Topography of the Heme Prosthetic Group of Cytochrome *b*-559 in the Photosystem II Reaction Center[†,‡]

G.-S. Tae[‖] and W. A. Cramer[*]

Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907

Received March 7, 1994; Revised Manuscript Received June 13, 1994[⊛]

ABSTRACT: The topography of the heme prosthetic group of cytochrome *b*-559 of the photosystem II reaction center was determined from measurement of the orientation of its α- and β-polypeptides in thylakoid membranes of spinach chloroplasts and in osmotically disrupted cells of the cyanobacterium *Synechocystis* sp. PCC 6803. The accessibility to trypsin proteolysis of an epitope located near the solvent-exposed N-terminus of the β-subunit was compared to that of the α-subunit, whose N- and C-termini had previously been localized from the trypsinolysis pattern to the stromal and lumenal sides of spinach thylakoid membranes, respectively [Tae *et al.* (1988) *Biochemistry 27*, 9075–9080; Vallon *et al.* (1989) *Biochim. Biophys. Acta 975*, 132–141]. The N-terminal epitope of the cyanobacterial β-subunit was modified by introducing a tridecapeptide epitope, previously found to be immunoreactive, from the C-terminal region of the spinach chloroplast α-subunit. This epitope had no homology with the cyanobacterial α-subunit. The cells with the hybrid β-subunit retained full photosynthetic activity. The intactness of membranes from osmotically shocked cyanobacteria was tested by trypsin inaccessibility to (a) the α-subunit C-terminus and (b) the manganese-stabilizing protein (MSP) of the oxygen-evolving complex that is on the lumenal side of the membrane. The loss after trypsinolysis of most of the β-subunit immunoreactivity, under conditions where (i) the α-subunit was cleaved near the N-terminus in both spinach thylakoids and osmotically shocked cyanobacterial membranes and (ii) the MSP protein in cyanobacteria was not disrupted, implied that the orientation of the β-subunit was parallel to that of the α-subunit in both kinds of membranes. This is consistent with expectations from the *cis*-positive rule for orientation of integral membrane proteins [von Heijne & Gavel (1988) *Eur. J. Biochem.* 74, 671–678] and the very positive E_m of cytochrome *b*-559 [Krishtalik *et al.* (1993) *Biophys. J. 65*, 184–195]. For this cytochrome, the polypeptide orientation uniquely determines the heme topography, because its coordinationn is *bis*-histidine and each polypeptide contains only one histidine, located near the N-terminal side of the single hydrophobic α-helix. The heme prosthetic group must therefore be located close to the stromal or cytoplasmic interface of the membrane.

The photosystem II reaction center complex includes the four major polypeptides D1, D2, and the α- and β-subunits of cyt *b*-559.[1] Studies on the structure of the cytochrome are of interest because (i) the topography of its α-subunit in the membrane is known (Tae *et al.*, 1988; Vallon *et al.*, 1989) and (ii) although the electron transport function is enigmatic (Cramer *et al.*, 1993), it has the unusual aspect that it is involved in a response to stress, particularly photoinhibition (Thompson & Brudvig, 1988; Nedbal *et al.*, 1992; Barber & De Las Rivas, 1993).

Cytochrome *b*-559 has the unique structure of a heme cross-linked dimeric integral membrane protein, based on (i) location and sequencing of a gene in the spinach plastid genome, *psbE*, with an 82 amino acid reading frame that codes for a 26-residue hydrophobic domain containing a single His residue near its N-terminal side; (ii) identification of a reading frame, *psbF*, starting 9 bases downstream of *psbE*, coding for a 38-residue polypeptide that also contained a 25-residue hydrophobic segment and one His residue (Herrmann *et al.*, 1984); (iii) a 1:1 stoichiometry (Widger *et al.*, 1985) of the *psbE* and *psbF* gene products, α- and β-subunits; and (iv) *bis*-histidine ligation of the heme (Babcock *et al.*, 1985) in the purified cytochrome (Widger *et al.*, 1984). Because the α- and β-subunits were recovered in 1:1 stoichiometry in a single fraction (Widger *et al.*, 1985), the unit for heme coordination would have to be an αβ heterodimer (Figure 1A) if there were only one cyt *b*-559 heme per reaction center (Miyazaki *et al.*, 1989; Buser *et al.*, 1992; Tang & Diner, 1994). Moreover, the single His residue in each polypeptide and the *bis*-histidine coordination would require that the two polypeptides have a parallel orientation in the membrane bilayer. However, an *in situ* stoichiometry of two high-potential *b*-559 hemes per PSII reaction center in thylakoids (Whitmarsh & Ort, 1984), two hemes per center in *Synechocystis* sp. PCC 6803 (MacDonald *et al.*, 1994), and a radiation cross-section in reaction centers corresponding to a dimeric cytochrome (Takahashi & Asada, 1989) suggest that the cyt *b*-559 heme to P-680 stoichiometry is two, but that one heme can be lost in more highly purified reaction center preparations. In the case of two hemes, the units for heme coordination and cytochrome structure could be α_2 and β_2 (Figure 1C), as well as $(\alpha\beta)_2$ (Figure 1B), and the involvement of the β-subunit creates additional possibilities for the topography because it could have an orientation parallel or antiparallel to the α-subunit.

[†] This study was supported by NIH GM-38323.

[‡] This paper is dedicated to Professor Achim Trebst on the occasion of his 65th birthday.

[*] To whom correspondence should be addressed.

[‖] Present address: Department of Biology, Dankook University, Anseo-Dong, Cheonan, Choongnam, Korea.

[⊛] Abstract published in *Advance ACS Abstracts,* August 1, 1994.

[1] Abbreviations: BSA, bovine serum albumin; Chl, chlorophyll; cyt, cytochrome; DCBQ, 2,5-dichloro-*p*-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; MSP, manganese stabilizing protein; NTP, nucleoside triphosphate; OEC, oxygen-evolving complex; PCR, polymerase chain reaction; PMSF, phenylmethanesulfonyl fluoride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TBS, Tris-buffered saline.

0006-2960/94/0433-10060$04.50/0

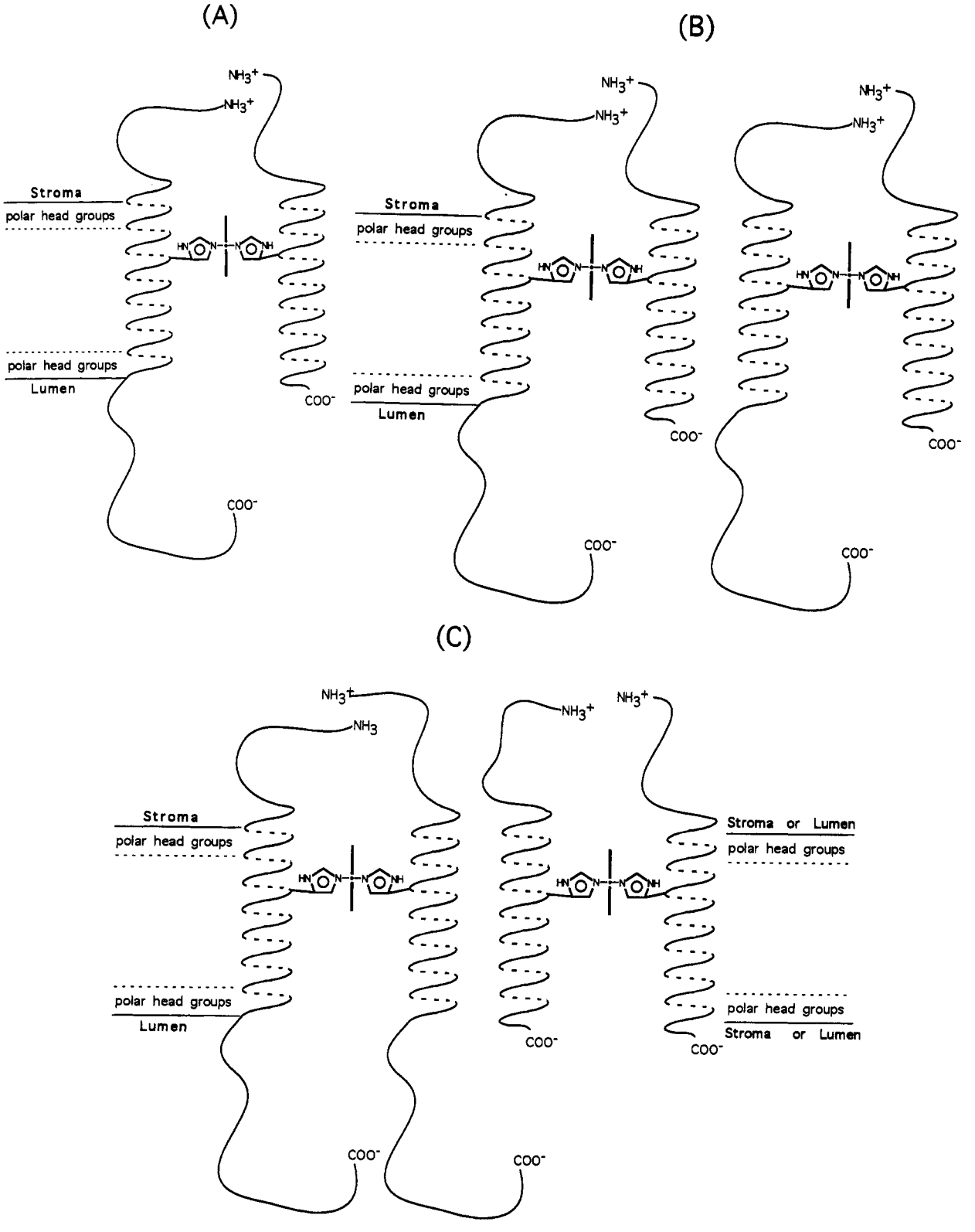

FIGURE 1: Models for orientation of (A) the unit $\alpha\beta$ heterodimer, (B) the diheme cyt *b*-559 $\alpha\beta$ heterodimer, and (C) α_2 and β_2 homodimers in the thylakoid membrane bilayer. The heme iron (symbolized by a dot in the middle of the vertical bar) is coordinated by His-22 and His-17 of the α- and β-subunits, respectively. The N- and C-termini of the α-subunit are positioned on the stromal and the lumenal sides of the membrane, respectively (Tae *et al.*, 1988; Vallon *et al.*, 1989).

Elucidation of the position of the hemes in the thylakoid membrane would help to solve the problem of the enigmatic function of this cytochrome because one will then have more information on the vectorial nature of the electron transport reaction(s) in which it is thought to participate. Because each polypeptide contains a single His residue located five residues from N-terminus of the hydrophobic domain, the orientation of these polypeptides in the membrane bilayer will determine the position of the heme in the thylakoid membrane. On the basis of the accessibility of protease to residues Arg-7 or Glu-6/Asp-11 of the α-subunit in intact thylakoid membranes probed with an epitope-specific antibody (Tae *et al.*, 1988)

and their relative access to the same or similar antibodies using immunoelectron microscopy (Vallon *et al.*, 1989), the N-terminus of the α-subunit was localized to the stromal side. As a consequence of this orientation, the single His residue in the α-subunit is located on the stromal side of the hydrophobic domain. Because the β-subunit contains a single His residue with a similar location in its hydrophobic domain, heme coordination by an $\alpha\beta$ heterodimer would require that both hemes be positioned on the stromal side. However, in the absence of a measurement of the orientation of the β-subunit, the possibility that one heme is coordinated by a β_2 homodimer allows the possibility that one heme could be located on the lumenal side (Figure 1C). Another possible structural arrangement that would allow a heme on each side of the membrane would be a dimer of fused tail-to-head α- and β-subunits (suggested by H. Pakrasi and J. Whitmarsh).

Molecular genetic techniques have been developed to introduce specific mutations in the photosystem II polypeptides of the photoheterotrophic cyanobacterium *Synechocystis* sp. PCC 6803 (Williams, 1988) in order to analyze structure and function relationships of the PSII complex. In the present study, the N-terminal domain of the cyt *b*-559 β-subunit (heptapeptide A1–Q7) in *Synechocystis* sp. PCC 6803 was replaced by an immunoreactive spinach epitope of the α-subunit (tridecapeptide R68–R80) using site-directed mutagenesis. On the basis of the accessibility of trypsin to both α- and β-subunits of cyt *b*-559 in thylakoid membranes from *Synechocystis* sp. PCC 6803, as well as spinach thylakoid membranes, the transmembrane orientation of both subunits of cytochrome *b*-559 in the thylakoid membrane has been determined. The location of the heme coordinated by these subunits is near the stromal or periplasmic side of the membrane.

MATERIALS AND METHODS

Preparation of Thylakoids. Spinach thylakoid membranes were prepared by osmotic shock from intact chloroplasts. The latter were made from approximately 35 g of leaves by (i) briefly homogenizing in a medium consisting of 0.33 M sorbitol, 5 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, 0.2% BSA, and 50 mM HEPES, pH 7.5; (ii) coarse filtration of the homogenate and centrifugation at ca. 1500*g* for 3 min (4 °C); and (iii) further purification on a Percoll gradient. The osmotic shock medium was as in Tae *et al.* (1988).

Site-Directed Mutagenesis of the psbF Gene in Synechocystis sp. PCC 6803. In order to introduce the spinach antigenic site (tridecapeptide R68–R80) (Tae *et al.*, 1988) to the cyt *b*-559 β-subunit to *Synechocystis* sp. PCC 6803, two synthetic oligonucleotides 5′-GGTAATTAACAATGCAACTTGATGAATTTAGTAGACGGGTTACTTATCCC-3′ and 5′-TGGTAATTATCAATGCGTTTTGACTCTTTTGGAACAACTTGATGAATTTAG-3′ were used as primers. The 21 nucleotides in the plasmid pCT901 (Tae *et al.*, 1988), corresponding to the first seven amino acid residues of the β-subunit (1-ATQNPNQ-7), were replaced by the DNA fragment encoding 13 amino acid residues (NH_2-RFDSLEQLDEFSR-COOH) using site-directed mutagenesis (Kunkel, 1985; Bebenek & Kunkel, 1989). The Pro at the original position 8 was also mutated to an Arg residue to facilitate cleavage by trypsin. Each of the synthetic phosphorylated oligonucleotides was annealed to single-stranded DNA (65 °C, 2 min) extracted from M13KO7 phage in 50 mM NaCl, 20 mM $MgCl_2$, and 20 mM Tris-HCl, pH 7.5, and the annealing mixture was cooled to 25 °C in 15–20 min. After the mutant oligonucleotide was annealed to the template, 10-fold concentrated synthesis buffer (5 mM each dATP, dGTP, dCTP, and dTTP, 10 mM ATP, 50 mM $MgCl_2$, 20 mM DTT, and 100 mM Tris-HCl, pH 7.9), Gene 32 product, T4 DNA polymerase, and T4 DNA ligase were added directly to the annealing mixture and incubated at 37 °C for 90 min to synthesize the mutated complementary DNA strand. The resulting closed heteroduplex DNA was transformed directly to the *Escherichia coli* MV1190 strain. Several colonies were picked and plasmids were isolated from individual colonies. The mutants were selected by direct DNA sequencing of the plasmid (Tabor & Richardson, 1987).

Transformation of the Mutated psbF Gene in pCT901 into the psbEF Deletion Mutant. Thirty milliliters of the *psbEF* deletion mutant (T1297; Pakrasi *et al.*, 1988) grown to (2–5) $\times 10^8$ cells/mL (A_{730} = 0.25 corresponds to 10^8 cells/mL) were harvested by centrifugation at room temperature (5000*g*, 10 min) and resuspended in 1 mL of fresh BG-11 medium containing 5 mM glucose. The mutated plasmid DNA (10 μL; 2 mg of DNA/mL) was added to T1297 cells and incubated for 4 h in a sterile Falcon tube (13 mL) under standard temperature and light conditions while shaking. The transformed cells were grown another 18 h while shaking after addition of 4 mL of BG-11 with 5 mM glucose. Cells were harvested by centrifugation (microcentrifuge, 10000*g*, 2 min), resuspended in 1 mL of fresh BG-11 medium/glucose, and spread on Petri dishes containing glucose (5 mM) and spectinomycin (25 μg/mL). Colonies of the transformed cells could be seen in 10–15 days.

Growth and Manipulation of Cyanobacterial Strains. The wild-type and the mutant strains of *Synechocystis* sp. PCC 6803 were grown either in liquid medium (BG-11) or an agar plates under constant illumination [30–50 μEinstein/(m^2·s)] at 30 °C (Rippka *et al.*, 1979; Williams, 1988). The 2-fold concentrated BG-11 medium and 3% (w/v) Bacto-agar (Difco, Detroit, MI) were separately autoclaved and mixed. The supplements such as 5 mM glucose, 25 μg/mL spectinomycin, or 20 μg/mL kanamycin were added when the agarose solution was cooled to 55–60 °C. The hybrid β-subunit mutant of cyt *b*-559, and the *psbEF* deletion mutant (T1297) were propagated and maintained on plates containing spectinomycin (25 μg/mL) or kanamycin (20 μg/mL), respectively.

Isolation of the Chromosomal DNA from Synechocystis sp. PCC 6803. One hundred milliliters of a late log-phase cell culture grown at 30 °C was harvested by centrifugation (5000*g*, 10 min), washed once with Tris–EDTA buffer (5 mM EDTA and 50 mM Tris-HCl, pH 7.5) and resuspended in saturated NaI solution (5 mL). Cells were heated to 65 °C for 20 min and centrifuged (10000*g*, 10 min). The sediment was washed once with Tris–EDTA buffer and resuspended in 10 mL of Tris–EDTA containing lysozyme (4 mg/mL). After incubation (37 °C, 45 min), 2% (w/v) SDS, 2% (w/v) *N*-laurylsarcosine, and 100 μg/mL proteinase K (745723, Boehringer) were added and incubated (50 °C, 1–2 h). The lysate was extracted twice with a 1:1 mixture of phenol and chloroform (1 h) and once with chloroform (1 h). The upper phase was precipitated with 0.1 volume of 7.5 M ammonium acetate, pH 7.8, and 2 volumes of 100% ethanol. The chromosomal DNA dried under vacuum was redissolved in 2 mL of TE buffer (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA) and 40 μL of 1 M $MgCl_2$.

Identification of Mutation Site by the Polymerase Chain Reaction. The *psbF* gene carrying the spinach cyt *b*-559 α-subunit epitope (R68–R80) at its 5′ end was amplified from the cyanobacterial genome, extracted from hybrid β-subunit mutant cells, by use of PCR with *Taq* DNA polymerase (Saiki

et al., 1985) in order to ascertain that the correct mutation had been introduced into the cyanobacterium. Two synthetic oligonucleotide 30-mers were used as primers for the DNA polymerization: (i) 5′-CCGAATTCGGTCAGGGACTAC-CGGCGAGCG-3′ (forward primer; G + C content, 60%), to hybridize the complementary strand upstream of the *psbE* gene including the start codon, and (ii) 5′-GAAGCTTC-CCAACACAGCCACCAACAATAG-3′ (reverse primer; G + C content, 60%), which was complementary to the region in the middle of the coding strand of the *psbL* gene, located immediately downstream of the *psbE* and *F* genes. Thirty PCR cycles (each 60 s at 94 °C for denaturation of the double-stranded DNA followed by 60 s at 55 °C and 60 s at 72 °C for hybridization and chain elongation, respectively), in a reaction medium (100 μL; Promega, M190A) including 0.8 mM dNTP, 2 mM $MgCl_2$, 1 μM forward primer, 1 μM reverse primer, 0.5 μg of chromosomal DNA, and 2 units of *Taq* DNA polymerase (Promega, M186A), were sufficient to generate several micrograms of amplified 0.5 kb DNA fragment. The amplified DNA fragments (0.5 kb) were passed through a resin (Promega, A7170) to remove the nonbound primers and sequenced directly with ^{32}P-labeled sequencing primer and sequencing-grade *Taq* DNA polymerase (Promega, Q4100). The sequencing primer (10 pmol) was labeled at 37 °C for 30 min using 10 pmol of [γ-^{32}P]ATP with T4 polynucleotide kinase (5 units) in 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM spermidine, and 50 mM Tris-HCl, pH 7.5, and then the reaction mixture was incubated at 90 °C for 3 min to inactivate the kinase activity. The ^{32}P-labeled sequencing primer was used directly without further purification. Sequencing-grade *Taq* DNA polymerase (5 units) was added to the sequencing reaction mix (final volume, 16 μL) containing 1.5 pmol of ^{32}P-labeled primer and the purified PCR-DNA template (4 pmol) in 2 mM $MgCl_2$ and 125 mM Tris-HCl, pH 9.0. Enzyme/primer/template mix (4 μL) was added to each of the dideoxynucleoside triphosphates (ddNTPs) (Promega, Q4100), and mineral oil (40 μL) was added to each tube. After 40 PCR cycles (each 30 s at 95 °C for denaturation, followed by 30 s at 42 °C and 60 s at 70 °C for annealing and chain elongation, respectively), the reaction mix was directly analyzed on a sequencing gel after addition of stop solution (10 mM NaOH, 95% formamide, 0.05% bromophenol blue, and 0.05% xylene cyanol).

Osmotic Shock Preparation of Cells of Synechocystis sp. PCC 6803. Ten liters of cells grown in BG-11 medium containing 5 mM glucose and 50 μg/mL spectinomycin were harvested in late log phase by centrifugation (7000*g*, 10 min). The pellet was washed once with 0.6 M sucrose, 5 mM EDTA, and 10 mM HEPES, pH 7.0, and resuspended to 1 g of cells/10 mL in the same buffer with 0.2% (w/v) lysozyme. The cells were incubated at 30 °C for 12 h under illumination [30–50 μEinstein/(m^2·s)] while shaking (Omata & Murata, 1985; Murata & Omata, 1988). Cells were harvested by centrifugation (7000*g*, 10 min), resuspended in 50 mM potassium phosphate, pH 6.8, 30 mM sodium citrate, and 0.2 mM $CaCl_2$ containing protease inhibitors (1 mM PMSF, 2 mM benzamidine, and 2 mM ε-aminocaproic acid) (Widger *et al.*, 1984), incubated on ice for 30 min, and harvested by centrifugation (7000*g*, 10 min). This osmotic shock step was repeated two times. The osmotically shocked cells were washed twice with the HEPES–NaCl solution (10 mM HEPES, pH 7.5, and 10 mM NaCl) and resuspended to 20 μg of Chl/mL in the HEPES–NaCl solution with 0.1 M sucrose for trypsin digestion.

Trypsinolysis and Thylakoid Membrane Extraction. The osmotically disrupted cells were incubated with trypsin (Chl: trypsin = 10:1) in 20 μg of Chl/reaction for the indicated times (0, 15, 30, and 60 min) at room temperature (ca. 20 °C). The digestion reactions were terminated by adding three different kinds of protease inhibitors (2 mM PMSF, 4 mM benzamidine, and 4 mM ε-aminocaproic acid). The trypsin-treated cells were added to a Beadbeater (Biospec Products, Bartlesville, OK) chamber (250 mL) filled to $^2/_3$ capacity with 0.1-mm diameter glass beads and broken in one 30-s cycle. After centrifugation (8000*g*, 10 min), most of the thylakoid membranes remain in the supernatant and were sedimented following the addition of 40 mM $CaCl_2$ by a second centrifugation step (25000*g*, 30 min). The resulting sedimennt was resuspended in 0.8 M sucrose and 50 mM MES, pH 6.0, centrifuged again (30000*g*, 20 min), and resuspended in 50 mM sucrose, 10 mM NaCl, and 10 mM HEPES, pH 7.5. The chlorophyll concentration was measured according to Lichtenthaler (1987).

Peptide Synthesis. The undecapeptide (NH_2-T-R-Q-E-L-P-I-L-Q-E-R-COOH) and the decapeptide (NH_2-T-I-D-R-T-Y-P-I-F-T-COOH), corresponding to residues 58–68 from the *psbE* polypeptide from *Synechocystis* sp. PCC 6803 and to residues 1–10 from the *psbF* polypeptide from spinach, respectively, were synthesized with an Applied Bioscience 430A automated solid-phase synthesizer at the Purdue University Biotechnology Center with standard techniques. The purity and the mass of the product were assayed by reverse-phase (water–acetonitrile) HPLC and mass spectroscopy, respectively.

Preparation of Antibody. Keyhole limpet hemocyanin was dissolved with an excess (molar ratio, 15–30) of synthetic peptide in 0.1 M potassium phosphate buffer (pH 7.5). Glutaraldehyde (20 mM, 0.5 mL) in phosphate buffer was added dropwise while the mixture was being stirred at room temperature, and the mixture was dialyzed overnight at 4 °C against 0.15 M NaCl and 0.10 M potassium phosphate buffer, pH 7.2. A total of 1.0 mL was mixed with an equal volume of complete Freund's adjuvant, aliquots of the resulting emulsion were injected into young female rabbits at multiple intradermal sites, and a booster was given after 4 weeks with a mixture of incomplete Freund's adjuvant.

Immunoblot Analysis. The mixture of proteins to be blotted was separated on SDS–PAGE, using a gel system of Schägger & von Jagow (1987) with a 9.9–19.8% (w/w) gradient acrylamide concentration (acrylamide:bisacrylamide = 8.25:1). The gel was incubated in 30 mM Tris-HCl, pH 8.0, including 17 mM boric acid, 2 mM SDS, and 20% methanol for 10–15 min at room temperature. Proteins were transferred to nitrocellulose paper (pore size 0.1 μm; Schleicher & Schuell) with a semidry transfer blotter (13 V constant voltage or 130 mA constant current, 3 h; Model TE70, Hoefer Scientific Instruments). The paper was washed in TBS buffer (0.15 M NaCl and 10 mM Tris-HCl, pH 7.5), incubated in a "Seal-a-Meal" bag with 10% milk casein on a nutator (1 h, room temperature), removed from the bag, and washed in TBS buffer. After nutation with antibody in TBS buffer containing 0.5% (w/v) bovine serum albumin (BSA) for 2–3 h, the paper was washed in TBS buffer containing 0.1% (w/v) Nonidet-40 and again incubated with a second antibody [goat anti-rabbit IgG conjugated with horseradish peroxidase (Type VI, Accurate Chemical and Scientific Corp.)] in TBS buffer containing 0.5% BSA. The paper was nutated for 1 h, washed in TBS buffer containing 0.1% (w/v) Nonidet-40, and stained

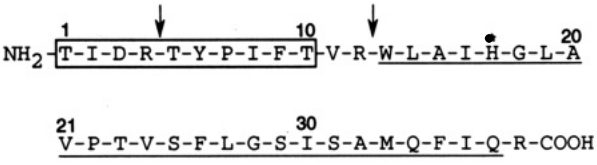

FIGURE 2: Amino acid sequence of spinach chloroplast cyt *b*-559 β-subunit. The amino acid sequence is written in one-letter code. The central hydrophobic domain of 25 residues is underlined; single His residue is noted (*); potential trypsin cleavage sites near amino and carboxy termini are marked by arrows; and the decapeptide epitope near the N teminus is boxed.

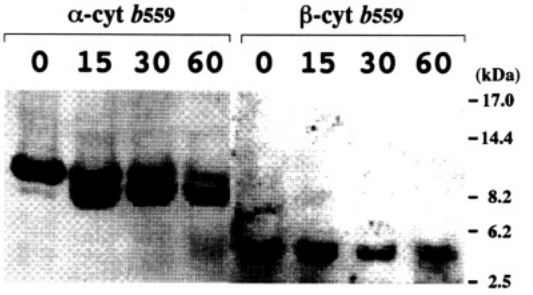

FIGURE 3: Trypsinolysis of the α- (left) and β-subunit (right) of cytochrome *b*-559 in intact thylakoid membranes. Thylakoid membranes were treated with trypsin [1:10 (w/w) trypsin: chlorophyll] for 15, 30, and 60 min at room temperature (Tae *et al.*, 1988) and analyzed by 15–20% gradient SDS–PAGE including 4 M urea. The gel was incubated in 10 mM Tris buffer (pH 8.0, 10 min, 25 °C) and transferred (130 mA, constant current, 30–60 min) onto the nitrocellulose paper (pore size 0.45 μm diameter). Each lane was loaded with the membrane equivalent of 10 μg of chlorophyll. Color reactions were performed with 0.017% 4-chloro-1-naphthol and 0.05% H_2O_2 in TBS.

for 10 min with 0.017% 4-chloro-1-naphthol and hydrogen peroxide in TBS buffer.

O_2 Evolution Activity. Steady-state electron transport activities of the wild-type and the hybrid β-subunit mutant were measured at 25 °C using a Clark-type O_2 electrode and a saturating actinic light intensity of 2000–2500 μEinstein/(m^2·s). Measurements were performed with cells (equivalent to 10 μg of Chl/mL) in BG-11 growth medium containing 0.25 mM DCBQ and 0.25 mM ferricyanide as the electron acceptor.

RESULTS

Effect of Trypsin on Reactivity of the Cyt b-559 β-Subunit with Antibody to Its N-Terminus. Antibody was generated to the synthetic decapeptide corresponding to the N-terminal region (the boxed sequence in Figure 2) of the cytochrome *b*-559 β-subunit in spinach chloroplasts. The two possible trypsin cleavage sites, Arg-4 and Arg-12, located near the NH_2 terminus, the latter perhaps bound to the membrane surface, are indicated by arrows. Trypsinolysis (>30 min, room temperature) of unstacked spinach thylakoid membranes (weight ratio of trypsin to chlorophyll, 1:10) caused a decrease in band intensity of 80–90% (5 trials) of the original M_r 4500 cyt *b*-559 β-subunit (Figure 3, lanes 3 and 4, β-cyt *b*-559). This digestion treatment caused more than half of the original M_r 9000 α-subunit to shift to a band of slightly smaller ($\Delta M_r \approx -800$) size (Figure 3, lane 3, 30-min incubation, α-cyt *b*-559), indicating that the thylakoids remained intact (Tae *et al.*, 1988) while the trypsin was accessible to the N-terminus of the α-subunit. However, only a small fraction of the antigenic sites of the cyt *b*-559 β-subunit remained after 30 min. Approximately the same fraction of β-subunit antigenic sites remained aftter long (60-min) incubation times, indicating that trypsin was accessible to the N-terminus of this β-subunit but was not able to completely digest the epitope. The N-terminal decapeptide of the β-subunit may retain partial antigenicity because the exposed trypsin cleavage site, Arg-4, is located near the middle of this segment. Removal of four residues may not eliminate all of the potential epitopes of this decapeptide.

Hybrid Mutant of the Cyt b-559 β-Subunit. The N-terminal region of the cytochrome β-subunit was elongated and modified in order to optimize its accessibility to, and reactivity with, the trypsin probe. The 21 nucleotides, encoding the first seven amino acid residues of the cyt *b*-559 β-subunit (1-ATQNPNQ-7) in *Synechocystis* sp. PCC 6803 (Figure 4), were replaced, using site-directed mutagenesis, by the sequence that corresponds to the tridecapeptide (68-RFD-SLEQLDEFSR-80), from the C-terminal α-subunit of spinach cyt *b*-559. This sequence was chosen because it was known to be immunoreactive and to involve only a small extension of the N-terminus of the wild-type β-subunit. It could be chosen because it was known not to react with the α-subunit of the cytochrome of *Synechocystis* (Vallon *et al.*, 1989). The Pro at position 8 was also mutated to an Arg residue. This additional Arg residue was introduced to facilitate the cleavage by trypsin of the spinach antigenic site on the N-terminal domain of the β-subunit in *Synechocystis*. The mutated *psbF* gene as well as the *psbE* gene and the spectinomycin resistance gene ($Spec^R$) in the pCT901 vector were transformed into the *psbEF* deletion mutant (T1297) to remove the background activity of cyt *b*-559 (Pakrasi *et al.*, 1988). The mutated *psbF* gene is believed to be incorporated into the chromosomal DNA through homologous recombination, resulting in the replacement of the kanamycin resistance gene (Km^R) with the $Spec^R$ gene. The presence of the nucleotide sequence encoding the spinach epitope (R68–R80), as well as an additional Arg residue, was confirmed by sequencing to be at the 5′ end of the *psbF* gene of the hybrid mutant β-subunit of *Synechocystis* sp. PCC 6803 (Figure 5).

Photoautotrophic Competence. The mutant was screened by the ability to grow on agar plates with spectinomycin. The presence of the spectinomycin resistance marker gene downstream of the *psbEF* gene was shown to have no effect on photosynthetic growth (Tae *et al.*, 1992). The growth rate of the hybrid mutant was similar to that observed for wild type under photoheterotrophic conditions (Table 1). The β-subunit hybrid mutant is able to grow photoautotrophically with a rate of O_2 evolution (H_2O to DCMU–ferricyanide) comparable to that of the wild type. These results imply that the insertional mutation of the N-terminal domain of β-subunit does not affect the structural and functional integrity of the photosystem II complex in the thylakoid membranes.

Intactness of Thylakoid Membranes Prepared by Osmolysis. The thylakoid membranes of *Synechocystis* sp. PCC 6803 were judged to be vesicular and intact by two criteria:

(1) Trypsin Accessibility to MSP. If the membranes were leaky to trypsin, it was expected that the extrinsic manganese stabilizing protein (MSP) of the oxygen-evolving complex (OEC) would be proteolyzed, since the 33-kDa polypeptide of the OEC in spinach thylakoids, corresponding to MSP in *Synechocystis* sp. PCC 6803, was known to be sensitive to trypsinolysis (Tae & Cramer, 1989). The osmotically shocked β-hybrid mutant cells were directly exposed to trypsin digestion

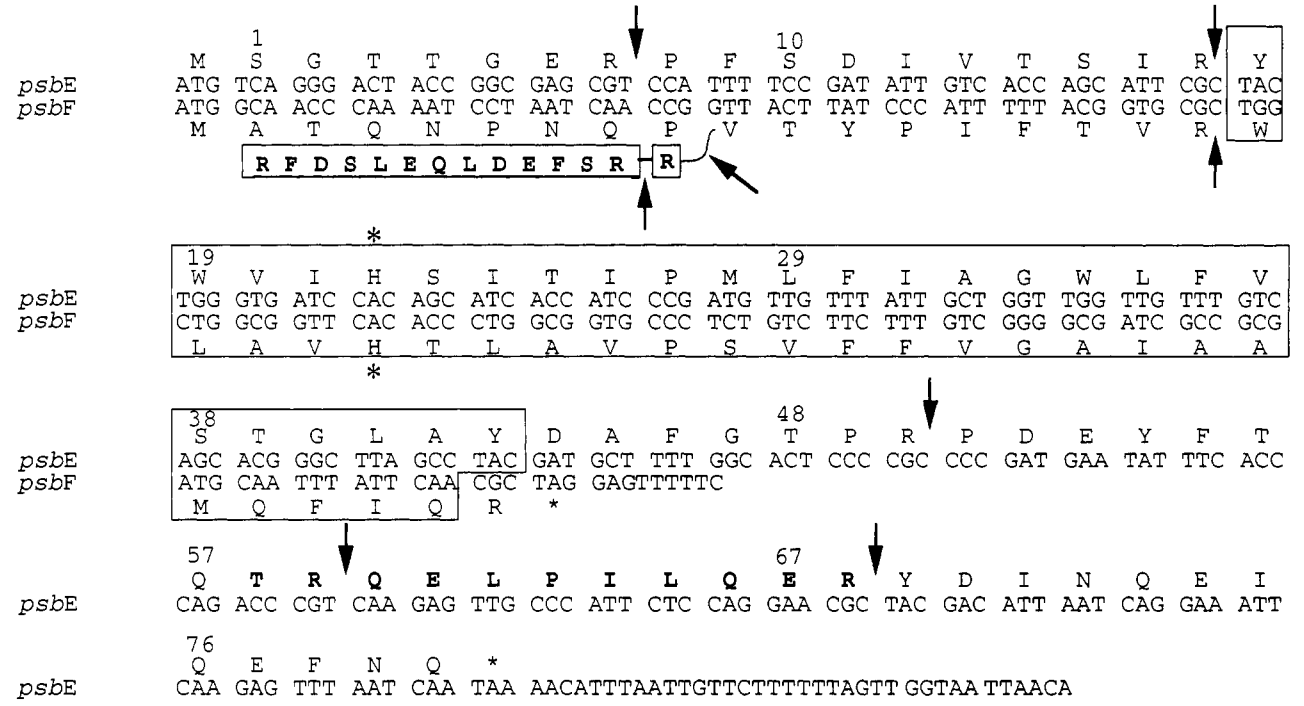

FIGURE 4: Nucleotide and amino acid sequences of the α- and β-subunits of cytochrome *b*-559 in *Synechocystis* sp. PCC 6803. The amino acid sequence is written in one-letter code. The central hydrophobic domain of 26 and 25 residues of the α- and β-subunits, respectively, is boxed. The single His residue on each subunit is noted (*). The amino acid sequence of the antigenic site on the α-subunit (T58–R68) was represented in bold letters. The spinach epitope (R68–R80) and the additional Arg residue on the mutant are shown in bold letters in boxes and connected directly to Val-9 of the original sequence of the β-subunit. The potential trypsin cleavage sites near amino and carboxy termini are marked by arrows.

without further mechanical cell breakage, and the trypsin-treated thylakoid membranes were extracted using glass beads following the addition of protease inhibitors. Scanning analysis (Figure 6, middle) of the Western blots indicated that ~100% and 80–90% (three trials) of MSP remained intact after trypsinolysis for 15 and 30 min [Figure 6 (top, left), lanes 2 and 3]. A somewhat larger amount (~30%) of MSP may be lost after 60 min [Figure 6 (top, left)]. Less than 5–10% of MSP was detected when the cells were broken by French press treatment or glass beads (data not shown). In experiments with the same membranes, the fraction of *b*-559 β subunit remaining after 15, 30, and 60 min of trypsinolysis is 50, 40, and 30% (Figure 6, bottom).

(2) Trypsin Accessibility to the Lumenal-Side C-Terminus of the α-Subunit. Antibody to the cyt *b*-559 α-subunit of *Synechocystis* sp. PCC 6803 was generated against the synthetic undecapeptide corresponding to the segment Thr-58–Arg-68 (Figure 4). The orientation of the α-subunit was previously determined in spinach thylakoids, in which the α-subunit spans the membrane once and its N- and C-termini are exposed to the stromal and lumenal sides, respectively (Tae *et al.*, 1988; Vallon *et al.*, 1989). Because the antigenic site (58-TRQELPILQER-68) of the α-subunit in *Synechocystis* is located near the C-terminal domain, which is exposed to the lumen (Figure 1A), trypsin digestion of Arg-7 and/or Arg-17 in the N-terminal region, and inaccessibility to the C-terminal epitope, is expected to result in a decrease in molecular weight but little loss of total protein. Exposure to trypsin of the osmotically shocked β-subunit hybrid mutant generated a smaller ($\Delta M_r \cong -2500$ Da) polypeptide which still contained the antigenic site of C-terminal domain [Figure 6 (top), lanes 2–4, α-cyt *b*-559]. Therefore, trypsin was accessible to Arg-7/Arg-17 located near the N-terminus of the α-subunit, based on the magnitude of the M_r shift, implying that its N- and C-termini are exposed to the cytoplasmic and lumenal sides of the thylakoid membrane, respectively. The effect of a 15-min trypsinolysis caused ≥50% of the original cyt *b*-559 α-subunit to shift to a band of smaller size [Figure 6 (top), lanes 2–4, α-cyt *b*-559]. In the case of the spinach thylakoid α-subunit, more than 50% of the subunit was found to be intact after a 15-min trypsinolysis (Tae *et al.*, 1988), indicating that Arg-7 on the N-terminal region of the cyt *b*-559 α-subunit in *Synechocystis* sp. PCC 6803 may be more exposed to trypsin digestion than in spinach thylakoids. Exposure of the membranes to trypsin for longer times, 30 and 60 min, did not cause any changes of the band intensities [Figure 6 (top), center blot, lanes 3 and 4]. Therefore, from the relative inaccessibility of the MSP and the lumenal side of the cyt *b*-559 α-subunit to trypsin, it is inferred that the cytochrome-containing membranes are vesicular and sealed.

Effect of Trypsin on Reactivity of the Cyt b-559 β-Subunit with Antibody to Its N-Terminus. Antibody to the C-terminal R68–R80 epitope used in the previous work to detect the cyt *b*-559 α-subunit in spinach thylakoids (Tae *et al.*, 1988) could be used to detect the β-subunit in the β-subunit hybrid mutant of cyt *b*-559 in *Synechocystis* sp. PCC 6803. Since two consecutive Arg residues (NH_2–RFDSLEQLDEFS**RR**-COOH) are designed at the C-terminus of the antibody recognition site (Figure 4, bold letters in boxes) to facilitate trypsin digestion, the accessibility of trypsin to these sites could be used to examine the orientation of the β-subunit in thylakoid membranes. The 15-min trypsinolysis caused a decrease of approximately 65% in band intensity [Figure 6 (top), lane 2, β-cyt *b*-559] compared to the control band (lane 1). Exposure for longer times, 30 and 60 min, to trypsin (lanes 3 and 4) caused a loss of approximately 80% of the antigenic site of the β-subunit. This indicated that trypsin was accessible to Arg-13 and Arg-14 (indicated by two arrows in Figure 4) of the N-terminal region of the β-subunit. The 15–20% of band intensity left after the 60-min trypsin digestion

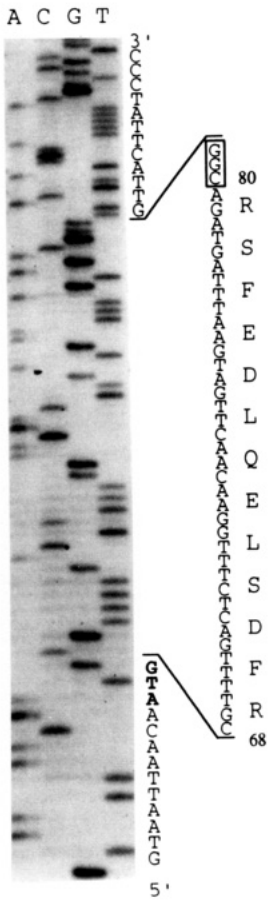

FIGURE 5: Identification by sequence of the hybrid mutation site on the *psbF* gene. The original seven amino acid residues from the N-terminal domain of the β-subunit were replaced by the 13-residue spinach epitope. The start codon (ATG) of the *psbF* gene is represented in bold letters, and the codon (CGG) for an additional Arg residue is in a box.

Table 1: Growth and O_2 Evolution Rates of the Wild-Type and the Hybrid β-Subunit Mutant of Cytochrome b_{559}

	wild type	mutant
Growth Rate[a] (Doubling Time, h)		
BG-11	27	24
BG-11 + glucose	14	14
O_2 Evolution ($H_2O \rightarrow$ DCBQ) (μmol of O_2/[(mg of Chl)·h])		
	279 ± 20[b]	269 ± 4

[a] Light intensity incident upon growth flasks: 25–30 μEinstein/(m^2·s). [b] Standard deviations calculated from three independent measurements.

may arise from the intact cells which trypsin could not penetrate even after the osmotic shock. Comparison of lanes 2 and 3 in Figure 6, for which there is no doubt of the intact vesicular nature of the membrane, indicates that the N-terminus of β-subunit is exposed to the cytoplasmic side of the thylakoid membrane in *Synechocystis* sp. PCC 6803.

DISCUSSION

Intramembrane Orientation of the Cyt b-559 α- and β-Subunits. The topographical orientation of the cyt *b*-559 α-subunit, an intrinsic component of the PS II reaction center in spinach thylakoid membranes, was previously determined by (i) proteolysis of potentially exposed epitopes and detection with an antibody to a synthetic tridecapeptide mimicking the C-terminal domain of the α-polypeptide (Tae *et al.*, 1988) and (ii) detection of the exposed antibody by gold labeling and immunoelectron microscopy (Vallon *et al.*, 1989). The

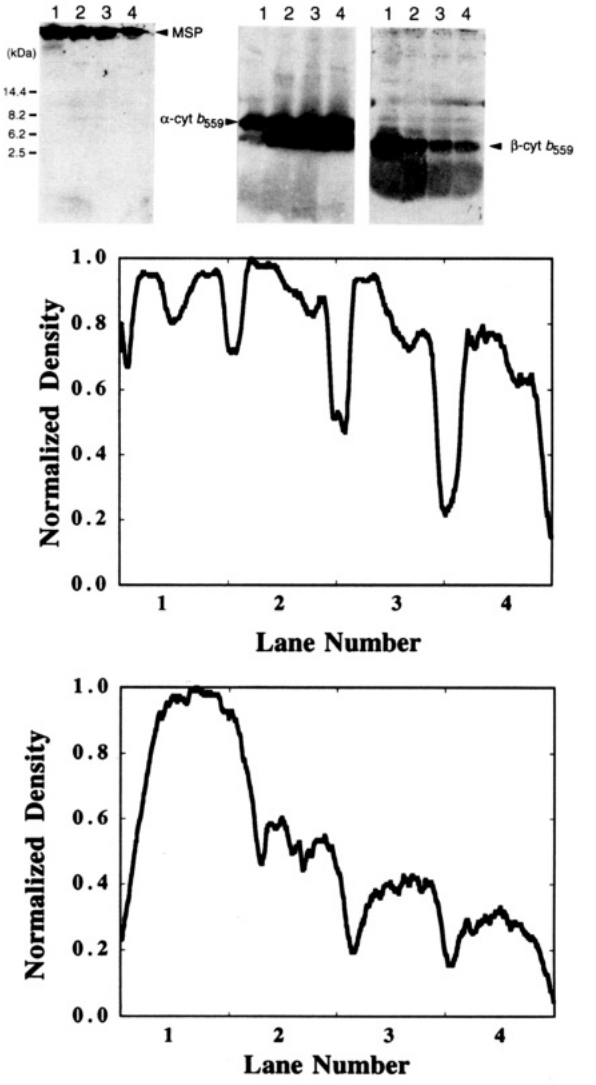

FIGURE 6: Trypsinolysis of the α- and β-subunits of cyt *b*-559 and MSP in osmotically shocked cells of *Synechocystis* containing the hybrid β-subunit mutant of cyt *b*-559. (Top) Pattern of trypsinolysis fragments. Osmotically disrupted β-subunit hybrid mutant cells were treated with trypsin (Chl:trypsin = 10:1) for 15, 30, and 60 min at room temperature while nutating and analyzed by 9.9–19.8% (w/w) gradient SDS–PAGE (acrylamide:bisacrylamide = 8.25:1). The gel was incubated in 30 mM Tris-HCl, pH 8.0, including 17 mM boric acid, 2 mM SDS, and 20% methanol for 10–15 min at room temperature and transferred (13 V constant voltage or 130 mA, constant current) to nitrocellulose paper (pore size, 0.1 μm diameter). Each lane was loaded with the membrane equivalent of 15 μg of Chl. Color reactions were performed with 0.017% 4-chloro-1-naphthol and 0.05% H_2O_2 in TBS. (Lower panels) Densitometry of lanes 1–4 showing trypsinolysis for t = 0, 15, 30, and 60 min for (middle) MSP and (bottom) β-cyt *b*-559. The major band in each blot was scanned using a Hewlett-Packard Scan Jet IIcx associated Desk Scan II software. The image was stored as a TIFF file, the image profile was obtained using NIH Image 1-537b7, and the image output was normalized and plotted with KaleidoGraph 3.0.2 (Abelbeck Software).

N-terminus of the cyt *b*-559 α-subunit was found by both approaches to be on the stromal side of the membrane, and this result was confirmed as a control in the present study (Figure 3). In experiments that utilized trypsin accessibility to an N-terminal epitope of the β-subunit, the band intensity on Western blots of the cyt *b*-559 β-subunit around M_r 4500 Da was also greatly decreased after trypsinolysis of intact thylakoid membranes (Figure 3). The N-terminal domain of the β-subunit (at least Arg-4) was found to be accessible to trypsin digestion, implying that the N-terminus of the β-subunit is exposed on the stromal side. A small fraction of

the antigenic sites of the β-subunit N-terminus remained after a longer incubation (60 min) in intact thylakoid membranes, whereas almost all of the parental band of the α-subunit shifted to a band of slightly smaller size, indicating that the trypsin digestion could not remove all of the epitopes near the decapeptide N-terminus of the β-subunit. This may be a result of cleavage after Arg-4, which is located in the middle of the 10-residue epitope, allowing retention of some of the antigenic reactivity in residues 5–10.

Intramembrane Orientation of the Cyt b-559 α- and β-Subunits in Synechocystis sp. PCC 6803. Replacement of eight residues of the N-terminal region of the cyt *b*-559 β-subunit with the tridecapeptide epitope (R68–R80) from the spinach thylakoid α-subunit C-terminus had little effect on the photoautotrophic growth rate and function in the resulting hybrid β-mutant (Table 1). Therefore, antibody to the C-terminal region (T58–R68) of the α-subunit and antibody to C-terminal epitope R68–R80 of the spinach cyt *b*-559 α-subunit were used to detect the α- and hybrid β-subunit (Figure 4) of cyt *b*-559, respectively. A critical step in the determination of membrane protein topological orientation using protease accessibility to antigenic sites is preparation of membranes through which the protease cannot penetrate. Cyanobacteria are classified as Gram-negative bacteria, in which the cell envelope is composed of outer and inner membranes with an intervening peptidoglycan layer. The cell wall of *Synechocystic* sp. PCC 6803 is resistant to breakage. Therefore, any mechanical forces applied to break the cells such as glass-bead grinding, French pressing, or "nebulizing" seemed to cause disruption of the structural integrity of the membranes, with the result that trypsin was able to penetrate the thylakoid membrane, as judged by its digestion of MSP, which is located on the lumenal side of the membrane (data not shown). Treatment of cells with a low ionic strength solution, however, permeabilized the outer cell membrane, allowing passage of trypsin. However, the cytochrome-containing internal membranes, including the thylakoid membranes, remained impermeant toward trypsin. Cytochrome *b*-559 was accessible to trypsin only from the cytoplasmic side of the membrane. It cleaved Arg residue(s) on the N-terminal region of the α-subunit, probably after Arg-7 (Tae *et al.*, 1988), generating two bands of slightly different M_r value [Figure 6 (top), center blot, α-cyt *b*-559] without affecting the MSP band intensities [Figure 6 (top), left blot, MSP]. These results imply that the cyt *b*-559 α-subunit, as in spinach thylakoids, spans the membrane once in *Synechocystis* sp. PCC 6803 and its N- and C-termini are exposed to the cytoplasmic and lumenal sides of the membrane, respectively. The intactness of the thylakoid membranes seemed to be mostly maintained during a trypsin digestion period up to 60 min and totally intact for 30 min, based on the preservation of the MSP band intensity and those of the sum of the parent–daughter cyt *b*-559 α-bands [Figure 6 (top), left and center blots]. The large decrease of intensity of the cytochrome β-subunit without an M_r shift, after a 15- and 30-min trypsinolysis, implied that its N-terminal domain is exposed to the cytoplasmic side of the thylakoid membrane (Figure 1).

From the analysis of the orientation of the β-subunit in both spinach thylakoid and *Synechocystic* membranes, it is inferred that the orientation of the cyt *b*-559 β-subunit is the same as the α-subunit with the N-terminus on the stromal or cytoplasmic side of the membrane. Because the position of the single histidine residue near the N-terminal end of the hydrophobic membrane-spanning α-helix is conserved in 15 known sequences of the α- and β-subunits (Cramer *et al.*, 1993), the location of the N-termini of both subunits on the stromal and cytoplasmic sides of the thylakoid membrane in chloroplasts and cyanobacteria implies that the cyt *b*-559 hemes are also on that side of the membrane (Figure 1).

One caveat about the present topographical analysis is that a significant fraction of the cytochrome *b*-559 subunits in thylakoids (Tae *et al.*, 1993) and perhaps in cyanobacteria, are nascent and not yet assembled in the photosystem II reaction center. The implication of the present work is that these unassembled subunits will have the same orientation in the membrane as those incorporated in the reaction center.

Heme Midpoint Potential; cis-Positive Rule. The experimentally determined position of the cyt *b*-559 hemes on the stromal or cytoplasmic side of the membrane described in the present work is in agreement with the predictions made by two different kinds of considerations: (i) the very positive midpoint redox potential [$E_m \approx +0.4$ V (Cramer & Whitmarsh, 1977)] of cyt *b*-559 can be explained by the large effect on the heme E_m of the positive helix dipole potential contributed by two parallel α-helices when the heme is in a hydrophobic niche on the stromal side of the membrane (Krishtalik *et al.*, 1993). Qualitatively, this criterion is also consistent with the existence of an α_2 or β_2 homodimer. (ii) This orientation of α- and β-subunits in an αβ heterodimeric unit obeys, or in the case of wild-type *Synechocystis* (depending on the positive charge, +0.5 – 1.0, of the N-terminal α-amino group) is at least neutral, the *cis*-positive rule of von Heijne and Gavel (1988) for orientation of integral membrane proteins. The αβ unit does not obey the rule if the β-polypeptide has an orientation opposite (i.e., with N-terminus on the lumenal side) to that previously established by Tae *et al.* (1988) for the cyt *b*-559 α-polypeptide. An additional conclusion from such considerations of the *cis*-positive rule is that the cyt *b*-559 should, in fact, assemble as an αβ unit. We note that the mutant construct of the β-subunit has three extra Arg on the N-terminal side, which increases the *cis*-positive bias by three units. Without these Arg in the mutant, the inferred orientation of the β-subunit would leave the αβ dimer neutral or favorably oriented, depending on the charge of the N-terminal α-amino group. The reverse orientation of the β-subunit in both wild type and mutant would be unfavorable by the criterion of the *cis*-positive rule. The N–C parallel orientation of α and β in spinach thylakoids, inferred from the data of Figure 3, is also favorable in terms of the distribution of positive charges. We also note that a β_2 homodimer with the N-terminus on the stromal side would also satisfy the dipole potential and *cis*-positive rules. However, the problems with an $\alpha_2\beta_2$ model have been discussed previously (Tae *et al.*, 1988) and in the introduction. In addition, the α-subunit orientation determined by Tae *et al.* (1988) would confer a positive charge distribution on an α_2 homodimer in spinach thylakoids that is unfavorable by 3–4 charge units.

Implications for Cyt b-559 Function from Heme Location. Cyt *b*-559 is an essential structural component of the PSII reaction center. Deletion of the *psbEF* genes, encoding the α- and β-subunits of cyt *b*-559 in *Synechocystis* sp. PCC 6803, resulted in loss of assembly of the PSII reaction center complex (Pakrasi *et al.*, 1988). Truncation of the COOH-terminal region (22-31 residues) of the α-subunit decreased the total number of properly assembled PSII complexes in the membrane (Tae & Cramer, 1992). A role in electron transport processes is suggested by the obligatory presence of the cyt *b*-559 in the reaction center. The observation that physiological reductants such as ascorbate can reduce cyt *b*-559 from the stromal side

of the membrane implies that the electrons needed for quenching long-lived $P680^+$ may be supplied by the high concentration of stromal reductants and transferred via a pathway including cyt *b*-559 that is located at the stromal interface partly for this purpose (Furbacher, 1993). A general constraint on electron transfer models in PSII that invoke a role of cyt *b*-559 in PSII cyclic electron transfer (Falkowski *et al.*, 1986), or in the transfer to an auxiliary chlorophyll (Thompson & Brudvig, 1988) or from a pheophytin (Nedbal *et al.*, 1992; Barber & De Las Rivas, 1993), is that the electron transfer between the heme and these components probably involves a long distance (≥15 Å) transfer because of the proximity of the heme to the stromal surface of the membrane.

ACKNOWLEDGMENT

We thank J. B. Heymann for the densitometric scans (Figure 6, middle and bottom) and helpful discussions, P. N. Furbacher and M. Ponomarev as well for helpful discussions, and V. Livingston for assistance with the manuscript.

REFERENCES

Babcock, G. T., Widger, W. R., Cramer, W. A., Oertling, W. A., & Metz, J. G. (1985) *Biochemistry 24*, 3638–3645.

Barber, J., & De Las Rivas, J. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 10942–10946.

Bebenek, K., & Kunkel, T. A. (1989) *Nucleic Acids Res. 17*, 5408.

Buser, C. A., Diner, B. A., & Brudvig, G. W. (1992) *Biochemistry 31*, 11441–11448.

Cramer, W. A., & Whitmarsh, J. (1977) *Annu. Rev. Plant Physiol. 28*, 133–172.

Cramer, W. A., Tae, G.-S., Furbacher, P. N. (1993) *Physiol. Plant. 88*, 705–711.

Falkowski, P. G., Fujita, Y., Ley, A., & Mauzerall, D. (1986) *Plant Physiol. 81*, 310–312.

Furbacher, P. N. (1993) Ph.D. Thesis, Purdue University, West Lafayette, IN, 100 pp.

Herrmann, R. G., Alt, J., Schiller, B., Widger, W. R., & Cramer, W. A. (1984) *FEBS Lett. 176*, 239–244.

Krishtalik, L. I., Tae, G.-T., Cherepanov, D. A., & Cramer, W. A. (1993) *Biophys. J. 65*, 184–195.

Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 488–492.

Lichtenthaler, H. K. (1987) *Methods Enzymol. 148*, 350–382.

MacDonald, G. M., Everly, M. E., Debus, R., Cramer, W. A., & Barry, B. (1994) *Biochemistry 33*, 4393–4400.

Miyazaki, A., Shina, T., Toyoshima, Y., Gounaris, K. & Barber, J. (1989) *Biochim. Biophys. Acta 975*, 142–147.

Murata, N., & Omata, T. (1988) *Methods Enzymol. 167*, 245–251.

Nedbal, L., Samson, G., & Whitmarsh, J. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 7929–7933.

Omata, T., & Murata, N. (1985) *Biochim. Biophys. Acta 810*, 354–361.

Pakrasi, H. B., Williams, J. C. K., & Arntzen, C. J. (1988) *EMBO J. 7*, 325–332.

Rippka, R., Deruelles, J., Waterbury, J. B., Herdman, M., & Stanier, R. Y. (1979) *J. Gen. Microbiol. 30*, 5387–5395.

Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, T. G., Horn, T. T., Mullis, K. B., & Erlich, H. A. (1985) *Science 230*, 1350–1354.

Schägger, H., & von Jagow, G. (1987) *Anal. Biochem. 166*, 368–379.

Tabor, S., & Richardson, C. C. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 4767–4771.

Tae, G.-S., & Cramer, W. A. (1989) *FEBS Lett. 259*, 161–164.

Tae, G.-S., & Cramer, W. A. (1992) *Biochemistry 31*, 4066–4074.

Tae, G.-S., Black, M. T., Cramer, W. A., Vallon, O., & Bogorad, L. (1988) *Biochemistry 27*, 9075–9080.

Tae, G.-S., Everly, R. E., Cramer, W. A., Madgwick, S. A. & Rich, P. R. (1993) *Photosyn. Res. 36*, 141–146.

Takahashi, L. M., & Asada, K. (1989) *Plant Cell Physiol. 30*, 915–921.

Tang, X.-S., & Diner, B. A. (1994) *Biochemistry 33*, 4594–4603.

Thompson, L. K., & Brudvig, G. W. (1988) *Biochemistry 27*, 6653–6658.

Vallon, O., Tae, G.-T., Cramer, W. A., Simpson, D., Hoyer-Hansen, G., & Bogorad, L. (1989) *Biochim. Biophys. Acta 975*, 132–141.

von Heijne, G., & Gavel, Y. (1988) *Eur. J. Biochem. 74*, 671–678.

Whitmarsh, J., & Ort, D. R. (1984) *Arch. Biochem. Biophys. 231*, 378–389.

Widger, W. R., Cramer, W. A., Hermodson, M., Meyer, D., & Gullifor, M. (1984) *J. Biol. Chem. 259*, 3870–3876.

Widger, W. R., Cramer, W. A., Hermodson, M., & Herrmann, R. G. (1985) *FEBS Lett. 191*, 186–190.

Williams, J. G. K. (1988) *Methods Enzymol. 167*, 766–778.