was measured as DCPIP reduction with H2O as electron donor. The Pm value, i.e., the concentration of herbicide inhibiting the rate of DCPIP reduction by 50%, was determined; the chlorophyll concentration was kept equal in all assays (Pucheu et al., 1984).

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