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LOCALIZATION OF THE REACTION SIDE OF PLASTOCYANIN FROM IMMUNOLOGICAL AND KINETIC STUDIES WITH INSIDE-OUT THYLAKOID VESICLES

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(1) The effect of four active antisera against plastocyanin on Photosystem I-driven electron transport and phosphorylation was investigated in spinach chloroplasts. Partial inhibition of electron transport and stimulation of plastocyanin-dependent phosphorylation were sometimes observed after adding amounts of antibodies which were in large excess and not related to the plastocyanin content of the chloroplasts. This indicates effects of the antibodies on the membrane. (2) The antibodies against plastocyanin neither directly nor indirectly agglutinated unbroken chloroplast membranes. (3) The plastocyanin content of right-side-out and inside-out thylakoid vesicles isolated by aqueous polymer two-phase partition from chloroplasts disrupted by Yeda press treatment was determined by quantitative rocket electroimmunodiffusion. Right-side-out vesicles retained about 25%, inside-out vesicles none of the original amount of plastocyanin. (4) The effect of externally added plastocyanin on the reduction of *P*-700 was studied by monitoring the absorbance changes at 703 nm after a long flash. In inside-out vesicles *P*-700 was reduced by the added plastocyanin but not in right-side-out vesicles and class II chloroplasts. These results provide strong evidence for a function of plastocyanin at the internal side of the thylakoid membrane.

Introduction

The structural organization of the components of photosynthetic electron transport within the thylakoid membrane is of importance for energy conversion by proton translocation [1] and field generation [2,3]. Vectorial translocation of electrons by the photoreactions from the inside to the outside (matrix side) of the thylakoid membrane is generally accepted (see Refs. 3 and 4). However, the results about the location of the reaction side of most cytochromes [5–7] and in particular of plastocyanin are contradictory. Plastocyanin functions as an immediate electron donor of *P*-700 [8,9].

Externally added plastocyanin was shown to restore electron flow in plastocyanin-deficient preparations of chloroplast thylakoids [10–12]. This could indicate a reaction site at the outside of the membrane. However, Hauska et al. [13] demonstrated that the added plastocyanin failed to restore phosphorylation and suggested that it induces an artificial electron flow through PS I. With an antiserum against plastocyanin these authors found no agglutination or inhibition of photoreactions in isolated chloroplast thylakoids. From these results and in analogy to the location of cytochrome *c* in mitochondria [14], it was concluded that plastocyanin in situ is located at the inside of the thylakoid membrane.

In support of this location is the finding of a

Abbreviations: DAD, 2,3,5,6-tetramethyl-*p*-phenylenediamine dihydrochloride; Tricine, *N*-tris(hydroxymethyl)methylglycine; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; PS, photosystem, Chl, chlorophyll.

slower reaction of endogenous plastocyanin with sulfonated phenazine methosulfate compared to phenazine methosulfate [15] in contrast to the inverse relationship of the reaction with solubilized plastocyanin [16]. Osmotic effects on the electron transport to PS I have also been interpreted as evidence for a reaction site of plastocyanin inside the osmotic barrier of the thylakoid [17]. The electrochromic absorbance changes in chloroplasts show no decrease during the fast electron transfer from plastocyanin to $P\text{-}700^+$ [18]. This is strong evidence for a location of plastocyanin on the same side, i.e., the inside [3,4] of the hydrophobic barrier of the thylakoid membrane as *P*-700.

However, several investigations seem to indicate a location of plastocyanin near or at the outside of the thylakoid membrane. This was suggested by Schmid et al. [19] who found that antibodies against plastocyanin did cause agglutination and inhibited electron transport of their chloroplasts. The same conclusion was drawn by Böhme [20] from inhibition of electron transport and agglutination of intact chloroplasts observed with purified immunoglobulin from a plastocyanin antiserum.

Under low-salt conditions, large membrane-impermeable polycations such as polylysine and histones [21,22] inhibit the photooxidation of cytochrome *f*. This effect has been attributed to the attraction of the positively charged polycations to the negatively charged plastocyanin located near the outer surface of the membrane. Smith et al. [23] compared the incorporation of the hydrophilic label diazonium benzenesulfonic acid into plastocyanin in situ and into isolated plastocyanin with that into the two photosystems. They found a relatively large amount of the label in plastocyanin in situ and concluded that plastocyanin is located in a hydrophilic cleft at the outside rather than behind the membrane. In support of this view, Nolan and Bishop [24] interpreted their experiments on the effect of amphotericin B on the thylakoid membrane.

In summary, both external and internal locations of plastocyanin seem to be supported by several independent investigations. Direct comparison of reactions at the inner and outer thylakoid surfaces should be possible after isolation of inside-out thylakoid vesicles [25]. This isolation has been done by using aqueous polymer two-phase partition, a separation method which separates membrane vesicles according to their surface properties [26]. The reversed sidedness of thylakoid vesicles has been demonstrated by proton [25] and electron flux measurements [27]. In addition, inside-out vesicles show convex protoplasmic and concave exoplasmic freeze-fracture faces [28], in contrast to right-side-out vesicles. Furthermore, the nativeness of inside-out membranes is indicated by the activity of the highly fragile water-splitting system [29]. The usefulness of these thylakoid vesicles has also been established by trypsinization experiments showing an internal location of the water splitting site [29].

We have reinvestigated the problem of the sidedness of plastocyanin by combining immunological and kinetic approaches with this preparation technique of thylakoid vesicles. Our results provide new evidence for an internal location of plastocyanin.

Materials and Methods

Preparation of right-side-out and inside-out thylakoid vesicles. Spinach was either grown at 19°C with about 12 000 lx illumination and a day length of 13 h or in the field. Spinach class II chloroplasts isolated as described previously [30] were used to prepare inside-out and right-side-out vesicles as described in Ref. 25. The T2 fraction contains right-side-out thylakoid vesicles (sidedness as in chloroplasts) while the B3 fraction contains mainly inside-out thylakoid vesicles. The inside-out vesicles are highly enriched in PS II [31], which in addition to the high density of exoplasmic face freeze-fracture particles [28], indicates an origin from grana partitions. In addition to the T2 and B3 fractions, we collected from the supernatant of the centrifugation for 30 min at $40\,000 \times g$ after the Yeda press treatment [25] a stroma lamellae fraction by 60 min centrifugation at $100\,000 \times g$ (100 k fraction) and stored it as well as its supernatant.

Inside-out vesicles with PS I and PS II in proportions equal to the average of the chloroplast lamellae have been isolated as described in Ref. 32. The procedure involves destacking of the thylakoids and randomization of the photosystems in the membrane in 10 mM Tricine (pH 7.4), 100 mM sucrose, followed by membrane pairing at pH 4.7 before passage through the Yeda press. This procedure and that

described above will be referred to as preparation methods L and H, respectively. Except for measurements of absorbance changes all fractions obtained by phase partition were diluted 1 : 5 with 10 mM sodium phosphate buffer (pH 7.4), 20 mM NaCl and centrifuged for 1 h at 150000 × *g* to remove polymers.

Preparation of plastocyanin. Plastocyanin was prepared from isolated spinach chloroplasts as described previously [33]. The eluate of the first DE-52 column was passed through a Sephadex G-25 column and used as crude plastocyanin. The concentration was determined from the absorbance at 597 nm using the difference extinction coefficient of $\Delta\epsilon_{597nm} = 4.9$ $mM^{-1} \cdot cm^{-1}$ [34]. The ratio of the absorbance at 278 nm to that at 597 nm was 3.8 and 1.6 for crude and purified plastocyanin, respectively. The removal of contamination present in crude plastocyanin was followed by immunoelectrophoretic analysis with antisera against crude plastocyanin. With these polyspecific sera purified plastocyanin showed only one precipitation arc.

Preparation of antisera. Two rabbits were immunized with crude plastocyanin by an intradermal primary injection of 3.2 mg protein as a paste with Freund's complete adjuvant (Difco). After 4 weeks the rabbits received an intraveneous booster injection of 1 mg protein in physiological buffer. With purified plastocyanin four rabbits were immunized by the same procedure except that 0.8 mg plastocyanin was used for both primary and booster injections. Other details are described in Ref. 35.

Immunochemical procedures. Immunoelectrophoretic analysis was carried out with the standard LKB/Camag equipment [36,37].

Agglutination experiments were carried out as described previously [35] by mixing 10 μl of either chloroplast or vesicle suspension containing about 0.1 mg Chl/ml with 5 μl serum. Before addition the serum was diluted eight times or more to avoid unspecific aggregation.

Indirect agglutination was performed according to the method of Coombs et al. [38] to test for bound antibodies. After incubation of vesicles with antiserum against plastocyanin for 1 min with gentle mixing, 5 μl of diluted antiserum against rabbit γ-globulin were added.

Quantitative rocket electroimmunodiffusion according to the method of Laurell [39] was done in agarose slab gels as described earlier [40] on microscope slides with the LKB/Camag equipment. The gels contained 0.8% agarose, 0.05 M Tris-barbiturate buffer, pH 8.8 (Camag, Berlin), and 0.1–0.4 ml antiserum per 10 ml gel. Chloroplasts or vesicle preparations were suspended in 10 mM NaCl and dissolved overnight at 4°C in 3.3% Triton X-100 and 1.6% deoxycholate at a concentration of about 0.5 mg Chl/ml. Samples of 8 μl were applied to the gels. The electrophoresis was run at 300 V/25 cm at about 12°C for 3 h. This was sufficient for full development of the rockets. Afterwards the gels were washed, dried and stained.

Characterization of the antisera. The relative titer of precipitating antibodies in the antisera was compared by rocket electroimmunodiffusion. One out of two rabbits and two (rabbits 146 and 147) out of four yielded large amounts of precipitating antibodies against crude and purified plastocyanin, respectively. The effect of varying amounts of antisera on soluble plastocyanin was determined from the oxidation rate of cytochrome *c* according to the enzymatic assay of Plesničar and Bendall [12]. The reaction mixture contained in 2.5 ml of 10 mM phosphate buffer (pH 7.0), 10 mM NaCl, 1 mM $MgCl_2$, 5 μM reduced cytochrome *c*, 0.1 mM methyl viologen, 0.75 nmol plastocyanin and PS I particles prepared as described before [41] at a concentration of 1 μg Chl/ml. Antisera were added to the stirred reaction mixture 2 min before the onset of the exciting red light (Schott filter RG 610/3 mm) of saturating intensity. The oxidation of cytochrome *c* was monitored at 418 minus 405 nm with an Aminco DW2 spectrophotometer in the dual-beam mode.

The specificity of the antisera against pure plastocyanin was tested by immunoelectrophoretic analysis with crude as well as with purified plastocyanin. Usually, only one precipitation arc was seen, even if crude plastocyanin was used. However, sometimes a second slower migrating antigen was also precipitated. Its amount was increased after KCN treatment of plastocyanin [15] (data not shown). We conclude that this antigen may be the apoprotein of plastocyanin. The antisera did not react with several other chloroplast proteins. We conclude that the sera contain antibodies against plastocyanin only, either the native or copper-free apoprotein; in this sense we

regard our sera to be monospecific. Serum 146 reacted with both forms of soluble plastocyanin and was therefore chosen for the agglutination experiments.

Assays of photophosphorylation and electron transport. Photophosphorylation was measured after preincubation of isolated chloroplast thylakoids [42] with antiserum for 15 min at 0°C in 60 mM Tricine-NaOH buffer, pH 8.5. The reaction mixture, at a temperature of 20°C, contained these chloroplasts at a chlorophyll concentration of 10 μg/ml, 1 mg bovine serum albumin, 50 mM Tricine-NaOH buffer, pH 8.5, 65 mM NaCl, 5 mM $MgCl_2$, 3 mM ADP, 2 mM phosphate with about 1 μCi of ^{32}P, 5 mM sodium ascorbate, 0.1 mM DAD and 0.2 mM anthraquinone-2-sulfonate. The incorporation of ^{32}P [43] was measured as described previously [44] using a Packard Tri-Carb Scintillation counter.

DAD-mediated electron transport through PS I [45] was monitored with a Clark-type electrode at 20°C as oxygen uptake. The reaction mixture contained in 4 ml chloroplasts at a concentration of 15 μg Chl/ml, 20 mM Tricine-NaOH, pH 7.6, 20 mM KCl, 3 mM $MgCl_2$, 0.5 mM NaN_3 and 15 μM DCMU. Antiserum was added to the stirred solution 5 min before 0.1 mM DAD and 2.5 mM sodium ascorbate were added, and the exciting red light (Schott filter RG 570/3 mm) of saturating intensity (800 W/m^2) was switched on.

The absorbance changes at 703 nm were measured with a single-beam flash photometer as described before [46], except that the electrical bandwidth ranged from d.c. to 10 kHz and the signals were stored directly in a TN 1 500 signal processor from Tracor. The amplitudes of the signals were corrected for dilution of the sample by added reagents by multiplying the stored data with a constant. The temperature of the sample was 22–24°C.

Chemicals. Poly(ethylene glycol) 4 000 was obtained from Union Carbide (New York, U.S.A.) and Dextran 500, batch number 3 447, from Pharmacia Fine Chemicals AB (Uppsala, Sweden). Cytochrome *c* from horse heart and ADP were purchased from Boehringer, digitonin from Merck, antiserum against rabbit γ-globulin from Behringwerke and agarose from Sigma.

Results

Influence of antisera against plastocyanin on photosynthetic reactions

The effect of antisera on soluble plastocyanin was determined from the oxidation rate of cytochrome *c* [12,13] by PS I particles [41]. The oxidation rate was proportional to the concentration of added plastocyanin up to 1.2 μM under our conditions. Tritations with antisera showed 50% inhibition of the activity of 0.75 nmol plastocyanin after addition of 40 and 80 μl (averages of three titrations) of sera 147 and 146, respectively. 3-fold amounts inhibited more than 90%. In agreement with these inhibiting amounts of antisera, we determined by rocket electroimmunodiffusion about twice the amount of precipitating antibodies in serum 147 compared to serum 146.

Photophosphorylation mediated by DAD has been shown to depend on plastocyanin in situ [4,15,47]. However, three out of four active antisera against plastocyanin stimulated the DAD-mediated phosphorylation up to 20%, as did the control sera. We added up to 10 μl serum/μg Chl. Assuming a content of one plastocyanin molecule/200 Chl molecules [12, 48,49] in our chloroplasts, this is 17- and 34-times more than that needed for 50% inhibition of soluble plastocyanin with sera 146 and 147, respectively.

PS I electron transport via plastocyanin mediated by DAD was investigated in unbroken chloroplast lamellae. Inhibition by our antisera was not observed except for antiserum 146, which inhibited up to 40% of the electron transport (results not shown). How-

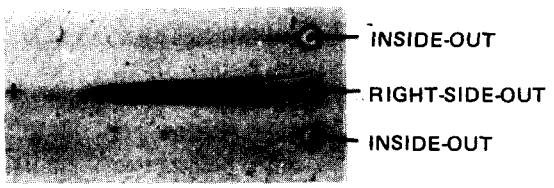

Fig. 1. Quantitative determination of the relative plastocyanin content in thylakoid vesicles of opposite sidedness by rocket electroimmunodiffusion. The holes were loaded with 5 μl of vesicle suspension of (top) inside-out vesicles containing 12 μg Chl (middle) right-side-out vesicles containing 14 μg Chl and (bottom) inside-out vesicles containing 7 μg Chl. The gel contained 0.1 ml of antiserum 146 per 10 ml agarose. Top and center, preparation method H; bottom, preparation method L. For details see Materials and Methods.

ever, to induce any inhibition an amount varying from 1 to 5 μl antiserum/μg Chl was necessary. This is about 2- to 9-times more than that needed for 50% inhibition of the plastocyanin activity if the same plastocyanin content of the chloroplasts is assumed as above. Aging of the chloroplasts at 0°C increased this lag phase in the titration curve. A similar lag of 0.5–10 μl antiserum/μg Chl in the titration curves as well as a lack of inhibition after aging of chloroplasts was observed in some of the earlier measurements of plastocyanin-dependent electron transport [19,20].

Agglutination studies with thylakoid preparations of opposite sidedness

Binding of antibodies to plastocyanin in situ without affecting its function would be consistent with the results above. This was tested by direct agglutination with all of our four antisera against plastocyanin as well as by indirect agglutination with antibodies against rabbit γ-globulin which could cross-link bound antibodies against plastocyanin [50]. All of our results were negative. Therefore, plastocyanin in situ is probably not accessible to antibodies.

The lack of agglutination would be consistent with location of plastocyanin either at the inside or in a small cleft, inaccessible to antibodies, at the outside of the thylakoid membrane. To distinguish between these possibilities we carried out agglutinaion studies with right-side-out and inside-out vesicle preparations obtained by both methods described in Materials and Methods [9] as well as with the stroma lamellae fraction (100k). No agglutination was observed with any of these vesicle preparations. Occasionally, however, inside-out vesicles showed weak direct and indirect agglutination immediately after preparation.

Plastocyanin content of different thylakoid vesicles

In all fractions separated after Yeda press treatment we determined the specific plastocyanin content by quantitative rocket electroimmunodiffusion. As shown in Table I, all fractions have a considerably lower content of plastocyanin than the starting class II chloroplasts. This indicates release of plastocyanin from the membranes by Yeda press treatment. However, in contrast to the fractions of inside-out vesicles (B3) retaining only about 5% of the original plastocyanin content, the fractions of right-side-out vesicles retain 15–32%. The stroma vesicles (100k), nearly all being right-side-out [30], retain 50–60% in agreement with previous results [51]. Including the plastocyanin determined in the supernatants of the vesicle pellets (values not shown), a recovery of 97% of the initial plastocyanin was calculated.

To estimate the actual amount of plastocyanin retained by inside-out vesicles, the rather high contamination of this fraction (B3) by right-side-out

TABLE I

SPECIFIC CONTENT OF PLASTOCYANIN IN CLASS II CHLOROPLASTS AND THYLAKOID VESICLES OF OPPOSITE SIDEDNESS

Plastocyanin was determined by rocket electroimmunodiffusion. The rocket length is normalized to the chlorophyll content of the added vesicles. A, with 0.4 ml antiserum 146 and B, with 0.1 ml antiserum 147 per 10 ml agarose gel; a rocket length of 24 and 46 mm, respectively, was observed with class II chloroplasts containing 3.76 μg Chl. Preparation method H involves Yeda press treatment of class II chloroplasts in the presence of 150 mM NaCl, whereas preparation method L involves unstacking of the membranes at low salt concentrations prior to the disruption (for details see Materials and Methods).

Preparation	Relative plastocyanin content (arbitrary units: mm rocket length/μg Chl)			
	Preparation method H		Preparation method L	
	A	B	A	B
Class II chloroplasts	6.38 (100%)	12.23 (100%)	5.71 (100%)	13.73 (100%)
(100k pellet)	3.45 (54%)	7.50 (61%)	– –	– –
Right-side-out vesicle fraction (T2)	1.52 (24%)	3.87 (32%)	1.14 (20%)	2.00 (15%)
Inside-out vesicle fraction (B3)	0.33 (5%)	0.54 (4%)	0.37 (6%)	0.56 (4%)

vesicles [28] has to be taken into account. We assumed the same contamination of our fraction obtained by preparation method H by 25% of right-side-out vesicles as determined previously from freeze-fracture studies [28]. The specific plastocyanin content of these contaminating vesicles is probably close to 1.52 as determined for our right-side-out vesicle fraction. These figures allow us to estimate the contribution of right-side-out vesicles to the specific plastocyanin content of our inside-out vesicle fraction to be 0.38 (0.25 × 1.52). This is very close to 0.33 determined for this fraction (Table I). Therefore, we

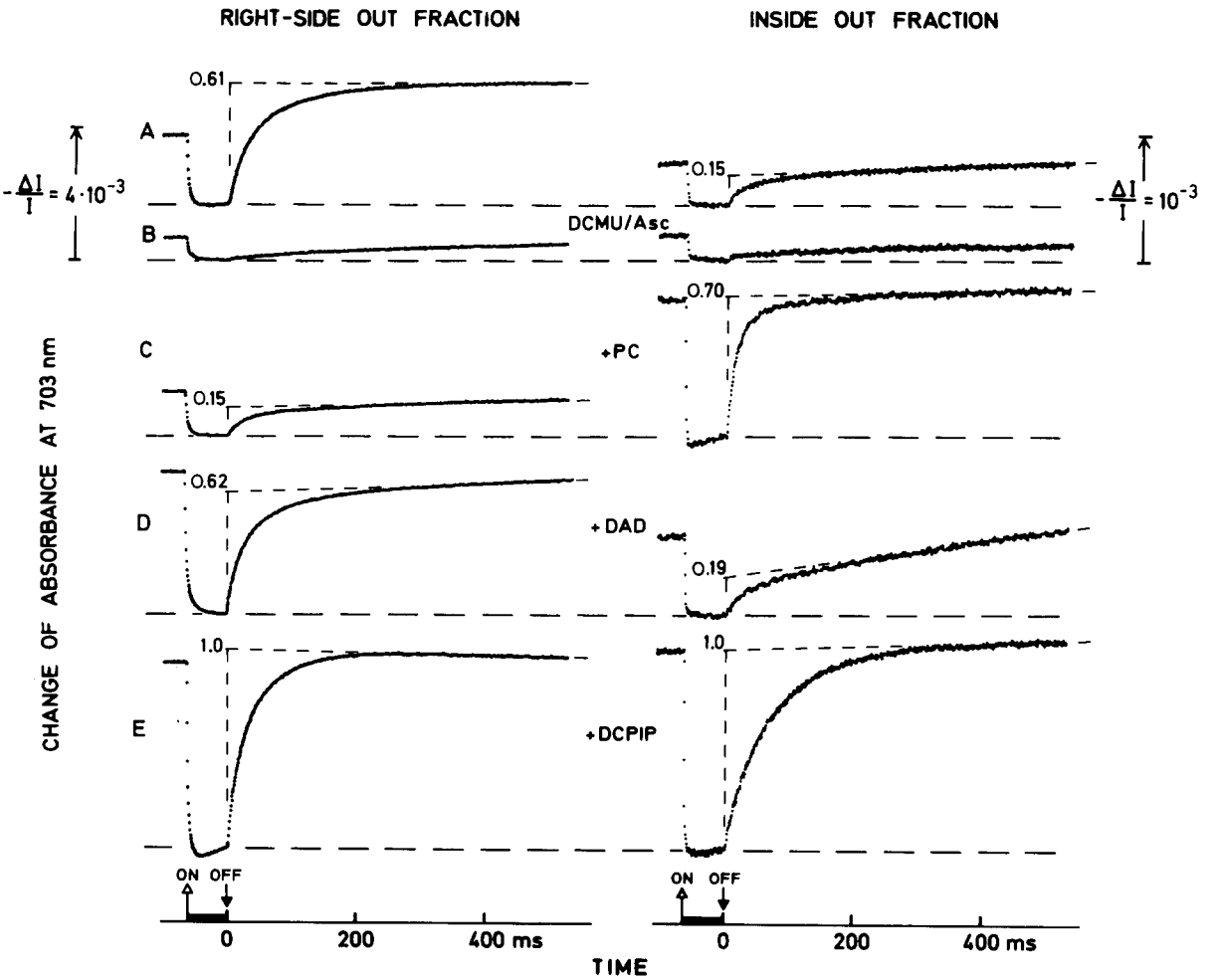

Fig. 2. Absorbance changes of *P*-700 induced by excitation with long flashes of saturating intensity of vesicles obtained by preparation method L. (Left) Right-side-out vesicle fraction (T2) at a chlorophyll concentration of about 30 μg/ml. (Right) Inside-out vesicle fraction (B3) at a chlorophyll concentration of about 8 μg per ml; approx. 4% of dextran, 7 mM sucrose and 3.3 mM phosphate buffer were introduced from the bottom phase (B3). To facilitate direct comparison of vesicle properties, the signals were normalized by a factor to equalize the amplitudes in the presence of 2,6-dichlorophenolindophenol. The high noise level of the right-hand traces is due to the low chlorophyll concentration. The two different scales refer to the amplitude of the left- and the right-hand transients, respectively. The transients are the average of five signals induced by blue flashes (Schott filter BG 23/6 mm) of 67 ms duration and an intensity of 340 W/m^2 at a repetition rate of 0.2 Hz. (A) The measuring solution contained 0.1 mM 9,10-anthraquinone-2-sulfonate as electron acceptor in addition to 20 mM Tricine-NaOH-buffer, pH 7.6, and 20 mM KCl. (B) Solution of A plus 17 μM DCMU and 1 mM sodium ascorbate. (C) Solution of B plus 0.62 μM plastocyanin. (D) Solution of B plus 0.1 mM DAD. (E) Solution of B plus 0.1 mM DAD and 0.1 mM 2,6-dichlorophenolindophenol. Asc, sodium ascrobate; DCPIP, 2,6-dichlorophenolindophenol; PC, plastocyanin.

conclude that inside-out vesicles lose their plastocyanin completely during the isolation procedure.

Reduction kinetics of P-700

Another approach to the location of plastocyanin is measurement of the reduction kinetics of *P*-700, which should depend on the accessibility of the donor side of PS I to plastocyanin. Fig. 2 shows the kinetics of absorbance changes of *P*-700 under various conditions in the right-side-out vesicle fraction (left-hand side) and in the inside-out vesicle fraction (right-hand side). During the long flash of saturating intensity *P*-700 is completely oxidized. The subsequent absorbance increase in the dark is due to the reduction of $P\text{-}700^+$. The amplitude of the fast phase of reduction was used to characterize the accessibility of *P*-700 to plastocyanin. The level of reduced *P*-700 at the onset of the long flash is determined by either the amount of electrons accumulated during the flash (Fig. 2A) [8] or the reduction rate in the dark (Fig. 2B–E) as well as by the intensity of the far-red monitoring light during the periodic dark time of 5 s between flashes.

The membrane-permeable reduced 2,6-dichlorophenolindophenol reduces $P\text{-}700^+$ directly [52], and therefore enables an estimation of total *P*-700 in the vesicle fractions independently of the presence of plastocyanin and the sidedness of the vesicles. Fig. 2E shows the corresponding signals in the two fractions. Their amplitudes were used to determine the relative amplitudes of the other signals. It should be mentioned that in the absence of an electron acceptor, the turnover of *P*-700 during the long flash was not limited by the Mehler's reaction [53] in the vesicles in contrast to chloroplasts (signals not shown).

Fig. 2C shows the effect of plastocyanin added to vesicle fractions in the presence of DCMU and ascorbate (cf. Fig. 2B). It induced a rapid reduction of only 15% of total $P\text{-}700^+$ in the right-side-out vesicle fraction as compared to 70% in the inside-out vesicle fraction. The difference clearly demonstrates the accessibility of the donor site of *P*-700 and its reaction with plastocyanin in the inside-out thylakoid fraction. Variation of the amount of added plastocyanin caused changes of the half-time of the rapid reduction, but did not change the amplitude.

Some plastocyanin is retained by right-side-out vesicles as reported above. If this plastocyanin is active it should enable fast reduction of $P\text{-}700^+$. Selective reduction of plastocyanin is possible by DAD which effectively donates electrons to oxidized plastocyanin but not to $P\text{-}700^+$ [4]. The time courses in Fig. 2D show that addition of DAD in the presence of DCMU and ascorbate (cf. Fig. 2B) causes fast reduction of 62 and 19% of total *P*-700 in the right-side-out and inside-out vesicle fraction, respectively.

These values are strikingly similar to those found for linear electron transport from water to *P*-700, as shown in Fig. 2A. The signals in Fig. 2A were measured in the absence of an external electron donor. They show in the right-side-out and inside-out vesicle fraction a reduction of 61 and 15%, respectively, of

TABLE II

PROPORTION OF $P\text{-}700^+$ REDUCED UNDER VARIOUS CONDITIONS IN THYLAKOID VESICLES OF OPPOSITE SIDEDNESS

Experimental conditions during the measurement of the absorbance changes at 703 nm and evaluation of the traces as in Fig. 2A, D and C, respectively. The numbers give the relative amplitudes in per cent related to the amplitudes found in the presence of 2,6-dichlorophenolindophenol and ascorbate (cf. Fig. 2E). The values of control chloroplasts were found in class II as well as in randomized unstacked thylakoids after low-salt treatment (method L). Addition of plastocyanin to these chloroplasts in the presence of DCMU and ascorbate did not induce fast reduction of *P*-700 but increased slightly the amplitude of the very slow reduction (cf. Fig. 2B) from about 7 to 11% of total *P*-700.

Rapid reduction of $P\text{-}700^+$ by	Control chloroplasts	Right-side-out vesicle fraction (T2)	Inside-out vesicle fraction (B3)
Active PSII	88	61; 56	15; 10; 18
DAD via residual plastocyanin	100	62; 67	19; 10
Added plastocyanin	0	15; 15	70; 68; 67

total $P\text{-}700^+$ by electrons accumulated between the two photoreactions during the long flash. The half-time of 30–50 ms of the reduction kinetics is considerably longer than that of 5–10 ms found in uncoupled chloroplasts (not shown, cf. Refs. 3 and 8). However, the inhibition by DCMU shown in Fig. 2B demonstrates that $P\text{-}700^+$ is reduced by electrons originating from PS II. The corresponding amount in our class II chloroplasts was 85–89% of total $P\text{-}700^+$ (Table II), in agreement with previous measurements [54]. Table II summarizes the results from several preparations, and shows good reproducibility of preparation method L [32] (see Materials and Methods).

The same observations concerning the different accessibility of *P*-700 to endogenous as well as externally added plastocyanin were made with right-side-out and inside-out vesicle fractions obtained by preparation method H [25]. However, the reproducibility of the values was not as good as that shown in Table II. This may be caused by variations in the purity of the inside-out vesicle fraction, and also by its high proportion of concavo-convex vesicles [28] hiding some inner surface from the surrounding.

Discussion

Previous results with antibodies against plastocyanin [13,19,20,55,56] as well as with chemical probes [21–24,33] have led to diverging conclusions about the location of plastocyanin in the thylakoid membrane. The conclusions were based on the assumption that these reagents do not permeate through membranes, which should be true particularly for large and hydrophobic antibodies. Therefore, we have reinvestigated the inhibiting and agglutinating effects of antibodies against plastocyanin in addition to two new approaches to the sidedness of plastocyanin.

The effects of antibodies against plastocyanin on photosynthetic reactions

Measurements of electron transport from DAD through PS I or of the associated phosphorylation can be used as tests of the activity of plastocyanin in situ [4,15]. We found slight stimulation of phosphorylation, in agreement with the results of Schmid et al. [19], with four of our antisera against plastocyanin as well as with two control sera. These results are not consistent with inhibition of plastocyanin in situ by antibodies. The stimulation of phosphorylation by all sera tested could arise from a decrease in the membrane permeability for protons.

In contrast to phosphorylation, electron transport from DAD through PS I has been inhibited by some antisera to a maximal extent of 40–50% [19,20] but not by other antisera against plastocyanin [13,55]. All of our four antisera had a high titer of precipitating antibodies against soluble plastocyanin. However, only one inhibited the electron transport rate up to 40%, in agreement with the previous results. This inhibition of electron transport together with simultaneous stimulation of phosphorylation has been ascribed to some external plastocyanin [13,55], which may be released by damaged chloroplast thylakoids [55,57]. The external plastocyanin was assumed to mediate a noncoupled artificial [58,55] electron transport competitive [19] with the coupled one via internal plastocyanin. Inhibition of external plastocyanin by antibodies would decrease the electron-transport rate but simultaneously increase the coupled one. However, we found that plastocyanin reacts only with *P*-700 in inside-out but not with *P*-700 in right-side-out vesicles or unbroken thylakoids (Table II). This allows us to exclude the possibility of such a competitive electron transport to *P*-700 from both sides of the thylakoid membrane in the same vesicle. Almost the same arguments hold for open appressed membrane pairs, e.g., at the end of grana stacks observed after osmotic shock of chloroplasts [59], after French press treatment [28,32,60] and also in vivo during chloroplast development [61]. *P*-700 was not reached by externally added plastocyanin in our unbroken thylakoids (Table II).

The amount of antiserum 146, necessary to induce some inhibition of electron transport, varied greatly and exceeded the plastocyanin content of the chloroplasts. This and the simultaneous stimulation of phosphorylation indicate that we do not fully understand the effects of serum components on chloroplast membranes. Modification of a component of the outer surface may, for example, lead to changes of the internal catalytic sites [35]. The same may be true for some other membrane-impermeable reagents.

Plastocyanin content of vesicles of opposite sidedness

The plastocyanin content of vesicles of opposite

sidedness is expected to provide evidence for the location of plastocyanin. We used the method of rocket electroimmunodiffusion, which allows quantitative determination of total plastocyanin irrespective of its activity or purity. Although the disruption of thylakoids used for preparation of the vesicles was carried out in the presence of an inert gas instead of compressed air and at a pressure 5–10-times lower than that used previously [49], only 25% of total plastocyanin was retained in the vesicles. We found that this plastocyanin was exclusively trapped in right-side-out vesicles. According to the size of the vesicles, the thylakoid membranes open up during the press treatment and reseal either as right-side-out or as inside-out vesicles [28,31,32]. It is probably during the transient open stage until the membranes reseal to right-side-out vesicles when about 70% of their plastocyanin content is lost (Table I). The low amount of plastocyanin in the inside-out vesicle fraction (Table I) originates from contamination with right-side-out vesicles. These results provide evidence for the location of plastocyanin inside the thylakoids where it may be free or loosely bound to the inner thylakoid surface. Recent results on the properties of the electron transfer from plastocyanin to P-700 in chloroplasts [62] are consistent with this conclusion. Location of plastocyanin in a small hydrophilic cleft at the outside of the thylakoid membrane [23] should have resulted in retention of plastocyanin by inside-out vesicles and is therefore not compatible with our result.

Agglutination studies with plastocyanin antibodies

Our attempts to agglutinate unbroken thylakoids with dilute antisera against plastocyanin yielded negative results in agreement with some earlier findings [13,55]. Negative results are also found with vesicles of opposite sidedness. For inside-out vesicles this can be ascribed to the absence of plastocyanin. The weak agglutination observed immediately after preparation of the inside-out vesicles indicates loose binding of plastocyanin to the original inside of the thylakoid. In contrast, the failure to agglutinate right-side-out vesicles indicates that the retained plastocyanin is not accessible to antibodies from the outer surface. These observations are consistent with location of plastocyanin inside the thylakoids.

Agglutination of intact chloroplasts by antibodies against plastocyanin has been previously observed [20] but was ascribed to unspecific binding of antibodies to the envelope of the chloroplasts [55]. With isolated thylakoid membranes precipitation of antibodies against plastocyanin in amounts equivalent to total plastocyanin has been shown [56] in contrast to our results. However, this agglutination was reported to depend on the preparation method of the thylakoids [19] and was found under conditions for maximal precipitation which do not allow exclusion with certainty of the possibility of membrane disturbance and thylakoid breakage. If conclusions concerning the location of membrane constituents are the aim of the experiment, agglutination tests should be done on microscope slides and the results judged in the microscope within 1–2 min [35]. During investigations of the transverse organization of the membrane with other nonpenetrating reagents the prerequisite impermeability throughout the incubation may also not be fulfilled under all conditions.

Accessibility of P-700 to plastocyanin in vesicles of opposite sidedness

Plastocyanin is a hydrophilic negatively charged protein at physiological pH values [34]. Therefore, it is ideal as a membrane-impermeable probe for the sidedness of its reaction site at the negatively charged thylakoid membrane [63,64]. Our results show that $P\text{-}700^+$ is reduced by added plastocyanin only in inside-out, but not in right-side-out vesicles (Fig. 2C) or in class II chloroplasts (Table II). The latter has been shown previously [49,65]. The minor effect observed with the right-side-out vesicle fraction is consistent with contamination with inside-out vesicles [28]. These results prove that the reaction site of plastocyanin is at the inside of the thylakoids. The orientation of PS I in the membrane is obviously not disturbed during the press treatment and separation of the vesicles. In contrast to this treatment, the addition of digitonin (data not shown) to chloroplasts makes most acceptor sites of *P*-700 accessible to added plastocyanin according to experiments carried out such as those in Fig. 2.

The right-side-out vesicle fraction obtained after membrane pairing at pH 4.7 [30] did not retain more than 20% of its original content of plastocyanin (method L, Table I). Nevertheless, about 60% of total $P\text{-}700^+$ was reduced by electrons from water in these

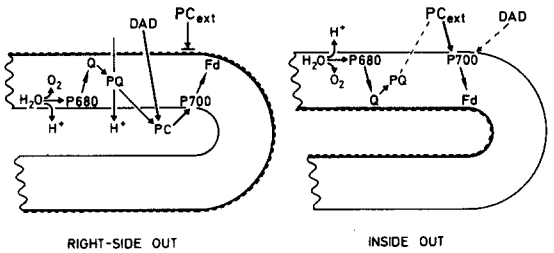

Fig. 3. Scheme of the electron pathways via plastocyanin to *P*-700 in right-side-out (left) and inside-out (right) vesicles. For details see text. PC_{ext}, externally added, reduced plastocyanin; PQ, plastoquinone; Q and Fd, primary electron acceptor of photoreaction II and I, respectively.

vesicles (Table II). The amount of reduced *P*-700$^+$ did not increase if electrons were supplied via DAD. This indicates that the same amount of plastocyanin reducing *P*-700$^+$ directly mediates also the electron flow between the two photoreactions in right-side-out vesicles. Fig. 3 outlines a scheme of the reactions. With respect to its decreased amount, plastocyanin has to funtion as a pool common to several Photosystems I and II in these vesicles. This supports previous evidence of interaction between PS I via plastocyanin in undisturbed chloroplast lamellae [62,66].

In conclusion, both the immunological and the kinetic studies with class II chloroplasts and in particular with vesicles of opposite sidedness localize the functional site of plastocyanin at the inner side of the thylakoid membrane.

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