

Mutational analysis of the structure and biogenesis of the photosystem I reaction center in the cyanobacterium *Synechocystis* sp. PCC 6803

(iron-sulfur center F_x /leucine zipper/photosynthesis/*psaB* gene/site-directed mutagenesis)

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Communicated by N. Edward Tolbert, October 19, 1992

ABSTRACT We have utilized the unicellular cyanobacterium *Synechocystis* sp. PCC 6803 to incorporate site-directed amino acid substitutions into the photosystem I (PSI) reaction-center protein PsaB. A cysteine residue (position 565 of PsaB) proposed to serve as a ligand to the [4Fe-4S] center F_x was changed to serine, histidine, and aspartate. These three mutants—C565S, C565H, and C565D—all exhibited greatly reduced accumulation of PSI reaction-center proteins and failed to grow autotrophically, indicating that this cysteine most likely does coordinate F_x , which is crucial for PSI biogenesis. Interestingly, the strain C565S accumulated significantly more PSI than the other two cysteine mutants and displayed photoreduction of the [4Fe-4S] terminal electron acceptors F_A and F_B . Mutations were also introduced into a leucine zipper motif of PsaB, proposed to participate in reaction-center dimerization. The mutants L522V, L536M, and L522V/L536M all exhibited wild-type characteristics and grew autotrophically, whereas the L522P mutation prevented PSI accumulation. These data do not provide support for a major structural role of the leucine zipper in reaction-center dimerization or in assembly of F_x . However, the amino acid substitutions incorporated were conservative and might not have perturbed the leucine zipper.

The photosystem I (PSI) reaction center represents a grand complex of protein subunits, both membrane-bound and extrinsic, which must assemble to bind cofactors that act to capture light energy and convert it into chemical energy (for a recent review, see ref. 1). Our goal is to gain insight into the interaction of protein subunits allowing the formation of the protein scaffold that binds electron-transfer components and their role in the chemical reactions accomplished by PSI. A heterodimer of homologous 82- to 83-kDa polypeptides, PsaA and PsaB, forms the reaction center of PSI, binding the chlorophyll (Chl) *a* dimer P700; and the electron acceptors A_0 , a Chl *a*; A_1 , probably a phylloquinone; and F_x , a [4Fe-4S] center; as well as ≈ 100 Chl antenna molecules (1). A third polypeptide, PsaC, binds the terminal electron acceptors F_A and F_B , both [4Fe-4S] centers (2). There are at least eight additional polypeptides associated with PSI, some with identified functions, others with unknown roles (1).

Although attempts to resolve the three-dimensional structure of PSI are progressing (3, 4), these data do not allow one to define precisely the protein structures important for the assembly and function of the reaction center. Therefore, we must rely on the current body of biochemical and biophysical data and analysis of evolutionary conservation to identify residues that may serve as important structural and/or functional components in the complex. The most convincing

evidence for the roles of particular residues in the PSI reaction center addresses the possible ligands to the [4Fe-4S] center F_x (5) and the role of a putative leucine zipper in the PsaA and PsaB proteins (6, 7). Cysteine residues, with few exceptions, serve as the ligands to [4Fe-4S] centers (5). There are only three conserved cysteine residues in PsaA and only two in PsaB. Two of the cysteines in PsaA are homologous to the PsaB cysteines, lying in a 12-residue sequence that is conserved in all the deduced amino acid sequences of *psaA* and *psaB* genes sequenced thus far (1). It has been proposed that F_x bridges the PsaA and PsaB polypeptides and is ligated by two cysteine residues from each of the reaction center proteins (5) (Fig. 1). Immediately adjacent to the conserved cysteine motif in both PsaA and PsaB are repeats of conserved leucines, spaced 7 residues apart and predicted to lie in α -helices (6, 7) (Fig. 1). The cysteine residues proposed to coordinate F_x lie in a position analogous to the DNA-binding region of regulatory proteins (6, 7).

We are using site-directed mutagenesis to investigate the roles of the aforementioned cysteine and leucine residues in the biogenesis and function of the PSI reaction center (Fig. 1). This genetic manipulation is most readily accomplished with the unicellular cyanobacterium *Synechocystis* sp. PCC 6803 (8, 9). PSI in the cyanobacteria is very similar to that from higher plants in both subunit composition and gene organization (1). By selecting under light-activated heterotrophic growth (LAHG) conditions (9), we have isolated segregated targeted inactivation mutants of both *psaA* and *psaB*, which encode the PSI reaction-center proteins in *Synechocystis* sp. PCC 6803 (10, 11). The ability to recover mutants lacking the PSI reaction center makes *psaA* and *psaB* viable candidates for site-directed mutagenesis.

We will describe *in vivo* site-directed amino acid changes in a PSI reaction-center protein. Site-directed mutations targeted to a proposed ligand of F_x and to a leucine zipper motif were incorporated into *psaB* and mutants were selected under LAHG conditions. Analysis of these mutants provides insight into the biogenesis and structure of the PSI reaction center.

MATERIALS AND METHODS

Chemicals and antibiotics were purchased from Sigma or Research Organics. Restriction and other enzymes were from New England Biolabs. Bacto-agar was from Difco. Nitrocellulose was from Schleicher & Schuell. [α - 32 P]dATP and [α - 35 S]thio[dATP] were from Amersham.

Experiments were performed with a glucose-tolerant strain of *Synechocystis* sp. PCC 6803, which was acclimated for

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Abbreviations: PSI, photosystem I; Chl, chlorophyll; LAHG, light-activated heterotrophic growth.

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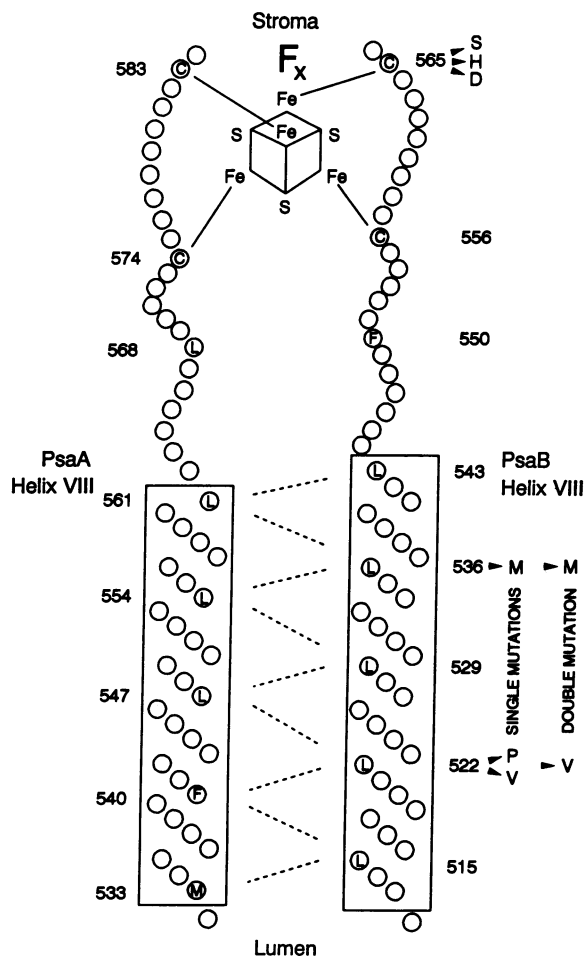


FIG. 1. Schematic diagram of portions of the PsaA and PsaB proteins from *Synechocystis* sp. PCC 6803 and of the [4Fe-4S] center F_x . Site-directed amino acid changes described in the text are indicated. Regions predicted to form α -helices are bounded by rectangles. Dashed lines represent the possible leucine zipper interaction.

growth on solid medium in the dark (8, 9). Except for tests for autotrophy, cells were grown at 30°C under LAHG conditions, as previously described (9), except a 10-min (rather than 5-min) pulse of light was used to ensure full illumination of all cultures in the growth chamber. Antibiotics were added, when appropriate, in the following concentrations: kanamycin, 5 $\mu\text{g}/\text{ml}$; spectinomycin, 20 $\mu\text{g}/\text{ml}$. Transformations were performed essentially as described (8), except in the case of strain $\Delta\text{B-RCPT}$, which was carefully maintained in dim light throughout the procedure, since it is very light-sensitive. Tests for autotrophic growth were performed using both liquid and solid medium without supplemental glucose in a chamber providing continuous light ($\approx 20 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$). To ensure that cells would grow in continuous light—i.e., that they were not light-sensitive—cultures were also grown in the light in medium with glucose. For those strains that exhibited light sensitivity, screens were used to reduce the light intensity to $\approx 3 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. Cultures for membrane isolation were grown in 15 liters of medium in carboys, bubbled with air. Cells were harvested with a continuous flow rotor (Sorvall) and were frozen at -70°C in BG-11 (8) with 15% (vol/vol) glycerol.

Nucleic acids were manipulated by standard methods (12), unless otherwise stated. Site-directed mutagenesis was performed with an oligonucleotide-directed *in vitro* mutagenesis kit as directed by the manufacturer (Amersham). DNA sequencing was performed with Sequenase version 2.0 poly-

merase as directed by the manufacturer (United States Biochemical). The polymerase chain reaction (PCR) was performed with AmpliTaq polymerase as directed by the manufacturer (Perkin-Elmer). Asymmetric PCR amplification was performed according to McCabe (13). The method for large-scale preparation of cyanobacterial DNA will be described elsewhere (L.B.S., N. R. Bowlby, S. L. Anderson, I. Sithole, and L.M., unpublished work). Small-scale preparations of DNA were performed on cells either scraped from solid medium or harvested from 50-ml liquid cultures (14).

Cells were broken with a bead beater (Biospec Products, Bartelsville, OK) and thylakoid membranes were isolated as described (10), except that 50 mM Tris at pH 8.3 was used in all solutions and the interval between bead-beater cycles was increased to 8 min. SDS/PAGE and immunoblotting were as described (10). To resolve PsaA and PsaB proteins, SDS/10% polyacrylamide gels were used; 17% gels were used to resolve PsaC and PsaD. Protein was assayed by the method of Lowry *et al.* (15). Antibodies raised in rabbits to the PsaA/B proteins from *Synechococcus* were previously described (16). Antibodies were raised in rabbits to PsaC or PsaD purified from *Escherichia coli* strains expressing, respectively, *psaC* from *Synechococcus* sp. PCC 7002 or *psaD* from *Nostoc* sp. PCC 8009 (17). PSI activity was measured in a Rank Brothers oxygen electrode (Cambridge, England) illuminated by projector lamps, using a modification of methods described by Izawa (18). Membranes equivalent to 2.5 μg of Chl were assayed in 1 ml of 50 mM Hepes, pH 7.5/10 mM NaCl/5 mM MgCl_2 at 25°C containing 1 mM KCN, 20 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea, 10 μg of superoxide dismutase (Boehringer Mannheim) per ml, 0.5 mM sodium ascorbate, 0.1 mM 2,6-dichloroindophenol, and 1.0 mM methyl viologen. Chl was extracted with methanol and quantified by using published extinction coefficients (19). A PSI complex was isolated from thylakoid membranes by using 1% (vol/vol) Triton X-100 and differential centrifugation, followed by sucrose density centrifugation in 0.1% Triton X-100 (5).

Room-temperature ESR spectroscopy was performed on a Bruker ER200D spectrometer, essentially as described (10). Low-temperature ESR spectroscopy was performed on a Bruker ECS-106 X-band spectrometer equipped with an Oxford liquid helium cryostat and temperature controller, essentially as described (20). Illumination was provided by a 150-W xenon lamp.

RESULTS

Recipient Strain. To allow for rapid segregation of mutations free of contamination from wild-type *psaB* DNA, a recipient strain of *Synechocystis* sp. PCC 6803 was engineered with the region targeted for mutagenesis deleted. A plasmid (pLS3531K) was constructed incorporating a fragment encoding kanamycin resistance (Km^R) from the plasmid pUC4K (21), flanked by a fragment encoding the 3' half of *psaA* and the 5' half of *psaB* from the plasmid pLS35 and a fragment containing the *psaB* 3' flanking region from the plasmid pLS31 (Fig. 2A). Wild-type *Synechocystis* sp. PCC 6803 was transformed with pLS3531K, and Km^R colonies were selected and segregated under LAHG conditions. Southern analysis confirmed complete segregation of the deletion mutation (data not shown). This strain ($\Delta\text{B-RCPT}$) exhibited the turquoise-blue color characteristic of cells lacking PSI due to genetic inactivation of either *psaA* or *psaB* (10, 11).

Site-Directed Mutagenesis. Oligonucleotides for site-directed mutagenesis of *psaB* were designed to effect the desired change in the coding sequence while also destroying or adding a restriction site. Site-directed mutations were made with the Amersham *in vitro* mutagenesis system and

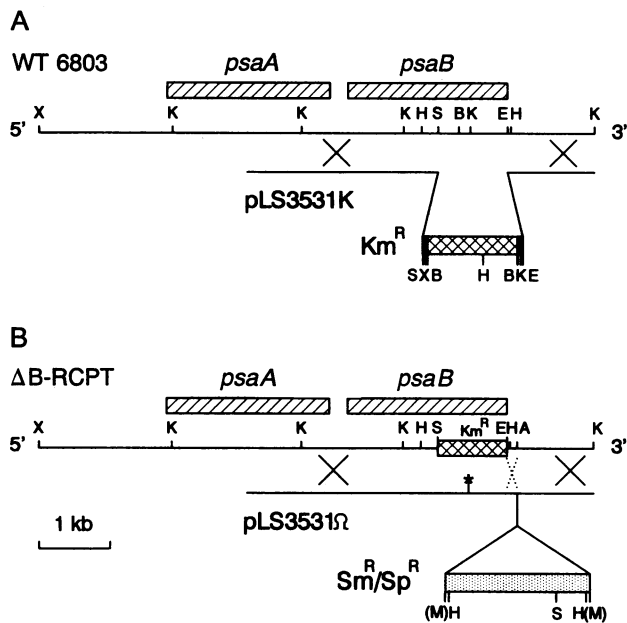


FIG. 2. (A) Map of the insert region from the plasmid pLS3531K and of the wild-type (WT) *Synechocystis* sp. PCC 6803 *psaA-psaB* operon. (B) Map of the insert region from the plasmid pLS3531 Ω and of the *psaA-psaB* region of the chromosome in strain Δ B-RCPT. Boxes represent protein-coding regions. Crosses indicate possible regions of homologous recombination. The star represents a site-directed mutation. Restriction sites: X, *Xba* I; K, *Kpn* I; H, *Hind*III; S, *Sph* I; B, *Bam*HI; E, *Eco*RI; M, *Sma* I. Sites in parentheses were destroyed in cloning.

single-stranded DNA generated from pLS15, which contains a 1.2-kbp *Hind*III fragment encoding the 3' half of *psaB* (22). Incorporation of the desired mutations was verified by restriction mapping and by DNA sequencing. A 940-bp *Afl* II-*Eco*RI fragment from each mutant pLS15 plasmid was subcloned into the *Afl* II/*Eco*RI sites of pLS35 (encoding all of *psaB*). The Ω fragment, which encodes resistance to spectinomycin and streptomycin (Sm^R/Sp^R), from the plasmid pHP45 Ω (23) was cloned into an *Afl* II site in pLS31 (encoding the 3' flanking region of *psaB*), forming the plasmid pLS31 Ω . An *Eco*RI fragment from pLS31 Ω was subcloned into the *Eco*RI site of pLS35 [forming pLS3515 Ω (Fig. 2B)] and the mutated pLS35 plasmids. pLS3531 Ω and the mutated variations of pLS3531 Ω were then used to transform the strain Δ B-RCPT and Sp^R colonies were selected under LAHG conditions.

The presence of the desired mutations in Sp^R colonies was verified by three different methods, each having particular advantages. Small-scale preparations of DNA were used as templates for conventional PCR amplification of the mutated

region of the *psaB* gene. The resulting PCR products were digested with the informative restriction enzymes, resolved by gel electrophoresis, and visualized by ethidium bromide staining (data not shown). Genomic DNA was digested with the informative restriction enzyme and other enzymes recognizing sites in this region and was subjected to Southern analysis using a fragment of *psaB* as a probe (data not shown). Finally, to verify integration of the proper sequence, the mutated region of *psaB* was amplified from CsCl gradient-purified genomic DNA by asymmetric PCR (13), and the primarily single-stranded PCR products were sequenced directly by using internal oligonucleotide primers (data not shown). In addition to initial verification of the mutants, DNA was isolated from samples taken from the large cultures to be used for phenotypic characterization to confirm that reversion had not occurred. In all of the cultures tested, no mixture of wild-type and mutant sequence or reversion of mutants to wild type was detected.

Characterization. Mutations were made in *psaB* as depicted in Fig. 1, changing the Cys565 to Ser (strain C565S), His (C565H), or Asp (C565D); changing the Leu522 to Val (L522V) or Pro (L522P); changing the Leu536 to Met (L536M); and introducing mutations at both positions 522 and 536 (L522V/L536M). To serve as a control, the strain Δ B-RCPT was transformed with the wild-type plasmid pLS3531 Ω (Fig. 2B), yielding the strain WT Ω . After genetic verification of each mutant strain, cells were tested for their ability to grow autotrophically in the light ($\approx 20 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) without glucose. The mutants C565S, C565H, C565D, and L522P, which all exhibited light sensitivity, failed to grow autotrophically, whereas L522V, L536M, and L522V/L536M grew autotrophically at near wild-type rates (Table 1). The rate of O_2 uptake by isolated membranes was measured in the presence of dichloroindophenol, an electron donor to PSI; methyl viologen, an electron acceptor from PSI; and inhibitors of PSII activity [3-(3,4-dichlorophenyl)-1,1-dimethylurea] and cytochrome oxidase activity (KCN) (18). The rates of O_2 uptake by isolated membranes from L522V, L536M, and L522V/L536M mutants were slightly lower than, but comparable to, that by WT Ω membranes (Table 1). The rates of O_2 uptake by isolated membranes from C565H, C565D, and L522P were all approximately equal and were very low relative to WT Ω (Table 1). The rates from these three strains were comparable to rates measured with isolated membranes from strain BDK8 (data not shown), in which the *psaB* gene has been inactivated (11). The rates from strain C565S were >2-fold higher than those from the other two cysteine mutants but were still significantly lower than that from WT Ω (Table 1).

The reduced rates of O_2 uptake measured for membranes from C565S, C565H, C565D, and L522P could have been the result of the lack of stable assembly of PSI or the assembly of PSI with reduced activity. Chl accumulation in *Synechocystis* sp. PCC 6803 reflects the accumulation of photo-

Table 1. Characterization of *psaB* mutants

Strain	Autotroph	Rate of O_2 uptake*	O_2 uptake per cell [†]	Chl per cell [‡]	Chl/protein in membrane [§]
WT Ω	Yes	2353 \pm 287	1624	0.69 \pm 0.10	10.71 \pm 2.47
C565S	No	765 \pm 185	459	0.60 \pm 0.21	12.98 \pm 4.01
C565H	No	289 \pm 30	127	0.44 \pm 0.01	8.78 \pm 1.62
C565D	No	309 \pm 60	164	0.53 \pm 0.02	8.41 \pm 2.62
L522V	Yes	1991 \pm 102	1234	0.62 \pm 0.08	11.20 \pm 1.88
L536M	Yes	1648 \pm 289	1467	0.89 \pm 0.23	16.36 \pm 2.84
L522V/L536M	Yes	1905 \pm 266	1372	0.72 \pm 0.09	12.29 \pm 1.97
L522P	No	274 \pm 34	142	0.52 \pm 0.02	8.42 \pm 1.63

*Micromoles of O_2 per mg of Chl per hr (average of at least five assays from at least two preparations).

[†]Product of O_2 uptake rate and Chl per cell.

[‡]Milligrams of Chl per OD₇₃₀ unit per liter (average of at least four assays from at least two cultures).

[§]Micrograms of Chl per mg of protein (average of at least two assays from at least two preparations).

synthetic reaction-center proteins (10). The strains C565H, C565D, and L522P all had reduced amounts of Chl, while accumulation in the other strains was in some cases slightly reduced, but comparable to that in WT Ω (Table 1). The mutants C565S, L522V, L536M, and L522V/L536M were green in color, while C565H, C565D, and L522P exhibited a turquoise-blue color (data not shown). Variability in the Chl determinations may have been introduced by differing accumulation of PSII or by the presence of free Chl.

To assay the accumulation of PSI proteins more precisely, immunoblotting of membrane proteins was performed with antibodies recognizing PsaA/B, PsaC, or PsaD (Fig. 3). The crossreaction of all three antibodies to protein from strains L522V, L536M, and L522V/L536M appeared to be approximately equal to that of wild type. All three antibodies recognized reduced levels of protein in membranes from C565S relative to wild type. However, greatly reduced amounts of the PsaA/B proteins were detected in membranes from C565H and C565D, and PsaA/B was undetectable in membranes from L522P. The PsaC and PsaD proteins were not detected by antibodies in membranes from C565H, C565D, or L522P. However, the PsaD antibody did recognize a protein of ≈ 11 kDa with equal intensity in all the samples. Very faint crossreaction to a protein with approximately the same molecular mass has been seen with different antiserum recognizing PsaD (10), suggesting the presence of another protein with an epitope in common with PsaD.

Room-temperature ESR spectroscopy was utilized to detect signal I, from P₇₀₀⁺ (data not shown). Data from room-temperature ESR spectroscopy correlated with the results of activity assays and immunoblotting. The spectra from L522V, L536M, and L522V/L536M were essentially the same as that of WT Ω , with a feature typical of signal I and of approximately equal intensity. The spectrum of membranes from strain L522P lacked any prominent features, once signal II (from Y_D⁺ in PSII) had been subtracted. The spectra of membranes from the three cysteine mutants included a feature of similar intensity, reduced relative to WT Ω , with a lineshape, linewidth, and approximate *g* value expected for signal I.

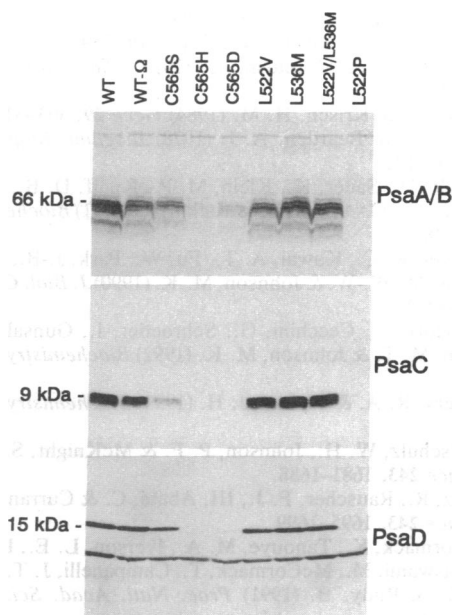


FIG. 3. Immunoblots of thylakoid membrane proteins, probed with antibodies recognizing PsaA/B, PsaC, or PsaD. Equal amounts of protein (150 μ g) were loaded in each lane. Molecular mass was estimated by comparison to migration of prestained molecular weight standards (Diversified Biotech, Newton, MA). WT, wild type.

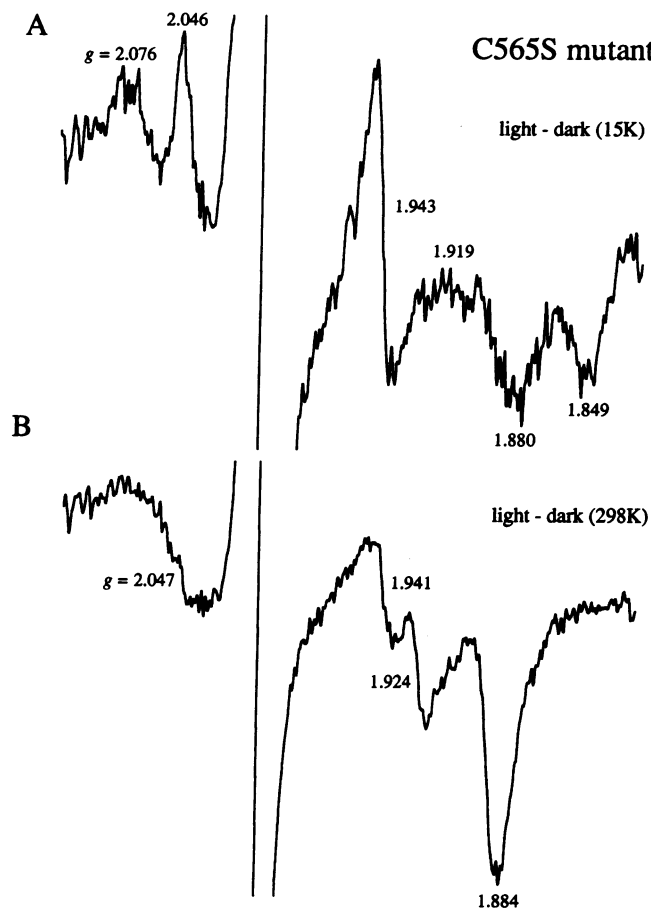


FIG. 4. Low-temperature ESR spectra of PSI complex isolated from the C565S mutant. Spectra were taken at 15 K under illumination and the dark spectrum was subtracted. (A) Spectrum of complex frozen in the dark and illuminated at 15 K. (B) Spectrum of complex illuminated at 298 K and frozen under illumination. The spectrum in A was multiplied by a factor of 2 relative to that in B.

ESR spectroscopy was also performed at cryogenic temperatures to detect signals generated by reduction of the [4Fe-4S] centers F_A and F_B bound by the PsaC protein. F_A is characterized by *g* values of 2.05, 1.94, and 1.86, whereas F_B has *g* values of 2.07, 1.92, and 1.89 (24). When both centers are reduced, their high-field and low-field signals merge, producing a spectrum with *g* values of 2.05, 1.94, 1.92, and 1.89 (24). The low-temperature spectra of membranes from strains L522V, L536M, and L522V/L536M were essentially wild-type, while F_A/F_B signals were not present in spectra of membranes from the strains C565H, C565D, and L522P (data not shown). Signals from photoreduced F_A/F_B were present in spectra of membranes from C565S (data not shown) and in spectra of PSI complex isolated from C565S membranes (Fig. 4). In both cases, the F_A and F_B resonances were present at reduced intensity, but with similar *g* values, relative to wild-type PSI complex.

DISCUSSION

Changes of Cys-565 of PsaB have profound effects on the stable assembly of PSI. The mutants C565S, C565H, and C565D all have greatly reduced accumulation of the PsaA, PsaB, PsaC, and PsaD polypeptides, as well as reduced or no PSI activity. Assaying for the ability of these strains to grow autotrophically is complicated by their sensitivity to continuous light above $\approx 3 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. These strains will grow in dim light ($< 3 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) with glucose, but the low light intensity in combination with reduced amounts of assembled

PSI may not allow autotrophic growth, even if photosynthetic electron transport is functional in the mutants. The mutant C565S does accumulate significantly more PSI than the other two cysteine mutants, reflected in higher Chl accumulation, higher O₂ uptake rates, and stronger signals on immunoblots. Electron transfer to the terminal electron acceptors in PSI, F_A and F_B, was detected in complexes from C565S, but the amount of F_A and F_B photoreduced was less than in wild-type complex on a Chl basis.

The three residue substitutions incorporated at position 565 represent the non-cysteine residues found to coordinate iron-sulfur centers in naturally occurring or mutant iron-sulfur proteins. Histidine serves as a ligand to the [2Fe-2S] center in the Reiske iron-sulfur protein from spinach (25). An aspartate serves as a ligand to one of the iron-sulfur centers in ferredoxin from *Pyrococcus furiosus* (26). Substitution of aspartate for cysteine by site-directed mutagenesis of the PsaC protein expressed in *E. coli* and reconstituted *in vitro* also causes an inactive [3Fe-4S] center to form, rather than a [4Fe-4S] center (20). Substitutions of serine for cysteine in center 2 of the *E. coli* fumarate reductase or in the *E. coli* dimethyl sulfoxide reductase prevent formation of [4Fe-4S] centers, although in the latter protein a [3Fe-4S] center does form (27, 28). It is important to note that all of these examples represent iron-sulfur centers with intrasubunit ligands. The F_x center is a rare example of intersubunit coordination of an iron-sulfur center.

The conservative mutations introduced into the leucine zipper motif of PsaB had no detectable effect on PSI accumulation or activity. All the assayed characteristics of the mutants L522V, L536M, and L522V/L536M were essentially wild-type.

The residue substitutions incorporated into the leucine zipper motif have been effective in perturbing leucine zippers in other proteins (29, 30). However, heptad repeats of leucines are also found in ion channels and do not appear to act primarily in subunit dimerization (31). Single valine-for-leucine substitutions in the *Drosophila* Shaker K⁺ channel protein altered properties of the voltage-dependent channel opening, implying a role of the repeated leucines in pore formation or channel gating (31).

In contrast to the introduction of conservative substitutions, the introduction of a proline at position 522 of PsaB had a deleterious effect on the biogenesis of PSI. In all aspects measured, this strain has the phenotype of BDK8, an inactivation mutant of *psaB* (11). The leucine-to-proline residue substitution probably disrupts the secondary structure of the eighth α -helix of PsaB.

Conclusions. Our data indicate that Cys-565 of PsaB serves as a ligand to the [4Fe-4S] center F_x and support the proposal that F_x bridges the PSI reaction-center proteins (5). Not only does F_x act as an electron-transfer component, but this [4Fe-4S] center also plays a major structural role in reaction-center dimerization and PSI complex stability. Our data do not definitively address the role of the leucine zipper, but suggest one of the following conclusions. (i) The mutations introduced (other than L522P) were too conservative to disrupt the leucine zipper interaction; (ii) the leucine zipper was disrupted but plays a minor role in PSI biogenesis, perhaps acting functionally in an as yet unmeasured capacity; or (iii) the leucine zipper does not form in the PSI reaction center of *Synechocystis* sp. PCC 6803.

We are grateful to Dr. N. R. Bowlby and Dr. G. T. Babcock (Department of Chemistry, Michigan State University) for room-

temperature ESR spectroscopy and for helpful discussions. We thank Dr. J. Guikema (Kansas State University) and Dr. D. A. Bryant (Pennsylvania State University, University Park, PA) for providing PsaA/B antibodies and for PsaC and PsaD proteins, respectively. This work was supported by the Department of Energy (DE-FG02-90-ER 20021 to L.M.), by Michigan State University Research Excellence Funds (to L.M.), and by the National Science Foundation (MCB-9205756 to J.H.G.).

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