Thylakoid Membrane Protein Topography

LOCATION OF THE TERMINI OF THE CHLOROPLAST CYTOCHROME b$_6$ ON THE STROMAL SIDE OF THE MEMBRANE

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The orientation of cytochrome b$_6$ in the thylakoid membrane and the question of whether the number of membrane spanning helices is an even or odd number was tested through the relative trypsin susceptibility of epitopes (Asp-5 to Gln-14) and (Ile-205 to Leu-214) at the NH$_2$ and COOH termini, respectively, of the 214-residue cytochrome b$_6$ polypeptide. A structure of the cytochrome with an even number of helices and the NH$_2$ and COOH termini on the stromal side of the membrane was inferred from the following: 1) cleavage of cytochrome b$_6$ by trypsin added to thylakoids occurs by removal of both of the exposed NH$_2$- and COOH-terminal epitopes. The epitopes at the termini were more sensitive to trypsin after prior treatment of thylakoids with carboxypeptidase A, indicating that these epitopes are shielded on the stromal side of the membrane by the COOH termini of other proteins. 2) Both epitopes were more trypsin-sensitive in thylakoid membranes than was cytochrome f that is only sensitive to trypsin acting on the lumen side of the membrane. 3) The NH$_2$- and COOH-terminal epitopes of cytochrome b$_6$ were also more sensitive to trypsin added to thylakoid membranes than were the oxygen-evolving complex 16- and 33-kDa proteins that are completely located on the lumen side. 4) The order of trypsin susceptibility was reversed in inside-out membranes, where the cytochrome NH$_2$- and COOH-terminal epitopes were less sensitive than the 16- and 33-kDa proteins. The decreased relative sensitivity of the cytochrome b$_6$ epitopes occurs in spite of a greater absolute sensitivity of these epitopes to trypsin in inside-out membranes. 5) The greater absolute sensitivity can be explained by a 4-helix model that includes trypsin-sensitive sites on the lumen side.

The multisubunit mitochondrial cytochrome bc$_1$, complex and the bc$_1$f complex of oxygenic photosynthesis occupy a central position in the respective electron transport chains, between protein complexes supplying low potential reducing equivalents (dehydrogenases and photosystem II, respectively) and those acting as the terminal oxidizing complex (cytochrome oxidase and the photosystem I reaction center, respectively) (Hauska et al., 1983; Cramer et al., 1987). The bc$_1$ complex of photosynthetic bacteria functions in a cyclic pathway in which the reaction center supplies both proton and oxidant on the n- and p-sides of the trans-membrane complex (Crofts, 1985). The primary sequences of cytochrome b of the mitochondrial and bacterial bc$_1$ complex (consisting of 379-434 residues except for trypanosomes (Hauska et al., 1988)), that will be referred to subsequently as “cytochrome b(bc$_1$)”, have a high degree of identity. Cytochrome b$_6$ is approximately half the size of cytochrome b(bc$_1$). The sequences of the chloroplast-cyanobacterial cytochromes b$_6$ are similar, but show a smaller degree of identity when compared to the sequences of cytochrome b(bc$_1$). The smaller 214-residue chloroplast cytochrome b$_6$ provided the key to identifying the 4 histidine ligands needed for heme coordination (Widger et al., 1984), since it only contains 4 His residues in hydrophobic domains and a total of only 5 in the spinach chloroplast sequence originally analyzed (Alt and Herrmann, 1984).

The distribution of hydrophobic segments in the heme-binding domain was found to be highly correlated between cytochrome b$_6$ and cytochrome b(bc$_1$) (Widger et al., 1984), leading to the inferences (a) that the 4 His residues used in heme coordination are located on two of the 19-23-residue membrane-spanning $\alpha$-helices, with a His pair coordinated to a heme on each side of the membrane that bridge these two helices (Widger et al., 1984; Saraste, 1984); and (b) five and nine helices are utilized, respectively, for the folding of the cytochrome b$_6$ (Fig. 1A) and cytochrome b(bc$_1$) polypeptides in the membrane bilayer, with the histidines coordinating the hemes residing on helices II and V. In these models, the amino acid composition of helix IV of cytochrome b$_6$ is unusually polar (1 Asp, 1 Glu, 1 Ser, 1 Thr, 2 Gly, and 3 Pro in the spinach chloroplasts). Subsequent analysis of the location in the cytochrome b(bc$_1$) polypeptide of inhibitor-resistant mutations in yeast (Di Rago and Colson, 1988; Di Rago et al., 1989) and mouse (Howell and Gilbert, 1988) mitochondria, and in the photosynthetic bacterium Rhodobacter capsulatus (Daldal, 1987; Daldal et al., 1989), indicated (a) that the cytochromes are oriented with the NH$_2$ terminus of these proteins on the n-side of the membrane, and (b) that these cytochromes contain eight membrane-spanning $\alpha$-helices instead of the nine originally proposed. This analysis was based on the use of inhibitors of the cytochrome bc$_1$ complex that are thought to specifically compete for quinone binding sites on the n- or p-side of the membrane. The data on inhibitor-resistant mutants would indicate that these cytochromes contain eight membrane-spanning $\alpha$-helices instead of the nine originally proposed.

The abbreviations used are: n- and p-side, stromal and lumen sides of the thylakoid membrane that have relatively negative and positive values, respectively; Hepes, 4-[(2-hydroxy)-1-piperazinethanesulfonic acid; OEC, oxygen-evolving complex; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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imply by analogy that the cytochrome b$_6$ polypeptide contains four trans-membrane helices (Fig. 1A) instead of five in the original model (Fig. 1B). One problem with the interpretation of the mutant data is that the action of n- and p-side inhibitors is not well defined because mutant loci are found that map in the center of the bilayer (cf. Fig. 4 in Daldal et al., 1989).

The genetic data for the bacterial and mitochondrial b cytochromes have not yet been checked by biochemical methods of topographical analysis. Furthermore, it is important to independently determine the topology of the cytochrome b$_6$ polypeptide because relative to cytochrome b$_6$ (bc$_1$) there are significant structural (Cramer et al., 1987) and functional differences. Regarding the latter, cytochrome b$_6$ unlike cytochrome b$_6$(bc$_1$) does not bind quinone photoaffinity probes (Doyle et al., 1989), and there is evidence that electron transfer between the hemes is not observed (Furbacher et al., 1989). The use of inhibitor-resistant mutants in cyanobacteria to check the topography of the cytochrome b$_6$ polypeptide will be difficult because of the absence or paucity of n-side inhibitors (Furbacher et al., 1989). The present work uses the accessibility/sensitivity to trypsin of epitopes on the cytochrome b$_6$ polypeptide as probes of its orientation in the membrane. A preliminary and abbreviated version of this work has been presented (Szczepaniak and Cramer, 1990).

**Materials and Methods**

**Preparation of Thylakoids—Inside-out, Right Side-out Membranes**

Preparation of Thylakoids—Spinach leaves grown on a 12-h light-dark cycle (50 g) were homogenized in 250 ml of buffer (0.3 M sucrose, 10 mM NaCl, 50 mM Hepes, pH 7.5) for about 5 s, filtered through four layers of cheesecloth, and subjected to centrifugation at 1000 x g (3 min, and immediately stopped by hand). The sediment was resuspended in 50 mM sucrose, 10 mM NaCl, and 50 mM Hepes, pH 7.5, centrifuged again at 1000 x g for 30 s, the sediment discarded, and the supernatant centrifuged (3000 x g, 5 min). The resultant centrifuge pellet was resuspended in homogenization buffer to which it was incubated for at least 30 min to ensure a high degree of unstacking prior to treatment with trypsin or carboxypeptidase.

**Assay of Chloroplast Stacking**—The relative degree of chloroplast thylakoid stacking was monitored by the ability of digitonin to disrupt the membrane systems (Chow and Barber, 1980; Chow et al., 1980). Chloroplasts (0.4 mg/ml) were resuspended in homogenization buffer, allowed to stand for 30 min in 0 °C, and then incubated with 0.5% digitonin at 0 °C for 30 min. The mixture was then diluted 6-fold with the same buffer and centrifuged at 10,000 x g for 30 min. The extent of stacking was related to the fraction of chlorophyll in the centrifuge pellet. Control chloroplasts were resuspended in buffer with 5 mM MgCl$_2$ (fully stacked, 67% of the membrane chlorophyll in the centrifuge pellet) and chloroplasts washed with 5 mM Hepes buffer, pH 7.5 (fully unstacked, 19% chlorophyll in pellet). By these criteria, the extent of thylakoid unstacking after incubation in homogenization buffer was approximately 76%.

**Inside-out and Right Side-out Thylakoid Vesicles**—Spinach leaves (100 g) were homogenized in 300 ml of buffer (0.5 M sucrose, 10 mM NaCl, 50 mM sodium phosphate buffer, pH 7.4), filtered through four layers of cheesecloth, and centrifuged (1000 x g, 3 min, immediately stopped by hand). Chloroplasts were incubated with homogenization medium, centrifuged (1000 x g, 10 min), and then incubated and centrifuged three times with osmotic shock medium (50 mM sucrose, 10 mM NaCl, 50 mM sodium phosphate buffer, pH 7.4). The final centrifuge sediments were resuspended in 5 mM MgCl$_2$, 5 mM NaCl, 10 mM sodium phosphate buffer, pH 7.4, and 100 mM sucrose at a chlorophyll concentration of 4.5 mg/ml. The stacked membranes were passed twice through a Yeda Press (nitrogen gas pressure, 1450 psi) and again twice after addition of EDTA (0.1 mM, pH 7.4, final concentration, 5 mM), and then centrifuged at 1000 x g for 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) polyethylene glycol 3350 (Sigma, P 3840), 10 mM Na-phosphate, pH 7.4, 5 mM NaCl, and 20 mM sucrose. After addition of thylakoid homogenate (4 mg of chlorophyll in 1 ml added to 24 ml), the phase system was mixed and centrifuged at 1500 x g (5 min). The top (T) and lower (B1) phase were collected and repartitioned with pure lower or upper phase, respectively, two more times to generate fractions T3 and B3 (notation of Andersson and Åkerlund, 1978) corresponding to right side-out and inside-out vesicles. Vesicles of each type were diluted with phase partition buffer and sedimented at 100,000 x g (50 min). Purified vesicles were resuspended in 0.3 M sucrose, 10 mM NaCl, and 10 mM Hepes, pH 7.5.

**Peptide Synthesis**

Two decapeptides, (I) NH$_2$-D-W-F-E-E-R-L-E-I-Q-COOH and (II) NH$_2$-R-I-K-Q-G-I-S-G-P-L-COOH, corresponding to epitopes spanning residues 5–14 and 205–214, respectively, of the 214-amino acid cytochrome b$_6$ polypeptide from spinach chloroplasts were synthesized and coupled to bovine serum albumin or keyhole limpet hemocyanin as previously described for the generation of antibodies in rabbits or chickens (Szczepaniak et al., 1989). The position of these epitopes in a 5- or 4-helix model for cytochrome b$_6$ folding in the membrane is shown (Fig. 1). In addition, antibody III to the entire cytochrome b$_6$ polypeptide was prepared to cytochrome b$_6$ electroeluted from an SDS gel of purified (Black et al., 1987) cytochrome b$_6$ complex.

**Protolysis**

Thylakoids (100 μl, 1 mg of chlorophyll/ml) were incubated at room temperature (about 20 °C) with chymotrypsin-free trypsin or carboxypeptidase A treated with diphenylcarbamyllchloride. The trypsin and carboxypeptidase reactions were terminated by addition of 5
mM phenylmethylsulfonyl fluoride and 5 mM o-phenanthroline, respectively.

**SDS-PAGE and Western Blotting**

The control and protease-treated thylakoid membranes were extracted with 90% acetone, recovered by centrifugation (4 min, 10,000 X g) at 4 °C, and were solubilized prior to electrophoresis in buffer containing 4 mM urea, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, and 50 mM Tris, pH 8.6, at a final chlorophyll concentration of 1 mg/ml. Proteins were separated electrophoretically in a gel system using 15% acrylamide (Piccioni et al., 1982). The gel was incubated in 0.025 M Tris, 0.190 M glycine, and 20% methanol, and the Western transfer was done at 100 mA for 2 h, or 150 mA for 30 min using a semidyadic transfer unit (Hoefer). The nitrocellulose paper (Amersham Hybond) was then soaked in Tris-buffered saline buffer (10 ml.

**RESULTS**

**Shielding of Cytochrome b$_6$ at the Stromal Surface**—Although the protease carboxypeptidase A by itself has no effect on cytochrome b$_6$ (Fig. 2, A-B, lane e compared to lane a), the efficacy of the digestion of epitopes I and II by trypsin added to thylakoid membranes was increased if the thylakoids were treated first with carboxypeptidase A. As seen by antibody (Western) analysis, cleavage of cytochrome b$_6$ by trypsin to a slightly smaller protein (ΔM$_r$ =$-1,000-2,000$; $M_r$ = 21,000–22,000 of cleaved product) occurred more completely if the membranes had been pretreated with the carboxypeptidase (compare lanes c and d in Fig. 2 to lane b). O-Phenanthroline was added to inhibit the carboxypeptidase before trypsin treatment so that the two proteases did not act simultaneously. When the order of protease addition was reversed, with trypsin added first and inhibited by phenylmethylsulfonyl fluoride before addition of carboxypeptidase, there was no increase in the extent of proteolysis by trypsin. Therefore, the access of trypsin to the terminal epitopes of cytochrome b$_6$ was increased by prior treatment with carboxypeptidase.

Thus, the segments of cytochrome b$_6$ that are exposed on the stromal membrane surface, including the NH$_2$- and COOH-terminal segments, are shielded by the COOH termini of other thylakoid proteins. A similar effect of carboxypeptidase on the trypsin accessibility to cytochrome b$_6$ was observed previously using heme stain for detection of cytochromes (Szczepaniak et al., 1989). The COOH terminus of cytochrome f in the b$_{6}$f complex is cleaved by carboxypeptidase A added to thylakoids, is exposed on the stromal surface (Willey et al., 1984), and could be involved in shielding cytochrome b$_6$.

Independently of the orientation of the COOH terminus of cytochrome b$_6$ in the membrane, the insensitivity of cytochrome b$_6$ to carboxypeptidase A is not unexpected, since the proline residue at the penultimate position of cytochrome b$_6$ should block the action of the carboxypeptidase (Ambler, 1967), and an earlier report to the contrary was probably due to a contaminating protease in the carboxypeptidase (e.g. chymotrypsin; Ambler, 1967).

The band seen at slightly smaller $M_r$ values (Δ$M_r$ = $-1,000–2,000$) in a Western blot using the antibody against the COOH-terminal and of cytochrome b$_6$ (Fig. 2B) must arise from cleavage at the NH$_2$ terminus. Although the NH$_2$-terminal epitope appears to be slightly more sensitive to trypsin than the COOH-terminal epitope in these experiments, as judged by the presence of the band running at a slightly smaller $M_r$ value in Fig. 2A compared to its absence in Fig. 2B (antibody to NH$_2$ epitope), the difference in sensitivity of the NH$_2$ and COOH termini must be small because it was not seen in all experiments (see below, Fig. 5).

**Relative Rate of Trypsin Proteolysis of Cytochrome b$_6$ in Thylakoids**—The accessibility/sensitivity to trypsin of epitope(s) III of the antibody to the entire cytochrome b$_6$, and epitopes I and II near the NH$_2$ and COOH termini, were compared in thylakoid membranes to that of epitopes of cytochrome f (Figs. 3 and 4) and the OEC extrinsic polypeptides (Figs. 5–7) located on the lumen side of the membrane. Most of the cytochrome f polypeptide, including all of its trypsin-sensitive sites, is also located on the lumen side of the membrane (Willey et al., 1984; Szczepaniak et al., 1989).

![Fig. 2. Effect of protease treatment on cytochrome b$_6$ in mostly stacked thylakoids.](https://example.com/fig2)

Thylakoids were incubated with carboxypeptidase A for 15 min, then with trypsin for 7.5 min at room temperature; lane a, control not treated with protease; lane b, trypsin-treated thylakoids (1:20 w/w), trypsin-chlorophyll; lanes c and d, sequential addition to the thylakoids of carboxypeptidase A (1:8 c), 1:1 d), w/w, carboxypeptidase A:chlorophyll, respectively, and then trypsin (1:20 w/w) after carboxypeptidase A action was terminated by o-phenanthroline; lane e, carboxypeptidase A-treated thylakoids (1:1 w/w, carboxypeptidase A:chlorophyll). Other conditions as in A.

![Fig. 2. Relative Rate of Trypsin Proteolysis of Cytochrome b$_6$ in Thylakoids.](https://example.com/fig2)

**Fig. 3. A–D, lanes b–i, show the effect in one experiment, using membranes pretreated with carboxypeptidase, of increasing trypsin:chlorophyll ratios and times of incubation on the integrity of cytochrome f (Fig. 3A), the COOH- and NH$_2$-terminal epitopes II and I of cytochrome b$_6$ (Fig. 3B and C), and epitope(s) III of cytochrome b$_6$ (Fig. 3D).** The greater sensitivity to trypsin of all of the cytochrome b$_6$ epitopes can be seen. A graph of the undigested epitopes as a function of the trypsin:chlorophyll ratio for several experiments is shown in Fig. 4. The data shown in Fig. 4 did not involve membranes pretreated with carboxypeptidase, but this did not affect the relative trypsin sensitivity of the different epitopes compared...
FIG. 3. A, effect of trypsin proteolysis of cytochrome f in carboxypeptidase A (treated 1:4 (w/w), protease:chlorophyll) in unstacked, thylakoids. After a 15-min incubation, the action of carboxypeptidase A was terminated by o-phenanthroline. The thylakoids were then incubated with trypsin for 7.5 min (1:100, 1:40, 1:20, 1:10 (w/w), trypsin:chlorophyll, lanes c, d, e, and f) at room temperature. Lane a, control; lane b, carboxypeptidase A-treated thylakoids. Membrane proteins were separated in 15% SDS-PAGE and cytochrome f was detected by Western blotting. Each lane was loaded with the membrane equivalent of 10 µg of chlorophyll. B, immunodetection of cytochrome bs trypsinolysis using antibody against epitope II, the COOH-terminal decapeptide, Ile-205 to Leu-214. Conditions as in A, except that the Western blot used the peptide-directed antibody. C, immunodetection of cytochrome bg trypsinolysis using antibody against NHB-terminal decapeptide, epitope I, Asp-5 to Gln-14. Thylakoids were incubated with trypsin for 7.5 min (1:100, 1:40, 1:20, 1:10 (w/w), trypsin:chlorophyll, lanes b, c, d, and e) at room temperature. Lane a, control. Carboxypeptidase A-treated sample not shown. Other conditions as in A, except that the Western blot used the peptide-directed antibody. D, immunodetection of cytochrome bs trypsinolysis using antibody III to the entire protein. Conditions as in A.

FIG. 4. Graph of relative trypsin sensitivity from the stromal side of the thylakoid membrane of epitopes of cytochromes f and bg and of the cytochrome bg NH2 and COOH termini, derived from data similar to those shown in Fig. 3 except that membranes were not treated with carboxypeptidase A. The relative protein concentrations were measured by densitometric analysis of photographic negatives of the Western blots. The density of all bands was in the linear range of spectrophotometric analysis. Epitopes of Cyt f (three experiments, standard deviation shown); ●, COOH terminus of cytochrome bg (data of Fig. 3E using carboxypeptidase-treated membranes not shown); ▲, NH2 terminus of cytochrome bg (data of Fig. 3C using carboxypeptidase-treated membranes not shown); ▲, entire protein of cytochrome bg (three experiments).

to each other. The values plotted for the undigested COOH-terminal epitope II and epitope(s) III of the whole protein are those of the band at the unshifted position in the gel, as shown in Fig. 3, B and D. The data in this experiment also indicate that (i) the virtually simultaneous cleavage ($\Delta M_r = -2,000$–$3,000$) of both NH2 and COOH termini by trypsin can be detected with antibody III to the intact protein (Figs. 3D and 5D; Table I); the loss of the NH2 epitope with increasing exposure to trypsin (Figs. 2B and 3C), without concomitant observation of a slightly smaller band, indicates that one does not observe cleavage of the COOH terminus alone. (ii) A small fraction of the cytochrome bg population starting in lane e (trypsin:chlorophyll = 0.05) of Fig. 3B shows cleavage of the NH2 terminus alone. The $\Delta M_r$ value of $-2,000$–$3,000$ for the slightly smaller component ($M_r$, 21,000) generated by trypsinolysis that reacts with antibody III to the intact cytochrome (Fig. 3D) is approximately twice that for antibody II to the COOH-terminal epitope (Fig. 3B), implying that antibody III detects cytochrome bg cleaved simultaneously at both termini. Antibody III must then react with at least one epitope that is resistant to the trypsin added to thylakoids, in addition to reacting with the NH2- and COOH-terminal epitopes. The presence of lower molecular size bands in these gels due to proteolysis at positions within the protein might have been anticipated. Such bands of $M_r > 3000$–$4000$ could have been detected, but were not found. The absence of such proteolytic cleavage products was noted previously in a study of bacteriorhodopsin proteolysis, and was attributed to destabilization by protease treatment of the membrane-embedded segments that allowed further access to protease and extensive proteolysis (Dumont et al., 1985).
FIG. 5. A, trypsin proteolysis of 16-kDa OEC extrinsic protein in unstacked thylakoids. Thylakoids were incubated with trypsin for 7.5 min (1:200, 1:100, 1:40, 1:20, 1:10 (w/w), trypsin:chlorophyll, lanes b, c, d, e, and f) for 15 min (1:10 (w/w), trypsin:chlorophyll, lane g), and for 30 min (1:10 (w/w), lane h) at room temperature. Lane a, control not treated with protease. Other conditions as in Fig. 3 except membranes were not pretreated with carboxypeptidase A. The 16-kDa polypeptide was detected by a Western blot using antibody to the entire protein. B, immunodetection of cytochrome $b_6$ trypsinolysis using antibody against the COOH terminus decapeptide, Ile-205 to Leu-214. Conditions as in A, except that the Western blot used the peptide-directed antibody. C, immunodetection of cytochrome $b_6$ trypsinolysis using antibody against NH$\text{$_2$}$-terminal decapeptide, Asp-5 to Glu-14. Conditions as in A, except that the Western blot used the peptide-directed antibody. D, trypsin proteolysis of cytochrome $b_6$. Conditions as in A except that the cytochrome $b_6$ was detected by Western blots using antibody to the entire protein.

TABLE I

| Trypsin/chlorophyll ratio (w/w) | 1:200 | 1:100 | 1:40 | 1:20 | 1:10 | 1:5 | 1:5 (15 min) |
|---------------------------------|-------|-------|------|------|------|----|--------------|
| Cyt $b_6$ (%)                   | 91$^a$ | 83    | 88   | 86   | 91   | 80 | 74           |
|                                 | (9.1) | (3.9) | (8.5)| (7.9)| (6.8)| (8.0)| (9.6)        |
| 16 kDa (%)                      | 92    | 89    | 84   | 82   | 73   | 66 | 56           |
|                                 | (2.6) | (2.4) | (6.9)| (10)| (10) | (2.9)| (3.3)        |

$^a$ Sum of parent $M_r$, 23,000 and product $M_r$, 21,000 bands.

$^b$ Relative to control not treated with trypsin-trypsin treatment for 7.5 min except for last column, 15 min; the values are the means of 4 to 5 different measurements, with the standard deviation in parentheses. Data from Fig. 5D.

The 16-kDa OEC polypeptide was also found to be relatively resistant to trypsin added to thylakoid membranes compared to epitopes I–III of cytochrome $b_6$ (15-min digestion, no carboxypeptidase A pretreatment) (Figs. 5 and 6). Trypsinolysis of epitope(a) III of cytochrome $b_6$ again results in a slightly smaller $M_r \approx 21,000$ product with the sum of the density of the protein bands in lanes b–f (Fig. 5D) equal to 85–90% of the control (Fig. 5D, lane a) (Table I), supporting the conclusion that the $M_r$, 21,000 band arose from simultaneous cleavage at both NH$\text{$_2$}$ and COOH termini. Thus, the loss of the $M_r$, 23,000 cytochrome $b_6$ caused by trypsinolysis of thylakoid membranes is caused by cleavage at the NH$\text{$_2$}$ and COOH termini and not to cleavage at another exposed site within the sequence (e.g. Lys-110 to Lys-111, Fig. 1).

The relative rate of cleavage of the $M_r$, 33,000 OEC polypeptide by trypsin also indicated a smaller accessibility of this OEC protein to trypsin relative to the cytochrome $b_6$ epitopes I–III, as summarized in the graph of Fig. 7.

The sensitivity/accessibility of the NH$\text{$_2$}$ and COOH-terminal epitopes of cytochrome $b_6$ to trypsin, relative to the
OEC polypeptides, is reversed in inside-out membranes (Figs. 8 and 9), compared to the intact thylakoids. The relative sensitivities to trypsin are (Fig. 8): cytochrome b₆ (NH₂) (epitope I) ≈ cytochrome b₆ (COOH) (epitope II) < 33 kDa < 16 kDa. The relative sensitivity of the 16- and 33-kDa polypeptides to each other in the inner membrane surface is as expected from their extractability from inside-out membranes and their relative proximity to the membrane-bound manganese of photosystem II (Kuwabara and Murata, 1983; Seibert et al., 1987). The smaller relative sensitivity of the cytochrome b₆ NH₂ and COOH epitopes I and II is observed in spite of a greater absolute sensitivity of the cytochrome b₆ epitopes to trypsin in the inside-out thylakoid membranes (Fig. 9). The greater absolute sensitivity of cytochrome b₆ detected by antibodies I and II would be expected if trypsin-sensitive sites (K-148, R-169) in the peptide loop between helices III and IV are exposed on the lumen side of the membrane, as predicted by the 4-helix model (Fig. 1A). This would not be expected from a 5-helix model (Fig. 1B) because in the latter model there are no trypsin sites on the lumen side. The absence of lower molecular weight products in these gels again implies that cytochrome b₆ may be destabilized in the membrane after initial proteolysis at an internal exposed loop, allowing further access to protease and extensive degradation (Dumont et al., 1985).

**DISCUSSION**

The relative sensitivity of epitopes of cytochrome b₆ and of other thylakoid membrane proteins to trypsin was used in the present work as an indicator of their sidedness and orientation in the membrane bilayer. The net sensitivity of an epitope will depend not only on its position in the membrane, but also on its intrinsic sensitivity (i.e. in the case of trypsin, number of accessible lysine and arginine residues), and on the extent of the shielding of these residues by other intrinsic and peripheral membrane proteins and the membrane surface. The shielding of cytochrome b₆ by the COOH termini of other proteins demonstrated here and in Szczepaniak et al. (1989) is an example of the latter effect.

The OEC proteins, particularly the 16-kDa polypeptide (very sensitive) and cytochrome f (relatively insensitive) represent two opposite ends of the spectrum with respect to their sensitivity to trypsin on the lumen side of the membrane. The relative insensitivity of cytochrome f to V8 protease, compared to the exposed lumen side loops of cytochrome b₆ was documented previously (Szczepaniak et al., 1989). The high intrinsic sensitivity of the OEC proteins to trypsin (Isogai et al., 1985; Jansen et al., 1987; Tae and Cramer, 1989) suggests that they would be expected to be more sensitive to trypsin than cytochrome f, and perhaps the most trypsin-sensitive of the proteins on the lumen side.

The dependence of each of the epitope or epitope systems of cytochrome b₆ and the reference proteins on the amount of trypsin added to thylakoids is not a single monophasic function (Figs. 4, 6, and 7). These graphs indicate that 30-50% of these epitopes are a relatively resistant fraction. At least part of the resistant fraction might be attributed to stacking of the membranes, because approximately 25% of the thylakoids were stacked ("Materials and Methods").

The comparison of the susceptibility of the NH₂- and COOH-terminal epitopes to trypsin in the intact thylakoids (order of susceptibility: cytochrome f < 16 kDa = 33 kDa < COOH-cytochrome b₆ = NH₂-cytochrome b₆), with that in inside-out thylakoid membranes (order of susceptibility: NH₂-cytochrome b₆ ≈ COOH-cytochrome b₆ < 33 kDa < 16 kDa),

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**Fig. 7. Relative trypsin sensitivity of epitopes of cytochrome b₆, cytochrome b₆ NH₂- and COOH-termini epitopes, and 33-kDa OEC extrinsic protein from the stromal side using mostly unstacked thylakoids.** Conditions as in Fig. 5. The points are the means of three different experiments. Epitopes of 35-kDa OEC extrinsic protein: NH terminus of cytochrome b₆ (NH*); COOH terminus of cytochrome b₆, and entire protein of cytochrome b₆; standard deviation representative of all cytochrome b₆ samples is shown.

**Fig. 8. Relative rate of trypsino- lysis of cytochrome b₆ and OEC extrinsic proteins (16 and 33 kDa) in inside-out vesicles.** A, immuno- detection of trypsino- lysis of cytochrome b₆ using antibody against the COOH-terminal decapetide epitope II. Mem- branes were treated with trypsin for 7.5 min (1:200, 1:100, 1:40, 1:20, 1:10 (w/w), trypsin:chlorophyll, lanes b, c, d, e, and f, respectively) and for 15 min (1:10 (w/w), trypsin:chlorophyll, lane g) at room temperature. Lane a, control, not treated with protease. B, immuno- detection of trypsino- lysis of cytochrome b₆ using the antibody against the NH₂-terminal decapetide, epitope I. Conditions otherwise as in A. C, immuno- detection of trypsino- lysis of 16-kDa OEC extrinsic protein. D, immuno- detection of trypsino- lysis of 33-kDa OEC extrinsic protein.
FIG. 9. Graph of sensitivity of cytochrome \( b_6 \) NH\(_2 \) and COOH termini relative to that of OEC extrinsic proteins (33 and 16 kDa) to trypsin added to inside-out vesicles. Data are the average of three experiments similar to the data shown in Fig. 8. Data from lane \( g \) not plotted. Relative protein concentrations measured by densitometric analysis as in Figs. 4, 6, and 7. Epitopes of: \( \Delta \), OEC 16-kDa protein; □, OEC 33-kDa protein; ■, cytochrome \( b_6 \), NH\(_2 \) terminus; and ●, cytochrome \( b_6 \), COOH terminus.

implies that both the NH\(_2 \) and COOH termini are on the stromal side of the membrane. This orientation is also implied by the comparison of the trypptic cleavage products of cytochrome \( b_6 \) by epitopes I, II, and epitope(s) III, indicating that this cleavage occurs predominantly by simultaneous removal of both termini. The number of membrane-spanning helices is therefore even and most likely four.

The main features of the 4-helix model are that (i) the two hemes cross-link helices II and IV, the heme on the lumen side (\( b_6 \)) coordinated by His-85 (helix II) and His-186 (helix II), and that on the stromal side (heme \( b_6 \)) by His-99 (helix II) and His-201 (helix IV). (ii) The 2 His residues on helix II are separated by 13 residues, and the 2 on helix IV by 14. The inter-His distance on helix IV is one more than in cytochrome \( b_{(bc)} \) and has been proposed to account for the more homogenous redox and spectral properties of the two hemes of cytochrome \( b_6 \) (Cramer et al., 1987). An additional unique intramembrane property of cytochrome \( b_6 \) is the arginine residue at position 86 in the bilayer next to His-85. This residue is apparently conserved in the chloroplast and cyanobacterial sequences, but not in cytochrome \( b_{(bc)} \), and is the only charged residue of cytochrome \( b_6 \) that appears to be in the hydrophobic membrane bilayer. (iii) Fairly long peripheral loops (about 25 and 42 residues, respectively) connect helices I–II and III–IV, on the lumen side of the membrane. The latter loop is analogous to a region of the cytochrome \( b_{(bc)} \) of \( Rb. \) capsulatus that has been inferred from the presence of a large number of p-side inhibitor-resistant loci, to be involved in quinone binding (Daldal et al., 1989). This is consistent with the model of Brasseur (1988) in which this peptide segment is proposed to be an amphipathic α-helix bound closely to the membrane surface. On the other hand, this III–IV interhelix loop was found to be much more accessible to V8 protease in permeabilized thylakoids than was cytochrome \( f \) (Szczepaniak and Cramer, 1989), implying that the interhelix loop III IV of cytochrome \( b_6 \) protrudes from the membrane surface. In addition, the binding of quinone photo-affinity labels to subunit IV and not cytochrome \( b_6 \) indicates that the former polypeptide and not the latter binds the quinol donor to the \( b_{f} \) complex (Doyle et al., 1989). In this case, the III IV interhelix loop of cytochrome \( b_6 \), unlike that of cytochrome \( b_{(bc)} \), would not be involved in the quinone binding function. The trypsin probe experiments of cytochrome \( b_6 \) in the thylakoid membrane reported here indicate that in terms

of orientation, topography, and heme binding, cytochrome \( b_6 \) is analogous to the heme-binding domain contained in the NH\(_2\)-terminal half of the larger cytochrome \( b \) of the cytochrome \( bc \) complex.

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