

Localization of Light-harvesting Complex II to the Occluded Surfaces of Photosynthetic Membranes

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Abstract. The photosynthetic membranes of green plants are organized into stacked regions interconnected by nonstacked regions that have been shown to be biochemically and structurally distinct. Because the stacking process occludes the surfaces of appressed membranes, it has been impossible to conduct structural or biochemical studies of the outer surfaces of the photosynthetic membrane in regions of membrane stacking. Although stacking is mediated at this surface, it has not been possible to determine whether membrane components implicated in the stacking process, including a major light-harvesting complex (LHC-II), are in fact exposed at the membrane surface. We have been able to expose this surface for study in the electron microscope and directly label it with antibodies to determine protein exposure. The appearance of the newly exposed outer stacked surface highlights the extreme lateral heterogeneity of the

photosynthetic membrane. The surface is smooth in contrast to the neighboring nonstacked surface that is covered with distinct particles. Although some investigators have suggested the existence of a cytochrome b_6/f -rich boundary region between stacked and nonstacked membranes, our results provide no structural support for this concept. To explore the biochemical nature of the occluded membrane surface, we have used an mAb against the amino terminal region of the LHC-II. This mAb clearly labels the newly exposed outer stacked surface but does not label the inner surface or the outer nonstacked surface. These experimental results confirm the presence of the amino terminal region of this complex at the outer surface of the membrane in stacked regions, and also show that this complex is largely absent from nonstacked membranes.

IN higher plants, the light reactions of photosynthesis occur within photosynthetic thylakoid membranes located in chloroplasts. Five major protein complexes located in thylakoid membranes are involved in the light reactions. These protein complexes include the two photochemical reaction centers, photosystem I and photosystem II (PS I and PS II, respectively),¹ a light-harvesting complex (LHC-II) associated primarily with PS II, a cytochrome b_6/f complex involved with electron transport, and an ATP synthase complex. The thylakoid membranes that contain these complexes adhere, or stack, at their outer surfaces, forming characteristic grana regions that are interconnected by nonstacked membrane regions. The two reaction centers, PS I and II, are spatially separated between these two regions, with PS I concentrated in nonstacked regions, and PS II predominantly located in stacked regions (Vallon et al., 1986; Andersson and Anderson, 1980). The majority of LHC-II is also found in stacked regions while the ATP synthase is located in nonstacked regions (Vallon et al., 1986; Allred and Staehelin, 1985). The location of the fifth complex, cytochrome b_6/f , is questionable but the majority of the evidence indicates it

is equally distributed throughout the membrane (Goodchild et al., 1985; Cox and Andersson, 1981).

Freeze-etching studies of thylakoid membranes reveal outer and inner surfaces that are rich in structural detail. The inner surface is covered with tetrameric particles that have been associated with the extrinsic oxygen-evolving proteins of PS II (Seibert et al., 1987; Simpson and Andersson, 1986), and are concentrated in the stacked (grana) regions of the membrane (Staehelin, 1976). The outer surface of nonstacked regions contains large particles identified as the CF_1 subunit of the ATP synthase (Miller and Staehelin, 1976) and smaller particles that may represent PS I. The only thylakoid surface that has not been studied in detail is the outer surface from the stacked region, which is hidden by the stacking process itself.

Membrane stacking has also made it impossible to confirm the exposure of macromolecular complexes at the thylakoid surface that may be responsible for stacking, including LHC-II. Although it is clear that the amino terminal region of LHC-II is critical for thylakoid stacking (Barber, 1982; Mullet, 1983), it has not been possible to confirm that this portion of the complex is exposed on the outer surfaces in stacked regions.

We have developed a technique to expose this previously

1. *Abbreviations used in this paper:* LHC, light-harvesting complex; PS, photosystem.

hidden outer surface for observation and analysis. The structure of this surface is remarkably smooth, with no apparent surface particles. This is quite different from the exposed outer surfaces from nonstacked regions that have numerous large particles interspersed with smaller particles. This striking lateral heterogeneity at the outer surface and the extreme smoothness of the stacked region suggests new restrictions on the lateral and transverse distribution of thylakoid membrane components.

The exposure of the outer stacked surface has enabled us to probe the location of LHC-II by immunolabeling. By applying antibodies directly to exposed membranes, we have been able to show that the amino terminus of this major chlorophyll-binding complex is indeed located at the outer membrane surface in stacked regions. These results support a model for the involvement of LHC-II in membrane stacking.

Materials and Methods

Exposure of Outer Stacked Surfaces

A suspension of spinach thylakoid membranes, isolated as described by Miller and Staehelin (1976), were placed on alcian blue (1 mg/ml)-treated glass coverslips in a low ionic strength buffer, containing 5 mM (*N*-morpholino)ethane sulfonic acid (MES) and 2 mM MgCl₂. The membranes were attached to the glass coverslips by a 5-min incubation in this buffer. The glass coverslips were then held by forceps so the attached membranes could be mechanically disrupted by a squirt of buffer (10 ml) from a syringe, (Fig. 1 *a*). The glass coverslips with bound membranes were then turned upside down on a moist filter paper, quick frozen in liquid freon 22, and stored under liquid nitrogen before replica formation. The coverslips were placed on a stage in a freeze-etching device (BAF 400; Balzers S. p. A., Milan, Italy), and freeze-dried, without prior fracturing, for 60 min, at -80°C. After freeze drying, the membranes were rotary shadowed at an angle of 23° with platinum followed by a coat of carbon. The resulting replica was washed in bleach and examined in an electron microscope (410; Philips Electronic Instruments, Inc., Mahwah, NJ).

Immunolabeling

Monoclonal antibodies (designated "MLH1") were donated by Dr. Sylvia Darr and have been previously characterized (Darr et al. 1986). Membranes that were attached to glass and mechanically disrupted as described above were immunolabeled with a 1:20,000 dilution of MLH1 (protein concentration of ascites fluid: 4×10^{-3} mg/ml) for 30 min, followed by several washes with 5 mM MES (pH 6.5), 2 mM MgCl₂, quick frozen, and prepared for EM as described above. In some experiments, the coupling factor was removed before labeling by incubating the membranes with 2 M NaBr (Nelson, 1986) for 20 min before mechanical disruption. These membranes were then labeled with MLH1, washed, and frozen as described above. Gold-labeling experiments were carried out by incubating MLH1-labeled membranes with an anti-mouse (IgG) gold complex (5 nm, 1:20 dilution, Janssen Life Science Products, Piscataway, NJ) for 30 min with several washes after each step and then frozen as described above. All labeling was done in duplicate with control experiments in which we incubated the membranes with normal mouse serum, at the same protein concentration as used for the MLH1 labeling.

Results

Characterization of the Outer Stacked Surface

We exposed the outer stacked surfaces of thylakoid membranes by mechanical disruption of membranes attached to glass coverslips (Fig. 1), as described in Materials and Methods. The mechanical force of this action is frequently sufficient to separate adhering membrane pairs, revealing the

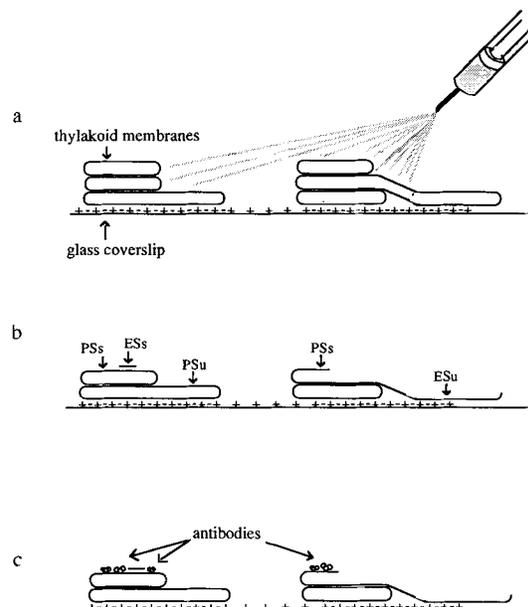


Figure 1. Schematic diagram of the technique used to expose and immunolabel the outer stacked surface. (*a*) Membranes that adhered to an alcian blue-treated glass coverslip are mechanically disrupted by a stream of buffer from a syringe. (*b*) The result is the exposure of all four surfaces, including the newly exposed outer stacked surface. The inner surfaces are labeled according to the convention of Branton et al. (1975): *ESu*, the inner surface in a nonstacked membrane region; *ESS*, the inner surface in a stacked region. Similarly, the outer surfaces are *PSs* and *PSu*. (*c*) The exposed membranes surfaces are then labeled with specific antibodies.

elusive outer surfaces from stacked regions of the thylakoid membrane system (Fig. 2). In control experiments where membranes are attached to the coverslip but not mechanically disrupted, the smooth surface is not observed. Throughout this report, we will refer to membrane surfaces using the convention described by Branton et al. (1975), as illustrated in Fig. 1 *b*.

This technique exposes an exceedingly smooth surface that can be identified as the outer stacked surface (*PSs*) by its position among other membrane surfaces. For example, occasionally a piece of an inner stacked surface, identifiable by its numerous tetramers, remains attached to the smooth outer surface (Fig. 2). Also, this smooth surface is frequently continuous with and surrounded by the distinct outer nonstacked surface (*PSu*, Fig. 2). There is a clear delineation between the extremely smooth outer stacked surface (*PSs*) and the particle-rich outer nonstacked surface (*PSu*). Fig. 2 shows both an inner surface fragment attached to the middle of the smooth outer surface, and a sharp transition from the outer stacked surface to the nonstacked surface.

An mAb (MLH1) reacts with a high degree of specificity against the amino terminal region of the major chlorophyll-binding protein of LHC-II that has a molecular mass of 26 kD (Darr et al., 1986). Immunoblotting with this antibody identifies a broad band with an apparent molecular mass of 26,000, as shown in Fig. 3. MLH1 does not react with any of several minor proteins of LHC-II or with LHC-I (Darr et al., 1986).

When thylakoid membranes, mechanically disrupted as

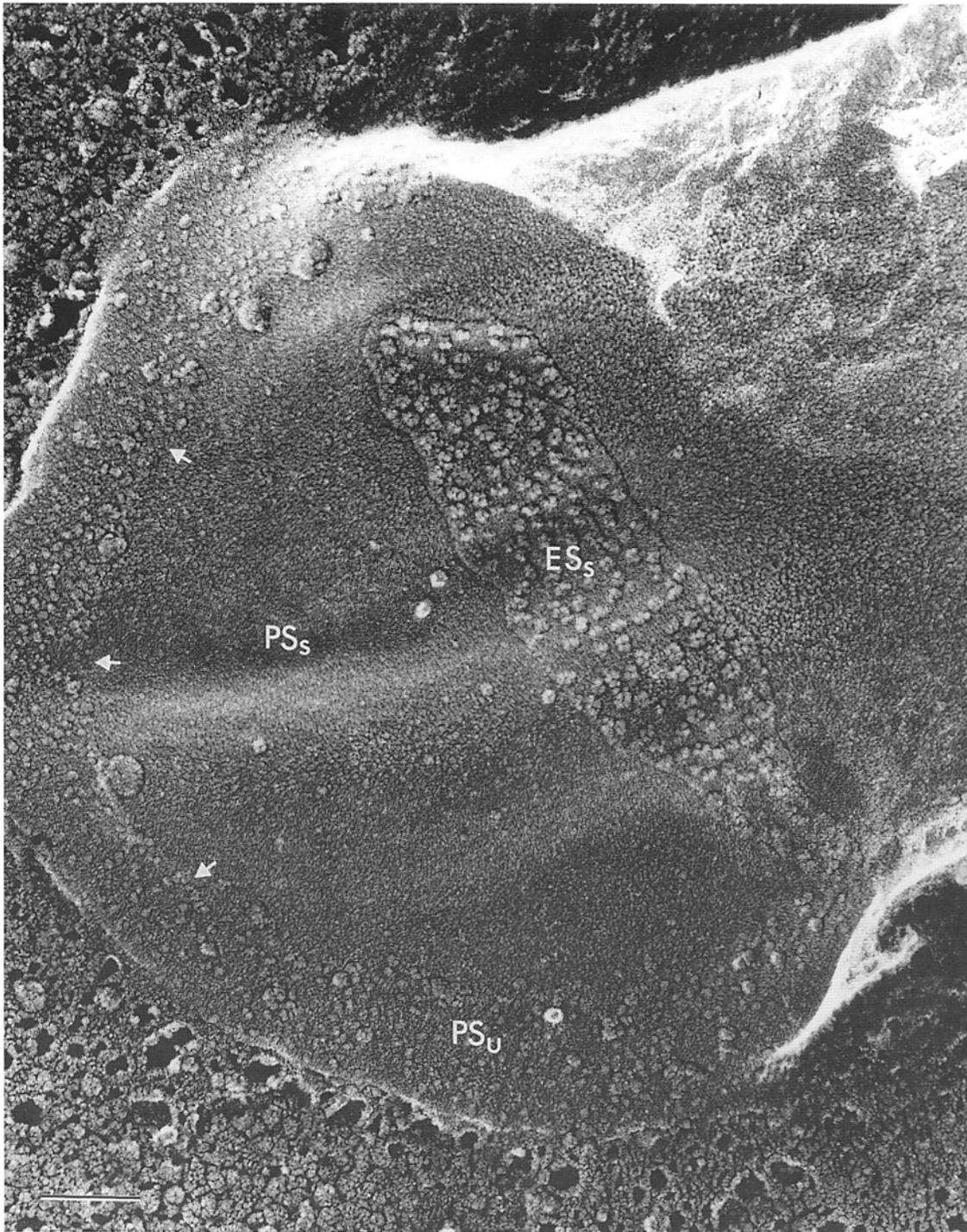


Figure 2. This electron micrograph enables us to identify the stacked outer surface, PS_s , by its positioning among the other membrane surfaces. A fragment of the inner surface (ES_s), identified by its tetramers, remains attached to this smooth stacked outer surface. In addition, this stacked outer surface is surrounded by and continuous with the nonstacked regions (PS_u). The numerous particles of the nonstacked regions abruptly end at the edge of the stacked region (arrows). Bar, 100 nm.

described above, are incubated with MLH1, the newly exposed outer stacked surface is clearly labeled (Fig. 4). There is no labeling on the membranes incubated with normal mouse serum (Fig. 4, *inset*). This smooth surface provides an ideal background for visualizing individual antibody molecules as illustrated in Fig. 4. The size and shape of these

IgG molecules are similar to other observations of IgG molecules freeze-dried on mica (Heuser, 1983).

The apparent absence of IgG labeling on the outer nonstacked surface may indicate that LHC-II is not located in this region of the membrane. However, one might suggest that the apparent absence of labeling is because of the numer-

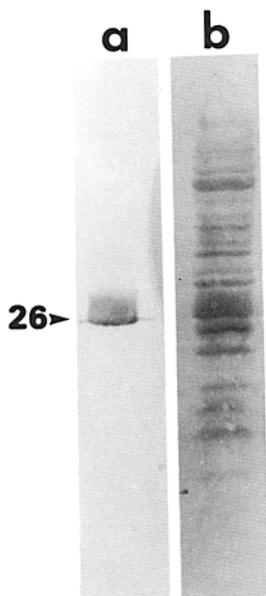


Figure 3. Immunoblot of the mAb MLH1, diluted 1:10,000 (lane *a*). This mAb reacts against the amino terminus of a LHC-II protein that has a molecular mass of 26 kD. The antibody was incubated against a thylakoid membrane preparation as shown in the Amido black lane (lane *b*).

ous small and large particles, including the coupling factor (Miller and Staehelin, 1976) that cover the outer nonstacked surface, interfering in the binding or visualization of the antibodies. To investigate this possibility, we removed the cou-

pling factor by a NaBr wash (Nelson, 1986) before labeling. This procedure did not change the labeling pattern (data not shown); IgG labeling was observed only on the outer stacked surface.

To further confirm the lack of labeling on the outer nonstacked surfaces, membranes labeled with MLH1 were also incubated with an anti-mouse gold complex. Any antibody hidden by the highly structured outer nonstacked surface would be made visible by the large, electron-dense gold complex. As seen in Fig. 5, the outer stacked surface is covered with the gold label while the PSu surface contains only a few scattered gold particles. When membranes are incubated with normal mouse serum followed by anti-mouse gold there are only a few scattered gold particle on the membrane surface (not shown).

Discussion

Exposure and Identification of the Appressed Surface

The outer stacked surface of the thylakoid membrane can not be directly observed by standard freeze-etching techniques. We have exposed the outer stacked surface by mechanical disruption and subsequently identified it by its position among the other well-characterized thylakoid surfaces. The

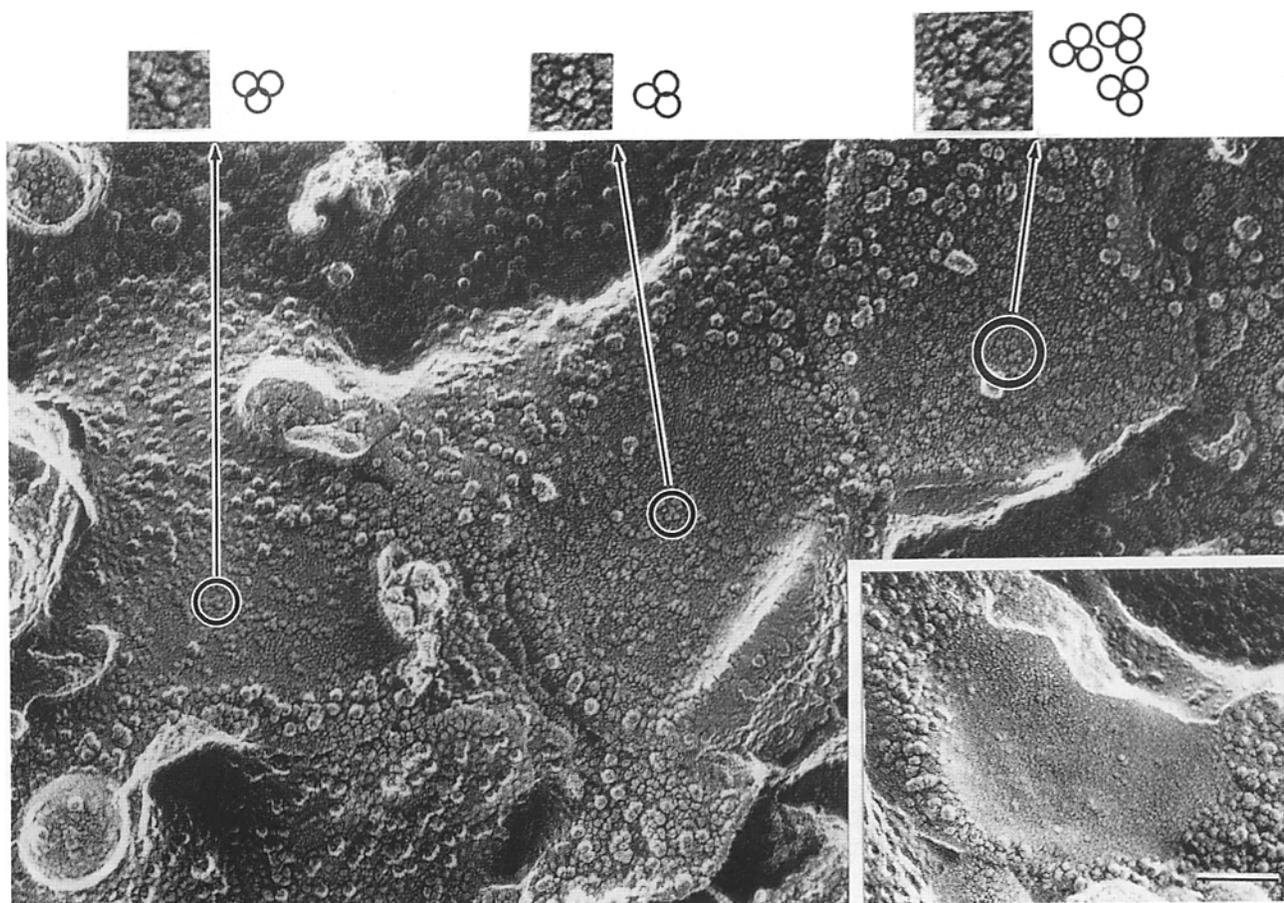


Figure 4. Outer stacked surfaces are intensely labeled with the mAb MLH1. Individual antibody molecules are clearly visible on the smooth outer stacked surface. The characteristic "Y" shape of the IgG molecules is seen at higher magnifications (*enlarged regions above micrograph*). There is no labeling on normal serum control-treated membranes (*inset*, same magnification) or on inner surfaces (not shown). Bar, 100 nm. (Enlarged regions are 2.5× the original magnification, or 280,000×).

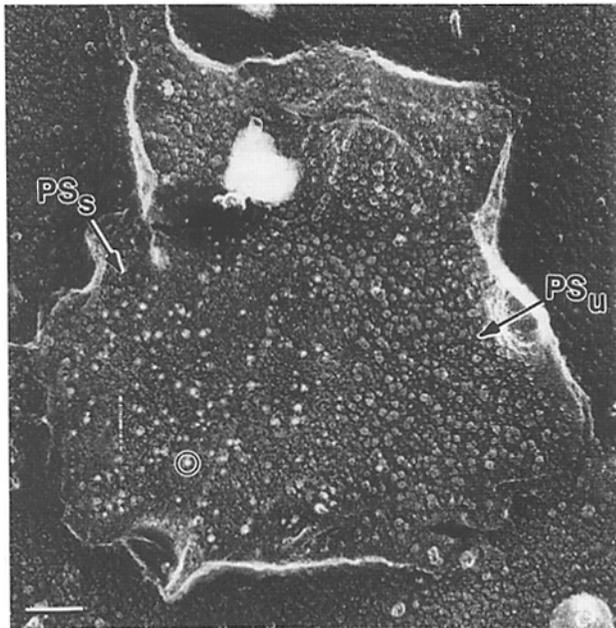


Figure 5. Thylakoid membranes labeled first with MLH1 and then by a goat anti-mouse antibody conjugated to colloidal gold. Many gold particles are visible on the outer stacked surface but only a few scattered gold particles are seen on the outer nonstacked surface. One gold particle is circled on the outer stacked surface. Bar, 100 nm.

outer stacked surface is continuous with and surrounded by the outer nonstacked surface. The numerous particles from the outer nonstacked surface abruptly end at the edge of a stacked region where the smooth outer stacked surface begins. The occasional fragment of an inner stacked surface that remains attached after mechanical disruption (Fig. 2) further confirms the identity of this new surface. The quick freezing process, which enables membranes to be frozen immediately after mechanical disruption, reduces the possibility that the smooth outer stacked surface is created by protein rearrangement. Previous work (Stachelin et al., 1977) reported the observation of a similar surface by partial unstacking of thylakoid membranes. However, this method did not eliminate the possibility of particle migration during the unstacking process and lacked conclusive evidence of its identification such as fragments of inner surface attached to the outer stacked surface.

Molecular Implications of Outer Stacked Surface Structure

The most striking feature of the outer stacked surface is its relative smoothness in comparison to the rest of the thylakoid surfaces. In contrast, the outer nonstacked surface is covered with 12.0- and 8.0-nm particles, while the inner surface contains numerous tetrameric particles. The smoothness of the outer stacked surface suggests that membrane polypeptides located in stacked regions have minimal outer surface exposure. This is consistent with predicted surface exposed polypeptide loops for LHC-II (Cashmore, 1984; Burgi et al., 1987), PS II (Alt et al., 1984; Deisenhofer et al., 1985) and the cytochrome b_6/f complex (Widger et al., 1984; Willey

et al., 1984) that do not exceed 70 amino acids in length (<8,000 D). Such polypeptide segments are probably too small to be seen in rotary-shadowed replicas. In addition, there is no evidence for extrinsic proteins associated with the outer stacked surface. In contrast, the large particle on the outer nonstacked surface is the extrinsic subunit of the ATP synthase, CF_1 (Miller and Staehelin, 1976) with a molecular weight in excess of 350,000. The tetrameric particle on the inner surface consists of extrinsic oxygen-evolving proteins with molecular weights ranging from 17,000 to 33,000 (Simpson and Andersson, 1986; Seibert et al., 1987).

The lack of a large surface structure on the outer stacked surface is consistent with several recent lines of data regarding LHC-II. The thickness of frozen hydrated two-dimensional crystals of LHC-II is only 4.8 nm (Lyon and Unwin, 1988). The structure of LHC-II in such crystals would easily fit into the membrane (5.0-nm width) without significant surface exposure. Furthermore, when LHC-II is cleaved by trypsin the protein only loses a small fragment corresponding to the first eight amino acids on the amino terminus (Mullet, 1983). Mullet's results (1983) suggest that only a small portion of the amino terminus of LHC-II is accessible to the enzyme. A minimal surface exposure for LHC-II is also supported by Burgi's experiments involving the phosphorylation of tyrosine residues of LHC-II (Burgi et al., 1987). In these experiments, none of the seven tyrosine residues located on the outer surface according to structural predictions were phosphorylated.

Immunolabeling

The MLH1 mAb is clearly visible on the outer stacked surface (Fig. 4), indicating that the amino terminus of LHC-II is exposed on this surface. There is no apparent labeling on the inner surface (not shown) or on the outer nonstacked surface (Fig. 4). Additional experiments involving CF_1 removal and gold labeling (Fig. 5) have confirmed the presence of bound MLH1 on the outer stacked surface and its absence from the outer nonstacked surface. Vallon et al. (1986) have localized 90% of LHC-II to stacked regions. Steinback et al. (1978) have shown that trypsin cleavage of intact membranes releases a 2-kD fragment from the LHC-II, which Mullet (1983) has identified as the amino terminus of this protein. Our results are consistent with these studies, adding evidence for an almost complete restriction of LHC-II to stacked regions and for the presence of its amino terminus on membrane surfaces directly involved in stacking.

In this paper, we have shown that individual antibody molecules can be directly visualized on a membrane surface without the need for secondary antibody conjugates. To our knowledge, this is the first such application of direct antibody labeling on a biological membrane. By eliminating the secondary antibody conjugate, more membrane surface structure is visible and less is obscured by a large labeling complex. The direct observation of antibodies also minimizes the distance between label and antigen, allowing for a more precise spatial localization. This method clearly works on a smooth membrane surface and should be applicable for more highly structured surfaces.

Domains within the Thylakoid Membrane

Work by many investigators supports the notion that thyla-

koid membranes display two distinct domains corresponding to the stacked and nonstacked regions of the membrane system. Anderson and Andersson (1980) have coined the term "lateral heterogeneity" to describe the situation. The striking exclusion of both large and small particles from the outer stacked surface (Fig. 2) supports this view. The absence of the large particle on the outer stacked surface supports earlier conclusions that ATP synthase is excluded from the stacked region (Vallon et al., 1986; Allred and Staehelin, 1985). If the smaller particle on the outer stacked surface is associated with PS I, then PS I is also restricted to the nonstacked regions. This correlates with thin-section immunolabeling (Vallon et al., 1986) and fractionation studies (Andersson and Anderson, 1980) that have shown PS I to be exclusively located to nonstacked regions.

The distribution of MLH1 labeling on outer thylakoid surfaces provides direct evidence of lateral heterogeneity. This antibody molecule is highly concentrated on smooth stacked surfaces with only a few scattered antibodies on nonstacked surfaces (Figs. 4 and 5). Therefore, the 26-kD protein of the LHC-II complex is almost exclusively located in the stacked regions.

There is good evidence that LHC-II molecules are responsible for membrane stacking. LHC-II molecules reconstituted in lipid bilayers have been shown to form stacks when cations are present (McDonnell and Staehelin, 1980). More specifically, the amino terminus of LHC-II has been shown to play a major role in membrane appression. Removal of this region by amino-peptidases severely disrupts membrane appression, possibly by increasing electrostatic repulsion between the adjacent membranes (Barber, 1982). For such a small amino terminal segment to affect membrane adhesion, the distance between the adhering membranes must be minimal. The fact that pieces of inner surface remain attached to the outer surface after mechanical disruption (Fig. 2), suggests such a substantial attraction between adjacent stacked membranes does exist.

A small distance would also limit the accessibility of soluble proteins to the space between stacked membranes. In addition, protein complexes with large outer surface protrusions would be prevented from migrating into the stacked region. In fact, it has been suggested that the exclusion of ATP synthase and PS I from the stacked regions might be because of steric hindrance created by these closely appressed membranes (Murphy, 1986). The CF₁ subunit of ATP synthase extends into the stromal space ~12 nm (Miller and Staehelin, 1976) while the ferredoxin NADP-reductase of PS I extends at least 5 nm. This exclusion of ATP synthase and PS I from stacked regions is confirmed by thin section immunolabeling (Vallon et al., 1986). On the other hand, the cytochrome b₆/f complex does not have any large protrusions on the outer surface (Murphy, 1986), and therefore would be free to migrate into the stacked region. This is supported by fractionation and immunological studies that localize this complex to both stacked and nonstacked regions (Anderson, 1982; Olive et al., 1986).

Our results do not support the proposal of Melis et al. (1986) that a cytochrome b₆/f-rich domain exists in the region between appressed and nonappressed membranes. Fig. 2 shows the regions between appressed and nonappressed membranes of both inner and outer surfaces. We have not observed a differentiated zone that might correspond to a

cytochrome b₆/f-rich domain in these micrographs or any of our preparations.

The two remaining complexes, LHC-II and PS II, do not have large exposed regions on the outer surface but have been predominantly localized within the stacked regions. It has been suggested LHC-II freely migrates into the stacked regions and then is held there by means of attractive interactions with other LHC-II molecules. LHC-II can migrate back to nonstacked regions only when these attractive forces are disrupted by phosphorylation (Bennett, 1983). Lateral attraction between LHC-II molecules has been demonstrated by the formation of sheetlike structures in the absence or presence of lipids (Li and Hollingshead, 1982). After migrating into the stacked regions, PS II may also become concentrated in this region by lateral attractive forces between PS II and LHC-II. The small amount of PS II that is found in nonstacked regions is believed to have fewer of these closely associated LHC-II molecules (Melis and Duysens, 1979; Thielen and Van Gorkum, 1981).

We have exposed a membrane surface that previously had been hidden by the appression of an adjacent membrane. The exposure of this surface has confirmed the structural heterogeneity of the photosynthetic membrane. The uneven distribution of MLH1 antibody molecules on the outer thylakoid surface has correlated with previous evidence for the lateral heterogeneity of protein complexes within the thylakoid membrane. The absence of distinct particles on the appressed surface suggests that the intrinsic protein complexes found in this region are minimally exposed. In addition, the smoothness of this surface and the apparent small distance between adjacent membranes provide support for the steric hindrance theory of lateral heterogeneity. Further analysis of this outer stacked surface may help in our understanding of the stacking process and the lateral segregation of protein complexes associated with the formation of grana. In the future we plan to use additional antibody probes to localize specific protein regions on the outer stacked surface as well as the other thylakoid surfaces. Finally, since this method is capable of exposing previously occluded membrane surfaces, it should be useful for structural studies of other appressed membrane systems, including cellular junctions.

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