Spectroscopic and Functional Characterizations of Cyanobacterium Synechocystis PCC 6803 Mutants on and near the Heme Axial Ligand of Cytochrome b<sub>559</sub> in Photosystem II*§

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The functional role of cytochrome (cyt) b<sub>559</sub> in photosystem II (PSII) was investigated in H22Kα and Y18Sα cyt b<sub>559</sub> mutants of the cyanobacterium Synechocystis sp. PCC6803. H22Kα and Y18Sα cyt b<sub>559</sub> mutant carries one amino acid substitution on and near one of heme axial ligands of cyt b<sub>559</sub> in PSII, respectively. Both mutants grew photoautotrophically, assembled stable PSII, and exhibited the normal period-four oscillation in oxygen yield. However, both mutants showed several distinct chlorophyll a fluorescence properties and were more susceptible to photoinhibition than wild type. EPR results indicated the displacement of one of the two axial ligands to the heme of cyt b<sub>559</sub> in H22Kα mutant reaction centers, at least in isolated reaction centers. The maximum absorption of cyt b<sub>559</sub> in Y18Sα mutant PSII core complexes was shifted to 561 nm. Y18Sα and H22Kα mutant PSII core complexes contained predominately the low potential form of cyt b<sub>559</sub>. The findings lend support to the concept that the redox properties of cyt b<sub>559</sub> are strongly influenced by the hydrophobicity and ligation environment of the heme. When the cyt b<sub>559</sub> mutations placed in a D1-D170A genetic background that prevents assembly of the manganese cluster, accumulation of PSII is almost completely abolished. Overall, our data support a functional role of cyt b<sub>559</sub> in protection of PSII under photoinhibition conditions in vivo.

Cytochrome b<sub>559</sub> (cyt b<sub>559</sub>)<sup>2</sup> is one of the essential components of the photosystem II (PSII) in higher plants, green algae, and cyanobacteria (1–5). cyt b<sub>559</sub> is a heme-bridged heterodimer protein that is composed of one α- and one β-subunit (encoded by the psbE and psbF genes) of 9 and 4 kDa, respectively. Each subunit provides a His ligand (His-22 residue of α- or β-subunit of cyt b<sub>559</sub>) for the non-covalently bound heme. In addition, cyt b<sub>559</sub> exhibits different redox potential forms: a high potential form with a midpoint redox potential around +400 mV, an intermediate potential form around +200–250 mV, and a low potential form with a midpoint redox potential about +50–100 mV (Refs. 5–9 and references therein). Several previous studies have proposed that cyt b<sub>559</sub> participates in secondary electron transfer pathways, which protect PSII from photoinhibition (5, 10–12). In these models the high potential form of cyt b<sub>559</sub> might donate its electron to reduce highly oxidized chlorophyll radical species generated in PSII reaction centers under the donor-side photoinhibitory conditions. On the other hand, cyt b<sub>559</sub> might accept an electron from the acceptor side of PSII (Q<sub>B</sub>, PQ, or phophytin (primary phophetin a electron acceptor) from generating damaging singlet oxygen species under the acceptor-side photoinhibitory conditions (13–15). In addition, a novel quinone-binding site (Q<sub>C</sub>) was identified in proximity to cyt b<sub>559</sub> in the new 2.9Å PSII crystal structure (4). The occupancy of this Q<sub>C</sub> site has been proposed to modulate the reox equilibration between cyt b<sub>559</sub> and the PQ pool (16, 17) or to involve in the exchange of PQ on the Q<sub>B</sub> site from the pool (4). Despite the recent progress in understanding the structure and function of PSII, the exact function of cyt b<sub>559</sub> in PSII remains unclear.

Prior mutagenesis studies with Synechocystis sp. PCC6803, Chlamydomonas reinhardtii, or Nicotiana tabacum showed no stable PSII reaction centers assembled in the absence of either cyt b<sub>559</sub> subunit (18–24). In addition, an early site-directed mutagenesis study with Synechocystis 6803 showed that substituting either of the heme axial ligands (His-22 of the α-subunit or His-22 of the β-subunit) with Leu severely diminished the assembly or stability of PSII (23). Furthermore, another site-directed mutagenesis study involving C. reinhardtii showed that the H22Y and H22M mutants of the cyt b<sub>559</sub> α subunit accumulated 10–15% of the PSII (compared with wild-type cells) and contained a disrupted heme pocket, whereas still retaining significant amounts of O<sub>2</sub> evolution activity (24). This
Site-directed Mutants on the Heme of cyt b_{559}

study concluded that a redox role for the heme of cyt b_{559} was not required for O2 evolution (24). Moreover, one recent study on the F265β cyt b_{559} tobacco mutant showed that the PQ pool was largely reduced in dark-adapted leaves of this F265β cyt b_{559} tobacco mutant. This study provided evidence that cyt b_{559} might mediate the oxidation of reduced PQ in the dark (15). Because the architecture of the thylakoid membrane in this tobacco mutant was significantly altered, the authors did not rule out the possibility that their result might be due to the thylakoid membrane global structural changes caused by a lower level of cyt b_{559} (15).

To study the exact role of cyt b_{559} in the secondary electron transfer pathway in PSII, we constructed a set of site-directed mutants of the cyt b_{559} heme ligands (His-22 of the α-subunit and His-22 of the β-subunit) with Synecocystis sp. PCC6803 cells (25). In these mutants, the His-22 residue of the α- or β-subunit was replaced with Met, Glu, Gln, Tyr, Lys, Arg, or Cys. O2 evolution and chlorophyll a fluorescence measurements revealed that only the H22Kα mutant grew photoautotrophically or photomixotrophically (supplemented with 5 mM glucose) as described in Table 1. Cultures were propagated at 30 °C with a dual pulse-amplitude modulation fluorometer (Walz).

EXPERIMENTAL PROCEDURES

Growth and Preparation of Synechocystis sp. PCC6803 Cells—Synechocystis cells were grown in BG-11 medium photoautotrophically or photomixotrophically (supplemented with 5 mM glucose) as described in Table 1. Cultures were propagated at 30 °C under a light intensity of 25–30 microeinsteins m^{-2}s^{-1} and were continuously bubbled with sterile, humidified air.

Measurement of Chlorophyll a Fluorescence at 295 K—Chlorophyll a fluorescence measurements at 295 K were performed with a dual pulse-amplitude-modulation fluorometer (Walz). The relative PSII content of cells on a chlorophyll basis was estimated from the total yield of variable chlorophyll a fluorescence (F_{max} - F_{0}) measured in the presence of DCMU and hydroxylamine according to Hung et al. (25). Experimental conditions for measurements of PSII fluorescence yield in the presence and absence of actinic light and decay of flash-induced variable fluorescence in response to a saturating flash given to wild-type and mutant cells are described in the legend to Fig. 1.

Measurement of Photosynthetic Oxygen Evolution—Steady-state rates of oxygen evolution were measured with a Clark-type oxygen electrode (YSI model 5331 oxygen probe) fitted with a water-jacketed cell as described previously (25). Oxygen evolution of cells (10 μg of Chl ml^{-1}) was measured in BG11 medium and in the presence of 2 mM potassium ferricyanide and 2 mM 2,6-dichloro-p-benzoquinone as electron acceptor. Oxygen evolution of PSII core complexes were measured in 40 mM Mes, pH 6.5, 15 mM MgCl2, 15 mM CaCl2, 10% (v/v) glycerol, 1 mM glycine betaine and in the presence of 1 mM potassium ferricyanide and 0.5 mM 2,6-dichloro-p-benzoquinone.

Measurement of Flash O2 Yield—Flash O2 yield measurements were performed using a bare platinum electrode that allows the centrifugation of cells upon the electrode surface as described previously (26, 27). For each measurement, cells of 3 μg/ml Chl in 500 μl of an HN buffer (10 mM Hepes-NaOH, pH 7.2, 30 mM NaCl) were centrifugally deposited at 1500 × g for 5 min onto the platinum surface of the electrode in a Sorvall HB-4 swing rotor. O2 signals were measured by recording the amperometric response of the electrode system to a train of 19 saturating xenon flashes at frequency of 4 Hz. Analysis of the oscillatory pattern of release of O2 from dark-adapted cells was performed assuming a four-state model (28, 29).

D1 Degradation Analysis—2 μg of Chl of cells was dissolved with 50 μl of Laemmli sample buffer plus 5% lithium dodecyl sulfate and incubated at 55 °C for 10 min (30). Electrophoresis was performed by using a 12–22% linear gradient polyacrylamide gel containing 6 M urea. After transfer onto polyvinylidene difluoride membrane, D1 protein was detected by using antiserum against the C-terminal part of D1 (25). Bands were visualized by using enhanced chemiluminescence reagents (PerkinElmer Life Sciences). The density of D1 protein bands was quantized by using Biospectrum 600 imaging system.

Polypeptide Compositions Analysis of Wild-type and Mutant PSII Core Complexes—Wild-type and mutants PSII core complexes were separated on a 12–22% 6 M urea SDS/PAGE gel. Heme staining was performed as described in Napoli et al. (31). cyt c_{550} Content—cyt c_{550} contents in wild-type and mutants PSII core complexes were determined from modeling the difference absorption spectra and applying an extinction coefficient of 25 mm^{-1}cm^{-1} to the cyt c_{550} peak and 41 molecules of Chl/PSII as described in Roose et al. (32).

Reduced Minus Oxidized Difference Spectra of cyt b_{559} in Wild-type and Mutant PSII Core Complexes—Optical absorption difference measurements were performed on suspensions of wild-type* and mutant PSII core complexes (20 μg/ml Chl) in 1 ml of MMNB buffer (25 mM Mes, 5 mM MgCl2, 10 mM NaCl, 1 mM glycine betaine, 0.03% n-dodecyl β-D-maltoside, pH 5.7) at room temperature on a Jasco V-560 UV-visible spectrophotometer with a bandwidth of 1.0 nm. Four-step redox titration was performed, as described in Bondarava et al. (15). The sample suspension was oxidized by adding 0.1 mM K3Fe(CN)6 (from a fresh made 10 mM stock solution), and the spectrum was recorded and stored as the oxidized spectrum. The reduction was performed by first adding hydroquinone (0.3 mM) followed by ascorbate (0.6 mM) and, finally, a few grains of dithionite.

Conditions for EPR Measurements—EPR spectra were obtained at X-band using a Bruker EMX spectrometer equipped with a Bruker TE102 cavity and an Advanced Research System continuous-flow cryostat (3.2–200 K). The microwave frequency was...
measured with a Hewlett-Packard 5246L electronic counter. The instrument settings are shown in the legend to Fig. 6.

Measurement of Fluorescence at 77 K—Fluorescence emission spectra were recorded with a fluorescence spectrometer (Jasco model FP-6500). All the measurements were carried out at 77 K using cell suspensions at a chlorophyll concentration of 20 μg/ml. The excitation light wavelength used for exciting chlorophyll was 435 nm (excitation bandwidth 5 nm, emission bandwidth 1 nm). The excitation light wavelength used for phycobilisomes was 600 nm (excitation bandwidth 3 nm, emission bandwidth 1 nm).

Construction of Double Mutants D170AD1/H22Kα cyt b_{559} and D170AD1/Y18Sα cyt b_{559}—Double mutants D170AD1/H22Kα and D170AD1/Y18Sα cyt b_{559} were constructed by transformation of the H22Kα and Y18Sα cyt b_{559} mutant plasmid into the host strain D170AD1 (31), respectively. Mutants were selected on solid media containing the antibiotic erythromycin and kanamycin (0.1 g/ml). The excitation light wavelength used for exciting cyt b_{559} was 600 nm (excitation bandwidth 3 nm, emission bandwidth 1 nm).

RESULTS

Growth, Oxygen Evolution Activity, and PSII Contents of Mutant Cells—The growth characteristics, light-saturated oxygen evolution activity, and PSII contents of the mutant strains that are discussed in this study are listed in Table 1. Both H22Kα and Y18Sα cyt b_{559} mutant cells grow photoautotrophically. The oxygen evolution activity of H22Kα and Y18Sα cyt b_{559} mutant cells was about 71 and 77% that of wild-type cells, respectively. The PSII contents of H22Kα and Y18Sα cyt b_{559} mutant cells were about 81 and 86% that of wild-type cells, respectively.

PSII Fluorescence Yield in the Presence and Absence of Actinic Light—H22Kα and Y18Sα cyt b_{559} mutant cells showed very distinct time-dependent, flash-induced transients of PSII fluorescence yield in the presence and absence of actinic light compared with wild-type cells (see Fig. 1). Wild-type cells showed the lowest $F_0$ (0.107) and the highest $F_v/F_m$ (0.417) (see Fig. 1A); Y18Sα mutant cells showed the high $F_0$ (0.157) and the low $F_v/F_m$ (0.349) (see Fig. 1B); H22Kα mutant cells also showed the high $F_0$ (0.135) and the low $F_v/F_m$ (0.320) (see Fig. 1C). In addition, the maximum fluorescence $(F_{M})$ in Y18Sα and H22Kα cyt b_{559} mutant cells was significantly increased during the actinic light illumination and then decrease, whereas the actinic light was turned off. In contrast, the $F_M$ value in wild-type cells kept almost constant before, during, and after the actinic light illumination. The results are highly reminiscent of the changes in maximal fluorescence yield that accompany with state transitions (Refs. 34 and 35 and references therein). Moreover, we also found that the $F_v/F_M$ value after the

| Strain | PS | O₂ evolution | PSII content | Reference |
|--------|----|--------------|--------------|-----------|
|        |    | % of wild type | % of wild type |           |
| Wild type | +  | 100 | 100 | This study |
| Y18Sα | +  | 77 ± 15 | 86 ± 10 | This study |
| H22Kα | +  | 71 ± 18 | 81 ± 16 | This study |
| D170AD1 | –  | 0 | 135 ± 24 | 33 |
| D170AD1/H22Kα | –  | 0 | 5 ± 2 | This study |
| D170AD1/Y18Sα | –  | 0 | 26 ± 4 | This study |

### TABLE 1

Summary of properties of cyt b_{559} mutant cells

Experimental conditions are described under “Experimental Procedures.” The average oxygen evolution rates of wild-type cells were 560 ± 77 μmol of O₂/mg of Chl-h. The average total yield of variable chlorophyll $a$ fluorescence $(F_{m'}/F_o)$ of wild-type cells was 0.120 ± 0.008. PS, photoautotrophic growth.
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The manganese cluster and QA were intact in Y18S flash in the absence of DCMU is shown in supplemental Fig. 1. DCMU is due mostly to the forward electron transfer from QA to QB and QB to QB and QB. Our results indicated that in Y18S mutant cells in response to a saturating single-turnover flash. In wild-type cells in the dark, the chlorophyll a fluorescence induced by a MT light pulse decayed to the steady-state fluorescence level that was slightly above the F_{0} level initially and went up gradually (in about 5 s) to the steady-state fluorescence level. The same effects were also observable in Y18S and H22Kα cyt b_{559} mutant cells after actinic light illumination was off for about 2 min in Fig. 1, B and C. Our results suggested that a fluorescence quencher was induced by the MT light pulses and decayed within about 10 s in a significant fraction of PSII of Y18Sα and H22Kα cyt b_{559} mutant cells.

Decay of Flash-induced Variable Fluorescence—The induction and decay of Q_{A} in wild-type and Y18Sα and H22Kα cyt b_{559} mutant cells in response to a saturating single-turnover flash in the absence of DCMU is shown in supplemental Fig. 1. The fluorescence decay in wild-type cells in the absence of DCMU is due mostly to the forward electron transfer from Q_{A} to Q_{B} and Q_{B} to Q_{B}. The fluorescence decay in Y18Sα and H22Kα cyt b_{559} mutant cells was very similar to wild-type cells, but there was a small increase in the slow phase component that could be attributed to the back reaction of Q_{A} with the donor-side components. Our results indicated that in Y18Sα and H22Kα cyt b_{559} mutant cells the forward electron transfer from Q_{A} to Q_{B} and Q_{B} was normal in the majority of mutant reaction centers. In addition, Y18Sα and H22Kα cyt b_{559} mutant cells showed very similar kinetics of chlorophyll a fluorescence decay to that in wild-type cells in response to a saturating single-turnover flash in the presence of DCMU (Y18Sα mutant data are not shown; for H22Kα mutant data, see Ref 25). Our results suggest that the manganese cluster and Q_{A} were intact in Y18Sα and H22Kα cyt b_{559} mutant cells.

Oxygen-flash Yield Experiments—The patterns of oxygen yield on the dark-adapted cyt b_{559} mutant and wild-type cells were measured on a bare platinum electrode under a sequence of saturating, single-turnover flashes given at a frequency of 4 Hz. The amplitude of the oxygen yield as a function of flash number is shown in supplemental Fig. 2. Both mutants exhibited the characteristic period-four oscillation in oxygen yield, indicating that the basic mechanism of water oxidation remains largely unchanged in these cyt b_{559} mutant reaction centers. To evaluate possible alternations in the transition probabilities associated with the four-step water oxidation mechanism,
TABLE 2
S-state cycling parameters

| Strain          | 10 min of dark; S-state distribution: S0/S1/S2/S3 | Misses α | Hits β | Double hits γ | Deactivation δ |
|-----------------|-----------------------------------------------|----------|-------|---------------|---------------|
| Wild type       | 26/73/1/0                                     | %        | %     | %             | %             |
| Y18Sα           | 29/68/3/1                                     | 14       | 83    | 2             | 1             |
| H22Kα           | 30/69/1/0                                     | 15       | 81    | 2             | 2             |

Cells were given a series of 20 preflashes before the 10-min dark period preceding the series of measuring flashes. Numerical analysis of the amplitudes was performed using a four-state model as described previously (28, 29).

Table 3) contain oscillatory patterns were analyzed using an eigenvector method to estimate the parameters α, β, γ, and δ, which correspond to misses, hits, double hits, and deactivations, respectively (28, 29). These results are summarized in Table 2. Our results showed that only relatively small alternations in the transition probabilities in these cyt b$_{559}$ mutant cells were detected with this analysis.

Susceptibility to Photoinhibition—Fig. 3 showed time course of the PSII activity (oxygen-evolution activity) and the D1 degradation in wild-type and Y18Sα and H22Kα cyt b$_{559}$ mutant cells under the high light condition (~500 microeinsteins m$^{-2}$ s$^{-1}$). The steady-state oxygen-evolution rates were decreased faster in Y18Sα and H22Kα mutant than in wild-type cells under the high light condition (Fig. 3A). In addition, after the first hour of illumination, the amount of D1 polypeptide was significantly increased in wild-type cells and about the same in H22Kα mutant cells but significantly decreased in Y18Sα mutant cells (Fig. 3B). After 2 h of high light illumination, the amount of D1 polypeptides in wild-type and H22Kα and Y18Sα cyt b$_{559}$ mutant cells were decreased to about 95, 70, and 35% of the original level (Fig. 3B). Therefore, our results demonstrated that H22Kα and Y18Sα cyt b$_{559}$ mutant cells were more susceptible to photoinhibition than wild-type cells under the high light condition.

Oxygen-evolution Activity and Polypeptide Compositions of Wild-type and Mutant PSII Core Complexes—The oxygen-evolution activity for wild-type and H22Kα and Y18Sα mutant PSII core complex was about 2100, 420–720, and 1120 μmol of O$_2$/mg of Chl/h, respectively (see Table 3). Polypeptide compositions of isolated wild-type and mutant PSII core complexes are shown in supplemental Fig. 3. The cyt c$_{559}$ contents in wild-type and H22Kα and Y18Sα mutant PSII core complexes were about 0.54, 0.37, and 0.22 per reaction center, respectively. Heme staining of cyt c$_{550}$ in wild-type and mutant PSII core complexes is shown in supplemental Fig. 4. The lower oxygen-evolution activity in Y18Sα and H22Kα mutant PSII core complex seemed to correspond to a lower amount of cyt c$_{550}$ associated with their PSII complex.

Determination of Different Potential Forms of cyt b$_{559}$ in Wild-type and Mutant PSII Core Complexes—To estimate the potential forms of cyt b$_{559}$ in wild-type and mutant PSII core complexes, we measure reduced minus oxidized optical difference spectra of the cyt b$_{559}$ heme in wild-type (A) H22Kα (B), and Y18Sα (C) cyt b$_{559}$ mutant oxygen-evolving PSII core complexes using four step titration (ferricyanide → hydroquinone → ascorbate → dithionite) (Fig. 4). A hydroquinone-reduced minus ferricyanide-oxidized absorption difference spectrum (HF, dash line) was used to estimate the HP form of cyt b$_{559}$, and an ascorbate-reduced minus hydroquinone-oxidized absorption difference spectrum (AH, thin solid line) was used to estimate the IP form of cyt b$_{559}$. Dithionite-reduced minus ascorbate-oxidized absorption difference spectrum (DA, thick solid line) of cyt b$_{559}$ contains both the HP form of cyt b$_{559}$ and cyt c$_{550}$. Because cyt c$_{550}$ does not contribute significantly at 559.5 nm of the difference spectrum (8, 29); therefore, we used the amplitude of dithionite-reduced minus ascorbate-oxidized absorption difference spectra at 559.5 nm to estimate the amount of the LP form of cyt b$_{559}$. Our results showed that oxygen-evolving wild-type PSII core complexes (Fig. 4A and Table 3) contain ~8% HP form, ~78% IP form, and ~14% LP form of cyt b$_{559}$. In contrast, H22Kα (Fig. 4B) and Y18Sα cyt

**TABLE 3**
Summary of properties of cyt b$_{559}$ mutant PSII core complexes

| Strains          | O$_2$ evolution activity | Redox forms$^a$ |
|------------------|--------------------------|----------------|
|                  | μmol of O$_2$/mg chl/h   | HP | IP | LP |
| Wild type        | 2100                     | 8  | 78 | 14 |
| H22Kα            | 1120                     | 9  | 12 | 79 |
| Y18Sα            | 420–720                  | 6  | 8  | 86 |

$^a$ Content of redox forms of cyt b$_{559}$ (in % of cyt b$_{559}$ ±7%) in wild-type and cyt b$_{559}$ mutant PSII core complexes.

**FIGURE 3.** Time course of the photoinhibition and the D1 degradation in wild-type (C) and H22Kα (A) and Y18Sα cyt b$_{559}$ mutant cells (D). Cells (10 μg of Chl/ml) were incubated in BG-11 medium and illuminated at ~500 microeinsteins m$^{-2}$ s$^{-1}$. Aliquots of the suspensions were taken at the times indicated. The oxygen evolution rates of wild-type and Y18Sα and H22Kα cyt b$_{559}$ mutant cells at time 0 were 573 ± 98, 499 ± 47, and 508 ± 32 μmol of O$_2$/mg Chl/h, respectively. Shown is a Western blot of polypeptides from cells taken at 0, 60, and 120 min of illumination using antibody against the C-terminal part of D1 protein.
Site-directed Mutants on the Heme of cyt b$_{559}$

$b_{559}$ mutant PSII core complexes (Fig. 4C) contain much more LP form (~79 and 86%, respectively), much less IP form, and HP form of cyt b$_{559}$ (Table 3).

The dithionite-reduced minus ferricyanide-oxidized difference spectra of the cyt b$_{559}$ heme in Y18Sα cyt b$_{559}$ PSII core complexes are shown in Fig. 5. Y18Sα cyt b$_{559}$ mutant PSII core complexes (thin dash line) gave rise to dithionite-reduced minus ferricyanide-oxidized difference spectra that can be attributed to redox-induced absorption changes of cyt b$_{559}$ and cyt c$_{550}$. On the other hand, difference spectra from Tris-washed Y18Sα cyt b$_{559}$ PSII core complexes (thick solid line) (that were depleted of manganese ions and extrinsic polypeptides, including cyt c$_{550}$) can be attributed to redox-induced absorption changes in cyt b$_{559}$. Our results showed that the maximum absorption of cyt b$_{559}$ in Y18Sα mutant PSII core complexes was shifted to 561 nm. In addition, the α-absorption band of cyt b$_{559}$ in H22Kα mutant PSII core complexes was significantly broadened (see Fig. 4B and Ref. 25).

**FIGURE 4.** The reduced minus oxidized difference spectra of cyt b$_{559}$ heme in oxygen-evolving wild-type (A) and H22Kα (B) and Y18Sα (C) cyt b$_{559}$ mutant PSII core complexes. DA, the dithionite-reduced minus ascorbate-oxidized difference spectrum (thick solid line). AH, the ascorbate-reduced minus hydroquinone-oxidized difference spectrum (thin solid line). HF, the hydroquinone-reduced minus ferricyanide-oxidized difference spectra (dotted line). A linear base-line correction has been applied to each difference spectrum that consists of a straight line connecting the spectral values at 540 and 580 nm.

**FIGURE 5.** The dithionite-reduced minus ferricyanide-oxidized difference spectra of cyt b$_{559}$ heme in oxygen-evolving (thin solid line) and Tris-washed (thick solid line) Y18Sα cyt b$_{559}$ mutant PSII core complexes. A linear base-line correction has been applied to each difference spectrum that consists of a straight line connecting the spectral values at 540 and 580 nm.
light-induced EPR signals for photooxidization of HP or some IP form of cyt $b_{559}$ in H22Kα and Y18Sα cyt $b_{559}$ mutant PSII core complexes under 77 K illumination (Fig. 6, B and C). Furthermore, to estimate the redox potential of the EPR signals for the high spin form of cyt $b_{559}$ in spectra of H22Kα cyt $b_{559}$ mutant PSII core complexes, we treated H22Kα cyt $b_{559}$ mutant PSII core complexes with 1 mM ascorbate and 2 mM dithionite and examine them by Continue-wave-EPR. We found that the EPR signals for the high spin form of cyt $b_{559}$ ($g_x = 6.11; g_y = 5.90$) were only slightly reduced by ascorbate but completely reduced by dithionite (data not shown). Therefore, the EPR signals of the high spin form of cyt $b_{559}$ in spectra of H22Kα cyt $b_{559}$ mutant PSII core complexes correspond mainly to the LP form of cyt $b_{559}$.

**General Properties of D170AD1 and cyt b$_{559}$ Double Mutants**—Several earlier studies proposed that the reduced HP form of cyt $b_{559}$ might donate its electron to highly oxidized chlorophyll radical species generated in PSII reaction centers under donor-side photoinhibitory conditions, for example, when the watersplitting reaction is inhibited (5, 11). Previous mutational analysis had shown the substitution of aspartate 170 of the D1 protein with alanine (D170A) prevents the assembly of a functional manganese cluster. To test the possible role of cyt $b_{559}$ under donor-side photoinhibitory conditions, we constructed D170AD1/H22Kα and D170AD1/Y18Sα double mutants. D170AD1 mutant cells are able to accumulate stable PSII to the wild-type level but cannot evolve any oxygen (33). Interestingly, our results showed that little PSII was present in D170AD1/H22Kα and D170AD1/Y18Sα double mutants (see Table 1).

**77 K Fluorescence Emission Spectra**—Fig. 8A shows the 77 K fluorescence emission spectra recorded from cells that were excited at 600 nm, where the phycobilin pigments preferentially absorb. In spectra of wild-type cells, fluorescence peaks at 655 nm, originating from allophycocyanin, and a peak at 690 nm, originating from PSII. In spectra of cyt $b_{559}$ mutant cells, a fluorescence peak was present at 660 nm, but the other peak was strongly enhanced and shifted to 686 nm. The emission peak at 686 nm originates from terminal phycobilin emitters (ApcE) or from CP43 (39–42). Therefore, the medium enhancement of emission peaks at 686 nm in Y18Sα and H22Kα cyt $b_{559}$ mutant cells indicated that the energy transfer...
from phycobilisomes to PSII reaction centers was partially inhibited or uncoupled in these mutant cells. These results are consistent with the findings that the maximal fluorescence yields are considerably lower in dark-adapted cells (Fig. 1). In addition, the strong enhancement of emission peaks at 686 nm in double mutant D170AD1/H22Kα cyt b559 and D170AD1/Y18Sα cells indicated that the energy transfer from phycobilisomes to PSII reaction centers is greatly diminished due to the loss of accumulated PSII in these mutant cells (see Table 1).

Fig. 8B shows the 77 K fluorescence emission spectra recorded from cells excited at 435 nm, where the chlorophyll molecule preferentially absorbs. In spectra of wild-type and D170AD1 and cyt b559 mutant cells, preferential excitation of

FIGURE 8. 77 K fluorescence emission spectra from wild-type, D170AD1, Y18Sα, H22Kα, D170AD1/H22Kα, and D170AD1/Y18Sα mutant cells. A, shown is excitation of chlorophyll at 435 nm (excitation bandwidth 5 nm; emission bandwidth 1 nm; spectra were normalized at 720 nm). B, shown is excitation of phycobilisomes at 600 nm (excitation bandwidth 3 nm; emission bandwidth 1 nm; spectra were normalized at 660 nm). All measurements were carried out at 77 K using cell suspensions at a chlorophyll concentration of 20 µg/ml.
chlorophyll at 435 nm resulted in three emission peaks: at ~686, ~694 and ~720 nm. According to previous studies, the emission peak at ~686 nm originated from CP43 or from terminal phycobilin emitters (ApCE) (39–42), whereas the origin of the emission peak at ~694 nm seems to originate from CP47 functionally coupled to the PSII reaction center. The emission peak at ~720 nm originates from Chl a in PSI (Refs. 39–42 and references therein). Therefore, the absence of the emission peaks at ~694 nm in spectra of D170AD1/Y185α and D170AD1/H22Kα double mutants was correlated with their very low PSII contents (see Table 1).

**DISCUSSION**

In this work we report spectroscopic and functional characteristics of Y185α and H22Kα cyt b_{559} mutants. Both cyt b_{559} mutants are able to grow photoautotrophically, assemble stable PSII reaction centers, and show the normal period-four oscillation in oxygen yield. In addition, both Y185α and H22Kα cyt b_{559} mutant PSII core complexes retained the heme of cyt b_{559} and gave rise to distinct optical absorption and EPR signals of cyt b_{559}. Therefore, these cyt b_{559} mutants, and their reaction centers appear eminently suitable to study the functional role(s) of cyt b_{559} in PSI.

The H22Kα cyt b_{559} mutant is very interesting because it is the only site-directed mutation of the heme axial ligands of cyt b_{559} that retains the ability to grow photoautotrophically and assembles a large amount of functional PSII (25). Our results showed that H22Kα mutant PSII core complexes gave rise to EPR signals (g_x = 6.11; g_y = 5.90), which are characteristic for the high spin form of cyt b_{559}. These EPR signals were also observed in R7α mutant PSII core complexes (36) and in PSI membranes treated with 2,3-dichloro-5,6-dicyano-p-benzoquinone (characterized with the g = 6.19 and g = 5.95 split signal) (37). It is generally believed that the high spin state (S = 5/2) corresponds to a five-coordinate complex of Fe^{III}PorL that has a square pyramidal structure where the Fe(III) ion is above the plane of the porphyrin (Ref. 34 and references therein). Most six-coordinate octahedral Fe^{III}PorL_{2} complexes have low spin (S = 1/2) (Ref. 37 and references therein). Therefore, the EPR signal for the high spin form of cyt b_{559} (g_x = 6.11; g_y = 5.90) in spectra of H22Kα cyt b_{559} mutant PSII core complexes indicates the loss of one of the two axial ligands to the heme of cyt b_{559} in their reaction centers (37). Furthermore, the EPR signals for the high spin form of cyt b_{559} (g_x = 6.10; g_y = 5.88) were completely reduced by dithionite but not significantly by ascorbate or hydroquinone (Ref. 37 and this study). In addition, our four-step redox titration results showed that LP form of cyt b_{559} is dominated in H22Kα cyt b_{559} mutant PSII core complexes. Therefore, the EPR signals for the high spin form of cyt b_{559} in the spectra of H22Kα cyt b_{559} mutant PSII core complexes corresponds to the LP form of cyt b_{559}. Overall, these results demonstrate that the mutational alteration in axial ligation of cyt b_{559} drastically influences redox properties of cyt b_{559}.

Previous mutagenesis studies have demonstrated that both axial ligations to the heme of cyt b_{559} are very important to the assembly and/or stability of PSII (18–25). It is not clear how the H22Kα cyt b_{559} mutant, which lacks one of its axial heme ligands, retains its ability to assemble a large amount of functional PSII and grow photoautotrophically as the wild type (25). One possibility is that the substitution on the lysine side may be able to interact with the nitrogen on the porphyrin of cyt b_{559} and other surrounding residues through electrostatic interactions and hydrogen bonding to complement the loss of one axial ligation to the heme of cyt b_{559} and, thus, stabilize the structure of cyt b_{559}. Alternatively, the side chain of the lysine 22 residue in the H22Kα cyt b_{559} mutant might serve as an axial ligand to the heme of cyt b_{559} in assembled PSII in vivo but not in isolated PSI core complexes under our in vitro experimental conditions. The third possibility is that the side chain of the lysine 22 residue in the H22Kα cyt b_{559} mutant might serve as an axial ligand to the heme of cyt b_{559} only during the early assembly of the PSII complex but not in the fully assembled PSI complex. Further analysis would be required to explore these possibilities.

In the recent 2.9 Å PSII crystal structural model (4), the phenolic oxygen atom of the tyrosine 18 residue on the α-subunit of cyt b_{559} is located in proximity (within 4 Å distance) to the heme cofactor and to one of its axial ligands (the His-22 residue on the α-subunit of cyt b_{559}). Y185α mutant PSII core complexes gave rise to EPR signals for both the low spin form (g_x = 2.94; g_y = 2.26) and high spin form (g_x = 6.11; g_y = 5.90) of cyt b_{559}. Our results indicated that one of the axial ligands was displaced in a significant fraction of reaction centers in Y185α mutant PSII core complexes. Our results indicate that the side chain of the tyrosine 18 residue on the α-subunit of cyt b_{559} plays a significant role in stabilization of the heme axial-ligation of the His-22 residue on the α-subunit of cyt b_{559}. In addition, our result also showed that the maximum absorption of α-absorption band of cyt b_{559} was shifted to 561 nm in H185α mutant PSII core complexes (see Fig. 6). Furthermore, the four-step redox titration experiments showed that the LP form of cyt b_{559} is the predominant form in oxygen-evolving Y185α PSII core complexes. Because the side chain of the tyrosine residue is more bulky and hydrophobic than that of the serine residue, this Tyr-18 → Ser mutation on the α-subunit of cyt b_{559} is expected to drastically alter the hydrophobic heme environment of cyt b_{559} and, thus, convert the redox potential of cyt b_{559} into the LP form (43). A previous study proposed that the structural difference between the HP form and LP form of cyt b_{559} arises from a deviation of the planes of the two axial histidine imidazole rings from a parallel orientation (44). Our result is not at odds with this early proposal (44).

Our chlorophyll a fluorescence and oxygen-flash yield results showed that the primary electron transfer in Y185α and H22Kα cyt b_{559} mutant cells is largely unaffected. The finding that dark-adapted mutant cells exhibit greater fluorescence quenching during brief saturating pulses and that this is reversed by moderate actinic illumination is an intriguing phenomenon that resembles the changes in fluorescence yield due to saturating light pulses that accompany state transitions (Refs. 34 and 35 and references therein). By analogy, the mutants appear to be more prone to adopting the state II configuration in the dark. It is worth noting that unlike other cyanobacterial strains, such as *Synechococcus*, *Synechocystis* is more refractory to forming the state II con-
Site-directed Mutants on the Heme of cyt b$_{559}$

figuration upon dark adaptation. Conceivably, the alteration in the cyt b$_{559}$ local structure caused by the mutations results in the activation of the state II configuration or that mutations impede the traffic of plastoquinol/plastoquinone exchange under high turnover conditions in a manner that activates state II configuration. Alternatively, the high $F_v$ value and the increase of the $F_M$ value during actinic light illumination could be attributed to the increased fluorescence emission from uncoupled phycobilisomes in Y18S$_\alpha$ and H22K$_\alpha$ cyt b$_{559}$ mutant cells (36). Further work examining these possibilities needs to be performed.

Previous studies on Synechocystis 6803 mutant strains showed that defects in the binding of extrinsic subunits of PSII largely altered the oxygen-flash yield and charge recombination fluorescence kinetics (45–47). H22K$_\alpha$ and Y18S$_\alpha$ mutant cells exhibited normal oxygen-flash yield and charge recombination fluorescence kinetics. Therefore, our results indicate that H22K$_\alpha$ and Y18S$_\alpha$ mutant cells contain intact extrinsic subunits in vivo. In addition, the cyt c$_{550}$ contents in wild-type and H22K$_\alpha$ and Y18S$_\alpha$ mutant PSII core complexes were about 0.54, 0.37, and 0.22 per reaction center, respectively. Our results suggest that cyt c$_{550}$ is very labile in Synechocystis 6803 PSII core complexes during isolation. Defects in the binding of extrinsic subunits in mutant PSII core complexes have been shown to influence the redox property of cyt b$_{559}$ (5). However, our results in Table 3 showed that H22K$_\alpha$ and Y18S$_\alpha$ mutant PSII core complexes contained much more LP form of cyt b$_{559}$ (~79 and ~86%, respectively) than oxygen-evolving or Tris-washed wild-type PSII core complexes (~14 and ~30%, respectively) (36). Tris-washed wild-type PSII core complexes were depleted of all extrinsic polypeptides. Therefore, our results suggest that the altered redox property of cyt b$_{559}$ in H22K$_\alpha$ and Y18S$_\alpha$ cyt b$_{559}$ mutant PSII core complexes is mainly due to the primary effect of mutations on cyt b$_{559}$.

Both Y18S$_\alpha$ and H22K$_\alpha$ cyt b$_{559}$ mutant cells exhibited the increased susceptibility to photoinhibition than wild-type cells. Several earlier studies proposed that the HP form of cyt b$_{559}$ might donate its electron to reduce highly oxidized chlorophyll radical species generated in PSII reaction centers under donor-side photoinhibitory conditions (5, 10), for example, when the water-splitting reaction is inhibited (5, 10). Our results showed that H22K$_\alpha$ and Y18S$_\alpha$ mutant PSII core complexes contained predominately the LP form (~79 and 86%, respectively) of cyt b$_{559}$ (see Table 3). Therefore, Y18S$_\alpha$ and H22K$_\alpha$ cyt b$_{559}$ mutants are expected to be vulnerable to donor-side photoinhibitory conditions (5, 10).

Interestingly, our results showed that D170AD1/Y18S$_\alpha$ and D170AD1/H22K$_\alpha$ double mutants accumulated little PSII as compared with D170AD1, Y18S$_\alpha$, H22K$_\alpha$ mutants, or wild type. One possible explanation of our results is that D170AD1/Y18S$_\alpha$ and D170AD1/H22K$_\alpha$ double mutants might suffer from donor-side photoinhibition under our normal growth conditions. Therefore, our results are not at odds with the proposed redox role of cyt b$_{559}$ under donor-side photoinhibitory conditions (5, 10). Alternatively, it has been reported previously that cyt b$_{559}$ is important to the assembly and/or stability of PSII (18–24). Therefore, D170AD1/Y18S$_\alpha$ and D170AD1/H22K$_\alpha$ mutations might induce structural changes of cyt b$_{559}$ that affect the assembly or stability of PSII in these double mutants.

On the other hand, our fluorescence results showed that in Y18S$_\alpha$ and H22K$_\alpha$ cyt b$_{559}$ mutant cells in the dark, the chlorophyll a fluorescence induced by a MT light pulse decayed to the $F_v$ level initially and then came back to a fluorescence level that is significantly higher than the $F_M$ (see Fig. 2, B and C). A similar but much stronger effect has been reported in a previous study on ΔpsbL tobacco mutant (48). The authors of this previous study attributed their result to the back electron flow from plastoquinol in ΔpsbL tobacco mutant and concluded that PsbL contributed to ensure unidirectional forward electron flow from Q$_\alpha$ to the PQ pool (48). Therefore, our results suggest that cyt b$_{559}$ might play a similar role as PsbL in modulation of electron flow from PSII to the PQ pool. In the recent 2.9 Å resolution x-ray crystallographic structural models of PSII, a novel PQ (Q$_C$) binding site was located in proximity to cyt b$_{559}$. The occupancy of this Qc site by PQ was proposed to be involved in exchange of PQ on the Q$_b$ site from the pool (4) or to modulate the redox equilibration between cyt b$_{559}$ and the PQ pool (16, 17). Therefore, Y18S$_\alpha$ and H22K$_\alpha$ mutations on cyt b$_{559}$ might induce a structural perturbation on this Qc site and, thus, affect exchange of PQ on the Q$_b$ site from the pool (4). Alternatively, a previous study proposed that cyt b$_{559}$ might function as a plastoquinol oxidase to keep the PQ pool and the acceptor-side of PSII from over-reduction in the dark (15). Over reduction of the PQ pool could as well lead to a change in $F_v/F_M$ (15). Therefore, impairment of this proposed plastoquinol oxidase function in Y18S$_\alpha$ and H22K$_\alpha$ cyt b$_{559}$ mutants might be also accountable for our results.

Overall, our results are consistent with a functional role of cyt b$_{559}$ in protecting PSII from the photoinactivation under acceptor-side and donor-side photoinhibitory conditions. Future spectroscopic and mutant studies on the functions of the QC and the cyclic electron transfer pathway (includes cyt b$_{559}$, Chlz, and Car molecules) in PSII will provide further structural and mechanistic insights into the exact function(s) of cyt b$_{559}$ in PSII.

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REFERENCES
1. Kern, J., and Renger, G. (2007) Photosynth. Res. 94, 183–202
2. Loll, B., Kern, J., Saenger, W., Zouni, A., and Biesiadka, J. (2005) Nature 438, 1040–1044
3. Ferreira, K.N., Iverson, T.M., Maghlaoui, K., Barber, J., and Iwata, S. (2004) Science 303, 1831–1838
4. Guskov, A., Kern, J., Gabdulkhakov, A., Broser, M., Zouni, A., and Saenger, W. (2009) Nat. Struct. Mol. Biol. 16, 334–342
5. Stewart, D. H., and Brudvig, G. W. (1998) Biochim. Biophys. Acta 1367, 63–87
6. Wada, K., and Arnon, D. I. (1971) Proc. Natl. Acad. Sci. U.S.A. 68, 3064–3068
7. Thompson, L. K., Miller, A. F., Buser, C. A., de Paula, J. C., and Brudvig, G. W. (1989) Biochemistry 28, 8048–8056
8. Roncé, M., Bousac, A., Zurita, J. L., Bottin, H., Sugiuira, M., Kirilovsky, D., and Ortega, I. M. (2003) J. Biol. Inorg. Chem. 8, 206–216
9. Kaminskaya, O., Kern, J., Shuvalov, V. A., and Renger, G. (2005) Biochim.
