Localization of Phycoerythrin at the Lumenal Surface of the Thylakoid Membrane in *Rhodomonas lens*

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Abstract. The thylakoids of cryptomonads are unique in that their lumens are filled with an electron-dense substance postulated to be phycobiliprotein. In this study, we used an antiserum against phycoerythrin (PE) 545 of *Rhodomonas lens* (gift of R. MacColl, New York State Department of Health, Albany, NY) and protein A–gold immunoelectron microscopy to localize this light-harvesting protein in cryptomonad cells. In sections of whole cells of *R. lens* labeled with anti–PE 545, the gold particles were not uniformly distributed over the dense thylakoid lumens as expected, but instead were preferentially localized either over or adjacent to the thylakoid membranes. A similar pattern of labeling was observed in cell sections labeled with two different antisera against PE 566 from *Cryptomonas ovata*. To determine whether PE is localized on the outer or inner side of the membrane, chloroplast fragments were isolated from cells fixed in dilute glutaraldehyde and labeled in vitro with anti–PE 545 followed by protein A–small gold. These thylakoid preparations were then fixed in glutaraldehyde followed by osmium tetroxide, embedded in Spurr, and sections were labeled with anti–PE 545 followed by protein A–large gold. Small gold particles were found only at the broken edges of the thylakoids, associated with the dense material on the lumenal surface of the membrane, whereas large gold particles were distributed along the entire length of the thylakoid membrane. We conclude that PE is located inside the thylakoids of *R. lens* in close association with the lumenal surface of the thylakoid membrane.

T HE cryptomonads are a small group of unicellular biflagellated algae that contain phycobiliproteins as their primary accessory pigments (Gantt, 1979). In addition to the cryptomonads, two other algal groups, the red algae and the blue-green algae (or cyanobacteria), also contain phycobiliproteins (Gantt, 1980; Glazer, 1980). In all three groups, the phycobiliproteins function as efficient harvesters of light energy for photosynthesis (Emerson and Lewis, 1942; Haxo and Blinks, 1949; Haxo and Fork, 1959). In addition, amino acid sequence analyses and immunological studies have shown that the phycobiliproteins of all three groups are evolutionarily closely related (Wehrmeyer, 1983; Guard-Friar et al., 1986).

However, the localization of the phycobiliproteins within the cryptomonad chloroplast is distinctly different from that in red algae and cyanobacteria and has not yet been fully elucidated. In both the red algae and cyanobacteria, the phycobiliproteins are organized in large macromolecular aggregates called phycobilisomes that are attached to the stromal surfaces of thylakoid membranes, or to the cytoplasmic surface of the cell membrane in a cyanobacterium lacking thylakoids. Phycobilisomes are not present on the stromal surfaces of the thylakoids in cryptomonad chloroplasts. Furthermore, cryptomonads are unique among algae and higher plants in that their thylakoid lumens contain dense material. These two distinct characteristics prompted Dodge (1969) and Wehr-

meyer (1970) to propose that the phycobiliproteins of cryptomonads are located in the intrathylakoid space. Support for this hypothesis was provided by Gantt et al. (1971) who found that the appearance of phycobiliproteins in the filtrates of protease-digested cells correlated with the disappearance of the electron-dense material in the thylakoid lumen. Rhiel et al. (1985) similarly observed that the loss of the lumenal dense material paralleled the loss of phycobiliproteins in nitrogen-deficient, high light–grown cells of *Cryptomonas maculata*. In addition, several studies (Faust and Gantt, 1973; Lichtlé, 1979; Thinh, 1983) have shown that growing cryptomonad cells under conditions of decreased light intensity leads to an increase in the cellular content of phycobiliproteins and to a concomitant increase in thylakoid lumen width.

Recently, Spear-Bernstein and Miller (1987) have shown by immunoelectron microscopy that phycoerythrin (PE)[1] 545 is localized inside the thylakoid lumen in the cryptophyte alga, *Rhodomonas lens*. They observed that the label was homogeneously distributed over the entire intrathylakoid space. We report here a different distribution of the labeled antigen. Using an antibody generated against PE 545 to label

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1. *Abbreviations used in this paper:* PE, phycoerythrin; PS II, photosystem II.

both sections of intact *R. lens* cells and isolated thylakoid fragments in vitro, we observed that PE antigens are closely associated with the lumenal surfaces of thylakoid membranes.

Materials and Methods

Cell Cultures

R. lens was obtained from the Culture Collection of Marine Phytoplankton, Bigelow Laboratory for Ocean Sciences (West Boothbay Harbor, ME), and grown in the liquid medium of Gantt et al. (1971). Cells used for ultrastructural immunocytochemistry of whole cells and isolated thylakoids, and for Western blot analyses were grown at $19 \pm 1°C$ under incandescent lamps ($8.6 \ \mu Es^{-1}m^{-2}$) on a 12-h light/12-h dark cycle and harvested at 1-2 h of the light cycle. Cells used in osmotic shock preparations were grown at room temperature in a northwest window. Log-phase cells were used in all experiments.

Antisera and Immunoblotting

Antiserum raised in rabbits against purified PE 545 from *R. lens* and two different rabbit antisera (designated A and B) against PE 566 from *Cryptomonas ovata* (Guard-Friar et al., 1986) were the generous gifts of Robert MacColl (New York State Department of Health, Albany, NY).

PE and other cellular proteins were extracted from *R. lens* cells by subjecting cells to three cycles of freezing and thawing in 0.1 M sodium phosphate buffer, pH 6.0. The extract was then clarified by centrifugation. B-PE from *Porphyridium cruentum* was purchased from Sigma Chemical Co. (St. Louis, MO). Polypeptides were separated essentially as described by Laemmli (1970) on 15% SDS-polyacrylamide gels containing 12.5% glycerol. The separated polypeptides were transferred electrophoretically to nitrocellulose filters (Towbin et al., 1979). After blocking with 3% BSA in TBST (50 mM Tris, pH 7.4, 150 mM NaCl, 0.1% Tween-20, and 1 mM sodium azide) for 1 h, blots were incubated overnight at room temperature in one of the anti-PE antisera diluted as follows with TBST plus 3% BSA: anti-PE 545, $1:10^6$; anti-PE 566A, $1:10^5$; anti-PE 566B, $1:5 \times 10^4$. The blots were washed several times with TBST minus NaN$_3$ and then incubated for 45 min in a 1:7,500 dilution of alkaline phosphatase-conjugated, goat anti-rabbit IgG (Promega Biotec, Madison, WI) in TBST plus 3% BSA. After several washes in TBST minus NaN$_3$, immunoreactive bands were visualized by incubating blots in the substrates nitro blue tetrazolium and 5-bromo-4-chloro-3 indolyl phosphate (Sigma Chemical Co.).

Protein A-Gold Preparation

Large colloidal gold particles (14-16 nm in diameter) were prepared according to the method of Frens (1973). Small diameter colloidal gold particles (3-12 nm in diameter) were prepared as described by Tschopp (1984). Both large and small diameter colloidal gold preparations were adjusted to pH 5.8-6.5 and then complexed to protein A as described by Roth et al. (1978). Protein A-gold complexes were collected at 25,000 rpm (large gold particles) or 30,000 rpm (small gold particles) in a rotor (type 40; Beckman Instruments [Canada] Inc., Mississauga, Ontario) at 4°C for 35 min. Sodium azide was added to the protein A-gold solutions at a final concentration of 0.02%.

To obtain a protein A-gold solution containing uniformly sized gold particles of 5-6 nm in diameter, the procedure of Slot and Geuze (1981) was followed. A protein A-small gold solution was layered on a 10-30% continuous sucrose gradient (11 ml vol, ~8 cm long) and centrifuged at 35,000 rpm in a Beckman Instruments (Canada) Inc. SW41Ti rotor for 1 h at 4°C. Fractions of 1 ml were collected from the red area of the gradient and dialyzed against PBS overnight at 4°C. Both sodium azide and polyethylene glycol (mol wt 20,000) were added to give final concentrations of 0.02% in each fraction. Diameters of gold particles from each fraction were measured on electron micrographs. Fractions containing 5-6-nm-diam gold particles were pooled and used for immunocytochemistry.

Postembedding Immunocytochemistry

For routine ultrastructural immunocytochemistry *R. lens* cells were fixed at 20°C for 2 h by adding a solution of 2% glutaraldehyde in 0.2 M sodium phosphate buffer, pH 7.0, plus 0.3 M sucrose to cell cultures in a 1:1 ratio. Cells were then collected by gentle centrifugation and rinsed in buffered

0.15 M sucrose and, subsequently, in buffer alone. After dehydration through a graded ethanol series and propylene oxide, some cells were embedded in Epon resin. Other cells were dehydrated in 25% ethanol at -5°C, then in 50, 75, and 95% ethanol at -18°C. Infiltration and embedding in Lowicryl K4M at -18°C were performed according to the following schedule: 95% ethanol/resin, 1:1, overnight; 95% ethanol/resin, 1:2, twice for 4 h each and then overnight; pure resin, 8 h and then overnight. Cells were transferred in fresh resin to Beem capsules, concentrated by centrifugation, and polymerized under ultraviolet light at -18°C for 24 h and then at 20°C for 48 h.

The width of thylakoids was increased by fixing cells in a solution of low osmolarity. This was accomplished by using a low glutaraldehyde concentration and by omitting sucrose from the fixative solution. Cells were fixed for 1 h at 4°C by adding a solution of 0.5% glutaraldehyde in 0.2 M sodium cacodylate buffer, pH 7.2, in a 1:1 ratio to cell cultures. Cells were collected, rinsed several times in buffer, and postfixed in buffered 1% osmium tetroxide for 1 h at 4°C. After several buffer rinses, cells were taken through a graded ethanol series and embedded in Spurr's epoxy resin.

Pale gold sections, cut with a diamond knife, were collected on Formvar-coated nickel grids. Grids were floated successively on drops of the following solutions: 10% (vol/vol) hydrogen peroxide, 20 min (osmicated cells only); PBS, 10 min; PBS containing 1% (wt/vol) ovalbumin or BSA (fraction V), 15 min; 1:100-1:2,500 dilution (Epon and Lowicryl sections) or 1:50-1:100 dilution (Spurr sections) of an anti-PE antiserum in PBS containing 1% ovalbumin or BSA, 30 min; and 1:10 (Epon and Spurr sections) or 1:20 (Lowicryl sections) dilution of protein A-small gold in PBS, 45 min. The following controls were also performed: (*a*) incubation in protein A-gold alone, omitting the antibody step; and (*b*) incubation with nonimmune rabbit IgG (Sigma Chemical Co.) in place of the antiserum. Epon and Lowicryl sections were stained with 2% uranyl acetate. Spurr sections were stained with uranyl acetate and Reynold's lead citrate or 2% potassium permanganate. Sections were viewed with an electron microscope (model 410; Philips Electronic Instruments, Inc., Mahwah, NJ) operated at 80 kV.

Thylakoid Preparation and Preembedding Immunocytochemistry

All procedures were carried out at 4°C unless noted otherwise. *R. lens* cells were fixed for 1 h by adding 0.5% glutaraldehyde in 0.2 M sodium cacodylate buffer, pH 7.2, in a 1:1 ratio to cell cultures. After two buffer rinses, free aldehyde groups were blocked by resuspending cells in buffered 0.1 M ammonium chloride for 45 min (Roth, 1983). Cells were rinsed again in buffer and then resuspended to a density of $1.5-2.0 \times 10^6$ cells/ml in sonicating buffer containing 10 mM Tricine, pH 7.5, and 1 M sucrose (Spear-Bernstein and Miller, 1985). Cells were sonicated until cell breakage, monitored by light microscopy, was 80-90%.

The sonicated cell fragments were slowly diluted with PBS and pelleted by centrifugation at 5,000 g for 10 min. The pellet was washed twice in PBS, once in PBS containing 0.2% (wt/vol) BSA (fraction V) (PBS/BSA), and resuspended in PBS/BSA. Anti-PE 545 antiserum was added to one aliquot of cell fragments at a final concentration of 1%. Antiserum was omitted from a second aliquot which served as a control. After an overnight incubation with continuous shaking, the cell fragments were pelleted as described above, washed twice in PBS/BSA at 20°C, and resuspended in PBS. Protein A-small gold (5-6-nm fraction) was added to both antibody-labeled and control cell fragments at a final concentration of 20%. Labeling was carried out at 20°C for 1.5 h. The suspensions were agitated every 10 min. Pellets were again collected, thoroughly washed in PBS, and fixed in a solution of 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, for 1 h at 20°C. After postfixation in buffered 1% osmium tetroxide for 1 h at 20°C, the cell fragments were dehydrated through a graded ethanol series and embedded in Spurr's epoxy resin.

Gold sections were cut with glass knives and processed for postembedding immunocytochemistry as described above, except that a 1:10 dilution of protein A-large gold in PBS was used to label these sections.

Quantitation of Gold Particles in Thylakoid Regions of R. lens Chloroplasts

To get an indication of PE antigen distribution in *R. lens* thylakoids, the average percent of gold particles labeling the following thylakoid regions was calculated from Epon and Lowicryl cell sections labeled with anti-PE 545 or anti-PE 566 followed by unfractionated protein A-small gold: outside the thylakoid lumen not touching the dense material of the lumen; outside the lumen touching the edge of the dense material; inside the lumen touching the edge of the dense material; inside the lumen not touching the edge of

the dense material. A gold particle was scored as touching the edge of the dense material of the lumen if the center of the gold particle was within the particle's radius of the edge.

Sections of osmotically shocked *R. lens* cells which had been postfixed in osmium tetroxide and labeled with anti–PE 545 followed by protein A–small gold (5–6 nm fraction) were used to further resolve the distribution of gold particles over the thylakoids. The average percent of gold particles labeling each of the following compartments was determined from 18 electron micrographs: in the interthylakoid space not touching the thylakoid membrane; outside the thylakoid touching the membrane; over the thylakoid membrane; in the thylakoid lumen touching the membrane; in the lumen not touching the membrane. Labeling over the stroma was negligible. A gold particle was considered to be touching the thylakoid membrane if the center of the particle was within ~2.8 nm (the radius of an average gold particle) of the membrane. A gold particle was considered to be over the membrane if the center of the particle lay over any part of the membrane. Data were generated from 978 gold particles. All micrographs were taken at a magnification of 16,500 and printed at a final magnification of 107,000.

Results

Anti–PE Antisera Recognize the β Subunit of PE 545 from R. lens

SDS-PAGE analysis of a protein extract obtained as described in Materials and Methods from low light–grown cells of *R. lens* reveals a prominent band at 18–19 kD (Fig. 1 *a*, lane *3*). On unstained gels and on nitrocellulose filters, this band is bright pink. *R. lens* PE 545 is an $\alpha\alpha'$ β_2 dimer with the β subunit having a molecular mass of 17.7–19.5 kD and the α subunits, 9.7–10.5 kD (MacColl et al., 1976). The prominent pink band is thus the β subunit of PE 545. Fig. 1 *a*, lane *3*, also shows that several bands are present in the 8–11 kD range where the α subunit of PE 545 would be located. Immunoblot analysis of these polypeptides using an antiserum against PE 545 of *R. lens* (Fig. 1 *b*, lane 2) and two different antisera directed against PE 566 from *C. ovata* (Fig. 1 *b*, lanes *4* and *6*) shows that all three antisera recognize the β subunit of PE 545, but do not show cross-reactivity with the α subunits. There is also a minor reaction with a higher molecular mass polypeptide band which may represent reactivity to a contaminant present in the protein preparations used to generate the antisera.

As a further test of the cross-reactivity of the three antisera, purified B-PE from the red alga *P. cruentum* was run as a control in each experiment. B-PE is an $\alpha_6\beta_6\gamma$ hexamer with the α and β subunits each having a molecular mass of 17.5 kD and the γ subunit being 30 kD (MacColl and Guard-Friar, 1987). SDS-PAGE reveals a broad band at 17–19 kD and a smaller band at 30 kD (Fig. 1 *a*, lane 2). On immunoblots, both bands are recognized by the anti–PE 545 antiserum (Fig. 1 *b*, lane *1*) and by each antiserum directed against PE 566 (Fig. 1 *b*, lanes *3* and *5*).

PE Antigens Are Present in the Thylakoid Regions of R. lens Chloroplasts

For immunolabeling, cells of *R. lens* were fixed in glutaraldehyde and embedded in either Epon or Lowicryl. After this procedure, the thylakoid lumens appear as dense bands that traverse the chloroplast, either as pairs (Fig. 2, *a* and *c*) or, when the pairs lie adjacent to one another, as deeper stacks (Fig. 2, *a*, *b*, and *d*). Since these cells were not postfixed with osmium tetroxide, the membranes limiting the dense thylakoid lumens appear as thin, electron-translucent lines (*arrows*, Fig. 2, *a* and *c*). The electron-translucent band
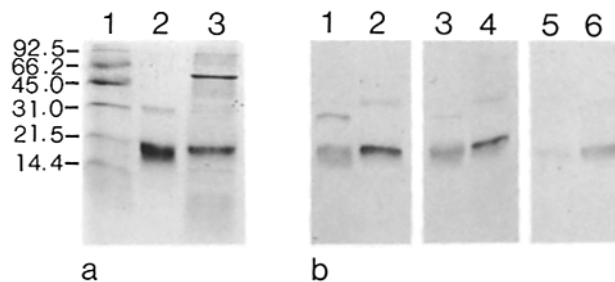

Figure 1. SDS-PAGE and immunoblotting of a protein extract from *R. lens* cells and purified B-PE from *P. cruentum*. (*a*) Coomassie blue-stained 15% SDS-polyacrylamide gel loaded with B-PE from *P. cruentum* (lane *2*) and a protein extract from *R. lens* obtained as described in Materials and Methods (lane *3*). Molecular mass standards (in kD) are shown in lane *1*. (*b*) Immunoblots showing reactivity of anti–PE 545 (lanes *1* and *2*), anti–PE 566A (lanes *3* and *4*), and anti–PE 566B (lanes *5* and *6*) with the α, β, and γ subunits of B-PE (lanes *1*, *3*, and *5*) and with the β subunit of PE 545 present in the *R. lens* protein extract (lanes *2*, *4*, and *6*). Variable amounts of the *R. lens* protein extract were loaded in lanes *2*, *4*, and *6* in order to generate immunoreactive signals of approximately equal intensities for the three antisera. The ratio of *R. lens* protein loaded in these lanes was ~1:2:7, respectively. Approximately 2 μg of B-PE were loaded in lanes *1*, *3*, and *5*. As a control, incubation in an anti–PE antiserum was omitted and blots were incubated in buffer alone. No staining was seen on these blots (data not shown).

seen between the lumens of a thylakoid pair includes not only the interthylakoid space but also the thylakoid membranes that border this space. Granular areas and regions of moderate electron density located between pairs of thylakoids correspond to stromal regions of the chloroplast.

Fig. 2 *a* shows a section through the chloroplast of *R. lens* that was incubated in anti–PE 545 followed by protein A–small gold. The thylakoids are densely labeled, whereas the chloroplast stroma is virtually free of gold particles. Only a few gold particles are seen over the cell's cytoplasm and peripheral ejectosomes or over the embedding resin. Upon closer inspection of the labeling pattern, it appears that the gold particles preferentially label the edges of the densely staining thylakoid lumens. In other words, much of the label seems to be closely associated with the thylakoid membranes.

Two different antisera raised against PE 566 from *C. ovata* were also used to label sections of *R. lens* (Fig. 2, *b* and *c*). The labeling patterns demonstrated by both of these antisera are the same as that obtained with anti–PE 545. Most of the gold particles label the thylakoids very near the electron-translucent regions that correspond to the thylakoid membranes.

Table I shows the relative distribution of gold particles over the thylakoids of *R. lens* cells fixed in the absence of osmium tetroxide, embedded in either Epon or Lowicryl, and labeled with one of the anti–PE antisera. The impression that PE antigens are closely associated with the edges of the dense lumenal material gained from the micrographs of anti–PE–labeled cell sections is corroborated by the results shown in Table I. Regardless of which anti–PE antiserum or which embedding resin was used, ~79–90% of the labeling is associated with the edges of the dense material of the thylakoid lumen. Low levels of labeling are seen in the lumen

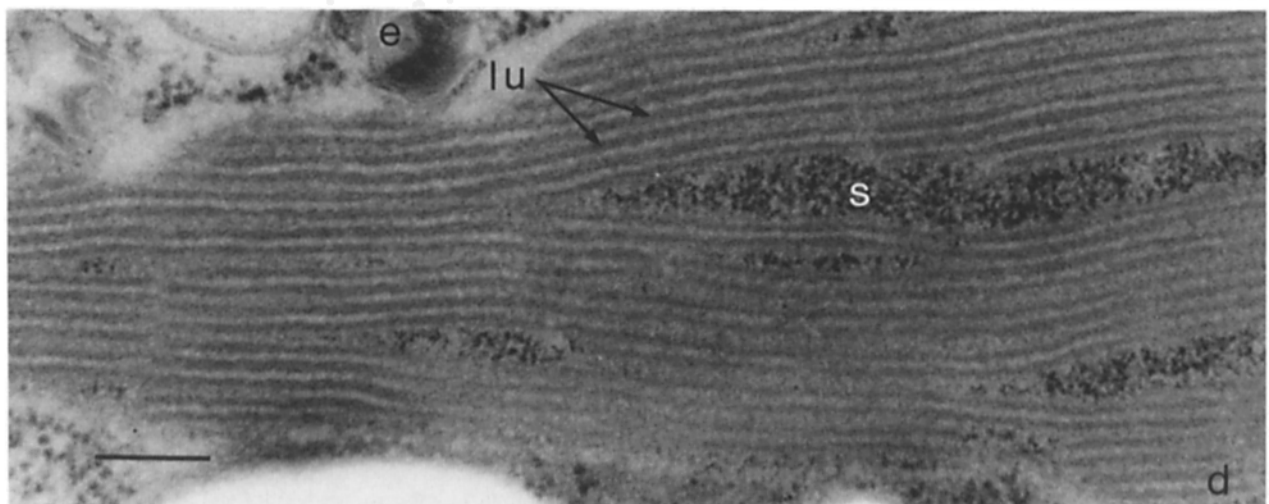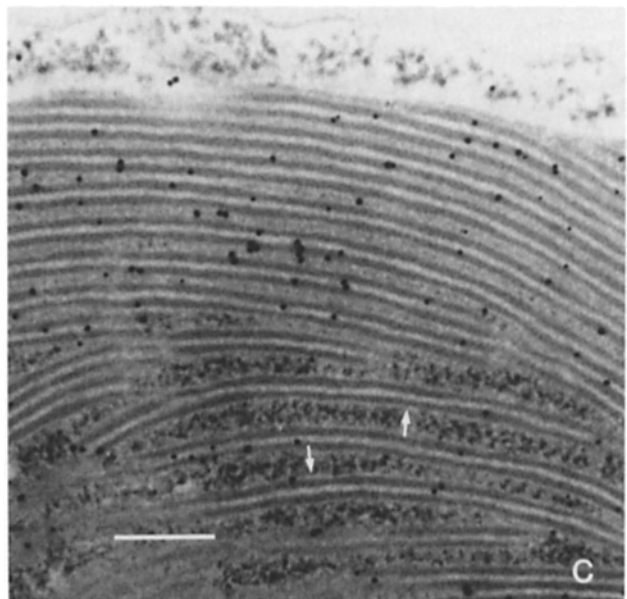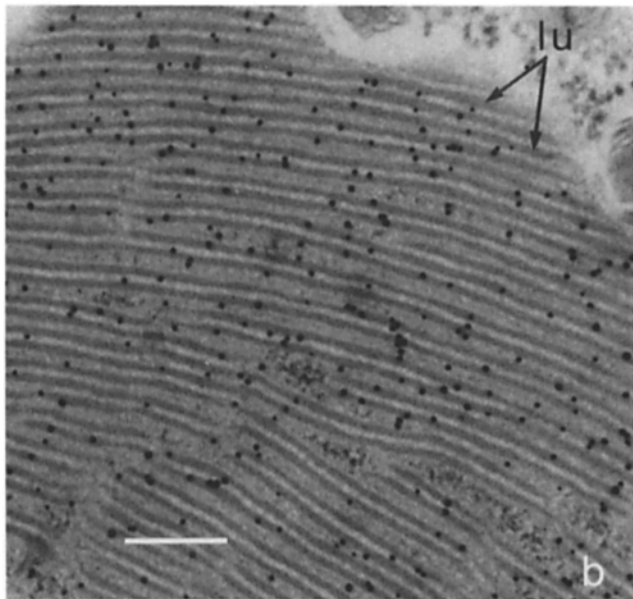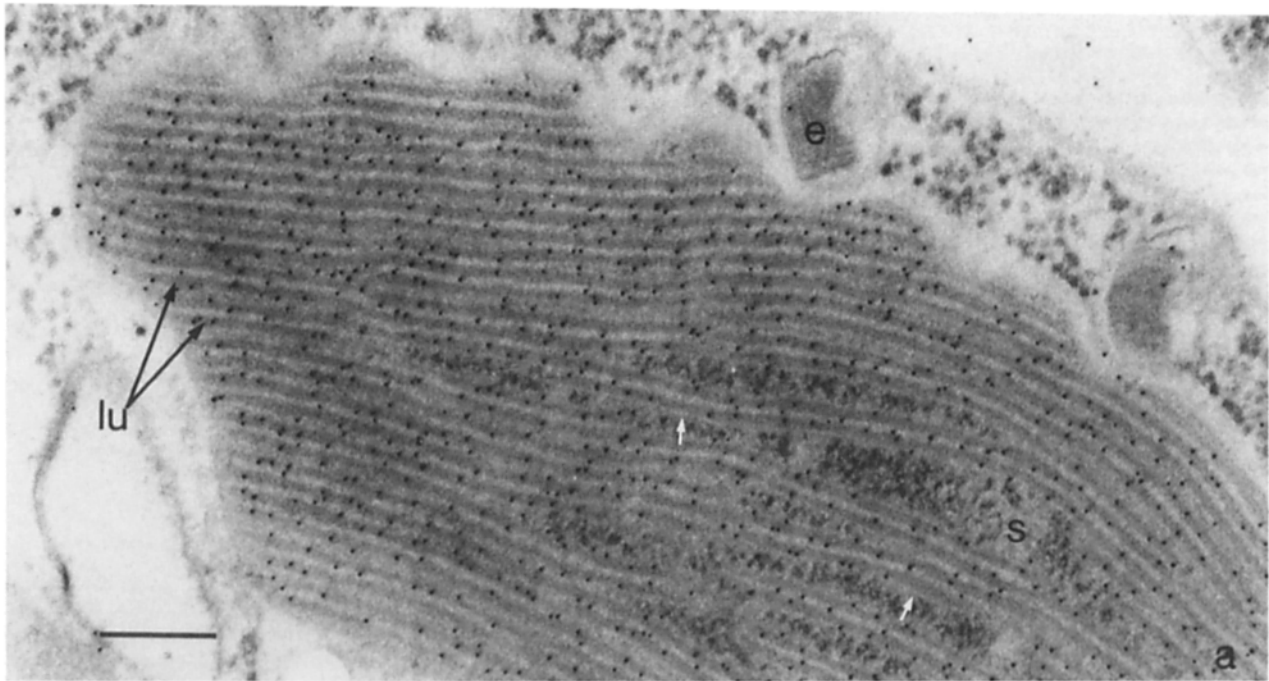**Table I. Percent Gold Particles Labeling Various Thylakoid Regions in Nonosmicated R. lens Cells**

| Embedding resin | Anti–PE* | Gold particles | | | |
| | | Outside lumen | | Inside lumen | |
		Not touching dense material	Touching edge of dense material	Touching edge of dense material	Not touching dense material
		%	%	%	%
Lowicryl K4M	545	9.7	30.4	48.4	11.5
	566A	8.0	30.5	49.7	11.8
	566B	6.9	36.4	53.3	3.4
Epon	545	6.8	39.7	48.0	5.4
	566A	7.7	41.1	47.2	4.2
	566B	13.2	40.7	45.3	0.8

* Anti–PE 545, antiserum directed against PE 545 from *R. lens*. Anti–PE 566A and B, two distinct antisera directed against PE 566 from *C. ovata*.

proper when anti–PE 566B was used to label cell sections, whereas the values obtained after labeling sections with anti–PE 545 or with anti–PE 566A are slightly higher. Few gold particles are seen over the stroma or interthylakoid space (outside the lumen not touching the dense material) after anti–PE labeling.

As a control, the antibody incubation step was omitted from the labeling procedure and sections were labeled with protein A–small gold alone. Under these conditions virtually no labeling is seen over the chloroplast of *R. lens* (Fig. 2 *d*).

Anti–PE 545 Antiserum Preferentially Labels R. lens Thylakoid Membranes

To determine the location of PE in *R. lens* thylakoids more precisely, cells were first fixed in a slightly hypotonic, buffered glutaraldehyde solution and then postfixed in osmium tetroxide. These conditions resulted in an increase in thylakoid lumen width and in densely stained thylakoid membranes, respectively (Fig. 3). These osmotically shocked cells contain thylakoids that are ~20–30% wider than the thylakoids of cells fixed under routine conditions. The section shown in Fig. 3 is labeled with anti–PE 545 and protein A–small gold (5–6-nm fraction). Unfortunately, the increase in membrane resolution gained by postfixation in osmium tetroxide results in a reduction in the number of antigenic sites accessible to the antiserum. However, the position of a gold particle is more easily determined in these slightly swollen and well-defined thylakoids. Most of the gold particles lie on or adjacent to the densely stained thylakoid membranes. A few particles, not associated with a membrane, are seen in the thylakoid lumens and in the interthylakoid spaces. Almost no labeling is seen over the chloroplast stroma.

When the PE 545-labeling pattern in osmotically shocked *R. lens* cells is quantitated, a nonrandom distribution of gold particles is seen (Fig. 4). Most of the gold particles (77%)

either label the thylakoid membranes or one of the membrane surfaces. Only ~20% of the gold particles are located over the thylakoid lumen not touching the membrane. Although the position of a gold particle could not be resolved as accurately in the thylakoids of cells fixed under routine conditions (1% glutaraldehyde and 0.15 M sucrose) and postfixed in osmium tetroxide, a similar labeling pattern of PE antigens was obtained. In these cell sections, 76.6% of the labeling is associated with the thylakoid membrane or one of its surfaces, while only 14.2% of the gold particles label the lumen proper (data not shown).

The results shown in Fig. 4 also indicate that almost 60% of the label is localized either directly over the thylakoid membrane or is associated with the lumenal surface of the membrane. Moreover, since the lateral resolution of an IgG molecule plus protein A–small gold is ~10.5 nm, a gold particle seen in the lumen proper may, in fact, also be labeling an antigen located at the lumenal surface of the membrane. The same argument could explain the labeling seen along the nonlumenal surfaces of thylakoid membranes. Thus, taken all together, these data not only indicate that anti–PE 545 antiserum preferentially labels *R. lens* thylakoid membranes, but they also suggest that PE 545 antigenic sites are located at the lumenal surface of the membranes.

As a control, nonimmune rabbit IgG was used in place of anti–PE 545 antiserum to label sections of osmicated, osmotically shocked *R. lens* cells. No specific labeling was observed in these sections. The same results were obtained when cell sections were incubated in protein A–small gold alone (data not shown).

PE 545 Antigens Are Associated with the Lumenal Surface of R. lens Thylakoid Membranes

To determine if PE 545 is indeed associated with the lumenal surface of *R. lens* thylakoid membranes, thylakoids were iso-

Figure 2. Localization of PE antigens in the chloroplast of nonosmicated *R. lens* cells embedded in Epon. (*a*) Anti–PE 545 labeling (5–6-nm gold particles). (*b*) Anti–PE 566A labeling (unfractionated small gold particles). (*c*) Anti–PE 566B labeling (unfractionated small gold particles). With each antiserum, most of the label is observed at the edges of the dense thylakoid lumens (*lu*) where the thylakoid membranes are located (*arrows*). The chloroplast stroma (*s*), the cell's cytoplasm, and the peripheral ejectosomes (*e*) are unlabeled. (*d*) Protein A–small gold alone. Virtually no labeling is seen over the chloroplast when incubation in antiserum is omitted. Bars, 0.2 μm.
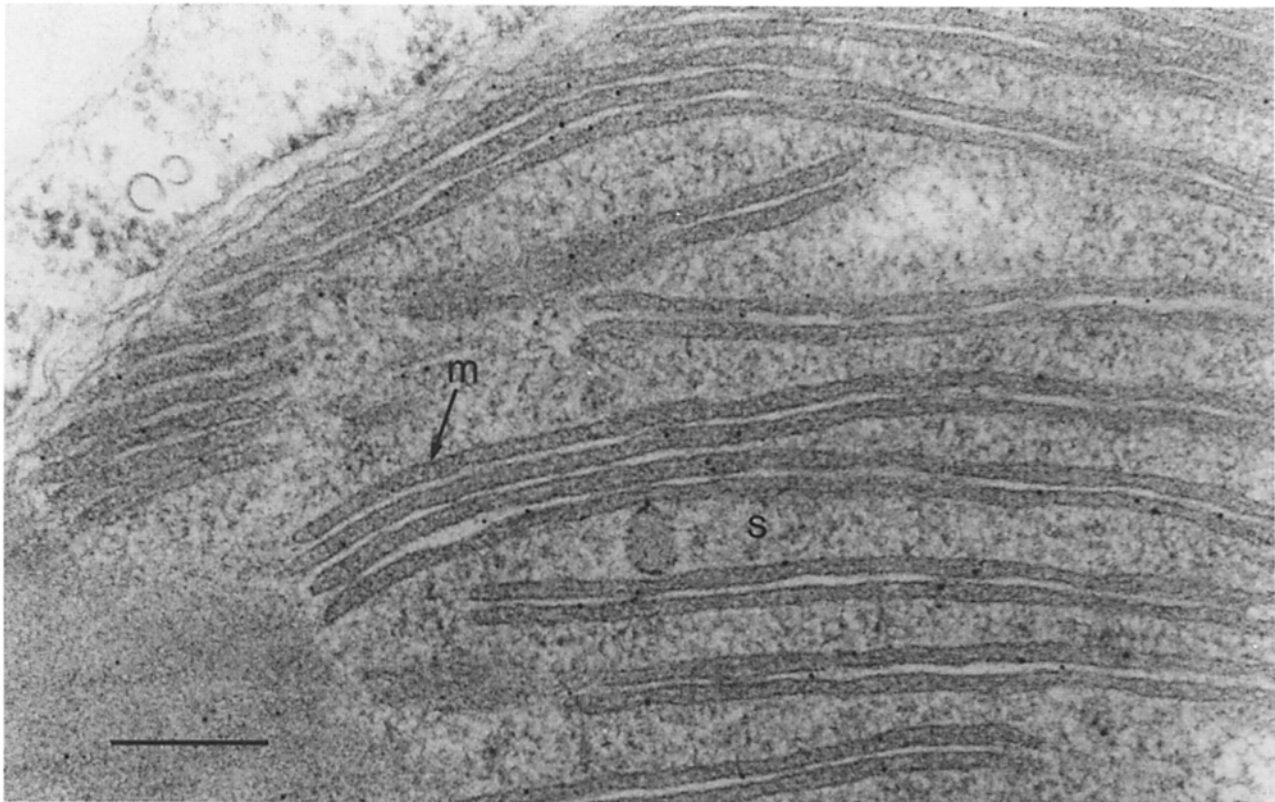
Figure 3. Localization of PE 545 antigens in osmotically swollen and osmicated *R. lens* cells. The majority of the small gold particles (5–6-nm fraction) lie on or adjacent to the densely stained thylakoid membranes (*m*). Much of the label is located at the lumenal surface of the membranes. Stromal regions (*s*) are unlabeled. Bar, 0.2 μm.
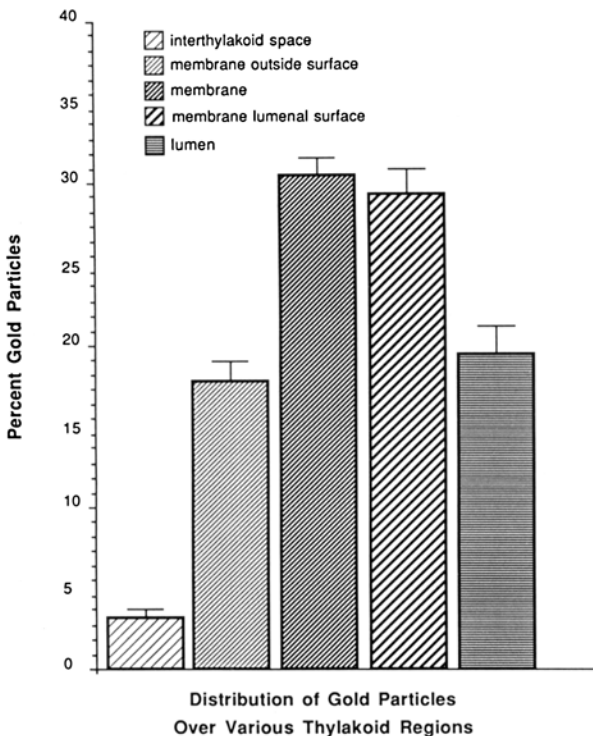

Figure 4. The distribution of gold particles over various thylakoid regions in osmotically swollen and osmicated *R. lens* cells. The location of a gold particle was determined as described in Materials

lated from cells fixed in dilute glutaraldehyde. The isolated chloroplast fragments were then labeled with anti–PE 545 followed by protein A–small gold (5–6-nm fraction) before embedding in Spurr. Sections of these in vitro–labeled, chloroplast fragments were either viewed directly or labeled with anti–PE 545 followed by protein A–large gold before viewing (Fig. 5).

In vitro labeling of *R. lens* chloroplast fragments with anti–PE 545 antiserum is seen only at the ends of broken thylakoids (small gold particles, Fig. 5, *a–c*). Small gold particles are not seen on the outside surfaces of the accessible thylakoid membranes (*arrowheads*, Fig. 5 *a*). Furthermore, it is evident that anti–PE 545 antiserum does not uniformly label the dense material at the broken edge of the thylakoids. Only broken thylakoid membranes are labeled with small gold particles (Fig. 5, *a–c*). Fig. 5 *c* shows clearly that anti–PE 545 does not label the bulk of the dense material spilling out of the broken thylakoids; the small gold particles are localized at the broken ends of the membranes.

An in vitro–labeled chloroplast fragment, in which one thylakoid membrane and some of the lumenal contents were removed during sonication, is shown in Fig. 5 *b*. Three small

and Methods. Note that the outside surface of a thylakoid membrane may border either the stroma or the interthylakoid space. Error bars, SEM.
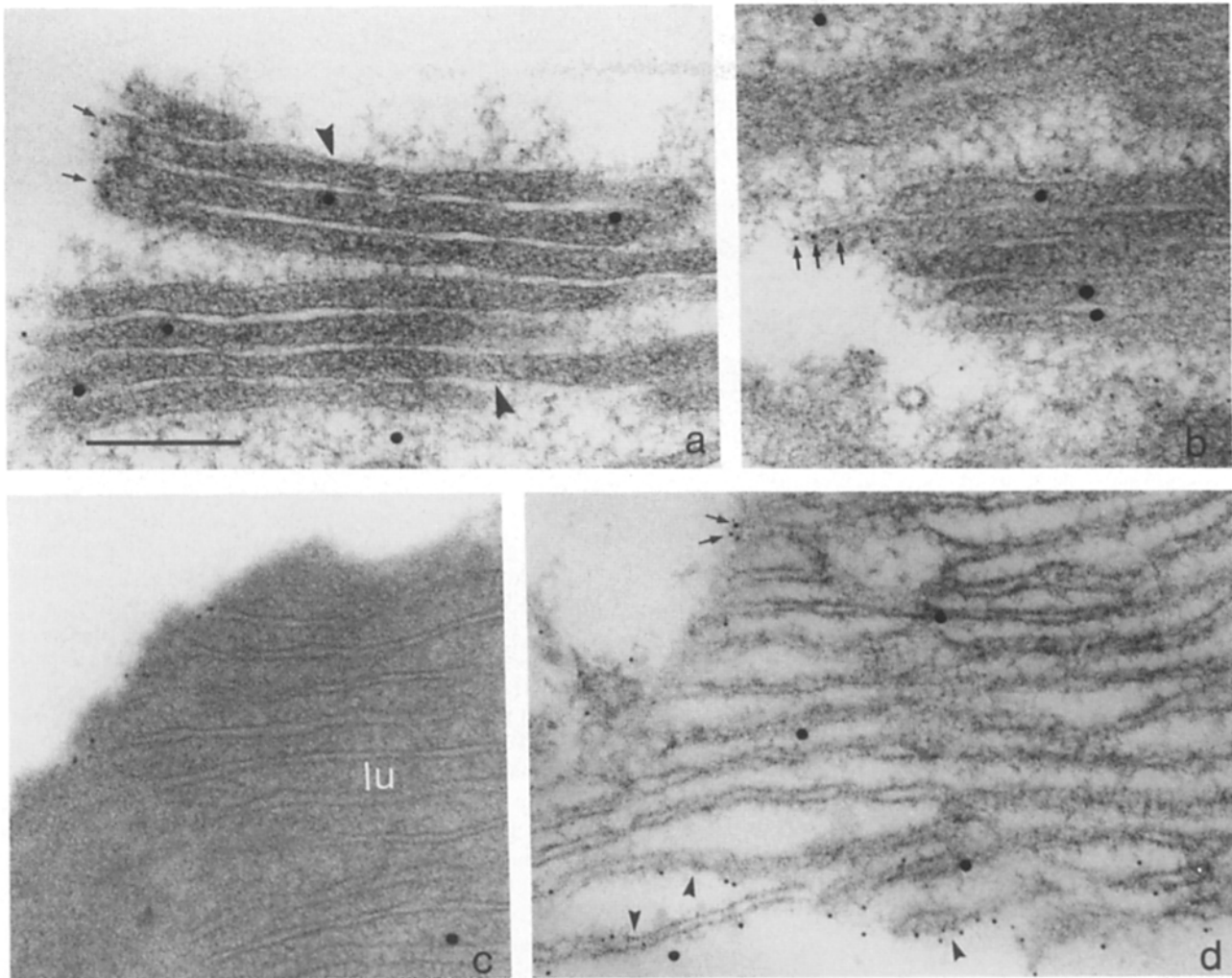
Figure 5. Localization of PE 545 antigens in isolated *R. lens* chloroplast fragments. Chloroplast fragments from glutaraldehyde-fixed *R. lens* cells were isolated and labeled in vitro with anti-PE 545 followed by protein A–small gold (5–6-nm fraction). After fixation and embedding, sections of these chloroplast fragments were labeled with anti-PE 545 followed by protein A–large gold. (*a*) Small gold particles label the ends of broken thylakoid membranes (*arrows*) but not the outside surfaces of the membranes (*arrowheads*). Several large gold particles are seen closely associated with the thylakoid membranes. (*b*) Three small gold particles (*arrows*) label the lumenal surface of the remaining membrane of a broken thylakoid. In vitro labeling is also seen at the broken ends of other thylakoid membranes. (*c*) The bulk of the dense material extruding from the lumens (*lu*) of broken thylakoids is not labeled with small gold particles; however, in vitro labeling is observed at the lumenal surface of broken thylakoid membranes. (*d*) Small gold particles label the ends of broken thylakoid membranes (*arrows*) and the lumenal surface (*arrowheads*) of the membranes when the dense material of the thylakoid lumen has been removed. Bar, 0.2 µm.

gold particles label the lumenal side of the remaining thylakoid membrane (*arrows*, Fig. 5 *b*). When the dense material of the thylakoid lumens was completely removed during sonication, in vitro labeling of PE 545 antigens is seen all along the lumenal surface of thylakoid membranes (Fig. 5 *d*). Note in Fig. 5 *d* that the paired membranes represent two thylakoid membranes and an interthylakoid space. The empty thylakoid lumens have become markedly swollen.

The postembedding, labeling pattern shown by the large gold particles indicates that PE 545 antigens are still accessible to the antiserum even after cell disruption and in vitro labeling. Label is seen along the length of the thylakoids and many of the large gold particles are located at or near the thylakoid membranes (Fig. 5, *a–d*).

No specific labeling of *R. lens* chloroplast fragments was seen when anti-PE 545 antiserum was omitted from the in vitro labeling procedure and chloroplast fragments were incubated in protein A–small gold alone (data not shown).

The results of these in vitro labeling experiments, in combination with the other work described above, clearly indicate that the PE 545 antigens recognized by the anti-PE 545 antiserum are closely associated with the lumenal surface of *R. lens* thylakoid membranes.

Rod-shaped Structures Are Present in R. lens Thylakoid Lumens

Rod-shaped structures are seen in favorable sections through the thylakoid lumens of both hypotonically fixed *R. lens* cells (Fig. 6, *a* and *b*) and sonicated chloroplast fragments (Fig. 6, *c* and *d*). These structures appear to be attached to the lu-
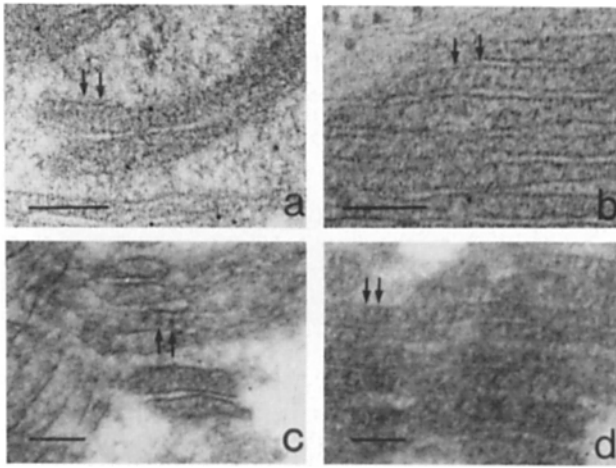
Figure 6. Rod-shaped structures in the thylakoid lumens of *R. lens*. Rod-shaped structures (*arrows*) extending partially or completely across the thylakoid lumens are observed in sections of both intact, osmotically swollen cells (*a* and *b*) and isolated chloroplast fragments (*c* and *d*). Bars, 0.1 μm.

menal side of one thylakoid membrane and may or may not extend completely across the lumen. The distance between adjacent rod-shaped structures appears greater in the fragmented chloroplasts than in intact cells (Fig. 6, compare *c* and *d* with *a* and *b*). This difference may be the result of partial extraction and/or the osmotic environment during the isolation of chloroplast fragments. It was not possible to determine if the anti-PE 545 antiserum labels the rod-shaped structures, since sections containing favorable profiles of the rods were quite rare.

Discussion

Localization of PE in the Thylakoid Lumen and Its Association with the Thylakoid Membrane

In this study we have demonstrated that PE is located in the thylakoid lumen in *R. lens*, that no PE is present on the nonlumenal surfaces of the thylakoid membranes, and finally that three different antisera raised against two spectrally distinct PEs preferentially label a population of PE antigens associated with the lumenal surface of the thylakoid membranes.

Dodge (1969) first suggested that the phycobiliproteins of cryptomonads were located in the thylakoid lumens and this hypothesis was subsequently supported by Gantt et al. (1971) who demonstrated that the dense lumenal material was protease sensitive. Recently, Spear-Bernstein and Miller (1987) have confirmed that PE is located in the intrathylakoid space of cryptomonads by immunogold labeling. However, in both our immunolabeling study and in the work of Spear-Bernstein and Miller (1987), a moderate level of labeling was observed on the nonlumenal, or outside, surfaces of the thylakoid membranes. Since the phycobiliproteins of cyanobacteria and red algae are always organized into phycobilisomes which are attached to the stromal surfaces of the thylakoid membranes, we wanted to be certain that there was not a population of PE attached to the outside surfaces of cryptomonad thylakoid membranes. To do this, we isolated chloroplast

fragments from *R. lens* cells which had been fixed in dilute glutaraldehyde and labeled these isolated thylakoids in vitro with anti-PE 545 followed by protein A-small gold. Subsequently, the thylakoids were postfixed and embedded in Spurr, and sections were viewed either directly or after labeling with the same antibody followed by protein A-large gold. No small gold particles were ever observed bound to isolated, intact thylakoids. Instead labeling was observed only when the thylakoid membranes had been broken so that the dense contents of the lumens spilled out, and even then the labeling was associated with the lumenal surface of the membranes. This in vitro labeling experiment shows conclusively that all the PE of *R. lens* is located inside the thylakoids. Finally, we observed that when cell sections of *R. lens* were labeled with anti-PE 545 or with one of two different antisera raised against PE 566 from *C. ovata*, gold particles were preferentially associated with the thylakoid membrane. We observed this nonhomogeneous distribution of label in cells fixed in glutaraldehyde alone or in glutaraldehyde followed by osmium tetroxide and in cells embedded in Spurr, Epon, and Lowicryl. Our most detailed analysis was made on cells fixed in a dilute glutaraldehyde solution so that the thylakoids were slightly swollen, and then postfixed in osmium tetroxide so that thylakoid membranes were visible. In these cells, 77% of the gold particles was either over the thylakoid membrane or touching it. In nonosmicated cells, ∼80–90% of the label touched the edges of the dense lumenal material where the membrane is located.

A close association between PE and the thylakoid membranes is anticipated since PE effectively transfers excitation energy to photosystem II (PS II) complexes located in the thylakoid membranes (Haxo and Fork, 1959; Lichtlé et al., 1980). Excitation energy is believed to be passed from phycobiliproteins to PS II by inductive resonance, and in order for this transfer to be efficient, donor and acceptor chromophores must be within 10 nm of each other. In addition, the results of a crystallization study of phycocyanin 645 from *Chroomonas* sp. have indicated that this phycobiliprotein is directly associated with thylakoid membranes in vivo (Morisset et al., 1984). Recently, Lichtlé et al. (1987) have isolated PE-PS II complexes from *Cryptomonas rufescens*. These complexes demonstrate high PS II activity and an absorbance maximum at 565 nm, characteristic of *C. rufescens* PE. Negative staining of the complexes revealed stacks of three or four discs attached to the outer surface of small vesicles. Lichtlé et al. (1987) proposed that the vesicles are inside out fragments of thylakoid membranes and that the discs are attached PE molecules. Our data confirms that a population of the PE present in *R. lens* is closely associated with the lumenal surface of the thylakoid membranes.

Spear-Bernstein and Miller (1987) have also studied the location of PE in *R. lens* using the same PE 545 antiserum obtained from Robert MacColl that we used. They, however, observed a uniform distribution of label over the entire intrathylakoid space. Only in cells treated with protease did they observe a preferential labeling of the thylakoid membranes. A possible explanation for the difference between our PE-labeling pattern and theirs is that the secondary antibody-gold probe they used had less lateral resolution (reportedly 30 nm) than the protein A-small gold probe (10.5 nm) we used. Moreover, Spear-Bernstein and Miller (1987) affinity purified and concentrated the PE 545 antiserum,

while we did not. Thus, it is possible that the antibody fraction they used may have been deficient in antibodies recognizing membrane-bound PE.

Nevertheless, the results of Spear-Bernstein and Miller's (1987) study clearly indicate that the entire thylakoid lumen of *R. lens* contains PE. We also observed a low to moderate level of labeling over the central region of the lumen. In several sections through the thylakoids of *R. lens*, we have observed rod-shaped structures extending across the lumen. Mörschel and Wehrmeyer (1979) observed similar structures in the thylakoid lumen of *Hemiselmis rufescens* and suggested that they were composed of phycobiliproteins. In our micrographs, the rods are 15–24 nm in length, similar to the 18–24 nm long stacks of discs found in cryptomonad PE–PS II complexes by Lichtlé et al. (1987). In their model of excitation energy transfer in cryptomonads, Lichtlé et al. (1987) have proposed that light energy is first collected by the "multiple pigment forms" (Mörschel and Wehrmeyer, 1975; Hiller and Martin, 1987) of PE in the lumen. Excitation energy is then transferred to the membrane-associated forms of PE and thence to PS II located in the thylakoid membrane. The rod-shaped structures we observed in *R. lens* thylakoid lumens may be homologous to the stacks of PE discs observed by Lichtlé et al. (1987). Each disc may represent a different form of the pigment which harvests light energy and ultimately passes it, via the membrane-associated PE disc, to PS II reaction centers. The results of our work and those of Spear-Bernstein and Miller's (1987) study are consistent with this model since labeling of PE antigens was observed closely associated with the lumenal surface of the thylakoid membranes (PE discs attached to the membrane) as well as over the center of the thylakoid lumen (PE discs distal to the membrane).

Evolution of Cryptomonad Phycobiliprotein Organization

Although the phycobiliproteins of cryptomonads are evolutionarily closely related to those of cyanobacteria and red algae, they also exhibit several unique characteristics. A given cryptomonad species contains a single phycobiliprotein, either PE or phycocyanin, which may exist in multiple forms. In contrast, cyanobacteria and red algae always contain phycocyanin and allophycocyanin and, in addition, these organisms may also contain PE or, in some cyanobacterial species, phycoerythrocyanin. The phycobiliproteins of cyanobacteria and red algae are organized in large macromolecular aggregates called phycobilisomes which are located on the stromal surface of the thylakoid membranes. In these complexes, light energy is transferred vectorially from the peripherally located molecules of PE or phycoerythrocyanin (if present) to phycocyanin and then to allophycocyanin molecules located in the core of the phycobilisome, adjacent to the thylakoid membrane. Excitation energy is then transferred from the phycobilisome to PS II complexes in the thylakoid membranes. Cryptomonad phycobiliproteins also transfer light energy to PS II complexes in the thylakoid membranes; however, cryptophycean phycobiliproteins are located inside the thylakoids where they may be organized into stacks of several discs. Since, with respect to the phycobiliproteins of cyanobacteria and red algae, cryptomonad phycobiliproteins transfer excitation energy to PS II complexes from the opposite side of the thylakoid membranes,

Gantt et al. (1971) suggested that cryptomonad thylakoid membranes might be inside out. However, Spear-Bernstein and Miller (1985) have demonstrated by freeze-fracture and freeze-etch analyses that cryptomonad thylakoid membranes are oriented in the same manner as those of other algae and higher plants.

How then could these differences between the organization of the phycobiliproteins in cyanobacteria and red algae and that seen in the cryptomonads have evolved? We wish to suggest a possible scenario. It is generally accepted that the chloroplasts of red algae have evolved from endosymbiotic cyanobacteria (Gray and Doolittle, 1982). The striking similarities in the organization of the phycobiliproteins in the phycobilisomes of these two groups, as well as the high amino acid sequence homology and immunological relatedness between cyanobacterial and red algal phycobiliproteins of the same spectral class, lend strong support to this hypothesis. The chloroplasts of cryptomonads must also be evolutionarily related to the cyanobacteria and red algal chloroplasts, for the phycobiliproteins of cryptomonads show immunological cross-reactivity and significant amino acid sequence homology with cyanobacterial and red algal phycobiliproteins (Glazer and Apell, 1977; Wehrmeyer, 1983; Guard-Friar et al., 1986). However, cryptomonad cells, which possess flagella, trichocysts, a unique rootlet system, and a distinctive periplast, are very different from the flagella-less red algae. Rather the cryptomonads are a distinct group of zooflagellates which phagocytosed a eukaryotic red-type alga and with time reduced it to a chloroplast and its surrounding structures (Gillott and Gibbs, 1980). Besides the two additional membranes surrounding the cryptomonad chloroplast, other vestiges of this eukaryote–eukaryote symbiosis are the nucleomorph (the vestigial nucleus of the red alga) and the starch grains and putative eukaryotic ribosomes (the cytoplasmic remnants) observed in the periplastidal compartment (Gibbs, 1981). The nucleomorph has been found to contain DNA (Ludwig and Gibbs, 1985; Hansmann et al., 1986) and a small RNA-containing nucleolus-like body (Gillott and Gibbs, 1980).

In the cyanobacteria, the genes for the colorless linker polypeptides which function in the assembly and the stability of the phycobilisome are located downstream from the phycocyanin and allophycocyanin gene sets (Conley et al., 1986; Houmard et al., 1986; Belknap and Haselkorn, 1987; Lomax et al., 1987). In the red algae, the genes for the phycobiliprotein subunits and the high molecular weight anchor polypeptide are encoded on the chloroplast genome (Egelhoff and Grossman, 1983; Grossman et al., 1986), whereas the genes for the linker polypeptides are located on the nuclear genome (Grossman et al., 1986). We propose that the original red alga phagocytosed by the cryptomonads contained phycobilisomes whose linker polypeptides were encoded by nuclear DNA. During the progressive reduction of the endosymbiont, the genes for these linker polypeptides were lost from the nucleomorph DNA. Before this loss, one or more mutations had allowed one of the phycobiliproteins to be directed into the thylakoid lumen. With the loss of the linker polypeptides, phycobilisomes were lost and present day cryptomonads evolved from the cell which had the phycobiliprotein in the lumen of its thylakoids. Guard-Friar et al. (1986) have shown that four of the six types of phycobiliproteins—two phycoerythrins and two phycocyanins—

found in the different cryptomonad species today are all immunologically related (in the cyanobacteria and red algae, phycocyanins and phycoerythrins are antigenically unrelated). In light of these data, Guard-Friar et al. (1986) have suggested that the six types of cryptomonad phycobiliproteins evolved from a common ancestor. This ancestral phycobiliprotein could have been the phycobiliprotein which crossed the thylakoid membrane and entered the lumen.

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