Articles

Thylakoid Membrane Protein Topography: Transmembrane Orientation of the Chloroplast Cytochrome *b*-559 *psb*E Gene Product†

Gun-Sik Tae,‡ Michael T. Black,‡,§ William A. Cramer,*,‡ Olivier Vallon,∥ and Lawrence Bogorad∥

Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907, and Department of Cellular and Developmental Biology, Harvard University, Cambridge, Massachusetts 02139

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ABSTRACT: Protease accessibility and antibody to a COOH-terminal peptide were used as probes for the in situ topography of the M_r 10 000 *psb*E gene product (α subunit) of the chloroplast cytochrome *b*-559. Exposure of thylakoid membranes to trypsin or *Staphylococcus aureus* V8 protease cleaved the α subunit to a slightly smaller polypeptide ($\Delta M_r \approx -1000$) as detected on Western blots, without loss of reactivity to COOH-terminal antibody. The disappearance of the parent M_r 10 000 polypeptide from thylakoids in the presence of trypsin correlated with the appearance of the smaller polypeptide with $\Delta M_r = -750$, the conversion having a half-time of approximately 15 min. Exposure of inside-out vesicles to trypsin resulted in almost complete loss of reactivity to the antibody, showing that the COOH terminus is exposed on the lumenal side of the membrane. Removal of the extrinsic polypeptides of the oxygen-evolving complex resulted in an increase of the accessibility of the α subunit to trypsin. These data establish that the α subunit of cytochrome *b*-559 crosses the membrane once, as predicted from its single, 26-residue, hydrophobic domain. The NH_2 terminus of the α polypeptide is on the stromal side of the membrane, where it is accessible, most likely at Arg-7 or Glu-6/Asp-11, to trypsin or V8 protease, respectively. As a consequence of this orientation, the single histidine residue in the α subunit is located on the stromal side of the hydrophobic domain. Because the β subunit contains a single histidine 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. If one heme is on the lumenal side, it must be coordinated by a β_2 homodimer whose orientation would be opposite to that of the α subunit.

The reaction center core of photosystem II consists of four polypeptide species, two of which are contributed by the subunits (α, β) of cytochrome *b*-559 (Nanba & Satoh, 1987). The conclusion that the chloroplast cytochrome *b*-559 has the unprecedented and unusual structure of a heme cross-linked dimer was based on (i) purification and sequence analysis of an M_r 10 000 polypeptide of the cytochrome (Widger et al., 1984a) and (ii) location and sequencing of a gene with an 83 amino acid reading frame, *psb*E, on the spinach plastid genome (Herrmann et al., 1984; Westhoff et al., 1985). The *psb*E gene product has a 26-residue hydrophobic domain containing a single His residue. (iii) This work also identified a reading frame, *psb*F, starting nine bases downstream, coding for a 39-residue polypeptide that also contained a 25-residue hydrophobic segment and one His residue (Herrmann et al., 1984). The presence of these two genes for cytochrome *b*-559 has been demonstrated in at least seven other oxygenic photosynthetic systems [e.g., Pakrasi et al. (1988)]. (iv) The NH_2-terminal sequence of an M_r 4000–6000 component isolated from the cytochrome *b*-559 preparation matched the prediction for the *psb*F gene (Widger et al., 1985). (v) The stoichiometry of the 9.2-kDa *psb*E and 4.3-kDa *psb*F gene products, the α and β subunits of the cytochrome, is 1:1 (Widger et al., 1985). (vi) The heme ligation in cytochrome *b*-559 is bis-histidine (Babcock et al., 1985). Therefore, since the *psb*E and *psb*F polypeptides each contain a single histidine residue, the heme must cross-link two polypeptides in a dimer structure (Widger et al., 1985; Cramer et al., 1986). Since the α and β subunits were recovered in 1:1 stoichiometry in a single fraction (Widger et al., 1984a, 1985), the unit for heme coordination would have to be an $\alpha\beta$ heterodimer if there were only one *b*-559 heme per reaction center. However, the numerous reports of a stoichiometry of two *b*-559 hemes per reaction center (Lam et al., 1983; Murata et al., 1984; Yamamoto et al., 1984) suggest that it is likely that the *b*-559 heme to P-680 stoichiometry is 2 but that one heme is readily lost, particularly in the more highly purified reaction center preparations (Nanba & Satoh, 1987). In the latter case, the units for heme coordination could be α_2 and β_2, as well as $(\alpha\beta)_2$. Arguments for the latter model are (i) that no indication was seen of two separate fractions eluting in detergent–2 M urea from a DEAE column, even though the α_2 and β_2 dimers differ by 10 charge units, and (ii) that the *b*-559 messenger RNA from the *psb*E and *psb*F genes is bicistronic (Herrmann et al., 1984).

Elucidation of the position of the hemes in the membrane would help to solve the problem of the function of this cytochrome, which is still enigmatic (Cramer et al., 1986; Thompson & Brudvig, 1988). Because each polypeptide contains a single His residue located five residues into the hydrophobic domain from the NH_2 terminus, the orientation of these polypeptides in the membrane bilayer will determine

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* To whom correspondence should be addressed.

‡ Purdue University.

§ Present address: Department of Chemistry, University of Edinburgh, Edinburgh EH9 3JJ, Scotland.

∥ Harvard University.

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Table I: Amino Acid Sequence of Spinach Chloroplast Cytochrome *b*-559 α Subunit (Widger et al., 1984a; Herrmann et al., 1984)[a]

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                  ↓  10                  20  *                30
NH2-S-G-S-T-G-E-R-S-F-A-D-I-I-T-S-I-R-Y-W-V-I-H-S-I-T-I-P-S-L-F-

                  40                  50                  60
I-A-G-W-L-F-V-S-T-G-L-A-Y-D-V-F-G-S-P-R-P-N-E-Y-F-T-E-S-R-Q-

                  ↓ 70                80↓
G-I-P-L-I-T-G-R-F-D-S-L-E-Q-L-D-E-F-S-R-S-F-COOH
                  |_____________________|
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[a] The amino acid sequence is written in one-letter code (Schulz & Schirmer, 1979). Central hydrophobic domain of 26 residues is underlined; peptide (R-68 → R-80) to which antibody was generated in this work is noted by a bracket; single His residue is noted (*); potential trypsin cleavage sites near NH_2 and COOH termini are marked by (↓).

the position of the two hemes in the membrane. The sequence of the *psb*E gene product, the α subunit of the cytochrome, predicts a 26-residue membrane-spanning α-helix (Table I, underlined segment) and polar 17- and 39-residue segments extending from the membrane on the NH_2- and COOH-terminal sides, respectively. Neither the sequence information nor recognition of the hydrophobic domain predicts the sidedness of the protein in the membrane. However, the extrinsic segments of the M_r 10000 α subunit could provide topographical information. Proteases were used to probe these potentially exposed regions, and an antibody to a tridecapeptide mimicking the COOH-terminal domain was used to monitor their effect.

Materials and Methods

Separation of Inside-Out and Right-Side-Out Thylakoid Vesicles by Phase Partitioning. Spinach thylakoids isolated from intact chloroplasts were resuspended in 50 mM sucrose, 10 mM NaCl, and 50 mM sodium phosphate buffer (pH 7.4) and were sedimented at 1000*g* for 10 min. This step was repeated two times, and the final pellets were resuspended in 5 mM $MgCl_2$, 5 mM NaCl, 10 mM sodium phosphate buffer (pH 6.5), and 100 mM sucrose at a chlorophyll concentration of 4.5 mg/mL. The stacked membranes were passed twice through a YEDA press at a nitrogen gas pressure of 1450 psi. The material was then passed twice more through the YEDA press after addition of EDTA (0.1 M, pH 7.4, final concentration 5 mM) and then centrifuged at 1000*g* (10 min) to remove starch. The separation of inside-out from right-side-out vesicles was accomplished by phase partitioning, using a two-phase polymer system whose composition was 5.7% (w/w) dextran T500, 5.7% (w/w) poly(ethylene glycol) 3350 (Sigma P-3640), 10 mM sodium phosphate buffer (pH 7.4), 5 mM NaCl, and 20 mM sucrose. After addition of thylakoid homogenate (4 mg of Chl[1] in 1 mL added to 24 mL) the phase system was mixed and centrifuged at 1500*g* (3 min). The upper (T1) and lower (B1) phases were collected and repartitioned with pure lower phase (B0) and upper phase (T0), respectively, at least two more times to generate fractions T3 and B3 (or T4 and B4 in some experiments) corresponding to RSO and ISO vesicles. Vesicles of each type were diluted with phase partition buffer and sedimented at 100000*g* (30 min, 4 °C). Purified vesicles were resuspended and homogenized in 50 mM sucrose, 10 mM NaCl, and 10 mM Hepes (pH 7.5) (Åkerlund & Andersson, 1983; Atta-Asafo-Adjei & Dilley, 1985).

[1] Abbreviations: Chl, chlorophyll; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; ISO and RSO, inside out and right side out; OEC, oxygen-evolving complex; PMSF, phenylmethanesulfonyl fluoride.

Assay of Purity of Inside-Out and Right-Side-Out Vesicles. ISO and RSO vesicles were prepared as discussed above and washed with 0.8 M Tris-HCl (pH 8.2). The proteins of each fraction (washed, unwashed ISO and RSO vesicles) were separated on a 15–20% gradient SDS–PAGE gel and transferred to nitrocellulose filter under the same conditions as discussed below (Immunoblot Analysis). The intensity of antibody-reactive bands on the nitrocellulose from the lumen-exposed extrinsic M_r 17000 polypeptide was determined by densitometric scanning, and the relative areas under the peaks were measured.

Preparation of Photosystem II Complex. Highly active intact thylakoid membranes were suspended in 300 mM sucrose, 15 mM NaCl, 5 mM $MgCl_2$, and 50 mM sodium phosphate (pH 6.9). An aqueous solution of Triton X-100 (20% w/v) was added under stirring to obtain a Triton X-100:Chl ratio of 20:1 (w/w). After incubation for 1 min, the suspension was centrifuged at 1000*g* (2 min), the pellet discarded, and the supernatant then centrifuged at 35000*g* (10 min). The resultant pellet was resuspended in 40 mM sodium phosphate buffer (pH 6.9) and centrifuged at 1000*g* (2 min). The supernatant was centrifuged at 35000*g* (10 min), and the final pellet had a Chl *a*/*b* ratio $\approx$ 2.0 (Kuwabara & Murata, 1982).

Removal of OEC Extrinsic Polypeptides from Photosystem II Complex. PSII complex (0.5 mg of Chl/mL) was exposed to 0.8 M Tris-HCl buffer (pH 8.2) and 0.3 M sucrose for 30 min in the light at 4 °C and centrifuged at 35000*g* (10 min) (Seibert et al., 1987). The pellet was washed once, with 5 mM $MgCl_2$ included in the reaction medium used for trypsin proteolysis (vide infra).

Exposure of Membranes to Protease. Thylakoids, membranes vesicles, or PSII complex was incubated with trypsin (Sigma, T-1005) or V8 protease (Boehringer, 791-156) in the respective membrane resuspension media at room temperature (ca. 20 °C) for the indicated times. The reactions were terminated by addition of a 2-fold excess of soybean trypsin inhibitor (Sigma, T-9003) or PMSF (0.5 mM), respectively.

Peptide Synthesis. The 13 amino acid peptide (tridecapeptide) NH_2-R-F-D-S-L-E-Q-L-D-E-F-S-R-COOH, corresponding to residues 68–80 from the 82 amino acid *psb*E polypeptide, was synthesized on an Applied Bioscience 430A automated solid-phase synthesizer at the Purdue University Biotechnology Center with standard techniques. The purity of the product was assayed by reverse-phase (water–acetonitrile) HPLC.

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 against 0.15 M NaCl and 0.01 M potassium phosphate buffer, pH 7.2, at 4 °C. A total of 1.5 mL was mixed with an equal volume of complete Freund's adjuvant, aliquots of the resulting emulsion were injected into young rabbits at multiple intradermal sites, and a booster was given after 5 weeks.

Immunoblot Analysis. Samples of protein to be blotted were electrophoresed, except where noted (Figure 1B), in a 15–20% gradient SDS–polyacrylamide gel. The gel was incubated in 10 mM Tris buffer (pH 8.0, 10 min, 25 °C). Polypeptides were transferred to nitrocellulose filter (Hybond-C) by electrophoresis (0.6 A, constant current, 3–4 h). The paper was washed in 0.1 M NaCl and 0.1 M sodium phosphate buffer (PBS, pH 7.0), incubated in Seal-a-Meal

bags with 10% milk casein on a nutator (2 h, 25 °C), removed from the bag, and washed in PBS. After equilibration with cytochrome *b*-559 antibody in 0.5% BSA and PBS for 2–3 h on the nutator and washing in PBS, the paper was reacted with a second antibody in 0.5% BSA and PBS and again nutated for 2–3 h. The filter was then washed in PBS and stained for 10 min with 0.017% 4-chloro-1-naphthol in PBS.

Results

Properties of the psbE Protein Sequence (*Table I*). The initiator methionine of the *psb*E gene product is posttranslationally cleaved so that the first residue in the 82-residue polypeptide in the sequence is NH_2-Ser (Widger et al., 1984a). The NH_2-terminal region contains a tryptic cleavage site at Arg-7 and two *Staphylococcus aureus* V8 protease sites at Glu-6 and Asp-11. Arg-17 is probably too close to the membrane surface to be accessible to proteases. If the NH_2-terminal region is accessible to these proteases, cleavage by trypsin would cause a decrease in molecular weight of approximately 800 (ΔM_r = −800), and V8 protease would have a similar effect. The COOH-terminal region contains potential trypsin and V8 protease sites, respectively, at R-50, R-59, R-68, and R-80 and at E-53, E-57, D-70, E-73, D-76, and E-77 (Table I). The spinach chloroplast cytochrome *b*-559 does not contain any Lys residues. A peptide spanning residues 68–80 was synthesized that extended between the Arg residues at positions 68 and 80, which are potential trypsin cleavage sites. An antibody to this peptide was generated as a potential probe of the topography of the cytochrome α subunit.

Properties of the Antibody to the α Subunit Carboxy-Terminal Peptide. In ELISA tests, the antibody reacted strongly with the peptide (Figure 1A). Preincubation of the peptide with the antibody at concentrations of 0.06 or 5 mg/mL completely inhibited this reaction and inhibited binding of the antibody to the membrane by about a factor of 3. The unrelated synthetic decapeptide PSI A1.5 that mimicked residues 230–239 (O. Vallon and L. Bogorad, unpublished data) of the spinach chloroplast *psa*A gene product (Krish et al., 1986) did not bind the antibody or inhibit its binding to the *b*-559 tridecapeptide or to the membrane. In immunoblot experiments (Figure 1B), the antibody reacted with a band of the expected M_r value in spinach thylakoids. Preincubation with the *psb*E tridecapeptide prevented binding of the antibody (lane 3, Figure 1B), whereas the PSI-A1.5 peptide had no effect (lane 2, Figure 1B). The antibody also reacts with the cytochrome *b*-559 from the higher plant chloroplasts of barley and tobacco, but not with the cytochrome in membranes from the flagellate *Cyanophora paradoxa* nor with the cyanobacterium *Synechocystis* sp. PCC 6803 (not shown). This is consistent with a high degree of sequence identity in the former that differ in this region only at residue-71, whereas the latter sequences differ considerably in the COOH-terminal regions.

Effect of Trypsin and V8 Protease on Size and Reactivity of Cytochrome b-559 α Subunit with Antibody to Its COOH Terminus. Trypsin and V8 protease digestion of the NH_2-terminal region should generate a slightly smaller ($\Delta M_r \simeq$ −700 to −1200) polypeptide that would react with antibody to the tridecapeptide. The effect of 15-min trypsinolysis of unstacked spinach thylakoid membranes is to cause approximately half of the original M_r 10000 cytochrome *b*-559 α subunit to shift to a band of slightly smaller size that also reacted with the antibody (Figure 2A). Exposure to trypsin for longer times, 30 and 60 min, caused an increasing loss of the parental M_r 10000 band and a corresponding accumulation of the slightly smaller product. After longer (120 min) incubation times, the trypsinolysis apparently disrupted the membrane, causing almost complete loss of reactivity. The magnitude of the ΔM_r shift, determined from calibration using low molecular weight standards on seven different gels, was ΔM_r = −762 ± 227 (Figure 2C), indicating that the hypothesis that the trypsin site is at Arg-7, and that Arg-17 is inaccessible, is likely to be correct. A similar cleavage of a small NH_2-terminal peptide was obtained by treatment with *S. aureus* V8 proteases for 15, 30, and 60 min (Figure 2B). The latter protease should cleave at the COOH end of both glutamic (Glu-6) and aspartic acid (Asp-11) residues under the conditions employed here (Drapeau et al., 1972; Houmard &

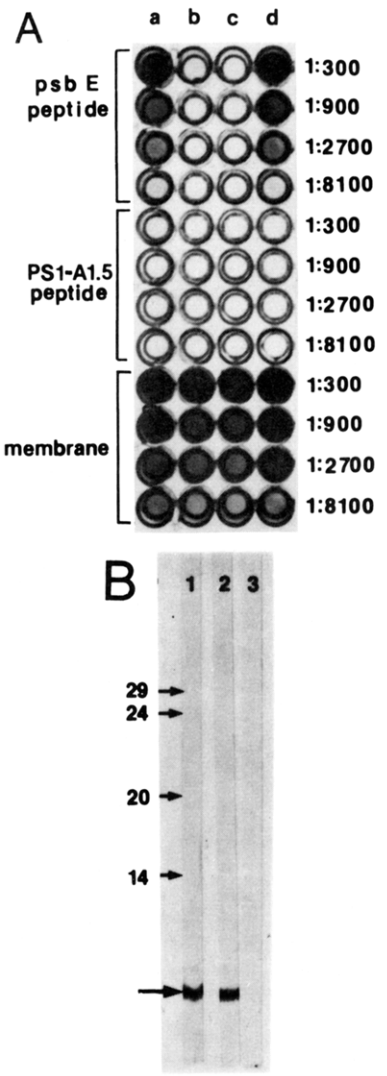

FIGURE 1: Specificity of antibody to cytochrome *b*-559 α subunit COOH terminus. (A) ELISA tests of *psb*E tridecapeptide and PSI A1.5 peptide and French press treated thylakoid membranes. (a) Antibody to *psb*E peptide; (b and c) *psb*E antibody preincubated with 5 and 0.06 mg/mL *psb*E peptide, respectively; (d) *psb*E antibody preincubated with 5 mg/mL PSI A1.5 peptide. For preincubations, antibody was diluted 1:40 in PBS containing the indicated amount of peptide, incubated 2 h at room temperature, and then made to the final dilution and transferred to the wells. Each group of four rows (1–4) corresponds to increasing dilution of the antibody, 1:300, 1:900, 1:2700, and 1:8100. (B) Western blot of spinach thylakoid membranes probed with *psb*E antibody (lane 1); *psb*E antibody preincubated with 5 mg/mL A1.5 peptide (lane 2) and 5 mg/mL *psb*E tridecapeptide (lane 3). Electrophoresis was on an 18% SDS–PAGE gel according to Thomas and Kornberg (1978). The protein was transferred to nitrocellulose filters according to Towbin et al. (1979). Immunoblot assays revealed with alkaline phosphatase were carried out following the manufacturer's instructions (ProMega Biotech.). Molecular weight standards are from the Coomassie-stained gel.

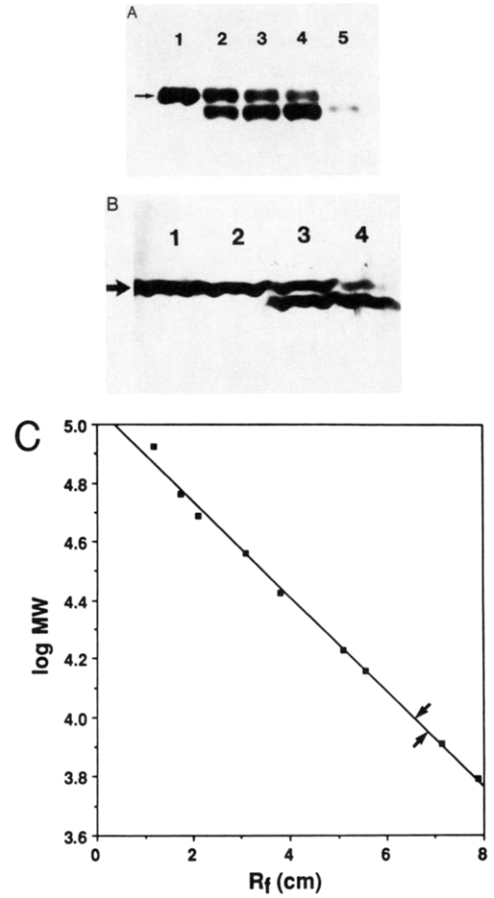

FIGURE 2: (A) Trypsinolysis and (B) V8 protease digestion of cytochrome *b*-559 in thylakoids. Thylakoids were treated with trypsin (1:10 weight ratio, trypsin:Chl) for 15, 30, 60, or 120 min or V8 protease (2:5 weight ratio, protease:Chl) for 15, 30, or 60 min, at room temperature, and analyzed by 15–20% gradient SDS–PAGE (cf. Materials and Methods). Each lane was loaded with the membrane equivalent of 10 μg of Chl. (C) The ΔM_r of the parental and cleaved protein bands (indicated by the arrows) detected after 15 min in trypsin was 762 ± 227 (n = 7), determined relative to the molecular weight standards (fructose-6-phosphate kinase, MW = 84 000; pyruvate kinase, MW = 58 000; fumarase, MW = 48 500; lactate dehydrogenase, MW = 36 500; triosephosphate isomerase, MW = 26 600; myoglobin, MW = 17 000; myoglobin fragment I + II, MW = 14 400; myoglobin fragment I, MW = 8200; myoglobin fragment II, MW = 6200).

Drapeau, 1972), but it appeared that only one V8 site was recognized.

Comparison of the areas under the two peaks in Western blots similar to that of Figure 2A, as a function of time in trypsin up to 60 min, showed that as the amount of material in the larger M_r 10 000 band decreased, indicated by the smaller area under this band, the area subtended by the smaller M_r component increased (Table II). It was concluded that the material in the smaller molecular weight component arose from the trypsinolysis of the larger.

Experiments with Inverted and Right-Side-Out Vesicles. When equal amounts of ISO and RSO membranes were loaded onto the gel (Figure 3, lanes 1 and 5, respectively, in which the ratio of cytochrome *b*-559 is 1.5:1), trypsin treatment for 15 min caused a large decrease in the amount of *b*-559 in the ISO membranes that could react with the COOH-terminal antibody (Figure 3, lane 2), whereas little or no change in the amount of antibody-reactive cytochrome was observed after 15- or 30-min reaction of trypsin with the RSO fraction (Figure 3, lanes 6 and 7). The accessibility of the OEC extrinsic 17-kDa polypeptide to Tris wash was used to assay the

Table II: Dependence of the Amount of Intact Cytochrome *b*-559 (M_r 10 000) and Trypsin-Cleaved *b*-559 (ΔM_r = −750) on Time of Exposure of Thylakoids to Trypsin

time in trypsin (min)[a]	area under parental cyt *b*-559 band (rel units)[b]	area under trypsin-cleaved cyt *b*-559 band (rel units)	total area (rel units)
control	100	0	100
15	44	36	80
30	23	51	74
60	16	73	89

[a] Trypsin:Chl ratio, 1:10 (w/w). [b] Area under bands on Western blots corresponding to parental and trypsin-cleaved cytochrome *b*-559 was measured by densitometry.

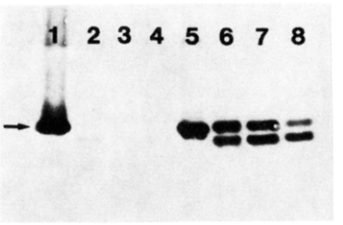

FIGURE 3: Trypsinolysis of inside-out (lanes 1–4) and right-side-out (lanes 5–8) thylakoid membranes. Inside-out (B3 fraction) and right-side-out (T3 fraction) membrane vesicles (cf. Materials and Methods) were treated with trypsin (trypsin:Chl = 1:10) for 15 (lanes 2 and 6), 30 (lanes 3 and 7), and 60 min (lanes 4 and 8). Each lane of the 15–20% gradient SDS gel was loaded with the membrane equivalent of 10 μg of Chl. Chl a/b ratio of inverted and right-side-out membranes was 2.15 and 3.30, respectively. The purity of the ISO and RSO membranes was assayed with the accessibility of the M_r 17 000 OEC extrinsic polypeptide to 0.8 M Tris washing (cf. Materials and Methods) and was 84% and 72%, respectively.

purity of the ISO and RSO fractions (Materials and Methods). The accessibility of this extrinsic polypeptide to Tris was much smaller in the population of RSO compared to that in ISO vesicles and was 28% compared to 84% in the experiment shown (Figure 3). Thus, the purity of the RSO and ISO vesicles in this experiment was 72% and 84%, respectively. The intactness and sidedness of the ISO and RSO membranes were also tested by use of the known accessibility of the cytochrome *f* COOH terminus to carboxypeptidase A in thylakoid membranes (Willey et al., 1984). The cytochrome *f* was sensitive to carboxypeptidase A, but not trypsin, in RSO vesicles and sensitive to trypsin in ISO, but not RSO, vesicles (A. Szczepaniak, G.-S. Tae, and W. A. Cramer, unpublished data). Cytochrome *b*-559 in ISO or RSO membranes was not affected by reaction with carboxypeptidase A or Y. The latter protease is not active toward cytochrome *f* (A. Szczepaniak, G.-S. Tae, and W. A. Cramer, unpublished data). Carboxypeptidase A is presumably inactive toward cytochrome *b*-559 because the Arg-80 near the *b*-559 COOH terminus blocks the action of the enzyme (Ambler, 1967).

The proteolyzed component of the α subunit in RSO membranes (Figure 3) displayed the same $\Delta M_r \approx -750$ shift that was shown in Figure 2. Two weakly staining components arising from uncleaved and proteolyzed α polypeptide were sometimes observed in the ISO membrane fraction, due to the contaminating RSO contribution of approximately 20–30% that can be present. The absence of the bands from the trypsin-treated ISO fraction in Figure 3 is a consequence of (i) the higher purity (84%) of the preparation and (ii) the photograph of the nitrocellulose paper not showing the two faint bands that were seen on the paper. The experiments described in Figures 2 and 3 show that the α subunit of cytochrome *b*-559 is oriented with COOH and NH_2 termini on the lumenal and stromal side, respectively. The complete or

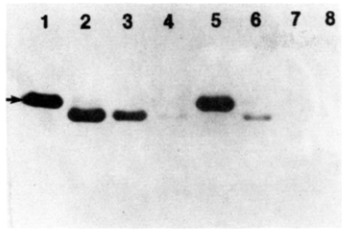

FIGURE 4: Effect of removal of OEC extrinsic polypeptides in PSII complex on accessibility of cytochrome *b*-559 to trypsin. Untreated (lanes 1–4) and Tris-washed (cf. Materials and Methods) (lanes 5–8) PSII complex. Control (lanes 1 and 5); trypsinolysis for 15 (lanes 2 and 6), 30 (lanes 3 and 7), and 60 min (lanes 4 and 8). Lanes loaded with equivalent of 10 μg of Chl. Chl *a*/*b* ratio of PSII complex, 2.1.

almost complete loss in ISO membranes of the antibody-reactive domain of the α subunit indicates that the two copies of the cytochrome *b*-559 α subunit in the reaction center have the same orientation. The conclusion concerning the orientation of the α subunit with the COOH terminus on the lumen side was first reached by immunogold labeling and visualization by electron microscopy, using a monoclonal antibody to the cytochrome. This antibody was subsequently found to have a specificity that overlapped that of the antibody to the COOH-terminal tridecapeptide described in the present work (O. Vallon, G.-S. Tae, W. A. Cramer, D. Simpson, G. Hoyer-Hansen, and L. Bogorad, unpublished results).

The COOH terminus of the cytochrome *b*-559 α subunit is shielded by the OEC extrinsic polypeptides. These three polypeptides, of M_r 17 000, 24 000, and 33 000, are known to stabilize or bind part of the OEC manganese cluster, to shield the manganese from exogenous reductants, and to provide an optimum environment for interaction between cations (manganese, calcium) and anionic (chloride) cofactors and the photosystem II core (Babcock, 1986). The three extrinsic polypeptides and the bound manganese can be removed by exposure to high concentrations of Tris buffer at alkaline pH [e.g., Yamamoto and Nishimura (1983)], as is another M_r 10 000 polypeptide (Ljundberg et al., 1984). Oxygen-evolving preparations made by the procedures of Berthold et al. (1981) or Kuwabara and Murata (1982) have been suggested to be open nonvesicular structures (Dunahay et al., 1984) that should be accessible to trypsin on both sides of the membrane as well as to Tris. The cytochrome *b*-559 α subunit in PSII complex prepared according to Kuwabara and Murata (1982) showed increased accessibility and/or sensitivity to trypsin after the alkaline–Tris treatment (Figure 4). The trypsin also cleaved the NH_2 terminus to the slightly shorter form, showing that the stromal side of these vesicles was accessible to the protease.

DISCUSSION

Topography of Cytochrome b-559 α Subunit. The following conclusions about the topographical orientation of the cytochrome *b*-559 α subunit polypeptide, an intrinsic component of the photosystem II reaction center, were obtained from Figures 2–4 and Table II: (i) The NH_2 terminus of the *b*-559 α subunit is on the stromal side of the membrane where it is accessible, at least as far as Arg-7, to added proteases that can cleave it after Arg-7 (trypsin), or Glu-6 or Asp-11 (*S. aureus* V8), to a discrete slightly shorter form. Since the SDS–PAGE system yields an M_r value for the *b*-559 α polypeptide that is close to its molecular weight of 9162, the conclusion from the observed ΔM_r shift of ca. −750 after trypsinolysis implies that Arg-7 is the site of proteolysis and that Arg-17 is too close to the membrane surface to allow accessibility of the protease. This result may be of general

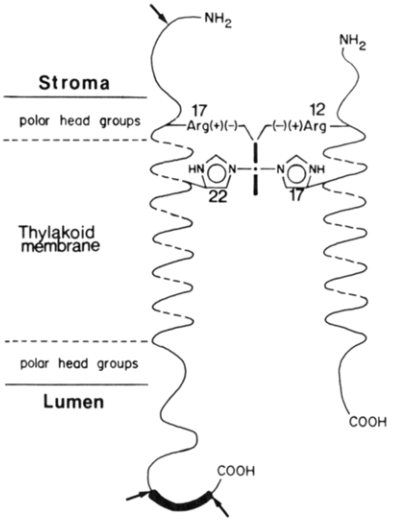

FIGURE 5: Model for orientation of the monoheme unit of cytochrome *b*-559 in the thylakoid membrane bilayer, α subunit (left) and β subunit (right), showing coordination of the heme iron (symbolized by dot in middle of vertical bar) by His-22 and His-17 of the α and β subunits, possible salt bridges to the heme propionic acids from Arg-17 and Arg-12, the trypsin cleavage sites (↓) on the stromal and lumen sides of the α subunit, and the antibody-reactive α subunit COOH-terminal peptide (bold segment).

interest because it is common for hydrophobic α-helical spans of membrane protein to be punctuated by basic residues at the aqueous interface which might be thought to be potential trypsin cleavage sites. (ii) The *b*-559 α subunit COOH terminus is partly shielded by the three OEC extrinsic polypeptides but exposed on the lumen side to trypsin at least as far as Arg-68. This is the first basic residue encountered from the COOH terminus at which cleavage would result in the loss of antibody reactivity. (iii) Furthermore, the conversion of more than $^3/_4$ of the parental NH_2 termini to the shortened form (Figure 2A,B) and the almost complete digestion by trypsin of the antibody site at the COOH terminus in ISO vesicles argue that all α subunits have the same orientation.

Implications of Polypeptide Topography for Heme Location. The above conclusion (iii) that all α polypeptides are similarly oriented indicates that a $(\alpha\beta)_2$ tetrameric model of two asymmetrically oriented heterodimers (Cramer et al., 1986) is probably not correct. The latter model was based on the inference that the αβ heterodimer was the most likely unit stucture for heme binding and also that the two hemes present in the PSII reaction center would be distributed across the membrane, one on each side of the bilayer in analogy with cytochrome b_6 (Widger et al., 1984b). Since the histidine heme ligands in α and β subunits, His-22 and His-17, respectively, are each positioned five residues into the hydrophobic bilayer, location of one heme on each side of the membrane would require an $\alpha_2\beta_2$ model for heme coordination, in which the α and β subunits would be oppositely oriented in the bilayer. If the heme is coordinated by a heterodimer unit, the two β subunits would have the same orientation as the α, with the NH_2 and COOH termini on the stromal and lumen sides, respectively, and both hemes would be located on the stromal side of the membrane. The orientation of the heme in a heterodimer unit, along with sites of trypsin reaction on the two sides of the membrane and the antibody-reactive peptide near the COOH terminus, is shown in Figure 5. Location of the two hemes on the stromal side of the membrane would be consistent with observations of equal accessibility of both hemes to exogenous ferricyanide (Horton & Cramer, 1974; Cramer & Whitmarsh, 1977), but not so

readily with a biphasic oxidation by ferricyanide (Selak et al., 1984). Other data on cytochrome *b*-559 properties, such as differential lability of the two hemes (Cramer & Whitmarsh, 1977) and room temperature photoreduction by PSII of only one heme (Whitmarsh & Cramer, 1977), would in this case require an asymmetry in the environment of the two hemes if they are on the same side of the bilayer.

It will be important to consider the topographical information on the cytochrome *b*-559 hemes when analyzing the structure of the PSII reaction center. Although there are many analogies between the reaction center of the purple photosynthetic bacteria and that of the plant/algal photosystem II (Michel & Deisenhofer, 1988), one important difference is that the latter contains the two cytochrome *b*-559 hemes, in addition to the reaction center chlorophyll(s), auxiliary Chl/pheophytin, and the bound quinone–Fe complex. It is obviously premature to discuss distances between the *b*-559 hemes and P-680 or the auxiliary Chl, although this is of interest because of the photooxidation of cytochrome *b*-559 by PSII at 77 K (Knaff & Arnon, 1969; Thompson & Brudvig, 1988). One distance parameter defined by the present work is that the heme ligand His-22 of the α subunit is positioned five residues, or approximately 7.5 Å, from the stromal end of its hydrophobic domain. The distal heme edge will extend 5 Å further into the bilayer from the position of coordination of the heme iron by the imidazole rings, so that it is approximately 10–15 Å from the stromal-side polar interface.

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