Site-directed Mutagenesis of the PsaC Subunit of Photosystem I

Nicolas Fischer*, Pierre Sétif§, and Jean-David Rochaix‡

From the §Departments of Molecular Biology and Plant Biology, University of Geneva, 30 quai Ernest-Ansermet, 1211 Geneva, Switzerland and the §CEA, Département de Biologie Cellulaire et Moléculaire, CNRS,URA 2096, C.E. Saclay, 91191, Gif-Sur-Yvette Cedex, France

The two [4Fe-4S] clusters F_A and F_B are the terminal electron acceptors of photosystem I (PSI) that are bound by the stromal subunit PsaC. Soluble ferredoxin (Fd) binds to PSI via electrostatic interactions and is reduced by the outermost iron-sulfur cluster of PsaC. We have generated six site-directed mutants of the green alga Chlamydomonas reinhardtii in which residues located close to the iron-sulfur clusters of PsaC are changed. The acidic residues Asp and Glu, which are located one residue upstream of the first cysteine liganding cluster F_B and F_A, respectively, were changed to a neutral or a basic amino acid. Although Fd reduction is not affected by the E46Q and E46K mutations, a slight increase of Fd affinity (from 1.3- to 2-fold) was observed by flash absorption spectroscopy for the D9N and D9K mutant PSI complexes. In the F_A, triple mutant (V49I/K52T/R53Q), modification of residues located next to the F_A cluster leads to partial destabilization of the PSI complex. The electron paramagnetic resonance properties of cluster F_A are affected, and a 3-fold decrease of Fd affinity is observed. The introduction of positively charged residues close to the F_B cluster in the F_B, triple mutant (I12V/T15K/Q16R) results in a 60-fold increase of Fd affinity as measured by flash absorption spectroscopy and a larger amount of PsaC-Fd cross-linking product. The first-order kinetics are similar to wild type kinetics (two phases with t_1/2 of <1 and ~4.5 μs) for all mutants except FB1, where Fd reduction is almost abolished with t_1/2 < 1 μs. These data indicate that F_B is the cluster interacting with Fd and therefore the outermost iron-sulfur cluster of PSI.

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‡§ From the Departments of Molecular Biology and Plant Biology, University of Geneva, 30 quai Ernest-Ansermet, 1211 Geneva, Switzerland and the CEA, Département de Biologie Cellulaire et Moléculaire, CNRS, URA 2096, C.E. Saclay, 91191, Gif-Sur-Yvette Cedex, France

The abbreviations used are: PSI, photosystem I; Fd, ferredoxin; EPR, electron paramagnetic resonance; EDC, N-ethyl-3-(3-dimethylaminopropyl) carbodiimide; sulfo-NHS, N-hydroxysulfosuccinimide ester; MOPS, 4-morpholinepropanesulfonic acid; WT, wild type; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

Photosystem I (PSI) is a multicomponent complex located in the thylakoid membranes of oxygenic photosynthetic organisms. This complex uses the energy of light to drive electron transfer from plastocyanin or cytochrome c₅₅₉ to ferredoxin (Fd) (1). In eukaryotes, PSI is composed of at least five chloroplast-encoded polypeptides (PsaA, -B, -C, -I, and -J) and six nucleus-encoded polypeptides (PsaD, -E, -F, -G, -H, and -K) (2). The two major subunits, PsaA and PsaB, bind the primary electron donor P700 (chlorophyll a dimmer) and the intermediate electron acceptors A₅ (chlorophyll a monomer), A₁ (phytoquinone), and F₅₆₅ ([4Fe-4S] cluster) (1). The terminal electron acceptors F_A and F_B are two [4Fe-4S] clusters coordinated by the 8–9-kDa subunit PsaC (3, 4). PsaC is highly conserved among species and contains two CXXCXXXXXCP motifs that are characteristic of [4Fe-4S] binding proteins (2). Cysteines 11, 14, 17, and 58 coordinate the iron atoms of cluster F_B whereas cysteines 21, 48, 51, and 58 coordinate cluster F_A (5). The midpoint potentials of F_A and F_B have been determined in spinach using low temperature electronic paramagnetic resonance (EPR) and are ~540 and ~590 mV, respectively (6, 7). It is, however, not clear whether these values of midpoint potentials are valid at physiological temperature (see Ref. 1 for a discussion). A structural model for the PsaC subunit has been proposed based on the crystal structure of the [2Fe2] ferredoxin from Peptococcus asaccharolyticus (Peptococcus aerogenes) (8, 9). The x-ray structure of PSI has been solved at a resolution of 4 Å in which the position of the three iron-sulfur clusters is clearly defined (10). One cluster is proximal to F_A at a distance of 15 Å, whereas the more distal cluster is at a distance of 22 Å from F_X. Interaction of the negatively charged Fd with the PSI complex is mediated by basic residues provided by the stromal subunits PsaC, -D, and -E, which are all required for efficient electron transfer to Fd (11–16). Studies by electron microscopy of the PSI-Fd covalent complex (17) and modelling studies (18) indicate that the Fd binding site is located close to the distal cluster and that the latter is the electron donating cluster to Fd, suggesting a linear electron pathway between F_B and Fd. The present resolution of the PSI structure is, however, not sufficient to determine the orientation of the PsaC subunit within the complex in particular because of the local 2-fold pseudosymmetry of this subunit (19, 20). Therefore the unambiguous assignment of F_A or F_B as being the proximal or distal cluster has not yet been possible from structural data. Several studies involving the selective chemical inactivation of F_B (21–23), in vitro reconstitution of PSI complex with mutated recombinant PsaC subunit (24, 25) and in vivo site-directed mutagenesis (26) have led to contradictory results, favoring either orientation of PsaC relative to the core complex. More recent work still leads to contradictory results with reconstitution of modified PsaC supporting F_A as the distal cluster (27, 28), whereas characterization of electron transfer properties of PSI after selective inactivation of cluster F_B provides evidence for F_B being the distal cluster (29, 30).

In this work, we have used in vivo site-directed mutagenesis of the PsaC subunit of the green alga Chlamydomonas reinhardtii to modify residues that are located close to the iron-sulfur clusters to alter the electron transfer properties of these mutant PSI complexes. We have previously used a similar approach to alter the properties of cluster F_A by modifying two

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positively charged residues located next to this cluster (31). Here we describe mutations close to F_{A} that alter its EPR properties and that lead to partial destabilization of the complex; we show that introduction of basic residues or removal of acidic residues close to F_{B} allows for stable assembly of the complex and leads to an increase of Fd affinity for the PSI complex. These results provide additional strong evidence that F_{B} is the cluster interacting with Fd and the terminal electron acceptor of PSI.

EXPERIMENTAL PROCEDURES

*C. reinhardtii* wild type and mutant strains were grown as described (32). If necessary, the media, Tris acetate phosphate medium (TAP medium) and high salt minimal medium, were solidified with 2% Bacto agar (Difco) and supplemented with spectinomycin (Sigma). Procedures for the preparation of recombinant plasmids and DNA sequencing were performed as described (33). The bacterial host used was *Escherichia coli* DH5α. *C. reinhardtii* total DNA was isolated as described previously (34). The FA_{1} and FB_{1} mutations were introduced in the *psaC* coding region by single tube polymerase chain reaction as described (35). The D9N, D9K, E46Q, and E46K mutations were introduced by a two-step amplification with pairs of complementary oligonucleotides that carry the desired mutation and introduce a restriction site into the sequence. The third base of the codon to be mutated carried a 2-fold degeneracy to generate DNA fragments carrying both mutations with a single pair of oligonucleotides. The following oligonucleotides were used: D9N and D9K, 5′-CGTAAAATTTCCAC/AACGCTGAT GTTTGAAC-3′ and 5′-GTCAAAATTTCCAC/GTCTGATTTAAA CG-3′ (MaeII site introduced); E46Q and E46K, 5′-CACCGTTCAAGCA CTCGA/AGACTCTCTCCGATGTG-3′ and 5′-CCACCTGACCTC TCTCCTGTTGAGCAGTTGA-3′ (Ddel site introduced); FA2, 5′-CGCA CTGAGACTCTTGTGGCTGACATGATTGAAAGCCTT-3′; and FB1, 5′-AAACGCGCTCACAAGGTTAACGGCAACGACCAATGAT CGTAAAT-3′. These mutagenic oligonucleotides were used for amplification together with oligonucleotides complementary to the 5′ and 3′ ends of the wild type and mutant restriction sites, respectively. The amplified DNA fragments were gel purified, digested with NdeI and BglII, cloned into the plasmid pBSEP5.8 (NdeI/BglII) and sequenced. This plasmid carries a 5.8-kilobase EcoRI-PstI fragment from the chloroplast EcoRI fragment R23 (36) with unique NdeI and BglII restriction sites introduced at the 5′ and 3′ ends of the *psaC* coding sequence (31). This vector contains also an *aadA* expression cassette at 580 nm and from spectinomycin resistance to wild type, indicating that they accumulate functional and/or present in reduced amounts. Growth properties of the transformants and control strains are shown in Table I. Both the FA_{2} and FB_{1} mutants are unable to grow photosensitive at 600 μm, suggesting a defect in photosynthetic activity. The *psaC* gene is present in the recipient strain, the presence of the FB_{1} and FA_{2} mutations in the transformants was checked by polymerase chain reaction and confirmed by sequencing of the amplification product (data not shown).

Analysis of the Transformants—Cells grown under dim light conditions (5 μE/m²/s) were dark adapted for 5 min before recording their fluorescence transients to determine whether they contained functional PSI (46). With the exception of FB_{1}, all the transformants displayed fluorescence transients comparable with wild type, indicating that they accumulate functional PSI (Fig. 1). The fluorescence transient of the FB_{2} strain is intermediate between those characteristic of PSI-deficient (like psaC−: aadA) and wild type strains. This suggests that the FB_{2} mutant accumulates PSI complexes that are not fully functional and/or present in reduced amounts. Growth properties of the transformants and control strains are shown in Table I. Both the FA_{2} and FB_{1} mutants are unable to grow photoautotrophically under 60 μE/m²/s illumination and are photosensitive at 600 μE/m²/s on TAP (acetate containing) medium, suggesting a defect in photosynthetic activity. The D9N, D9K, E46Q, and E46K mutations do not affect the growth properties of the transformants.

We determined the level of steady-state accumulation of the PSI complex by Western blot analysis of total cells extracts with antibodies raised against the PsaA, D, and E subunits of described (44), probed with specific antibodies, and revealed by enhanced chemiluminescence using the ECL kit (Amersham Pharmacia Biotech).

RESULTS

Site-directed Mutagenesis of the *psaC* Gene—We targeted two classes of residues in the PsaC subunit. First, we mutated Asp^{9} and Glu^{46}, which are located one residue upstream of the first cysteine liganding clusters F_{A} and F_{B}, respectively. Asp^{9} has been proposed to mediate docking of PsaC on the core complex by interacting electrostatically with basic residues of PsaA or PsaB (24). To test this hypothesis but also to assess whether these residues participate in determining the properties of the iron-sulfur clusters, we introduced the following changes into the *psaC* subunit: D9N, D9K, E46Q, and E46K. In another set of mutants we exchanged three amino acids located between cysteines liganding F_{A} by the corresponding residues located close to F_{B} and *vice versa*. The following triple mutations were designed: 112VT15K/Q16R and V49I/K52T/R53Q (for simplicity these sets of mutations will be referred to as FB_{1} and FA_{2}, respectively). We used oligonucleotides carrying the desired mutations together with oligonucleotides flanking the *psaC* coding sequence to generate via polymerase chain reaction mutated versions of the *psaC* gene (see “Experimental Procedures” for details). The amplified products were cloned into a vector comprising a 5.8-kilobase DNA fragment of the chloroplast genome containing the *psaC* gene and the aadA cassette that confers spectinomycin and streptomycin resistance to *C. reinhardtii* (37). The constructs were introduced into the chloroplast genome of *C. reinhardtii* via biofilm transfection (38). The D9N, D9K, E46Q, and E46K mutations were transformed into a wild type strain, whereas FB_{1} and FA_{2} were transformed into a strain in which the *psaC* coding region had been deleted (45). The transformants were selected on TAP medium plates supplemented with 150 μg/ml spectinomycin under low light conditions (5 μE/m²/s). Growing colonies were restreaked several times on the same selective medium and analyzed. The D9N, D9K, E46Q, and E46K mutations modify a restriction site in the genome. The homoplasmicity of the mutant genomes was verified by Southern blot analysis (data not shown, see “Experimental Procedures” for details). Because no wild type copy of the *psaC* gene is present in the recipient strain, the presence of the FB_{1} and FA_{2} mutations in the transformants was checked by polymerase chain reaction and confirmed by sequencing of the amplification product (data not shown).
PSI. Fig. 2 shows that PSI accumulates normally in the mutants except in the FA2 strain in which PSI is reduced to 20–30% of wild type level, as determined by PhosphorImager quantification (data not shown). This result shows that the FA2 mutations destabilize the complex, whereas all the other mutations have essentially no effect on PSI steady-state accumulation. The data also suggest that the PSI complex present in the FB1 strain is not fully functional, because the growth phenotype and fluorescence transient of this strain cannot be explained by a reduced PSI level (Fig. 1 and Table I).

Low Temperature Light-induced EPR Spectra of PSI Complexes— The PSI complex was purified by solubilization of thylakoid membranes with β-dodecylmaltoside and sucrose density gradient centrifugation. Integrity of the PSI complex was checked by Western blot analysis and flash absorption spectroscopy at 820 nm (data not shown). Below 30 K, PSI undergoes a stable light-induced charge separation between P700' and (FA,FB)2 that can be observed by EPR spectroscopy. When samples are incubated in the dark with DCPIP to reduce P700' and frozen in darkness, photoreduction of PSI leads to the formation of FA2 and FB2 that can be distinguished by their respective g values (FA2, 2.047, 1.945, and 1.857; FB2, 2.07, 1.932, and 1.878). Fig. 3 shows light-induced EPR spectra of PSI complexes isolated from wild type and the different mutants measured after 2 min of illumination at 15 K and recorded in darkness (g values of FA2 and FB2 in the upper spectrum are indicated by continuous and dotted lines, respectively). Two other signals at g ~ 1.91 and 2.11 were previously ascribed to a modified form of FA2, which is only observed in C. reinhardtii (16). Signals were normalized to the same concentration of intact PSI as measured by absorption changes at 820 nm. The amount of charge separation is the same (∼10%) in all samples as measured by double integration of similar spectra recorded under nonsaturating conditions (2 mW microwave power). All the spectra gave g values similar to wild type values except for the FA2 mutant, for which a new signal on the left side of the spectra was observed.

Table I

Growth phenotypes of wild type and mutants

| Strain | TDL | TL | THL | HSML | HSMHL |
|--------|-----|----|-----|------|-------|
| WT     | ++  | ++ | ++  | ++   | ++    |
| psaC::aadA | +   | +  | +   | +    | +     |
| D9K    | ++  | ++ | ++  | +    | +     |
| D9N    | ++  | ++ | ++  | +    | +     |
| E46Q   | ++  | ++ | ++  | +    | +     |
| E46K   | ++  | +  | +   | +    | +     |
| FB1    | +   | +  | +   | -    | -     |
| FA2    | +   | +  | +   | -    | -     |

High Affinity Binding of Ferredoxin to Mutant PSI

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Fig. 1. Fluorescence transients of dark adapted cells of wild type, D9K, D9N, E46K, E46Q, FB1, and FA2 and from a strain (psaC::aadA) in which the psaC coding sequence has been disrupted with the aadA selectable marker (58). Cells were grown in dim light (5 μE/m²/s) and dark adapted before the measurements.

Fig. 2. Immunoblot analysis of total cell extracts of wild type and the FB1, FA2, D9K, D9N, E46K, and E46Q mutants and of a strain lacking psaC (ΔC). Cells were grown in liquid TAP medium in low light (5 μE/m²/s). 15 μg of total proteins were loaded per lane. Proteins were separated by SDS-polyacrylamide gel electrophoresis, electroblotted on nitrocellulose membrane, and probed with antibodies raised against the subunits PsA, PsD, and PsE of PSI. An antibody raised against the D1 protein of photosystem II was also used as loading control.
High Affinity Binding of Ferredoxin to Mutant PSI

Fig. 3. Light-induced EPR spectra of PSI complexes isolated from wild type and from the E46K, E46Q, D9K, D9N, FB1, and FA2 mutants. Each of these spectra corresponds to the difference between spectra recorded after a 2-min illumination at 14 K and in darkness. The large signal around g = 2.00 (mostly P700+ ) is not shown. Spectra correspond to approximately 3 μM PSI. EPR conditions were as follows: temperature, 15 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 20 mM Tricine, pH 8.0, in the presence of 5 mM sodium ascorbate and 25 μM DCPIP. Tubes were incubated at room temperature for 2 min in darkness before freezing in darkness.

decay of carotenoid triplet states. Therefore, the signals shown in the upper part of Fig. 5 correspond to electron transfer from (FA′, FB′ ) to Fd. When absorption transients are measured with wild type PSI and Fd (Fig. 5, left upper panel, trace WT), three phases of Fd reduction are observed: the two fastest phases (t1/2 of <1 μs and about 4.5 μs) were identified as first-order components because their half-times are unaffected by Fd concentration and their amplitudes increase together with Fd concentration; the slowest phase was identified as a slower second-order component because its rate depends linearly upon Fd concentration (16, 31). The first-order phases are ascribed to electron transfer within a PSI-Fd complex formed prior to the laser flash, whereas the second-order phase is due to diffusion-limited Fd reduction by PSI that does not initially bind Fd. At 580 nm, the amplitude of the 4.5-μs phase is about 2-fold larger than the submicrosecond phase in WT PSI. The WT kinetic trace can be compared in the upper part of Fig. 5 with traces obtained with the different mutants. Traces can be directly compared as they are normalized to identical PSI concentrations, except for FB1 ([Fd] = 0.39 μM) (see legend to Fig. 5). On the time scale that is shown, the amplitudes after a few tens of a microsecond can be ascribed mostly to first-order Fd reduction, whereas the “slope” that is visible afterward in all cases except for FB1 is due to second-order Fd reduction. From the figure, it can be seen that the different PSI complexes can be arranged in the following ascending order of Fd affinity: FA2 < WT ~ E46K (~ E46Q) < D9K (~ D9N) < FB1. The WT spectrum was in both case multiplied by a factor 0.65. Dif. indicates FA2 = 0.65*WT.

Fig. 4. Light-induced EPR spectra of PSI complexes isolated from wild type and from the FA2 mutant under conditions where the signals corresponding to FA are barely detectable. EPR conditions were as follows: temperature, 9 K; microwave power, 20 mW; modulation amplitude, 1 mT; microwave frequency, 9.419 GHz. The samples were treated as in Fig. 3. The two lower spectra correspond to the weighted difference of the wild type and FA2 spectra from this figure (dif. 1) and from Fig. 3 (dif. 2). The wild type spectrum was shown in the lower part of Fig. 5 for WT and FB, and as summarized in Table II. Compared with WT (Kd ~ 7.5 μM), Fd part of the spectrum is clearly visible. To characterize this signal, EPR light minus dark difference spectra of wild type and FA2 PSI were recorded at 9 K and 20 mW microwave power (Fig. 4). Under these conditions, the FA+ signals are saturated and become hardly visible (compare WT spectra in Figs. 3 and 4). Comparison of the wild type and FA2 spectra in Fig. 4 indicates that, in the FA2 mutant, the new signal is most probably due to a modified FA–. The two lower spectra in Fig. 4 represent a weighted difference between the FA2 and wild type spectra recorded at 9 K (Fig. 4, dif. 1) and 15 K (Fig. 4, dif. 2) in which the wild type spectrum has been multiplied by a factor of 0.65 to minimize the normal FA+ signals. These two difference spectra share common features and three g values for the modified FA– signals can be determined around 2.08, 1.915, and 1.87. Assuming no significant saturation at 15 K of any of the FA– signals, the above weighted differences would mean that in 65% of the complexes, FA– is as in the wild type, whereas 35% feature a modified FA–.

Ferredoxin Reduction by PSI—Electron transfer from PSI to Fd has been studied in the cyanobacterium Synechocystis sp. PCC 6803 and in C. reinhardtii (16, 31, 40, 41). When isolated PSI complexes are illuminated by a saturating laser flash, Fd reduction is accompanied by an absorption change in the 400–600 nm range, which was shown to correspond to electron transfer from (FA′, FB′) to Fd (40, 41). This process was studied mostly at 480 and 580 nm in the cyanobacterial system, whereas the measurements are restricted to a single wavelength (580 nm) in C. reinhardtii because of the large antenna absorption at 480 nm. A signal recorded in the absence of Fd was subtracted from that recorded in its presence, to eliminate contributions from other processes, i.e., P700+ formation and
prior to the flash, at concentrations that are suitable for such a

concentrations were 4.07 mM for WT, E46K, E46Q, D9K, D9N, FB1, and FA2 mutants and suspended in 20 mM Tricine, pH 8.0, 0.03% dodecylmaltoside, 30 mM NaCl, 5 mM MgCl2, 2.2 mM sodium ascorbate, and 20 mM DCPIP. Concentrations between 0.185 and 0.2 mM of PSI were used for the actual measurements, but the data were normalized to identical PSI concentration of 0.193 mM for better comparison. Ferredoxin (from C. reinhardtii) concentrations were 4.07 μM for WT, E46K, and FA2, 4.00 μM for D9K and D9N, and 0.39 μM for FB1. The transients measured with the E46Q and D9N PSI are not shown because they are identical to the ones obtained with the E46K and D9K PSI, respectively. Lower part, the sum of amplitudes of first-order components at 580 nm was plotted as a function of Fd concentration for WT and FB1. The data were fitted assuming a simple binding equilibrium between PSI and Fd (continuous curves), resulting in the following fitting parameters: WT, Kd = 8.2 mM, asymptotic ΔA = 1.4 × 10^{-4}; FB1, Kd = 0.125 mM, asymptotic ΔA = 2.0 × 10^{-4}.

### TABLE II

**Characteristics of wild type and mutant PSI complexes**

| Strain   | Dissociation constant for PSI-Fd interaction | First-order reduction of Fd half-times | Second-order rate constant of Fd reduction |
|----------|---------------------------------------------|---------------------------------------|------------------------------------------|
|          | (μM)                                       | (μM)                                  | (μM)                                    |
| WT       | 7.5 ± 1.5                                   | <1 μM (35)                            | 2.5 × 10⁻³                                |
| D9N      | 4.5 ± 1.5                                   | 1.5 ± 1.5 (65)                        | 1.9 × 10⁻³                               |
| D9K      | 4.5 ± 1.5                                   | <1 μM (35)                            | 5.5 ± 1.5 (65)                           |
| E46Q     | 8 ± 1                                       | <1 μM (35)                            | 5.5 ± 1.5 (65)                           |
| E46K     | 7.5 ± 1.5                                   | <1 μM (35)                            | 5.5 ± 1.5 (65)                           |
| FB1      | 0.125 ± 0.02                                | <1 μM (90)                            | Not measurable                           |
| FA2      | 21 ± 2                                      | <1 μM (35)                            | 2.1 × 10⁹                                |

affinity is decreased about 3-fold in FA2, is not changed in E46Q and E46K, and is increased about 1.5-fold in D9N and D9K. A huge increase in Fd affinity of about 60-fold is observed for FB1 compared with WT. The half-times of the two first-order components and their relative amplitudes are very similar in WT PSI and all mutants except FB1, where the submicrosecond component is largely dominating (Table II and Fig. 5). Second-order rate constants are almost identical for the different complexes (Table II). However, it could not be determined for FB1 because essentially all PSI is complexed with Fd prior to the flash, at concentrations that are suitable for such a determination.

### FIG. 5

**Ferredoxin reduction measured by flash-induced absorption changes at 580 nm with wild type and mutant PSI complexes.** Upper part, the transients (1-ms full time scale) were obtained after subtraction of traces recorded in the absence of ferredoxin from traces recorded in its presence (average of 64 or 128 traces in each case) so that the curves correspond only to electron transfer from (FA, Fb) to ferredoxin. PSI complexes were prepared from WT, E46K, E46Q, D9K, D9N, FB1, and FA2 mutants and suspended in 20 mM Tricine, pH 8.0, 0.03% dodecylmaltoside, 30 mM NaCl, 5 mM MgCl2, 2.2 mM sodium ascorbate, and 20 mM DCPIP. Concentrations between 0.185 and 0.2 mM of PSI were used for the actual measurements, but the data were normalized to identical PSI concentration of 0.193 mM for better comparison. Ferredoxin (from C. reinhardtii) concentrations were 4.07 μM for WT, E46K, and FA2, 4.00 μM for D9K and D9N, and 0.39 μM for FB1. The transients measured with the E46Q and D9N PSI are not shown because they are identical to the ones obtained with the E46K and D9K PSI, respectively. Lower part, the sum of amplitudes of first-order components at 580 nm was plotted as a function of Fd concentration for WT and FB1. The data were fitted assuming a simple binding equilibrium between PSI and Fd (continuous curves), resulting in the following fitting parameters: WT, Kd = 8.2 mM, asymptotic ΔA = 1.4 × 10⁻⁴; FB1, Kd = 0.125 mM, asymptotic ΔA = 2.0 × 10⁻⁴.

### FIG. 6

**Western blot analysis of PSI complexes isolated from wild type and FB1 mutant 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. Presence of activated Fd is indicated at the top of each lane. Positions corresponding to the free and cross-linked polypeptides are shown. Asterisks indicate products whose sizes are consistent with double cross-linking events (see text). The open circle indicates a cross reacting signal.

**Chemical Cross-linking of Fd to PSI**—Chemical cross-linking has been used in different organisms to study the interaction between soluble Fd and PSI. Cross-linking of subunits PsaD, -E, -C, and -H to Fd has been demonstrated (16, 47–49). When using the zero-length cross-linker EDC, carboxyl groups are activated and can react with amino groups mainly provided by lysines. Residues Lys35 of PsaC and Lys106 of PsaD were identified as being the cross-linking site on these subunits by site-directed mutagenesis and protein sequencing, respectively (16, 47). In C. reinhardtii, replacement of the Lys35 residue of PsaC by negatively charged amino acids resulted in a dramatic reduction of affinity of Fd for the mutants complexes and to the loss of cross-linking (16). We performed a similar experiment with wild type and FB1 PSI complexes (Fig. 6). Purified Fd was incubated with EDC and sulfo-NHS to activate its carboxyl groups (see "Experimental Procedures"). The excess of reagent was then removed by gel filtration, and the activated Fd was eluted. PSI particles were incubated with or without activated Fd and analyzed by Western blotting. In this way no internal cross-link between PSI subunits because of free cross-linker can occur, and all cross-linking products that are observed involve activated Fd. Clearly the amount PsaC-Fd cross-linking product is significantly increased with the FB1 mutant PSI, thus confirming the higher affinity of Fd for this complex (Fig.
The PsaD-Fd and PsaE-Fd cross-links are also detected with the FB1 complex, showing that interactions between these subunits and Fd does occur. Higher molecular weight cross-linking products are also present with sizes that are consistent with the presence of PsaD-Fd-PsaC and PsaE-Fd-PsaC double cross-links (indicated by asterisks in Fig. 6). Interestingly, this putative PsaE-Fd-PsaC signal is clearly stronger with the FB1 complex compared with wild type, and the PsaE-Fd signal is conversely weaker in the mutant, suggesting that the altered Fd-PSI interaction increases the amount of double cross-linking reaction (Fig. 6, lower panel).

DISCUSSION

Approaches to study electron transfer by mutagenesis of the cysteines that coordinate the iron-sulfur clusters of PsaC were unsuccessful in the green alga *C. reinhardtii* because the PSI complex was severely destabilized in these mutants (50). In this respect the PSI of *C. reinhardtii* appears to be more sensitive to alterations in the PsaC subunit compared with cyanobacterial systems. However, in the present work we have generated six mutant strains of *C. reinhardtii* in which residues located close to the \(4\text{Fe}-4\text{S}\) clusters have been modified. Strikingly, a triple mutation close to the \(\text{FA}_2\) cluster in the \(\text{FA}_2\) mutant leads to partial reduction of PSI accumulation, whereas the reciprocal modification next to \(\text{FB}_1\) or mutations on Asp9 and Glu46 have no effect on PSI levels. This result, together with other studies (16, 31), shows that it is possible to manipulate *in vivo* the structure of PsaC in *C. reinhardtii* despite the essential role that this subunit plays for PSI accumulation.

Low temperature EPR spectroscopy of the purified PSI complexes revealed that the \(\text{FA}_2\) mutant complex features an altered spectrum revealing a modified form of \(\text{FA}_2\). Interestingly, two of the mutated residues had already been targeted in a previous study in which a K52S/R53A double mutant of *C. reinhardtii* was generated (31). These changes were made to mimic the situation of the green sulfur bacteria *Chlorobium limicola*, in which the homolog of the PsaC subunit has a serine and an alanine at these positions, and \(\text{FB}_1\) was claimed to be preferentially photoreduced at low temperature in this organism (Ref. 51; see also, however, Ref. 52). Indeed, a preferential reduction of \(\text{FB}_1\) was observed in the *C. reinhardtii* mutant and tentatively explained by a modification of the redox potential of \(\text{FA}_2\). This effect was attributed to the removal of two positively charged residues close to \(\text{FA}_2\) that could lower its potential leading to an increase of \(\text{FB}_1\) reduction. In the light of the present study, this model appears to be overly simplistic because the \(\text{FA}_2\) (V49I/K52T/R53Q) mutations also remove these residues between the last cysteine of the first cysteine binding motif and the first cysteine of the second cysteine binding motif, respectively. Lys48 is the 14th of these 26 residues in PsaC. Residues 14 and 15 of the 27 intercysteine sequence of *Sulfolobus* are colored in cyan for showing the approximate position of Lys48. Two orientations of PsaC were assumed, which are similar to the ones described in Fig. 4 of Ref. 56. In the upper and lower parts, cluster \(\text{FA}_2\) is the distal and proximal cluster, respectively. In each part, the lower cluster lies behind the upper cluster, which means, according to the current structural knowledge of the PSI stromal side, that PsaD and PsaE are lying “behind” and “in front” of the page, respectively. 

![Fig. 7. Model of the PsaC subunit based on the structure of the [4Fe-4S] Fd of *Sulfolobus* sp. but without the N-terminal zinc binding motif using Rasmol V2.5 (R. Sayle, Glaxo). Only a backbone representation with α-carbon atoms is shown. Assuming structural similarity, residues of *Sulfolobus* ferredoxin were labeled according to the PsaC sequence. This is straightforward for residues close to the cysteine binding motifs (Asp9, Ile12, Thr15, Glu46, Val49, Lys35, and Arg53). PsaC and the *Sulfolobus* ferredoxin contain 26 and 27 residues between the last cysteine of the first cysteine binding motif and the first cysteine of the second cysteine binding motif, respectively. Lys35 is the 14th of these 26 residues in PsaC. Residues 14 and 15 of the 27 intercysteine sequence of *Sulfolobus* are colored in cyan for showing the approximate position of Lys35. Two orientations of PsaC were assumed, which are similar to the ones described in Fig. 4 of Ref. 56. In the upper and lower parts, cluster \(\text{FA}_2\) is the distal and proximal cluster, respectively. In each part, the lower cluster lies behind the upper cluster, which means, according to the current structural knowledge of the PSI stromal side, that PsaD and PsaE are lying “behind” and “in front” of the page, respectively.](image)

population with submicrosecond electron transfer between \(\text{FA}_2\) and \(\text{FB}_1\), if the first possibility holds or a single type of PSI-Fd complex with submicrosecond reduction of Fd if the second possibility holds. Whatever explanation is correct, both electron transfer steps, between \(\text{FA}_2\) and \(\text{FB}_1\) and between \(\text{FB}_1\) and Fd, occur mostly in the submicrosecond time range in the mutant \(\text{FB}_1\). The large submicrosecond phase observed with the \(\text{FB}_1\) complex therefore indicates that Fd is mainly bound in a single orientation in its binding pocket, in which fast electron transfer to Fd is occurring (Fig. 5 and Table II). The increase in affinity of Fd for the \(\text{FB}_1\) PSI complex was also confirmed by chemical cross-linking experiments, showing that more PsaC-Fd cross-linking product is formed with the mutant complex. Whether the increase in PsaC-Fd cross-linking is due to a new interac-
tion between Lys$^{15}$ and an acidic residue of Fd or to increased interaction of Lys$^{35}$ with its partner cannot be assessed from the present experiments.

Fd binding to PSI is mediated by electrostatic interaction of negatively charged residues of Fd with basic amino acids of the stromal PsaC, -D, and -E subunits (13, 15, 16). The FB$_1$ mutation introduces a lysine and an arginine between two of the cytostines that coordinate cluster F$_B$. FA$_2$ is the counterpart of the FB$_1$ mutation, because it suppresses a lysine and an arginine in the vicinity of F$_A$. This mutation leads to some decrease in Fd affinity, but the effect is only 3-fold compared with the 60-fold increase that is observed in FB$_1$. Moreover, the first-order kinetics of Fd reduction are not substantially affected in FA$_2$, contrary to FB$_1$. These results strongly favor a model in which F$_B$ is close to the Fd binding site and therefore the outermost cluster of PSI. One or both of the basic residues that are introduced in mutant FB$_1$ can probably form new electrostatic interaction(s) that lead to the high affinity observed.

Removal of Asp$^9$ leads to a slight increase in affinity, indicating that this residue is also close to the Fd-PSI interaction domain. However, no difference could be observed between the D9N and the D9K mutations, suggesting that rather than forming a new electrostatic interaction, these mutations contribute in rendering the local environment more positive. Because Asp$^9$ is close to F$_B$, this result is also consistent with the orientation of PsaC with F$_B$ as the terminal electron acceptor. However, this result does not agree with the proposal that Asp$^9$ is involved in the electrostatic binding of PsaC to one of the core subunits of PSI (PsaA or PsaB) (24, 27). We do not observe any effect of the D9N or D9K mutations on PSI assembly or on subunit composition, which would be expected if Asp$^9$ mediates binding of PsaC to PSI. On the contrary, the effect we observe on Fd reduction indicates that Asp$^9$ is exposed at the surface of PSI. The discrepancy might be due to some improper orientations of recombinant mutated PsaC during reconstitution that could impede complex formation or could lead to "wrong" complexes that are not competent for efficient electron transfer. The reduction of PSI accumulation to 20–30% of wild type could impede complex formation or could lead to "wrong" complexes that are not competent for efficient electron transfer. The discrepancy might be due to some improper orientations of recombinant mutated PsaC during reconstitution that could impede complex formation or could lead to "wrong" complexes that are not competent for efficient electron transfer.

The alternative orientation (Fig. 7, lower panel) would be very difficult to reconcile with our data. The results of this mutagenesis study clearly confirm the orientation of the PsaC subunit proposed recently based on chemical inactivation of F$_B$ and suggests a linear electron flow F$_B$ → F$_A$ → F$_D$ → Fd (29, 30).

Interestingly the growth properties and the fluorescence transients of the FB$_1$ mutant indicate that its PSI complex is only partially functional. Western blot analysis reveals that PSI accumulates normally and EPR spectroscopy indicates that at low temperature stable charge separation can be achieved in this complex and that the properties of the iron-sulfur clusters are not altered (Figs. 2 and 3). The 60-fold smaller dissociation constant ($K_d$) of the Fd-FB$_1$ complex can be due to a reduced $k_{off}$ constant or to an increased $k_{on}$ constant ($K_d = k_{off}/k_{on}$) or to a contribution of both. Because the increase in affinity appears to limit photosynthetic activity, the $k_{off}$ may be the limiting factor in this mutant. Unfortunately this could not be checked because the affinity is so high that the second-order rate constant $k_{on}$ of Fd reduction could not be measured (see "Results"). In a recent detailed analysis of PsaD- and PsaE-deficient mutants of Synechocystis PCC 6803, the function of these two subunits in Fd reduction could be better defined (13). The effects observed on $k_{on}$ and $k_{off}$ in these mutants indicated that PsaD may be involved in guiding Fd to its binding site, whereas PsaE may be responsible for maintaining the PSI-Fd complex. The increase of the putative PsaC-Fd-PsaE cross-link in the FB$_1$ mutant is compatible with the proposal that Fd is retained on PSI allowing more time for interaction with PsaE and successful cross-linking to occur. Whether the tight binding of Fd to the complex is limiting the overall electron transfer or whether other deleterious effects within the terminal part of PSI cause the in vivo phenotype of the FB$_1$ strain is still under investigation.

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