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Localization of Membrane Proteins in the Cyanobacterium *Synechococcus* sp. PCC7942[1]

Radial Asymmetry in the Photosynthetic Complexes

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Localization of membrane proteins in the cyanobacterium *Synechococcus* sp. PCC7942 was determined by transmission electron microscopy utilizing immunocytochemistry with cells prepared by freeze-substitution. This preparation procedure maintained cellular morphology and permitted detection of cellular antigens with high sensitivity and low background. *Synechococcus* sp. PCC7942 is a unicellular cyanobacterium with thylakoids organized in concentric layers toward the periphery of the cell. Cytochrome oxidase was localized almost entirely in the cytoplasmic membrane, whereas a carotenoprotein (P35) was shown to be a cell wall component. The major photosystem II (PSII) proteins (D1, D2, CP43, and CP47) were localized throughout the thylakoids. Proteins of the Cyt b_6/f complex were found to have a similar distribution. Thylakoid luminal proteins, such as the Mn-stabilizing protein, were located primarily in the thylakoid, but a small, reproducible fraction was found in the outer compartment. The photosystem I (PSI) reaction center proteins and the ATP synthase proteins were found associated mostly with the outermost thylakoid and with the cytoplasmic membrane. These results indicated that the photosynthetic apparatus is not evenly distributed throughout the thylakoids. Rather, there is a radial asymmetry such that much of the PSI and the ATPase synthase is located in the outermost thylakoid. The relationship of this structure to the photosynthetic mechanism is discussed. It is suggested that the photosystems are separated because of kinetic differences between PSII and PSI, as hypothesized by H.-W. Trissl and C. Wilhelm (Trends Biochem Sci [1993] 18: 415–419).

The structure of the photosynthetic membrane (the thylakoid) has been subjected to a wide variety of investigations (Albertsson et al., 1990; Olive and Vallon, 1991). The thylakoids in the chloroplasts of higher plants and algae are highly complex, specialized membranes consisting of two compartments, the grana and the stromal lamellae, which interconnect grana stacks. They perform interrelated photochemical and redox reactions, based on the functions of discrete supramolecular assemblies of proteins, pigments, and electron carriers. Distinctions are now made between appressed and stroma-exposed membranes or specific domains such as appressed membranes, the margins of the grana and stroma, the grana end membranes, and the zone between the grana and the stroma lamellae (Albertsson et al., 1990). The combination of biochemical and morphological studies have shown that PSI and PSII are differentially located within these different membrane domains, resulting in a heterogeneous chloroplast membrane system. This separation of the photosystems has been termed lateral heterogeneity (Albertsson, 1987; Albertsson et al., 1991; Anderson and Andersson, 1988; Anderson et al., 1988; Anderson and Goodchild, 1987; Anderson and Melis, 1983). Specifically, PSII is located primarily in the grana (appressed) regions, whereas PSI is located mostly in the stroma membranes and at the margins of the grana stacks (Anderson and Melis, 1983). Recognition that this lateral heterogeneity occurs in chloroplast membranes has led to a great deal of activity in an attempt to describe how it relates to the operation of the two-photosystem photosynthetic mechanism. Many studies have been aimed at understanding the stacking phenomenon in the grana, but results of this research have led to explanations as to how, but not why, stacking occurs.

The cyanobacteria are photosynthetic prokaryotes that contain Chl *a* (as well as the accessory phycobiliproteins) and are capable of an oxygenic photosynthesis that is virtually identical with that of higher plant chloroplasts. A great deal of evidence has accumulated to demonstrate that the proteins from cyanobacteria are very closely related to those of chloroplasts and that the primary light reactions are fundamentally the same in both systems (Crouse et al., 1985; Giovannoni et al., 1988; Blankenship, 1992). However, very little information is available concerning the distribution of photosynthetic complexes in cyanobacteria. We report here the localization of photosynthetic membrane complexes in the cyanobacterium *Synechococcus* sp. PCC7942. This strain has three to four photosynthetic lamellae arranged concentrically around the periphery of the cell. In this communication, we will demonstrate the precise localization of membrane proteins in this cyanobacterium through the use of freeze-sub-

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Abbreviations: MSP, Mn-stabilizing protein; TBS, Tris-buffered saline.

stitution specimen preparation for transmission EM in combination with immunocytochemistry. We will show that all of the photosynthetic supramolecular complexes are not distributed evenly throughout the membranes. Rather, the ATP synthase and PSI are localized toward the periphery of the cell and mostly in the thylakoid that is closest to the cytoplasmic membrane. At the same time, the PSII are distributed more evenly throughout the thylakoid membranes. This gives rise to a concept of "radial" heterogeneity in cyanobacteria that closely resembles the lateral heterogeneity in chloroplasts.

MATERIALS AND METHODS

Organism and Culture Conditions

The unicellular coccoid cyanobacterium *Synechococcus* sp. PCC7942 was grown in a modified BG-11 medium (Allen, 1968) under conditions described previously (Sherman and Sherman, 1983). Cell numbers were determined using a Petroff-Hausser Cell Counting Chamber.

EM—Chemical Fixation, Freeze-Substitution, and Immunocytochemistry

Cells and isolated membranes for immunocytochemistry were prepared by chemical fixation using the van Tuinen and Riezman (1987) procedure as previously described (Reddy et al., 1988) or the freeze-substitution procedure described by Reddy et al. (1993). The chemically fixed cells and isolated membranes were pelleted in 2% type VI agarose (Sigma). The resulting 2-mm blocks were dehydrated in a graded series of ethanol concentrations (35, 50, 70, 90, 100%) and infiltrated with two changes of LR White resin for a total of 16 h at room temperature on a rotator. The specimens were transferred to gelatin capsules containing fresh resin and polymerized for 24 h at 55°C in a nitrogen-saturated environment, under a vacuum of 635 mm Hg.

For the freeze-substitution preparation, the cells were concentrated, resuspended in 1% agarose (type VI, Sigma), and spread thinly onto glass slides. Small squares of the cell-agarose sheet were quick frozen by plunging them into liquid propane that was cooled to −190°C by liquid nitrogen. The specimens were transferred to −80°C where, during the next few days, the cellular water was gradually replaced with an organic solvent. For immunocytochemistry, we determined that ethanol was the best freeze-substitution solvent. The cells were then infiltrated with and embedded in Lowicryl HM20 and photopolymerized by UV light for 48 h at 4°C, followed by an additional 48 h at room temperature.

Immunocytochemical localization of antigens in cyanobacteria was performed utilizing TBS, which contained 20 mM Tris-HCl, pH 7.4, 150 mM NaCl. To this buffer was added 1% BSA (TBS-B), 0.3% Tween 20 (TBS-T), or both 0.3% Tween 20 and 1% BSA (TBS-TB). Thin sections (approximately 80–100 nm) on carbon-coated, Formvar copper or nickel grids were incubated in TBS-TB for 20 min to block nonspecific immunoglobulin sites. Grids were then placed on 50-μL drops of primary antibody diluted in TBS-B to the appropriate concentration as determined by trial and incubated in a moist chamber for approximately 15 h at 4°C. Between changes of solution, excess liquid was drawn off the grids with filter paper, taking care not to let the grids dry. The grids were removed from the antibody and washed for a total of 15 min at room temperature by flotation on TBS-T placed in the wells of spot plates. They were incubated on TBS-TB for approximately 5 min prior to a final 1-h incubation on a dilute solution of secondary antibody consisting of 8-to 10-nm colloidal gold-IgG complex diluted in TBS-TB (15 nm of gold conjugated to protein A for Fig. 1, a and b). The colloidal gold was prepared by the sodium citrate-tannic acid method (Slot and Geuze, 1985), followed by conjugation to goat anti-rabbit IgG (Sigma). A commercially prepared IgG-colloidal gold conjugate (Energy Beam Sciences, Agawam, MA) was used as a control. The grids were floated on TBS-T, jet-washed with double-distilled H_2O, and dried. They were stained with uranyl acetate and lead citrate prior to examination in a Philips 400 transmission electron microscope at an accelerating voltage of 80 KeV.

The small gold particles used to visualize the proteins yield high resolution but are sometimes difficult to visualize if the concentration of the label is low. Thus, selected electron micrographs were scanned into an Apple Macintosh Quadra 950 computer at a resolution of 300 dots per inch using a Sharp JX-610 flatbed scanner and video enhanced by adjusting contrast and brightness in Adobe Photoshop 2.5.1. The diameter of the gold particles was enlarged 2-fold to increase visibility. The image files were printed on a Mitsubishi CP210U dye-sublimation printer using Professional Output Manager software from Visual Business Systems (Atlanta, GA). All microscopy and computer image processing was carried out in the Electron Microscopy Center in the School of Agriculture, Purdue University.

Membrane Isolation

Synechococcus sp. PCC7942 cells were harvested, resuspended to approximately 3×10^8 cells/mL in 50 mM Mes (pH 6.5), 1 mM benzamidine, 1 mM caproic acid, 1 mM PMSF, and broken by three passages through a French pressure cell at 16,000 psi. DNase I and $MgCl_2$ were added to the lysates to a final concentration of 50 μg/mL and 6 mM, respectively, and the lysates were incubated on ice (4°C) for 1 h. EDTA was added to a final concentration of 10 mM, and unbroken cells and large pieces of cell debris were cleared from the lysate by centrifugation in a Beckman J2–21 centrifuge at 5,500*g* for 5 min. The cleared lysate was pelleted at 180,000*g* for 2 h (4°C) in a Beckman L8–70M ultracentrifuge. Cell membrane components were prepared by a modification of the Suc gradient purification previously described (Omata and Murata, 1984). The pelleted membranes were homogenized into 50 mM Mes buffer and loaded directly onto Suc step gradients (0.5, 0.9, 1.5, 2.2, and 2.67 M Suc). The gradients were centrifuged at 265,000*g* for 16 h (4°C) in a Beckman L8–70M ultracentrifuge. Envelopes were harvested from the 2.2/2.67 M Suc layer interface, thylakoids from the center of the green band within the 1.5 M Suc layer, and cytoplasmic membranes from the 0.5 M Suc layer. Membrane fractions were centrifuged at 265,000*g* for 2 h (4°C), and the pellets were homogenized into 50 mM Mes buffer and repel-

leted in a Beckman TL100 tabletop ultracentrifuge at 100,000*g* for 15 min (4°C).

Antibodies

Antibodies against these membrane proteins were obtained from various sources and were prepared from proteins eluted from SDS polyacrylamide gels. In the case of the photosynthesis proteins, we generally used more than one antibody preparation. We were interested in four criteria when evaluating the antibodies: (a) the reaction with the appropriate band on a western blot at a dilution usable for immunocytochemistry, (b) low background on the embedding resin, (c) low background in the cell, and (d) high reactivity with the membrane protein. All of the antibodies against photosynthesis proteins have been analyzed against subcellular fractions, and we are confident that they react against the specified component. Most important, we used the antibodies at a concentration that yielded a single band, under typical development conditions, on western blots (data not shown). However, antibodies that yield one specific band on western blots can vary dramatically in their immunocytochemical properties. Some antibodies have high backgrounds with a particular resin or demonstrate nonspecific binding to other cellular components (even if this binding was not obvious on the western blots). Thus, a single band on a western blot is a necessary, but not sufficient, factor for the antibody to meet the above criteria. The antibodies described in this paper generated high and specific reactivity for both western blots and immunocytochemistry.

Antibodies against the following proteins were used in this study and were obtained from the sources indicated: (a) P35, a carotenoid-binding protein from *Synechococcus* sp. PCC7942 prepared in this laboratory by George Bullerjahn and Kaz Masamoto; (b) Cyt oxidase subunit I from *Anacystis nidulans*, from Günter Peschek, University of Vienna; (c) ATP synthase β-subunit from *Chlamydomonas reinhardtii*, from Bruce Selman, University of Wisconsin; (d) PSI apoproteins (PsaA and PsaB proteins) from *Synechococcus* sp. PCC7942, prepared by James Guikema, Kansas State University; (e) PSII apoprotein PsbA (D1) from *Synechococcus vulcanus*, from H. Koike, Riken, Tokyo, Japan; (f) PSII apoprotein PsbD (D2) from *Synechococcus* sp. PCC7942 (C-terminal 46 amino acids), from Susan Golden, Texas A & M; (g) PSII apoproteins PsbB (CP47) and PsbC (CP43), from M. Ikeuchi and Y. Inoue, Riken, Tokyo, Japan; (h) PSII protein PsbO (MSP) from spinach, from Tom Kuwabara, Toho University, Japan; (i) Cyt *f* from maize, from William Taylor, University of California, Berkeley.

We always used the greatest dilution possible with a given antibody. This is routine for homologous antibodies but much more difficult when heterologous sources are used. This is where the freeze-substitution procedure became most valuable. Immunochemistry on freeze-substituted cells permitted antibody dilutions that were 10- to 100-fold more dilute than for chemically fixed cells. Thus, we were able to use dilutions that were similar to (or even less dilute than) the dilutions used on western blots. These conditions generated high reactivity and low background.

RESULTS

Envelope Proteins

One goal of this research was to develop immunocytochemical procedures that would permit routine, qualitative determination of membrane protein localization. Our previous results indicated that the chemical fixation procedure of van Tuinen and Riezman (1987) gave reasonable immunolabeling with homologous antibodies but did not maintain normal cellular morphology. As illustrated in Figure 1, a, b, and d, chemical fixation normally resulted in separation of the cell wall from the underlying cytoplasmic membrane. There also appeared to be a loss of cell wall structural integrity resulting in a scalloped appearance with occasional discontinuity. The cytoplasm appeared uneven because of dense areas with clumped ribosomes and lighter staining areas that contained fiber networks (possibly nucleic acid) and vacuoles. Significant cell shrinkage occurred in some preparations (cf. a and b to d in Fig. 1). In contrast, the almost instantaneous freezing, followed by substitution of intracellular water and simultaneous fixation or stabilization by organic solvents, resulted in a smooth, continuous cell wall tightly adhered to the cytoplasmic membrane (Fig. 1c). The ribosomes were evenly distributed in the cytoplasm, although they were slightly more concentrated close to the thylakoid membranes that encircle the outer regions of the cell. Carboxysomes were more centrally located, and there were few vacuoles or open spaces in the cytoplasm.

An example of immunolocalization following chemical fixation is presented in Figure 1, a and b, which shows the distribution of P35, a carotenobinding protein isolated in this laboratory from *Synechococcus* sp. PCC7942. Western blots of isolated membranes have demonstrated that this protein is located in the cell wall fraction, and this is amply verified by the immunocytochemistry. Virtually all of the gold particles ($\geq$95%) are located over the cell wall, and the cellular and resin background labeling is essentially nonexistent. Although the chemical fixation procedure caused the cell wall to pull away from the cell, this feature was used to advantage for this and some other envelope proteins to clarify the site of localization.

Cyt oxidase also was localized to the outer compartment (Fig. 1, c and d). The results with Cyt oxidase were also unequivocal, with $\geq$95% of the gold located over the envelope. In this case, most of the gold appeared over the cytoplasmic membrane (in Fig. 1 cf. c and d with a and b). This was evident in both freeze-substituted (Fig. 1c) and chemically fixed (Fig. 1d) cells and correlated extremely well with the biochemical results for *Synechococcus* sp. (Peschek et al., 1989). We conclude that Cyt oxidase is located in the cytoplasmic membrane, although there appeared to be appreciable label in the outer membrane in both c and d in Figure 1. Although we are confident to assign P35 to the cell wall (the outer membrane of the cell envelope) and Cyt oxidase to the cytoplasmic membrane (the inner membrane of the cell envelope), this precise delineation is not always possible. In iron-deficient cells, the membrane system often decreases to a single thylakoid, in addition to the cytoplasmic membrane and the outer membrane (Sherman and Sherman, 1983). Immunologically, the differences among the cytoplasmic

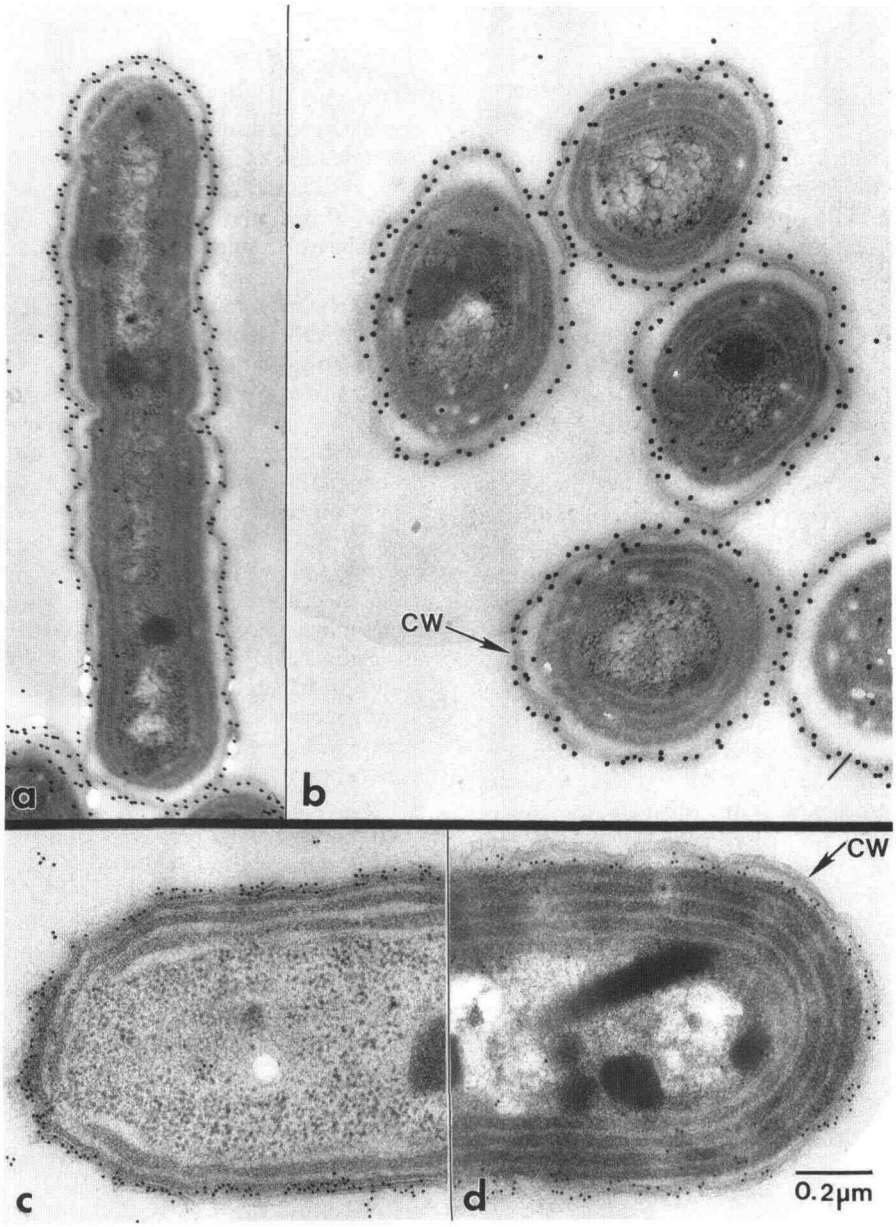

Figure 1. Localization of proteins to the outer compartment of *Synechococcus* sp. PCC7942 by immunocytochemistry. a and b, P35, a carotenobinding protein; dilution, 1:5,000; chemical fixation. c, Cyt oxidase; dilution, 1:4,000; freeze-substitution. d, Cyt oxidase; dilution, 1:500; chemical fixation. CW, Cell wall.

membrane, cell wall, and periplasm of the cell envelope in this stress condition were not clear. We often concluded, therefore, that the protein was localized in the envelope (which includes the cytoplasmic membrane, the periplasmic space, and the cell wall) without definitively assigning it to a particular membrane.

There are three other considerations that must be analyzed when we assign a protein to a specific compartment. First, the antibody is a relatively large, flexible molecule. The entire complex (gold-primary antibody-secondary antibody) can span 30 to 40 nm; thus, our resolution was no better than ± 30 nm, given the propensity of the complex to flop in one direction or another. Therefore, it was difficult to differentiate among the separate sections of the envelope. Second, there was the problem of contamination mentioned earlier. We have tried to rule out this problem in many ways, including the use of several antibody preparations made against a single antigen. Nonetheless, the presence of a minor, yet highly immunoreactive, contaminant cannot be rigorously excluded. Finally, the proteins are synthesized in the cytoplasm, and it is difficult to differentiate between biosynthesis and contamination by a cytoplasmic protein. In most cases, there was essentially no gold in the cytoplasm. Where such material was present, it was most often seen over the carboxysomes,

implying contamination with ribulose-1,5-bisphosphate (see below).

Thylakoid Proteins

We have determined the location of several proteins of the four major photosynthetic complexes (PSI, PSII, Cyt b_6/f, and ATP synthase). The most striking results were for ATP synthase and PSI, as shown in Figures 2 to 4. The localization of ATP synthase was obtained by use of an antibody prepared against the *C. reinhardtii* chloroplast ATP synthase β subunit (Fig. 2). The results were unequivocal—the gold was located predominantly at the cell periphery and was over either the envelope or the thylakoid proximal to the cytoplasmic membrane. The resin background was low, and there was little gold found in the other cellular regions. It is interesting to note that the gold label often appeared to form clusters that were evenly spaced along the cell periphery (arrowheads in Fig. 2, b and d). We performed a number of controls to determine whether this clustering was an artifact, including the use of gold conjugates from different sources and different preparations of gold tagged to the secondary antibody.

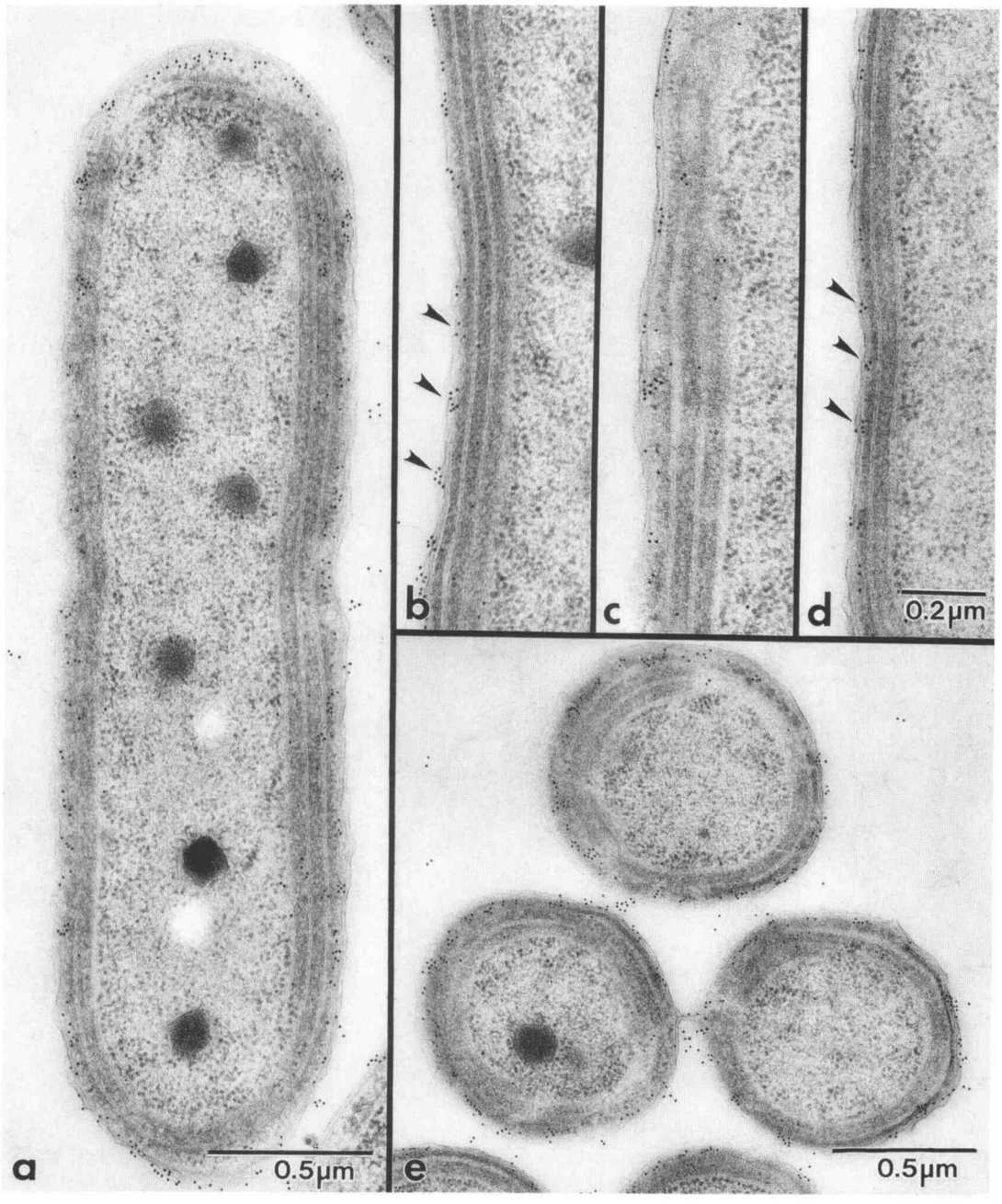

Figure 2. Localization of ATP synthase to membranes in *Synechococcus* sp. PCC7942 with antibody against the β subunit; dilution, 1:5,000; freeze-substitution. The arrowheads highlight regions of antibody clustering.

The use of these different preparations with many primary antibodies convinced us that this clustering occurred reproducibly with specific antibodies (e.g. ATP synthase) and was not an artifact.

We checked the validity of these in situ experiments by analyzing the localization in chemically fixed, isolated membranes. Two examples are presented in Figure 3, which shows the labeling of isolated membrane fractions with antibodies against Cyt oxidase (Fig. 3, a and b) and ATP synthase (Fig. 3, c–e). As indicated in "Materials and Methods," we separated the cellular membranes into envelope, cytoplasmic membrane, and thylakoid fractions. The Cyt oxidase antibody reacted very strongly with the cytoplasmic membrane fraction (Fig. 3a), weakly with the envelope fraction (data not shown), and not at all with the thylakoids (Fig. 3b). These results corroborate the in situ results. The ATP synthase antibody reacted moderately well with the envelope (Fig. 3c), strongly with the cytoplasmic membrane (Fig. 3d), and moderately well with the thylakoids (Fig. 3e). We noted that only about 25% of the thylakoid vesicles reacted with the ATP synthase antibody, in keeping with the in situ location of the gold particles. Again, the results with isolated membranes were similar to what was obtained in situ, and we conclude that the ATP synthase is located in the cytoplasmic membrane and in thylakoids (but to a greater extent in the outermost thylakoid).

The single most surprising result was the localization of PSI, determined with an antibody prepared against the PsaA/

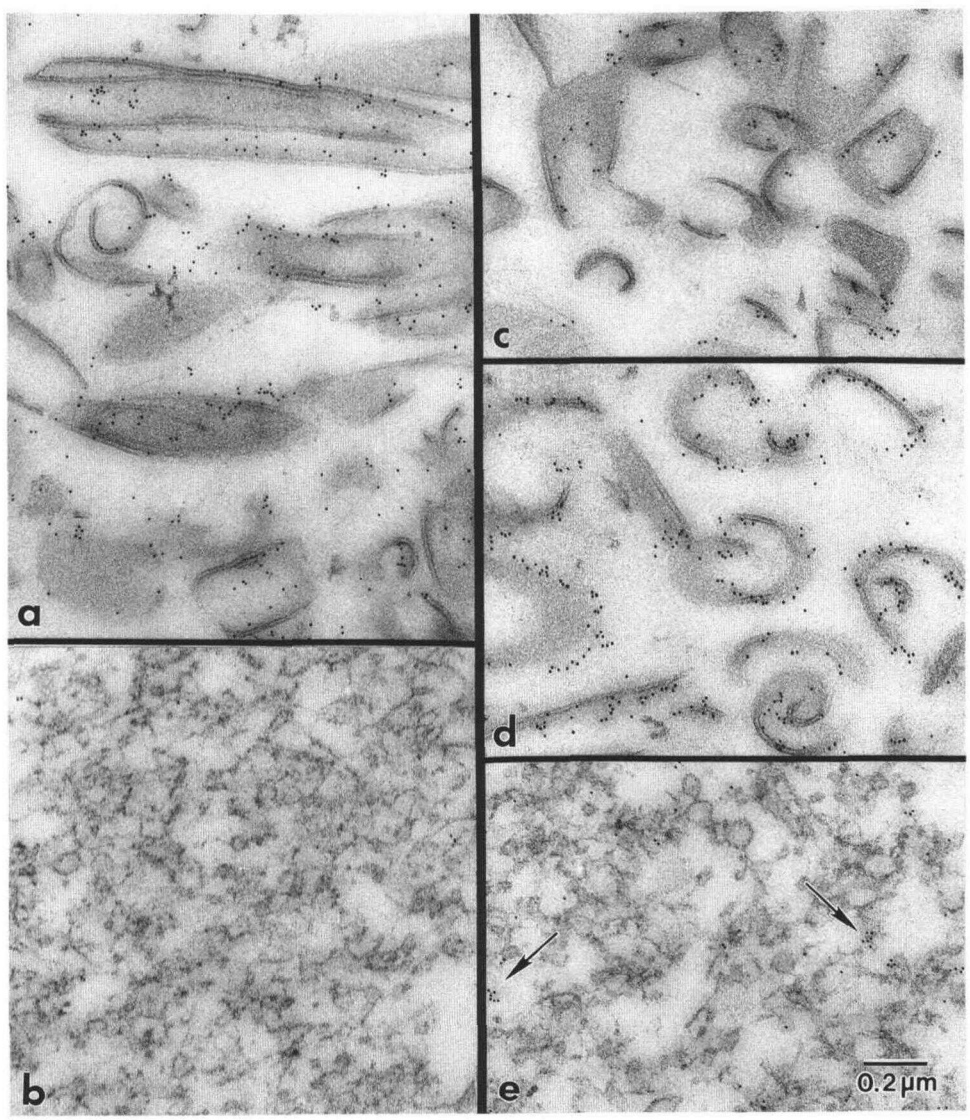

Figure 3. Gold labeling of isolated membranes of *Synechococcus* sp. PCC7942 that were chemically fixed prior to immunocytochemistry. a and b, Cyt oxidase antibody reacted against cytoplasmic membranes (a) and thylakoid membranes (b); dilution, 1:500. c to e, ATP synthase antibody reacted against cell walls (c), cytoplasmic membranes (d), and thylakoids (e); dilution, 1:100. The arrows in e point to regions of gold binding that are clustered, similar to what is found in situ in Figure 2.

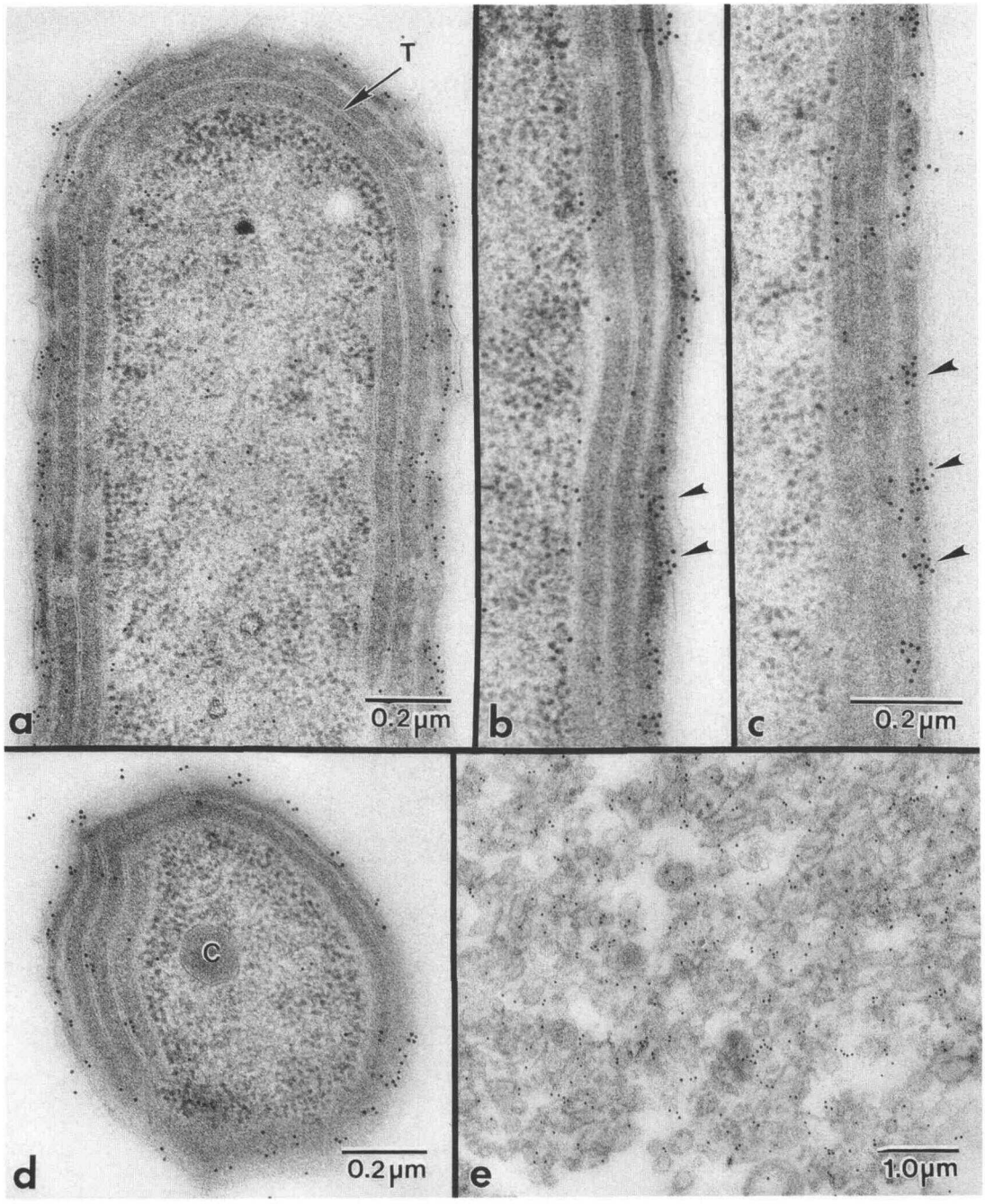

Figure 4. Localization of PSI reaction center proteins, PsaA/B, in *Synechococcus* sp. PCC7942. a to d, Freeze-substituted cells; dilution, 1:4,000. e, Chemically fixed, isolated membranes; dilution, 1:100. The arrowheads highlight regions of antibody clustering. T, Thylakoid.

B apoproteins (the PSI reaction center proteins) from *Synechococcus* sp. PCC7942. As shown in Figure 4, a to d, a high percentage of the gold was located over the outer thylakoid and the proximal envelope, often forming clusters similar to the ATP synthase distribution (arrowheads, Fig. 4, b and c). In this case, there were some gold particles over the inner thylakoids, but most of the immunoreactivity appeared at the cell periphery. Analysis of isolated membranes indicated that the antibody reacted strongly with the thylakoid fraction (Fig. 4e) and moderately with the envelope (data not shown). In this case, the antibody interacted with one-third to one-half of the vesicles in the thylakoid fraction, consistent with the reaction to a single membrane in situ. We conclude that there is radial asymmetry in the cellular distribution of ATP synthase and PSI, with the majority of these complexes in the outer thylakoid.

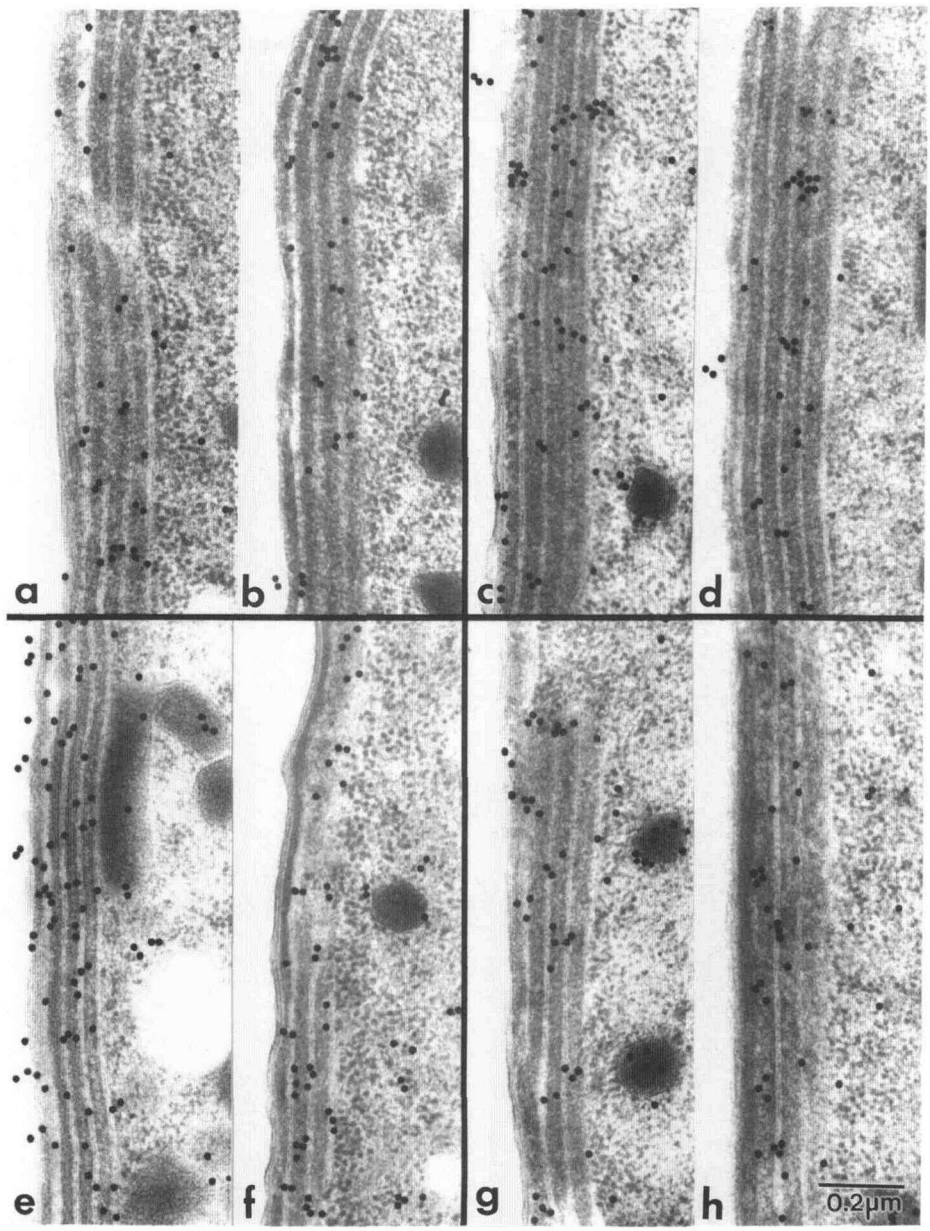

Figure 5. Computer-enhanced electron micrographs demonstrating the localization of PSII proteins in freeze-substituted *Synechococcus* sp. PCC7942. a and b, D1 antibody; dilution, 1:500. c and d, CP43 antibody; dilution, 1:500 (c) and 1:1000 (d). e and f, D2 antibody; dilution, 1:1000 (e) and 1:2000 (f). g and h, CP47 antibody; dilution, 1:100 (g) and 1:500 (h).

The PSII and Cyt b_6/f complexes were localized, as expected, throughout the thylakoids, and only some label was associated with the envelope. The micrographs in Figures 5 and 6 are computer enhanced, and, therefore, the gold particles could be enlarged for greater clarity. As shown in Figure 5, the PSII proteins D1 (Fig. 5, a and b), D2 (Fig. 5, e and f), CP43 (Fig. 5, c and d), and CP47 (Fig. 5, g and h) were, more or less, evenly distributed throughout all of the thylakoid lamellae. The amount of labeling of these PSII proteins appeared low, especially when compared to the PSI labeling shown in Figure 4. In fact, labeling was successful only with specimens that were prepared by freeze-substitution. We believe that it is difficult to make quantitative comparisons between the level of reactivity of antibodies against PSI and PSII complexes. The PSII antibodies are mostly heterologous, whereas the PSI antibody is homologous. On the other hand, this differential reactivity may be a real reflection of the low PSII:PSI ratio (1:3) of *Synechococcus* sp. PCC7942 cells. Because of such uncertainties, we made no further attempt to quantitate PSI versus PSII.

Figure 6, a and b, demonstrate that the Cyt b_6/f complex is also localized throughout the thylakoids. The antibody

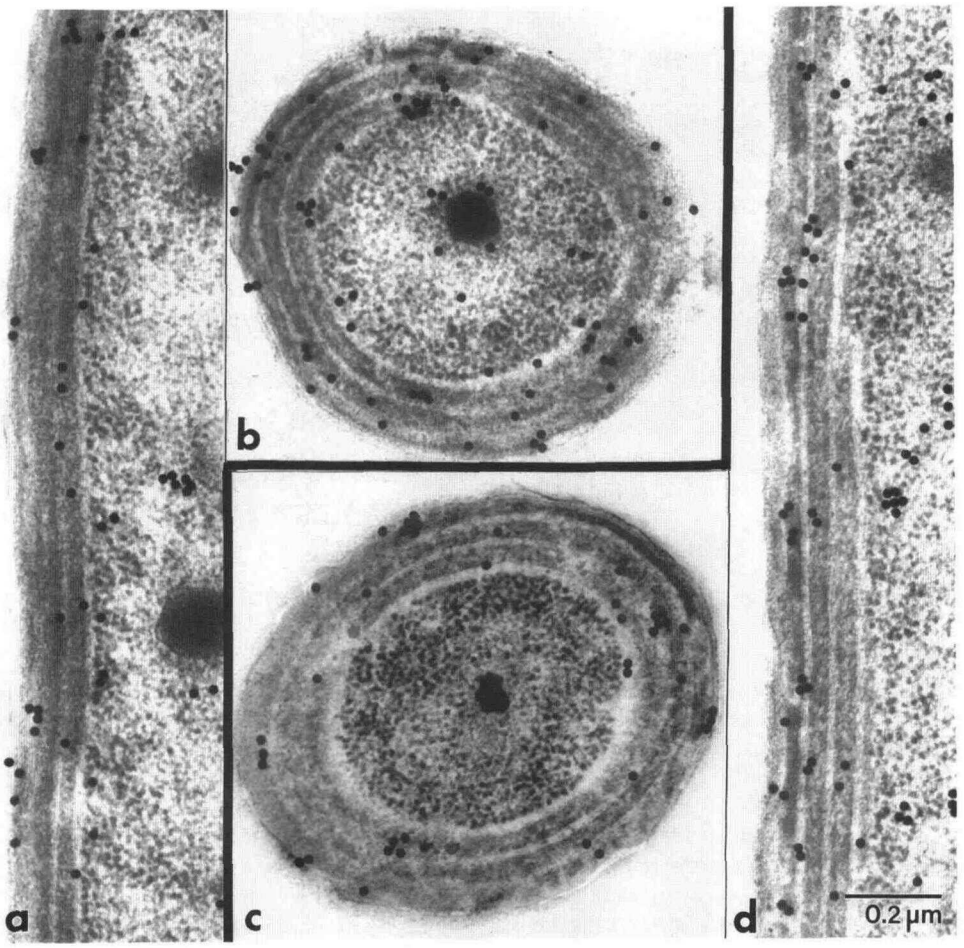

Figure 6. Computer-enhanced electron micrographs demonstrating the localization of photosynthetic membrane proteins in freeze-substituted *Synechococcus* sp. PCC7942. a and b, Cyt *f* antibody; dilution, 1:100. c and d, Antibody for PsbO protein, the MSP; dilution, 1:1000 (c) and 1:2000 (d).

against Cyt *f* (Fig. 6, a and b) and against Cyt b_6 (data not shown) showed some clustering, as is evident in both a and b of Figure 6. Finally, the antibody against the *psbO* gene product (MSP, an extrinsic luminal membrane protein) was distributed throughout the thylakoids (Fig. 6, c and d), similar to the intrinsic membrane proteins of PSII. However, approximately 5 to 10% of the gold particles were found over the envelope, indicating that some MSP may be targeted to the envelope.

DISCUSSION

The results presented above demonstrate that immunocytochemistry of freeze-substituted cyanobacteria can be used to localize membrane proteins (both integral and associated) with high precision. The localization of P35 and Cyt oxidase to the outer membrane and cytoplasmic membrane, respectfully, brings the number of proteins localized exclusively in the envelope compartment in *Synechococcus* sp. PCC7942 to five. This includes two proteins induced upon growth in iron-deficient media, IrpA (Reddy et al., 1988) and MapA (Webb et al., 1994), and a carotenobinding protein, CbpA (Reddy et al., 1989), that is induced under high-light conditions. It is evident that precise localization utilizing immunocytochemistry can be determined in both chemically fixed and freeze-substituted cells but that freeze-substitution provides a 10- to 100-fold increase in antibody reactivity. Reactivity of some antibodies in chemically fixed cells is so low as to be insignificant, whereas the same antibody gives significant localization when used with freeze-substituted cells. An additional advantage of freeze-substitution is a much reduced possibility of translocation of luminal proteins during specimen preparation. This method, in combination with low-temperature embedding in Lowicryl resin, may either preserve the integrity of the antigens or expose them more fully, thus allowing the use of significantly more dilute antibody titers and yielding more specific reactions. These findings generated a high degree of confidence in the technical capabilities inherent in this procedure and our ability to localize proteins to particular membrane regions.

A highly schematic model of our findings is represented in Figure 7, which is based on the earlier cyanobacterial membrane models from Gantt (1980), Giddings et al. (1983), Nierzwicki-Bauer et al. (1983), and Nicholls et al. (1992).

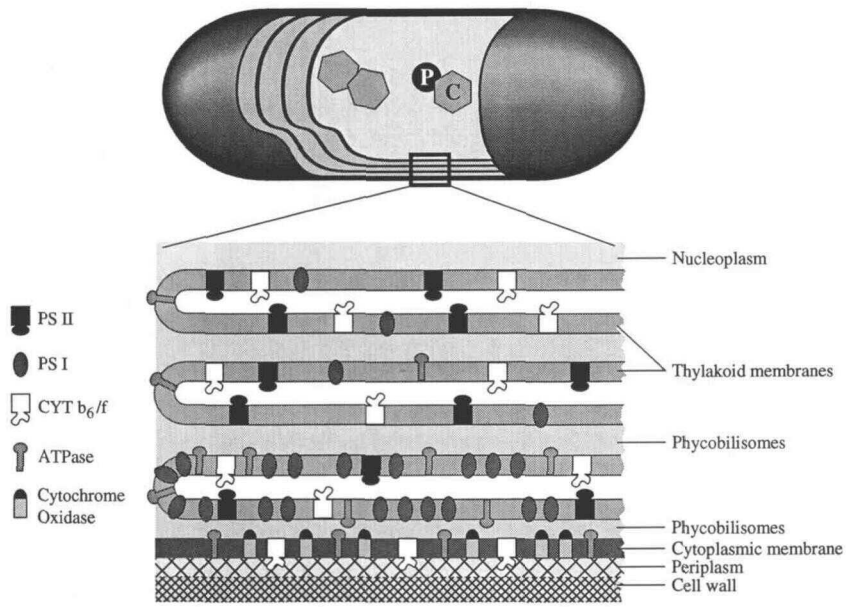

Figure 7. A schematic model of membrane structure in the cyanobacterium *Synechococcus* sp. PCC7942. The upper part of the diagram, based on the model of Nierzwicki-Bauer et al. (1983), is a cutaway of the cell that shows the different membrane layers and the nucleoplasm with carboxysomes (C) and a polyphosphate granule (P). The diagram highlights two of the main conclusions drawn from the immunocytochemistry: the cytoplasmic membrane contains a complete respiratory chain and the outer thylakoid contains a higher proportion of PSI and ATP synthase complexes. For simplicity, the phycobilisomes are indicated only as the region between the membrane leaflets.

There are two salient features that we will discuss, the first of which concerns the cytoplasmic membrane. Our results suggest that the cytoplasmic membrane has a complete respiratory chain, including Cyt oxidase, a Cyt b_6/f-like complex, and ATP synthase. Our immunocytochemistry findings support the biochemical results of Nicholls et al. (1992), who reached a similar conclusion. The importance of this respiratory pathway as a major metabolic pathway in *Synechococcus* strains remains to be determined. In addition, the immunocytochemistry does not necessarily indicate that the thylakoids and cytoplasmic membrane have identical ATP synthase and Cyt b_6/f complexes but only that the complexes are immunologically similar.

The most unique and controversial finding from our results is that the thylakoid leaflets do not have an identical composition. Instead, the thylakoid closest to the cytoplasmic membrane has a higher percentage of PSI and ATP synthase compared to the inner thylakoids. We believe that the individual membrane sacs have fenestrae and are appressed, thus permitting movement of molecules through the cytoplasm or continuity of the luminal space for development of pH gradients (see Nierzwicki-Bauer et al. [1983] for a more thorough discussion of cyanobacterial internal organization). The complexes are depicted as randomly distributed in any particular membrane, a feature that obviates the need to develop a mechanism for complex-complex interaction in the plane of the membrane. Indeed, it is possible that functional and structural interactions between complexes occur in the thylakoid lumen. The accumulation of PSI and ATPase in the outermost thylakoid and the more even distribution of PSII and Cyt b_6/f are consistent with the lateral heterogeneity thought to exist in chloroplast thylakoids.

The results raise the question of whether or not there is a functional asymmetry for PSI and PSII. For example, is PSI more active in the outer membrane than in the inner membrane? Conversely, is PSII more active in the inner membrane? Does the structural asymmetry reflect steps in biosynthesis that result in the accumulation of PSI in the outermost membrane but a more even distribution of the PSII, which is turned over more rapidly? Although we cannot answer these questions yet, we have additional evidence for structural asymmetries in the thylakoids. Using the low ionic strength, nondenaturing gel system of Allen and Staehelin (1991), we have analyzed the Chl-protein complexes of *Synechococcus* sp. PCC7942. We can identify up to 22 green bands, including 13 PSI-containing bands, on these gels (T. Troyan and L. Sherman, unpublished observations). We believe that this complexity reflects the lateral heterogeneity described here. Similarly, we find very few green bands that contain both PSI and PSII, which is also consistent with this model of membrane structure.

We would like to emphasize that these results may be significant only for cyanobacteria, such as *Synechococcus* sp. PCC7942, with concentric thylakoids. We have performed similar experiments on strains, such as *Synechocystis* sp. PCC6803 and *Cyanothece* sp. ATCC51142, that have thylakoids that protrude radially into the cytoplasm from two or three poles along the cytoplasmic membrane. In these cells, the PSI and ATPase antibodies act quite similarly to the PSII and Cyt b_6/f antibodies and evenly decorate the thylakoids (data not shown). These cyanobacteria may have a different form of localized compartmentalization, because each of the thylakoids contains a more equal distribution of the photosynthetic complexes. Such results give us additional confidence that the antibodies are good and that the results are valid. Nonetheless, it is possible that the organization in *Synechococcus* sp. PCC7942 prevents the antibody from attaching to exposed protein domains within the phycobilisome layer. This possibility will be tested in the future on specific mutants that lack phycobilisomes.

PsbO (the luminal PSII protein involved with O_2 evolution) had a small percentage of the gold particles associated with the envelope. This finding correlates with our western blots and with the previous results of Smith et al. (1992). We also

found, in agreement with Smith and Howe (1993), that PSI membrane-associated proteins PsaD and PsaE are located on the cytoplasmic membrane to a small degree (data not shown). These results may indicate that the localization of thylakoid-luminal proteins is not absolutely precise and that some proteins are targeted to the cytoplasmic membrane or that the two membranes interact at specific points. We also found that a small percentage of the gold particles for all four PSII proteins were located on the outer compartment (Fig. 5, e–h). However, we are uncertain about the significance of these findings. Our results need to be compared with those of Smith and Howe, who presented western blot evidence for the presence of D1, but not CP43 or CP47, in the cytoplasmic membrane (Smith and Howe, 1993). We cannot confidently make localization determinations based on a small percentage of the overall label. Such experiments should first be performed on deletion mutants in which the authentic antigen is missing.

The localization of PSI and ATP synthase has ramifications in many important areas, of which we would like to highlight three: (a) the relationship of membrane organization in cyanobacteria and chloroplasts, (b) the photosynthetic mechanism and the functional relationships of PSI and PSII, and (c) stoichiometry of PSI to PSII. Our results are particularly valuable for the comparison of membrane morphology between cyanobacteria and chloroplasts. It is now evident that the lateral heterogeneity found in chloroplasts has a parallel in a prokaryotic ancestor. It was established in the 1980s that PSII are located in the grana and that PSI and ATP synthase are located in the stroma and in the nonappressed and the end regions of the grana. More recently, models have been developed to show the distribution of PSIα and PSIIα (which have relatively larger antennae) versus PSIβ and PSIIβ (Andreasson and Albertsson, 1993). This scheme indicates that the grana are composed of all of the PSIIα and PSIα (with a larger proportion of PSIIα), whereas the stroma have PSIIβ and PSIβ (with a larger proportion PSIβ) (Svensson et al., 1991). We can now hypothesize that this organization first developed in cyanobacteria—the radial heterogeneity of PSI and ATP synthase in cyanobacteria eventually became the lateral heterogeneity of chloroplasts. Since it is very likely that the thylakoids in cyanobacteria are interconnected, what we have described is actually a more extreme form of lateral heterogeneity.

Why would the photosystems occupy virtually separate and discrete domains and what implication does this have for the photosynthetic mechanism? Such a concept would be hard to integrate with the earlier view of the photosynthetic apparatus, in which the photosystems were close together (≤60Å), and demonstrated a certain degree of pairing that led to electron transport between individual pairs of photosystems. Such ideas were tested by Emerson enhancement studies and interpreted to conclude that there existed a significant population of specifically linked PSII and PSI (Malkin et al., 1986). However, more recent studies from Malkin's laboratory (Malkin and Braun, 1993) have generated the opposite conclusion. The newer results support the view of functionally separated photosystems and are consistent with the structural studies that separate PSI from PSII.

A physical reason for this separation was proposed recently by Trissl and Wilhelm (1993) based on the kinetic differences between PSI and PSII. The authors postulated that PSII is a slow photosystem, whereas PSI is a fast photosystem. If the two systems are permitted to be in excitonic contact, the PSI could drain off the excitation energy from PSII because PSI has long-wavelength-absorbing pigments and faster trapping kinetics. They speculated that PSII would operate at much reduced capacity if it were mixed with PSI. One way to overcome this difficulty is to generate a high PSII:PSI ratio, and this is accomplished in certain eukaryotic algae (Trissl and Wilhelm, 1993). However, the cyanobacteria normally have a very low PSII:PSI ratio (0.3–0.5); therefore, physical separation may be required. Trissl and Wilhelm (1993) also pointed out the importance of the phycobilisome-PSII coupling to produce excitonically independent units, but physical separation may be equally likely. It should be noted that the proteins destined for the PSII and Cyt b_6/f complexes are synthesized in the cytoplasm; the inner membranes may represent the insertion points of newly made proteins intended for these complexes. Since PSI turns over more slowly, these complexes may accumulate to a greater extent in the outermost thylakoid.

The concept of photosystem spatial separation is consistent with our results and with other structural studies and provides a framework for the variation in stoichiometry of the photosystems (PSII:PSI ratio). Trissl and Wilhelm also hypothesized that systems with fully or partially mixed photosystems will have: (a) suboptimal regulation of energy distribution, (b) significantly higher ratios of PSII:PSI, and (c) significantly lower ratios of maximum fluorescence to initial fluorescence. This is precisely what we find in membranes from iron-deficient cells. In this case, there is only one membrane lamella within the cell (Sherman and Sherman, 1983) and the PSII:PSI ratio is about 1.0 and the maximum fluorescence to initial fluorescence is approximately 1.2 to 1.5. This may provide the reason for the induction of the *isiA* gene, which codes for CP43′. This is a Chl-protein similar to CP43 that acts as a weak antenna for both PSI and PSII (Burnap et al., 1993). The inefficient antenna may prevent PSI from draining excitonic energy from PSII and allow for a higher PSII:PSI ratio and closer spatial association without energetic detriment.

Such alterations in membrane surface area and photosystem stoichiometry might also occur during modification of other environmental factors such as light quality and light intensity. Gantt and co-workers (Mustardy et al., 1992) developed a procedure to determine the quantitative in situ labeling of the photosystems, which they applied to membranes of the phycobilisome-containing red alga *Porphorydium cruentum*. This procedure could help determine the density of the photosystems in the different membranes after they have been separated biochemically. Future lines of research will be to separate and identify various membrane domains of cyanobacterial thylakoids to determine the PSI and PSII content and to investigate the physical relationship of the thylakoids to the cytoplasmic membrane.

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