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CHLOROPLAST MEMBRANE SIDEDNESS

LOCATION OF PLASTOCYANIN DETERMINED BY CHEMICAL MODIFIERS

D. D. SMITH, B. R. SELMAN*, K. K. VOEGELI**, G. JOHNSON and R. A. DILLEY

Purdue University, Department of Biological Sciences, W. Lafayette, Ind. 47907 (U.S.A.)

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SUMMARY

Intact grana and stroma membranes (outer membrane absent) and detergent or sonication disrupted thylakoid membranes were treated with the hydrophilic covalent chemical modifiers [^{35}S]diazonium benzene sulfonic acid ([^{35}S]DABS) and [^{14}C]glycine ethylester plus 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluenesulfonate (CDIS). Plastocyanin was purified using column chromatography followed by polyacrylamide gel electrophoresis and the incorporation of [^{35}S]DABS and [^{14}C]glycine ethylester into plastocyanin was determined by slicing the gels and counting the radioactivity in the plastocyanin band. Plastocyanin isolated from thylakoids disrupted prior to chemical modification binds two to four times as much of either modifier than the plastocyanin isolated from intact chloroplasts. This ratio is five to ten times lower than the ratio expected for a component buried behind the permeability barrier of a membrane. The data suggest that plastocyanin is partially exposed at the external surface of the thylakoid membrane rather than being completely buried in, or behind, the lipo-protein membrane.

INTRODUCTION

The structural organization of the electron transport components within the chloroplast membrane has become increasingly important in understanding phosphorylation as well as electron transport (for a review, see ref. 1). Electron microscopy and antibody studies have located ribulose diphosphate carboxylase [2, 3] coupling

Abbreviations: DABS, diazonium benzene sulfonic acid; CDIS, 1-cyclo-hexyl-3-(2 morpholinoethyl)-carbodiimide metho-*p*-toluene sulfonate; SDS, sodium dodecyl sulfate.

* Present address: Department of Biochemistry, University of Wisconsin, Madison, Wisc., U.S.A.

** Present address: Département de Physiologie Végétale, Université de Genève, 3, Place de l'Université 1211 Genève 4, Switzerland

factor [3, 4] ferredoxin-NADP-oxidoreductase [5], and the ferredoxin reducing substance (FRS) [6, 7] on the outer surface of the chloroplast membrane. However, antibodies made against Photosystem II particles [8] and cytochrome *f* [9] have little or no effect on electron transport in intact thylakoids, indicating that the components are buried beneath the surface and are inaccessible to externally added reagents. Biochemical and electron microscopy studies indicate that Photosystem I is on the external surface of thylakoid membranes while most of Photosystem II is in the internal region [10, 11].

The location of plastocyanin in the chloroplast membrane is a subject of controversy due to the conflicting data in the literature. Under low salt conditions, polycations bind to the chloroplast membrane and inhibit the photooxidation of cytochrome *f* [12, 13]. These data were interpreted as an indication that plastocyanin is located on the outer surface of the chloroplast membrane.

Hauska et al. [14] reported that the antibody to plastocyanin does not cause an agglutination of the membrane and has no effect on either electron transport or phosphorylation. However, if the antibody was added during sonication, both functions were inhibited. Sonication of particles (previously depleted of plastocyanin) in the presence of plastocyanin restored both electron transport and phosphorylation. Although the addition of plastocyanin following sonication restored electron transport, it failed to restore phosphorylation. On the basis of these experiments, Hauska et al. ([14], see also ref. 9) have suggested that plastocyanin is located on the inside of the thylakoid membrane. Quite different results were more recently obtained by Schmid et al. [15] also using antibodies to plastocyanin. They found that the antibody did cause agglutination and inhibited electron transport, suggesting that plastocyanin must be at least partially exposed at the membrane exterior surface.

We have examined the location of plastocyanin in the chloroplast membrane by treating intact and disrupted thylakoids with the hydrophilic chemical modifiers diazonium benzene sulfonic acid (DABS), (a chemical modification reagent with broad reactivity), and 1-cyclohexyl-3-(2 morpholinoethyl)-carbodiimide metho-*p*-toluene sulfonate (CDIS) plus glycine ethylester (for modification of carboxyl and phosphate groups). The rationale for this approach rests on the fact that sonication or detergent-disrupted chloroplasts quantitatively lose the plastocyanin normally associated with them [16]. Therefore, the chemical modifiers given after the disruption treatment will have complete access to the plastocyanin. Comparing the incorporation of the chemical modifiers in the control and disrupted situations can, therefore, give a measure of the extent to which the plastocyanin is normally buried. Due to their net charges, neither DABS, CDIS nor glycine ethylester would be expected to readily cross a lipophilic membrane [17–19]. Under short reaction times, chemical modification should be mostly limited to those groups exposed to the media and, for intact lamellae, only those groups exposed at the outer surface of the membranes.

Evidence which can be used as a basis of comparison is the DABS labeling of Photosystems I (externally located) and II (buried). In addition, data for comparison are available from the DABS labeling of mitochondrial F_1 coupling factor on submitochondrial vesicles in either the normal (F_1 on the outside) or inverted (F_1 on the inside) membrane vesicle orientation [20]. Labeling ratios of near 20 : 1 were found in both above-mentioned studies.

EXPERIMENTAL METHODS

Chloroplast isolation

Spinach chloroplasts were isolated as previously described [22] and resuspended in buffer containing 20 mM tricine-KOH (pH 7.6), 10 mM NaCl and 0.3 M sucrose or 0.4 M sorbitol. Chlorophyll was assayed as described by Arnon [23].

Chloroplast disruption

Chloroplasts were disrupted prior to treatment with chemical modifiers either by sonic oscillation (Bronson sonication-power setting 5) for 1 min or by incubation with 1 % Triton X-100 for 30 min. Both procedures were performed at 0 °C at 1–2 mg chlorophyll/ml in the resuspension buffer.

Chemical modifications

A. Diazotization. Diazotization was performed in the dark at 0 °C using either intact or disrupted chloroplasts at 1.0 mg chlorophyll/ml in a total volume of 15–20 ml of 10 mM NaCl/20 mM K_2HPO_4, pH 7.6. Carrier-free [^{35}S]DABS (prepared as described in ref. 11) was added to a final concentration of 1 mM and the reaction quenched after 1.0 min by the addition of an equal volume of 0.2 M histidine, pH 6.0, or by dilution with 3 vols. of buffer. Reaction mixtures were subsequently added to 200 ml −15 °C acetone to begin the plastocyanin isolation procedure.

B. Carbodiimide activation and glycine ethylester addition. CDIS-activated carboxyl esterification (see ref. 24) with [^{14}C]glycine ethylester was performed in the dark at 0 °C in 10 ml of a mixture containing chloroplasts (intact or disrupted) at 2.0 mg chlorophyll/ml, 0.4 M sorbitol, 20 mM tricine, 10 mM NaCl, 10 mM KCl, 20 mM CDIS and 0.4 mM [^{14}C]glycine ethylester (specific activity $1.32 \cdot 10^7$ cpm/mol). The pH was adjusted to 6.0 with 0.1 M HCl and the reaction initiated by the simultaneous addition of CDIS and glycine ethylester. The reaction was terminated after 15 min by the addition of the mixture to 200 ml pre-chilled (−15 °C) acetone.

Isolation of plastocyanin

The acetone precipitates from the quenched reaction mixtures were collected by centrifugation, air dried, and the plastocyanin isolated as previously described [16]. This procedure gave a preparation that is quite variable (from 20 to 50 % as judged by SDS gel electrophoresis) in its purity for plastocyanin.

Pure plastocyanin was prepared from spinach leaves as described by Lien and Bannister [25] and ran as a single band on sodium dodecyl sulfate (SDS) gel electrophoresis.

Plastocyanin bioassay

Plastocyanin activity was measured as described by Plesnicar and Bendall [26] in reaction mixtures containing in 3.0 ml, chloroplasts at 5 μg chlorophyll, 0.5 % digitonin, 12 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), 0.5 mM methylviologen, 50 μM reduced (using dithionite) cytochrome *c*, 10 mM KH_2PO_4/KOH (pH 7.0) buffer, 10 mM NaCl, 1 mM $MgCl_2$, and a sample containing plastocyanin. Cytochrome *c* oxidation was measured at 550 nm in a Beckman Acta III spectrophotometer fitted for side illumination (see ref. 16).

The amount of plastocyanin recovered was expressed as the rate of absorbance change per min at 550 nm (due to the cytochrome *c* oxidation) obtained with 5 μg chlorophyll. As shown by Plesnicar and Bendall [26], the rate of cytochrome *c* oxidation is a linear function of added plastocyanin.

Determination of membrane permeability to sulfanilic acid

The permeation of sulfanilic acid, a precursor for DABS which has an $-NH_2$ group rather than the diazo ($-N=N^+$) group, was determined using the three-phase silicone oil technique described by Gaennslen and McCarty [27]. The light vs. dark permeation of sulfanilic acid was compared to both methylamine, a compound known to be accumulated in the light, and inulin, a compound known not to penetrate the chloroplast membrane [28].

Polyacrylamide gel electrophoresis

SDS gel electrophoresis was performed essentially as described by Laemmli [29] with the following modifications: the running gels were 15 cm long and of 12 % acrylamide; the final concentration of Tris · HCl, pH 8.8, was 0.19 M; final concentrations of 0.03 % *N,N,N',N'*-tetramethylethylenediamine and 0.05 % ammonium persulfate were used for polymerization; the electrode buffer contained 0.05 M Tris, 0.38 M glycine, and 0.1 % sodium lauryl sulfate at pH 8.3. The gels were run at 1.5 mA/tube. The gels were fixed in 12.5 % trichloroacetic acid, stained in a mixture of aqueous 0.4 Coomassie brilliant blue (R-250)-ethanol-glacial acetic acid (45 : 45 : 10, by vol.). Destaining was accomplished by several changes of a solution containing 25 % ethanol and 10 % glacial acetic acid. The gels were then scanned at 570 nm using a gel scanner attachment for a Beckman Acta III spectrophotometer. The initial experiments (see ref. 21) used 8 % acrylamide and 10 cm gels, giving somewhat less resolution. A shoulder often occurred on the plastocyanin band using the shorter, less dense gels that was clearly separated from the plastocyanin on the higher resolution gels used in the present work.

There is a possibility that a non-plastocyanin peptide may run with plastocyanin in the SDS gels and account for some of the isotope counts. To check this point, we first electrophoresed the crude plastocyanin fractions from the DEAE columns on urea-containing acrylamide gels, where the peptides migrate mainly according to their native charge. The plastocyanin band was cut out and electrophoresed on dissociating SDS gels (as above) where the peptides migrate according to their molecular weight, the charge being predominantly that due to the adhering SDS. We refer to this as the two-gel system technique. The urea-containing disc gels were prepared with a 1 cm, 3 % acrylamide stacking gel containing 0.19 M Tris · HCl (pH 6.8) and 6 M urea; a 7.5 cm, 6 % running gel with the same concentrations of buffer and urea, but at pH 8.8; upper and lower reservoir buffers containing 6 M urea, 0.05 M Tris, 0.38 M glycine. Purified plastocyanin was co-electrophoresed on the urea gels with the treated and control experimental samples. The bands in the experimental gels corresponding to the position of the pure plastocyanin were cut out and placed on top of an SDS disc running gel, 7.5 cm long with a composition as described for the SDS gels above. The stacking gel was then polymerized around the piece from the urea gel. Pure plastocyanin was electrophoresed in companion gels. These SDS gels were then sliced and counted as above.

Determination of the incorporation of label into plastocyanin

From the gel traces, the relative amounts of plastocyanin in each sample could be estimated by the area (determined gravimetrically from the cut-out of the gel trace) under the peak associated with plastocyanin. These bands were sliced out and soaked in 1.0 ml of 90 % NCS tissue solubilizer (Amersham/Searle-Nuclear-Chicago Corp.) in water (v/v) at 50 °C for 2 h. The vials were cooled to room temperature and scintillation cocktail, tritosol [30] or Bray's cocktail (4 g PPO and 0.1 g POPOP per 1 toluene), added to 10 ml. Radioactivity was measured in a Beckman model DPM-100 liquid scintillation counter. We were able to relate the area under the plastocyanin peak to the radioactivity in a corresponding band and calculate a relative specific activity for the incorporation of ^{35}S label into plastocyanin.

Due to the low specific activity of [^{14}C]glycine ethylester, it was necessary to use larger gels, 12×90 mm, for the electrophoresis of the [^{14}C]glycine ethylester labeled samples. The plastocyanin content of samples used for these analyses was estimated by running the usual smaller gels with treated samples and pure plastocyanin. The amount of plastocyanin/mg area from the purified plastocyanin gels could then be used to determine the amount of plastocyanin/mg protein loaded on the treated samples. The plastocyanin bands from the larger gels were sliced out, incubated with 2.0 ml of 90 % NCS, and counted as before. Using this procedure we were able to fairly accurately determine the incorporation of [^{14}C]glycine ethylester into plastocyanin. An additional problem encountered was that after treatment of plastocyanin with CDIS plus glycine ethylester, it ran as two bands on SDS gel electrophoresis. The specific activity for the incorporation of [^{14}C]glycine ethylester into plastocyanin, as reported here, is the summation of both bands.

Protein concentration in crude preparations was determined as described by Lowry et al. [31]. The concentration of pure plastocyanin was determined using the extinction coefficient $9.8 \cdot 10^3$ l/mol · cm at 597 nm [32].

RESULTS

Membrane penetration by sulfanilic acid

There is evidence in the literature that DABS cannot penetrate either red blood cell membranes [17] or mitochondrial membranes [20], and because CDIS is a sulfonated derivative, Uribe [18] and McCarty [19] have argued that it cannot cross chloroplast membranes. Because of their high reactivity, the permeability of DABS or glycine ethylester (plus CDIS) (expressed as the distribution of the soluble material) cannot be measured directly. Nevertheless, we have examined the permeability of the DABS precursor, sulfanilic acid, using the silicone oil technique described by Gaennslen and McCarty [27] and compared it with the penetrability of inulin and methylamine. It is evident from Table I that illuminated chloroplasts accumulate about 10-fold more methylamine than chloroplasts kept in the dark (when corrected for inulin counts carried into the lower phase). This result is expected from the light-induced pH gradient [27]. Comparing columns III and IV, it can be seen that a certain amount of inulin (0.9 %, column IV) is carried through the silicone layers with the sedimenting chloroplasts in the presence of sulfanilic acid in the dark. However, there is essentially the same amount of [^{35}S]sulfanilic acid carried down with the chloroplasts (column III). There is about 20 % more of either inulin (1.1 %) or sulfanilic acid (1.0 %)

TABLE I

THE PENETRABILITY OF SULFANILIC ACID TO CHLOROPLAST LAMELLAE

The experiments were performed using the three phase system described by Gaennslen and McCarty [23]. The upper phase contained in 0.1 ml: 50 mM KCl, 2.5 mM $MgCl_2$, 20 mM tricine-KOH buffer (pH 8.0), 30 μM phenazine methosulfate and chloroplasts equivalent to 20 μg chlorophyll. The middle phase contained 0.1 ml of a (2 : 1) mixture of silicone oils Versilube F-50 and Versilube SF-96(50) (a generous gift from the General Electric Corp. Waterford, N.Y.). The lower phase contained 0.1 ml of 10 % sucrose and 1.0 % Triton X-100. In addition, the upper phase contained either (i) 1.0 mM [^{14}C]methylamine (specific activity $5.52 \cdot 10^5$ cpm/μmol), (ii) [^{14}C]inulin (equivalent to $8.32 \cdot 10^5$ cpm/ml) plus 1.0 mM methylamine, (iii) 2.5 mM [^{35}S]sulfanilic acid (specific activity $2.2 \cdot 10^5$ cpm/μmol), and (iv) [^{14}C]inulin (equivalent to $1.1 \cdot 10^6$ cpm/ml) plus 2.5 mM sulfanilic acid. Aliquots from the upper phase were counted before centrifugation to determine the total counts. The chloroplasts suspensions were either kept in the dark or illuminated for 1.0 min before and during centrifugation. After centrifugation for 18 s, the microfuge tubes were frozen, the bottom phase cut off, and the radioactivity determined by liquid scintillation as in methods (chlorophyll was bleached by heating the sample for 2–3 h with 0.1 ml 30 % hydrogen peroxide at 70 °C).

	Compound added to upper phase			
	I [^{14}C] Methylamine	II [^{14}C]Inulin plus methylamine	III [^{35}S] Sulfanilic acid	IV Sulfanilic acid plus [^{14}C]-Inulin
Total cpm applied to upper phase	$5.52 \cdot 10^4$	$8.32 \cdot 10^4$	$5.74 \cdot 10^4$	$10.69 \cdot 10^4$
Total cpm found in the lower phase				
(a) Illuminated chloroplasts	$7.00 \cdot 10^3$	$9.8 \cdot 10^2$	$5.83 \cdot 10^2$	$1.15 \cdot 10^3$
(b) Dark chloroplasts	$1.26 \cdot 10^3$	$9.8 \cdot 10^2$	$4.8 \cdot 10^2$	$9.12 \cdot 10^2$
Percent of compound added to the upper phase found in the lower phase				
(a) Illuminated chloroplasts	12.7 %	1.2 %	1.0 %	1.1 %
(b) Dark chloroplasts	2.3 %	1.2 %	0.8 %	0.9 %

carried down with the chloroplasts in the light. As in the dark, the amount of sulfanilic acid going with the chloroplasts can be accounted for by the apparent entrappment of some of the boundary aqueous material that carries the non-penetrating inulin down through the silicone with the chloroplasts. Therefore, we conclude that neither the light nor the dark samples show a tendency to either accumulate or non-specifically absorb sulfanilic acid.

Identification of plastocyanin by SDS electrophoresis

Fig. 1, trace A, shows a typical SDS gel electrophoresis profile for the combined fractions eluted from a DEAE column containing 80 % of the total plastocyanin present in isolated chloroplasts (based on 1 plastocyanin per 300 chlorophylls [26]). There are several prominent bands in this extract and it clearly cannot be considered a pure plastocyanin preparation. However, a major band does occur at 11 000 daltons and by comparing the profile of this extract with the profile of pure plastocyanin (trace B) it is evident that the extract does contain a large proportion of plastocyanin and that the plastocyanin polypeptide occurs in a section of the gel free from other

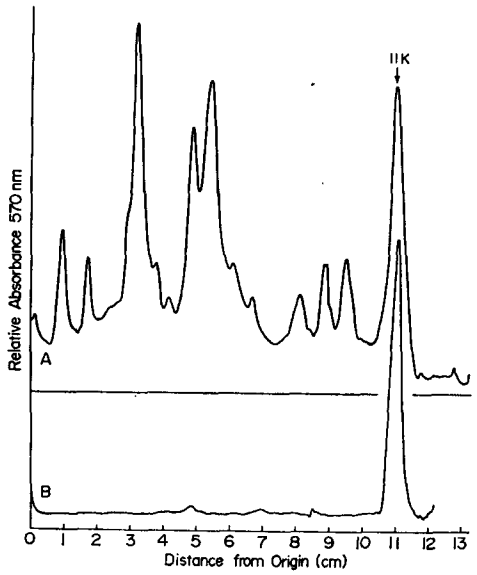

Fig. 1. Gel electrophoresis patterns of crude and pure plastocyanin preparations in single gel system (SDS). Trace A: spinach chloroplasts, at 1.0 mg chlorophyll/ml, were sonicated for 1.0 min in a total volume of 20 ml. The plastocyanin was isolated and the active fractions from a DEAE column were pooled and electrophoresed as described in Experimental Methods, using 15 cm long, 12 % acrylamide gels. Trace B: gel electrophoreses pattern of pure plastocyanin as in A. Numbers refer to molecular weights in 10^3 daltons.

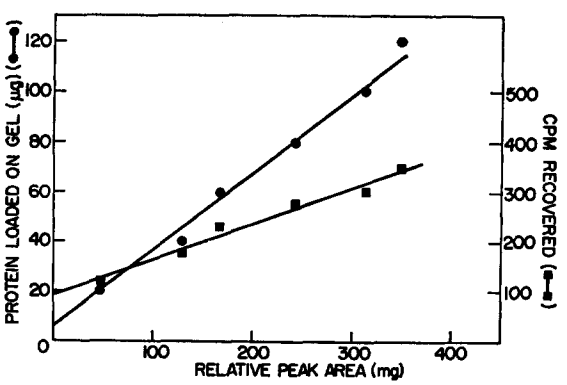

Fig. 2. Relative plastocyanin peak area as a function of the amount of protein loaded on disc-gels and the radioactivity recovered from the plastocyanin band. Sonicated spinach chloroplasts were treated with [^{35}S]DABS, and the plastocyanin isolated, electrophoresed and counted as detailed in Experimental Methods.

contaminants. The plastocyanin peak is symmetrical and easily separated from higher molecular weight material for the determination of radioactivity incorporation.

Relationship between the amount of protein loaded on disc-gels, the plastocyanin peak area, and the radioactivity recovered

To determine the relative specific activity for the incorporation of radioactive labels into plastocyanin, it is necessary to demonstrate that the procedures used are quantitative. Both the amount of protein loaded on disc-gels and the radioactivity recovered from the plastocyanin should be linear with respect to the relative plastocyanin peak area. Fig. 2 shows a typical recovery plot for a single experiment in which disrupted chloroplasts were treated with [^{35}S]DABS and the plastocyanin fraction isolated and electrophoresed. Both the amount of protein applied to the gel and the cpm recovered from the plastocyanin bands are linearly proportional to the area under the plastocyanin peak traced out by the spectrophotometer.

TABLE II

[^{35}S]DABS INCORPORATION INTO PLASTOCYANIN FROM INTACT AND DISRUPTED THYLAKOIDS

Spinach chloroplasts, at 1.0 mg chlorophyll/ml, where used without disruption for the controls or were fractionated with 1 % Triton X-100 for 30 min or sonicated for 1.0 min (where indicated) prior to treatment with 1 mM [^{35}S]DABS in a total volume of 20.0 ml. The plastocyanin was isolated and the specific activity of DABS binding was determined as detailed in Experimental Methods. Experiment 5 is data from plastocyanin isolated by the double gel electrophoresis.

Experiment	Electrophoresis	Treatment	Relative plastocyanin activity*	Relative specific activity**
1	Single gel	Triton	18	57.0
		Control	19	20.0
2	Single gel	Triton	19	55.0
		Control	18	21.0
3	Single gel	Sonication	19	51.0
		Control	19	20.2
4	Single gel	Sonication	18	72.0
		Control	18	19.0
5	Double gel	Triton	20	20.3 †
		Control	19	5.4

* This refers to the spectrophotometric assay for plastocyanin contained in the combined fractions from the DEAE columns. The activity is expressed as $\Delta A_{550} \times 10^2$/min per 5 μg chlorophyll (change in absorbance per assay mixtures, containing 0.2 ml plastocyanin sample).

** This refers to the radioactivity recovered in the plastocyanin band of the electrophoresis gels as cpm/mg-area plastocyanin.

† When corrected for the different specific activity of the [^{35}S]DABS used in the double gel experiment compared to the single gel experiments, these relative specific activity values become 52.8 and 14.0 for the triton and control treatments respectively.

Distribution of [^{35}S]DABS in plastocyanin isolated from intact and disrupted chloroplasts

1. *Single gel SDS disc gel electrophoresis.* The results for several experiments in which intact and disrupted thylakoids were treated with [^{35}S]DABS followed by the isolation of the plastocyanin by one-dimensional SDS disc gel electrophoresis are summarized in Table II. The data illustrate two main points: (1) both Triton solubilization and sonication of chloroplast membranes expose plastocyanin to the diazonium reagent more than in intact thylakoids (2) the exposure of plastocyanin to DABS by either Triton solubilization of the membrane or sonication does not lead to an inactivation of plastocyanin (measured as the stimulation of cytochrome *c* oxidation). The ratio of the incorporation of DABS into plastocyanin released from the membrane by sonication or detergent treatment prior to adding DABS compared to the incorporation into membrane bound plastocyanin was from 3 to 4.

Typical gel scans of the single SDS gels are shown in Fig. 1, trace A; it is apparent that the plastocyanin runs as a symmetrical band, well resolved from the band on the high molecular weight side. In ref. 21, (Fig. 1, trace A) it is seen that the shorter, less dense gels used in the experiments had a shoulder on the plastocyanin band. This band is well resolved away from the plastocyanin using the present 12 % acrylamide, 15 cm gels.

2. *Two gel polyacrylamide disc gel electrophoresis.* The possibility cannot be eliminated that the peak in the plastocyanin preparation which migrates in SDS disc gels the same distance as pure plastocyanin contains a contaminant of an identical molecular weight. To further diminish the possibility that this was occurring, a two-gel electrophoresis separation system was used on plastocyanin preparations from membranes modified with [^{35}S]DABS. The plastocyanin preparations partially purified using DEAE column chromatography were first run on urea gels. Fig. 3, trace A, shows a scan of a urea gel which was loaded with a preparation from membranes treated with Triton X-100 prior to modification with [^{35}S]DABS. Separation in this gel system is primarily on the basis of the charge of the polypeptides. The scan in Fig. 3, trace A, is characteristic of the separation produced by these gels. A large amount of material stayed near the top of the gel. The bottom of the gel contains one predominant band which migrates the same distance as purified plastocyanin (Fig. 3, trace B). This band was sliced out and placed on top of an SDS gel as described in Experimental Methods. Fig. 4, trace A, shows a scan of the SDS gel loaded with the band removed from the urea gel represented by Fig. 3, trace A. This scan is representative of the results we found with many gels; it contains a single symmetrical peak which migrates the same distance as purified plastocyanin. These SDS gels were sliced, and counted as previously described.

Experiment 5 on Table II shows the data from the two-gel experiment. The ratio of the incorporation of DABS into plastocyanin released from the membrane by detergent treatment as compared to the incorporation into membrane bound plastocyanin was 3.76. The "relative specific activity" of the plastocyanin was lower after electrophoresis in urea and SDS gels. However, as noted in Table II, if corrected for the differences in the specific activity of the two [^{35}S]DABS preparations, the cpm in the double gel experiment correspond to approximately the same relative amount of DABS bound per mg area of plastocyanin as in the single gel experiments. In the two gel system, the ratio of the incorporation of [^{35}S]DABS into the plastocyanin of

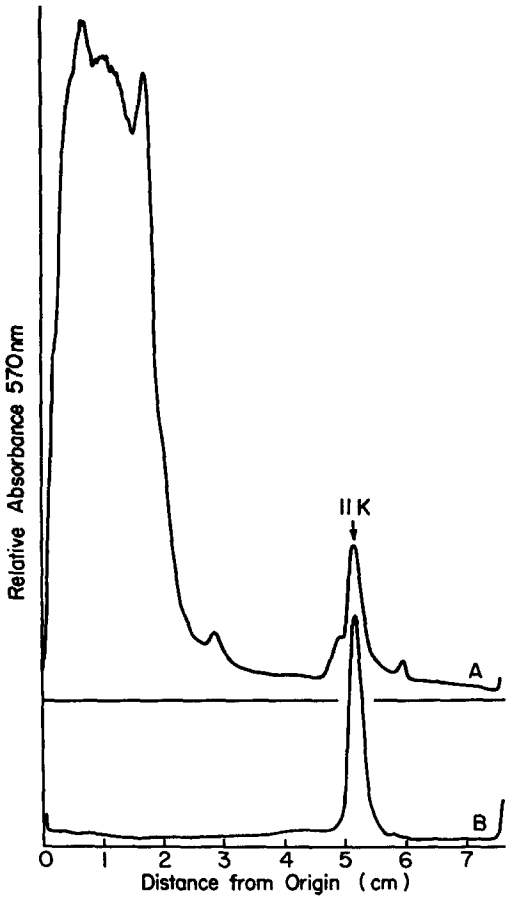

Fig. 3. Scans of urea gels of crude and pure plastocyanin preparations. Trace A: spinach chloroplasts were treated with 1 % Triton X-100 for 30 min. The plastocyanin was isolated and the active fractions pooled and electrophoresed as described in Experimental Methods. Trace B: scan of urea gel loaded with pure plastocyanin and electrophoresed on a companion gel with the crude preparation shown in trace A.

disrupted chloroplast membranes to the incorporation in control membranes was completely consistent with the ratio calculated with data from the single SDS gel technique.

CDIS catalyzed incorporation of [^{14}C]glycine ethylester into plastocyanin

CDIS is a hydrophilic carbodiimide that reacts with carboxy and phosphate groups. In the presence of a nucleophile, such as a primary amine, a carbodiimide activated carboxyl forms a stable amide bridge covalently linking the amine to the carboxyl carbon, subsequently releasing a substituted urea [24]. CDIS and glycine ethylester were chosen for their hydrophilic properties and the sulfonated derivative of the carbodiimide would not be expected to cross a lipid membrane [18, 19].

Experiments in which isolated plastocyanin was reacted with CDIS, glycine

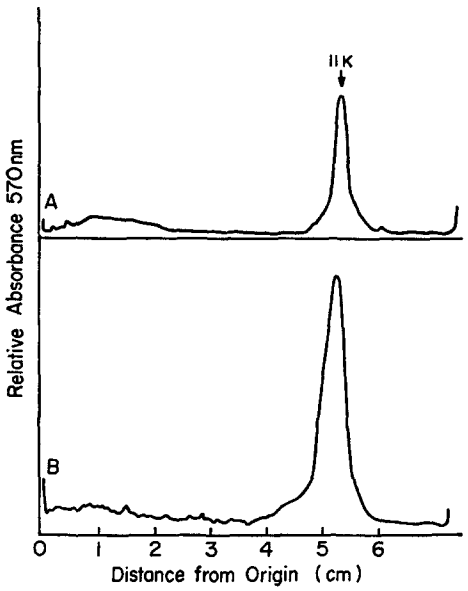

Fig. 4. Scans of SDS gels used in the two-gel electrophoresis system. Trace A: the plastocyanin band from the gel represented in Fig. 3, trace A, was cut out and placed on top of an SDS gel as described in Experimental Methods. Trace B: scan of pure plastocyanin electrophoresed on a companion gel with the crude preparation shown in trace A.

TABLE III

GLYCINE ETHYLESTER INCORPORATION INTO PLASTOCYANIN ISOLATED FROM INTACT AND SONICATED CHLOROPLASTS

Spinach chloroplasts, at 2.0 mg chlorophyll/ml, were sonicated (where indicated) for 1.0 min prior to treatment with 20 mM CDIS plus 0.2 mM [^{14}C]glycine ethylester in a total volume of 10 ml; the other components of the reaction mixture are as indicated in Experimental Methods. The plastocyanin was isolated by the single SDS electrophoresis step and the specific activity determined as detailed in Experimental Methods.

Experiment	Treatment	Total plastocyanin recovered (mg)*	Specific activity (cpm · 10^{-4}/mg plastocyanin)	$\frac{\mu\text{mol glycine ethylester}}{\mu\text{mol plastocyanin}}$
1	Control	1.52	2.06	0.033
	Sonicated	1.53	8.14	0.13
2	Control	0.84	6.58	0.11
	Sonicated	0.86	16.3	0.26

* Determined from the area under the curve of a gel scan. The bioassay for plastocyanin used for the DABS experiments is not applicable in this case because the CDIS inactivates the plastocyanin. Note that in Experiment 1, we pooled all of the plastocyanin containing fraction from the DEAE cellulose column, while in Experiment 2 we used only the peak fractions.

ethylester and both compounds, indicated that CDIS is necessary for [^{14}C]glycine ethylester to react with plastocyanin (as shown in ref. 21). However, unlike DABS, the activation reaction with CDIS (plus or minus glycine ethylester)inhibits the activity of plastocyanin. Pure plastocyanin was incubated as described in the Methods section, with 20 mM CDIS and/or 0.2 mM [^{14}C]glycine ethylester for 1 hr at 0 °C, reisolated and the relative activity of the plastocyanin in the bioassay was determined. Plastocyanin from incubation mixtures containing CDIS alone had 36 % of the relative activity (in the bioassay) of plastocyanin incubated with glycine ethylester. In the presence of CDIS and glycine ethylester the relative activity was 34 % that of plastocyanin incubated with glycine ethylester alone. In the absence of an added nucleophile, it is possible that an endogenous nucleophile, e.g. lysine or arginine, cross-links with an activated carboxyl, rendering the plastocyanin inactive. This cross-linking might also explain the two bands seen when plastocyanin, reacted with CDIS and glycine ethylester, is electrophoresed on SDS polyacrylamide gels.

Table III summarizes the data from several experiments in which intact and sonicated thylakoids were treated with CDIS and [^{14}C]glycine ethylester and the plastocyanin fractions isolated. Although both fractions contained the same total amount of plastocyanin, the total activity of the plastocyanin in the bioassay recovered from sonicated chloroplasts was only about 70 % of the control. Apparently, this difference reflects the ability of CDIS to inactivate plastocyanin when it is free in solution. Table III shows that the plastocyanin isolated fron sonicated chloroplasts (prior to the chemical modification treatment) contains 3–4 fold more label (ratios range from 2.5 to 4.5) than the plastocyanin isolated from intact lamellae.

DISCUSSION

Techniques were developed that permitted a quantitative recovery of plastocyanin and a measure of the relative specific activity for the labeling of plastocyanin with either a sulfonated diazonium (DABS), or a sulfonated carbodiimide plus a nucleophilic reagent (CDIS+glycine ethylester). Both the DABS and CDIS are probably non-penetrating in the chloroplast membrane although this has not been directly shown in the chloroplast system. Evidence indicating that DABS is non-penetrating has been presented for red blood cells [17] and mitochondria [20]. In the chloroplast system, sulfanilic acid, a precursor in the DABS synthesis, does not penetrate the membrane significantly more than inulin (Table I), a non-penetrating molecule. The similarity of DABS and sulfanilic acid makes it quite reasonable to assume that DABS does not penetrate through the membrane in the time periods used for the DABS labeling (usually 1 min or less). Significant DABS labeling of plastocyanin in intact membranes is believed not to be due to penetration by DABS to the inside. We are carrying the assumption further in assuming that the CDIS+glycine ethylester couple do not appreciably penetrate through the membrane in the much longer (15 min) incubation period.

The 12 % acrylamide, 15 cm long (Fig. 1, trace A) SDS gels from which the data in Table II, experiments 1-4, were derived, clearly separated the peptide that in a previous series of experiments (using 8 % acrylamide, 10 cm long gels; see ref. 21) sometimes ran as a shoulder on the plastocyanin band. The ratio of radioactivity in the plastocyanin isolated from Triton or sonicated membranes compared to control

(intact) membranes (about 2 to 4 fold) remained about the same in both series of experiments. This suggests that the radioactivity data were not being spuriously influenced by a putative impurity. However, to further assure that the plastocyanin band was homogeneous, the two-gel polyacrylamide electrophoresis approach was used. It is extremely unlikely that an impurity would electrophorese with plastocyanin in both gel systems. Using the two-gel system, we find the same pattern of DABS labeling of plastocyanin in the control membranes, compared to Triton X-100 or sonication-treated membranes as with the single SDS system. The results with both DABS and CDIS plus glycine ethylether (Table II and III) show that when the plastocyanin is released from the chloroplast membranes with Triton X-100 or sonication prior to derivatization, plastocyanin binds 2 to 4 fold more label than the plastocyanin isolated from membranes derivatized while intact.

The criteria we are using to evaluate whether plastocyanin is in the inside osmotic space or more toward the membrane exterior is a comparison to the DABS labeling of Photosystems I (exterior) and II (interior), and to DABS labeling of mitochondrial coupling factor in regular or inverted orientation mitochondrial particles [20]. The ratios obtained for Photosystem I/Photosystem II labeling range from near 10 [33, 34] to near 20 [11], and Schneider et al. [20] found that inverted submitochondrial particles (coupling factor on the outside) bound about 20 fold more DABS to the coupling factor than intact "right side out" membranes (having the coupling factor on the inside). By these criteria, we interpret our data as indicating that plastocyanin is not buried behind the lipid barrier of the thylakoid membrane, and it is not as completely exposed at the outer surface as the coupling factor protein.

While the antibody work of Hauska et al. [14] suggests that plastocyanin is "buried" within the osmotic space, the antibody work of Schmid et al. [15] indicates that endogenous plastocyanin is accessible, under some conditions, for reaction with antibody, consistent with the results reported here.

The stacked grana region might restrict the accessibility to the water soluble reagents, resulting in a lower level of derivatization of plastocyanin in the control case compared to the sonication or Triton X-100 treatments. This does not seem likely, in view of the fact that DABS labeling of stacked membranes was found to be quite similar to the labeling that occurred with low salt-suspended, unstacked membranes [11]. However, that situation would tend to reduce the DABS incorporation into plastocyanin in the control membranes but have no effect on the detergent or sonication treated membranes. Hence, the DABS labeling ratio as we express it would be artifactually too high, which would mean that plastocyanin is even more exposed at the outer surface than we are suggesting.

Taking the present findings, the above considerations, and published data into account, we are led to the conclusion that plastocyanin is partially buried in the membrane lipoprotein matrix, but more toward the external surface than the internal surface. The hypothesis that plastocyanin is located toward the exterior of the membrane is consistent with the membrane model of Arntzen et al. [10] visualizing Photosystem I located on the outer "half" of an asymmetric membrane, with Photosystem II mostly on the interior portion. Being closely associated with the Photosystem I reaction center, plastocyanin, if in the inner osmotic space, could not readily interact with *P*-700, unless *P*-700 itself was close to the interior side of the membrane, which is not likely [1].

These data do not exclude the possibility that plastocyanin may occur in more than one site, one of which is more externally located than the other. However, most of the plastocyanin would have to be partially exposed at the external membrane surface, in order to account for the labeling we have obtained.

Because plastocyanin is so freely water soluble, indicating that charged, polar amino acid residues [35] probably face at least part of the protein exterior, the membrane region surrounding the location of plastocyanin must also be, in part, a polar, charged region. One might picture this site as a hydrophilic cleft on the exterior portion of the membrane. Berzborn [36] has suggested from antibody agglutination studies that the grana membrane external surface may have clefts in which the ferridoxin-NADP reductase enzyme is located.

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