

Topography of the Chloroplast Cytochrome b_6 : Orientation of the Cytochrome and Accessibility of the Lumen-Side Interhelix Loops

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Z. Naturforsch. **44c**, 453–461 (1989); received January 27, 1989

Dedicated to Professor Achim Trebst on the occasion of his 60th birthday

Electron Transport, Photosynthesis, Membrane Proteins, Topography, Cytochrome b_6 - f Complex

The topography of chloroplast cytochromes f and b_6 was probed with proteases carboxypeptidase A (CpA), trypsin, and *Staph. aureus* V8. The cytochrome and its proteolytic products were detected by heme stain and, in most experiments, by immunoreaction. In thylakoids, the only protease that significantly affected the intactness of cytochrome f was CpA that caused a small ($\Delta M_r = -1-2000$) decrease in the apparent molecular weight. In SDS-treated thylakoids, both trypsin and V8 degraded cytochrome f . The inferred topography of cytochrome f , with the COOH-terminus on the stromal (n) side, one membrane-spanning α -helix near the COOH-terminus, and most of the Cyt f mass on the lumen (p) side, is consistent with that previously inferred by others.

Cytochrome b_6 was not sensitive to CpA, but was more sensitive to trypsin and V8 protease than cytochrome f , cytochrome b -559, or the 17 kDa OEC extrinsic protein. Trypsin caused a small decrease in size of cytochrome b_6 , which was observed using whole protein antibody as a single smaller band ($\Delta M_r \approx 2000$) or two smaller discrete bands ($\Delta M_r = -1000$ and 2500 , respectively) which, unlike the untreated protein, did not react with antibody generated to a peptide mimicking Asp-5–Gln-14 near the NH₂-terminus. These shortened tryptic fragments were attributed to cleavage after R-10 and K-23 near the NH₂-terminus, implying an orientation with the NH₂-terminus on the stromal side of the membrane. The sensitivity of cytochrome b_6 toward this trypsin cleavage was increased if the membranes were first incubated with CpA, showing that the NH₂-terminal region of cytochrome b_6 is masked by the COOH-terminal domain of one or more thylakoid proteins.

Under conditions where degradation of cytochrome b -559, cytochrome f , or the 17 kDa OEC extrinsic protein on the lumen side of the membrane was small ($\leq 10-20\%$), V8 protease degraded 80–90% of cytochrome b_6 . The main polypeptide fragment generated by V8 protease had an apparent molecular weight $M_r = 16,000$, reacted with antibody to a peptide mimicking Ala-146–Asp-155, and was more pronounced in membranes treated with SDS. The fragment could then result from cleavage at two sites, Glu-74 and/or Glu-166. It is concluded that the loop of cytochrome b_6 protruding from the lumen side of the membrane between helices III and IV, and possibly that between helices I and II as well, contains a single Glu residue conferring a sensitivity to V8 protease greater than that of the lumen-side mass of cytochrome f which has many more Glu residues. This implies a lumen-skeletal structure in which one or two loops of cytochrome b_6 on the lumen side are more exposed to the aqueous phase than cytochrome f .

Introduction

The cytochrome b_6 - f *trans*-membrane complex functions in the electron transport chain between

Abbreviations: Chl, chlorophyll; DCMU, 3-(3'-dichlorophenyl)-1',1'-dimethylurea; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; HPLC, high performance liquid chromatography; NQNO, 2-*n*-nonyl-4-hydroxyquinoline-N-oxide; OEC, oxygen-evolving system; PMSF, phenylmethanesulfonyl fluoride; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; TBS, Tris-buffered saline.

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Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/89/0500–0453 \$ 01.30/0

PS II and PS I in oxygenic photosynthesis [1]. In chloroplasts, it has been found to contain four polypeptides of $M_r > 10,000$ [2, 3]. Three of these (cytochrome f , b_6 , and the [2Fe–2S] Rieske center) contain redox centers, and three (cytochrome f , b_6 , and subunit IV) are chloroplast encoded. A fifth M_r 20,000 polypeptide in the complex has been proposed for membranes of *C. reinhardtii* [4]. Hypotheses can be made for the folding pattern in the membrane bilayer of such membrane proteins from (i) their amino acid sequences, usually determined for the NH₂-terminal region of the protein, and inferred for the remainder from the nucleotide sequence of the respective genes, and (ii) calculation ("hydropathy") of the distribution of long (20–25 residue) hydro-



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phobic segments associated with these sequences [5]. From the structural studies on bacteriorhodopsin [6, 7] and the bacterial photosynthetic reaction center [8, 9], as well as comparison of average helix length in intrinsic membrane *versus* soluble proteins [10], these long hydrophobic segments are believed to span the 30–40 Å hydrophobic membrane bilayer as α -helices.

This kind of analysis can provide initial hypotheses for structure, although it provides no information on the sidedness of the membrane protein. In the case of the bacterial reaction center L and M polypeptides, the prediction of five membrane spanning helices [11, 12], although not their precise boundaries, turned out to be accurate [8, 9]. For cytochrome *f*, inspection of the 285 residue sequence in pea [13] or spinach [14] chloroplasts indicated only one long hydrophobic span, residues 251–270 near the COOH-terminus of the protein. Shorter hydrophobic spans can be visualized when the “hydropathy” program utilizes a smaller averaging interval [15].

Cytochrome *b₆* contains four His residues in hydrophobic domains that are conserved in the cytochrome *b* of the *b-c₁* complex of mitochondria from many phyla [16–18]. The hydropathy plots for these cytochromes were found to be mathematically highly correlated [16]. This led to the prediction that the four His residues used to coordinate the two hemes of the cytochrome are located on two of the membrane-spanning helices, with a His pair co-ordinated to a heme on each side of the membrane bilayer and thereby cross-linking the two helices (Fig. 1). Heme cross-linking of bilayer-spanning helices of membrane-bound cytochromes occurs in the chloroplast PS II reaction center cytochrome *b-559* [19, 20], is predicted to occur in cytochrome oxidase subunit I [21], and thus may be a common structural motif in intrinsic membrane-spanning cytochromes.

Subsequent analysis of the location in the mitochondrial cytochrome *b* polypeptide of inhibitor-resistant mutations to DCMU and antimycin in yeast [22] and mouse [23] mitochondria, and to myxathiazol and stigmatellin in the photosynthetic bacteria [24], implied (i) that the heme binding domain of these *b*-cytochromes contained eight membrane-spanning α -helices instead of the nine originally proposed. The segment removed from the bilayer and proposed to reside in the polar phase in the model inferred from the mutation work [22, 23] was helix

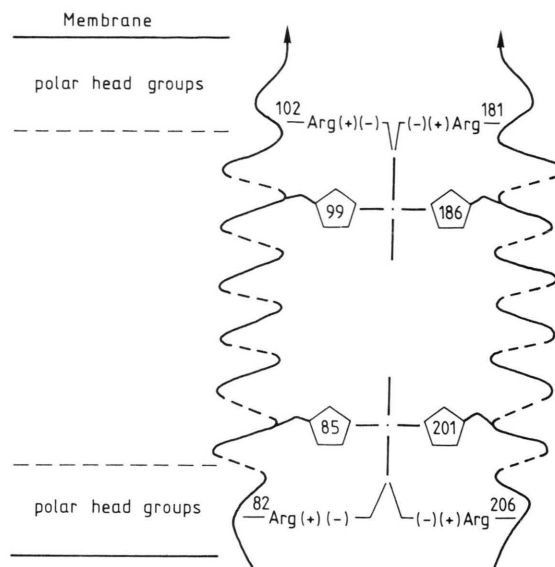


Fig. 1. Double heme cross-linked model of cytochrome *b₆*. The *trans*-membrane orientation is shown of two hemes coordinated by two pairs of intramembrane His residues located on both sides of two membrane-spanning α -helices.

IV in the original model [16, 17]. It does not contain His residues. Extrapolation of these results to the structure of the chloroplast cytochrome *b₆* would imply that it has four membrane-spanning α -helices with the two heme-binding His residues on helices II and IV (Fig. 2A), rather than the five originally proposed with the critical His residues on helices II and V (Fig. 2B). It was also predicted from the location of DCMU- and antimycin A-resistant mutants that the NH₂-terminus of the mitochondrial and bacterial cytochromes is on the n-side (stromal side)* of the membrane. These data for the mitochondrial and bacterial cytochromes, that rely on a specific sidedness of action of particular inhibitors, have not been checked by other methods of topographical analysis. The use of inhibitor-resistant mutants to study the topography of the cytochrome *b₆* will be somewhat more difficult since antimycin A does not clearly affect its response [1]. Furthermore, it has been argued that electron transfer does not occur in chloroplasts from heme *b_p* to heme *b_n* [25], so that it may not be possible to locate inhibitor-resistant loci in the cytochrome *b₆* gene analogous to the antimycin A- and DCMU-resistant loci in mitochondria and photosynthetic bacteria. The action of the inhibitor,

NQNO, often proposed to resemble antimycin in its action on cytochrome b_6 , appears to be not readily classified as n-side or p-side [25].

Previous studies on the topography of cytochrome b_6 concluded (i) the cytochrome is more sensitive to trypsin added from the p-side (lumen side)*, because of increased loss of heme-stained products in inside-out, as opposed to right side-out membranes [26]; (ii) the COOH-terminus is on the n-side, as inferred by an apparent sensitivity of the heme-stained cytochrome to CpA [26], (iii) the cytochrome was sensitive to the non-specific protease, pronase E, added from the n-side, although it could not be labeled from this side by the membrane-impermeant reagent, TNBS [27].

Methods

Chloroplast preparation

Spinach leaves (50 g) were homogenized in 250 ml buffer (0.3 M sucrose, 10 mM NaCl, 50 mM Hepes, pH 7.5) for about 5 sec, filtered through four layers of cheesecloth, then subjected to centrifugation at $1000 \times g$ (3 min), and immediately stopped by hand. The sediment was resuspended in 50 mM sucrose, 10 mM NaCl and Hepes, pH 7.5, centrifuged again at $1000 \times g$ for 30 sec, the sediment discarded, and the supernatant centrifuged at $3000 \times g$ for 5 min. The resultant pellet was resuspended in homogenization buffer for trypsin or carboxypeptidase A treatment, or in 50 mM bicarbonate buffer, pH 7.8, 0.3 M sucrose, for *Staph. aureus* protease V8 digestion. In the latter buffer, the V8 protease specifically cleaves on the COOH-side of glutamic acid residues [28].

Peptide synthesis

Two decapeptides, $\text{NH}_2\text{-D-W-F-E-E-R-L-E-I-Q-COOH}$ and $\text{NH}_2\text{-A-V-K-J-V-T-G-V-P-D-COOH}$, corresponding to residues 5–14 and 146–155, respectively, in the 214 amino

acid cytochrome b_6 polypeptide from spinach chloroplasts [18], were synthesized on an Applied Bioscience 430 A automated solid-phase instrument at the Purdue University Biotechnology Center using standard techniques. The purity of the products was assayed by reverse-phase (water–acetonitrile) HPLC.

Preparation and purification of antibodies

The cytochrome b_6f complex isolated from spinach chloroplasts [3] was subjected to preparative gel electrophoresis. After a short (1 h) staining period, the band corresponding to cytochrome b_6 was cut from the gel and protein was electroeluted using the Elutrap of Schleicher & Schüll and the Laemmli system buffer [29] with 0.1% SDS in the upper reservoir buffer. The preparation routinely yielded a single protein band in the region of interest.

The protein (about 1 mg/ml) was mixed with an equal volume of complete Freund's adjuvant. One ml (0.5 mg of protein) of the resulting emulsion was injected into young rabbits at multiple intradermal sites. The first booster with 0.2 ml of protein solution (1 mg/ml) mixed with an equal volume of Freund's incomplete adjuvant was given three weeks later. Similar boosts were given at two additional three week intervals. Sera were collected one week after the last booster injection.

Preparation of peptide-directed antibody. Five mg of keyhole limpet hemocyanin (KLH) or bovine serum albumin (BSA), was mixed with a 30 M excess of synthetic decapeptide, Asp-5–Gln-14 or Ala-146–Asp-155 in 2 ml 0.1 M potassium phosphate buffer, pH 7.5. Glutaraldehyde (20 mM, 10 ml) in phosphate buffer was added dropwise while the mixture was stirred at room temperature. The reaction was permitted to proceed with gentle agitation at room temperature for 30 min. The mixture was dialyzed overnight against 0.15 M NaCl and 0.01 M potassium phosphate buffer, pH 7.2, 4 °C. The solution of coupled KLH or BSA containing 1 mg of protein was mixed with an equal volume of complete Freund's adjuvant and injected into rabbits. Two boosters of 0.5 mg coupled protein were administered after 5 and 7 weeks using incomplete Freund's adjuvant.

γ -Globulins from the serum were obtained by ammonium sulfate precipitation [30, 31] and further purified by affinity chromatography using cyto-

* In order to use a common notation for all energy transducing membranes, the n- and p-side are defined as those with relatively negative and positive values, respectively, of the $\bar{\mu}_{\text{H}^+}$.

chrome b_6f complex coupled to CNBr-activated Sepharose 4B (Pharmacia).

solved in water at a concentration of 1 mg/ml and also stored at -20°C .

Proteolysis

Thylakoids (100 μl , 1 mg Chl/ml) were incubated with trypsin (Sigma, T-1005), CpA (Boehringer, 103-223), or V8 protease (Boehringer 291-156) in the respective membrane resuspension media at room temperature (*ca.* 20°C) for the indicated times with the chlorophyll/protein ratios detailed in the figure legends. The trypsin and V8 protease reactions were terminated by addition of 5 mM PMSF, and the carboxypeptidase reaction by 5 mM *o*-phenanthroline. The membranes were immediately washed with 90% acetone, recovered by centrifugation (4 min, $10,000\times g$) at 4°C , and then solubilized prior to electrophoresis.

Electrophoresis

Samples (10–20 μg /Chl) were solubilized in 4 M urea, 2% SDS, 10% glycerol, 5% mercaptoethanol, 50 mM Tris buffer, pH 8.6 [32], with 15% acrylamide. The gel was either stained with Coomassie Brilliant Blue R or used for immunoblotting. The gel was washed for 15 min in a blotting solution containing 0.025 M Tris, 0.193 M glycine and 20% methanol [33] and the transfer carried out using a current of 100 mA for 2 h. The nitrocellulose (Hybond-C) paper was then soaked in TBS buffer (10 mM Tris, pH 7.5, 0.15 M NaCl) with 0.25% gelatin and 5% powdered non-fat dry milk for at least 3 h and usually overnight. After equilibration with cytochrome b_6 antibody in 0.25% gelatin and TBS for 2 h, and washing in TBS containing the detergent 0.05% Nonidet P-40 (Accurate Chemical Scientific Corp.), the paper was reacted with second antibody conjugated to peroxidase in 0.25% gelatin and TBS. After a 2 h incubation, the paper was washed in TBS containing 0.05% Nonidet P-40 and stained for 10 min with 0.017% *n*-chloro-1-naphthol in TBS.

The stock trypsin solution was maintained at 10 mg/ml in 10^{-3} M HCl, where it was stable at -20°C for at least several weeks without loss of activity. The precise concentration was established by measuring the absorbance at 280 nm ($A_{1\text{cm}}^{1\%} = 15.4$). The trypsin was diluted just before use to 1 mg/ml in 50 mM Hepes, pH 7.5, containing 50 mM CaCl_2 added to retard autolysis [34]. V8 protease was dis-

Results

The four and five helix models for folding of the cytochrome b_6 polypeptide are shown in Fig. 2A, B, with the sidedness not specified. The charged residues are noted that are potential sites for trypsin (R, arginine; K, lysine) and *Staph. aureus* V8 protease (E, glutamic acid). It is assumed that in intact thylakoids the proteases cannot act at the charged residues immediately bordering the membrane-spanning helices even if the boundaries of these helices are not known precisely. This assumption appeared to be valid in a technically similar study on the topography of cytochrome b -559 [35].

1. Effect of carboxypeptidases and trypsin on cytochrome f and b_6 in thylakoid membranes

Cytochrome f is resistant to trypsin added to thylakoid membranes, but as shown by Willey *et al.* [13], is cleaved to a slightly smaller size ($\Delta M_r = 1-2000$) by carboxypeptidase A. Surprisingly, it is resistant to the less specific protease, carboxypeptidase Y (data not shown), perhaps because of the larger size of the latter. Successive treatment of the membranes with trypsin and carboxypeptidase A does not affect the characteristic pattern obtained with either protease alone (Fig. 3, top).

Carboxypeptidase A (CpA) has no effect on cytochrome b_6 , as measured by heme-stain (Fig. 3, bottom) or by immunoblot (not shown), in contrast to the result obtained by Mansfield and Anderson [26], but a result expected in retrospect since the Pro residue at the penultimate position should block CpA [36]. Little effect of trypsin on cytochrome b_6 is shown in Fig. 3, although as shown below, higher concentrations of trypsin have a definite effect. The efficacy of the trypsin at these lower concentrations is increased, however, if the membranes are treated first with CpA. *o*-Phenanthroline was added as an inhibitor of CpA before trypsin treatment so that the two proteases did not act simultaneously. Reversing the order of protease addition, with successive addition of trypsin inhibitor and CpA had no effect, showing that the access of trypsin was increased by prior treatment with CpA. Thus, the COOH-terminal region of one or more thylakoid proteins, possi-

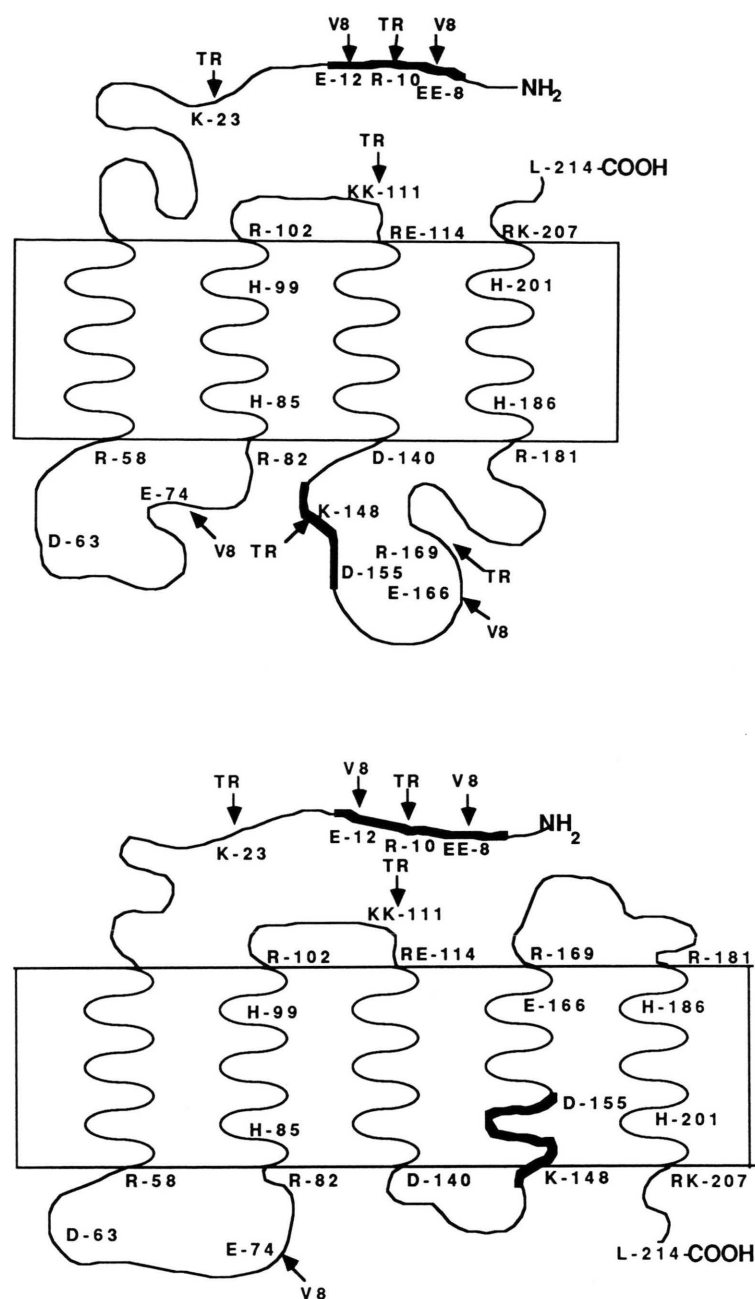


Fig. 2. Four (A) and five (B) helix models of cytochrome *b₆* in the membrane bilayer. The position is noted (i) of the charged residues that are possible sites for the action of trypsin (on K, R) or V8 protease (on E under these experimental conditions), and (ii) of the peptide segments to which polyclonal antibody was raised for detection of proteolytically generated peptide fragments of cytochrome *b₆*.

bly including cytochrome *f*, shields a part of the cytochrome *b₆* protein that includes the NH₂-terminal peptide region.

Successive treatment of thylakoid membranes with CpA and trypsin resulted in generation of two bands of slightly smaller molecular weight ($\Delta M_r \approx -1000$

and 2500) (Fig. 3), as would be predicted by the presence of R-10 and K-23 (Fig. 2) near the NH₂-terminus. This implied that the NH₂-terminus of a major part of the cytochrome *b₆* population is exposed on the stromal side of the membrane. It is not always possible to resolve both of the slightly smaller *b₆* bands

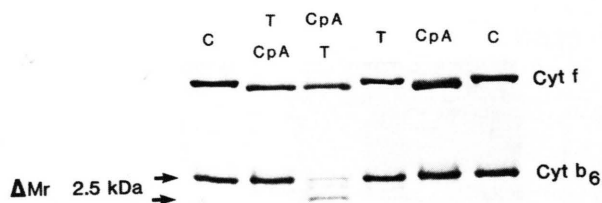


Fig. 3. Effect of protease treatment on cytochrome f and cytochrome b_6 in intact thylakoids. Thylakoids were treated with trypsin and carboxypeptidase A (protease:Chl, <1:40 and 1:2, respectively). Heme-stained gel showing cytochrome f (upper bands) and cytochrome b_6 (lower bands). Protease incubation for 15 min at room temperature, trypsin and carboxypeptidase A action terminated by addition of PMSF (5 mM) and *o*-phenanthroline (5 mM), respectively. Lane: C, control; T, trypsin; CpA/T, sequential addition to the thylakoids of carboxypeptidase A, and then trypsin after CpA action was terminated by *o*-phenanthroline; T/CpA, sequential treatment of thylakoids, first with trypsin, then after trypsin action was terminated by PMSF, with CpA. Each lane was loaded with the membrane equivalent of 25 μ g Chl.

on the gel, as shown by the effect of increasing concentrations of trypsin on cytochrome b_6 immuno-detected by Western blotting (Fig. 4A). The simultaneous decrease of the parental band and concomitant increase in the relative intensity of the cleaved poly-

peptide (Fig. 4B) implies a parent-daughter relation. However, no daughter bands can be detected when antibody for the Western blot is used that is directed to the peptide (Fig. 2) extending between residues Asp-5 and Gln-14 (Fig. 4C). This confirms that tryp-

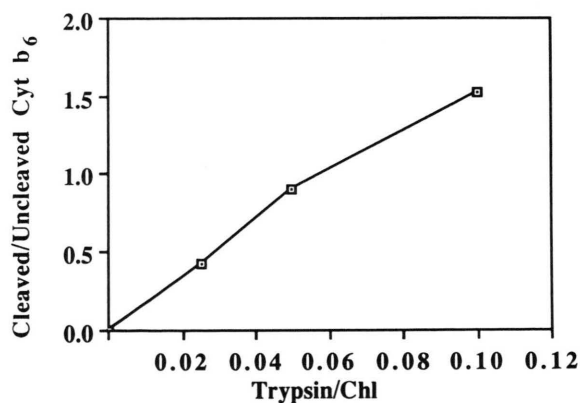
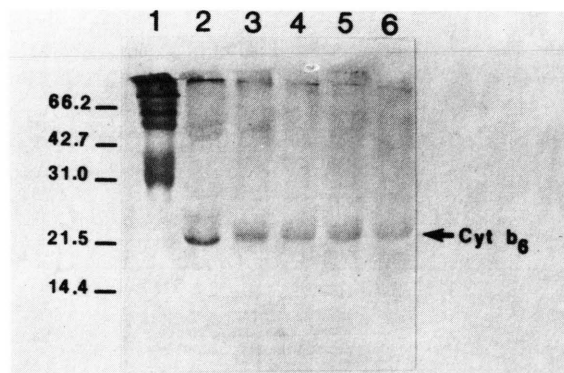
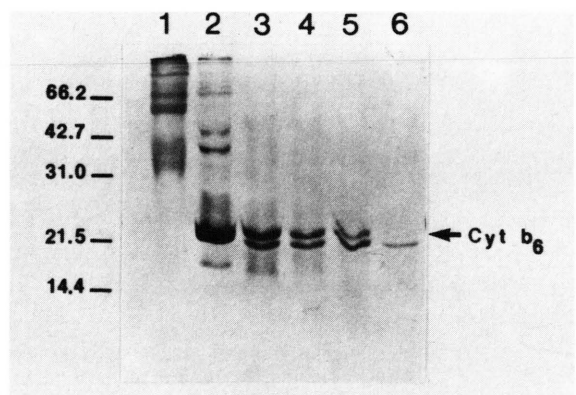


Fig. 4. Trypsinolysis of cytochrome b_6 in intact thylakoid membranes. (top left) Thylakoids were treated with trypsin for 15 min (1:40, 1:20, 1:10 or 1:5 weight ratio, trypsin:Chl lanes 3–6, respectively) at room temperature. Membrane polypeptides were separated in 15% SDS-PAGE and cytochrome b_6 was detected by a Western blot using polyclonal antibody. Each lane was loaded with the membrane equivalent of 10 μ g Chl. Lane 1, prestained SDS molecular weight markers (Sigma); lane 2, control. For precise molecular weight determination, SDS-PAGE standards (low molecular weight, Bio-Rad) were used: lysozyme, MW = 14,400; soybean trypsin inhibitor, 21,500; carbonic anhydrase, 32,000; ovalbumin, 42,700; bovine serum albumin, 66,200. (bottom left) Ratio of intensities of daughter/parent band as function of time. (top right) Immunodetection of trypsinolysis products using antibody against the decapeptide, Asp-5–Gln-14. Conditions and SDS gel the same as in (A), except that the Western blot used the peptide-directed antibody.

sin acts on the R-10 and/or K-23 sites, removing all or most of the peptide region with which that peptide-directed antibody can react. The loss of total intensity implies that the trypsin also acts on at least one other site that is a major epitope. If it is assumed that R-102, R-113, and R-206, K-207 are too close to the bilayer to allow protease accessibility, this trypsin-sensitive major epitopic site would be KK-111 (Fig. 2). The existence of the latter double Lys residue on the stromal side may explain the extensive degradation of cytochrome *b₆* seen in lanes 5 and 6 of Fig. 4. Under the conditions used in lane 5 (15 min, 1:10 ratio of trypsin:Chl), the loss of cytochrome *f*, cytochrome *b-559*, and the 17 kDa OEC extrinsic protein was 25–30%, <20%, and 20–25%, respectively. The sensitivity of cytochrome *b₆* to trypsin degradation is much greater in the presence of a small amount of detergent added to permeabilize the membrane (data not shown) as in the data in the right-hand lanes of Fig. 5, 6.

2. Effect of V8 protease

The lack of effect of trypsin (Fig. 3) or V8 protease (Fig. 5, lanes 1–5) on cytochrome *f* in intact thylakoids implies that no charged sites are accessible from the stromal side, confirming the model with only one membrane span previously proposed for cytochrome *f* [13, 14]. The action of protease on the

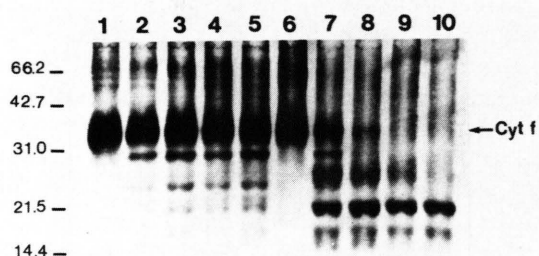


Fig. 5. V8 proteolysis of cytochrome *f*. V8 protease digestion of cytochrome *f* in intact (lanes 1–5) and SDS (0.2%) treated (lanes 6–10) thylakoid membranes. Thylakoids were incubated with V8 protease at room temperature: lanes 1, 6, controls, not treated with protease; lanes 2, 7, 15 min, 1:40 (w/w, protease:Chl); lanes 3, 8, 15 min, 1:20; lanes 4, 9, 30 min, 1:5; lanes 5, 10, 60 min, 1:5. Other conditions as in Fig. 4.

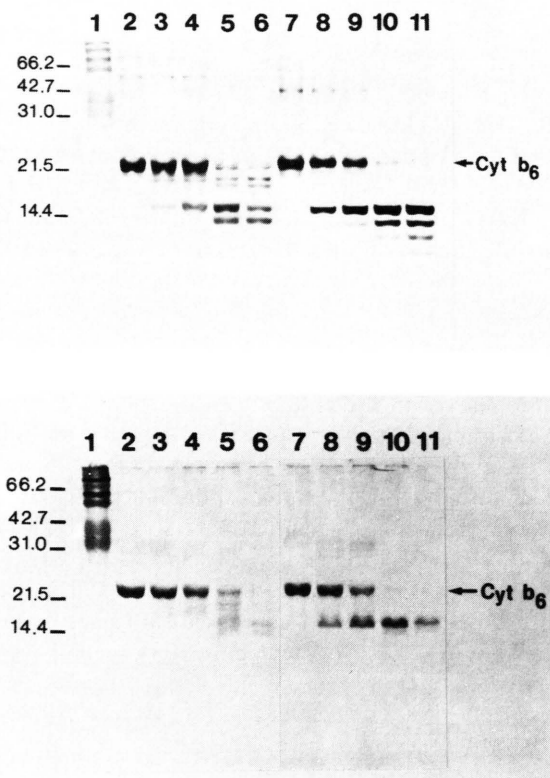


Fig. 6. V8 proteolysis of cytochrome *b₆*. (A) V8 protease digestion of cytochrome *b₆* in intact (lanes 2–6) and SDS (0.2%) treated thylakoids (lanes 7–11). Thylakoids were incubated with V8 protease at room temperature: lane 1, prestained molecular weight markers; lanes 2 and 7, controls not treated with protease; lanes 3, 8, 15 min, 1:40 (w/w, protease:Chl); lanes 4, 9, 15 min, 1:20; lanes 5, 10, 30 min, 1:5; lanes 6, 11, 60 min, 1:5. Other conditions as in Fig. 4. (B) Conditions as in (A), except that the Western blot used antibody against the peptide Ala-146–Asp-155.

lumen side of the membrane was tested with detergent-permeabilized membranes. When the protease is added to the thylakoid membranes in the presence of SDS (0.2%), the action of V8 protease on the lumen side causes complete loss of the parental band and the appearance of a major stable product at $M_r \approx 21,500$ (Fig. 5, lanes 6–10). The SDS was found not to affect the activity of the V8 protease in control experiments with casein (not shown).

Using V8 in the presence or absence of SDS, cytochrome *b₆* is again more sensitive to the protease (Fig. 6A, lanes 1–6) than is cytochrome *f*. Lanes 3–6 and 8–11 show the dependence of the proteo-

lysis on increasing protease concentration and time of incubation. A 15 min incubation (1:20, protease:Chl) causes the appearance of an M_r 15,800 (± 250 , $n = 4$)* product that also reacts with antibody directed against the peptide Ala-146–Asp-155 (Fig. 6B). The M_r 15,800 product appears more quickly when the protease is added in the presence of SDS (Fig. 6A, lanes 7–11). An M_r 14,000 (± 400 , $n = 4$) product appears clearly along with the M_r 15,800 band in lanes 5 and 10 in the absence and presence of SDS after 30 min (1:5, V8:Chl), with both of these bands having about twice the density in the presence of SDS. Both of these peptide fragments, as well as larger fragments that result from cleavage at E-8 or E-12, react with the antibody to the 146–155 peptide, although in the SDS-treated membranes the only clearly reactive band is the M_r 15,800. In the presence of the detergent, two additional smaller discrete proteolytic fragments are seen at M_r 12,000 (± 500 , $n = 4$) in lanes 10, 11, and at M_r 10,500 (± 800 , $n = 3$) in lane 11 of Fig. 6. These bands are disregarded in the subsequent discussion since in the lanes where they appear, cytochrome b_6 is completely degraded.

Discussion

Cytochrome f

The observations on cytochrome f topography are consistent with existing data and models [13, 14], with the COOH-terminus on the stromal side of the membrane, the protein spanning the bilayer with one α -helix spanning the approximate domain of residues 251–270 and the rest of the cytochrome f mass on the lumen side. Cytochrome f is the only thylakoid membrane protein whose topography is known that has its NH_2 -terminus on the lumen side of the membrane. The one surprise is the relative inaccessibility of the large lumen-size mass of cytochrome f to protease. The insensitivity to V8 added to the stromal side is consistent with cytochrome f spanning the membrane only once. Cytochrome f has 17 Glu residues on the NH_2 -side of the hydrophobic span, so that generation of the stable M_r 21,500 product by V8 protease treatment implies that most of the residues are masked. It had previously been shown that cytochrome f was much more accessible to proteinase K [13] and somewhat more sensitive to trypsin [26] in inside-out compared to right side-out vesicles

[13]. This is as expected if most of the cytochrome mass and consequently a larger number of potential protease sites are on the lumen side.

Cytochrome b_6

Using detection by heme stain it was also shown that cytochrome b_6 was about twice as sensitive as cytochrome f to trypsin in inside-out vesicles [26]. The use of heme stain alone does not allow a conclusion about protein sensitivity to the protease treatment since cytochrome b_6 is intrinsically more labile toward heme loss than is cytochrome f .

The conclusions reached in this study on the sidedness of the cytochrome b_6 polypeptide and on the likelihood of a four vs. a five-helix model are in agreement with the topographical conclusions from the studies of inhibitor-resistant cytochrome b mutants in yeast and mouse mitochondria [22, 23, 37], and the preliminary studies in *Rb. capsulata* [24]. The NH_2 -terminus of cytochrome b_6 is accessible to trypsin on the n-(stromal) side of the membrane. With this orientation, the five-helix model (Fig. 5B) predicts only one protease site, one for V8 acting at E-74 between helices I and II, on the p-(lumen) side. There would be no obvious trypsin sites on the lumen side in the five-helix model. The four-helix model (Fig. 2A) predicts that there should be three additional sites, two for trypsin and one for V8 protease, in the loop between helices III and IV. The increased sensitivity of cytochrome b_6 to trypsin in detergent-treated membranes (data not shown), as well as with inside-out membranes [26], indicates that the four-helix model is more likely. This conclusion is supported by studies of the discrete peptide fragments generated by V8 protease added to thylakoids. With the NH_2 -terminus on the stromal side, the M_r 16,000 and M_r 14,000 fragments generated by V8 protease must arise from cleavage (i) at Glu-166, generating two fragments because of partial cleavage at E-12, E-8, or (ii) from cleavage at Glu-166 and E-74. The exact nature of these peptide fragments awaits the sequencing of their NH_2 -termini.

The explanation of the extensive degradation of cytochrome b_6 at sites on the lumen side, from protease added on the stromal side, is that at high protease concentrations and/or prolonged incubation, the thylakoids are somewhat leaky to the protease. Yet the degree of this leakiness, measured by degradation of cytochrome f , cytochrome b -559, and the 17 kDa OEC protein, is quite small, especially con-

* n , number of trials.

sidering the large number of Glu residues in the latter two proteins, 17 in Cyt *f* [14] and 12 in the 17 kDa protein [38]. Assuming that the cytochrome *b₆* polypeptide has only one orientation in the bilayer, the greater sensitivity of cytochrome *b₆* would imply that at least one of its lumen-side interhelix loops is more accessible to the aqueous phase than is cytochrome *f*. The detailed structure of these loops is of interest. Both may be involved in a lumen-skeletal structure that is important for H⁺ translocation and binding of plastocyanin. In addition, the loop joining helices III and IV contains a myxathiazol-resistant mutation (at the locus equivalent to A-146 in cytochrome *b₆*) in

the mouse mitochondrial cytochrome [23], and is therefore presumably part of the quinone-binding site on the p-side of the bilayer.

Acknowledgements

This work was supported by U.S. NIH grant GM-38323. We thank Profs. B. Andersson and D. W. Krogmann, respectively, for antibody to the extrinsic 17 kDa polypeptide and to cytochrome *f*, Mr. G.-S. Tae for helpful discussions, and Ms. Janet Hollister for bringing order from chaos in the manuscript.

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