Fast Electron Transfer from Cytochrome c_6 and Plastocyanin to Photosystem I of *Chlamydomonas reinhardtii* Requires PsaF[†]

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ABSTRACT: To study the function of the PsaF subunit of photosystem I (PSI), the interactions between plastocyanin or cytochrome c_6 and PSI isolated from wild-type and a PsaF-deficient mutant of *Chlamydomonas reinhardtii* have been examined using cross-linking techniques and flash absorption spectroscopy. We show that efficient electron transfer from both plastocyanin and cytochrome c_6 to PSI depends on PsaF. A remarkable feature of the PSI complex of *C. reinhardtii* is that both plastocyanin and cytochrome c_6 reduce $P700^+$ with first-order kinetics and a half-time of 3 μs, which is unique among photosynthetic organisms examined.

The photosystem I (PSI)[1] complex functions as a light-driven oxidoreductase that transfers electrons from plastocyanin to ferredoxin in higher plants, most algae, and cyanobacteria. In some cyanobacteria and algae, the type I copper protein plastocyanin can be replaced by a class I *c*-type cytochrome, depending on the relative availability of copper and iron in the culture medium (Wood, 1978; Ho & Krogmann, 1984; Sandmann, 1986; Merchant & Bogorad, 1986). The PSI reaction center is a membrane-bound complex consisting of 13−14 polypeptide subunits. The three-dimensional structure of PSI from the cyanobacterium *Synechococcus elongatus* has been determined by X-ray crystallography at a resolution of 6 Å (Krauss *et al.*, 1993) and more recently of 4 Å (Krauss *et al.*, 1996). P700 of PSI is localized near the lumenal surface of the thylakoid membrane and is therefore accessible to the lumenal electron donor proteins plastocyanin and cytochrome c_6. Cross-linking results suggest that PsaF is involved in docking of plastocyanin and cytochrome c_6 to the PSI complex (Wynn & Malkin, 1988; Wynn *et al.*, 1989; Hippler *et al.*, 1989). The conformations of the cross-linked and authentic plastocyanin−PSI complexes appear to be similar based on the fast kinetics of reduction of $P700^+$ with a half-time of 13−15 μs observed in the cross-linked complex (Hippler *et al.*, 1989). This half-time is comparable to that found in intact thylakoids (Haehnel & Witt, 1971; Haehnel *et al.*, 1989), with digitonin−PSI particles (Bottin & Mathis, 1985, 1987), and with PSI-200 particles (Drepper *et al.*, 1996) at high plastocyanin concentrations.

The function of the PsaF subunit in PSI remains elusive [see Golbeck (1992)]. This subunit was found to be associated with the LHCI complex of PSI (Anandan *et al.*, 1989; Bassi *et al.*, 1992; Scheller & Møller, 1990). Removal of the PsaF subunit from a plant-derived PSI complex by mild detergent treatment impairs the electron transfer from plastocyanin to PSI (Bengis & Nelson, 1977). Surprisingly, the specific deletion of the *psaF* gene in cyanobacteria did not affect photoautotrophic growth (Chitnis *et al.*, 1991), and the *in vivo* measured electron transfer rate between cytochrome c_{553} and PSI was the same as in wild-type (Xu *et al.*, 1994). In contrast, the electron transfer reaction between plastocyanin and $P700^+$ was shown to be considerably reduced within whole cells of the 3bF strain of *Chlamydomonas reinhardtii* which lacks the *psaF* gene (Farah *et al.*, 1995). Mass spectroscopic analysis of tryptic peptides of plastocyanin and of the cross-linked product of plastocyanin and PsaF from spinach revealed that the PsaF subunit appears to be cross-linked with one of its N-terminal Lys residues to the conserved acidic amino acids 42−44 and 59−61 of plastocyanin (Hippler *et al.*, 1996). A region close to the N-terminal end of PsaF could form an amphipathic α-helix, whose positively charged face may interact with plastocyanin (Hippler *et al.*, 1996).

The half-time of fast electron transfer between plastocyanin and PSI in the unicellular alga *Chlorella* was measured to be 4 μs (Delosme, 1991), whereas no corresponding fast phase could be observed in the cyanobacterium *Synechocystis* (Hervàs *et al.*, 1994) and in the thermophilic cyanobacterium *Synechococcus elongatus* (Hatanaka *et al.*, 1993). Since the PsaF subunits of these cyanobacteria lack the positively charged N-proximal region [for amino acid sequences, see Hippler *et al.* (1996)], the question arises whether this segment evolved to allow fast electron transfer between plastocyanin and PSI in higher plants and algae.

The electron transfer from cytochrome c_6 to $P700^+$ was found to display a first-order kinetic component with a half-time of 8 μs in the green alga *Monoraphidium braunii* and of 4 μs in the cyanobacterium *Anabaena sp.* PCC7119, whereas no first-order microsecond phase could be detected

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[1] Abbreviations: chl, chlorophyll; cytc, cytochrome c_6; FWHM, full width at half-maximum; Mops, 3-(*N*-morpholino)propanesulfonic acid; pc, plastocyanin; PSI, photosystem I.

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when plastocyanin was the electron donor (Hervàs *et al.*, 1995). In the cyanobacterium *Anabaena sp.* PCC7119, cytochrome c_6 and plastocyanin are positively charged with an isoelectric point of 9 (Medina *et al.*, 1993). In contrast, these two proteins have acidic isoelectric points (ranging from 3.7 to 5.6) in *Synechocystis*, *Chlamydomonas*, and *Monoraphidium* (Hervàs *et al.*, 1994; Kerfeld *et al.*, 1995; Campos *et al.*, 1993).

Here, we have examined the interaction of cytochrome c_6 and plastocyanin with PSI from wild-type and a PsaF-deficient mutant of *C. reinhardtii*. The kinetics of PSI reduction reveal a fast phase with a half-time of 3 μs for both electron donor proteins. This fast phase depends on the PsaF subunit since the deletion of PsaF results in a strong decrease of the rates of electron transfer from plastocyanin and cytochrome c_6 to $P700^+$.

EXPERIMENTAL PROCEDURES

Strains and Media. *C. reinhardtii* wild-type and mutant strains were grown as decribed (Harris, 1989). Tris−acetate−phosphate medium (TAP) was soldified with 2% Bacto agar (Difco).

Isolation of Plastocyanin and Cytochrome c_6. The isolation of plastocyanin and cytochrome c_6 followed the prodecure of Merchant and Bogorad (1986) and Kerfeld *et al.* (1995), respectively, with modifications. Both donor proteins were purified from 15 L of a *C. reinhardtii* cell culture, grown mixotrophically in TAP medium in the presence of copper for plastocyanin or without copper for cytochrome c_6 as described in Harris (1989). As a second anion exchange column, a Poros HQ column, equilibrated with 20 mM Tricine, pH 7.8, 10 mM KCl, was used in the purification protocol of cytochrome c_6. Cytochrome c_6 was retained on the column and was eluted by a NaCl gradient (50−300 mM). The cytochrome c_6 containing fractions were pooled and concentrated on an Amicon YM 3 membrane and further purified on a Sephacryl S200 column (60 × 1.5 cm). This step was repeated. The last step in the purification protocol of plastocyanin also included a Sephacryl S200 rather than a Sephadex G50 column. The concentrations of plastocyanin and cytochrome c_6 were determined spectroscopically using an extinction coefficient of 4.9 mM^{-1} cm^{-1} at 597 nm for the oxidized form of plastocyanin (Katoh *et al.*, 1962) and 20 mM^{-1} cm^{-1} at 552 nm for the reduced form of cytochrome c_6 (Wood, 1978).

Isolation of Thylakoid Membranes and of the PSI Complex. Cell-wall-deficient wild-type and mutant cells were broken by passage through a "BioNeb" cell disruptor (Glas-Col) applying a pressure of 15 psi from a nitrogen tank. The isolation of thylakoids purified by centrifugation through a sucrose step gradient followed published procedures (Chua & Bennoun, 1975). The thylakoid membranes were diluted with H_2O, pelleted by centrifugation at 20000*g*, and resuspended in H_2O at a chlorophyll concentration of 0.8 mg/mL. The membranes were solubilized with 0.9% (w/v) dodecyl β-maltoside on ice for 20 min and then centrifuged to remove the undissolved membranes. The PSI extracts were loaded on a sucrose density gradient and centrifuged as described (Takahashi *et al.*, 1991). The lower bands containing the PSI particles were collected and diluted 3 times (20 mM Hepes, pH 7.5). The PSI particles were pelleted by centrifugation at 250000*g* for 3 h and resuspended in 20 mM Hepes, pH 7.5. A further solubilization of the PSI particles at a concentration of 0.5 mg of chl/mL was achieved with 0.15% (w/v) dodecyl β-maltoside and 0.2% (w/v) Zwittergent 16 on ice for 1 h. The solubilized particles were loaded on a 0.4−1.0 M sucrose density gradient, containing 0.05% (w/v) dodecyl β-maltoside, and centrifuged at 200000*g* in a TI60 (Beckman) fixed-angle rotor for 16 h. The lower bands were collected and concentrated as described above for the PSI particles. Chl concentrations were determined according to Porra *et al.*, (1989).

SDS−PAGE and Western Analysis. SDS−PAGE (15.5% T, 2.66% C) was carried out according to Laemmli (1970). After the electrophoretic fractionation, the proteins were electroblotted onto nitrocellulose and incubated with antibodies as described (Hippler *et al.*, 1989). Plastocyanin and cytochrome c_6 antibodies were a kind gift from S. Merchant, and the PsaA antibody was a kind gift from K. Redding. The membrane was immunodecorated with a donkey anti-rabbit IgG−horseradish peroxidase, and the immune complexes were detected by enhanced chemiluminescence using the ECL kit (Amersham).

Cross-Linking Procedure. PSI particles at a concentration of 0.1 mg of chl/mL were cross-linked with 20 μM plastocyanin or cytochrome c_6 in 30 mM Hepes, pH 7.5, 3 mM $MgCl_2$, and 1 mM ascorbate, using 5 mM EDC (*N*-ethyl-3-[3-(dimethylamino)propyl]carbodiimide) and 10 mM sulfo-NHS (*N*-hydroxysulfosuccinimide). The solution was incubated for 45 min in the dark. The reaction was terminated by adding ammonium acetate to a final concentration of 0.2 M, and then diluted 50-fold and pelleted at 200000*g*. for 45 min. The cross-linked particles were resuspended in 20 mM Hepes, pH 7.5, 0.05% Triton X-100.

Flash Absorption Spectroscopy. Flash-induced absorbance changes were measured in a single-beam spectrophotometer. The measuring light from a 100 W tungsten−halogen lamp was passed through a 820 nm interference filter (8 nm FWHM) and a Schott RG 780 filter before the cuvette (path length 1.2 mm) holding the sample. An identical pair of filters was used to protect the detecting silicone photodiode (1 cm^2) from fluorescence light. The output of the photodiode was amplified with an electrical bandwidth ranging from direct current to 0.1 or 1 MHz with direct current offset compensation. Flash excitation was provided by a ruby laser (695 nm, 25 ns FWHM). In all experiments, flashes of near-saturating intensity were used. Signals were recorded with a Tektronix (RTD) digitizer. The 2048 channels of the data memory were partitioned into segments with different sampling rates in order to resolve the kinetics in the microsecond and millisecond time range. When signal averaging was used, the time interval for dark adaptation of the sample between successive flashes was chosen sufficiently long to allow a complete return to equilibrium and is given in the figure legends. A total of 2−16 individual signals were averaged after inspection of kinetic traces for uniformity. A sum of two or three single-exponential components was determined as the least-squares fit to the measured absorbance transients using a modified Marquardt algorithm installed by Dr. P. Sétif.

Oxidized plastocyanin has an absorption band at 770 nm which yields an extinction coefficient of 1200 cm^{-1} M^{-1} at 820 nm (Katoh *et al.*, 1962), that is, 18% of that of P700 at 820 nm (ϵ = 6400 M^{-1} cm^{-1}, determined for P700 from spinach; Hiyama & Ke, 1972). Plastocyanin, oxidized by the reduction of $P700^+$, is very slowly reduced by ascorbate

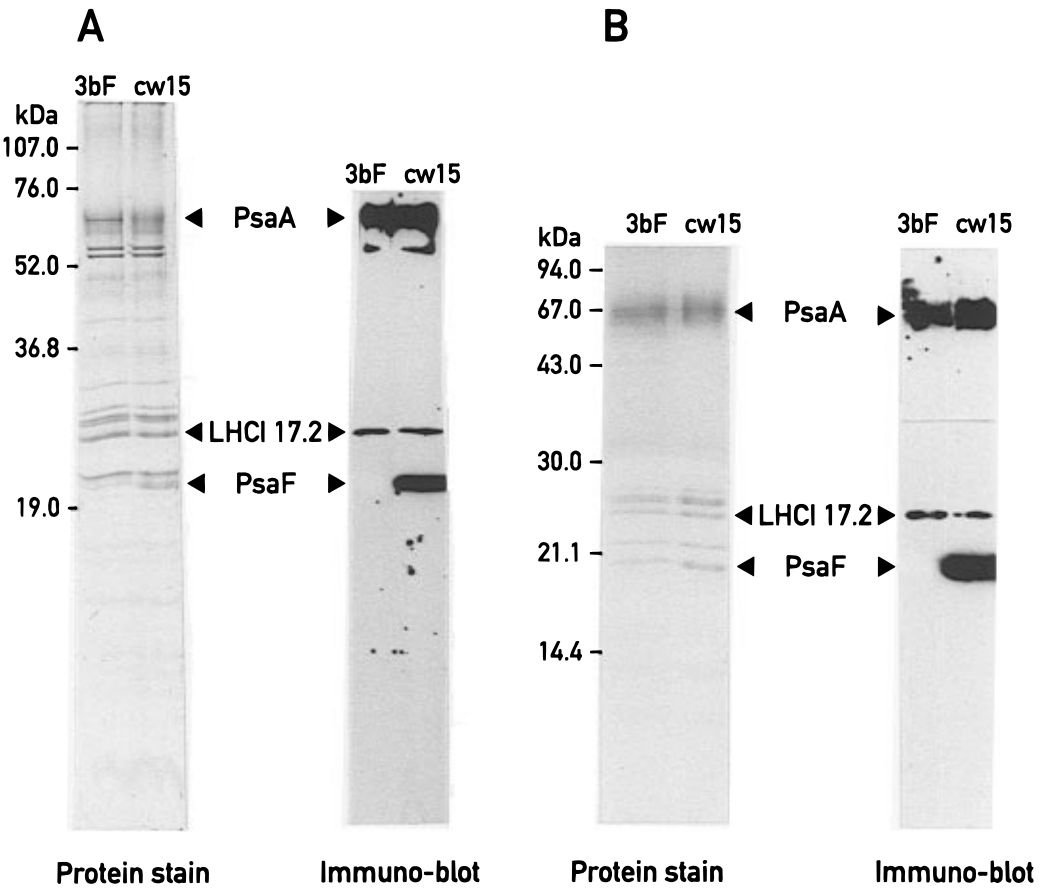

FIGURE 1: Purified PSI complex from the PsaF-deficient mutant lacks only the PsaF subunit. SDS−PAGE analysis of PSI particles from wild-type and the 3bF mutant strains. (A) Isolated PSI particles from solubilized thylakoid membanes. (B) PSI particles solubilized with 0.15% (w/v) dodecyl β-maltoside and 0.2% (w/v) Zwittergent 16. 2 μg of chl was loaded in each lane. The molar ratio of chl *a*/chl *b* was 7.0 and 6.5 for wild-type and 3bF, respectively. Protein staining was performed with Coomassie brillant blue. The protein blot was probed with anti-PsaA, anti-LHCI 17.2, and anti-PsaF antibodies.

and will contribute to the slower phase of absorption recovery (Katoh, 1979). This is taken into account for the evaluation of the kinetic data.

RESULTS

Isolation of a Functional PSI Complex from the 3bF Mutant Lacking PsaF. The isolation of a functional PSI complex from the 3bF mutant is a prerequisite for a detailed *in vitro* study of the electron transfer from plastocyanin and cytochrome c_6 to PSI. The isolation of an enriched PSI fraction from the mutant was achieved using mild cell breakage followed by the isolation of thylakoids and subsequent detergent solubilization (Figure 1A) These PSI particles were used for cross-linking experiments and electron transfer measurements since the photochemical properties of the PSI complex are impaired after further purification (N. Fischer, P. Sétif, and J. D. Rochaix, personal communication). In order to compare the stability of the PSI complex from wild-type and the PsaF-deficient mutant, the PSI particles were solubilized further with low detergent concentrations. The subsequent fractionation by sucrose density gradient centrifugation removed impurities from the isolated PSI complex (Figure 1B). Comparison of the protein patterns of the PSI particles from wild-type (CW15) and the 3bF mutant by SDS−PAGE revealed that they are similar, except for a band with a molecular mass of 20 kDa, which is missing in the mutant PSI complex (Figure 1A, B). Western analysis, using anti-PsaF antibodies, demonstrated that this band represents, as expected, the PsaF subunit of PSI. Further analysis with antibodies against PsaA, one of the large PSI reaction center subunits, and against the LHCI subunit 17.2 (Bassi *et al.*, 1992) showed that these polypeptides are present at the same level in the purified wild-type and mutant PSI particles (Figure 1A,B). A similar result was obtained with another LHCI polypeptide, subunit 18 (data not shown). Immunoblots performed with antibodies against the PSI subunits PsaD, -C, and -E (data not shown) further confirmed that the subunit composition in wild-type and mutant PSI particles is the same except for the lack of PsaF in the latter. Since the amount of LHCI subunits bound to the wild-type and mutant PSI complex is the same, we conclude that PsaF is not required for stable binding of LHCI to the PSI complex.

Cross-Linking of Plastocyanin or Cytochrome c_6 to PSI Depends on PsaF. To examine the role of the PsaF subunit in the interactions between plastocyanin or cytochrome c_6 and PSI, cross-linking experiments were performed. Purified plastocyanin and cytochrome c_6 were incubated separately with wild-type and mutant PSI complexes in the presence of the cross-linking reagents EDC and NHS. After cross-linking the products were fractionated by SDS−PAGE and identified by immunoblotting using antibodies against plastocyanin, cytochrome c_6, and PsaF. The results obtained with wild-type PSI complex demonstrate that PsaF can be cross-linked with plastocyanin (Figure 2, lanes 3 and 7) as well as with cytochrome c_6 (Figure 2, lanes 1 and 6), resulting in

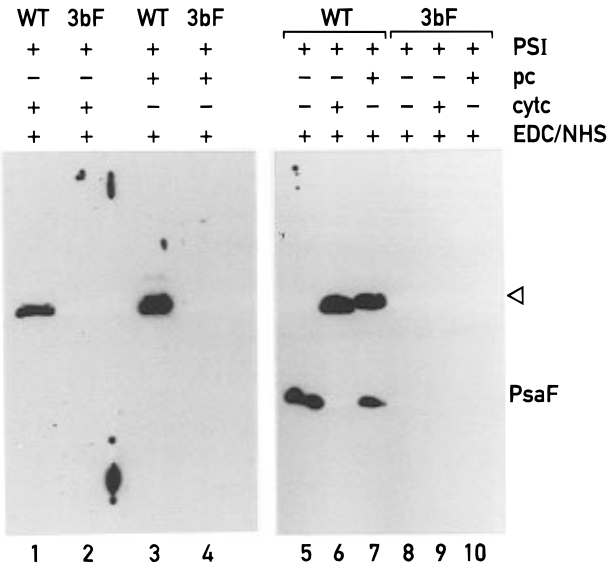

FIGURE 2: Plastocyanin and cytochrome c_6 can be cross-linked to the PSI complex from the wild-type, but not from the 3bF mutant strain. 2 μg of chl was loaded in each lane. Soluble plastocyanin and cytochrome c_6 were removed by centrifugation from the cross-linked PSI particles. After SDS−PAGE, the gel was immunoblotted. The antibodies used were anti-cytochrome c_6 (lanes 1 and 2), anti-plastocyanin (lanes 3 and 4), and anti-PsaF antibodies (lanes 5−10).

new bands with a molecular mass of 29 kDa and of 28.5 kDa, respectively, indicating a 1:1 stoichiometry between PsaF and plastocyanin or cytochrome c_6. While no free PsaF is detected after cross-linking with cytochrome c_6 (Figure 2, lane 6), not all of PsaF is cross-linked in the assay with plastocyanin (Figure 2, lane 7), indicating that the cross-linking of PsaF with cytochrome c_6 is more efficient than with plastocyanin. As a control, only free PsaF was detected in the wild-type PSI complex incubated in the absence of plastocyanin and cytochrome c_6 with the cross-linking reagents (Figure 2, lane 5).

In contrast, no cross-linking could be obtained with the PSI complex from the PsaF-deficient strain (Figure 2, lanes 2, 4, 8, 9, 10). The observation that neither plastocyanin nor cytochrome c_6 reacted with other polypeptides of the PSI complex from 3bF during the cross-linking procedure demonstrates the specificity of the interaction between PsaF and the two electron donor proteins.

Efficient Electron Transfer from Plastocyanin or Cytochrome c_6 to P700 Requires the Presence of the PsaF Subunit of PSI. Laser flash spectroscopy was used to investigate the electron transfer from plastocyanin or cytochrome c_6 to PSI *in vitro*. Figure 3 shows absorbance transients at 820 nm of PSI particles from wild-type (A and C) and the PsaF-deficient mutant (B and D) induced by a laser flash in the presence of 5 μM plastocyanin (Figure 3A,B) or 5 μM cytochrome c_6 (Figure 3C,D). The half-times of the dominant kinetic phase of the electron transfer from plastocyanin and from cytochrome c_6 to $P700^+$ of 3.6 ms and 3.7 ms, respectively, in wild-type are increased in the PsaF-deficient mutant to 290 ms and 126 ms, respectively. Hence, in the absence of PsaF, the second-order rate constants of electron transfer are diminished 80- and 34-fold for plastocyanin and cytochrome c_6, respectively. These differences are even more pronounced at lower salt concentrations, but disappear at high concentrations of $MgCl_2$ (see Figure 4). The absence of PsaF appears to have a stronger effect on the interaction

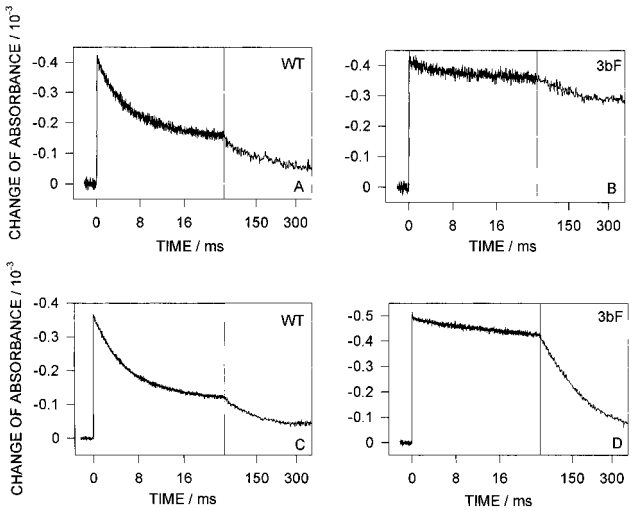

FIGURE 3: Electron transfer from plastocyanin or cytochrome c_6 to P700 is slower in the 3bF mutant. Absorbance transients at 820 nm induced by a laser flash in PSI particles from wild-type (A and C) and 3bF (B and D) in the presence of soluble 5 μM plastocyanin (A and B) and 5 μM cytochrome c_6 (C and D). The PSI particles had molar ratios of chl *a*/chl *b* of 8.5 and 6.5 and chl/P700 ratios of 170 and 192 (estimated by flash-induced absorbance changes, using an extinction coeficient of P700 at 820 nm, determined for spinach; see Experimental Procedures) for wild-type and 3bF, respectively. The cuvette contained PSI particles at 90 μg of chl/mL in 10 mM $MgCl_2$, 30 mM Mops, pH 7.0, 0.1 mM methylviologen, 1 mM ascorbate, 0.02 mM diaminoduorene (DAD), and 0.05% (w/v) dodecyl β-maltoside. The absorbance transients are the result of two flashes. The solid vertical lines separate regions of the time axis, recorded with different sampling rates.

between PSI and plastocyanin than between PSI and cytochrome c_6. The slow kinetics of electron transfer from plastocyanin and cytochrome c_6 to $P700^+$ from the PsaF-deficient mutant indicate also that stable charge separation occurred in these PSI particles, since the half-time for the back electron transfer from the internal iron−sulfur cluster F_X to $P700^+$ is about 1 ms [see Golbeck (1992)]. This result demonstrates the integrity of the PSI particles isolated from the PsaF-less mutant. The electron transfer from plastocyanin and cytochrome c_6 to $P700^+$ from wild-type gives rise in addition to a slower component with a half-time of 183 or 161 ms and amplitudes of about 30% of the total signal, respectively. These half-times are comparable to the values found for electron transfer from plastocyanin and cytochrome c_6 to PSI lacking PsaF, suggesting that this very slow kinetic component represents electron transfer to damaged PSI complex.

Figure 4 shows the second-order rate constant of $P700^+$ reduction by plastocyanin and cytochrome c_6 in PSI particles from wild-type and the PsaF-deficient mutant 3bF as a function of the $MgCl_2$ concentration. For wild-type PSI particles, a maximal reaction rate is observed at $MgCl_2$ concentrations between 0.4−1.0 mM and 1−10 mM for plastocyanin and cytochrome c_6, respectively. For both proteins, the maximal reaction rate decreases from a value of about 6.5×10^7 M^{-1} s^{-1} for plastocyanin and 3.5×10^7 M^{-1} s^{-1} for cytochrome c_6 to a value almost 2 orders of magnitude lower at 1 M $MgCl_2$. At these high salt concentrations, the rates are similar to those obtained with PSI particles from the PsaF-deficient mutant, which increase with increasing salt concentration.

Fast Electron Transfer Occurs between Plastocyanin or Cytochrome c_6 and P700 at High Concentrations of Both

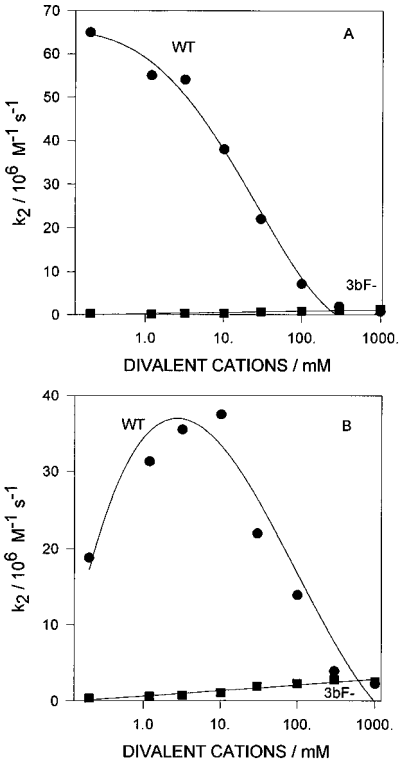

FIGURE 4: Salt dependence of electron transfer from plastocyanin and cytochrome c_6 to P700. Salt dependence of the second-order rate constant (k_2) for the interaction of plastocyanin (A) and cytochrome c_6 (B) with PSI particles from wild-type and 3bF (for conditions, see Figure 3).

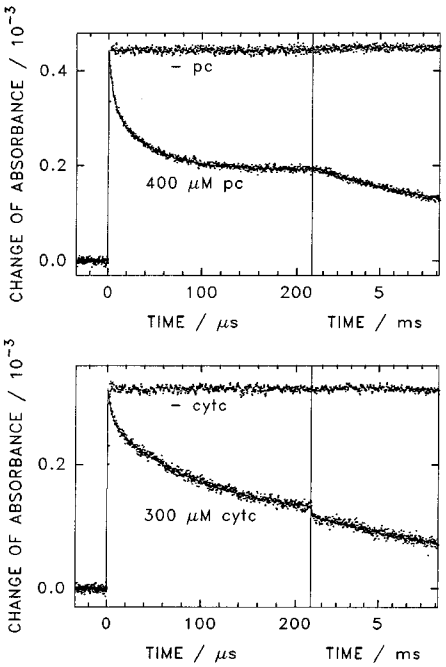

FIGURE 5: Fast electron transfer from plastocyanin or cytochrome c_6 to P700. Absorbance transients at 820 nm induced by a laser flash in PSI-particles from wild-type in the presence of 400 μM plastocyanin and 300 μM cytochrome c_6 and 3 mM $MgCl_2$. Conditions were as in Figure 3. The absorbance transients are the result of four flashes. The absorbance transients in the absence of donor proteins are shown as a control. These latter signals were normalized to the initial absorbance changes in the presence of donor proteins.

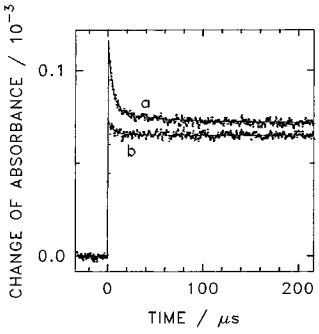

FIGURE 6: Fast electron transfer occurs in the cross-linked complex between cytochrome c_6 and PSI. Absorbance transients at 820 nm induced by a laser flash in PSI particles cross-linked with cytochrome c_6 . Conditions were as in Figure 3 except that the concentration of the cross-linked PSI particles was 15 μg of chl/mL. The transients were measured in the presence of 1 mM sodium ascorbate (a) or after the addition of 4 mM ferricyanide (b). The absorbance transients represent the average of 16 individual signals.

Donor Proteins. The electron transfer reactions between PSI and plastocyanin or cytochrome c_6 were further investigated using higher concentrations of the donor proteins. Figure 5 shows the absorbance transients at 820 nm induced by a laser flash for PSI particles in the presence of 400 μM plastocyanin and 300 μM cytochrome c_6, respectively. The time course can be deconvoluted into three different kinetic components of the $P700^+$ reduction. The fast component with a constant half-time of 3 μs for both proteins and a variable amplitude $A(1)$ reflects a first-order electron transfer, the rate of which is independent from the concentration of the donor proteins. This first-order process can therefore be attributed to the electron transfer reaction within a preformed complex between plastocyanin or cytochrome c_6 and PSI. The intermediate component with an amplitude $A(2)$ shows a half-time that decreases with increasing concentrations of reduced plastocyanin or cytochrome c_6, as known for second-order reactions between soluble reactants (data not shown). The half-time of the electron transfer from plastocyanin and cytochrome c_6 to P700 (Figure 5) is 28 μs and 67 μs, respectively, resulting in second-order rate constants of 6.1×10^7 M^{-1} s^{-1} and 3.4×10^7 M^{-1} s^{-1}, respectively. The amplitude $A(1)$ increases with increasing concentrations of reduced donor protein at the expense of $A(2)$ and is found to be 45% and 33% of $A(1)$ and $A(2)$ at 400 μM plastocyanin and 300 μM cytochrome c_6, respectively. The third very slow component with an amplitude of about 30% of the total signal is less affected by the concentration of plastocyanin or cytochrome c_6 and could be due to damaged PSI centers. With PSI preparation from spinach, this very slow kinetic component, that is almost independent from the plastocyanin concentration, is found to vary between 10% and 24% (Bottin & Mathis, 1985; Nordling *et al.*, 1991; Sigfridsson *et al.*, 1996; Drepper *et al.*, 1996). A more detailed analysis of the electron transfer between plastocyanin or cytochrome c_6 and PSI will be presented elsewhere (M. Hippler, F. Drepper, and J. D. Rochaix, unpublished results).

The Cross-Linked Complex between Cytochrome c_6 and PSI Is Competent in Fast Electron Transfer. To test whether the cross-linked complex between cytochrome c_6 and PSI is functional, the laser-induced absorbance transients of P700 in this complex were measured under reducing conditions (Figure 6, trace a). These measurements show that the cross-linked complex is competent in electron transfer. The fast component has a half-time of 3 μs with an amplitude corresponding to 33% of the total absorbance change. This half-time is the same as that found for the complex formed

Table 1: Properties of Electron Transfer from Plastocyanin and Cytochrome c_6 to PSI[a]

	plastocyanin	cytochrome c_6
half-time (μs) of first-order kinetic component (av of 6 measurements)	2.8 ± 0.3	3.3 ± 0.1
k_2(wild-type PSI) (M^{-1} s^{-1})	6.5×10^7 (0.4 mM)	3.5×10^7 (3 mM)
k_2(PSI lacking PsaF) (M^{-1} s^{-1})	1.32×10^6 (1 M)	2.52×10^6(1 M)

[a] The values of k_2 refer to the maximum obtained at a $MgCl_2$ concentration that is indicated in parentheses.

in vitro, indicating that the cross-linking preserves the conformation of the complex required for fast electron transfer. Addition of 4 mM ferricyanide diminishes the amplitude of the fast reduction phase and the total amplitude of the P700 absorbance change to 6% and 65%, respectively, compared to the total amplitude in the absence of ferricyanide (Figure 6, trace b). The susceptibility of the fast phase to more oxidizing conditions is expected if cytochrome c_6 is the electron donor and proves that this fast phase is due to electron transfer from the cross-linked cytochrome c_6 to P700.

DISCUSSION

The availability of a nuclear mutant of *C. reinhardtii* lacking the PsaF subunit of PSI has greatly helped us in assessing the role of this subunit in electron transfer on the donor side of PSI. In this study, we used this system to purify PSI particles from wild-type and from the PsaF-deficient strain. These PSI particles were used together with purified plastocyanin and cytochrome c_6 to characterize the electron transfer from these donors to P700 *in vitro*.

The PsaF Subunit Is Important for Efficient Electron Transfer from Plastocyanin and Cytochrome c_6 to PSI. The specificity of the interaction between plastocyanin or cytochrome c_6 and the PsaF subunit of PSI from *C. reinhardtii* is shown by the fact that both electron donors are cross-linked in a 1:1 stoichiometry to PsaF and that they do not react with any other PSI subunit, when the PSI complex lacks the PsaF subunit.

The biphasic kinetics of electron transfer from both plastocyanin and cytochrome c_6 to $P700^+$ in *C. reinhardtii* correspond to a preformed complex leading to a fast first-order phase and a bimolecular reaction resulting in a second-order kinetic phase. Similar results have been reported previously for the plastocyanin−PSI interaction for higher plants (Drepper *et al.*, 1996). The PsaF subunit plays an important role for the formation of the fast electron transfer complex as shown by the fact that in *C. reinhardtii* its absence causes a dramatic decrease of the rate constants of $P700^+$ reduction by 1−2 orders of magnitude (Figure 4, Table 1). A comparable effect on the rate constant of reduction of $P700^+$ was found with spinach inside-out-vesicles, when the oxidizing site of PSI was denaturated by high concentrations of Triton X-100, believed to cause a loss of the PsaF subunit (Ratajczak *et al.*, 1988). However, the effect of additional damage to PSI is difficult to assess in these experiments.

In marked contrast, the electron transfer from plastocyanin or cytochrome c_6 to P700 was reported to follow a strict second-order reaction in the cyanobacterium *Synechocystis* 6803 (Hervàs *et al.*, 1994). A further difference with *C. reinhardtii* is that the electron transfer from both electron donors to PSI was the same in the presence or absence of PsaF (Hippler *et al.*, 1996).

The differences in the salt dependence of the electron transfer between plastocyanin or cytochrome c_6 and PSI from wild-type and the PsaF-deficient strain strongly suggest that electrostatic interactions between the positively charged PsaF subunit and the two negatively charged donor proteins are important for efficient electron transfer. It is noteworthy that at higher ionic strength the electron transfer to PSI is more inhibited from plastocyanin than from cytochrome c_6 (Figure 4). This can be correlated with the finding that in the absence of the PsaF subunit, electron transfer to PSI is more impaired from plastocyanin than from cytochrome c_6 to PSI. The *in vivo* rate constant of electron transfer from plastocyanin to the PSI reaction center was decreased 20-fold in the PsaF-deficient mutant relative to wild-type (Farah *et al*., 1995). The corresponding decrease of the maximal rate constants determined *in vitro* is about 100-fold. To what extent the differences observed *in vitro* and *in vivo* measurements are due to differences in ionic strength remains to be investigated.

Fast Electron Transfer from Plastocyanin and Cytochrome c_6 to P700 in C. reinhardtii. A remarkable feature of the PSI complex of *C. reinhardtii* is that both plastocyanin and cytochrome c_6 reduce $P700^+$ with first-order kinetics and a half-time of 3 μs (Table 1). This suggests that these two proteins have a similar orientation in the binding pocket of PSI with respect to electrostatic and hydrophobic interactions. Athough these proteins have different primary structures and carry distinct redox cofactors, the distributions of acidic patches and hydrophobic surfaces are similar (Kerfeld *et al.*, 1995; Frazao *et al.*, 1995). It is therefore possible that the electron transfer from the heme of cytochrome c_6 occurs also via the hydrophobic northern part of cytochrome c_6 to $P700^+$ as shown for plastocyanin (Haehnel *et al.*, 1994).

The fast electron transfer within the cross-linked complex between cytochrome c_6 and PSI in *C. reinhardtii* shows that cross-linking conserves the orientation of the PsaF subunit and cytochrome c_6 like in the authentic complex and demonstrates the importance of the PsaF subunit for intra-complex electron transfer. Although about 100% of cytochrome c_6 are cross-linked to the PsaF subunit of PSI (Figure 2), 33% of the cross-linked PSI are able to perform fast electron transfer to P700. Whether the remaining 67% of nonfunctional cross-linked cytochrome c_6 are due to disoriented cytochrome c_6 within the cross-linked complex or to intrinsic properties of the cross-linked complex remains open. The 1:1 stoichiometry between PsaF and cytochrome c_6 in the active cross-linked complex demonstrates also that cytochrome c_6 reacts as a monomer with the PSI complex and not as a dimer, as suggested by Kerfeld *et al.* (1995).

Evolutionary Aspects. C. reinhardtii appears to be the first photosynthetic organism examined capable of using both plastocyanin and cytochrome c_6 to transfer electrons to $P700^+$ at a fast rate with a half-time of 3 μs. Whereas in higher plants a negatively charged plastocyanin is able to reduce PSI with first-order kinetics, a fast reduction phase is also found for the interaction of PsaF with a positively charged cytochrome c_6 in the cyanobacterium *Anabaena sp.* PCC 7119. The PsaF subunit of *Anabaena sp.* PCC 7119 (Nyhus *et al.*, 1992; Ziegler *et al.*, 1995) lacks a positively charged segment found in photosynthetic eucaryotic organisms which was shown to interact with plastocyanin in spinach (Hippler *et al.*, 1996) and plastocyanin and cytochrome c_6 in *C.*

reinhardtii (this study). It was suggested that this PsaF domain evolved to enable the formation of a stable complex competent for fast electron transfer from negatively charged plastocyanin to PSI in eucaryotic organisms (Hippler *et al.*, 1996). Our data strongly suggest that it also enables fast electron transfer from the negatively charged cytochrome c_6 to PSI. The reason why higher plants have maintained only plastocyanin as electron donor for PSI during evolution remains unclear, especially considering the fact that cytochrome c_6 donates electrons to P700 as fast as plastocyanin.

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REFERENCES

Anandan, S., Vainstain, A., & Thornber, J. P. (1989) *FEBS Lett. 256*, 150−154.
Bassi, R., Soen, S. Y., Frank, G., Zuber, H., & Rochaix, J.-D. (1992) *J. Biol. Chem. 267*, 25714−25721.
Bengis, C., & Nelson, N. (1977) *J. Biol. Chem. 252*, 4564−4569.
Bottin, H., & Mathis, P. (1985) *Biochemistry 24*, 6453−6460.
Bottin, H., & Mathis, P. (1987) *Biochim. Biophys. Acta 892*, 91−98.
Campos, A. P., Aguiar, A. P., Hervás, M., Regalla, M., Navarro, J. A., Ortega, J. M., Xavier, A. V., De la Rosa, M. A., & Teixeira, M. (1993) *J. Eur. Biochem. 216*, 329−341.
Chitnis, P. R., Purvis, D., & Nelson, N. (1991) *J. Biol. Chem. 266*, 20146−20151.
Chua, N.-H., & Bennoun, P. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 2175−2179.
Delosme, R. (1991) *Photosynth. Res 29*, 45−54.
Drepper, F., Hippler, M., Nitschke, W., & Haehnel, W. (1996) *Biochemistry 35*, 1282−1295.
Farah, J., Rappaport, F., Choquet, Y., Joliot, P., & Rochaix, J.-D. (1995) *EMBO J. 14*, 4976−4984.
Frazao, C., Soares, C. M., Corrando, M. A., Pohl, E., Dauter, Z., Wilson, K. S., Hervás, M., & Golbeck, J. H. (1992) *Annu. Rev. Plant Physiol. Plant Mol. Biol. 43*, 293−324.
Haehnel, W., Döring, G., & Witt, H. T. (1971) *Z. Naturforsch. 26B*, 1171−1174.
Haehnel, W., Ratajczak, R., & Robenek, H. (1989) *J. Cell Biol. 108*, 1397−1405.
Haehnel, W., Jansen, T., Gause, K., Klösgen, R. B., Stahl, B., Michl, D., Huvermann, B., Karas, M., & Herrmann, R. G. (1994) *EMBO J. 13*, 1028−1038.
Harris, E. H. (1989) *The Chlamydomonas Sourcebook.* Academic Press, San Diego.
Hatakana, H., Sonoike, K., Hirano, M., & Katoh, S. (1993) *Biochim. Biophys. Acta 1141*, 45−51.
Hervás, M., Ortega, J. M., Navarro, J. A., De la Rosa, M. A., & Bottin, H. (1994) *Biochim. Biophys. Acta 1184*, 235−241.
Hervás, M., Navarro, J. A., Díaz, A., Bottin, H., & De la Rosa, M. A. (1995) *Biochemistry 34*, 11321−11326.
Hippler, M., Ratajczak, R., & Haehnel, W. (1989) *FEBS Lett. 250*, 280−284.
Hippler, M., Reichert, J., Sutter, M., Zak, E., Altschmied, L., Schröer, U., Herrmann, R. G., & Haehnel, W. (1996) *EMBO J. 15*, 6374−6384.
Hiyama, T., & Ke, B. (1972) *Biochim. Biophys. Acta 267*, 170−171.
Ho, K. K., & Krogmann, D. W. (1984) *Biochim. Biophys. Acta 766*, 310−316.
Katoh, S. (1979) *Encycl. Plant Physiol., New Ser. 5*, 247−252.
Katoh, S., Shiratori, I., & Takamiya, A. (1962) *J. Biochem. 51*, 32−40.
Kerfeld, C. A., Anwar, H. A., Interrante, R., Merchant, S., & Yeates, O. T. (1995) *J. Mol. Biol. 250*, 627−647.
Krauss, N., Hinrichs, W., Witt, I., Fromme, P., Pritzkow, W., Dauter, Z., Betzel, C., Wilson, K. S., Witt, H. T., & Saenger, W. (1993) *Nature 361*, 326−331.
Krauss, N., Schubert, W. D., Klukas, Fromme, P., Witt, H. T., & Saenger, W. (1996) *Nat. Struct. Biol. 3*, 965−973.
Laemmli, U. K. (1970) *Nature 227*, 680−685.
Medina, M., Díaz, A., Hervás, M., Navarro, J. A., Gómez-Moreno, C., De la Rosa, M. A., & Tollin, G. (1993) *Eur. J. Biochem. 213*, 1133−1138.
Merchant, S., & Bogorad, L. (1986) *Mol. Cell. Biol. 6*, 462−469.
Nordling, M., Sigfridsson, K., Young, S., Lundberg, L. G., & Hansson, O. (1991) *FEBS Lett. 291*, 327−330.
Nyhus, K. J., Ikeuchi, M., Inoue, Y., Whitmarsh, J., & Pakrasi, H. B. (1992) *J. Biol. Chem. 267*, 12489−12495.
Porra, R. J., Thompson, W. A., & Kriedemann, P. E. (1989) *Biochim. Biophys. Acta 975*, 384−394.
Ratajczak, R., Mitchell, R., & Haehnel, W. (1988) *Biochim. Biophys. Acta 933*, 306−318.
Sandmann, G. (1986) *Arch. Microbiol. 21*, 6366−6375.
Scheller, H. V., & Møller, B. L. (1990) *Physiol. Plant. 78*, 484−494.
Sigfridsson, K., Young, S., & Hansson, Ö (1996) *Biochemistry 35*, 1249−1257.
Takahashi, Y., Goldschmidt-Clermont, M., Soen, S.-Y, Franzén, L. G., & Rochaix, J.-D. (1991) *EMBO J. 10*, 2033−2040.
Wood, P. M. (1978) *Eur. J. Biochem. 87*, 9−19.
Wynn, R. M., & Malkin,R. (1988) *Biochemistry 27*, 5863−5869.
Wynn, R. M., Omaha, J., & Malkin, R. (1989) *Biochemistry 28*, 5554−5560.
Xu, Q., Xu, L., Chitnis, V., & Chitnis, P. (1994) *J. Biol. Chem. 269*, 3205−3211.
Ziegler, K., Schütz, M., Zimmermann, R., & Lockau, W. (1995) Sequence submitted to EMBL/GENBANK/DDBJ, entry AVPSAFJ.

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