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Colocalization of Polyphenol Oxidase and Photosystem II Proteins

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ABSTRACT

Polyphenol oxidase (PPO) appears to be ubiquitous in higher plants but, as yet, no function has been ascribed to it. Herein, we report on the localization of PPO based upon biochemical fractionation of chloroplast membranes in *Vicia faba* (broad bean) into various complexes and immunocytochemical electron microscopic investigations. Sucrose density gradient fractionations of thylakoid membranes after detergent solubilization reveals that PPO protein (by reactivity with anti-PPO antibody) and activity (based upon ability to oxidize DL-dihydroxyphenylalanine) are found only in fractions enriched in photosystem II (PSII). Furthermore, of the PSII particles isolated using three different protocols utilizing several plant species, all had PPO. Immunogold localization of PPO on thin sections reveals exclusive thylakoid labeling with a distribution pattern consistent with other PSII proteins (80% grana, 20% stroma). These data strongly indicate that PPO is at least peripherally associated with the PSII complex.

PPO[1] (EC 1.10.3.1) is a ubiquitous enzyme in higher plants (2, 20). It is a copper-containing enzyme, which catalyzes the oxidation of phenols to the respective quinones. PPO is found in the chloroplast in healthy plant cells, although it is synthesized in the cytoplasm under nuclear control (10). The function(s) of this enzyme is, however, entirely unclear; various roles have been postulated for it, such as: a mediator of the Mehler reaction, an enzyme playing a role in host defense mechanisms, or as a mediator of photosynthetic electron transport (16). Unfortunately, there is no conclusive evidence for any of these roles (20).

Much of the confusion concerning a role for PPO stems from uncertainty of the subchloroplastic localization of the enzyme (20). Cytochemical localizations performed on numerous species all reveal accumulation of the osmiophilic DOPA-quinone polymer in the thylakoid lumen (4, 19). Unfortunately, as is the case with many cytochemical reagents, the reaction product diffuses easily so that the product may move along the lumen of the thylakoid from the point of reaction; thus, the cytochemical reaction, although pinpointing the location to the thylakoids, may not accurately reflect the subchloroplastic localization of the enzyme. In this report, we have taken two approaches to the subthylakoidal distribution of PPO. In the first, immunocytochemistry of PPO at the EM level allows one not only to localize an antigen of interest, but also to determine its relative distribution by quantification of immunogold particle distributions. Second, we utilized thylakoid fractionation protocols with subsequent assay of PPO activity to determine into which photosystem particle PPO fractionates. These two protocols have given us a much clearer picture of where PPO is localized in the thylakoid membrane.

MATERIALS AND METHODS

Plant Material

Vicia faba (broad bean) was grown in the greenhouse in a soil/sand/peat mixture (1:1:1, v/v). Seeds of the same seed lot were grown in both Stoneville, MS, and New Orleans, LA, for microscopy (Stoneville) and biochemical fractionation (New Orleans).

Chloroplast Isolation

After washing in ice-cold water, leaves were minced into ice-cold grinding buffer (50 mM Hepes/KOH [pH 7.6], supplemented with 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 330 mM sorbitol, and 5 mM sodium ascorbate) and were ground using a Tekmar[2] homogenizer (Tekmar Co., Cincinnati, OH) at a setting of 6 for 1 min. The brei was filtered through eight layers of cheesecloth and one layer of Miracloth and chloroplasts were concentrated by centrifugation at 1000*g* for 3 min. Further purification of chloroplasts or subchloroplastic fractions followed protocols outlined by Steinback *et al.* (15), Dunahay *et al.* (5), or Berthold *et al.* (3). Chl concentrations were estimated by the method of Arnon (2).

Electrophoresis

Subchloroplast fragments were solubilized in 0.1 M Tris/HCl (pH 7.6), containing 2% (w/v) LDS, 10% (v/v) glycerol, and 10% (w/v) sucrose by stirring on ice for at least 30 min. Residual membrane fragments were removed by 13,000*g*

[1] Abbreviations: PPO, polyphenol oxidase; DOPA, DL-dihydroxyphenylalanine; LDS, lithium dodecyl sulfate; LHC, light harvesting Chl complex.

[2] Mention of a trademark or proprietary product does not constitute a guarantee or waranty of this product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products that may also be suitable.

centrifugation for 10 min at 4°C. Supernatant fractions were loaded onto 10% (w/v) acrylamide slab gels prepared according to Laemmli (9) with LDS substituted for SDS. Gels were either stained for protein using Coomassie brilliant blue or were stained for PPO activity (as DOPA oxidase) as described below. Alternatively, fractions recovered from sucrose density gradients were adjusted to 2% LDS and loaded directly onto gels.

In some cases proteins were electrophoretically transferred to nitrocellulose using a semidry transfer apparatus (Hoeffer, San Francisco, CA) according to manufacturer's recommendations. Transferred proteins were then immunodetected or the nitrocellulose replica was stained for enzyme activity as described below.

Activity Staining

PPO activity was determined (as DOPA oxidase) by incubating gels in 0.1 M cacodylate (pH 7.2) containing 0.025 M DOPA. Aeration was provided by bubbling compressed air through the solution during staining, as described by Lax *et al.* (10). Alternatively, a nitrocellulose replica of the electrophoretically transferred proteins was placed on a pad of filter paper saturated with the above DOPA solution. Care was taken to ensure that the pad of filter paper was saturated but that there was no excess solution. The nitrocellulose replica was removed from the pad immediately upon formation of the dark oxidation product to prevent band broadening through diffusion of the product.

Immunocytochemistry

Lowicryl Resin

Leaves of 30 d old *Vicia faba* plants were cut into small (1 mm^2) pieces in ice-cold 3% (v/v) glutaraldehyde in 0.05 M Pipes (pH 7.4) on dental wax and then transferred to vials of the same for an additional 2 h on ice. The sections were washed with cold distilled H_2O and dehydrated in ethanol in the cold. Specimens were transferred from ethanol into 50% Lowicryl at 4°C overnight and then transferred to 3 full day changes of 100% Lowicryl at 4°C. After 3 d of 100% Lowicryl, the samples were transferred to BEEM capsules. The capsules were filled to the brim with Lowicryl resin and then capped. A cone of aluminum foil with 45° sides was supported on ice and the capsules with their specimen ends down, were placed in the cone. The capsules on ice were placed 20 cm from a UV light source for 4 to 5 h with a rotation of the capsule around its long axis over the course of the 4 to 5 h. The plastic was further polymerized at room temperature for 18 to 24 h before thin sections were cut.

Sections of about 90 nm were cut with an almost dry knife edge of a Delaware Diamond Knife (Bear, DE) on a Reichert Ultracut ultramicrotome. Sections were picked up on uncoated 300 mesh gold or nickel grids and processed for immunocytochemistry using the protocol of Vaughn (17). Sections from the same block face were used for all analyses so as to eliminate block to block and tissue to tissue variations. Antibodies to chloroplast coupling factor 1, P700 Chl *a* protein, plastocyanin, rubisco, and PPO used in this study were those described previously (17, 18). Antiserum to Cyt *f* was prepared to purified spinach antigen in rabbits. Only a 34 to 36 kD protein was recognized in Western blots utilizing this sera (KC Vaughn, unpublished data). Controls for specificity included deletion of primary antiserum, replacement of primary antisera with preimmune sera, or use of a nonchloroplastic sera (monoclonal antitubulin) for the specific chloroplast antisera.

L. R. White Resin

Small leaf pieces were fixed in 3% (v/v) glutaraldehyde in 0.05 M Pipes (pH 7.4) for 2 h, washed twice in 0.01 M cacodylate (pH 7.2) (15 min each), and postfixed in 2% OsO_4 in cacodylate for 2 h, all at 4°C. Samples were dehydrated in ethanol and embedded in L. R. White resin (soft grade). Immunogold labeling followed the protocols of Vaughn (17) with anti-PPO at a dilution of 1:80.

Morphometric Analysis

For analysis of colloidal gold distribution, a minimum of 30 micrographs for each antibody localization were examined. Gold particles were counted as either in the unappressed region (the grana end membranes, the edge of the grana stacks, and the stroma lamellae) or appressed membrane regions (internal areas of the grana stacks), or in the stroma (unassociated with membranes). Data are expressed as percentage of labeling in each region. All micrographs for morphometric analysis were photographed at ×20,000 and are from two separate labeling experiments and two separate fixations.

Cytochemical Staining

The protocols for cytochemical staining of leaf sections for PPO are as described previously (18).

RESULTS AND DISCUSSION

When membrane complexes of thylakoid membranes isolated from *Vicia faba* were solubilized by the method of Steinback *et al.* (15) and subjected to sucrose density gradient centrifugation, and subsequent LDS-PAGE, the resultant separation was identical to that reported previously (15) with spinach. PSI was isolated in the densest region of the gradient with PSII isolated in the upper third of the gradient (Fig. 1). Inspection of Figure 1 shows the enrichment of the PSI-associated CP1 protein in the densest region of the sucrose gradient, whereas PSII LHC Chl proteins were enriched in the upper one-third of the density gradient. When a duplicate gel was activity stained for polyphenol oxidase activity, only those fractions which are enriched in PSII complexes showed PPO activity (arrowheads, Fig. 1B). Moreover, when a nitrocellulose replica was immunodetected with antisera to PPO protein, the 45 kD active species was detected only in those fractions enriched in PSII (Fig. 1C). An additional immunoreactive band of 67 kD was visualized and corresponds to the inactive PPO bands previously described by Flurkey (6). Similar findings were obtained with the other species (tobacco, pea, lettuce, and spinach) tested (data not shown). In contrast to the results of Lieberei and Biehl (11), who found that PPO

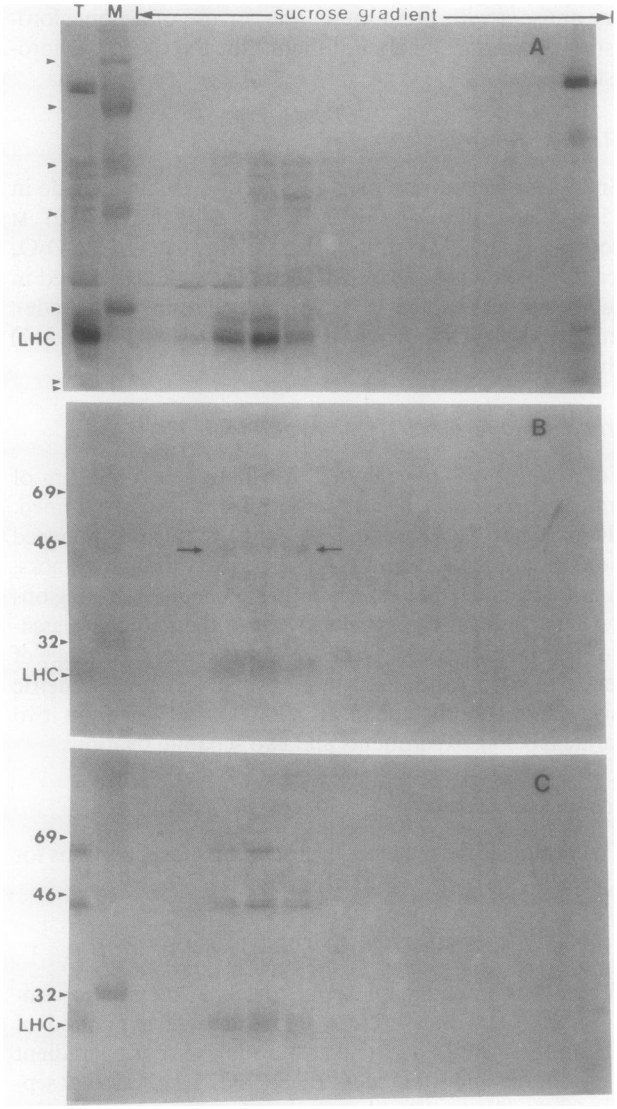

Figure 1. Electrophoretic separation of fractions of *V. faba* membrane complexes from Triton X-100 containing sucrose gradients. A, Coomassie blue-stained slab gel; B, activity stained nitrocellulose replica of an identical gel; C, immunodetection with antibody to PPO of a nitrocellulose replica of an identical gel. T, Total thylakoid preparation loaded onto gradient; M, mol wt standards. Gradient fractions are from the top (left) to the bottom (right). Note the PPO activity (arrows, B) and protein (confirmed immunologically, C) only in those fractions enriched in what was a green-colored band, LHC (PSII). Small arrowheads represent molecular mass standards as follows: myosin, 200 kD; phosphorylase *b*, 92.5 kD; BSA, 69 kD; ovalbumin, 46 kD, carbonic anhydrase, 32 kD; trypsin inhibitor, 20.5 kD; and lysozyme 14.3 kD.

was associated with PSI, we found that all of the PPO is associated with PSII. The separations afforded by this more gentle procedure allow a cleaner separation of the two photosystems. Because of the disparity of these results with those of former workers (11), we undertook to isolate PSII particles by a variety of other detergent solubilization methods using different detergents and protocols to eliminate the possibility that this was a mere fortuitous association of the PPO activity with the solubilized photosystems. Figure 2 shows the enrichment of PSII particles isolated from *Vicia* during the solubilization using the techniques of Berthold *et al.* (3), and the concomitant retention of PPO activity. These results were also found regardless of the plant species chosen (data not shown). Further confirmation of the biochemical fractionations was obtained using a third independent technique, that of Dunahay *et al.* (5), for the isolation of PSII particles (not shown). Membrane fragments enriched in PSII from several other species (tobacco, pea, lettuce, and spinach) also had PPO activity (data not shown).

We utilized immunocytochemistry of fixed tissue sections to eliminate the possibility of contamination of PSII membranes by PPO during the extractions using biochemical fractionation techniques. Immunolocalization of the enzyme also precluded the diffusion of reaction products that obscure

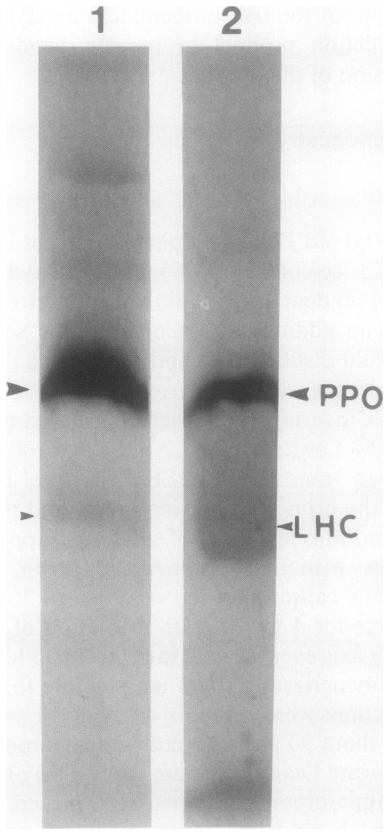

Figure 2. PPO activity stained LDS-PAGE gel of PSII membranes from *Vicia* prepared using high concentrations of Triton X-100 using the method of Berthold *et al.* (3). Lane 1, crude thylakoid preparation; lane 2, final PSII preparation. Note the enrichment of PSII associated LHC Chl complex and the retention of PPO activity in these particles.

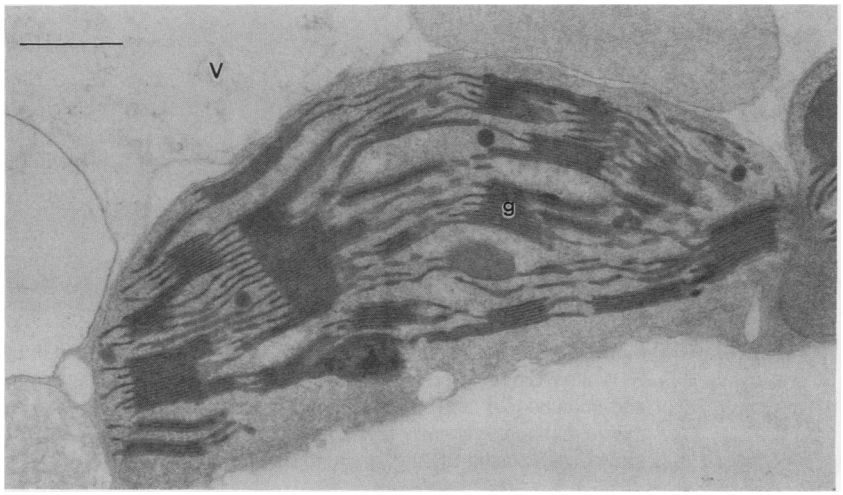

Figure 3. Cytochemical localization of polyphenol oxidase in *V. faba* mesophyll cells. Strong reaction is noted along the photosynthetic lamellae but is absent from the stroma and chloroplast envelope. Bar = 1.0 μm. V, Vacuole; g, granum.

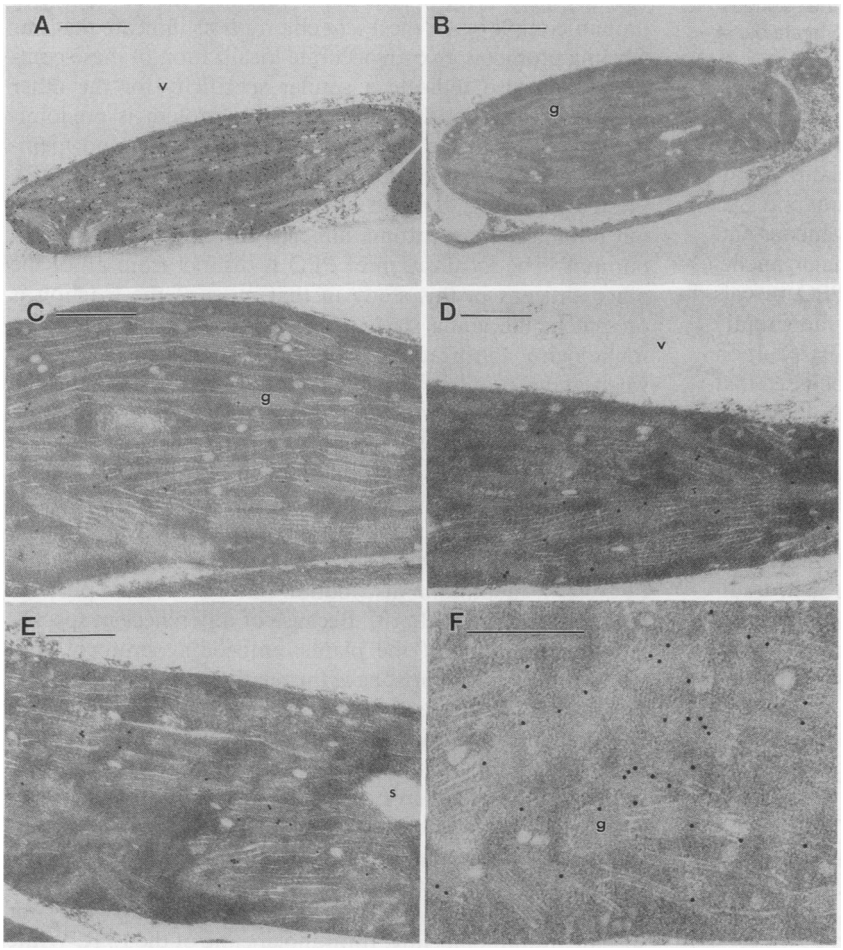

Figure 4. Immunocytochemical localizations of chloroplast proteins in *Vicia* leaves embedded in Lowicryl via the quick polymerization protocol of Vaughn (17). A, Rubisco (1:40); B, coupling factor 1 (both α and β subunits recognized) (1:40); C, P700 Chl *a* protein (1:80); D, plastocyanin (1:160); E, Cyt *f* (1:60); F, polyphenol oxidase (1:80). Dilution factors following the protein are dilutions of the primary antisera used for the localization. Bar = 1.0 μm in A and B, 0.5 μm in C to F.

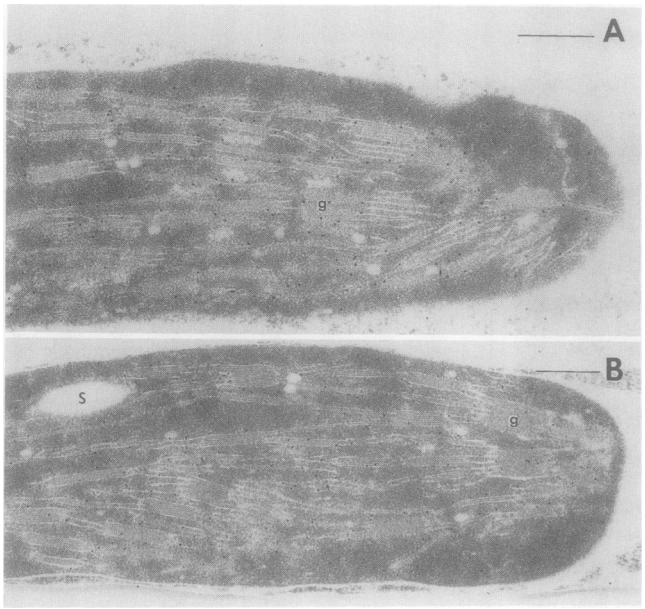

Figure 5. Micrographs from the separate immunogold labeling of PPO on *V. faba* leaf segments embedded in Lowicryl resin. In both experiments, areas of grana lamellae (g) are labeled, but the strongest labeling tends to be over those stacks cut obliquely. s, Starch; bar = 0.5 μm.

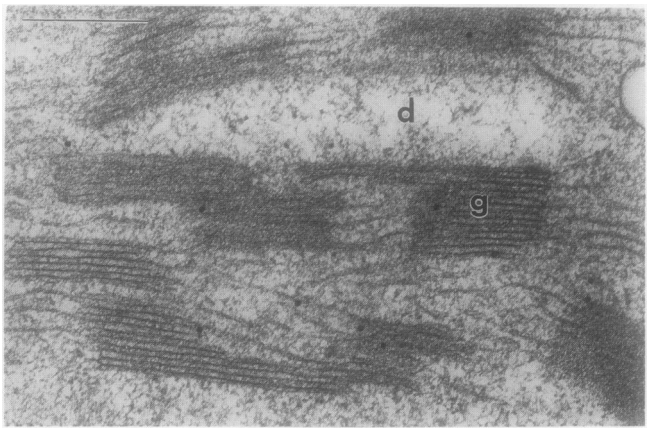

Figure 6. Area of *V. faba* chloroplast that has been embedded in L. R. White resin restored to antigenicity with a m-periodate treatment, and immunogold labeled. Labeling is present primarily in grana lamellae, although generally toward the ends of the stacks. d, Area of stroma with plastid DNA; g, granum. Bar = 0.5 μm.

localization when performing cytochemical detections of PPO. Chloroplasts of *V. faba* are typical higher plant chloroplasts, with clearly defined grana stacks and unappressed stroma thylakoids. The stroma is relatively homogeneous and starch grains are present mainly in the center of the organelle. Cytochemical staining of the leaf sections for PPO reveals strongly stained thylakoids with the reaction apparently strongest in the lumen of the membrane (Fig. 3). Based upon the density of the staining, we and others have concluded that PPO activity was present equally in all of the thylakoid membranes (8, 11, 20). This result is seemingly in conflict with our results from the biochemical fractionation which would have predicted a predominantly granal staining if PPO were associated with PSII.

Leaf tissue embedded in Lowicryl resin without osmication appears much different from tissue prepared through traditional electron microscopic protocols in that the membranes stand out in negative relief. The localization of rubisco in the stroma of the chloroplast (Fig. 4A) and chloroplast CF1 along the grana end membranes and stroma lamellae (Fig. 4B) is well accepted (1, 14). The same localizations of these two protein complexes in the *Vicia* chloroplasts indicate that our labeling protocols give an accurate localization of these complexes and also indicate a similar specificity for the other immunolocalizations. The P700 Chl *a* protein is predominantly located in the stroma lamellae and grana end membranes (Fig. 4C), but not exclusively, like CF1 (Fig. 4B). Plastocyanin (Fig. 4D) and Cyt *f* (Fig. 4E) are found throughout both grana and stroma lamellae in relatively equal proportions. The localization of PPO is distinct from all of the other antigens in this study in that most of the reaction is present in the grana stacks (Fig. 4F). Unlike the other immunological localizations, PPO labeling was most obvious when grana profiles were cut revealing cross-sections of the grana stacks (*i.e.* showing rounded profiles) rather than when the sections were cut perpendicular to the stack. In these profiles, immunogold is found preferentially toward the edge of the grana stack (Fig. 5). A summary of the distribution of labeling for each of these proteins is presented in Table I. From the morphometric measurements, it is obvious that PPO has a membrane distribution like PSII-related proteins reported previously (1, 13). Because of differences in species, growth conditions of the plants, embedding protocol, and antisera, direct comparisons of the relative distributions found in our study and those of others are not possible. However, the relative distribution of labeling is very similar between these studies and ours (1, 12, 13).

Table I. *Distribution of Colloidal Gold Particles in Thylakoids of Iowicryl Embedded Chloroplasts*

Antigen	Distribution	
	Appressed	Nonappressed
	%	
CF_1	0	100
P700	14	86
Plastocyanin	53	47
PPO	79	21
Cyt *f*	57	43

Tissues embedded in Lowicryl resin, while retaining high antigenicity, have relatively poor structural preservation. Tissues that have been osmicated and embedded in L. R. White resin have much greater structural detail but only rubisco, Cyt *f* (weakly), and PPO retained enough antigenicity for specific immunolabeling. Immunolabeling of the L. R. White embedded material also revealed labeling of PPO mainly in the grana stacks (Fig. 6). Quantitation of the gold labeling of the tissue embedded in the L. R. White resin gave an 84 to 16% distribution of grana to stroma lamellae labeling, similar

to that observed in the Lowicryl-embedded tissue (Table I). Thus, the distribution of PPO immunolabeling in Lowicryl is not an anomaly of the embedding in that resin.

The difference observed between the cytochemical and immunological staining indicates that one of the protocols may be in error. The cytochemical staining protocol results in the production of a dark DOPA-quinone product which is still slightly soluble and could move along the thylakoid lumen. This postulated movement is especially likely considering that the samples were incubated in cold DOPA substrate solution for 18 h and then transferred into fresh substrate at room temperature for a further 30 to 90 min. Even with only 16 to 24% of the PPO in the stroma lamellae, this prolonged incubation should result in strong reactions over the entire thylakoid, obscuring the grana/stroma differences.

The results of the immunocytochemical investigations serve to confirm the findings using the biochemical fractionations and indicate that the association of the PPO with the PSII membranes during fractionation is not fortuitous. These results could explain the recent findings that PSII membranes contain more copper than could be accounted for by the presence of plastocyanin in those preparations and makes, at least partially, suspect the identification of LHC as having the excess copper in this photosystem (7, 14). Because PPO is a known copper-containing enzyme (2, 20), its presence in PSII could easily account for the previously found extra copper in PSII particles described by Sibbald and Green (14). These data may also explain the lack of PPO in agranal bundle-sheath plastids, which lack the PSII core complex. While the function of PPO in the chloroplast remains unresolved, the finding of PPO in PSII particles increases the need to assess any role that PPO may play in the electron transport of that photosystem, whether structural or functional.

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