Lateral Distribution and Diffusion of Plastocyanin in Chloroplast Thylakoids

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Abstract. The lateral distribution of plastocyanin in the thylakoid lumen of spinach and pea chloroplasts was studied by combining immunocytochemical localization and kinetic measurements of P700[+] reduction at high time resolution. In dark-adapted chloroplasts, the concentration of plastocyanin in the photosystem I containing stroma membranes exceeds that in photosystem II containing grana membranes by a factor of about two. Under these conditions, the reduction of P700[+] with a halftime of 12 μs after a laser flash of saturating intensity indicates that to >95% of total photosystem I a plastocyanin molecule is bound. An analysis of the labeling densities, the length of the different lumenal regions, and the total amounts of plastocyanin and P700 shows that most of the remaining presumable mobile plastocyanin is found in the granal lumen. This distribution of plastocyanin is consistent with a more negative surface charge density in the stromal than in the granal lumen. During illumination the concentration of plastocyanin in grana increases at the expense of that in stroma lamellae, indicating a light-driven diffusion from stroma to grana regions. Our observations provide evidence that a high concentration of plastocyanin in grana in the light favors the lateral electron transport from cytochrome b_6/f complexes in appressed grana across the long distance to photosystem I in nonappressed stroma membranes.

THE membrane protein complexes involved in photosynthetic electron transport are heterogeneously distributed in higher plant chloroplasts with photosystem (PS)[1] II in appressed and PS I in nonappressed thylakoid membranes (5, 6) in contrast to the cytochrome (cyt) b_6/f complex, which is uniformly distributed throughout these thylakoid membrane regions (2, 4, 11). This organization of the complexes is probably a strategy to regulate the energy transfer to the photosystems, but requires a long-range electron transport from PS II to PS I that is not fully understood (for review, see reference 15). In addition to plastoquinol, the hydrophilic protein plastocyanin may be sufficiently mobile to shuttle fast enough across the long distances. Plastocyanin functions between cyt b_6/f and PS I in the lumen (17), a continuous space inside the thylakoid membrane system. The average distance between PS I and cyt b_6/f in nonappressed and cyt b_6/f in appressed membranes is ~20 and 200 nm, respectively. The longer distance from cyt b_6/f in appressed membranes may result in a considerably slower turnover of these complexes, as compared with that of cyt b_6/f in nonappressed membranes at a given shuttle speed of plastocyanin, and provides a problem in our understanding of photosynthetic electron transport. Therefore, a localization of plastocyanin in the lumen of appressed grana and its fast diffusion would be essential for an efficient function of these cyt b_6/f complexes in linear or cyclic electron transport. However, experimental results seem not to be consistent with this concept.

The kinetics of cyt f oxidation at partial inhibition of plastocyanin indicate a limited mobility of plastocyanin (16), which may be expected from the lumenal distance of 2–4 nm between thylakoid membranes in the light (29), which is not greater than the molecular dimensions of plastocyanin (10). Plastocyanin has also been shown to form a complex with PS I (18, 8). An inhomogeneous distribution may be expected from the lateral differences in the membrane composition and surface charges in stroma, grana, and exposed grana regions of the lumen. Digitonin fragmentation of thylakoids suggested a preferential location of plastocyanin in stroma lamellae (34).

Immunogold labeling has successfully been used to localize all integral complexes of thylakoids (2, 27, 30, 41). Here we have used this technique to investigate the distribution of the soluble plastocyanin in the thylakoid lumen. Plastocyanin is visualized directly in embedded, thin-sectioned chloroplasts of spinach and pea leaves in the dark and in the light by using monospecific polyclonal rabbit IgG directed against spinach plastocyanin followed by incubation with gold-conjugated protein A. The labeling pattern indicates that plastocyanin is preferentially located in the stromal lumen in the dark and moves laterally from stroma to grana regions in the light.

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1. *Abbreviations used in this paper:* cyt, cytochrome; PS, photosystem.

Materials and Methods

Chloroplasts and Antibodies

Spinach (*Spinacia oleracea*) was grown in hydrocultures and peas (*Pisum sativum*) were grown on vermiculite. Intact chloroplasts were isolated on a discontinuous Percoll (Pharmacia Fine Chemicals, Piscataway, NJ) gradient (22). Spinach plastocyanin was isolated from chloroplasts as described (36). Oxidized plastocyanin had an absorbance ratio $A_{278\ nm}/A_{597\ nm}$ of 1.2. Antibodies were raised in rabbits by an intradermal primary injection of 1 mg spinach plastocyanin in 1 ml PBS (137 mM NaCl, 1.5 mM KH_2PO_4, 7.9 mM Na_2HPO_4, 12.7 KCl, pH 7.3) homogenized with 1 ml CFA (Calbiochem-Behring Corp., La Jolla, CA). After 5 wk, 1 mg plastocyanin in 1 ml 150 mM sterile NaCl was intravenously injected. The whole serum was prepared by allowing the blood to clot at room temperature for 2.5 h followed by centrifugation of the supernatant for 20 min at 4,000 g. The IgG fraction of the preimmune serum and the antisera were isolated similar as described (28) on protein A-Sepharose CL4B (Sigma Chemical Co., St. Louis, MO). The IgG fractions identified by monitoring the absorbance at 280 nm during elution with 0.1 M glycine-HCl, pH 3.0, were passed through a gel filtration column (PD10; Pharmacia Fine Chemicals) equilibrated with 0.1 M potassium phosphate buffer, pH 7, and concentrated by centrifugation for 60 min at 7,000 g through an ultrafiltration membrane (Centricon C10; Amicon Corp., Danvers, MA) to the initial volume of the whole serum. The serum showed a good cross-reaction with pea plastocyanin, analyzed as described by Ouchterlony (31).

Gel electrophoresis was carried out in the presence of SDS on a polyacrylamide gradient from 10.2 to 15.4% (SDS-PAGE) using the Laemmli buffer system (23), except that 2 mM EDTA was present. The gels were stained with Coomassie Brilliant Blue R250 (Serva Fine Biochemicals Inc., Garden City Park, NY). Electroblotting (40) on nitrocellulose (BA85, Schleicher & Schuell, Keene, NH) of the gels was with an improved electrode arrangement (13). Free areas of the nitrocellulose were blocked by incubation with a solution of 2% (wt/vol) BSA in PBS-buffer, pH 7.4, for 30 min followed by staining of the antibodies with gold particles bound to protein A (Sigma Chemical Co.) as described (39). The monodisperse gold particles were prepared by reduction of tetrachlorogold with trisodium citrate after the given procedure for 15 nm gold (12).

Determination of Total Plastocyanin

The amount of plastocyanin in spinach chloroplasts and thylakoids was determined by quantitative rocket electroimmunodiffusion according to the method of Laurell (25) in agarose gels on microscope slides as described (17) except that the gels contained 0.83% (wt/vol) agarose, 0.2 M NaH_2PO_4, 0.2 M Na_2HPO_4, 3% (wt/vol) polyethylene glycol 20,000, 5 mM EDTA, and 2.8% antiserum. Chloroplasts were dissolved in 1.6% (wt/vol) Triton X-100 at a chlorophyll concentration of 0.4 mg/ml.

Immunocytochemistry

Dark adapted pieces of spinach or pea leaves (1 × 1 mm) were incubated for 30 min at room temperature with a mixture of 1% (wt/vol) glutaraldehyde and 4% formaldehyde either in the dark or during continuous illumination. The inactivation of antigenic sites of isolated plastocyanin by increasing concentrations of glutaraldehyde and formaldehyde was tested by dot blots on nitrocellulose (7). The rather high concentration of 1% glutaraldehyde showed no considerable decrease in antibody binding and ensured optimal cross-linking of proteins (24). After fixation, specimens were washed twice in 0.1 M cacodylate buffer, pH 7.4, and PBS, pH 7.4, dehydrated in an ascending series of dimethylformamide and embedded in Lowicryl K4M (42). Ultrathin sections were cut with a diamond knife and mounted on 200-mesh copper grids. Immunocytochemical labeling was performed by floating grids serially, section side down, on 10-μl droplets placed on wax sheets containing the following solutions:

(a) 0.2% glycine in PBS, pH 7.4, and/or 1% BSA, pH 7.4 to block nonspecific binding sites and to quench aldehydes present at the section surface. Treatment was carried out for 15 min followed by two 5-min washes in PBS.

(b) After this pretreatment, the grids were placed on drops with an appropriate dilution of the primary antibodies (0.1 μg/ml) in PBS, pH 7.4, containing 1% BSA followed by three 5-min washes in PBS.

(c) After removing unbound antibodies by proper washings, the grids were floated on drops containing gold-labeled protein A and then given three 5-min washes in PBS (pH 7.4) and two 5-min rinses in distilled water.

Monodisperse gold sols were prepared by reduction of $HAuCl_4$ with trisodium citrate as reducing agent (12). Protein A was linked to gold particles following the protocol of Roth (37). The gold sols could be stored at 4°C in PBS/1% BSA/0.02% azide for up to 4 wk with no loss of immunoreactivity. Before use, the conjugates were briefly centrifuged to remove aggregated gold particles and were routinely examined by negative staining.

(d) Labeled grids were dried, stained with aqueous uranyl acetate and lead citrate before examination with a Philips electron microscope 209 at 60 kV.

(e) The specificity of immunostaining was controlled by omitting the antibody or using preimmune IgG instead. In control experiments the number of gold particles was negligible and comparable to that observed outside the cross sections of chloroplasts with immunolabeled specimens (Figs. 2 and 3).

### Quantification of the Electron Microscopic Observations

Electron micrographs with cross-sections of chloroplasts with clearly resolved grana and stroma membranes were analyzed independently on two sets of glossy prints at a magnification of ∼100,000. The length of the lumen in grana stacks, exposed grana, and stroma lamellae (see Fig. 4) was measured. Each gold granule and its attribution to one of the regions, near (i.e., at a distance less than the diameter of a granule) or on top of stroma membranes (N_s), near but outside a grana stack (N_0), with its center on top of the first (N_1), the second (N_2), and the third or higher numbered grana thylakoid (N_3) was individually marked to minimize possible errors. The numbering was started at each of the two exposed grana thylakoids of a grana stack. In Table I, $N_0 + N_1$ and $N_2 + N_3$ are given as granules at exposed grana and grana, respectively. The distances for histograms (Figs. 5 and 7) were estimated at a magnification of 630,000 with a resolution of ∼2 nm. Assuming a Poisson distribution of labeled and unlabeled plastocyanin, the best estimate of the labeling density with the lowest variance is obtained by summarizing the counts in a given region of all chloroplasts analyzed and dividing this sum by the sum of the lumenal length of that region. The significance of differences was tested at an error level of 1%.

Flash Photometric Experiments

Intact chloroplasts were suspended at a concentration of 20 μg chlorophyll/ml in 0.2 M sorbitol, 5 mM $MgCl_2$, 10 mM NaCl and 15 mM Hepes buffer, pH 7.5. The dark-adapted sample was excited by pulses of saturating intensity from a frequency-doubled Nd-YAG laser of 534 nm light, 10 ns duration (full width at half maximum) and an energy of 10 mJ. The measuring light was provided by a flash lamp passed through an interference filter of 703 nm. During the plateau of nearly constant light intensity, the absorbance changes were monitored with a silicon photodiode for 50 μs. Changes in the intensity of the monitoring flash were subtracted. The signals were digitized with a transient recorder (model 6200; Biomation, Cupertino, CA) at a dwell time of 50 ns and averaged in a signal processor model TN1500; (Tracor, Inc., Instrument Group, Austin, TX).

Results

The specificity of antibodies raised against spinach plastocyanin was examined in immunoblotting experiments. Fig. 1 shows in lane *3* and *4* the polypeptide pattern after SDS-PAGE of spinach and pea thylakoids, respectively. In the immunoblots of these lanes (lanes *1* and *2*, respectively) the antibody to spinach plastocyanin reacted with a single band in both plants showing that it is monospecific and that it cross-reacts efficiently with pea plastocyanin. The position of the single bands at ∼14 kD is higher than the actual molecular mass of 10.5 kD of plastocyanin but agrees with the position of the bands of the isolated plastocyanins. The total amount of plastocyanin in spinach chloroplasts was determined by rocket immunoelectrophoresis using spinach plastocyanin for calibration at concentrations between 0.6 and 5.0 μM. We found 2.16 ± 0.22 μM (n = 6) in the samples containing 0.4 mg chlorophyll/ml which is equal to a molar ratio of chlorophyll to plastocyanin of 205 ± 20.
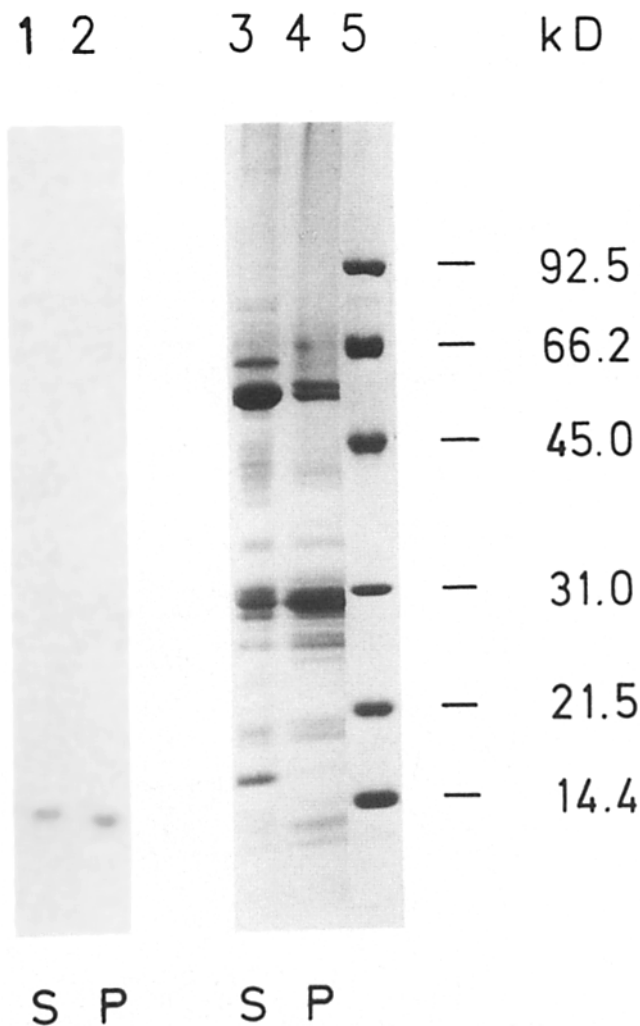
Figure 1. Characterization of antibodies raised against spinach plastocyanin. Lanes *3* and *4*, electrophoretogram of spinach (*S*) and pea (*P*) thylakoid membranes, respectively, stained with Coomassie Brilliant Blue (10–15% SDS-PAGE). Lane *5*, marker proteins. Lanes *1* and *2*, immunoblot analysis of the plastocyanin antibody as revealed by protein A-gold binding after transfer of the spinach (*S*) and pea (*P*) thylakoid proteins, respectively, to nitrocellulose.

We studied the distribution of plastocyanin in pieces of leaves from spinach and from pea both in the dark and in the light. Immunolabeling of thin sections of spinach and pea leaves is shown in Figs. 2 and 3, respectively. Gold granules are found almost exclusively at the thylakoid membranes. The electron micrograph of the dark adapted spinach chloroplast in Fig. 2 *a* shows considerably less granules in the grana stacks than the illuminated one in Fig. 2 *b*. A similar distribution is found in Fig. 3, *a* and *b* for pea. However, a conclusion about light-induced changes of the distribution of plastocyanin needs a detailed analysis and consideration of the following aspects: (*a*) three lumenal regions differ from each other in the adjacent membranes, grana, exposed grana, and stroma; (*b*) the length of the antibody-protein A-gold granule complex limits the resolution; and (*c*) evaluation of statistics of the distribution and significance of differences.

Fig. 4 illustrates the three lumenal regions included in our analysis, the grana region with appressed membranes on either side, the exposed grana region with an appressed membrane on one and the nonappressed end membrane on the other side, and the stroma region with nonappressed stroma lamellae on both sides. The tightly curved margins of the grana membranes do not contribute significantly to the length of the lumen and are not included but may contain some PS I (43).

The distance between the site of a plastocyanin molecule and the attached gold granule has to be known to judge the accuracy of an assignment of a granule either to the lumen of exposed grana or to a neighboring grana. The histogram of the distance between the middle of the thylakoid lumen and the center of a gold granule is shown in Fig. 5 for stroma thylakoids where the attribution of a granule is unambiguous. It indicates a maximal distance of 17 nm in illuminated and dark samples. We have also determined the distance between grana (including two membranes and the lumen) and found 13.9 ± 1.9 (25 grana stacks) and 15.5 ± 1.9 nm (34 grana stacks) in the light and the dark, respectively, in agreement with previous results (29). These values indicate that a gold granule bound to plastocyanin localized in a granum may be found not further than at the lumen of a neighboring granum. In a first approximation, the number of gold granules found at a grana thylakoid but bound to plastocyanin of a neighboring one may assumed to be equal for all grana thylakoids and cancel each other. Therefore, the granules were attributed in our analysis directly to the grana lamellae where they are observed in the micrographs except that granules in the stroma near end membranes were added to the number at exposed grana to give the total number of plastocyanin in the lumen of exposed grana.

The analysis of several sets of electron micrographs is shown in Table I. The data indicate in the dark an approximately twofold higher labeling density of plastocyanin in the stroma than that in grana. In both plants, illumination decreases the labeling density of plastocyanin in the stroma and increases that in grana. The overall labeling density is the same in the dark and light. This indicates that the binding between the antibody and plastocyanin is not different in grana and stroma and that the labeling density is proportional to the concentration of plastocyanin. In pea, the labeling density is slightly lower than in spinach as expected for IgG raised against spinach plastocyanin. An Ouchterlony diffusion in agarose (31) (not shown) indicated at least one antigenic site of spinach plastocyanin in addition to those of pea plastocyanin. Granules at the margins cannot be attributed to lumenal regions. They represent <10% of the total counts. The averaged ratio of the length of appressed to nonappressed membranes (see Fig. 4) is estimated from the lumenal lengths as 58:42 and 52:48 in spinach and pea, respectively.

Plastocyanin Bound to Photosystem I

It has previously been shown that plastocyanin bound to PS I reduces oxidized P700, the reaction center chlorophyll, with a halftime of 10–14 μs (18, 8). If the flash is short enough to avoid double excitation of PS I, then the amplitude of this kinetic component of P700$^+$ reduction gives the fraction of total P700 associated with plastocyanin. The kinetics
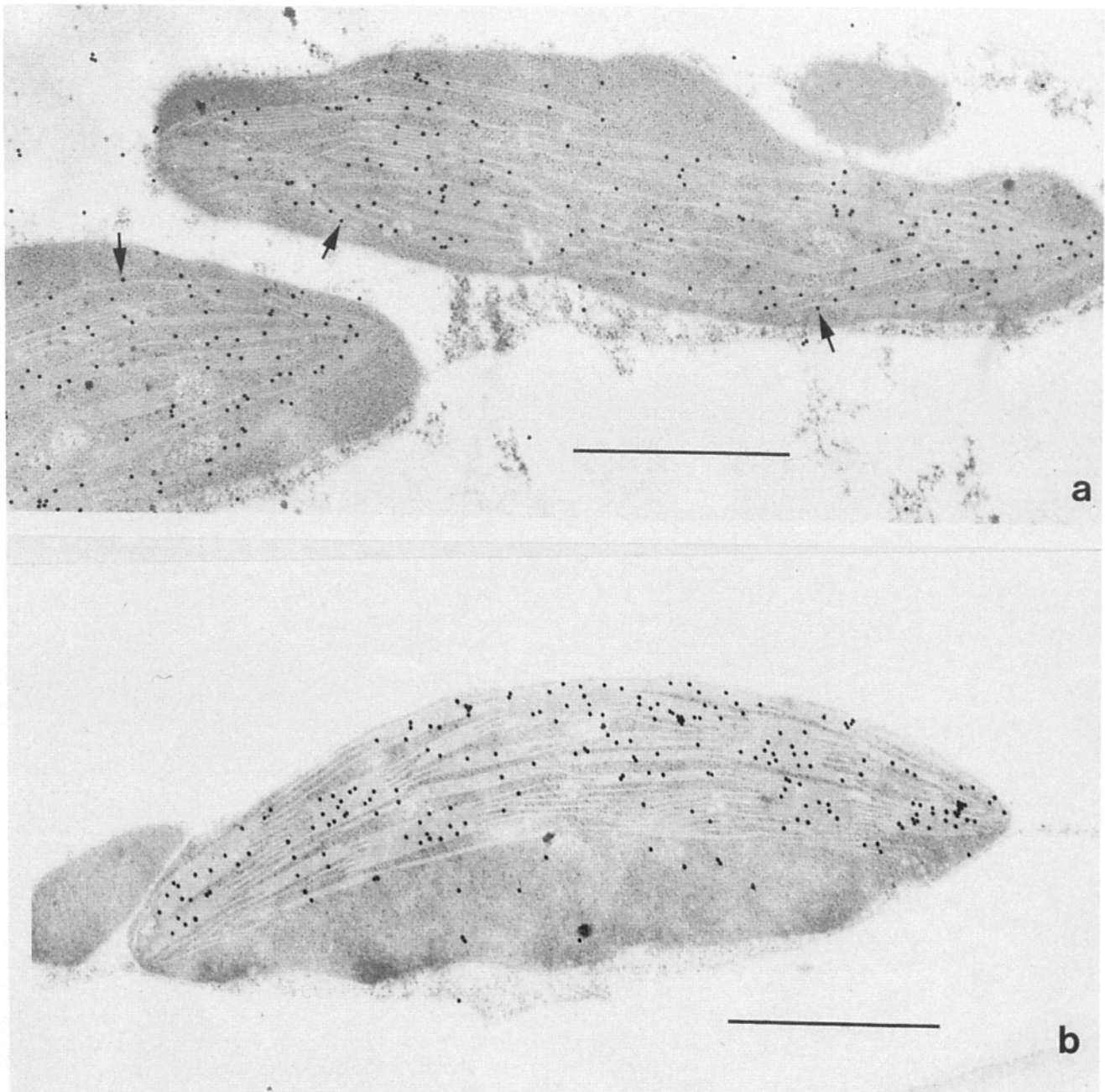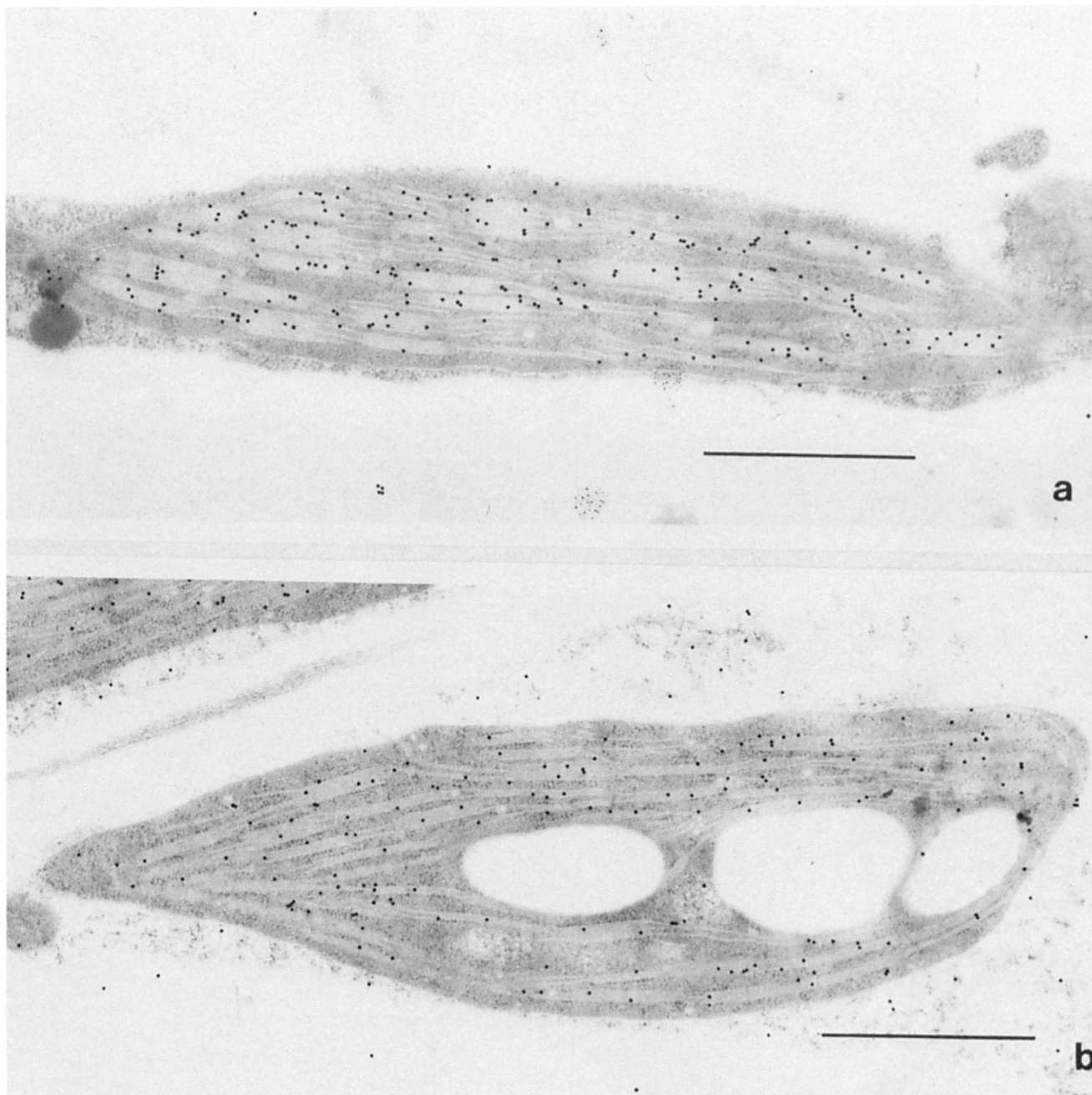
Figure 2. Immunogold localization of plastocyanin on Lowicryl sections of dark adapted spinach leaves. (*a*) Dark-adapted sample; (*b*) sample illuminated during fixation with formaldehyde/glutaraldehyde. *Arrowhead,* gold granules near stroma lamellae as used for the analysis in Fig. 5. Bar = 1 μm.

of the absorbance change of P700 in intact chloroplasts are shown in Fig. 6. The immediate decrease of the absorbance at 703 nm at the origin of the time axis is due to the oxidation of P700 during the laser flash. The subsequent increase of absorbance follows a first-order time course and is evidence for a reduction of >95% of total P700$^+$ by bound plastocyanin with a half-time of 11.3 μs. This indicates that in dark-adapted chloroplasts at least an amount of reduced plastocyanin equivalent to the amount of total P700 is associated with nonappressed membrane regions. A molar ratio of chlorophyll to total P700 of 670:1 can be estimated from the amplitude in Fig. 6 and a differential absorbance coefficient at 703 nm of 64 mM^{-1}cm^{-1}.

Plastocyanin in Exposed Grana

The labeling densities in Table I are not precise enough to indicate if the situation in exposed grana is different from that in grana. In an approach that is independent of membrane length we have analyzed the plastocyanin distribution perpendicular to the membranes in grana stacks assembled from six or more thylakoids. The histograms are shown in Fig. 7. Assuming that the maximal distance between the lumen and a gold granule is the same in exposed grana and stroma (Fig. 5) and taking into account the thickness of grana in the dark and in illuminated samples we estimate a ratio of plastocyanin in exposed grana ($N_0 + N_1$) to the average

1400

Figure 3. Immunogold localization of plastocyanin on Lowicryl sections of pea leaves. (*a*) Dark-adapted sample; (*b*) sample illuminated during fixation with formaldehyde/glutaraldehyde. Bar = 1 μm.

in grana thylakoids $(N_2 + N_3)/2$ of 0.98 and 0.84 in the dark and light, respectively. At variance with the definition given above N_3 represents the number of granules only at the third grana thylakoid. The values are in good agreement with the respective ratios of the labeling densities in Table I of 1.07 and 0.9. Statistically the difference is not significant.

Discussion

Heterogeneous Distribution of Plastocyanin

This study shows that plastocyanin is heterogeneously distributed in the dark in spinach and pea thylakoids with an almost twofold concentration in the stromal lumen as com-

pared with the granal one. The overall labeling density of this soluble protein is comparable to that reported for the integral cyt b_6/f complex (1) and PS I (41). The distribution of plastocyanin in the dark seems to be unfavorable for a turnover of cyt b_6/f complexes in appressed grana separated by long distances from PS I. The low concentration in grana could limit the turnover of cyt b_6/f in appressed as compared with the turnover in non-appressed membrane regions at low light intensities, and cyt b_6/f complexes in close proximity to PS II could have a function which is different from that of cyt b_6/f complexes near PS I as discussed (3). However, the fast reduction kinetics of P700$^+$ measured in dark-adapted chloroplasts permit a more detailed analysis.

LUMEN

EXPOSED
GRANA
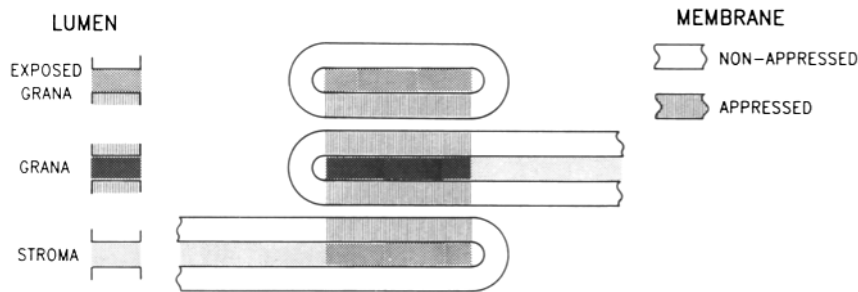
GRANA

STROMA

MEMBRANE

⊃ NON–APPRESSED

▶ APPRESSED

Figure 4. Scheme of thylakoids with three different regions of the lumen as included in the analysis for Table I.

Localization of Bound and Mobile Plastocyanin

The kinetics in Fig. 6 indicate that a reduced plastocyanin molecule is bound to almost every PS I. Therefore in the lumen of both stroma and exposed grana a fraction of plastocyanin proportional to the amount of PS I is immobilized by the complex, whereas the remainder should be more mobile. The relative amount of PS I (and of bound plastocyanin) is approximated by the fraction of total nonappressed membranes in these regions. The total length of nonappressed membranes is the sum of the lumenal length of exposed grana plus twice that of stroma (see Fig. 4). The values for dark adapted spinach chloroplasts in Table I give relative to total P700 0.74 and 0.26 for PS I in stroma and in end membranes, respectively. The amount of plastocyanin is proportional to the number of gold granules, N_g, N_e, and N_s in the lumen of grana, exposed grana, and stroma, respectively,

$$N_g \cdot f = PC_g' \tag{1}$$
$$N_e \cdot f = PC_e' + PC_e^b \tag{2}$$
$$N_s \cdot f = PC_s' + PC_s^b \tag{3}$$

where PC_s^b and PC_e^b are the fraction of total plastocyanin bound to PS I in stroma and end membranes, respectively, and PC_g', PC_e', and PC_s' are the fraction of total plastocyanin in grana, exposed grana, and stroma, respectively, that is not

bound to PS I. Dividing Eqs. 1 and 2 by Eq. 3 eliminates the constant, f. To estimate the individual fractions of plastocyanin, two more relations are needed. One is the amount of bound plastocyanin relative to P700, which is found (Fig. 6) to be near one ($PC_e^b + PC_s^b = 1$). The other is the amount of total plastocyanin in thylakoids relative to P700. A molar ratio of plastocyanin to P700 of 3.27 for spinach chloroplasts is given by the ratio of chlorophyll to plastocyanin and chlorophyll to P700 of 205 and 670, respectively. The value is in agreement with the previous determinations of two (14) and four (35). Table II summarizes the estimations based on our data.

At the ratio of plastocyanin to P700 of 3.27 the concentration of potentially mobile plastocyanin in grana exceeds that in stroma lamellae almost by a factor of two and the electron transfer from cyt b_6/f in grana to PS I does not seem as unfavorable as discussed above. At a ratio of two (14), the minimal value consistent with the data in Table I, almost all plastocyanin not bound to PS I would be located in the granal lumen.

This distribution of mobile plastocyanin is not consistent with the negative surface charge density of -0.037 $C \cdot m^{-2}$ (26) and -0.02 $C \cdot m^{-2}$ (21, 36) reported for PS II and the oxidizing site of PS I, respectively, which suggests an inverse distribution of the negatively charged plastocyanin. However, the surface charge density of PS I is likely to be a local

Table I. Distribution of Gold Granules Labeling Plastocyanin in the Thylakoid Lumen

Chloroplast		Region of lumen	Total counts	Total length of lumen	Labeling densities		Total counts[‡]/ total length of lumen
Sample	No.				Average*	Grana/ stroma	
				μm	counts/μm		counts/μm
Spinach		Grana	959	637	1.5 ($\pm$0.2)		
Dark	13	Exposed grana	444	274	1.6 ($\pm$0.4)	0.68	1.7
		Stroma	854	391	2.2 ($\pm$0.6)		
Spinach		Grana	1,153	572	2.0 ($\pm$0.5)		
Light	13	Exposed grana	379	206	1.8 ($\pm$0.3)	1.25	1.8
		Stroma	649	417	1.6 ($\pm$0.5)		
Pea		Grana	396	397	1.0 ($\pm$0.5)		
Dark	13	Exposed grana	337	255	1.3 ($\pm$0.6)	0.45	1.5
		Stroma	815	376	2.2 ($\pm$0.5)		
Pea		Grana	236	177	1.3 ($\pm$0.4)		
Light	8	Exposed grana	190	128	1.5 ($\pm$0.3)	0.81	1.5
		Stroma	326	207	1.6 ($\pm$0.3)		

* Total counts divided by total length of lumen. The mean $\pm$ SD in parentheses (see Materials and Methods) is given for the number (*No.*) of chloroplast cross-sections analyzed.
‡ Sum of total counts in the three regions of lumen divided by the sum of the total length of lumen in these regions.
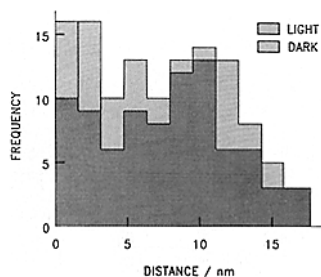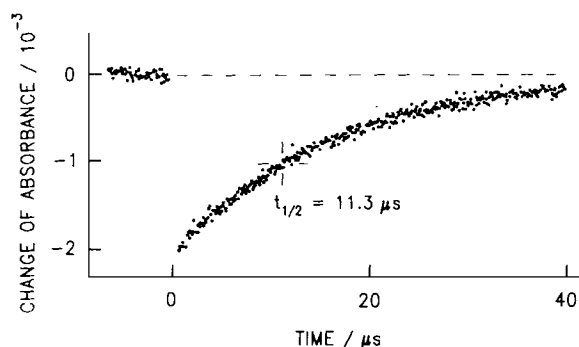
Figure 5. Distribution of the distance between the lumen of stroma lamellae and the center of gold granules in dark-adapted and illuminated spinach leaf sections. Only gold granules near clearly separated stroma lamellae were considered (see *arrowheads* in Fig. 2).

Figure 7. Distribution of gold granules perpendicular to the thylakoid plane in grana stacks assembled from six or more thylakoids in the dark (*A*) and during illumination (*B*). The origin of the abscissa is at the stromal surface of the exposed thylakoid. The horizontal bar at the top indicates the thickness of thylakoids as given in the text. N_1 identifies the exposed thylakoid, N_2 and N_3 the adjacent stacked thylakoids. For details see text.

one and may be too low as compared with stroma membranes, presumably due to a positively charged subunit of PS I. This subunit is required for an efficient electron transfer from plastocyanin to PS I (36). If the binding site at PS I is occupied by plastocyanin with its eight negative charges (10), then the remaining plastocyanin is likely to distribute in the lumen preferentially to regions with the lowest negative surface charge density. Our data suggest that the surface charge density at the inner surface of appressed membranes is lower than that of nonappressed membranes.
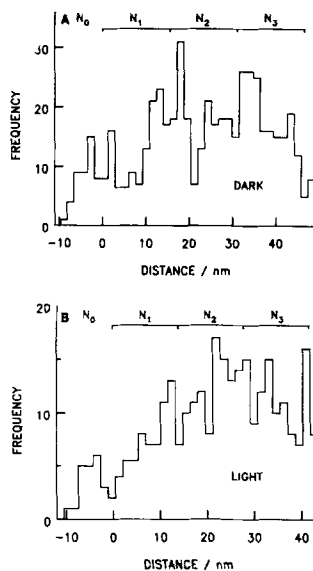
In the analysis of mobile and bound plastocyanin, the binding probability of the antibodies has been assumed to be the same for all plastocyanin molecules. But binding of plastocyanin with negative residues to the subunit of PS I (36) would hide a patch of high antigenic potential. Therefore the fraction of gold granules attributed to bound plastocyanin may be too high and the difference between mobile plastocyanin in grana and stroma lamellae may be smaller than shown in Table II, columns 3 and 4. However, the overall labeling density remained constant at the light-induced changes of the distribution (Table I). This could indicate that either plastocyanin remains bound to PS I after its oxidation in the light or binding of plastocyanin to P700 has a minor effect on immunolabeling, e.g., due to strong binding to the antibody or exposed antigenic determinants when it is bound.

Light-induced Changes of the Plastocyanin Distribution

During illumination, the plastocyanin concentration in grana

increases at the expense of that in stroma regions (Table I). This diffusion may be due to two effects: (*a*) during illumination, the lumenal pH decreases to a value of ~4.7 (38) and negative groups become protonated. The surface charge density in grana should decrease to a larger extent than that in the stroma region. (*b*) In strong illumination plastocyanin becomes oxidized by limitation of the electron transport rate at the cyt b_6/f complex. If the binding of the oxidized form is not as strong as that of the reduced form, there would be more mobile plastocyanin which could distribute between the lumenal regions. Bottin and Mathis (9) have shown that bound plastocyanin is rapidly replaced after its oxidation by reduced plastocyanin.

The labeling density in dark-adapted chloroplasts should indicate the actual distribution of plastocyanin. However, in the light during the incubation with glutaraldehyde for EM, the distribution of plastocyanin may change before its fixation. Extensive investigations of the effect of glutaraldehyde in chloroplasts (for review, see reference 33) have shown that

Figure 6. Light-induced absorbance changes of P700 monitored at 703 nm in spinach chloroplasts. At $t = 0$, the sample was excited by a laser flash of saturating intensity. 200 signals induced at distances of 1.5 s were averaged. The duration of the monitoring light was 0.1 ms. The halftime of the P700⁺ reduction of 11.3 μs is indicated.

Table II. Distribution of Plastocyanin Bound to Photosystem I and of Residual Plastocyanin between Different Lumenal Regions of Spinach Chloroplasts in the Dark

Molar ratio* of plastocyanin/ P700	Region of lumen	Plastocyanin		Relative concentration‖ of "mobile" plastocyanin
		Bound to PS I‡	Not bound to PS I§	
	Grana	–	1.39	1.00
3.27	Exposed grana	0.26	0.38	0.64
	Stroma	0.74	0.50	0.59

Amounts of plastocyanin are given relative to total P700.

* Estimated from rocket immunoelectrophoresis and the amplitude in Fig. 6.
‡ Estimated by multiplying the fraction of total P700 reduced rapidly by bound plastocyanin with the fraction of nonappressed membranes in the region of lumen (see text).
§ Calculated from the total counts in Table I and Eqs. 1–3.
‖ Plastocyanin not bound to PS I divided by the lumenal length.

thylakoid membranes are immobilized but retain their capacity to maintain a light-induced proton gradient (32). Except for a rapid inactivation of ATP synthase, glutaraldehyde stabilizes the function of the integral protein complexes (19) but inhibits linear electron transport in intact chloroplasts specifically by interaction with plastocyanin (20). These effects suggest that the internal proton concentration and the oxidized level of plastocyanin generated by the electron transport will not decrease before most of the proteins are crosslinked. Therefore, the concentration ratio of plastocyanin in grana versus stroma in the light may be slightly lower and the extent of the light-induced diffusion of plastocyanin from stroma to grana regions may be even greater than our experimental value.

The light-driven diffusion of plastocyanin increases its concentration to higher values in grana than estimated in Table II. This will increase the second order reaction rate with cyt b_6/f in this region and the turnover of these complexes in strong light. It is not known how much plastocyanin remains bound to PS I in the light but it is evident that the fraction of mobile plastocyanin in grana regions increases at the onset of illumination. The problem of the diffusion of plastocyanin across the long distances from cyt b_6/f in grana to PS I in stroma would be compensated by a high local concentration. Although this study cannot provide information on the diffusion coefficient, the distribution of plastocyanin is that expected for an efficient oxidation of cyt b_6/f in grana in linear electron transport. The electron transfer from cyt b_6/f in stroma and exposed grana via plastocyanin to PS I involves only short distances across the lumen to the opposite membrane and in nonappressed membranes also to neighboring PS I complexes in the same membrane and could be rapid even at a decreased concentration of plastocyanin in the light. In conclusion the turnover of cyt b_6/f in grana and that in stroma regions would approach each other in the light. Our results provide evidence for the involvement of cyt b_6/f in grana and the functional organization of linear photosynthetic electron transport.

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