Photosynthetic Water Oxidation in Cytochrome b_{559} Mutants Containing a Disrupted Heme-binding Pocket*

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The role of cytochrome b_{559} in photosynthetic oxygen evolution has been investigated in three chloroplast mutants of Chlamydomonas reinhardtii, in which one of the two histidine axial ligands to the heme, provided by the α subunit, has been replaced by the residues methionine, tyrosine, and glutamine. Photosystem two complexes functional for oxygen evolution could be assembled in the methionine and tyrosine mutants up to ~15% of wild type levels, whereas no complexes with oxygen evolution activity could be detected in the glutamine mutant. PSII supercomplexes isolated from the tyrosine and methionine mutants were as active as wild type in terms of light-saturated rates of oxygen evolution but in contrast to wild type contained no bound heme despite the presence of the α subunit. Oxygen evolution in the tyrosine and methionine mutants was, however, more sensitive to photoinactivation than the WT. Overall, these data establish unambiguously that a redox role for the heme of cytochrome b_{559} is not required for photosynthetic oxygen evolution. Instead, our data provide new evidence of a role for cytochrome b_{559} in the protection of the photosystem two complex in vivo.

Photosystem two (PSII) is a light-driven water-plastoquinone oxidoreductase located within the thylakoid membrane of oxygenic photosynthetic organisms (1, 2). The study of this complex is important not only because of the unique chemistry involved in the photosynthetic oxidation of water to dioxygen but also because PSII has been identified as a weak link in the photosynthetic electron transport chain (3, 4). PSII, and in particular the D1 subunit, is prone to irreversible light-induced damage, sometimes termed photoinhibition, which, unless repaired, causes a reduction in net rates of photosynthesis (5).

Cytochrome b_{559} (Cyt b_{559}) is a ubiquitous component of PSII and is found in the most minimal PSII complex capable of light-induced charge separation, the so-called D1/D2 complex described by Nanba and Satoh (6). It is composed of two small hydrophobic polypeptides, termed α (PsbE) and β (PsbF), each of which spans the membrane once. Unlike other cytochromes, Cyt b_{559} possesses a marked heterogeneity in the midpoint redox potential of its heme cofactor, with high, low, and intermediate potential forms described (7). Recent structural data obtained from cyanobacterial PSII have confirmed an αβ heterodimeric structure (8) with the heme molecule ligated by single His residues in each of the subunits, as predicted earlier from EPR spectroscopy (9). Whether one or two Cyt b_{559} are present per PSII in vivo is under debate, although a variety of recent evidence suggests just one (7).

Despite recent advances in understanding the structure and biochemistry of PSII, the role of Cyt b_{559} remains unclear. Suggested functions within PSII include a redox role in photosynthetic water oxidation (10), an involvement in the early steps of assembly of PSII (11, 12), and the protection of PSII from photoinactivation both before (13, 14) and after the assembly of the manganese cluster (15–17). Enzymatic activities such as a plastoquinol oxidase (18) and a superoxide dismutase (19) have also been proposed.

The analysis of Cyt b_{559} with regard to PSII function, particularly in vivo, has been hindered, however, by the lack of appropriate Cyt b_{559} mutants that still accumulate PSII. For example, previous attempts to manipulate the heme-binding pocket of the cytochrome through mutation of one or both of the His axial ligands to Leu in the cyanobacterium, Synechocystis 6803, led to the loss of PSII from the membrane (20).

Here we describe the characterization of Cyt b_{559} mutants created in the green alga Chlamydomonas reinhardtii, a widely used eukaryotic model to study photosynthesis (21). We have focused our studies on one of the two His ligands to the heme, residue His^{200} of the α subunit, encoded by the psbE gene. Based on the assumption that the binding of heme to Cyt b_{559} is important for assembly of PSII (20), we included in our choice of mutants residues that could still coordinate the heme (such as Met and Tyr) but may perturb its redox properties and possibly affect PSII function.

To aid our characterization of these mutants, we have developed a procedure for the isolation of oxygen-evolving PSII-LHCII supercomplexes from wild type (WT) C. reinhardtii. This type of complex, recently isolated from a PSI-deficient strain of C. reinhardtii and characterized by electron microscopy (22), consists of a dimer of PSII core complexes, surrounded by chlorophyll-binding subunits, that retains the extrinsic subunits associated with the water-oxidizing complex (PsO, PsP, and PsQ) and is highly active in oxygen evolution (23). The results presented here demonstrate unambigu-

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¶ The abbreviations used are: PSI and PSII, photosystem one and two, respectively; Cyt b_{559}, cytochrome b_{559}; WT, wild type; kb, kilobase pair(s); PCR, polymerase chain reaction; µE, microeinsteins; Mes, 4-morpholinoethanesulfonic acid; DM, dodecylmaltoside; Chl, chlorophyll; LHC, light-harvesting complex.
ously that Cyt b559 heme is not required for photosynthetic oxygen evolution and that mutation of the ε subunit His ligand leads to enhanced sensitivity of PSII activity to light stress.

**EXPERIMENTAL PROCEDURES**

**Strains and Growth Conditions—** *C. reinhardtii* strain CC-125 was used as the wild type strain and was obtained from the Chlamydomonas Genetics Center (Department of Botany, Duke University, Durham, NC). Cells were grown in Tris-acetate phosphate (TAP) medium or high salt minimal medium as described in Ref. 11. Strains obtained after chloroplast transformation were kept on TAP medium supplemented with 100 μg/ml spectinomycin, 50 μg/ml ampicillin, and 10 μM 3-(3,4-dichlorophenyl)-1,1-dimethyl (TAPAS D plates). 3-(3,4-Dichlorophenyl)-1,1-dimethyl was included to prevent a selection pressure for photoautotrophy.

**Recombinant Plasmin and in Vitro Mutagenesis—** Plasmid pF3 contained the psbE gene with upstream and downstream 3′-flanking sequences of 1.75 and 1.97 kb, respectively (11). To select for transformed *C. reinhardtii* cells, the aadA-selectable marker (obtained as a 1.9-kb EcoRI/SmaI fragment from plasmid pUC-aptX-A (24), which conveys resistance to spectinomycin and streptomycin, was inserted into an EcoRI restriction site of plasmid pF3, located 323 base pairs upstream of the psbE translation initiation codon. Oligonucleotide-mediated mutagenesis of the psbE gene was performed using a modified PCR-based polymerase chain reaction (PCR)—“megaprimers” approach (25, 26). Synthetic mutagenic oligonucleotides (Amersham Pharmacia Biotech) 5′-ACCGTAATCTCAATAAACCCAG-3′, 5′-ACGGTAATCATTTGGAATTACACCCAG-3′, and 5′-ACCGTTACTATATAAATACCCAG-3′ were used to create mutants H23M, H23Q, and H23Y, respectively. These primers were designed to delete a restriction site for the enzyme AflII and to create a novel KpnI site (underlined), along with the His3 mutation (Fig. 1A). The PCRs were performed in a PerkinElmer Life Sciences thermocycler using essentially the method described in Ref. 27 except that Pfu DNA polymerase (Stratagene) was used. The final weight fraction of about 1.3 kb was digested with SacI and AclI and ligated to SacI/AclI-restricted pF3aad to give the transforming plasmids. The sequence of the cloned insert was determined using an ABI/PerkinElmer 317 automated sequencer to confirm the absence of unwanted mutations.

**Transformation of C. reinhardtii—** Plasmid DNA precipitated onto gold particles (1.0-μm diameter) was introduced into the chloroplast genome of *C. reinhardtii* CC-125 using a biolistic method, employing a Bio-Rad Biolistic PDS-1000/He system (28). Transformants were selected on TAP medium supplemented with 100 μg/ml spectinomycin, 50 μg/ml ampicillin, and 10 μM 3-(3,4-dichlorophenyl)-1,1-dimethyl (TAPAS D plates). 3-(3,4-Dichlorophenyl)-1,1-dimethyl was included to prevent a selection pressure for photoautotrophy.

**Preparation of PSI-LHCII Supercomplexes—** PSI-LHCII supercomplexes of *C. reinhardtii* were prepared employing a method developed originally for spinach (23). Discontinuous gradients of 34 ml from 5.5-20% (w/v) sucrose were poured in 2-ml steps of 0.9% (w/v) sucrose increments. The gradients were prepared on ice from freshly made stock solutions of 5 and 20% (w/v) sucrose in buffer MMNB1 (25 mM Mes/KOH, pH 5.7, 5 mM MgCl2, 10 mM NaCl, 2 mM betaine) containing 0.035% (v/v) deionized water (H2O). *C. reinhardtii* thylakoid membranes were brought to a final volume of 1.5 ml containing 0.5 mg/ml chlorophyll and 25 mM DM in buffer MMNB2 and quickly solubilized by pipetting five times with a micropipette. Solubilization of thylakoids from mutant strains was carried out at 35 mM DM (H23M) and 30 mM DM (H23Y). Freshly prepared sucrose gradients were overlaid with the solubilized thylakoids; a volume of 0.7 ml containing a total amount of 0.35 mg of chlorophyll was loaded per gradient. The gradients were centrifuged at 131,000 × g for 13–15 h at 4 °C in a Beckman SW-28 swing-out rotor. Brakes were switched off at 24 × g during deceleration at the end of the centrifuge run. The gradients showed three major chlorophyll-containing bands, the lower of which corresponded to the PSI-LHCII supercomplex, the middle and upper bands contained the monomeric forms of PSI and LHCII, respectively. The fractions were concentrated by centrifugation in Centricon YM-100 centrifugal filter devices (Amicon) at 1500 × g. Samples were flash frozen in liquid nitrogen and stored at −80 °C. Chlorophyll was quantified according to Ref. 31, and absorption spectra were recorded at room temperature using a Shimadzu MPS-2000 spectrophotometer.

**Gel Electrophoresis and Immunodetection—** The protein composition of thylakoids and thylakoid membrane fractions was analyzed on 10% polyacrylamide gels, containing 0.7 M urea, using the Tricine-SDS-PAGE system described in Ref. 33. Prestained Low Molecular Weight Standards (Bio-Rad) were run alongside samples. Protein bands were visualized by staining gels with 0.1% (w/v) Coomassie Brilliant Blue R-250. Immunoblotting was performed as described in Ref. 11 using enhanced chemiluminescence.

**Assay of Cyt b559 and Heme Content in PSI Supercomplex Preparations—** Redox difference spectra were recorded as dithionite-reduced minus ferricyanide-oxidized spectra within the 520–600-nm region of the spectrum. PSI-LHCII supercomplex samples at 20 μg of Chl/ml in buffer MMNB1 containing 0.03% (w/v) DM were oxidized by adding 1 mM ferricyanide, and the spectrum was recorded and stored as a base line. A few grams of Na2SO4 were then added, and the reduced spectrum was recorded to ensure complete reduction of Cyt b559 more Na2SO4 was added, and another spectrum was recorded. A redox difference extinction coefficient of 2.34 mM−1 cm−1 at 559 nm was used to determine the heme concentration (37).

**Heme** was assayed as the reduced pyridine heme using the method described for samples with low heme concentrations by Berry and Trumpower (34). 0.5 ml of assay solution (200 mM NaOH, 40% (v/v) pyridine), 3 μl of 1 M K3Fe(CN)6, and 0.5 ml of PSI-LHCII supercomplex sample containing 20 μg of Chl in buffer MMNB1, 0.03% (w/v) DM were thoroughly mixed in a 1-ml cuvette. The oxidized spectrum was recorded and stored as the base line. A few grams of Na2SO4 were then added, and the spectrum of the reduced pyridine heme chromophore was recorded immediately before effects of dithionite on the base line were accumulated. Several successive spectra were measured to ensure that the conversion to the pyridine heme chromophore had been complete. The dual wavelength difference coefficient ε559-s-540 = 23.98 mM−1 cm−1 was used in Ref. 34 was used to determine the heme concentration.

**EPR Spectroscopy—** Cryogenic EPR spectra were collected on a Varian E-line EPR spectrometer equipped with an Oxford Instruments ESR 900 liquid helium cryostat. Spectrometer conditions were as follows: microwave frequency, 9.28 GHz; microwave power, 0.03 milliwatts (Chl/Car), 2 milliwatts (Cyt b559, Qa), or 5 milliwatts (S2 multiline); magnetic field modulation amplitude, 4 G (Chl/Car) or 20 G (Cyt b559, Qa, S2 multiline); temperature, 30.0 K (Chl/Car), 10.0 K (Cyt b559, Qa), or 6.0 K (S2 multiline). The Chl/Car and Cyt b559 signals were observed at a magnetic field of 3400 G. With these settings, the EPR spectra obtained for the PSI-LHCII supercomplex sample contained the two primary features previously reported by Mattig et al. (36). Trapping and subsequent reduction of the PSI-LHCII supercomplex sample using dithionite produced a broad spectrum with a broad absorption at 3400 G.

**Photoinhibition Measurements—** *C. reinhardtii* cells grown with air
The use of Procedures developed by Boynton and co-workers (35) (see "Experimental Procedures") to generate mutant psbE is monocistronic and is not part of a psbEFLJ operon (36). To select for transformants, a spectinomycin resistance cassette was inserted upstream of the psbE gene (Fig. 1A). After bombardment of wild type cells with a plasmid carrying a mutant psbE gene linked to the spectinomycin resistance cassette, the site-directed mutation was incorporated into the psbE gene on the chloroplast genome through homologous recombination. The WT control strain in these experiments was set to generate mutant psbE using primers flanking the psbE gene (data not shown). The psbE gene in the transformants was sequenced to verify that the engineered base changes were present in the chloroplast genome of the mutants (data not shown). DNA sequencing also confirmed that no other spontaneous mutations were incorporated into the psbE gene during construction of the mutants.

**RESULTS**

**Construction of PsbE His<sup>23</sup> Site-directed Mutants**—The mutants H23M, H23Q, and H23Y of the α subunit of Cyt b<sub>559</sub> were constructed in C. reinhardtii using the biolistic technique developed by Boynton and co-workers (35) (see "Experimental Procedures"). The use of C. reinhardtii rather than cyanobacteria to generate psbE mutants is simplified by the fact that the psbE is monocistronic and is not part of a psbEFLJ operon (36). To select for transformants, a spectinomycin resistance cassette was inserted upstream of the psbE gene (Fig. 1A). After bombardment of wild type cells with a plasmid carrying a mutant psbE gene linked to the spectinomycin resistance cassette, the site-directed mutation was incorporated into the psbE gene on the chloroplast genome through homologous recombination. The WT control strain in these experiments was designated PF3aad#1 and contains the spectinomycin-resistance cassette upstream of a wild type copy of psbE. (Fig. 1B, which shows a DNA gel blot of total DNA isolated from the mutants H23M, H23Q, and H23Y, the wild type control strain PF3aad#1, and the wild type, confirms that the transformants were homoplasmic so that all copies of the chloroplast genome contained the engineered mutations. The psbE-specific probe hybridized to a 1.2-kb HindIII/KpnI fragment in the WT but to a 0.8-kb HindIII/KpnI fragment in PF3aad#1 and unresolved 0.40- and 0.41-kb fragments in the His<sup>23</sup> mutants due to insertion of a new KpnI site (Fig. 1A). Homoplasmicity of the mutants was further confirmed by PCR analysis using primers flanking the psbE gene (data not shown). The psbE gene in the transformants was sequenced to verify that the engineered base changes were present in the chloroplast genome of the mutants (data not shown). DNA sequencing also confirmed that no other spontaneous mutations were incorporated into the psbE gene during construction of the mutants.

**TABLE I**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Growth properties and PSII activities in cells from the wild type and mutant strains</th>
<th>Hours per doubling</th>
<th>$F_v/F_m$</th>
<th>Oxygen evolution&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Autotrophic&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Mixotrophic&lt;sup&gt;b&lt;/sup&gt;</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>Wild type (CC-125)</td>
<td>57</td>
<td>10</td>
<td>0.71</td>
<td>100</td>
</tr>
<tr>
<td>PF3aad#1</td>
<td>59</td>
<td>10</td>
<td>0.74</td>
<td>100</td>
</tr>
<tr>
<td>H23M</td>
<td>No growth</td>
<td>15</td>
<td>0.30</td>
<td>5–15</td>
</tr>
<tr>
<td>H23Q</td>
<td>No growth</td>
<td>13</td>
<td>0.43</td>
<td>5–15</td>
</tr>
<tr>
<td>H23Y</td>
<td>No growth</td>
<td>15</td>
<td>0.08</td>
<td>Not detected</td>
</tr>
</tbody>
</table>

<sup>a</sup> In HSM medium.

<sup>b</sup> In TAP medium.

<sup>c</sup> $F_v$ is variable chlorophyll fluorescence, and $F_m$ is maximum chlorophyll fluorescence.

<sup>d</sup> Light-saturated rates of oxygen evolution in the presence of DCBQ/ferricyanide. Value for WT (CC-125) was ~120 μmol of O<sub>2</sub> · mg chlorophyll<sup>−1</sup> · h<sup>−1</sup>.

bubbling and stirring at an incident light intensity of 20–50 μEm<sup>−2</sup>s<sup>−1</sup> in TAP medium until their mid to late exponential phase (OD<sub>500</sub> of 0.6–0.8) were placed in flat glass dishes at 25 °C, stirred, and subjected to heat-filtered low light illumination of 100 μEm<sup>−2</sup>s<sup>−1</sup> or high light illumination of 1000 μEm<sup>−2</sup>s<sup>−1</sup> (provided by an apparatus equipped with a 1-kilowatt halogen lamp). Lincomycin (cell culture-tested; Sigma) at 100 μg/ml was then added to some of the samples to inhibit chloroplast protein synthesis. 8-ml samples were taken at set intervals during a time course of 4–5 h, and oxygen evolution was measured.
immunoblotting confirmed the presence of D1 and D2 in light-grown cultures of the H23M and H23Y strains at about 10–25% the levels found in the WT strains (data not shown). The levels of PsbE were also reduced in these mutants to 10–25% of WT (data not shown). Together, these results indicate that the heme ligand, His<sup>23</sup>, is not absolutely required for assembly of functional PSII complexes, although its replacement does reduce the accumulation of PSII within the membrane. For the H23Q mutant, no oxygen evolution was detected, and the steady-state levels of D1 and D2 were 1–10% of WT levels (data not shown). PsbE could not be detected immunologically in mutant (less than 10% of WT levels). To investigate the possibility that the reduced level of PSII in the His<sup>23</sup> mutants was due to the effect of the light on accumulation of PSII, cultures grown in the dark were also examined, and similar results to those grown in the light, described above, were obtained (data not shown).

**Isolation of a PSII Supercomplex from WT C. reinhardtii**—Preliminary attempts to characterize Cyt<sub>b</sub> and possibility that the reduced level of PSII in the His<sup>23</sup> mutants was due to the effect of the light on accumulation of PSII, cultures grown in the dark were also examined, and similar results to those grown in the light, described above, were obtained (data not shown).

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**Solubilization of WT C. reinhardtii thylakoid membranes** followed by sucrose density gradient centrifugation allowed the partial resolution of three pigment bands similar to the results obtained for spinach (23) (Fig. 2A). On the basis of absorbance spectra (Fig. 2B), SDS-PAGE, and immunoblotting, the lowest band (of greatest molecular mass) could be assigned to a PSII-LHCII supercomplex, the middle band to a PSI-LHCII supercomplex (PSI), and the upper band to LHCII. The C. reinhardtii PSI supercomplex gave a similar room temperature absorbance spectrum to the spinach PSII supercomplex with a red peak at 676 nm (Fig. 2B) and also contained a similar Chl a/b ratio of 3.1:1 (23). Further work is required to confirm whether the PSII supercomplex isolated here is structurally analogous to previous described supercomplexes (22, 23). SDS-PAGE and immunoblotting experiments using specific antisera allowed many of the protein components to be identified in this preparation (Fig. 2C). The level of contamination by PSI in this preparation was estimated by quantitative immunoblotting, using an anti-PsaA serum, to be −10% on a chlorophyll basis. The rate of oxygen evolution from the PSI supercomplex using 2,6-dichloro-p-benzoquinone and ferricyanide as electron acceptors was −1090 ± 30 μmol of O<sub>2</sub>/mg of Chl<sup>−1</sup>h<sup>−1</sup>.

**Oxygen-evolving PSII Supercomplexes Isolated from the H23M and H23Y Mutants Do Not Contain Heme**—PSII supercomplexes could be isolated from the H23Y and H23M mutants, with yields consistent with the reduced amount of oxygen evolution activity and levels of D1/D2 subunits in whole cells of these strains. Absorbance spectra are shown in Fig. 3A. The oxygen-evolution rates for both types of preparation were determined to be −740 ± 40 μmol of O<sub>2</sub>/mg Chl<sup>−1</sup>h<sup>−1</sup>. Because these preparations were contaminated by more PSI than the WT sample (estimated in PsaA blots to be at about 30% on a chlorophyll basis for the mutants compared with 10% for the WT control), the oxygen-evolving activity per mutant PSII center was estimated to be similar to the WT. Increased contamination by the PSI-LHCII supercomplex was also detected by SDS-PAGE (data not shown). Immunoblots confirmed the presence of the extrinsic proteins PsbO, PsbP, and PsbQ in PSI supercomplexes isolated from PF3aad#1, H23Y, and H23M as well as the presence of the PsbE subunit (Fig. 4) and, as expected, the D1, D2, CP47, and CP43 subunits (data not shown).

For the WT PSII supercomplexes, a dithionite-reduced minus ferricyanide-oxidized optical difference spectrum revealed a peak at 559 nm, consistent with the presence of Cyt<sub>b559</sub> (Fig. 3B). This value together with a full width at half-maximum of 11–12 nm indicated that no other cytochromes were present in the preparation. In contrast, no such signal was observed for the preparations isolated from the H23Y and H23M mutants (Fig. 3B). Similar results were obtained using a pyridine hemochrome assay to detect heme in the PSI samples. WT samples gave an absorbance band with λ<sub>max</sub> = 556 nm as expected (34), whereas no signals were observed with the mutants (Fig. 3C). After taking into account the level of contamination by PSI, the Chl/Cyt<sub>b559</sub> ratio in the WT preparation (obtained using both types of assay for the cytochrome) was estimated to be about 130–150.

**Characterization of the Mutant PSII Complexes by EPR Spectroscopy**—EPR spectroscopy was also used to confirm the absence of heme in supercomplexes isolated from the H23Y mutant. The EPR spectra of dark-adapted and 77 K-illuminated
PSII supercomplexes from the WT control clearly showed the $g_0$ and $g_2$ turning points of the Cyt $b_{559}$ signal (Fig. 5A) and showed that Cyt $b_{559}$ was already fully oxidized in the dark. On the other hand, no dark-oxidized or 77 K-photooxidizable heme was detected in the H23Y supercomplex (Fig. 5A). Nor were any new EPR signals observed in the dark spectrum or the light minus dark difference spectrum recorded for H23Y, indicating that no altered photooxidizable form of Cyt $b_{559}$ is functioning in the H23Y mutant.

Upon illumination at 77 K, under conditions when the manganese cluster can no longer be oxidized (15), EPR spectra showed the photoaccumulation of Car$^+/$/Chl$^2_2$ (not shown) as well as the reduced electron acceptor QA$^+/$/Chl$^2_2$ (broad negative signal at $\sim3800$ G in Fig. 5A) for both PF3aad#1 and H23Y. These spectra indicate that the usual electron transfer reactions are functioning similarly in both heme-deficient and WT PSII-LHCII supercomplexes and indicate that the secondary electron donors are active in both supercomplexes. Both PF3aad#1 and H23Y supercomplexes displayed a normal S2 state multiline EPR signal from the tetramanganese cluster generated by illumination at 200 K (Fig. 5B), indicating that the tetramanganese cluster was not influenced by the mutation and further supporting the conclusion that both PSII preparations possess a highly intact and active OEC. Thus, the initial electron transfer reactions associated with water oxidation in PSII appear to be independent of the presence of a redox-active Cyt $b_{559}$ heme.

PSII Activity in the His$^{23}$ Mutants Is More Susceptible to Photoinactivation—To investigate whether mutation of PsbE His$^{23}$ leads to strains that are more sensitive to photoinactivation, light-saturating rates of oxygen evolution were measured as a function of time, from aliquots of cells taken from low light-grown cultures that were then exposed to either high or moderate light intensities. The artificial electron acceptors ferricyanide and 2,5-dimethyl-p-benzoquinone were used so that rates of oxygen evolution were a measure only of PSII activity rather than the complete photosynthetic electron transport chain.
Heme-deficient Photosystem Two

Fig. 5. EPR spectra of PSII-LHCII supercomplexes isolated from PF3aad#1 and H23Y mutant. Samples contained 1.7 mg ml⁻¹ (PF3aad#1) and 1.9 mg ml⁻¹ chlorophyll (H23Y). A, spectra obtained from dark-adapted samples (Dark) were subtracted from spectra obtained after illuminating at 77 K (Light) to give difference spectra (Light-minus-dark). B, S₂ multiline spectra obtained by subtracting spectra of dark-adapted samples (in S₁ state) from those of samples illuminated at 200 K (in S₂ state).

Fig. 6 shows that when cells of the WT control, PF3aad#1, were exposed to moderate intensity white light (100 µEm⁻²s⁻¹), there was little loss of PSII activity during the time course of the experiment either in the absence or presence of lincomycin. Under the latter condition, protein synthesis is blocked in the chloroplast, so loss of PSII activity is a monitor of the damage to PSII that is normally repaired by de novo protein synthesis. In contrast, after a lag period, the H23Y mutant showed a dramatic decrease in PSII activity under these moderate light conditions both in the presence and absence of lincomycin (Fig. 6). PSII activity in the H23Y mutant is thus more susceptible to photoactivation than in the WT. Also, the absence of an effect of lincomycin indicated that the rate of de novo synthesis, and assembly of PSII in the mutant was unable to match PSII inactivation under these moderate light conditions. Under high light, PSII activity was again more rapidly lost in the H23Y mutant than in the WT. In the presence of lincomycin, the light-induced loss of PSII activity from WT cells occurred with a half-time of ~20 min, whereas PSII activity in the H23Y strain was undetectable after 25 min. The H23M mutant was even more sensitive to photoactivation than the H23Y mutant (data not shown).

**DISCUSSION**

The mutants described here are the first in which heme-binding within PSII has been perturbed without losing the ability to assemble PSII. This has allowed us to investigate the participation of Cyt b₅₅₉ in water oxidation and in protection from photoactivation, functions that have been proposed based mainly on experiments conducted on PSII samples in vitro.

Our analyses have been helped by the development of a method to isolate large oxygen-evolving PSII complexes depleted in PSI and devoid of cytochromes besides Cyt b₅₅₉. On the basis of size and polypeptide composition and by comparison with results from higher plants and a PSI-deficient strain of *C. reinhardtii*, this complex appears to be a PSII-LHCII supercomplex. It is thought likely that because a relatively rapid and gentle solubilization procedure is used to release this complex from the membrane, the PSI supercomplex represents the organization of PSII in vivo (23). In this report, we characterize mutant strains that possess ~10–25% of WT levels of PSII; however, a combination of the solubilization conditions described here plus an affinity purification strategy such as His tagging should in principle allow the rapid isolation of PSII supercomplexes from mutants expressing even lower amounts of PSII.

A *Redox-active Cyt b₅₅₉ Is Not Needed for Water Oxidation*—We have demonstrated using optical methods that PSII supercomplexes isolated from the H23Y and H23M mutants contain undetectable amounts of heme yet retain largely WT levels of oxygen-evolving activity. At present, there is no biochemical procedure to remove heme selectively from PSI without inhibiting oxygen evolution. Thus, the mutants described in this paper represent an important advance in the study of Cyt b₅₅₉ in that they provide a selective means for investigating heme-depleted PSII complexes. Low temperature EPR spectroscopy confirmed the absence of heme in the H23Y mutant and also indicated that no altered photooxidisable form of Cyt b₅₅₉ was operating in the *psbE* mutant. Additional EPR exper-
ments strongly implied that the absence of heme in the H23Y supercomplex does not inhibit or alter electron transfer events occurring between the donor and the acceptor side of PSII. First, at 77 K, when electron donation from the Mn4 cluster to P680" is blocked, illumination generated a charge-separated state between Car" or Chl7 and QX in the heme-deficient supercomplex as in the WT. Second, illumination at 200 K produced a normal multiline EPR signal of the S2 state of the Mn4 cluster of both the wild type and the heme-deficient supercomplex. Therefore, the above spectroscopic data strongly argue against the concept that Cyt b559 has a direct role in water oxidation or is linked to the function of the Mn4 cluster in the initial step of this process (10).

Role of Cyt b559 in the Assembly of PSII—Our results do not allow us to draw conclusions on the requirement of heme for the assembly of PSII. It is possible that the His23 mutants bind heme in vivo, to allow assembly of PSII, but lose the heme during the relatively mild detergent treatment used to isolate the PSII supercomplex. Unfortunately, the low levels of PSII in the His23 mutants prevented an accurate estimation to be made of the Cyt b559 heme content in thylakoid membranes by either EPR or optical methods. For other membrane-bound cytochromes, mutation of the axial ligand can sometimes lead to retention of the heme with altered ligation (38) or loss of heme as in the case of cytochrome c oxidase (39). In the latter instance, it was not possible to isolate heme-deficient complexes from the membrane using detergent (39), possibly because of the need for heme to stabilize the tertiary structure of the complex (40). Other possibilities that cannot be ruled out for the His23 mutants include compensation for the missing heme by insertion of an alternate cofactor or metal ion and stabilization of dimer formation between the α and β subunits by hydrogen bonding or other interactions. We, therefore, do not exclude at this stage the interesting possibility that a chlorophyll molecule has replaced the heme molecule in the Cyt b559 mutants. Alternatively, because Cyt b559 is likely to be located on the periphery of the PSII supercomplex (8), heme binding may not play a crucial role in maintenance of the quaternary structure. Nevertheless, it is clear that mutation of the His ligand leads to loss of heme in supercomplexes isolated from the H23Y and H23M. This is compatible with an αβ heterodimeric structure for the cytochrome, as confirmed by recent structural studies (8) rather than earlier models involving α2 and β2 homodimers (7).

Mutation of a single histidine ligand to the heme to either Gln (described here) or to Leu (20) causes a drastic reduction in the accumulation of the PSII reaction center subunits, D1 and D2, in the membrane. Similarly, truncation of 31 or 22 residues from the C terminus of the PsbE subunit in Synechocystis 6803 leads to reduced levels of assembled PSII, despite expression of the psbE gene at WT levels (41). Like the H23 mutants described here though, the decreased number of PSII complexes that did assemble were active in oxygen evolution (41). This is consistent with a role for Cyt b559 in the early steps of assembly of PSII as suggested previously from analysis of a psbE null mutant of C. reinhardtii and more recently from biochemical studies on the assembly of PSII in a greening system (12). That the levels of PSII are not increased in dark grown cultures of the mutants described here argues against a light-induced reduction in levels, a possibility not excluded in previous analyses of cyanobacterial mutants.

Whether there is one or two Cyt b559 per PSII complex in C. reinhardtii thylakoid membranes is unresolved. However, a Chl/Cyt b559 ratio of 130–150 in the PSII-LHClII supercomplex isolated here would broadly favor a stoichiometry of 1 Cyt b559 per isolated PSII complex, since the number of chlorophylls per PSII monomer, albeit from a spinach supercomplex, is about 100 (42). A stoichiometry of 2 Cyt b559 per PSII monomer would mean a total antenna size of about 260–300 chlorophylls per monomer, of which the bulk (220–260) are present in chlorophyll a/b-binding proteins outside the Chl a-containing PSII core complex. Such a scenario is unlikely, given the low Chl a/b ratio of the antenna system (43) and the determined Chl a/b ratio of the supercomplex of about 3:1:1.

Role of Cyt b559 in Protecting PSII from Damage in Vivo—Importantly, the His23 mutants are more sensitive to photoinhibition than the WT. This is therefore the first evidence from mutational studies of Cyt b559 to support a role for the cytochrome in the protection of PSII. Because the intensity of light that gave half-saturation of oxygen evolution in the mutant cells was similar to that for the WT (data not shown), it is unlikely that the mutants show enhanced photoactivation purely because of the reduced levels of PSII compared with WT.

A number of PSII mutants show water-oxidation activity that is more sensitive to photoinhibition than WT at high light irradiances. They can be subdivided into two classes. The first, exemplified by the psbO null mutant of Synechocystis 6803 (44), includes mutants where the PSII complex has been perturbed so that the rate of irreversible damage has increased compared with the WT. A second class of mutant, which includes a psbH null mutant of Synechocystis 6803 (44) and a psbT" null mutant of C. reinhardtii (45), shows similar rates of light-induced damage to the WT but has a less efficient PSII repair pathway. Blocking the PSII repair cycle by the addition of an inhibitor of chloroplast protein translation, such as lincomycin, allows these two classes to be differentiated. On the basis of the data shown in Fig. 6, the H23Y mutant shows an increased rate of damage to PSII relative to the WT. By comparing the effect of lincomycin on loss of PSII activity, the repair cycle also appears less efficient in the mutant. PSII in a C. reinhardtii mutant lacking the PsbP protein is known to be more sensitive to photoinactivation (46). However, loss of binding of extrinsic proteins to PSII is unlikely to be the cause of the enhanced rates of damage in the His23 mutants, since PsbP, PsbO, and PsbQ are retained at WT levels in the mutant PSII supercomplexes (Fig. 4).

Our results thus far do not permit us to draw conclusions on the reason for the enhanced sensitivity to photoinhibition. Although oxygen evolution in the PSII complexes occurs at WT rates in the H23M and H23Y mutants, it is always possible that there is a minor structural perturbation to PSII in vivo that leads to enhanced rates of damage in the mutants. Alternatively, a protective function involving the heme may have been compromised in the mutants. Two types of model have been proposed to explain how Cyt b559 may photoprotect PSII (7). In one, Cyt b559 acts as an emergency electron donor or acceptor within the reaction center in situations where there is an imbalance in electron flow into and out of PSII. Interconversion of the cytochrome between its high and low potential states strongly implied that the absence of heme in the H23Y supercomplex does not inhibit or alter electron transfer events occurring between the donor and the acceptor side of PSII. First, at 77 K, when electron donation from the Mn4 cluster to P680" is blocked, illumination generated a charge-separated state between Car" or Chl7 and QX in the heme-deficient supercomplex as in the WT. Second, illumination at 200 K produced a normal multiline EPR signal of the S2 state of the Mn4 cluster of both the wild type and the heme-deficient supercomplex. Therefore, the above spectroscopic data strongly argue against the concept that Cyt b559 has a direct role in water oxidation or is linked to the function of the Mn4 cluster in the initial step of this process (10).
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Photosynthetic Water Oxidation in Cytochrome\textsubscript{b} 559 Mutants Containing a Disrupted Heme-binding Pocket
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