CHLOROPLAST MEMBRANES OF THE GREEN ALGA

ACETABULARIA MEDITERRANEA

II. Topography of the Chloroplast Membrane

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ABSTRACT

The localization of the chlorophyll-protein complexes inside the thylakoid membrane of Acetabularia mediterranea was determined by fractionating the chloroplast membrane with EDTA and Triton X-100, by using pronase treatment, and by labeling the surface-exposed proteins with $^{125}$I. The effects of the various treatments were established by electrophoresis of the solubilized membrane fractions and electron microscopy. After EDTA and pronase treatment, the membrane structure was still intact. Only the two chlorophyll-protein complexes of 67,000 and 125,000 daltons and an additional polypeptide of 35,000 daltons are part of this membrane fraction. More than 50 polypeptides were found in the membrane before the EDTA and pronase treatment. The 125,000 dalton complex seems to be buried inside the lipid layer. The 23,000 dalton subunit of the 67,000 dalton complex is largely exposed to the surface of the EDTA-insoluble membrane and only the chlorophyll-binding subunit of 21,500 daltons is buried inside the lipid layer.

In recent years much research in photosynthesis has been focused on the localization of functional parts of the electron transport system in the thylakoid membrane (37). However, our knowledge of the spatial arrangement of these components is still limited. Although the structure of the thylakoid membrane has been studied extensively with the electron microscope and a number of different structural components have been described (10, 15, 22, 24, 27-30), the function of most of these membrane components is still uncertain. By using specific agents like antibodies directed against surface-exposed membrane components, it has become possible to localize some functional parts (8, 9, 16, 18, 22). However, only a few elements of the membrane have been located, and little is known about the arrangement of the other membrane components.

In recent studies of membrane architecture, especially of erythrocytes, a number of reagents have been used which are unable to penetrate intact cells or closed membrane vesicles, but which form stable addition products to proteins or which hydrolyze peptide bonds (7, 12, 13, 20, 31, 36, 38, 39). The modified membranes were then generally treated with detergents and the solubilized membrane proteins were separated electrophoretically on polyacrylamide gels. The accessibility of a given protein to the reagent is demonstrated by the labeling pattern or by a reduction in its molecular weight. Reagents which have been used in this way include various proteases (7, 36) and $^{125}$I,
as generated by lactoperoxidase (13, 20, 31, 39).

We have used two of these methods, treatment with pronase and iodination, to analyze the topography of the chloroplast membrane in the green alga *Acetabularia mediterranea*. The spatial arrangement of the different membrane components was also determined by fractionating the chloroplast membrane with the detergent Triton X-100 and EDTA. In previous work, two of the major membrane components, the chlorophyll-protein complex of photosystem I (PSI) and the light-harvesting chlorophyll-protein complex of photosystem II (PSII), were isolated from this chloroplast membrane and their polypeptide components were identified (2, 3). The light-harvesting chlorophyll-protein complex of PSII had a molecular weight of 67,000 daltons (67 kdaltons) and contained two different subunits of 23 and 21.5 kdaltons (2, 3). The chlorophyll-protein complex of PSI had a molecular weight of 125 kdaltons and could be dissociated into free chlorophyll and a major polypeptide of 79 kdaltons (3). In the present study, we describe the arrangement of the different subunits of the two chlorophyll-protein complexes inside the chloroplast membrane.

**MATERIALS AND METHODS**

The cultivation of *A. mediterranea*, the isolation of washed chloroplast membranes, the protein and chlorophyll determinations, the fluorescence measurements, and the electrophoretic separation and molecular weight estimation of the chloroplast membrane proteins were performed as described elsewhere (2).

**Pronase Treatment of the Chloroplast Membrane**

The chloroplast membranes were incubated with different concentrations of pronase (pronase R, free of nucleases, Calbiochem, San Diego, Calif.) in 0.1 M Tris-HCl, pH 7.6, for 15 min at 25°C unless otherwise stated (membrane concentration 100 μg chlorophyll per ml). The incubation was stopped by the addition of 4 vol of ice-cold 0.1 M Tris-HCl, pH 7.6, 100 μg phenylmethylsulfonylfluoride (PMSF) (Sigma Chemical Co., St. Louis, Mo.) per ml and the suspension was washed twice with the Tris-PMSF buffer and once with distilled water and lyophilized. All the centrifugation steps were done at 2°C.

**Enzymatic Iodination of the Chloroplast Membrane**

Either washed chloroplast membranes or EDTA-insoluble membrane residues were suspended in 0.1 M Tris-HCl, pH 7.6, at a concentration of 50 μg chlorophyll per ml unless otherwise stated. The membranes were incubated according to the method of Hubbard and Cohn (20). 1 ml of the incubation mixture contained 50 μCi carrier-free 125I and 4 mU lactoperoxidase, 3 mU glucose oxidase, and 5 μmol glucose. The activity of glucose oxidase and lactoperoxidase was determined as described by Hubbard and Cohn (20) except that the assay was performed at pH 7.6 instead of 7.2.

The membrane suspension was incubated for 20 min at room temperature. For controls, lactoperoxidase was omitted from the complete iodination system. The incubation was stopped by the addition of 1 vol 0.1 M Tris-HCl, pH 7.6, 2% bovine serum albumin according to Arntzen et al. (4). The membranes were washed at least five times with 0.1 M Tris buffer and were either resuspended in distilled water and freeze-dried or precipitated with 2 vol of 10% trichloroacetic acid (TCA) and collected on glass fiber filters.

The radioactivity present in the labeled membranes was measured by counting the material on the glass filter in a gamma counter. The labeled membrane proteins were solubilized and separated electrophoretically as described (2). After electrophoresis, the gel was sliced into 1 mm slices and counted in a gamma counter. Duplicates of the gel were stained for protein with Coomassie blue and destained according to Fairbanks et al. (14).

**Thin Sectioning**

Isolated chloroplast membranes were fixed in 3% glutaraldehyde in 1 mM Tris-HCl, pH 7.5, for 30 min. The membranes were postfixed 1 h in 2% aqueous OsO4, dehydrated through a graded series of acetones, and embedded in Spurr's resin. Sections were cut on a Reichert Ultramicrotome (American Optical Co., Buffalo, N.Y.) and stained in uranyl acetate and lead citrate. Sections were examined in a Philips 300 electron microscope.

**Freeze-Fracturing**

Isolated chloroplast membranes, fixed in glutaraldehyde as described above, were gradually infiltrated with glycerol to a final concentration of 30% vol/vol over the course of an hour. Individual samples were frozen in liquid Freon 12 and stored in liquid nitrogen. Replicas were prepared according to the method of Moor and Mühlethaler (25) on a Balzers Freeze-Etching device (Balzers High Vacuum Corp., Santa Ana, Calif.). Specimen temperature was −107°C, and the fractured surface was replicated with only minimal etching after the last pass of the microtome knife. Successful replicas were cleaned in bleach and chromic acid and examined in a Philips 300 electron microscope.

**RESULTS**

**Fractionation of the Chloroplast Membrane of *A. mediterranea***

In our previous work we have been able to fractionate the chloroplast membrane of *A. medi-
*A. terranea* by treatment with Triton X-100 into two subfractions, a fragment $B_w$ with PSII properties and a second fraction $C_w$ which resembled PSI (2) (Fig. 1). Each of these two fractions had distinct and unique complements of polypeptides, indicating an almost complete separation of one from the other. Because of the large number of polypeptides, isolation and identification of the individual polypeptides was difficult.

In the present study an additional fractionation procedure was used before the treatment of the chloroplast membrane with Triton X-100. First, the washed chloroplast membrane was extracted with EDTA and 2-mercaptoethanol. After the centrifugation of the membrane suspension, the supernatant was removed and the remaining EDTA-insoluble membrane was then incubated with Triton X-100. This membrane suspension was centrifuged on sucrose density gradients and separated into two submembrane fractions. Finally, the polypeptides of each of these two fractions were separated electrophoretically on polyacrylamide gels. (Fig. 1).

**EXTRACTION OF CHLOROPLAST MEMBRANE PROTEINS WITH EDTA AND 2-MERCAPTOETHANOL:** Isolated chloroplast membranes were washed twice with 0.1 M Tris-HCl, pH 7.6. The washed chloroplast membranes were suspended in 0.1 M Tris-HCl, pH 7.6, 50 mM 2-mercaptoethanol (20 μg chlorophyll/ml buffer) and dialyzed against the EDTA buffer. After 17,000 x g, 20 min centrifugation, the EDTA-soluble proteins were removed and the EDTA-insoluble membrane was then incubated with Triton X-100. This membrane suspension was centrifuged on sucrose density gradients and separated into two submembrane fractions. Finally, the polypeptides of each of these two fractions were separated electrophoretically on polyacrylamide gels. (Fig. 1).

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**Figure 1** Protocol for the fractionation of the chloroplast membranes of *A. mediterranea*. Details are given in Materials and Methods and Results.

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twice against 2 liters of 0.1 M Tris-HCl, pH 7.6, 50 mM 2-mercaptoethanol, 1 mM EDTA as described previously (1). Then the suspension was centrifuged for 10 min at 1,000 g. Both the resulting supernate and pellet were further purified. The supernate was centrifuged at 17,000 g for 20 min, dialyzed against distilled water, and freeze-dried. This fraction was called “EDTA-soluble chloroplast membrane proteins.” The green pellet was suspended in 0.1 M Tris-HCl, pH 7.6, and centrifuged for 10 min at 1,000 g. The sediment was called “EDTA-insoluble chloroplast membranes” (Fig. 1).

The washed chloroplast membranes of A. mediterranea were solubilized in 0.2% sodium dodecyl sulfate (SDS) and could be resolved by SDS polyacrylamide gel electrophoresis into approximately 57 different protein fractions with molecular masses ranging from 10 to 190 kdaltons. After the solubilization of the membrane with 0.2% SDS, part of the chlorophyll was still bound to three chlorophyll-protein complexes of 125, 67, and 21.5 kdaltons. By treatment of the washed chloroplast membranes with EDTA, 2-mercaptoethanol, and Tris-HCl, pH 7.6, a large number of the proteins were released from the membrane. No chlorophyll but approximately 40% of the protein was solubilized and appeared in the EDTA-containing supernatant solution (Table I). This effect of EDTA on the membrane was demonstrated by comparison of the protein:chlorophyll ratios of the washed and EDTA-insoluble chloroplast membranes. The ratio of 6.8 for the washed membrane fraction decreased to 3.7 after the EDTA treatment (Table I). Electrophoretic analysis of the EDTA-soluble and insoluble membrane proteins revealed that approximately 39 or two-thirds of the chloroplast membrane proteins were solubilized by the EDTA treatment and could be separated quantitatively from the remaining chloroplast membranes (Fig. 2 A). The EDTA-insoluble chloroplast membranes contained all three chlorophyll-protein complexes and in total at least 18 different polypeptides (Fig. 2 B).

**Fractionation with Triton X-100:** The EDTA treatment of the washed chloroplast membrane resulted in the solubilization and almost complete separation of a large number of polypeptides from the remaining membrane. In a second fractionation step, this EDTA-insoluble membrane was incubated with 1% Triton X-100 (Fig. 1). The resulting suspension could be resolved by sucrose gradient centrifugation into three different chlorophyll-containing bands A, B, and C (Fig. 1) at approximately the same positions in the gradient as the three fractions of Triton X-100-treated washed chloroplast membranes (2).

Fraction A of the EDTA-insoluble membrane had a chlorophyll a:b ratio similar to that of the unfragmented membrane and contained no electrophoretically detectable protein. Furthermore, no structure was detected under the electron microscope.

Fraction B of the EDTA-insoluble membrane (B_{EDTA}) was enriched in chlorophyll b (Table I). The fluorescence of fraction B_{EDTA} at room temperature was high, compared to that of the unfraccionated membrane. After fraction B_{EDTA} had been solubilized in a solution of 0.2% SDS and the proteins had been electrophoretically separated, three chlorophyll bands appeared. The fastest migrating chlorophyll band contained protein-free chlorophyll-SDS while the two other bands contained chlorophyll bound to protein (Fig. 2 D). The molecular weights of these chlorophyll-binding proteins were 67 and 21.5 kdaltons. After staining the gel with Coomassie blue, only four protein bands were found: (A and B) two chlorophyll-binding proteins of 67 and 21.5 kdaltons, (C) a strongly stained protein band of 23 kdaltons, and (D) a broad band of approximately 47 kdaltons (Fig. 2 D). Since both the 23 and 21.5 kdal-

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**Table I**

Distribution of Chlorophyll and Protein in the Fractions Produced by EDTA and Triton X-100 Treatment of A. mediterranea Chloroplast Membranes

| Fractions                          | Chlorophyll a:b | Chlorophyll | Protein | Protein/chlorophyll |
|-----------------------------------|-----------------|-------------|---------|---------------------|
| Washed chloroplast membranes      | 1.22            | 100         | 100     | 6.82                |
| EDTA-insoluble chloroplast membranes | 1.20           | 84          | 45.4    | 3.7                 |
| EDTA-soluble chloroplast membrane proteins | -              | -           | 39      | -                   |
| Fraction B of the EDTA-insoluble membrane | 0.54         | 9.5         | 6.8     | 4.9                 |
| Fraction C of the EDTA-insoluble membrane | 4.3           | 12.9        | 12.1    | 7.9                 |
FIGURE 2. Electrophoresis of various membrane protein fractions after EDTA treatment of the chloroplast membrane of *A. mediterranea*. The gel was stained with Coomassie blue and shows the protein pattern of the EDTA-soluble protein fraction (A), the EDTA-insoluble membrane (B), and the fractions $C_{EDTA}$ (C), and $B_{EDTA}$ (D). Arrows indicate the position of the chlorophyll-binding proteins. (E) Marker proteins *Escherichia coli* RNA polymerase subunits $\beta'$ (165,000), $\beta$ (155,000) and $\alpha$ (39,000), carbonic anhydrase (29,000), RNase (13,700) and cytochrome c (12,500).

Electrophoretic analysis of the EDTA-soluble protein fraction (A) revealed a single band with a molecular weight of approximately 125,000, corresponding to the chlorophyll-binding protein. This band was not present in the EDTA-insoluble membrane fraction (B). The fractions $C_{EDTA}$ (C) and $B_{EDTA}$ (D) showed a complex pattern of polypeptides with molecular weights ranging from 10 to 70,000. Arrows indicate the position of the chlorophyll-binding proteins.

The Topography of Polypeptides of the Chloroplast Membrane of *A. mediterranea*

The effect of Pronase on the Chloroplast Membrane: Upon treatment of the washed chloroplast membranes of *A. mediterranea* with EDTA and 2-mercaptoethanol, approximately 40% of the membrane-bound protein was solubilized and could be removed quantitatively from the remaining membrane. The residual EDTA-insoluble membrane was then incubated for 15 min at 25°C with increasing concentrations of pronase (1, 10, and 100 µg). Incubation with 100 µg pronase per ml resulted in solubilization of 20% more of the membrane-bound protein (Table II).

The membranes treated with different concentrations of pronase were dissolved in 0.2% SDS
and the protein mixture was analyzed electrophoretically on a polyacrylamide gel. Before Coomassie blue staining of the gel, four chlorophyll-containing bands appeared in preparations of both the untreated control and pronase-treated samples. These chlorophyll-containing bands corresponded to the three chlorophyll-protein complexes of 125, 67 and 21.5 kdaltons and the free chlorophyll zone. Even at a concentration of 100 μg per ml, pronase did not seem to alter the electrophoretic behavior of any of the three chlorophyll-protein complexes.

However, Coomassie blue staining of the gel revealed that pronase treatment significantly altered most of the other membrane proteins. At a concentration of 1 μg pronase per ml most of the proteins except for the chlorophyll-proteins shifted to lower molecular weights. At a concentration of 10 μg pronase per ml, the number of detectable protein bands in the gel decreased significantly, and at a concentration of 100 μg pronase per ml only the three chlorophyll-binding proteins of 125, 67, and 21.5 kdaltons and a polypeptide of 35 kdaltons were detected in the gel (Fig. 3 A). The 35 kdalton protein component could not be detected in the electrophoretic protein pattern of the untreated control of EDTA-insoluble membranes. Therefore, this polypeptide probably represents a fragment of a higher molecular weight protein which was partially degraded in the presence of pronase. During the incubation of the membrane with 100 μg pronase per ml only the chlorophyll-binding 21.5 kdalton subunit of the 67 kdalton chlorophyll-protein complex remained intact; the 23 kdalton subunit was gradually degraded and in some samples it completely disappeared (Fig. 3 A).

Similar results were obtained when washed membranes without the preceding EDTA extraction step were directly incubated with pronase. Again only three of the 50-57 proteins of the washed membrane were undegraded after treatment with 100 μg pronase per ml.

There is evidence that in addition to the three undegraded chlorophyll-binding proteins and the 35 kdalton protein fragment, other smaller fragments are embedded in the pronase-treated membrane. The Coomassie blue-stained protein material in the unresolved front band increased significantly after pronase treatment (Fig. 3). This band contains fragments of molecular weights lower than approximately 9,000. These fragments seemed to be part of originally larger membrane-bound proteins. A large portion of these proteins was degraded by pronase and removed from the membrane. However, some parts of these proteins seemed to be unaccessible to the enzyme and remained membrane-bound.

The combined EDTA and pronase treatments did not seem to destroy the membrane structure as visualized in the electron microscope. Thin sections of the isolated membranes showed no detectable changes in the membrane structure caused by either EDTA extraction or pronase treatment (Fig. 4a-c). When the membranes were examined by freeze-fracturing, a technique which exposes membrane interiors by fracturing them along an internal plane (10), two complementary faces (labeled PF and EF after the system of Branton et al. (11)) were exposed (Fig. 5a), which looked very similar to those observed in other species (10, 15, 24, 27). EDTA extraction of the membrane caused no change in the appearance of the fracture faces (Fig. 5b). Pronase treatment,
while it did leave intact the basic structure of the thylakoid, caused a noticeable reduction in both the size and number of particles on the E fracture face (Fig. 5c).

**The Iodination of the Chloroplast Membrane:**

In the presence of H$_2$O$_2$, lactoperoxidase catalyzes the iodination of tyrosyl residues in proteins (26). This method has been employed to label exposed proteins on membranes. Since the iodination occurs via an enzyme substrate complex between the protein substrate and the lactoperoxidase and since the enzyme is relatively large (mol wt 78,000) (32), it is generally thought that the iodination reaction is limited to the surface-exposed part of the membrane.

In the method of Phillips and Morrison (31), H$_2$O$_2$ was added directly to the reaction mixture. However, it has been shown that with this method it is difficult to maintain a constant low concentration of H$_2$O$_2$ during the incubation and thus to avoid damages to the biological structure by an excess of H$_2$O$_2$ (13). Subsequently a milder iodination procedure using glucose and glucose oxidase as an H$_2$O$_2$-developing system was described by Hubbard and Cohn (20). Under these conditions damage of the membrane by H$_2$O$_2$ was largely reduced.

We have used the procedure of Hubbard and Cohn to iodinate the chloroplast membranes of *A. mediterranea*. The reaction was almost completely dependent on the presence of lactoperoxidase in the reaction mixture (Table II). Without this enzyme, the iodination of the membrane was reduced by 96-97%. However, without the H$_2$O$_2$-generating system, the iodination of the membrane was not reduced when the reaction was performed in the light (Table III). In the dark, the reaction was inhibited to 76% of the complete system. In the light, the chloroplast membranes seem to develop sufficient amounts of H$_2$O$_2$ to support the continued iodination.

The conditions for the iodination of surface-exposed proteins were originally developed for erythrocyte membranes. Modifications of these conditions, for example a change in the concentration of membrane material, may lead to the labeling of additional proteins at various depths of the membrane (39). Therefore, we tried to optimize the conditions for the iodination of the surface in chloroplast membranes.

The effect of different incubation conditions on the specificity of surface labeling was tested by determining the amount of radioactivity found in the EDTA-soluble proteins. These proteins are probably removed only from the surface of the chloroplast membrane. The concentration of $^{125}$I was kept constant and the concentration of the chloroplast membranes was increased from 25 to 400 $\mu$g chlorophyll per ml. The specific radioactivity of the iodinated membrane dropped sharply with increasing membrane concentrations (Fig. 6). However, the percentage of label found in the EDTA-soluble proteins remained approximately constant under all these different conditions (Fig. 6). This result indicates that over the range tested, the variation in the membrane concentration did not influence the percent of label found in surface-exposed proteins. This conclusion was further supported by comparison of the electrophoretic labeling pattern of the different EDTA-insoluble membrane samples. At membrane concentrations between 25 and 100 $\mu$g chlorophyll per ml the distribution pattern of protein-bound $^{125}$I looked very similar. At concentrations of 200 and 400 $\mu$g chlorophyll per ml the specific radioactivity of the membrane proteins was too low to be detected in the gel. The follow-
FIGURE 4 Electron micrograph of the washed (a), EDTA-insoluble (b), and the pronase-treated (c) chloroplast membrane of *A. mediterranea*. Magnification × 23,300.
FIGURE 5  Freeze-fracture faces of the washed (a), EDTA-insoluble (b), and pronase-treated (c) chloroplast membrane of A. mediterranea. Magnification × 71,000.
TABLE III
Controls for the Iodination Reaction*

| Reagents deleted                                      | Incubation in the light | Incubation in the dark |
|-------------------------------------------------------|-------------------------|------------------------|
| None                                                  | $3.52 \times 10^4$ (100%) | $3.29 \times 10^4$ (100%) |
| Glucose and glucose oxidase                           | $3.39 \times 10^4$ (96%) | $2.52 \times 10^4$ (76%) |
| Lactoperoxidase                                       | $0.11 \times 10^4$ (3.2%) | $0.095 \times 10^4$ (2.9%) |
| Glucose, glucose oxidase, and lactoperoxidase         | $0.098 \times 10^4$ (3.0%) | $0.067 \times 10^4$ (2.1%) |

* The iodination was done as described in Materials and Methods.

The specificity of the iodination reaction was further tested by fractionating the washed chloroplast membrane and determining the amount of radioactivity in each of the submembrane fractions (Table III). Not all the $^{125}$I was bound to protein. Approximately 30% of the radioactivity could be extracted from the membrane with acetone and was probably bound to lipids. EDTA treatment of the washed membrane solubilized only 30% of the label (Table III). The specific radioactivity of the EDTA-soluble proteins was only slightly higher than that of the acetone-extracted total membrane (Table III). This result does not agree with results obtained with other iodinated chloroplast membranes. It has been reported that in peas and lettuce, EDTA washing of the chloroplast membrane removes approximately 90% of the radioactivity from the membrane, although only 10-15% of the protein has been solubilized (4). Since $^{125}$I reacts preferentially with tyrosine (20), the relatively low specific radioactivity of the EDTA-soluble proteins in A. mediterranea might be explained by a relatively low content of this amino acid in these proteins. The iodinated EDTA-insoluble membrane fraction was treated for 15 min with 100 μg pronase per ml at 25°C. Under these conditions a large portion of the label in this membrane fraction was solubilized (Table III). The pronase-treated membrane contained 30-35% of the total radioactivity. Most of this radioactivity could be solubilized with 90% acetone and only approximately 5% of the total radioactivity was recovered in the acetone-insoluble material of the pronase-treated membrane fraction.

The EDTA-soluble proteins comprise approximately 40% of the total protein of washed membranes. If these proteins were surface bound as assumed earlier, their removal should influence the accessibility of the remaining proteins in the EDTA-insoluble membrane. This possibility was tested in the following experiments.

Washed membranes were iodinated as described. The labeled membranes were then extracted with EDTA and 2-mercaptoethanol and the EDTA-soluble proteins were removed. The remaining EDTA-insoluble membranes were treated with 0.2% SDS and the solubilized proteins separated electrophoretically. The distribution pattern of radioactivity in the gel indicates that in the washed membranes none of the EDTA-insoluble proteins were preferentially labeled (Fig. 7a). The large peak in the unresolved front band coincides with the zone of free chlorophyll and probably contains $^{125}$I bound to lipids. This peak disappears in samples which have been extracted with 90% acetone before electrophoresis. There were a number of other small peaks, but

Figures 6 and 7a. The effect of various membrane concentrations on the amount of $^{125}$I incorporated into the total membrane (●—●) and into the EDTA-soluble proteins (▲—▲) of A. mediterranea.
Figure 7 Electrophoresis of [125I]-lactoperoxidase-treated chloroplast membranes of A. mediterranea. (a) Washed chloroplast membranes were first iodinated and then extracted with EDTA. The EDTA-insoluble membranes were either solubilized and separated electrophoretically (○—○) or treated with 100 μg pronase per ml before solubilization of the membrane fraction and electrophoretic analysis of the membrane components (▲—▲). The gel was sliced into 1 mm sections and the radioactivity determined as described in Materials and Methods. The arrows indicate the position of the chlorophyll-binding proteins in the gel. (c) The washed chloroplast membranes were first extracted with EDTA and the EDTA-insoluble membranes were iodinated as described in Materials and Methods. The other experimental conditions were as described in Fig. 7a. (b) The polypeptide composition of the EDTA-insoluble chloroplast membrane before (2) and after (1) pronase treatment as revealed by Coomassie blue staining of the separated proteins in the gel.
most of them could not be identified. Only the peak of approximately 23 kdaltons could be related to the two heavily stained bands of 23 and 21.5 kdaltons.

All these small peaks disappeared after the prelabeled membranes were treated with pronase (100 μg/ml) (Fig. 7a). The 125, 67, and 21.5 kdalton chlorophyll-protein complexes and the polypeptide fragment of 35 kdaltons were recovered in the gel (Fig. 7b), but none of them contained any detectable radioactivity. The small peak of radioactivity retained on the gel after pronase treatment coincides with the position of the 23 kdalton polypeptide, which was partially degraded. The intensity of the radioactivity peak in the unresolved front band did not change. Obviously the protein-bound radioactivity was removed after pronase treatment and was not shifted to the position of this band.

In a second experiment, the chloroplast membrane was iodinated only after the EDTA-soluble proteins had been removed. If these latter proteins are at the surface, their removal could lead to a change in the accessibility of the remaining proteins of the EDTA-insoluble membrane. Indeed, the pattern of radioactivity of the EDTA-insoluble membrane proteins shows significant changes (compare (a) and (c) in Fig. 7). In the EDTA-insoluble chloroplast membrane, three main polypeptides became preferentially labeled with 125I: two peaks of 48 and 45 kdaltons and a major peak, which comigrated with the two polypeptides of 23 and 21.5 kdaltons. Although on this gel it was not possible to relate the radioactivity of the main peak to either one of these two polypeptides, it became evident after the pronase treatment of the membrane that the radioactivity in this peak was only associated with the 23 kdalton polypeptide (Fig. 7c). Parallel to the disappearance of radioactivity in this peak, the 23 kdalton polypeptide was degraded and disappeared (Fig. 7b). On the other hand, the 21.5 kdalton chlorophyll-binding polypeptide was not affected by the pronase treatment. The radioactivity in the unresolved front band, thought to contain protein fragments, increased after pronase treatment; some of the label is likely to be bound to the short fragments of degraded proteins which were embedded in the membrane and protected against complete destruction. These fragments were too small to be resolved on the gel and their radioactivity appeared at the same position as the label bound to the lipids.

These results indicate that the EDTA-soluble proteins were indeed on the surface and that they blocked, at least partially, the iodination of other membrane proteins. Only after the EDTA-soluble proteins were removed, the three proteins of 48, 45, and 23 kdaltons became accessible to 125I and were preferentially labeled. It is reassuring that both pronase treatment and iodination had no effect on the same group of membrane components. The chlorophyll-protein complexes of 125, 67, and 21.5 kdaltons and the fragment of 35 kdaltons were neither labeled with 125I nor digested by pronase.

In the EDTA-insoluble membrane residue, the 23 kdalton polypeptide was accessible to both the lactoperoxidase and the pronase, while the 67 and 21.5 kdalton chlorophyll-binding proteins were shielded from these probes. Since it has been shown that the 23 and the 21.5 kdalton polypeptides are components of the 67 kdalton chlorophyll-protein complex (2, 3), one would expect to find a reaction of these two probes not only with the subunit but also with the undissociated whole chlorophyll-protein complex. One might assume that the label found in the 23 kdalton region of the gel is not bound to the 23 kdalton subunit of the chlorophyll-protein complex but to a second polypeptide of a similar molecular weight which cannot be separated from the subunit electrophoretically and which is also digested by pronase. However, the label in the 23 kdalton region of the gel appears not only after electrophoretic separation of the proteins of the iodinated EDTA-insoluble membrane, but also after electrophoresis of the solubilized subfragment BEDTA of the iodinated membrane. This fraction contains only an unidentified protein of 47 kdaltons and the 67 kdalton chlorophyll-protein complex and its two subunits of 21.5 and 23 kdaltons. Thus, the label found in the 23 kdalton region of the gel seems indeed to be associated with the 23 kdalton subunit of the chlorophyll-protein complex.

A reaction of the two surface probes with both the subunit of 23 kdaltons and the undissociated 67 kdalton chlorophyll-protein complex could only be expected if all of the 67 kdalton chlorophyll-protein is equally accessible to iodination and pronase action. This seems not to be the case. The 67 kdalton chlorophyll-protein complex was isolated electrophoretically from an iodinated and pronase-treated membrane and rerun electrophoretically with high SDS. Under these conditions the complex is completely dissociated into its two
subunits of 23 and 21.5 kdaltons (2, 3). Both subunits, the 21.5 and also the 23 kdalton polypeptides were undegraded and their molecular ratio was preserved. Neither of the two subunits contained any detectable radioactivity. Obviously, the two subunits in the undissociated complex of the EDTA- and pronase-treated membrane were protected against both probes.

Since a large part of the membranes is stacked, one may assume that the intact 67 kdalton chlorophyll-protein complexes are localized within the stacked areas of the membrane and thus are shielded from the surface agents. So far, we have not been able to unstack the membranes completely to expose all the membrane surface. Even under conditions which cause unstacking in chloroplast membranes of other plants (21, 28), the membranes of Acetabularia chloroplasts were still stacked.

**DISCUSSION**

The chloroplast membrane of *A. mediterranea* was fractionated by the combined treatment with EDTA and Triton X-100 into different complements of polypeptides. EDTA treatment had been applied to chloroplast membranes earlier. Some proteins such as the coupling factor and a Ca\(^{2+}\)-dependent ATPase (5, 19, 40) were dissolved from the membrane by EDTA, but the bulk of proteins remained bound to the membrane structure even after EDTA treatment, but the bulk of proteins remained bound to the membrane structure even after EDTA treatment. In contrast, approximately 40% of the membrane protein or two thirds of the membrane polypeptide species in *A. mediterranea* were dissolved by a solution of EDTA and 2-mercaptoethanol.

The question arises whether in *A. mediterranea* the EDTA-soluble proteins were part of the chloroplast membrane or whether they were unspecifically attached to the membrane during the isolation step and represented normally soluble contaminants. There is evidence that at least part of the solubilized proteins were originally components of the membrane. Fractions B\(_m\) and C\(_m\) of the washed chloroplast membranes that had not been extracted with EDTA before the Triton X-100 treatment, contained a large number of polypeptides which can be extracted by EDTA solutions alone. It seems unlikely that those EDTA-soluble proteins that were found in Triton X-100 fragments of washed membranes would still be part of the purified membrane fractions after the detergent treatment if they were only nonspecific contaminants.

In recent years, new techniques have been applied to the study of protein topography in membranes (7, 12, 20, 23, 31, 36, 38). In this study we have used two of these techniques to describe the arrangement of proteins in the chloroplast membrane of the green alga *A. mediterranea*, enzymatic iodination and pronase treatment, in addition to the fractionation of the membrane by EDTA and Triton X-100 treatment. It became evident that each of these two methods had drawbacks and that reliable conclusions could be drawn only after a combination of all three approaches.

The iodination of protein-bound tyrosyl residues by lactoperoxidase is widely believed to be a protein-specific surface probe. However, our results show that not all of the \(^{125}\text{I}\) was bound to protein, but that a significant part of it was also found in lipids. A further drawback of this method was the fact that \(_I\) reacts preferentially only with a single amino acid (20). As a result, the distribution of label among the iodinated polypeptides may reflect not only the difference in accessibility of a given protein, but also the relative content of the reactive group on the surface of the polypeptide. For instance, the specific radioactivity of the EDTA-soluble proteins was only slightly higher than that of the acetone-extracted original membrane, although other results suggested that these proteins are surface-exposed.

The second probe, pronase, seemed to interact specifically with surface proteins under conditions where the action of pronase could be stopped rapidly by immediate freezing and lyophilizing of the washed sample. Otherwise, degradation of the remaining membrane-bound proteins during the following handling of the membranes would slowly continue.

Despite these limitations, both probes proved to be useful tools for the study of membrane architecture in the chloroplast of *A. mediterranea*.

Pronase degraded most of the membrane proteins. Both the size and number of particles on the fracture face were reduced, but electron microcopy of thin sections revealed no gross alterations of the membrane. In the pronase-treated membrane only the 125, 67, and 21.5 kdalton chlorophyll-binding proteins and the fragment of 35 kdaltons were unaffected by the enzyme and seemed to be buried inside the lipid layer of the membrane.

This conclusion was supported by results obtained after the iodination of washed or EDTA-insoluble membranes. None of the proteins that
were shielded from pronase contained significant amounts of label. Both probes seemed to be unable to penetrate the membrane and reach these buried proteins.

There was further evidence that the two probes reacted preferentially only with the membrane surface. Some of the EDTA-insoluble membrane proteins became available to enzymatic iodination only after the EDTA-soluble proteins had been removed from the membrane. This result suggests again that proteins became fully accessible to the surface probes only after their exposure to the surface.

The localization of the different protein groups was based on different approaches. Since each of these techniques gave similar results, we believe that our data reflect the true arrangement of membrane components rather than artifacts.

Using the EDTA and pronase treatment, we were able to differentiate between three groups of membrane proteins (Fig. 8). After EDTA treatment, approximately three-fifths of the originally

![Diagram of the effects of EDTA and pronase treatment on the chloroplast membrane of A. mediterranea.](image)

**Figure 8** Schematic description of the effects of EDTA and pronase treatment on the chloroplast membrane of *A. mediterranea*. The washed chloroplast membrane before (*a*) and after the treatment with EDTA (*b*) and pronase (*c*).
membrane-bound protein species were solubilized and could be removed quantitatively from the remaining EDTA-insoluble membrane. The EDTA-soluble proteins are probably linked by bivalent cations to the hydrophilic part of the intermediary proteins (Fig. 8a). However, it is also possible that the bivalent cations are not directly involved in the binding of these proteins but rather induce some conformational changes in the protein molecule. These conformational changes could, for instance, expose hydrophobic parts of the proteins and allow an interaction with the hydrophobic interior of the membrane. These interactions cannot be very strong, since the reduction in the concentration of free bivalent cations, for example by the addition of chelating agents such as EDTA, always leads to the solubilization of the EDTA-soluble proteins (Fig. 8b).

After pronase treatment, only the chlorophyll-protein complex of PSI and the chlorophyll-binding subunit of the light-harvesting chlorophyll-protein complex of PSII remained intact, while the rest of the EDTA-insoluble proteins was degraded. Hence, in addition to the class of EDTA-soluble proteins, there is a second group of hydrophobic proteins including only the 125 and 21.5 kdalton chlorophyll-binding proteins which seem to be buried inside the lipid layer of the membrane (Fig. 8c).

There was evidence that after pronase treatment not only the 35 kdalton fragment but also other small fragments of the pronase-sensitive proteins remained inside the membrane. This third group of intermediary proteins seems to be linked with one part to the lipid layer probably by hydrophobic interaction, while the rest of the molecules remain outside the lipid layer, where they can be reached by the surface probes. The intermediary proteins could link certain EDTA-soluble proteins to a buried protein, thus forming a functional unit.

Most of our results are in accordance with the fluid mosaic membrane model of Singer and Nicolson (34). However, these authors discriminated only between two categories of membrane proteins, the peripheral and integral proteins. The peripheral proteins correspond to the EDTA-soluble proteins, while the definition of the integral proteins includes only the intermediary proteins but not the buried proteins. According to this model, the stability of the membrane derives from thermodynamic factors which are responsible for the sequestering of hydrophobic or nonpolar groups in the lipid phase of the membrane (34). A completely buried protein could only exist inside the lipid layer if it contained a minimum of exposed ionic amino acid residues. Since all of the hydrophobic proteins analyzed so far contain substantial numbers of ionic residues it is argued that these proteins cannot be completely buried because too much free energy would be required to embed the ionic residues within the hydrophobic interior of the membrane (35). However, it is not known whether all of the ionic amino acid residues are exposed to the surface of the molecule. It is possible that the effective number of hydrophilic groups of a protein is much smaller than its number of ionic amino acids. Thus, such a protein may be buried inside the membrane even though it contains a relatively large number of ionic amino acids. A buried protein could further be stabilized inside the lipid layer by the interaction with exposed ionic groups of other buried or intermediary proteins. In the chloroplast membrane of A. mediterranea the few proteins buried inside the lipid layer do not seem to be isolated but rather to be clustered together with intermediary and EDTA-soluble proteins, forming larger functional units. For instance, one of the buried chlorophyll-binding polypeptides of 21.5 kdaltons, together with the intermediary polypeptide of 23 kdaltons, forms the chlorophyll-protein complex of 67 kdaltons. This complex, together with an additional minor component, can be isolated from the EDTA-insoluble membrane as a structural unit. In the washed chloroplast membranes, this unit, with several EDTA-soluble proteins, forms the more complex, functional unit of PSII which can be isolated from the washed membranes by detergent treatment (2).

In several studies of chloroplast membranes, a number of components have been described which are exposed at the outer surface (8, 9, 18, 22). Generally, the interpretation of these experiments has been based on the assumption that the original outer membrane surface in chloroplasts was preserved in isolated membrane preparations. In our own study, we were unable to distinguish between the outer and inner membrane surface. Although in the washed, EDTA-insoluble, and pronase-resistant membrane preparations most of the membranes were stacked and therefore their original sidedness would seem to be preserved, a significant amount of membranes—especially after the pronase treatment—seemed to have changed their sidedness and to have formed smaller vesicles.
Thus, before a definite description of the two surfaces of the chloroplast membranes in *Acetabularia* can be presented, it will be necessary to isolate homogeneous preparations of right side-out or inside-out vesicles.

The interpretation of our results is further complicated by the fact that many chloroplast membranes were stacked. Within the stacked areas the attack by EDTA, pronase, and lactoperoxidase may be limited, thus introducing artifacts. The EDTA-soluble proteins were removed quantitatively from the remaining EDTA-insoluble chloroplast membranes. One could argue that perhaps part of the EDTA-insoluble proteins remained bound only to the membrane structure because they were localized within the stacked area and were thereby protected against the EDTA treatment. However, in this case the proteins should be protected not only against EDTA but also against pronase treatment and iodination, which they are not. The only surface-exposed protein which seems to be protected against the surface probes within the stacked membrane regions is the 23 kdalton subunit of the 67 kdalton chlorophyll-protein complex.

Freeze-fracturing of the chloroplast membrane in *A. mediterranea* revealed two different faces which could be distinguished by the number and size of their particles. These two faces looked very similar to those of freeze-fractured chloroplast membranes in other plants (10, 15, 24, 27). Several attempts have been made to identify the nature of the freeze-fracture particles, and it has been suggested that they may consist of protein (6, 29). Removal of surface proteins by EDTA washing of spinach thylakoids does not change the appearance of these faces (30). Even in the EDTA-insoluble chloroplast membrane of *Acetabularia*, the two faces look unchanged, although approximately 40% of the membrane-bound proteins has been solubilized by the EDTA extraction. Pronase treatment, however, causes a distinct change in the appearance of one of the fracture faces (Fig. 4b,c). However, the interpretation of this change is complicated by a number of factors. The most important problem is our observation that after pronase treatment, fragments of polypeptides remain bound to the membrane. Although a large part of the pronase-sensitive proteins was digested and solubilized, the pronase-resistant fragments of these proteins may still be assembled into structures of the thylakoid membrane which appear intact and unaffected by the pronase treatment. Also complicating our analysis is the fact that after pronase treatment many regions of the membrane preparations are highly vesiculated and show some variation between different E fracture faces.

Comparison of the results presented here with recent studies by other workers on a chlorophyll b-lacking barley mutant (17) suggests an explanation of our results. This barley mutant lacks the light-harvesting chlorophyll-protein complex of PSII, but its thylakoid membrane fracture faces seem to be identical to those of wild-type barley. It has long been apparent that the particles visible on thylakoid fracture faces, especially the EF face which contains particles roughly 150 Å in diameter, were too large to correspond to single polypeptides. Our observations, as well as those of Henriques and Park (17), are consistent with the idea that the chlorophyll-protein complex of PSII comprises only a part of a large complex of protein and lipid making up the large particle on the E fracture face. If the 21.5 kdalton subunit of the 67 kdalton chlorophyll-protein complex were at the center of this particle, protected from pronase treatment, then the persistence of smaller particles in the EF face after pronase treatment of the membrane is understandable.

Similarly, if only the chlorophyll-binding protein were missing from the particle, its other non-chlorophyll-binding constituents could conceivably assemble into a virtually indistinguishable structure in the thylakoid, despite the fact that the chlorophyll-binding component of the light-harvesting structure buried near the membrane center was lacking, as it is in the chlorophyll b-lacking mutant of barley (17). It is therefore possible that in *Acetabularia* the particle on the EF face represents a large assembly of polypeptides and other molecules with the 21.5 kdalton component of the 67 kdalton chlorophyll-protein complex at its center. Since the 67 kdalton chlorophyll-protein complex is part of the PSII (2), the particles on the E fracture face may contain the PSII. This hypothesis is supported by the observations that in stacked regions of the chloroplast membranes both the PSII activity (33) and the particles on the E fracture face (15, 24, 28) are concentrated. Nevertheless, further information on membrane structure and organization is required before a definitive identification of these particles can be made.

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