The PsaC subunit of photosystem I provides an essential lysine residue for fast electron transfer to ferredoxin

Nicolas Fischer, Michael Hippler,
Pierre Sétif¹, Jean-Pierre Jacquot² and
Jean-David Rochaix³

Departments of Molecular Biology and Plant Biology, University of Geneva, 30 quai Ernest-Ansermet, 1211 Geneva 4, Switzerland, ¹CEA, Département de Biologie Cellulaire et Moléculaire, CNRS, URA 2096, C.E. Saclay, 91191 Gif-sur-Yvette Cedex and ²Université de Paris Sud, IBP-630, 91405 Orsay Cedex, France

³Corresponding author

e-mail: Jean-David.Rochaix@molbio.unige.ch

PsaC is the stromal subunit of photosystem I (PSI) which binds the two terminal electron acceptors F_A and F_B. This subunit resembles 2[4Fe-4S] bacterial ferredoxins but contains two additional sequences: an internal loop and a C-terminal extension. To gain new insights into the function of the internal loop, we used an *in vivo* degenerate oligonucleotide-directed mutagenesis approach for analysing this region in the green alga *Chlamydomonas reinhardtii*. Analysis of several *psaC* mutants affected in PSI function or assembly revealed that K₃₅ is a main interaction site between PsaC and ferredoxin (Fd) and that it plays a key role in the electrostatic interaction between Fd and PSI. This is based upon the observation that the mutations K₃₅T, K₃₅D and K₃₅E drastically affect electron transfer from PSI to Fd, as measured by flash-absorption spectroscopy, whereas the K₃₅R change has no effect on Fd reduction. Chemical cross-linking experiments show that Fd interacts not only with PsaD and PsaE, but also with the PsaC subunit of PSI. Replacement of K₃₅ by T, D, E or R abolishes Fd cross-linking to PsaC, and cross-linking to PsaD and PsaE is reduced in the K₃₅T, K₃₅D and K₃₅E mutants. In contrast, replacement of any other lysine of PsaC does not alter the cross-linking pattern, thus indicating that K₃₅ is an interaction site between PsaC and its redox partner Fd.

Keywords: electron transfer/ferredoxin/photosystem I/
PsaC

Introduction

In oxygenic photosynthetic organisms, conversion of light energy into chemical energy is achieved by multiprotein-pigment complexes located in the thylakoid membranes. Photosystem I (PSI) performs the light-induced electron transfer from plastocyanin or cytochrome *c*₆ (Cyt_c) to Fd. PSI complex is composed of at least five chloroplast-encoded subunits (PsaA, -B, -C, -I and -J) and six nuclear-encoded polypeptides (PsaD, -E, -F, -G, -H and -K) (Golbeck, 1992). The primary electron donor (P700) and the intermediate acceptors (A₀, A₁ and F_X) are bound by

the two major subunits PsaA and PsaB (Brettel, 1997), whereas PsaC binds the two terminal electron acceptors (F_A and F_B) (Hayashida *et al.*, 1987; Høj *et al.*, 1987; Oh-Oka *et al.*, 1987). This 8–9 kDa protein is located on the stromal side of PSI and contains two CxxCxxCxxxCP motifs that are the ligands of the [4Fe-4S] clusters F_A and F_B. The position of these clusters is clearly defined in the PSI crystal structure at 6 and 4 Å resolution, placing one cluster close to F_X and the other closer to the stromal surface (Krauss *et al.*, 1993, 1996). Assigning an identity (F_A or F_B) to the clusters within the PSI structure is still uncertain. PsaC is a ferredoxin-like protein (Dunn and Gray, 1988). However, it contains two domains which are absent from 2[4Fe-4S] ferredoxins: an internal loop between the two [4Fe-4S] cluster binding motifs and a C-terminal extension (Figure 1). These additional sequences have been proposed to interact with the PsaA/B heterodimer and the PsaD subunit, respectively (Naver *et al.*, 1996). PsaC has been shown to be essential for *in vivo* assembly of PSI in *Chlamydomonas reinhardtii*. Disruption of the gene or mutagenesis of cysteines liganding the [4Fe-4S] clusters destabilizes the PSI complex in this organism (Takahashi *et al.*, 1991, 1992). The PsaD and PsaE subunits are located on the stromal side of PSI and interact with PsaC (Jansson *et al.*, 1996). PsaD is required for stable assembly of PsaC and PsaE in PSI complex reconstitution assays (Zhao *et al.*, 1990; Li *et al.*, 1991; Naver *et al.*, 1995). Chemical cross-linking revealed a direct interaction between PsaD and Fd (Zanetti and Merati, 1987; Zilber and Malkin, 1988; Andersen *et al.*, 1992a; Lelong *et al.*, 1994). Inactivation of PsaD demonstrated its role for efficient Fd reduction (Chitnis *et al.*, 1989; Xu *et al.*, 1994a). PsaE has also been shown to interact with ferredoxin:NADP⁺-oxidoreductase as well as Fd and to be required for Fd reduction (Andersen *et al.*, 1992b; Rousseau *et al.*, 1993; Sonoike *et al.*, 1993; Xu *et al.*, 1994a). The PsaF subunit provides a docking site for plastocyanin and Cyt_c on the luminal side of PSI, and is essential for their fast photo-oxidation in *C.reinhardtii*, but not in cyanobacteria (Xu *et al.*, 1994b; Farah *et al.*, 1995; Hippler *et al.*, 1996, 1997). Interaction between the transmembrane domain of PsaF and the stromal subunit PsaE has been reported (Xu *et al.*, 1994b; Jansson *et al.*, 1996). In the current model of PSI three subunits appear to be involved in the reduction of Fd. PsaD and PsaE are involved in the correct docking of Fd to PSI and PsaC coordinates the [4Fe-4S] clusters from which an electron can be transferred to Fd.

To gain new insights into the function of the internal loop of PsaC which is absent from 2[4Fe-4S] ferredoxins and might therefore be important for the function of this subunit, we performed *in vivo* degenerate oligonucleotide-directed mutagenesis of this loop in *C.reinhardtii*. This approach has led to the identification of lysine 35 as a

Fig. 1. Schematic representation of the PsaC polypeptide. The two C-C-C-C-P motifs liganding the [4Fe-4S] clusters are indicated. The C-terminal extension and the internal loop are boxed. The wild-type sequence and the possible changes introduced by degenerate oligonucleotide-directed mutagenesis are indicated for the two halves of the internal loop. The positions of the three lysine residues of the PsaC polypeptide of *C.reinhardtii* are shown by closed triangles.

key residue for Fd reduction. Replacement of this lysine with neutral or negatively charged amino acids abolishes fast electron transfer to Fd and limits photosynthetic growth of the mutant strains. Furthermore, we show that Fd can be cross-linked to PsaD, PsaE and PsaC and our data strongly suggest that the cross-linking-site of PsaC is lysine 35.

Results

Degenerate oligonucleotide-directed mutagenesis of the internal loop of PsaC

Because there is no obvious target for site-directed mutagenesis to study the function of the internal loop of PsaC, we chose a mutagenesis approach for the random assortment of defined mutations within a short stretch of amino acids. In this study we defined the additional loop of PsaC as the ten amino acids E₂₇MVPWDGCKA₃₆ and mutated the first five and last five residues separately. Oligonucleotides for each half of the loop were designed with a 2-fold degeneracy of one base of each codon to be mutated (see Materials and methods). In this way, the wild-type or mutant residue was introduced at any given position within the loop. For each half of the loop, the possible changes are shown in Figure 1. Because of the possibility that multiple mutations might often lead to the destabilization of the PSI complex, we designed the oligonucleotides such that *psaC* genes with single and double mutations should represent about 47% of the mutated *psaC* copies if one assumes that the oligonucleotides used anneal with the same efficiency. The partially degenerate portion of the oligonucleotides is flanked by 15 bases of homologous sequence on each side to allow polymerase chain reaction amplification (see Materials and methods for details). The amplified DNA, containing a population of *psaC* genes with different combinations of mutations, was digested with *Nde*I and *Bgl*II and inserted into plasmid pBSEP5.8 *aadA* [*Nde*I/*Bgl*II]. This plasmid contains a 5.8 kb *Eco*RI-*Pst*I fragment from the chloroplast DNA fragment R23 (Rochaix, 1978) with two unique *Nde*I and *Bgl*II restriction sites located at the 5' and 3' ends of the *psaC* coding sequence. The *aadA*

expression cassette was inserted at a *Sal*I site 800 bp upstream of *psaC* (Takahashi *et al.*, 1992). This selectable marker confers spectinomycin and streptomycin resistance to *C.reinhardtii* (Goldschmidt-Clermont, 1991).

The two plasmid libraries of mutated *psaC* genes corresponding to the two parts of the loop (loop-1 and loop-2) were introduced via biolistic transformation (Boynton *et al.*, 1988) into the chloroplast of a *C.reinhardtii* strain in which the *psaC* coding sequence had been deleted (Fischer *et al.*, 1996). Transformants were selected on TAP plates supplemented with 150 µg/ml of spectinomycin and maintained in dim light (5 µE/m²/s).

Analysis of the transformants reveals the importance of residue K₃₅

Transformants were restreaked once on selective media and could be directly analysed. As no wild-type copy of the *psaC* gene is present in the recipient strain, only the mutated version of *psaC* is expressed and its phenotype can be characterized readily. Forty-eight transformants obtained with each library were tested for their capacity to grow on TAP (permissive for mutants of *C.reinhardtii* deficient in photosynthesis) or HSM (minimal) plates under different light regimes. Fluorescence transients of cells grown in dim light (5 µE/m²/s) and dark adapted before the experiment were measured to determine if they had a functional PSI complex (Bennoun and Delepelaire, 1982). Transformants with altered growth or fluorescence phenotypes as well as several transformants with wild-type phenotype were identified and further characterized. Total genomic DNA was isolated from these strains and the *psaC* gene amplified by a two-step asymmetric PCR to generate single stranded DNA (see Materials and methods). The amplified DNA was directly sequenced to identify mutations in the loop region. Table I shows the results of this analysis for 11 transformants of the loop-1 mutagenesis and 14 of the loop-2 mutagenesis. All other transformants examined had either integrated a wild-type *psaC* or only the *aadA* selectable marker without *psaC*.

Results of loop-1 mutagenesis. Transformants L1–39 and L1–47 have characteristic phenotypes of PSI-deficient strains and carry triple mutations M₂₈K/V₂₉D/P₃₀S and M₂₈K/P₃₀S/W₃₁S, respectively. The fact that transformants L1–1 and L1–6 contain two of these three mutations (V₂₉D/P₃₀S and P₃₀S/W₃₁S, respectively), but show no altered growth or fluorescence phenotype, strongly suggests that the mutation M₂₈K is responsible for the PSI-deficient phenotype (Table IA). Western blot analysis of total cell extracts confirmed that in transformants L1–39 and L1–47, the PSI complex was strongly destabilized (data not shown). From a similar subtractive comparison of the different combinations of mutations, it appears that transformants assembling PSI but having a defect in growth phenotype always carry the E₂₇V/V₂₉D mutations.

Results of loop-2 mutagenesis. The same type of comparison for this set of mutants showed that all the transformants affected in growth carry the mutation K₃₅T and are unable to grow on HSM under high light conditions. Conversely, in transformant L2–31 all residues of loop-2 are mutated except K₃₅ and this transformant is able to grow on HSM under high light, although its growth rate

Table I. *In vivo* phenotypes of strains carrying mutations in the additional loop of PsaC**(A) Loop-1 mutagenesis**

							Growth					
	27			31	mut.	Fluo.	T _{DL}	T _L	T _{HL}	HSM _{DL}	HSM _L	HSM _{HL}
WT	E	M	V	P	W	0	WT	+	+	+	+	+
DC	–	–	–	–	–	–	PSI	+	–	–	–	–
L1–1	E	M	D	S	W	2	WT	+	+	+	+	+
L1–6	E	M	V	S	S	2	WT	+	+	+	+	+
L1–11	E	M	V	S	W	1	WT	+	+	+	+	+
L1–12	V	M	V	S	W	2	WT	+	+	+	+	+
L1–16	E	M	V	S	W	1	WT	+	+	+	+	+
L1–19	V	M	D	S	W	3	WT	+	+	+	slow	–
L1–34	E	M	D	S	W	2	WT	+	+	+	+	+
L1–35	V	M	D	P	S	3	WT	+	+	–	slow	–
L1–37	V	M	V	S	S	3	WT	+	+	+	+	+
L1–39	E	K	D	S	W	3	PSI	+	–	–	–	–
L1–47	E	K	V	S	S	3	PSI	+	–	–	–	–

(B) Loop-2 mutagenesis

							Growth					
	32			36	mut.	Fluo.	T _{DL}	T _L	T _{HL}	HSM _{DL}	HSM _L	HSM _{HL}
WT	D	G	C	K	A	0	WT	+	+	+	+	+
DC	–	–	–	–	–	–	PSI	+	–	–	–	–
L2–1	V	D	C	K	A	2	WT	+	+	+	+	+
L2–2	V	G	C	T	A	2	WT	+	+	+	+	–
L2–3	D	G	S	K	T	2	WT	+	+	+	+	+
L2–5	V	G	S	K	A	2	WT	+	+	+	+	+
L2–9	V	G	C	T	T	3	WT	+	+	+	+	–
L2–10	D	G	C	T	T	2	WT	+	+	+	+	–
L2–11	V	G	S	K	A	2	WT	+	+	+	+	+
L2–12	V	D	S	T	A	4	WT	+	+	slow	+	–
L2–17	V	G	S	T	A	3	WT	+	+	+	+	–
L2–22	D	G	S	T	A	2	WT	+	+	+	+	–
L2–24	D	G	S	T	T	3	WT	+	+	+	+	–
L2–27	V	G	C	T	A	2	WT	+	+	+	+	–
L2–31	V	D	S	K	T	4	WT	+	+	+	+	slow
L2–32	D	D	S	T	A	3	WT	+	+	+	+	–

DC, deletion of *psaC*; mut., number of mutations; Fluo., fluorescence phenotype in dim light; T, (TAP) rich medium; HSM, minimal medium; light conditions: DL, 5 $\mu\text{E}/\text{m}^2/\text{s}$; L, 60 $\mu\text{E}/\text{m}^2/\text{s}$; HL, 600 $\mu\text{E}/\text{m}^2/\text{s}$.

is reduced compared with wild-type (Table IB). This suggests that the mutation K_{35}T is causing the growth defect. Accordingly, K_{35} was subjected to further mutagenesis.

Random mutagenesis of residue K_{35}

We designed an oligonucleotide with complete degeneracy of the codon corresponding to K_{35} . Only adenosine was excluded in the third position of the codon to avoid the occurrence of two out of three stop codons. Amplification, cloning and transformation of this new library containing *psaC* genes randomly mutated at position 35 was performed as described above. We analysed 32 transformants and selected four with altered growth and/or fluorescence phenotypes. Fluorescence transients and growth phenotypes on plates as well as the mutation at position 35 for each transformant are shown in Table II. Strains carrying mutations K_{35}T , K_{35}D and K_{35}P displayed fluorescence transients that were very similar to wild-type, suggesting that PSI is functional in these strains (data not shown). Transformants containing the mutation K_{35}T grew significantly slower on HSM plates under high light. The K_{35}P mutation leads to a slightly more severe phenotype as it impairs growth on HSM plates under high light. The most

affected transformant contains the mutation K_{35}D . This strain is photosensitive under high light and its growth rate is reduced on HSM at 60 $\mu\text{E}/\text{m}^2/\text{s}$. We also measured the doubling time of these different strains in liquid HSM media under low light conditions. It can be seen that none of the mutations present at position 35 affects the growth rate (Table II). Western blot analysis was performed on total extracts of cells grown in dim light to measure the accumulation of the PSI complex (Figure 2). It is apparent that the K_{35}T , K_{35}P and K_{35}D mutants accumulate high levels of PSI, indicating that the mutations do not markedly affect the stability of the complex. In contrast, a stop codon at position 35 in one of the transformants leads to a PSI-deficient phenotype (Table II). Western blot analysis showed that truncation of the PsaC polypeptide at position 35 completely destabilizes the PSI complex (data not shown).

These mutant strains were obtained by random mutagenesis as described above and only one transformant (two in the case of K_{35}T) was obtained for each mutation. The phenotypes observed could be due to additional chloroplast or nuclear mutations that might have occurred during chloroplast transformation. To exclude this possibility, we amplified the *psaC* genes containing the K_{35}T , K_{35}P and

Table II. *In vivo* phenotypes of *K*₃₅ mutants

Strain	Fluo.	T _{DL}	T _L	T _{HL}	HSM _L	HSM _{HL}	Doubling time (h) (HSM 10 $\mu\text{E}/\text{m}^2/\text{s}$)
WT	WT	+	+	+	+	+	32 $\pm$ 4
DC	PSI ⁻	+	–	–	–	–	∞
<i>K</i> ₃₅ P	WT	+	+	+	+	–	35 $\pm$ 5
<i>K</i> ₃₅ T	WT	+	+	+	+	slow	31 $\pm$ 7
<i>K</i> ₃₅ D	WT	+	+	–	slow	–	30 $\pm$ 6
<i>K</i> ₃₅ E	WT	+	+	–	slow	–	33 $\pm$ 6
<i>K</i> ₃₅ R	WT	+	+	+	+	+	29 $\pm$ 7
<i>K</i> ₃₅ Stop	PSI ⁻	+	–	–	–	–	∞

DC, deletion of the *psaC* gene; Fluo., fluorescence phenotype in dim light; T, TAP medium; DL, 5 $\mu\text{E}/\text{m}^2/\text{s}$; L, 60 $\mu\text{E}/\text{m}^2/\text{s}$; HL, 600 $\mu\text{E}/\text{m}^2/\text{s}$.

Fig. 2. Immunoblot analysis of total cell extracts of wild-type, the *K*₃₅T, *K*₃₅R, *K*₃₅D, *K*₃₅E and *K*₃₅P mutants and of a strain lacking *psaC* (ΔC). Cells were grown in liquid TAP medium in low light (5 $\mu\text{E}/\text{m}^2/\text{s}$). 15 μg of total protein were loaded per lane. Proteins were separated by SDS–PAGE, electroblotted on nitrocellulose membrane and probed with antibodies raised against subunits PsA, PsD, PsE and PsF.

*K*₃₅D mutations and cloned them into the pBSEP5.8 *aadA* [*Nde*I/*Bgl*III] transformation vector. After sequencing of the *psaC* gene to confirm the presence of the mutations at *K*₃₅ and the absence of any undesired mutation that might have been introduced during amplification, these genes were inserted into the chloroplast genome of *C. reinhardtii* by transformation. Analysis of several individual transformants for each mutation showed that their growth phenotype was indeed due to the mutation present in the *psaC* gene.

Site-directed mutagenesis of *K*₃₅

To further test the role of the positive charge provided by *K*₃₅, we constructed the *K*₃₅E and *K*₃₅R mutant strains by site-directed mutagenesis. Amplification with specific oligonucleotides, cloning and transformation of *C. reinhardtii* was performed as described above. Several transformants for each mutation were obtained and analysed. Clearly, the *K*₃₅E change leads to a growth deficient phenotype under high light, as observed for the *K*₃₅D mutant, whereas the *K*₃₅R mutant grows under all conditions tested (Table II). In both mutants, PSI accumulates to high levels (Figure 2). These results indicate that replacement of *K*₃₅ by a negatively charged residue severely limits photosynthetic growth under high light, whereas replacement by another positively charged amino acid restores the wild-type growth phenotype. In agreement

with an electrostatic role of *K*₃₅, its replacement by a neutral residue leads to an intermediate phenotype.

Fd reduction by PSI

Electron transfer from PSI to Fd has already been studied in the cyanobacterium *Synechocystis* sp. PCC 6803 and in *C. reinhardtii* (Sétif and Bottin, 1994, 1995; Fischer *et al.*, 1997). Upon P700 oxidation by a saturating laser flash, Fd reduction is accompanied by an absorption change in the 460–600 nm region in the cyanobacterial system. With *C. reinhardtii* PSI, the measurements were restricted to a single wavelength (580 nm), because its larger antenna size precludes measurements at other wavelengths. In the absence of Fd, an absorption change at 580 nm is also observed (see Materials and methods). This contribution was subtracted from the signals recorded in the presence of Fd so that the kinetic curves shown in Figure 3 correspond solely to electron transfer from $[\text{F}_\text{A}\text{F}_\text{B}]^-$ to Fd. When absorption transients were measured at 580 nm with wild-type PSI and Fd, three phases of Fd reduction were observed (Figure 3, curve a): two first order phases, one with $t_{1/2} < 1 \mu\text{s}$ and a second with $t_{1/2} = 4.5 \pm 1.5 \mu\text{s}$, and a slower phase due to second order electron transfer, where the rate is proportional to the ferredoxin concentration. The half-times of the first order phases are independent of ferredoxin concentration, but their amplitudes increase with ferredoxin concentration. At 580 nm, the 4.5 μs phase has an amplitude 2-fold larger than that of the submicrosecond phase. The first order components are ascribed to electron transfer within a PSI–Fd complex formed before flash excitation. Linear fitting of the sum of the amplitudes of the first order components as a function of Fd concentration (assuming a simple binding equilibrium between Fd and PSI) allows one to determine the dissociation constant for the wild-type complex ($K_d = 6\text{--}9 \mu\text{M}$, data not shown). A linear fit of the observed rate of the slowest phase as a function of Fd concentration was also performed, providing a second order rate constant of $2.3 \times 10^8/\text{M}/\text{s}$ for the wild-type PSI complex (data not shown).

The same experiments were performed with the *K*₃₅T, *K*₃₅D, *K*₃₅E and *K*₃₅R PSI complexes. Figure 3A shows that the first order components are completely absent with the *K*₃₅T, *K*₃₅D and *K*₃₅E PSI complexes (curves c, d and e). We estimated the lower limit of the dissociation constants for these mutant complexes: $K_d > 300 \mu\text{M}$ (see Materials and methods for estimation). In contrast, two first order phases with $t_{1/2}$ similar to wild-type are present

Fig. 3. Ferredoxin reduction measured by flash-induced absorption changes at 580 nm with wild-type and mutant PSI complexes. The transients were obtained after subtraction of traces recorded in the absence of ferredoxin from traces recorded in its presence. The contribution of P700⁺ decay, which is different in the presence or absence of ferredoxin, was also subtracted so that the curves correspond only to electron transfer from [F_AF_B]⁻ to ferredoxin. PSI complexes were prepared from wild-type (a), K₃₅R (b), K₃₅T (c), K₃₅D (d) and K₃₅E (e) and suspended in 20 mM Tricine pH 8.0, 0.03% β-DM, 30 mM NaCl, 5 mM MgCl₂, 2.2 mM sodium ascorbate and 20 μM DCPIP. Traces are the average of 128 measurements with and without ferredoxin. A delay of 3 s was used between two consecutive flashes. The kinetics shown in panels (A) and (B) correspond to 0.14 μM PSI, as measured by absorption changes at 820 nm. Concentrations of ferredoxin from *C.reinhardtii* are 16.2 μM for K₃₅T, K₃₅D, K₃₅E and K₃₅R, and 17.3 μM for wild-type. The full scales in panels (A) and (B) are 100 μs and 50 ms, respectively. Panel (C) shows the dependence of the observed rate of ferredoxin reduction on the concentration of ferredoxin: K₃₅T (●); K₃₅D (+) and K₃₅E (▲). Note the different scales used. A linear fit of the data is also shown. It corresponds to $k_{\text{obs}} \text{ (s}^{-1}\text{)} = [\text{Fd}] \times (4.5 \times 10^7 \text{ M/s})$ for K₃₅T, $k_{\text{obs}} \text{ (s}^{-1}\text{)} = [\text{Fd}] \times (6.3 \times 10^6 \text{ M/s})$ for K₃₅D and $k_{\text{obs}} \text{ (s}^{-1}\text{)} = [\text{Fd}] \times (2.8 \times 10^6 \text{ M/s})$ for K₃₅E.

in the experiments performed with the K₃₅R PSI complex (Figure 3A, curve b). The ratio of the submicrosecond and the microsecond phases are also similar between wild-type and K₃₅R PSI, whereas the amplitudes of these first order phases are slightly larger in this mutant PSI complex, indicating that the affinity of Fd for this PSI complex is higher ($K_d = 4.5\text{--}6.5 \mu\text{M}$). Clearly, the replacement of K₃₅ by neutral or negatively charged amino acids drastically affects the affinity of Fd for PSI, showing that the positive charge provided by this residue is important for formation of the PSI-Fd complex. The second order component is also altered in the K₃₅T, K₃₅D and K₃₅E PSI complexes (Figure 3B). Linear fitting of the dependence of the observed rates on Fd concentration provides rate constants of $4.5 \times 10^7 \text{ M/s}$, $6.3 \times 10^6 \text{ M/s}$ and $2.8 \times 10^6 \text{ M/s}$ for K₃₅T, K₃₅D and K₃₅E, respectively (Figure 3C),

whereas the second order component observed with the K₃₅R PSI complex is the same as for wild-type. The positive charge provided by K₃₅ appears, therefore, to be important for the precise positioning of Fd on the reducing side of PSI, which allows for fast electron transfer. Removal of this positive charge or its replacement by negative charges leads to 5-fold and 35- to 80-fold decrease of the electron transfer rate, respectively. Properties of the wild-type and mutant PSI-complexes are summarized in Table III.

Light induced EPR spectra of PSI at low temperature

Stable charge separation between P700⁺ and [F_AF_B]⁻ can be achieved in PSI at low temperature. Below 30 K, this charge-separated state can be observed by electron paramagnetic resonance (EPR) when the samples are incubated in the dark at room temperature with ascorbate and DPIP to reduce P700⁺, and frozen in darkness. At low temperature, photoreduction of PSI leads to the formation of F_A⁻ or F_B⁻, which can be distinguished by their different EPR g-values (F_A⁻: 2.047, 1.945 and 1.857; F_B⁻: 2.07, 1.932 and 1.878). The difference spectra obtained from EPR measurements after 2 min illumination at 14 K and in darkness are shown in Figure 4. Signals were normalized to the same concentration of slowly decaying P700⁺ as measured by absorption changes at 820 nm (data not shown). After normalization to P700⁺, spin quantitation of the three signals gave similar values. The g-values are the same for the five types of PSI, within a margin of error of ± 0.002 . Vertical arrows ($g = 2.11$ and 1.911) indicate signals that have been observed only in PSI from *C.reinhardtii* and which are presumably due to a modified form of F_A⁻ (Fischer *et al.*, 1997). These data show that the mutant PSI complexes contain intact F_A and F_B iron-sulfur clusters and are able to perform stable charge separation between P700⁺ and [F_AF_B]⁻.

Chemical cross-linking of Fd with *C.reinhardtii* PSI particles

Interaction of Fd with several subunits of PSI has been demonstrated by chemical cross-linking in different organisms. In the cyanobacterium *Synechocystis* PCC 6803, Fd was shown to cross-link to PsaD, and the cross-linking site was identified (Lelong *et al.*, 1994). In higher plants, cross-linking has been reported between Fd and PsaD in spinach (Zanetti and Merati, 1987; Zilber and Malkin, 1988); in barley, Fd can be cross-linked to the PsaD, PsaE and PsaH subunits (Andersen *et al.*, 1992a). We performed similar experiments to determine which PSI subunits could be cross-linked to Fd in *C.reinhardtii*. Fd was purified from wild-type cells and incubated with *N*-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and *N*-hydroxysulfosuccinimide ester (sulfo-NHS) to activate its carboxyl groups (see Materials and methods). The excess of reagent was then removed by gel filtration, and the activated Fd was eluted. PSI particles purified from wild-type cells were incubated with or without activated Fd. In this way, no internal cross-link between PSI subunits due to free cross-linker can occur and any cross-linking product has to involve activated Fd. The cross-linked sample and the control PSI were separated by SDS-PAGE. Western blot analysis performed with antibodies raised

Table III. Characteristics of wild-type and mutant PSI

	WT	K ₃₅ T	K ₃₅ D	K ₃₅ E	K ₃₅ R
Dissociation constant for PSI-ferredoxin interaction	6.0–9.0 μ M	>300 μ M	>300 μ M	>300 μ M	4.5–6.5 μ M
Half-times of first-order rates of ferredoxin reduction	<1 μ s	not observed	not observed	not observed	<1 μ s
	$4.5 \pm 1.5 \mu$ s				$4.5 \pm 1.5 \mu$ s
Second-order rate constant of ferredoxin reduction	$2.3 \pm 0.5 \times 10^8$ /M/s	4.5×10^7 /M/s	6.3×10^6 /M/s	2.8×10^6 /M/s	$2.3 \pm 0.5 \times 10^8$ /M/s

Fig. 4. Light-induced EPR spectra of PSI complexes isolated from wild-type and the K₃₅R, K₃₅T, K₃₅D and K₃₅E mutants. Each of these spectra corresponds to the difference between spectra recorded after 2 min illumination at 14 K and in darkness. The large signal around $g \approx 2.00$ (mostly P700⁺) is not shown. Spectra correspond to approximately 5 μ M PSI. Vertical arrows ($g = 2.11$ and 1.911) mark signals that have been observed only in PSI from *C.reinhardtii*, presumably due to a modified form of F_A⁻. EPR conditions: temperature 14 K; microwave power 20 mW; modulation amplitude 1 mT; microwave frequency 9.419 GHz. The spectra shown are the sum of eight scans before and after illumination. All samples were prepared in Tricine, 20 mM pH 8.0, in the presence of 5 mM sodium ascorbate and 25 μ M DCPIP. Tubes were incubated at room temperature for 2 min. in darkness before freezing in darkness.

against the PsaD, PsaE and PsaC polypeptides revealed that Fd could be cross-linked to all three subunits (Figure 5, lanes 2 and 7). To our knowledge, this is the first report of a direct interaction between Fd and the PsaC subunit by chemical cross-linking.

We performed the same experiment with PSI particles isolated from the K₃₅D, K₃₅T, K₃₅E and K₃₅R mutants and from a strain carrying the changes K₅₂S/R₅₃A in the PsaC subunit (Fischer *et al.*, 1997). No cross-linking can be observed between Fd and the PsaC subunit of the K₃₅T, K₃₅D, K₃₅E and K₃₅R mutant PSI complexes, whereas

Fig. 5. Western blot analysis of PSI complexes isolated from different strains and cross-linked to Fd. The cross-linked and control samples were separated on three 15% polyacrylamide gels and electroblotted on nitrocellulose membranes. The blots were incubated with antibodies raised against PsaC, PsaD and PsaE polypeptides [panels (A), (B) and (C), respectively]. Mutated residues in the PsaC subunit and the presence of activated Fd are indicated at the top of each lane. Positions corresponding to the free and cross-linked polypeptides are shown. Note that the difference in the position of the PsaC–Fd cross-linking product shown in lane 7 is due to differences in resolving gel size.

this cross-link is maintained with the K₅₂S/R₅₃A PSI (Figure 5A). Strikingly, the introduction of an aspartate or a glutamate at position 35 almost completely eliminates the PsaD–Fd and PsaE–Fd cross-links (Figure 5B and C, lanes 3 and 8). However, when the immunoblot shown in Figure 5 was overexposed, faint signals corresponding to the PsaD–Fd and PsaE–Fd cross-links could be observed with the K₃₅D PSI (data not shown). The K₃₅T mutation also strongly affects the PsaD–Fd and, to a lesser extent, the PsaE–Fd cross-links (Figure 5B and C, lane 4). Whereas no cross-linking between Fd and PsaC can be observed with the K₃₅R PSI complex, cross-linking of Fd to PsaD and PsaE is similar to wild-type PSI (Figure 5, lane 9). Although it is difficult to quantify cross-linking signals precisely, it appears that there is more PsaD–Fd and PsaE–Fd cross-linking in the K₃₅R than in the wild-type PSI complex, in agreement with the higher affinity of Fd for this mutant complex as measured by flash absorption spectroscopy. The cross-linking patterns obtained with the K₅₂S/R₅₃A and wild-type PSI particles are identical,

indicating that the interaction between Fd and PSI is not altered in this mutant. These data show that changes of residue K₃₅ of PsaC drastically affect the cross-linking of Fd to subunits forming the reducing site of PSI and support the spectroscopic data indicating that the binding of Fd to the PSI complex is altered in these mutants. Carboxyl groups activated by EDC/sulfo-NHS react mainly with the ε-NH₂ group of lysine residues (Grabarek and Gergely, 1990). Amongst the three lysines of the PsaC subunit of *C.reinhardtii*, K₅₂ is clearly not involved in the cross-linking to Fd. To test the role of the third lysine (K₆, see Figure 1) in the cross-linking between Fd and PSI we analysed a strain with the K₆N mutation which was obtained by PCR-mediated random mutagenesis of the *psaC* gene (N.Fischer and J.-D.Rochaix, unpublished results). Figure 5A (lane 6) shows that PSI particles isolated from this mutant display the same cross-linking patterns with PsaC, PsaD and PsaE as wild-type. Therefore, the specific disappearance of the PsaC–Fd cross-link observed only when K₃₅ is changed, strongly supports the view that this residue is the cross-linking site between Fd and PsaC.

The signal corresponding to the PsaC–Fd cross-link (Figure 5A) is weak compared with the cross-linking products between Fd and PsaD or PsaE (Figure 5B and C). This raises the possibility that PsaC is less accessible to activated Fd, but could also be explained if the major epitope recognized by the polyclonal antibody includes the additional loop of PsaC, which is masked by the covalently bound Fd. The observation that free PsaC from the mutants L1–19 and L1–35 carrying triple mutations in this region is weakly recognized by this antibody is compatible with this possibility (data not shown).

We also tested whether activated Fd could react with other subunits of PSI. Surprisingly, a cross-linking product was revealed by Western blot analysis with an antibody raised against PsaF (Figure 6). This subunit has been reported to interact with PsaE and might have an exposed stromal domain (Xu *et al.*, 1994b; Jansson *et al.*, 1996; Kruip *et al.*, 1997). A cross-linking product between the soluble electron acceptor flavodoxin and PsaF has also been observed (Mühlenhoff *et al.*, 1996). To determine if this interaction was an artefact or if Fd indeed contacts a stromal portion of PsaF, we performed competition experiments with Fd and Cytc. The latter is an alternative luminal soluble electron carrier that reduces P700⁺, and is induced instead of plastocyanin when copper is limiting (Merchant and Bogorad, 1986). When the PSI complex was incubated in the presence of EDC/sulfo-NHS with Fd, a PsaF–Fd cross-link appeared (Figure 6A, lane 4). However, when both Fd and Cytc were present in the cross-linking reaction, only a band corresponding to the PsaF–Cytc product could be detected (Figure 6A, lanes 5 and 6). The absence of a high molecular weight product consisting of PsaF–Fd–Cytc, and the competition of Fd binding to PsaF by Cytc, indicate that the PsaF–Fd cross-link is due to a non-specific interaction between Fd and the luminal binding site of the PsaF subunit for Cytc. The proposed binding site contains several lysines for electrostatic interaction with Cytc or plastocyanin (Hippler *et al.*, 1996, 1997). Thus, our data suggest that these lysines can react non-specifically with Fd.

Fig. 6. Western blot analysis of PSI complexes cross-linked to Fd and/or Cytc. The samples were separated and blotted as described in Figure 6 and the blots incubated with antibodies raised against PsaF. Positions corresponding to the free and cross-linked polypeptides are indicated. Signals corresponding to free Cytc are due to heme staining by the chemiluminescence reagents used in these experiments. (A) Wild-type PSI complex after incubation with Fd and/or Cytc in the presence of the chemical cross-linkers EDC/sulfo-NHS as indicated on top of each lane. (B) Mutated residues in the PsaC subunit and the presence of activated Fd are shown at the top of each lane.

Discussion

Identification of K₃₅ of PsaC as an important residue by degenerate oligonucleotide-directed mutagenesis.

In this study, we attempted to obtain new insights into the function of a 10 amino acid stretch of the PsaC subunit, which is absent from 2[4Fe-4S] bacterial ferredoxins. We used a PCR-based mutagenesis approach to randomly introduce specific mutations in this region. Analysis of the transformants and sequencing of their *psaC* gene revealed several combinations of mutations affecting PSI function. Comparison of these combinations clearly showed that the K₃₅T change was responsible for a deficient growth phenotype under high light conditions and that K₃₅ was a promising candidate for further mutagenesis. A combination of two mutations, E₂₇V/D₂₉V, also appeared to affect PSI activity, and the function of these residues is currently under investigation. Further, the mutation M₂₈K does not allow stable assembly of the PSI complex *in vivo*. This mutagenesis approach proved to be very helpful for identifying potentially important residues within a defined region, with considerably less effort than a systematic site-directed mutagenesis strategy.

K₃₅ is important for Fd reduction and binding to PSI

We have mutated residue K₃₅ and identified four mutations K₃₅P, K₃₅T, K₃₅D and K₃₅E, which limit photoautotrophic growth of *C.reinhardtii*, although they do not appear to significantly alter PSI stability or assembly *in vivo*. In contrast, the growth properties of the K₃₅R mutant and wild-type are indistinguishable under the conditions tested in this study. Spectroscopic measurements of Fd reduction with PSI particles isolated from K₃₅T, K₃₅D and K₃₅E mutant strains demonstrate that the affinity of Fd for its

binding site on PSI is drastically diminished in these mutants, whereas it is similar to, and even higher than wild-type with the K₃₅R PSI complex. Binding of the negatively charged Fd to PSI is thought to be mediated by electrostatic interactions with the PsaD subunit. One interaction site revealed by chemical cross-linking was shown to be K₁₀₆ in *Synechocystis* sp. PCC 6803 (Lelong *et al.*, 1994). Surprisingly, replacement of this lysine by a neutral amino acid had little effect on the affinity of Fd. Replacement of K₁₀₆ by a cysteine resulted in a 10-fold increase of the dissociation constant, but the first order electron transfer reaction was unaffected (Hanley *et al.*, 1996). In contrast, removal of the positive charge provided by K₃₅ of PsaC eliminates the first order electron transfer and increases the dissociation constant >30-fold. This demonstrates that K₃₅ of PsaC is important for PsaC–Fd interaction and for fast electron transfer. Our results also show that an arginine residue can replace K₃₅. Interestingly, K₃₅ is conserved in all PsaC subunits studied so far except for *Cyanophora paradoxa* in which an arginine is found at this position (Rhiel *et al.*, 1992). The second order rate constant is reduced 5-fold when a neutral amino acid is present at position 35. However, a 35- to 80-fold reduction is observed when a negatively charged residue is introduced, suggesting that repulsion of negative charges occurs in the latter case. The large decrease in the second order rates observed when neutral or negatively charged residues are present at position 35 indicates that K₃₅ is involved in the electrostatic interaction which leads to proper diffusion-dependent recognition between the two partners, PSI and Fd. The 35- and 80-fold reductions found for K₃₅D and K₃₅E, respectively, appear to be limiting for photoautotrophic growth at light intensities higher than 60 $\mu\text{E}/\text{m}^2/\text{s}$. However, none of the mutations of K₃₅ affects photoautotrophic growth under low light conditions, suggesting that an 80-fold reduction in electron transfer rate from PSI to Fd is not limiting for the overall electron transport, although it is not clear to what extent this rate is affected *in vivo*. A similar situation has been described for a *C.reinhardtii* strain lacking the PSI subunit PsaF, in which a 20-fold reduction of the rate of electron transfer from reduced plastocyanin to P700⁺ *in vivo* does not appear to limit photoautotrophic growth (Farah *et al.*, 1995). Measurements performed *in vitro* with isolated PSI complex of this mutant showed that at optimal salt concentrations the reduction in electron transfer rate was 80-fold (Hippler *et al.*, 1997).

Fd can be cross-linked to PsaC

The interactions between Fd and PSI have been studied in different organisms, and chemical cross-linking of Fd to PsaD, PsaE and PsaH subunits has been reported (Zanetti and Merati, 1987; Zilber and Malkin, 1988; Andersen *et al.*, 1992a; Lelong *et al.*, 1994). Our results show that Fd can be cross-linked to the PsaD, PsaE and PsaC subunits of the PSI complex from *C.reinhardtii*. Although cross-linking of the alternative electron acceptor flavodoxin to PsaC has been shown to occur (Mühlenhoff *et al.*, 1996), this is the first report of a direct interaction between PsaC and its redox partner Fd.

Cross-linking experiments performed with PSI complexes carrying a mutation at each of the three lysine residues of the PsaC subunit of *C.reinhardtii* show that

cross-linking between Fd and PsaC is lost only when K₃₅ is modified. Although we cannot formally exclude cross-linking between the less reactive amino-terminus of PsaC and Fd, it is very unlikely that this interaction would only be affected by mutations at K₃₅. In addition, cysteine 34 is exposed to the surface of PSI, as determined by chemical modification experiments (P.Chitnis, personal communication), confirming that K₃₅ is exposed. Therefore, the data strongly suggest that K₃₅ is the cross-linking site between Fd and the PsaC subunit. Cross-linking of Fd to PsaD and PsaE is reduced when K₃₅ is changed to neutral or negatively charged amino acids, suggesting that the three cross-linking sites are part of the same binding pocket. The observation that high levels of cross-linking between PsaD, PsaE and Fd are found with the K₃₅R PSI complex indicate that the positive charge provided by arginine allows Fd to fit into its binding site. Interestingly, three and two first-order phases of electron transfer from PSI to Fd have been measured in *Synechocystis* sp. PCC 6803 and *C.reinhardtii*, respectively (Sétif and Bottin, 1994, 1995; Fischer *et al.*, 1997). These different phases may be ascribed to different PSI–Fd complexes leading to first order electron transfer with different kinetics. The observation that Fd can be cross-linked independently to the PsaD, PsaE and PsaC subunits is compatible with the view that Fd could adopt different positions within its binding pocket.

A new model of the reducing side of PSI

PsaD was considered as the Fd-docking subunit of PSI because it was found to cross-link to Fd (Lelong *et al.*, 1994). The fact that mutations of K₁₀₆ of PsaD, involved in the cross-linking of this subunit to Fd, have little effect on the binding of Fd to the PSI complex (Hanley *et al.*, 1996), the finding that PsaE is required for efficient Fd reduction (Rousseau *et al.*, 1993; Xu *et al.*, 1994a) and the present study revealing the role of K₃₅ of PsaC in this process, demonstrate that binding of Fd to the PSI complex is not achieved solely by the PsaD subunit. We propose a new model for the reducing side of PSI, in which the binding of the negatively charged Fd to PSI is mediated by electrostatic interactions with positively charged residues of the PsaC, PsaD and PsaE subunits. Among the site-directed changes introduced so far into these three subunits, none had such a dramatic effect on electron transfer to Fd as the changes of K₃₅. The function of the PsaC subunit is to bind the terminal electron acceptors of PSI. Our results show that PsaC also provides an important residue for electrostatic interaction with Fd to bring these two proteins in close contact for fast electron transfer between the terminal electron acceptors [F_AF_B] and the iron-sulfur cluster of Fd.

Materials and methods

Strains and media

C.reinhardtii wild-type and mutant strains were grown as described (Harris, 1989). If necessary the media [Tris acetate phosphate medium (TAP) and high salt minimal medium (HSM)] were solidified with 2% Bacto agar (Difco) and supplemented with spectinomycin (Sigma).

Nucleic acid techniques

Procedures for the preparation of recombinant plasmids and DNA sequencing were performed as described (Sambrook *et al.*, 1989). The

bacterial host used was *E. coli* DH5 α . *C. reinhardtii* total DNA was isolated as described previously (Rochaix *et al.*, 1988). Degenerate oligonucleotide-directed mutagenesis was performed using the following 42mer oligonucleotides: 5'-TTTAGATGTTTGTAG(A/T)AA(T/A)GG(T/A)T(C/T)CAT(G/C)GGATGGTTGTAAAGC-3' (loop-1 mutagenesis) and 5'-AATGGTTCCATGGG(A/T)TG(G/A)T(T/A)GTA(A/C)A(G/A)-CTAGTCAAATGGCGT-3' (loop-2 mutagenesis). Site-directed mutagenesis was performed using the following 30mer oligonucleotides: 5'-GCCATTTGACTAGCAGACAACCATCCCAT-3' (K₃₅R) and 5'-GCCATTTGACTAGTTTCGACAACCATCCCAT-3' (K₃₅E). Single tube PCR as described by Picard *et al.* (1994) was used with each mutagenic oligonucleotide, together with two oligonucleotides complementary to the 5' and 3' ends of *psaC* and carrying *NdeI* and *BglII* restriction sites, respectively. Extensive mutagenesis of lysine 35 was performed by the same method with the following 33mer oligonucleotide 5'-CCATGGGATGGTTGT(A/C/G/T)(A/C/G/T)(C/G/T)GCTAGTCAAATGGCG-3'. The amplified DNA fragments were gel purified, digested with *NdeI* and *BglII* and cloned into the plasmid pBSEP5.8 *aadA* [*NdeI/BglII*]. This plasmid contains a 5.8 *EcoRI-PstI* fragment from the chloroplast *EcoRI* fragment R23 (Rochaix, 1978) with two unique *NdeI* and *BglII* restriction sites introduced at the 5' and 3' ends of the *psaC* coding sequence (Fischer *et al.*, 1997). This vector contains also an *aadA* expression cassette conferring spectinomycin and streptomycin resistance to *C. reinhardtii* (Goldschmidt-Clermont, 1991), inserted at a *SalI* site 800 bp upstream of *psaC*. Two-step asymmetric PCR amplification was used to generate partially single stranded DNA as described in Dorit *et al.* (1997). The amplified DNA was phenol extracted, precipitated and used directly for dideoxy sequencing.

Chloroplast transformation in *C. reinhardtii* was carried out as described (Boynton *et al.*, 1988) with a helium driven particle gun adapted from the one described by Finer *et al.* (1992). Wild-type cells were grown at 25°C in liquid TAP medium and plated on TAP plates supplemented with 150 μ g/ml spectinomycin. Once the plates were dry, cells were bombarded with tungsten microprojectiles coated with the appropriate DNA. The bombarded cells were incubated for two weeks at 25°C under low light (5 μ E/m²/s). Growing colonies were restreaked on fresh TAP plates containing 150 μ g/ml spectinomycin and characterized.

Thylakoid membranes and PSI complex purification were performed as described in Fischer *et al.* (1997). Integrity of the wild-type and mutant PSI particles was tested by measuring the flash-induced absorption changes at 820 nm. These measurements showed that the PSI complexes had retained all the redox cofactors and were able to perform stable charge separation between [F_AF_B]⁻ (data not shown).

Isolation of ferredoxin and cytochrome *c*₆

Ferredoxin was purified from 15 liters of a *C. reinhardtii* cell culture, grown mixotrophically in TAP medium. The isolation of ferredoxin followed the procedure of the isolation of cytochrome *c*₆ (Hippler *et al.*, 1997). After the Sephacryl S200 column (60×1.5 cm), ferredoxin-containing fractions were collected and passed over a DEAE-Sepharose CL6B, that was equilibrated with 20 mM Tricine pH 7.8, 10 mM KCl (TK-buffer). Ferredoxin was retained on the column and was eluted by a KCl gradient (50–600 mM). The ferredoxin-containing fractions were pooled, diluted with TK-buffer to obtain a salt concentration of ~20 mM KCl and concentrated on an Amicon YM 3 membrane. The isolated ferredoxin had an absorbance ratio A₄₂₀/A₂₇₅ of 0.4. The concentrations of ferredoxin was determined spectroscopically using an extinction coefficient of 9.4/mM/cm at 423 nm for the oxidized form of ferredoxin (Fee and Palmer, 1971). Recombinant Fd from *C. reinhardtii* was prepared according to Jacquot *et al.* (1997). This recombinant ferredoxin contains an additional alanine at the N-terminus and the T₇S mutation. These changes do not appear to alter its properties (Rogers *et al.*, 1992).

Flash-absorption and electron paramagnetic resonance (EPR) spectroscopy

Decay kinetics of P700⁺ at 820 nm and ferredoxin reduction at 580 nm were recorded at 296 K with a microsecond time resolution as described previously (Sétif and Bottin, 1994, 1995). For these measurements, both authentic and recombinant *C. reinhardtii* Fd were used and gave identical results. In the absence of Fd, an absorption change at 580 nm is also observed, and the contribution of P700⁺ decay is different in the presence or absence of Fd (for a detailed description, see Sétif and Bottin, 1995). These contributions were subtracted from the signals shown in Figure 4. Low temperature EPR spectroscopy was performed with a Bruker ESR300D X-band spectrometer equipped with an Oxford Instruments helium cryostat. Estimation of the lower limit of K_d of Fd for PSI in the mutants was determined as follows:

$K_d = [\text{PSI}]_{\text{free}} \times [\text{Fd}]_{\text{free}} / [\text{PSI-Fd}]$. Under conditions where there is little or no complex formation, $[\text{Fd}]_{\text{free}} = [\text{Fd}]_{\text{total}}$ and $[\text{PSI}]_{\text{free}} = [\text{PSI}]_{\text{total}}$. The above equation can be rewritten: $K_d = [\text{Fd}]_{\text{total}} / \text{proportion of PSI forming a complex}$. From the signal to noise ratio of our experiments, we can estimate that the above proportion is less than 5% for a ferredoxin concentration of 16 μ M, which means: $K_d > 16/0.05 = 320 \mu\text{M}$.

Chemical cross-linking

N-ethyl-3-(3-dimethylaminopropyl) carbodiimide, (EDC) and *N*-hydroxysulfosuccinimide ester (sulfo-NHS) were purchased from Sigma. Native Fd at a concentration of 100 μ M was activated with 5 mM EDC, 10 mM sulfo-NHS in 10 mM MOPS pH 6.5 and incubated at room temperature for 20 min. The sample was then loaded on a PD10 column (Pharmacia) equilibrated with 30 mM HEPES pH 7.5. The brown band corresponding to Fd was eluted with 30 mM HEPES pH 7.5 and concentrated on a YM5 centricon centrifugation unit (Amicon). PSI particles at a concentration of 0.1 mg chlorophyll/ml were incubated with 10 μ M activated Fd in 1 mM ascorbate, 0.03% β -dodecyl maltoside (CalBiochem), 3 mM MgCl₂ and 30 mM HEPES pH 7.5 for 1 h at room temperature in the dark. For competition between Fd and Cyt_c in cross-linking experiments, PSI particles at a concentration of 0.1 mg chlorophyll/ml were incubated with 20 μ M Cyt_c and/or 10 μ M Fd together with 5 mM EDC and 10 mM sulfo-NHS, under the same conditions as described above.

SDS-PAGE was carried out according to Laemmli (1970). After electrophoresis, proteins were electroblotted onto nitrocellulose as described in Hippler *et al.* (1989), probed with specific antibodies and revealed by enhanced chemiluminescence using the ECL kit (Amersham).

Acknowledgements

We thank K.Redding for the gift of antibodies against PsaA and for stimulating discussion and N.Roggli for drawings and photography. M.H. acknowledges the support of a long-term fellowship from the Human Frontier Science Program. This work was supported by a grant from the Human Frontier Science Program to P.S. and J.-D.R. and grant 31.34014.92 from the Swiss National Fund to J.-D.R.

References

- Andersen, B., Koch, B. and Scheller, H. V. (1992a) Structural and functional analysis of the reducing side of photosystem I. *Physiol. Plant.*, **84**, 154–161.
- Andersen, B., Scheller, H. V. and Moller, B. L. (1992b) The PSI-E subunit of photosystem I binds ferredoxin: NADP⁺- oxidoreductase. *FEBS Lett.*, **311**, 169–174.
- Bennoun, P. and Delepelaire, P. (1982) Isolation of photosynthetic mutants in *Chlamydomonas*. In Edelman *et al.* (eds), *Methods in Chloroplast Molecular Biology*. Elsevier Biomedical Press, pp. 25–38.
- Boynton, J. E., Gilham, N. W. and Harris, E. H. (1988) Chloroplast transformation in *Chlamydomonas* with high velocity microprojectiles. *Science*, **240**, 1534–1538.
- Brettel, K. (1997) Electron transfer and arrangement of the redox cofactors in photosystem I. *Biochim. Biophys. Acta*, **1318**, 322–373.
- Chitnis, P. R., Reilly, P. A. and Nelson, N. (1989) Insertional inactivation of the subunit II of photosystem I from the cyanobacterium *Synechocystis* sp. PCC 6803. *J. Biol. Chem.*, **264**, 16381–16385.
- Dorit, R. L., Ohara, O., Hwang, C. B.-C. (1997) Direct sequencing of PCR products. In Ausubel, F. A., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. C., Smith, J. A. and Struhl, K. (eds), *Current Protocols in Molecular Biology*, John Wiley and Sons, New York, pp. 15.2.1–15.2.11.
- Dunn, P. P. J. and Gray, J. C. (1988) Localisation and sequence of the gene for the 8 kDa subunit of photosystem I in pea and wheat chloroplast DNA. *Plant Mol. Biol.*, **11**, 311–319.
- Farah, J., Rappaport, F., Choquet, Y., Joliet, P. and Rochaix, J.-D. (1995) Isolation of *psaF*-deficient mutant of *Chlamydomonas reinhardtii*: efficient interaction of plastocyanin with photosystem I reaction center is mediated by the PsaF subunit. *EMBO J.*, **14**, 4976–4984.
- Fee, J. A. and Palmer, G. (1971) The properties of parsley ferredoxin and its selenium-containing homolog. *Biochim. Biophys. Acta*, **245**, 175–195.
- Finer, J. J., Vain, P., Jones, M. W. and McMullen, M. D. (1992) Development of the particle inflow gun for DNA delivery to plant cells. *Plant Cell Reports*, **11**, 323–328.

- Fischer, N., Stampacchia, O., Redding, K. and Rochaix, J.-D. (1996) Selectable marker recycling in the chloroplast. *Mol. Gen. Genet.*, **251**, 373–380.
- Fischer, N., Sétif, P. and Rochaix, J.-D. (1997) Targeted mutations in the *psaC* gene of *Chlamydomonas reinhardtii*: preferential reduction of F_B at low temperature is not accompanied by altered electron flow from photosystem I to ferredoxin. *Biochemistry*, **36**, 93–102.
- Golbeck, J.H. (1992) Structure and function of PSI. *Annu. Rev. Plant Physiol. and Plant Mol. Biol.*, **43**, 293–324.
- Goldschmidt-Clermont, M. (1991) Transgenic expression of aminoglycoside adenine transferase in the chloroplast: a selectable marker for site-directed transformation of *Chlamydomonas*. *Nucleic Acids Res.*, **19**, 4083–4089.
- Grabarek, Z. and Gergely, J. (1990) Zero-length cross-linking procedure with the use of active esters. *Anal. Biochem.*, **185**, 131–135.
- Hanley, J., Sétif, P., Bottin, H. and Lagoutte, B. (1996) Mutagenesis of the ferredoxin cross-linking site: modification of positively charged amino acids. *Biochemistry*, **35**, 8563–8571.
- Harris, E.H. (1989) *The Chlamydomonas Source Book: a Comprehensive Guide to Biology and Laboratory Use*. Academic Press, Inc., San Diego, CA.
- Hayashida, N., Matsubayashi, K., Shinozaki, M., Inoue, K. and Hiyama, T. (1987) The gene for the 9 kDa polypeptide, a possible apoprotein for the iron-sulfur centers A and B of the photosystem I complex, in the tobacco chloroplast DNA. *Curr. Genet.*, **12**, 247–250.
- Hippler, M., Ratajczak, R. and Haehnel, W. (1989) Identification of the plastocyanin binding subunit of photosystem I. *FEBS Lett.*, **250**, 280–284.
- Hippler, M., Reichert, J., Sutter, M., Zak, E., Altschmied, L., Schröer, U., Herrmann, R.G. and Haehnel, W. (1996) The plastocyanin binding domain of photosystem I. *EMBO J.*, **15**, 6374–6384.
- Hippler, M., Drepper, F., Farah, J. and Rochaix, J.-D. (1997) Fast electron transfer from cytochrome *c*₆ and plastocyanin to photosystem I of *Chlamydomonas reinhardtii* requires Psf. *Biochemistry*, **36**, 6343–6349.
- Høj, P.B., Swendsen, I., Scheller, H.V. and Møller, B.L. (1987) Identification of a chloroplast-encoded 9 kDa polypeptide as a 2[4Fe-4S] protein carrying centers A and B of photosystem I. *J. Biol. Chem.*, **262**, 12676–12684.
- Jacquot, J.P., Stein, M., Suzuki, A., Liottet, S. and Miginiac-Maslow, M. (1997) Residue Glu 91 of *Chlamydomonas reinhardtii* is essential for electron transfer to ferredoxin thioredoxin reductase. *FEBS Lett.*, **400**, 293–296.
- Jansson, S., Andersen, B. and Scheller, H.V. (1996) Nearest-neighbour analysis of higher plant photosystem I holocomplex. *Plant. Physiol.*, **112**, 409–420.
- Krauss, N., Hinrichs, W., Witt, I., Fromme, P., Pritzkow, W., Dauter, Z., Betzel, C., Wilson, K.S., Witt, H. and Saenger, W. (1993) Three-dimensional structure of system I of photosynthesis at 6 angstrom resolution. *Nature*, **361**, 326–331.
- Krauss, N., Schubert, W.D., Kuklas, O., Fromme, P., Witt, H. and Saenger, W. (1996) Photosystem I at 4 Å resolution represents the first structural model of a joint photosynthetic reaction center and core antenna system. *Nat. Struct. Biol.*, **3**, 965–973.
- Kruip, J., Chitnis, P.R., Lagoutte, B., Rögnér, M. and Boekema, E.J. (1997) Structural organization of the major subunits in cyanobacterial photosystem I. *J. Biol. Chem.*, **272**, 17061–17069.
- Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680–685.
- Lelong, C., Sétif, P., Lagoutte, B. and Bottin, H. (1994) Identification of the amino acids involved in the functional interaction between photosystem I and ferredoxin from *Synechocystis* sp. PCC 6803 by chemical cross-linking. *J. Biol. Chem.*, **269**, 10034–10039.
- Li, N., Zhao, J., Warren, P.V., Warden, J.T., Bryant, D. and Golbeck, J.H. (1991) Psf is required for the stable binding of Psf to the photosystem I core protein in *Synechococcus* sp. PCC 6301. *Biochemistry*, **30**, 7853–7872.
- Merchant, S. and Bogorad, L. (1986) Regulation by copper of the expression of plastocyanin and cytochrome *c*552 in *Chlamydomonas reinhardtii*. *Mol. Cell. Biol.*, **6**, 462–469.
- Mühlhoff, U., Zhao, J. and Bryant, D.A. (1996) Interaction between photosystem I and flavodoxin from the cyanobacterium *Synechococcus* sp. PCC 7002 as revealed by chemical cross-linking. *Eur. J. Biochem.*, **235**, 324–331.
- Naver, H., Scott, M.P., Andersen, B., Møller, B.L. and Scheller, H.V. (1995) Reconstitution of barley photosystem I reveals that the N-terminus of PSI-D subunit is essential for tight binding of PSI-C. *Physiol. Plant.*, **95**, 19–26.
- Naver, H., Scott, M.P., Golbeck, J.H., Møller, B.L. and Scheller, H.V. (1996) Reconstitution of Barley photosystem I with Modified PSI-C allows identification of domains interacting with PSI-D and PSI-A/B. *J. Biol. Chem.*, **271**, 8996–9001.
- Oh-Oka, H., Takahashi, Y., Wada, K., Matsubara, H., Ohya, K. and Ozeki, H. (1987) The 8 kDa polypeptide in photosystem I is a probable candidate of an iron-sulfur center protein coded by the chloroplast gene *frxA*. *FEBS Lett.*, **218**, 52–54.
- Picard, V., Ersdal-Badju, E., Lu, A. and Bock, S.C. (1994) A rapid and efficient one-tube PCR-based mutagenesis technique using Pfu DNA polymerase. *Nucleic Acids Res.*, **22**, 2587–2591.
- Rhiel, E., Stirewalt, V.L., Gasparich, G.E. and Bryant, D.A. (1992) The *psaC* genes of *Synechococcus* sp. 7002 and *Cyanophora paradoxa*: cloning and sequence analysis. *Gene*, **112**, 123–128.
- Rochaix, J.-D. (1978) Restriction endonuclease map of the chloroplast DNA of *Chlamydomonas reinhardtii*. *J. Mol. Biol.*, **126**, 597–617.
- Rochaix, J.-D., Mayfield, S., Goldschmidt-Clermont, M. and Erickson, J. (1988) Molecular biology of *Chlamydomonas*. In Shaw, C.H. (ed.), *Plant Molecular Biology: A Practical Approach*. IRL Press, Oxford, pp. 253–275.
- Rogers, W.J., Hodges, M., Decottignies, P., Schmitter, J.-M., Gadal, P. and Jacquot, J.-P. (1992) Isolation of a cDNA fragment coding for *Chlamydomonas reinhardtii* ferredoxin and expression of the recombinant protein in *Escherichia coli*. *FEBS Lett.*, **3**, 240–245.
- Rousseau, F., Sétif, P. and Lagoutte, B. (1993) Evidence for the involvement of PSI-E subunit in the reduction of ferredoxin by photosystem I. *EMBO J.*, **12**, 1755–1765.
- Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Sétif, P. and Bottin, H. (1994) Laser flash absorption spectroscopy study of ferredoxin reduction by photosystem I in *Synechocystis* sp. PCC6803: evidence for submicrosecond and microsecond kinetics. *Biochemistry*, **33**, 8495–8504.
- Sétif, P. and Bottin, H. (1995) Laser flash absorption spectroscopy study of ferredoxin reduction by photosystem I: spectral and kinetic evidence for the existence of several photosystem I-ferredoxin complexes. *Biochemistry*, **34**, 9059–9070.
- Sonoike, K., Hatanaka, H. and Katoh, S. (1993) Small subunits of photosystem I reaction center complexes from *Synechococcus elongatus*. II. The *psaE* gene product has a role to promote interaction between the terminal electron acceptor and ferredoxin. *Biochim. Biophys. Acta*, **1141**, 45–51.
- Takahashi, Y., Goldschmidt-Clermont, M., Soen, S.-Y., Franz, L.-G. and Rochaix, J.-D. (1991) Directed chloroplast transformation in *Chlamydomonas reinhardtii*: insertional inactivation of the *psaC* gene encoding the iron sulfur protein destabilizes photosystem I. *EMBO J.*, **10**, 2033–2040.
- Takahashi, Y., Goldschmidt-Clermont, M. and Rochaix, J.-D. (1992) Directed mutagenesis of chloroplast gene *psaC* in *Chlamydomonas reinhardtii*. In Murata, N.E. (ed.), *Research in Photosynthesis Vol.III*. Kluwer Academic Publishers In Proc., pp. 393–396.
- Xu, Q., Jung, Y.S., Chitnis, V.P., Guikema, J.A., Golbeck, J.H. and Chitnis, P.R. (1994a) Mutational analysis of photosystem I polypeptides in *Synechocystis* sp. PCC 6803: subunit requirements for the reduction of NADP⁺ mediated by ferredoxin or flavodoxin. *J. Biol. Chem.*, **269**, 21512–21518.
- Xu, Q., Xu, L., Chitnis, V.P. and Chitnis, P.R. (1994b) Function and organization of photosystem I in a cyanobacterial mutant strain that lacks Psf and Psf subunits. *J. Biol. Chem.*, **269**, 3205–3211.
- Zanetti, G. and Merati, G. (1987) Interaction between photosystem I and ferredoxin: identification by chemical cross-linking of the polypeptide which binds ferredoxin. *Eur. J. Biochem.*, **169**, 143–146.
- Zhao, J., Warren, P.V., Li, N., Bryant, D.A. and Golbeck, J.H. (1990) Reconstitution of electron transport in photosystem I with Psf and Psf proteins expressed in *Escherichia coli*. *FEBS Lett.*, **276**, 175–180.
- Zilber, A. and Malkin, R. (1988) Ferredoxin cross-links to a 22 kDa subunit of photosystem I. *Plant Physiol.*, **88**, 810–814.

Received July 18, 1997; revised December 2, 1997;
accepted December 3, 1997