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Topographical studies of the polypeptide subunits of the thylakoid cytochrome b_6-f complex

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The orientation of specific polypeptides of the cytochrome b_6-f complex with respect to the chloroplast stromal phase has been studied using trinitrobenzenesulfonate (TNBS) and pronase E as impermeant modifying reagents. Of the four polypeptides of the complex (33, 23, 20 and 17 kDa), only cytochrome f was labeled by ^{14}C-TNBS in unfractionated membranes. However, to a varying degree, all of the constituent polypeptides were sensitive to pronase digestion and, in the case of cytochrome f, it was possible, by immunoblotting techniques to identify several degradation products. These results are discussed in relation to the organization of the cytochrome complex in thylakoid membranes and argue for an exposure to the stromal phase of all of the polypeptides, while functional considerations indicate that at least cytochrome f and the Rieske iron-sulfur protein have a possible transmembrane organization.

Introduction

The cytochrome b_6-f complex is an integral protein complex found in thylakoid membranes that catalyzes electron transfer from reduced quinones, such as plastohydroquinone. to plastocyanin and other high-potential electron acceptors [1,2]. The availability of a highly purified preparation of this complex [1] has allowed for detailed studies of structure-function relationships regarding both electron transport and proton translocation [3–6].

The purified cytochrome complex contains cytochrome f, cytochrome b_6, the Rieske iron-sulfur protein (20 kDa), and one additional polypeptide of unknown function [2]. One plastoquinone molecule has also been found associated with the complex [7]. While much recent work has concentrated on understanding electron-transport processes involving the complex, little is known concerning the organization of the individual polypeptide components of the complex in relation to their orientation in the thylakoid membrane, and this problem has been considered in the present study.

Two membrane impermeant modification procedures, trinitrobenzenesulfonate (TNBS) labeling and pronase E digestion, have been used to probe the topography of the cytochrome b_6-f complex in unfractionated thylakoids. ^{14}C-TNBS has been used to label exposed polypeptides and the extent of modification followed by SDS-polyacrylamide gel electrophoresis in conjunction with fluorography. Pronase digestion of surface-exposed polypeptides has been analyzed utilizing SDS-polyacrylamide gel electrophoresis in conjunction with immunoblotting procedures involving specific antibodies to components in the cytochrome com-

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Abbreviations: TNBS, trinitrobenzenesulfonate; Chl, chlorophyll; Cyt c, cytochrome c; LHCP, light-harvesting chlorophyll-protein complex.

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plex. Our results are discussed in relation to the orientation of specific polypeptides in the cytochrome b_6-f compelx with respect to the chloroplast stromal phase.

Materials and Methods

Preparation of chloroplast membranes

Chloroplast thylakoid membranes were prepared from freshly harvested greenhouse-grown spinach. Leaves were homogenized in a Waring blender in a solution containing 0.3 M sucrose/10 mM NaCl/50 mM potassium phosphate buffer (pH 8) and the resulting slurry was filtered through filtering silk. Chloroplasts were collected by centrifugation of the filtrate at $3000 \times g$ for 1 min and resuspended in a 1 : 10 dilution of blending solution. Thylakoid membranes were isolated by centrifugation at $35\,000 \times g$ for 10 min and washed once with 0.3 M sucrose and 30 mM potassium phosphate buffer (pH 8). The membranes were resuspended in the same solution at a chlorophyll concentration of 1 mg/ml.

Chemical modification with TNBS

Thylakoid membranes (5 mg chlorophyll) were modified by reaction with 10 μCi [^{14}C]TNBS (Research Products International, Inc.; Mt. Prospect, IL; 45–55 μCi/mmol) for 3 h at 4°C. The membranes were washed twice by centrifugation with 20 mM Tricine-KOH buffer (pH 8) to remove unreacted [^{14}C]TNBS.

Pronase digestion of thylakoid membranes.

Thylakoid membranes were resuspended in a solution containing 0.3 M sucrose and 30 mM potassium phosphate buffer (pH 8) at a chlorophyll concentration of 1 mg/ml. The suspension was incubated at 27°C for 20 min in the presence of pronase E (Protease type XIV; Sigma Chem. Co.). The treated membranes were washed extensively by centrifugation (at least 4 times) with ice-cold incubation medium. These washings were sufficient to stop the reaction by pronase, since it has been found that this enzyme is inactive at 4°C (results not shown).

Preparation of cytochrome b_6-f complex

[^{14}C]TNBS modified membranes or those modified in the presence of pronase were fractionated by detergent solubilization using *n*-octyl-β-D-glucopyranoside/cholate followed by ammonium sulfate fractionation and centrifugation on sucrose gradients according to the procedure of Hurt and Hauska [8] for the isolation of the cytochrome b_6-f complex.

SDS-polyacrylamide gel electrophoresis, heme-staining, immunoblotting and fluorography

Modified thylakoids and the resolved cytochrome b_6-f complex were analyzed by SDS-polyacrylamide gel electrophoresis on slab gels (1.5 mm thick) according to the method of Chua [9] using 10–15% gradient gels. Samples were solubilized prior to electrophoresis in sample buffer containing 2% SDS/2% β-mercaptoethanol/2% glycerol/50 mM Tris-HCl (pH 8). Molecular weight standards were bovine serum albumin, ovalbumin, chymotrypsinogen, cytochrome *c* and bovine trypsin inhibitor.

Gels were prepared for fluorography according to the method of Laskey and Mills [10] as modified by Burkhardt et al. [11] or stained specifically for heme using the procedure of Thomas et al. [12] to identify the cyt *f* and cyt b_6 containing polypeptides. In some instances, gels were electroblotted to nitrocellulose paper and specific polypeptides detected using antibodies raised in rabbits against the different polypeptides of b_6-f complex. We routinely used the BioRad Immuno-Blot (GAR-HRP) Assay Kit (colorimetric method) to detect areas of antigen-antibody reaction according to the instructions from the manufacturer.

Antibody

Antibodies to individual polypeptides of the cytochrome complex were obtained after injection of individual subunits (isolated from the maize complex by preparative SDS-polyacrylamide gel electrophoresis) into white rabbits. Initial injections were made in Freund's complete adjuvant and booster injections were made in Freund's incomplete adjuvant. Serum samples were collected by standard procedures and used without purification.

Results

TNBS and pronase as impermeant membrane probes

Previous studies from our laboratory on the

membrane organization of Photosystem I have utilized [^{14}C]TNBS and pronase E as modifying reagents [13]. The former reacts primarily with free amino groups under mild conditions while the latter is a non-specific protease that will digest surface-exposed polypeptide regions. In the course of this and previous work on the topography of Photosystem I and the cytochrome b_6-f complex using pronase E, it has been possible to establish a number of observations that indicate the effects of pronase E on thylakoids are not the consequence of gross alterations in membrane integrity. (1) No loss of plastocyanin, a protein known to be localized in the chloroplast lumenal space, occurred, even after treatment with the highest concentrations of pronase E used in this work. (2) No loss of cytochrome f (approx. 650 Chl/cyt f) occurred from pronase-treated thylakoids, based on oxidized minus reduced chemical difference spectra. (3) Studies on Photosystem I have shown no change in the Chl/P-700 ratio after pronase treatment or no alteration in the low-temperature fluorescence properties of Photosystem I. The latter result indicates no loss or dissociation of chlorophyll from the Photosystem I complex due to membrane alteration arising from pronase treatment. Moreover, activities, such as P-700 photooxidation and its subsequent dark reduction by plastocyanin, were unaffected in Photosystem I complexes prepared from pronase-treated thylakoids. These results indicate that the protease had no access to the lumenal portions of the complex where reduced plastocyanin interacts with P-700$^+$. An effect of pronase on Photosystem I donation by reduced plastocyanin was observed when the isolated complex was directly treated with pronase. (4) The Photosystem I complex prepared from thylakoids treated with increasing amounts of pronase shows a series of discrete peptides on SDS-polyacrylamide gel electrophoresis, and these are immunologically related to the reaction center polypeptide of approx. 62 kDa. This would be expected if the membrane served as a barrier to the penetration of the protease, and the enzyme only digested surface-exposed portions of polypeptides. When the isolated Photosystem I complex is treated with the protease, discrete peptides are not observed, and SDS-polyacrylamide gel electrophoresis shows a smeared profile resulting from unhindered random digestion of accessible peptides. (5) Not all polypeptides of thylakoid membranes display the same sensitivity to pronase E digestion. Some polypeptides of the Photosystem I complex are totally resistant to pronase, indicating no substantial membrane degradation. On the whole, these results indicate that the thylakoid membrane acts as a barrier to penetration of pronase E, and that the effects of the proteolytic enzyme on such membranes is the result of a removal of exposed portions of proteins, and not the consequence of the loss of membrane integrity. We have therefore used pronase E, as well as TNBS, under the specific conditions developed in our previous study to modify stroma-exposed components of thylakoids and have isolated the cytochrome b_6-f complex from modified membranes to investigate effects specific to this membrane complex.

[^{14}C]TNBS labeling of the subunits of the cytochrome b_6-f complex

The results of a study of the components of the cytochrome b_6-f complex isolated from [^{14}C]TNBS-labeled thylakoids are shown in Fig. 1. Lane 1 shows the Coomassie-blue staning profile

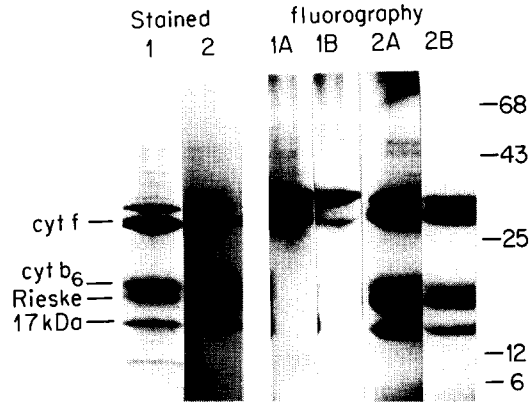

Fig. 1. [^{14}C]TNBS labeling of subunits of the cytochrome b_6-f complex. Cytochrome b_6-f complex was prepared from spinach thylakoids modified with [^{14}C]TNBS at 4°C. Likewise, an isolated cytochrome b_6-f complex was modified directly with [^{14}C]TNBS. The cytochrome complex was analyzed by electrophoresis on SDS-polyacrylamide gel electrophoresis and radioactive polypeptides were identified by fluorography. The Coomassie blue profiles of these complexes are presented in lanes 1 and 2, respectively. The corresponding fluorographs (lanes 1A, 1B or 2A, 2B) were taken at different exposure times. Lane 1A, 3 weeks; lane 1B, 1 week; lane 2A, 1 week; and lane 2B, 3 days.

of the cytochrome complex with its four main subunits: cytochrome *f* (33 kDa), cytochrome b_6 (23 kDa), the Rieske iron-sulfur protein (20 kDa) and a 17 kDa polypeptide [1,2]. A band migrating above cytochrome *f* may be the ferredoxin-NADP reductase, as discussed by Clark et al. [14]. Lanes 1A and 1B are fluorographs taken at two different exposures to detect [^{14}C]TNBS in the polypeptide subunits. The only labeled subunits in the complex is cytochrome *f*, although the 'putative' reductase band also shows heavy labeling. No TNBS labeling of the three other polypeptides of the cytochrome complex is detected even when fluorographs are overexposed (lane 1A). If an isolated cytochrome b_6-*f* complex (lane 2) is reacted with [^{14}C]TNBS and subsequently analyzed by fluorography (lanes 2A and 2B), all the subunits are modified by [^{14}C]TNBS. These results indicate that groups reactive towards [^{14}C]TNBS are present in all the polypeptides of the complex, but are only available in thylakoids in a single polypeptide (cytochrome *f*).

Pronase sensitivity of cytochrome f in thylakoids

The effect of increasing concentrations of pronase E on thylakoid polypeptides is shown in Fig. 2. The left-hand portion of the figure shows the Coomassie-blue staining pattern and indicates extensive degradation of numerous polypeptides. To analyze for cytochrome *f*, the gel was stained specifically for heme (right-hand panel of Fig. 2). In the absence of pronase, a heme-staining band which has previously been shown to be associated with cytochrome *f* [1] is present at approx 33 kDa. Even at low concentrations of pronase (3–10 μg), there is a decrease in the heme-staining intensity as the cytochrome is digested by pronase. At higher concentrations of pronase (30–300 μg), little or no heme-staining band remains associated with cytochrome *f*.

Pronase sensitivity of the cytochrome cytochrome b_6-f in thylakoids

In order to study the effect pronase digestion on the other components of the cytochrome complex, the complex was isolated by the procedure of Hurt and Hauska [8] from thylakoids treated with increasing amounts of pronase and the constituent polypeptides analyzed by SDS-polyacrylamide gel electrophoresis.

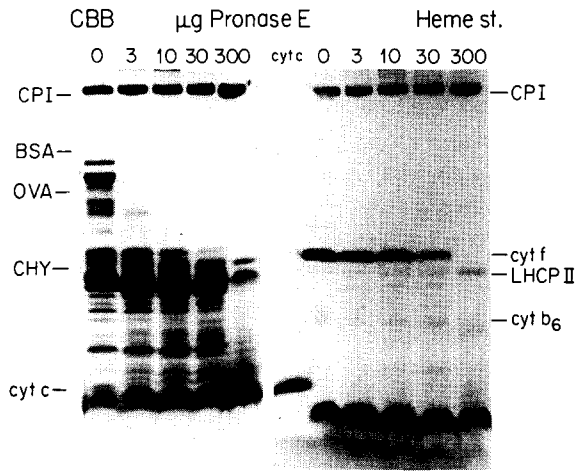

Fig. 2. Sensitivity of cytochrome *f* to pronase in spinach thylakoids. Thylakoids were incubated at 27°C for 20 min in the presence of the indicated pronase concentrations (0–300 μg/ml). Polypeptides of the thylakoids were analyzed by SDS-polyacrylamide gel electrophoresis and stained with Coomassie blue (left panel) or specifically for heme (right panel). BSA, bovine serum albumin, OVA, ovalbumin; CHY, chymotrypsinogen.

As shown in Fig. 3 (left panel), the Coomassie blue staining patterns indicate a substantial degradation of cytochrome *f* in membranes treated

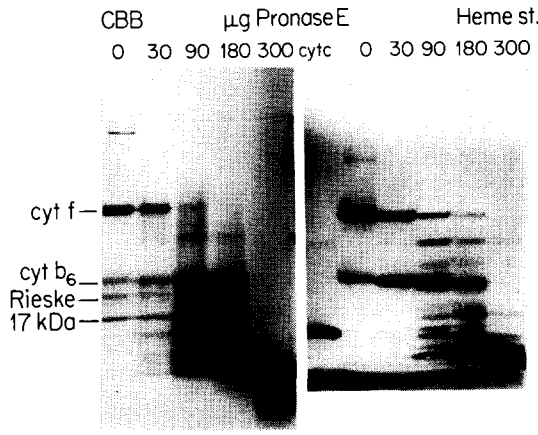

Fig. 3. Cytochrome b_6-*f* complex prepared from pronase treated thylakoids. Thylakoids were treated with the indicated concentrations of pronase (0–300 μg/ml) at 27°C for 20 min. Cytochrome b_6-*f* complex from treated membranes was prepared according to the procedures outlined by Hurt and Hauska [8]. Constituent polypeptides of the complex were analyzed by SDS-polyacrylamide gel electrophoresis. Gels were stained with Coomassie brilliant blue (CBB) (left panel) or specifically for heme (right panel). Equal amounts of cytochrome *f* were loaded on each lane.

with 30–90 μg pronase. It is important to mention that we find substantial differences in the pronase used in this work such that concentrations required for modification varied significantly with different lots of the enzyme. Thus, the results in Figs. 2–4 are not directly comparable in terms of pronase concentrations, although the results in any one experiment can be compared. Heme-staining (Fig. 3, right panel) shows that a series of lower molecular-weight polypeptides are produced (particularly evident at 90 and 180 μg pronase) in the complex isolated from pronase-treated membranes, some of which comigrate with cytochrome b_6. The larger number of heme-staining bands observed in this figure but not evident in the analysis of unfractionated thylakoids (Fig. 2) is due to the increased sensitivity obtained through the analysis of a purified complex versus unfractionated membranes.

Because of the possible comigration of Coomassie-blue staining bands from degraded polypeptides with other constitutent polypeptides of the complex, it is difficult to draw conclusions concerning the sensitivity to pronase of the lower molecular weight components of the complex. It appears that some degradation of the Rieske iron-sulfur protein occurs at the lowest concentration of pronase used (see also Fig. 4), while the 17 kDa polypeptide appears more resistant to pronase. At the highest concentration of pronase, however, degradation of all subunits has occurred, and a strong Coomassie-blue staining region appears below 12 kDa.

Analysis of the cytochrome b_6-f complex from pronase-treated membranes by immunoblotting

In order to analyze specific polypeptides of the cytochrome b_6-f complex from pronase-treated thylakoids, a colorimetric immunoblotting procedure was employed utilizing antibodies to individual polypeptides of the complex. As shown in Fig. 4A, when the complex isolated from pronase-treated membranes is probed with an antibody to cytochrome f, a series of smaller cross-reacting peptides are formed as a result of digestion. These are particularly evident at 90 and 180 μg pronase. With 300 μg pronase, only low molecular-weight cross-reacting products (less than 12 kDa) are detected. In this particular experiment there is little degradation of cytochrome f when 30 μg pronase was used during the treatment.

The available antibody to cytochrome b_6 shows some reaction towards the 20 kDa polypeptide associated with the Rieske iron-sulfur center, as shown in Fig. 4B (control). At a pronase concentration of 30 μg, however, there is degradation of the 23 kDa and 20 kDa polypeptides, and this degradation increases markedly as the pronase concentration is increased (Fig. 4B). Similar results are obtained with an antibody to the 20 kDa Rieske protein (Fig. 4C) in that degradation of this polypeptide is obvious even at the lowest concentration of pronase used (30 μg). A small zone of contamination below the Rieske protein makes identification of smaller digestion products difficult to define.

In Fig. 4D is an analysis using an antibody specific for the 17 kDa subunit of the cytochrome complex. A small amount of degradation appears after treatment with 30 μg pronase and a lower molecular-weight staining band appears after degradation with 90 and 180 μg of pronase. In contrast to the results with the other subunits, no cross-reacting material remains after treatment

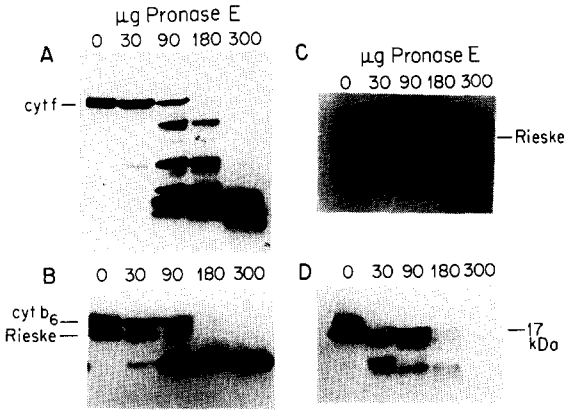

Fig. 4. Immunological identification of polypeptides and fragments associated with different subunits of the isolated b_6-f complexes from pronase-treated thylakoids. The cytochrome b_6-f complex from pronase-treated thylakoids were resolved on SDS-polyacrylamide gel electrophoresis. The gels were electroblotted on nitrocellulose paper and allowed to immunoreact with antibody raised against cytochrome f (panel A), cytochrome b_6 (panel B), the Rieske-protein (panel C), or the 17 kDa polypeptide (panel D). Areas of antigen-antibody reaction were detected colorimetrically using a commercial immunoblot assay kit.

with the highest concentration of pronase used (300 μg pronase).

Discussion

In the present work, two experimental approaches have been used to study the possible stromal orientation of the polypeptide subunits of the cytochrome b_6-f complex. TNBS labeling and degradation by the proteolytic enzyme, pronase E, have been previously utilized to study the transmembrane topography of the chloroplast Photosystem I complex [13] and with both these procedures, it was shown that they specifically modify stromal-exposed portions of thylakoid polypeptides [13]. The present study indicates that, to a varying degree, all of the polypeptide constituents of the cytochrome b_6-f complex have regions exposed to the stromal phase. This conclusion is based on labeling of cytochrome f by TNBS and by partial pronase digestion of all the polypeptides of the cytochrome complex in unfractionated thylakoids.

While the function of all the polypeptide constituents of the cytochrome complex is not yet known, several polypeptides have been associated with well-characterized electron-transfer carriers. The 33 kDa polypeptide is the apoprotein of cytochrome f and this carrier is believed to accept electrons from the Rieske iron-sulfur center and to transfer them to plastocyanin [2,15]. The latter is localized in the chloroplast lumenal space [15,16] and thus a portion of the cytochrome f polypeptide must be accessible to the lumen in order to interact with plastocyanin.

Several recent reports have considered the transmembrane orientation of cytochrome f. Bricker and Sherman [17] found trypsin-sensitivity of cytochrome f in unfractionated maize membranes, while Willey et al. [18] reported no effect of trypsin, but proteolytic digestion by other enzymes (chymotrypsin, proteinase K and carboxypeptidase) in studies with pea thylakoids. The findings of Mansfield and Bendall [19] showed almost no reactivity of cytochrome f towards pronase E in right-side out vesicles (stromal side) but the removal of a 1 kDa segment of the cytochrome by this enzyme in inside-out vesicles (lumenal side). The recent model of Willey et al. [18] and Alt and Hermann [20], based mostly on hydropathy plots, show one transmembrane segment present along with a short (14 amino acids) segment exposed on the stromal exposed surface and a large (approx. 250 amino acids) lumenal exposed portion.

In our current study, we have confirmed that cytochrome f has a stromal-exposed portion as TNBS labeling of the polypeptide is observed in unfractionated thylakoids. Results of pronase digestion are more difficult to interpret in terms of the above model. Several heme-staining as well as immunological cross-reacting bands associated with cytochrome f are detected after pronase treatment of thylakoids. It is significant that the digestion of cytochrome f does not occur at a pronase concentration at which other polypeptides of the cytochrome complex show significant degradation. This result would argue for a screening of a substantial part of the cytochrome f subunit by neighboring polypeptides and that alteration of the latter allows a greater accessibility of pronase to cytochrome f. This mechanism would allow for a series of immunologically related polypeptides to be produced that are dependent on the sites of pronase digestion.

The Rieske iron-sulfur center has been shown to be associated with a 20 kDa polypeptide [21] and although this polypeptide is unreactive towards TNBS, it is readily digested by pronase. Since the Rieske center is the presumed electron donor to cytochrome f, a portion of the protein must be lumenal-exposed in relation to this function, and the protein would also appear to have a transmembrane segment. Amino acid sequence data for this nuclear-encoded protein are not yet available and comparisons with a predicted structure cannot be made.

The remaining two polypeptides of the cytochrome complex (23 and 17 kDa) also show segments localized on the stromal side of the membrane. The 23 kDa polypeptide has been shown to be the apoprotein of cytochrome b_6 [2,8] and five membrane-spanning loops have been predicted on the basis of a hydropathy plot for this protein [22,23]. The 17 kDa polypeptide is of unknown function and shows the most resistance to pronase digestion of any of the polypeptides of the cytochrome complex. This resistance could arise from large segments of the protein being transmem-

brane or from a screening of exposed portions of the protein by overlapping sections of neighboring polypeptides. Recent sequence analyses have predicted three membrane-spanning regions for this peptide [23,24] and during digestion with pronase some low molecular-weight products were detected by immunoblotting but the resolution was not sufficient to define the molecular weight of these products. Our results do, however, indicate that the 17 kDa polypeptide has stromal-exposed regions.

In summary, all of the polypeptide constituents of the cytochrome b_6-f complex have stromal-exposed regions. While two of these proteins (the Rieske iron-sulfur protein and cytochrome f) have functions which lead one to expect a lumenal-exposed region as well, the localization of lumenal portions for the 17 kDa polypeptide and cytochrome b_6 have not yet been verified although transmembrane orientations for these two polypeptides have also been predicted.

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