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Insertion of the N-terminal Part of PsaF from *Chlamydomonas reinhardtii* into Photosystem I from *Synechococcus elongatus* Enables Efficient Binding of Algal Plastocyanin and Cytochrome c_6*

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A strain of the cyanobacterium *Synechococcus elongatus* was generated that expresses a hybrid version of the photosystem I subunit PsaF consisting of the first 83 amino acids of PsaF from the green alga *Chlamydomonas reinhardtii* fused to the C-terminal portion of PsaF from *S. elongatus*. The corresponding modified gene was introduced into the genome of the *psaF*-deletion strain FK2 by cointegration with an antibiotic resistance gene. The transformants express a new PsaF subunit similar in size to PsaF from *C. reinhardtii* that is assembled into photosystem I (PSI). Hybrid PSI complexes isolated from these strains show an increase by 2 or 3 orders of magnitude in the rate of $P700^+$ reduction by *C. reinhardtii* cytochrome c_6 or plastocyanin in 30% of the complexes as compared with wild type cyanobacterial PSI. The corresponding optimum second-order rate constants (k_2 = 4.0 and 1.7×10^7 M^{-1} s^{-1} for cytochrome c_6 and plastocyanin) are similar to those of PSI from *C. reinhardtii*. The remaining complexes are reduced at a slow rate similar to that observed with wild type PSI from *S. elongatus* and the algal donors. At high concentrations of *C. reinhardtii* cytochrome c_6, a fast first-order kinetic component ($t_{1/2}$ = 4 μs) is revealed, indicative of intramolecular electron transfer within a complex between the hybrid PSI and cytochrome c_6. This first-order phase is characteristic for $P700^+$ reduction by cytochrome c_6 or plastocyanin in algae and higher plants. However, a similar fast phase is not detected for plastocyanin. Cross-linking studies show that, in contrast to PSI from wild type *S. elongatus*, the chimeric PsaF of PSI from the transformed strain cross-links to cytochrome c_6 or plastocyanin with a similar efficiency as PsaF from *C. reinhardtii* PSI. Our data indicate that development of a eukaryotic type of reaction mechanism for binding and electron transfer between PSI and its electron donors required structural changes in both PSI and cytochrome c_6 or plastocyanin.

One of several minor differences found in the otherwise remarkably conserved electron transfer chains of oxygenic photosynthesis of cyanobacteria, algae, and land plants concerns the type of electron carrier proteins used to transfer electrons from the cytochrome b_6/f complex to photosystem I and the way they interact with PSI[1] (1, 2). All cyanobacteria investigated utilize a cytochrome c_6 as a soluble periplasmic electron carrier. In several cases, *e.g. Synechococcus elongatus,* cytochrome c_6 is the only electron carrier in the periplasma and is constitutively expressed (1–3). Other cyanobacteria like *Anabaena* sp. PCC 7119 and *Synechocystis* sp. PCC 6803 and most algae examined, however, use both cytochrome c_6 and the copper-containing plastocyanin as alternative periplasmic electron carrier proteins (4–8). In these organisms, they are differentially expressed depending mostly on the relative availability of copper and iron in the culture medium (5, 8). In contrast, plastocyanin is expressed constitutively in photosynthetic land plants that lack cytochrome c_6. Thus, in the evolution of oxygen-evolving organisms a tendency to replace the originally used *c*-type cytochrome by plastocyanin is clearly discernible.

The PSI complex functions as a light-driven oxidoreductase that transfers electrons from cytochrome c_6 or plastocyanin to ferredoxin or flavodoxin (see Refs. 9 and 10 for a review). According to the established atomic structure of PSI from *S. elongatus*, the primary donor of PSI, P700, is located within the highly conserved reaction center core close to the periplasmic surface of the photosynthetic membrane (9, 11, 12). Two horizontal helixes l and l′, attributed to the PSI core subunits PsaA and PsaB, are thought to form a recognition site for binding of the periplasmic electron carriers (11, 12). However, despite the high degree of structural conservation of the PSI core subunits in all oxygen-evolving organisms, the mechanism of interaction between plastocyanin or cytochrome and PSI varies in different species.

In higher plants, electron transfer from plastocyanin to $P700^+$ is a biphasic process that includes a first-order kinetic component with a half-life of about 12 μs which is attributed to electron transfer from plastocyanin to $P700^+$ within a stable complex between plastocyanin and PSI formed prior to the photooxidation of P700 (13–15). Biochemical studies indicate that the PsaF subunit of PSI is involved in the formation of this complex (16–18). Similar first-order kinetic components with half-lives of about 3 μs are observed for the reduction of $P700^+$ by both plastocyanin and cytochrome c_6 in the green alga *Chlamydomonas reinhardtii* (19). Electron transfer from both donors to PSI from a *psaF*-deficient mutant of *C. reinhardtii*

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[1] The abbreviations used are: PSI, photosystem I; cyt c_6, cytochrome c_6; Pc, plastocyanin; PsaF, subunit III of photosystem I; *psaF*, gene of the PsaF subunit; P700, primary donor of photosystem I; PCR, polymerase chain reaction; Mops, 4-morpholinepropanesulfonic acid; kb, kilobase pairs.

was shown to be drastically slower indicating that PsaF is essential for efficient electron transfer from both plastocyanin and cytochrome c_6 to PSI (19, 20). Similar first-order kinetics have also been observed for PSI reduction by plastocyanin or cytochrome *c* in the green algae *Chlorella* and *Monoraphidium braunii* (21, 22). This suggests that the formation of complexes between PSI and these electron donors which involve the PsaF subunit is likely to occur generally in algae and plants. In contrast, in the cyanobacterium *Synechocystis* sp. PCC 6803, the reduction of $P700^+$ by cytochrome c_6 or plastocyanin is a second-order process and fast phases which can be attributed to electron transfer within a stable preformed complex could not be detected (3, 23–25). In addition, spectroscopic investigations of PSI complexes from *S. elongatus* and *Synechocystis* sp. PCC 6803 lacking the PsaF subunit show that the absence of PsaF does not affect the rates of $P700^+$ reduction by cytochrome c_6 (3, 25).

It has been shown that the PsaF subunit of PSI from spinach cross-links at one of its N-terminal lysines between residues 10–23 or 24–51 to the conserved acidic amino acids 42–44 and 59–61 of plastocyanin, respectively (26). This region close to the N-terminal end of PsaF could form an amphipathic α-helix, whose positively charged face may interact with plastocyanin (26). Amino acid sequence comparison, however, shows that a 27-amino acid domain of this well conserved N terminus of PsaF from plants and algae is missing in cyanobacteria (9, 27). It was therefore suggested that the introduction of this new N-terminal domain into algal and plant-type PsaF may be responsible for the formation of the complex between PSI and cytochrome c_6 or plastocyanin that is characteristic for PSI from algae and plants (26). In order to test this hypothesis we have introduced a chimeric *psaF* gene containing the N-terminal coding region from the green alga *C. reinhardtii* into the genome of a *psaF*-deletion strain of *S. elongatus* and report the functional characterization of a cyanobacterial photosystem I complex carrying an algal type PsaF subunit.

EXPERIMENTAL PROCEDURES

In Vitro Site-directed Mutagenesis, Molecular Cloning Strategies, and Vectors—DNA manipulations were performed in *Escherichia coli* strain XL1 blue according to standard protocols (28). Integrative cartridge vectors were constructed by modifications of a genomic 2.45-kb *Bam*HI/*Xho*I fragment from *S. elongatus* carrying the *psaF*/*psaJ* operon subcloned into pBSC $M13^+$ (29) (Fig. 1). A DNA construct, pCRSEF/2, was constructed in which the region of the *psaF* gene from *S. elongatus* encoding the mature PsaF subunit was substituted completely by the *psaF* region from *C. reinhardtii* (30). First, an *Eco*RV site was introduced at codons 24 and 25 of *psaF* (*i.e.* codons 1 and 2 of the mature PsaF from *S. elongatus*), and the termination codon was modified to create an *Xba*I site. These modifications were introduced by inverse PCR in the presence of oligonucleotides SEF1 (5′-TGCGATATCAGCGGAGGCTAGG-3′) and SEF2 (5′-TCTCTAGATTTGCTGTTTGTTG-3′) to create the vector pSEF1. Second, a *psaF* cDNA clone from *C. reinhardtii* (30) was modified by PCR in the presence of oligonucleotides CRF1 (5′-CCGATATCGCGGGCCTGACC-3′) and CRF2 (5′-CAGCTAGCGGGGAGACACGG-3′) to create an *Eco*RV site at codons 63 and 64 (*i.e.* codons 1 and 2 of the mature PsaF) and an *Nhe*I site at the termination codon. This fragment was inserted into the *Eco*RV and *Xba*I sites of pSEF1 to produce the plasmid pCRSEF/2 which carries the *psaF*/*psaJ* operon from *S. elongatus* in which the part of the *C. reinhardtii psaF* gene encoding the entire mature PsaF is fused in frame to the presequence of *psaF* from *S. elongatus*.

In addition, a second plasmid was constructed in which the codons for amino acids Ile64 to His145 of PsaF from *C. reinhardtii* were inserted in frame between codon Asp24 and codon Ala83 of *psaF* from *S. elongatus*. First, the genomic *Cla*I/*Xho*I fragment from *S. elongatus* was modified to create a *Bal*I site at codons 81 and 82 by PCR in the presence of oligonucleotide FBal (5′-CTTGGCCATGCCGGTGATTTTC-3′) and the M13 reverse primer. This PCR fragment was inserted into the *Bal*I and *Xho*I sites of vector pCRSEF/2 to create vector FBalCRF/2. The fusion part of the DNA construct was sequenced. Finally, the *Sma*I fragment of pHP45Ω (31) carrying the streptomycin/spectinomycin resistance genes was inserted into the *Hpa*I site of pCRSEF/2 and FBalCRF/2, generating plasmids pCRSEF/3 and FBalCRF/3 which served as integrative vectors for the genetic manipulation of *S. elongatus*.

Culturing Conditions, Transformation, and Selection of S. elongatus—Wild type and mutant strains from *S. elongatus* were cultivated as described (32). Genetic manipulations of the *psaF*/*psaJ* locus from *S. elongatus* were carried out using the $psaF^-/Km^r$ strain FK2, which carries a *psaF* gene disrupted by a kanamycin resistance marker (32). Prior to transformation, FK2 was grown in medium D supplemented with 40 μg/ml kanamycin. Cells were transformed by electroporation in the presence of plasmids pCRSEF/3 or FBalCRF/3 essentially as described previously, selected for streptomycin resistance in liquid cultures, and colony-purified on solid media (32). From these initial clones, strains with the desired Km^s/Sm^r phenotype were selected by replica plating on solid media containing either 2 μg/ml streptomycin or 25 μg/ml kanamycin. Cells carrying the *C. reinhardtii psaF* gene were identified by either immunoblot analysis of photosynthetic membranes isolated from small scale cultures using anti-*C. reinhardtii* (20) or anti-*S. elongatus* PsaF antibodies[2] or by Southern blot analysis of genomic DNA as described (32).

Isolation of Protein Components from S. elongatus and C. reinhardtii—Photosystem I complexes were extracted from PSII-depleted membranes by 0.6% w/v β-dodecyl maltoside and purified by centrifugation in sucrose gradients as described (33, 34). PSI from *C. reinhardtii* was isolated as described (19), and plastocyanin and cytochrome c_6 were isolated from *C. reinhardtii* following the protocol of Ref. 6 with modifications described in Ref. 19. Cytochrome c_6 from *S. elongatus* was isolated essentially according to Ref. 35. Plastocyanin and cytochrome c_6 concentrations were determined spectroscopically using absorption coefficients of $\epsilon_{597\ nm}$ = 4.9 mM^{-1} cm^{-1} and $\epsilon_{552\ nm}$ = 20 mM^{-1} cm^{-1}, respectively (19).

Analytical Methods, Cross-linking Procedures, and Immunoblot Analysis—For the fast immunoblot analysis of photosynthetic membranes, 3.5-ml cultures of *S. elongatus* were grown to OD_{750} = 1, harvested by centrifugation, washed once in 1 ml of HMCM buffer (20 mM Hepes, pH 7.8, 10 mM $CaCl_2$, 5 mM $MgCl_2$, 0.5 M mannitol), resuspended in 50 μl of HMCM buffer, and frozen. The thawed suspension was incubated with 2 mg/ml lysozyme for 30 min at 48 °C and frozen. Cells were then lysed by the addition of 10 volumes of MCM (MMCM minus mannitol), and the photosynthetic membranes were recovered by centrifugation in a microcentrifuge at 4 °C for 10 min at maximum speed. Membranes were washed once in 500 μl of MCM supplemented with 0.1% sulfobetain 10, pelleted by centrifugation, and resuspended in SDS loading buffer at a concentration of approximately 0.25 mg of chlorophyll/ml.

Cytochrome c_6 and plastocyanin were chemically cross-linked to photosystem I essentially as described (19); PSI particles at a concentration of 0.1 mg of chlorophyll/ml in 30 mM Hepes, pH 7.5, 3 mM $MgCl_2$, and 1 mM ascorbate were incubated in the presence of 20 μM plastocyanin or cytochrome c_6 with 5 mM *N*-ethyl-3-(3-diaminopropyl)carbodiimide and 10 mM *N*-hydroxysulfosuccinimide for 45 min in darkness. The reactions were terminated by addition of ammonium acetate to a final concentration of 0.2 M and diluted 4-fold. PSI complexes were sedimented by centrifugation at 200,000 × *g* for 45 min and resuspended in 20 mM Hepes, pH 7.5, 0.05% Triton X-100. Analytical SDS-polyacrylamide gel electrophoresis was carried out using 15 or 16.5% (w/v) polyacrylamide gels (36). For immunoblot analysis, PSI complexes equivalent to 4 μg of chlorophyll and membrane preparations equivalent to 10 μg of chlorophyll were analyzed. Western blots and antibody incubations were carried out essentially as described (36). Immunodetection reactions were performed using anti-rabbit IgG antibodies linked to horseradish peroxidase followed by enhanced chemiluminescence detection (ECL, Amersham Pharmacia Biotech).

Flash-absorption Spectroscopy—Flash-induced absorption changes at 817 nm were measured at 296 K on a single beam spectrophotometer essentially as described (15). Flash excitation was performed using a frequency-doubled Nd:YAG laser (5 ns full width at half maximum). The measuring light was provided by a luminescence diode (Hitachi HE8404SG, 40 milliwatts, 30 nm full width at half maximum), filtered through an 817-nm interference filter (9 nm full width at half maximum), and passed through a cuvette with an optical path length of 1 cm that contained 200 μl of sample. For flash-absorption experiments, PSI reaction centers were suspended in the presence or absence of cytochrome c_6 or plastocyanin at a standard concentration of 50 μM chlorophyll in 30 mM Mops, pH 7.0, supplemented with 0.05% β-dodecyl

[2] U. Mühlenhoff, unpublished.

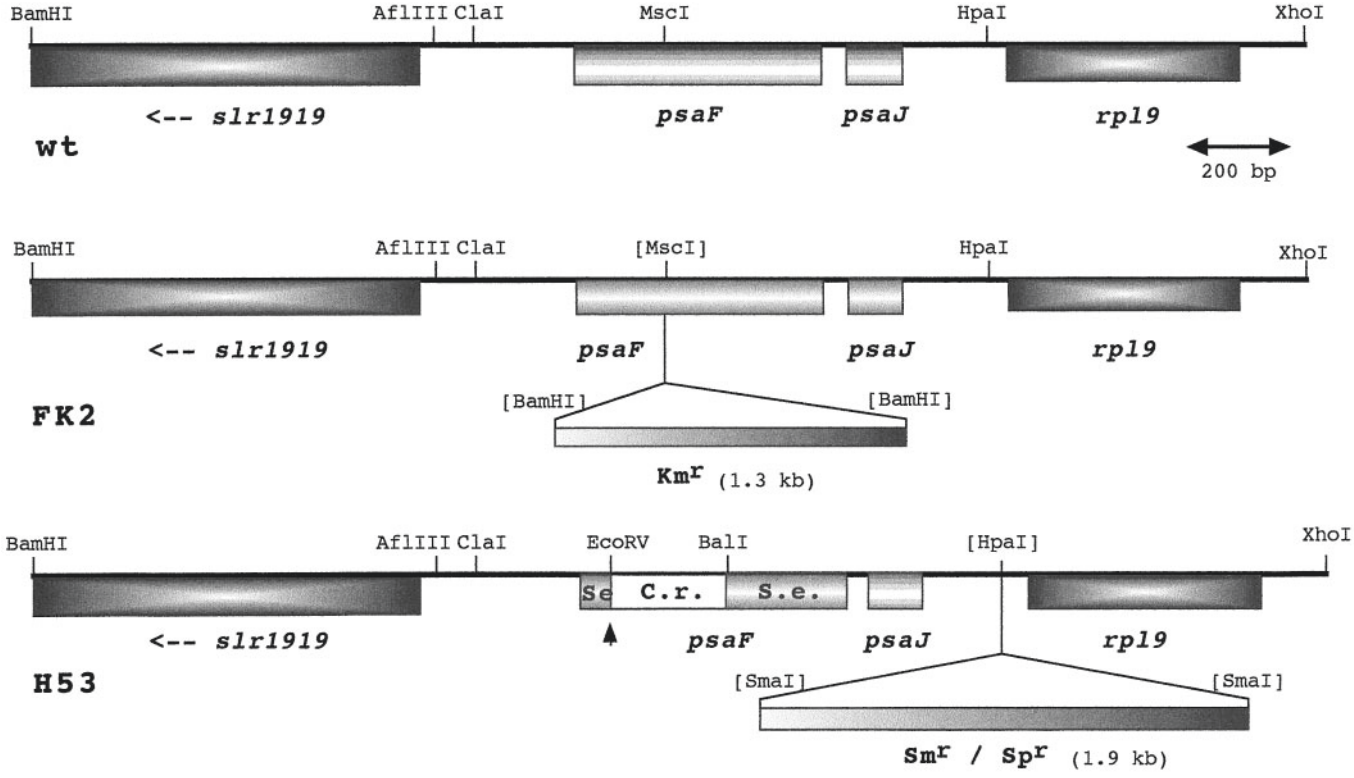

FIG. 1. **Construction of an integrative vector for the complementation of the *psaF/psaJ*-deletion strain FK2 from *S. elongatus* with the *psaF* gene from *C. reinhardtii*.** The physical maps of the *psaF*/*psaJ* operon in wild type and the FK2 strain are shown in the *upper* and *central part*. The structure of the integrative vector FBalCRF/3 used to generate the *S. elongatus* strain H53 that contains a hybrid *psaF* gene is displayed in the *lower part*. Vector FBalCRF/3 contains a *psaF* gene consisting of the first 83 codons of the mature PsaF subunit of *C. reinhardtii* inserted between the region encoding the presequence and the C terminus of *psaF* from *S. elongatus* (*S.e.*). A gene conferring resistance to streptomycin and spectinomycin has been included for phenotypic selection. Portions of the *psaF* gene originating from *C. reinhardtii* (*C.r.*) are displayed in *white,* and the cleavage site of the signal peptidase is indicated by an *arrow*. For DNA sequences see Refs. 29 and 30. The Sm^r/Sp^r gene cassette was obtained from pHP45Ω (31); the Km^r gene originated from pRL161 (32). Restriction sites in *brackets* were lost during construction. For details of the cloning procedures see "Experimental Procedures."

maltoside, 0.2 mM methyl viologen, 0.1 mM diaminodurene, 1 mM sodium ascorbate, and $MgCl_2$ as indicated in the figure legends. For measurements at high donor concentrations (Fig. 6), a cuvette with 3-mm optical path length was used that contained 30 μl of sample. Four individual signals were averaged, and the resulting kinetic traces were fitted to a sum of one or two exponential components and a constant offset with the program GNUPLOT (Unix version 3.5, Williams), performing nonlinear least squares fitting using the Marquart-Levenberg algorithm.

RESULTS

Vector Construction—To generate an *S. elongatus* strain carrying the *psaF* gene from *C. reinhardtii*, integrative vectors were introduced into the *S. elongatus* strain FK2 that carries a *psaF* gene interrupted by a kanamycin resistance cassette and thus served as a *psaF*-free background ((32) see Fig. 1, *middle part*). Vector constructions were performed using the genomic fragment from *S. elongatus* carrying the *psaF*/*psaJ* locus ((29) see *top* of Fig. 1) as follows. First, we took into account that the PsaF subunit is expressed as a precursor protein carrying an N-terminal export sequence required for the translocation of the N terminus of the protein into the periplasma. In order to be efficiently recognized by the cyanobacterial export apparatus, the hybrid *psaF* gene should therefore contain the entire cyanobacterial export sequence and the ASA-D cleavage site of the export protease. Second, in cyanobacteria, *psaF* and *psaJ* are arranged in an operon. In order to avoid any interference with the expression of the PsaJ subunit which is likely to be involved in the binding of PsaF to PSI, the organization of the *psaF*/*psaJ* operon should be maintained (25) (see Fig. 1).

As shown in Fig. 1 (*bottom*) and Fig. 2, the integrative vector FBalCRF/3 carries a gene encoding a hybrid PsaF protein that contains the cyanobacterial signal sequence (up to codon Asp-24 of *S. elongatus psaF*), the N-terminal domain of PsaF from *C. reinhardtii* (30) (*i.e.* between codons Asp-63/Ile-64 to codon His-145), and the hydrophobic C-terminal part of the cyanobacterial subunit (starting with codon Ala-60 of *S. elongatus psaF*) which is assumed to anchor the protein to the hydrophobic core of PSI. A streptomycin/spectinomycin resistance gene cassette was inserted at the *Hpa*I site located 165 base pairs downstream of *psaJ*, well downstream of the transcribed region of the *psaF*/*psaJ* operon (see "Experimental Procedures" for details of the construction). In addition to FBalCRF/3, a vector, pCRSEF/3, was constructed in which the complete region of the *psaF* gene from *S. elongatus* encoding the entire mature PsaF subunit was replaced by the corresponding part of *psaF* from *C. reinhardtii* (see "Experimental Procedures"). However, following the introduction of this vector into *S. elongatus*, *C. reinhardtii* PsaF was not recovered in PSI although it was verified by Southern blot analysis that the gene was cointegrated together with the antibiotic resistance marker and inserted correctly into the genome (not shown).

Characterization of the Mutant H53 Expressing a Chimeric PsaF Subunit—Following the electroporation of the *psaF*-deletion strain FK2 from *S. elongatus* in the presence of the plasmid pFBalCRF/3, streptomycin-resistant transformants with the desired Km^s/Sm^r phenotype were selected by replica plating on solid media, and those expressing the chimeric *C. reinhardtii-S. elongatus* PsaF protein were screened by immunoblot analysis using anti-*C. reinhardtii*-PsaF antibodies (not

A

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                                                        EcoRV
ATGCGTCGATTCTTAGCCCTACTCCTTGTCTTGACCCTGTGGCTAGGTTTTACCCCCCTAGCCTCCGCTGATATCGCGGGCCTGACCCCC      90
   M  R  R  F  L  A  L  L  L  V  L  T  L  W  L  G  F  T  P  L  A  S  A  D  I  A  G  L  T  P

TGCTCCGAGAGCAAGGCTTACGCCAAGCTGGAGAAGAAGGAGCTGAAGACCCTGGAGAAGCGCCTGAAGCAGTACGAGGCGGACAGCGCC      180
   C  S  E  S  K  A  Y  A  K  L  E  K  K  E  L  K  T  L  E  K  R  L  K  Q  Y  E  A  D  S  A

CCCGCCGTGGCCCTGAAGGCCACCATGGAGCGCACCAAGGCCCGCTTCGCCAACTACGCTAAGGCCGGCCTGCTGTGCGGCAACGACGGT      270
   P  A  V  A  L  K  A  T  M  E  R  T  K  A  R  F  A  N  Y  A  K  A  G  L  L  C  G  N  D  G

                                                BalI
CTGCCCCACCTGATTGCTGACCCCGGCCTGGCCCTGAAGTATGGCCATGCCGGTGATTTTCTCATTCCCAGTGTGCTCTTCCTGTACATC      360
   L  P  H  L  I  A  D  P  G  L  A  L  K  Y  G  H  A  G  D  F  L  I  P  S  V  L  F  L  Y  I

GCTGGCTGGATTGGCTGGGTGGGCCGCGCCTACTTGATTGCCGTGCGCAACAGCGGCGAAGCCAATGAAAAAGAAATCATTATTGATGTT      450
   A  G  W  I  G  W  V  G  R  A  Y  L  I  A  V  R  N  S  G  E  A  N  E  K  E  I  I  I  D  V

CCCCTTGCCATCAAATGTATGCTCACGGGTTTTGCTTGGCCTTTGGCCGCCCTCAAGGAGCTGGCATCGGGCGAACTCACCGCCAAAGAT      540
   P  L  A  I  K  C  M  L  T  G  F  A  W  P  L  A  A  L  K  E  L  A  S  G  E  L  T  A  K  D

AACGAAATCACTGTTTCCCCTCGCTAG   630
   N  E  I  T  V  S  P  R
```

B

```
S.elongatus:      DVAGLVPCKDSPAFQKR----------------AAAAVNTTADPASGQKRFERYSQA--LCGEDGLPHLVVDGRLS----R
C.reinhardtii:    DIAGLTPCSESKAYAKLEKKELKTLEKRLKQYEADSAPAVALKATMERTKARFANYAKAGLLCGNDGLPHLIADPGLALKYGH
S.elongatus H53:  DIAGLTPCSESKAYAKLEKKELKTLEKRLKQYEADSAPAVALKATMERTKARFANYAKAGLLCGNDGLPHLIADPGLALKYGH
P.purpurea:       EVAGLIPCKDSAAFNKRMVNSVKKLQARLAKYDANTPPALALNKQIEKTKTRFATYGRAGLLCGTDGLPHLISDGRWS----R
                    *** **  * *  *                        *            **  *  *  *** ******  *

S.elongatus:      AGDFLIPSVLFLYIAGWIGWVGRAYLIAVRNSGEANEKEIIIDVPLAIKCMLTGFAWPLAALKELASGELTAKDNEITVSPR
C.reinhardtii:    AGEVFIPTFGFLYVAGYIGYVGRQYLIAVKGEAKPTDKEIIIDVPLATKLAWQGAGWPLAAVQELQRGTLLEKEENITVSPR
S.elongatus H53:  AGDFLIPSVLFLYIAGWIGWVGRAYLIAVRNSGEANEKEIIIDVPLAIKCMLTGFAWPLAALKELASGELTAKDNEITVSPR
P.purpurea:       AGDFVFPGLLFLYITGWIGWVGRGYLLSVAKTSKPTEKEIILDVPLAVKFMSSGFAWPLAAWQEFSSGQLIAPNDDITVSPR
                  **    *   ***  * ** *** **  *        ********** *    *  *****  *   * *      ******
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FIG. 2. **Sequence analysis of *psaF* from the *S. elongatus* strain H53.** *A,* nucleotide and deduced amino acid sequence of the hybrid *psaF* gene from vector FBalCRF/3. The part of the amino acid sequence originating from *C. reinhardtii* is *underlined*. *Eco*RV and *Bal*I restriction sites were introduced during construction of the vector and are not present in wild type *S. elongatus psaF*. *B,* amino acid sequence alignment of the mature PsaF from *C. reinhardtii* (30), *S. elongatus* wild type (29), and strain H53, and *Porphyra purpurea* (39). Conserved amino acids are indicated by *asterisks,* and conserved lysine residues at the N-terminal part of the algal PsaF which are functionally involved in the binding of cytochrome c_6 and plastocyanin (37) are *underlined*.

shown, see "Experimental Procedures"). The organization of the *psaF*/*psaJ* locus of one of these strains, H53, was investigated by Southern blot analysis shown in Fig. 3. First, upon hybridization with a 500-base pair fragment from a *C. reinhardtii psaF* cDNA clone carrying part of the gene encoding the mature PsaF, a single 6.3-kb genomic *Eco*RV restriction fragment is detected that is not present in *S. elongatus* wild type nor in strain FK2 (Fig. 3*A*). The same DNA fragment is detected in H53 DNA after hybridization with a probe carrying the Sm^r/Sp^r genes inserted into pFBalCRF/3 (Fig. 3*B*). Taken together, these blots indicate that the region of the *psaF* gene of *C. reinhardtii* has been inserted with the antibiotic marker gene into the genome of *S. elongatus.* Furthermore, when the blots were probed with the *Cla*I/*Xho*I fragment carrying the *psaF*, *psaJ,* and *rpl9* genes, only single *Eco*RV fragments of ~13 and 14.5 kb are observed in DNA from wild type and strain FK2. The size difference is due to the presence of the Km^r marker gene in FK2 (Fig. 3*C*, see Fig. 1). For strain H53, however, two *Eco*RV fragments of ~8.0 and 6.3 kb are detected, indicating that a new *Eco*RV site has been introduced into the *psaF*/*psaJ* locus of H53. Since the Sm^r/Sp^r marker genes do not contain an *Eco*RV site, the existence of a new *Eco*RV restriction site together with the Sm^r/Sp^r genes at the *psaF*/*psaJ* gene locus indicates that the entire part of the *psaF* gene that originates from *C. reinhardtii* has been introduced into strain H53, because *C. reinhardtii psaF* is flanked by a new *Eco*RV site and the Sm^r/Sp^r genes in the integrative vector pFBalCRF/3 (Fig. 1). Finally, when the blots were probed with a fragment carrying the Km^r marker present in strain FK2, no hybridization signal was observed in DNA from strain H53 (Fig. 3*D*). This result indicates that H53 represents a fully segregated mutant in which the original *psaF*/*psaJ* gene locus of strain FK2 that carried a kanamycin resistance gene has been completely replaced by the new *psaF* gene at the *psaF*/*psaJ* gene locus in all copies of the polyploid genome. Essentially the same conclusion was obtained by a similar Southern blot analysis that was carried out using *Eco*RI-restricted DNA (not shown).

The expression pattern of the *psaF* genes in *S. elongatus* wild type and strains FK2 and H53 was monitored by Western blot analysis of photosynthetic membranes and isolated photosystem I complexes using anti-*C. reinhardtii*-PsaF antibodies (Fig. 4). This antibody recognizes the cyanobacterial PsaF subunit in membranes and photosystem I preparations in of wild type *S. elongatus*. As expected from the deduced sequences, the apparent mass of the cyanobacterial PsaF subunit is 15 kDa, approximately 3 kDa smaller than its algal counterpart (Fig. 4, *lanes 1, 4,* and *5*). In strain FK2, the corresponding subunit is absent (Fig. 4, *lanes 2* and *6*). However, in membranes and PSI from strain H53 a new version of the PsaF protein is detected which exceeds the original cyanobacterial PsaF subunit by 2.5 kDa in mass and is only slightly smaller than PsaF from *C. reinhardtii* (Fig. 4, *lanes 3* and *7*). Thus, the modified *psaF* gene of strain H53 is expressed, and the hybrid PsaF protein is assembled

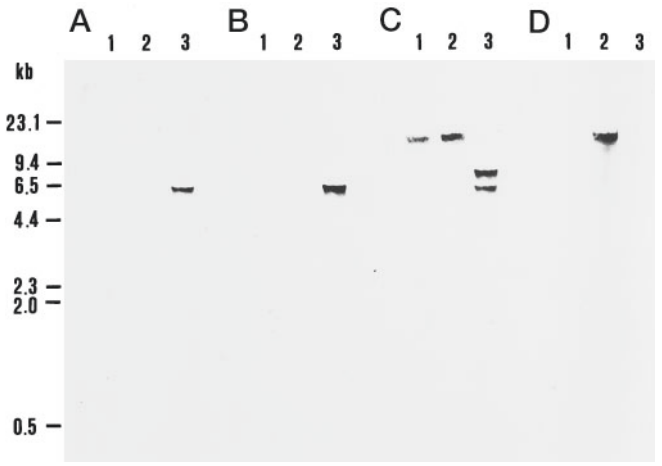

FIG. 3. **Southern analysis of genomic DNA from wild type and mutant strains of *S. elongatus*.** *Lane 1,* wild type DNA; *lane 2,* DNA from strain FK2; *lane 3,* DNA from strain H53. DNA samples were restricted by *Eco*RV separated on a 0.8% agarose gel and transferred to nylon membranes. The blots were probed with the following biotinylated DNA probes: *A,* PCR fragment of a *psaF* cDNA clone from *C. reinhardtii* encoding the part for the mature PsaF; *B,* the *Sma*I fragment of plasmid pHP45Ω containing the Sm^r/Sp^r genes; *C,* *Cla*I/*Xho*I fragment containing *psaF*/*psaJ* from *S. elongatus;* *D*, *Hin*cII fragment of plasmid pRL161 carrying Km^r gene. *Bars* to the *left* indicate the positions of the λ-*Hin*dIII restriction fragments.

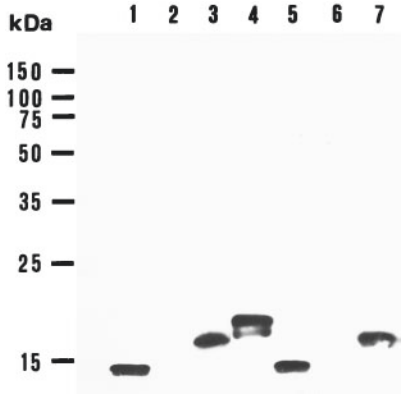

FIG. 4. **Immunoblot analysis of membranes and photosystem I preparations from wild type *S. elongatus* and strains FK2 and H53 probed with antibodies against PsaF from *C. reinhardtii*.** *Lane 1, S. elongatus* wild type membranes; *lane 2*, membranes from strain FK2; *lane 3,* membranes from strain H53; *lane 4*, *C. reinhardtii* PSI; *lane 5*, *S. elongatus* wild type PSI; *lane 6*, PSI from strain FK2; *lane 7,* PSI from strain H53. Membrane samples equivalent to 10 μg of chlorophyll and PSI preparations equivalent to 4 μg of chlorophyll were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. The blot was probed with an anti-*C. reinhardtii*-PsaF antiserum and developed by luminol detection. *Bars* to the *left* indicate the position of recombinant molecular mass marker proteins (Sigma) whose molecular masses (in kDa) are indicated.

into the cyanobacterial photosystem I complex. In addition, the fact that the protein is very similar in size to the mature algal PsaF subunit indicates that the protein is exported across the photosynthetic membrane and processed correctly by the transit peptidase. Essentially the same results were obtained by Western blot analysis using anti-*S. elongatus*-PsaF antibodies, with the exception that these antibodies do not detect *C. reinhardtii* PsaF (not shown). However, the visualization of this new PsaF subunit in PSI particles by a general staining technique was not unambiguously possible since the protein comigrates with the abundant allophycocyanin and phycocyanin subunits of phycobilisomes in SDS-polyacrylamide gels. Upon removal of these contaminations by ion-exchange chromatography, PSI preparations were obtained that no longer show an increased rate of $P700^+$ reduction in the presence of cytochrome c_6 described below and are thus no longer suited for further investigations (not shown). In this respect it has been reported for PSI from plants and algae that the fast electron transfer from plastocyanin to $P700^+$ is impaired in preparations purified by ion-exchange chromatography, an effect that is correlated with the loss of the PsaF subunit from the reaction centers (18, 19). Apparently, the hybrid PSI complexes from *S. elongatus* strain H53 show a similar behavior.

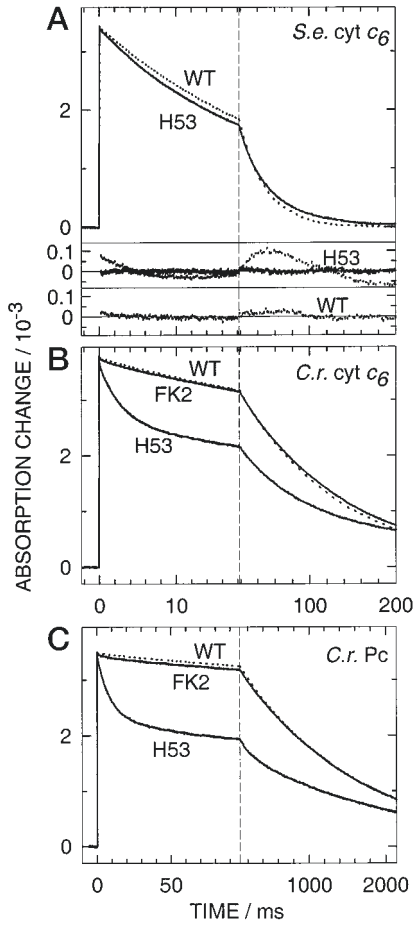

FIG. 5. **Flash-induced absorption changes measured at 817 nm with isolated photosystem I complexes from wild type *S. elongatus* and from the mutant strains FK2 and H53.** Kinetic traces were recorded in the presence of cytochrome c_6 from *S. elongatus* (*S.e.*) (*A*), cytochrome c_6 from *C. reinhardtii* (*C.r.*) (*B*), and plastocyanin from *C. reinhardtii* (*C*). The concentrations of reduced donor protein were 10 μM. $MgCl_2$ was present at 1 mM (*A*), or 10 mM (*B* and *C*). Transients obtained with PSI from wild type *S. elongatus* (*WT*) are displayed on *dotted lines,* and those obtained with PSI from the mutant strains FK2 and H53 are shown on *continuous lines*. The *vertical dashed lines* separate regions recorded on different time scales. In order to be directly comparable, the kinetic traces of the mutant PSI were normalized to the same initial amplitude as for wild type PSI. (The differences in amplitude was <10%.) Residuals for fits of the traces of $P700^+$ reduction of PSI from wild type *S. elongatus* and strain H53 by cytochrome c_6 from *S. elongatus* with one (*dotted trace*) or two exponential components (*solid trace*) are shown in the *panels* directly *below A*. Results of fits: *S. elongatus* H53 PSI, two components, $t_{½}$ = 7.2 ms (30%) and $t_{½}$ = 27 ms (70%) (*solid trace*); wild type PSI, one component, $t_{½}$ = 20 ms (for details see text.)

Cytochrome c_6 from C. reinhardtii Forms a Complex with the Hybrid PSI from S. elongatus H53—In order to study the properties of the hybrid PsaF subunit within PSI from the H53 strain, electron transfer from cytochrome c_6 and plastocyanin to PSI was monitored by flash absorption spectroscopy. Fig. 5 shows the absorbance transients at 820 nm of PSI particles

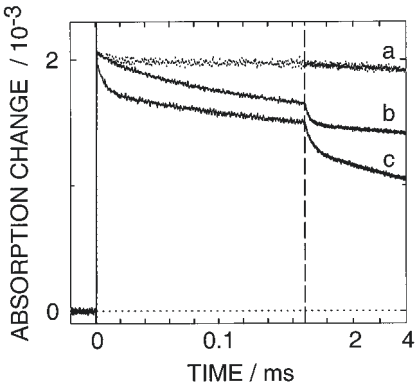

FIG. 6. **Flash-induced absorption changes measured at 817 nm with isolated photosystem I complexes from the *S. elongatus* strain H53 in the presence of 300 μM plastocyanin (*trace b*) or cytochrome c_6 (*trace c*).** *Trace a* (*dotted line*) shows the transient signal of a control experiment recorded with PSI from *S. elongatus* H53 in the absence of soluble donors. The kinetic traces are the average of four measurements in the presence 100 μM PSI and 3 mM $MgCl_2$. The *vertical dashed lines* separate regions recorded on different time scales.

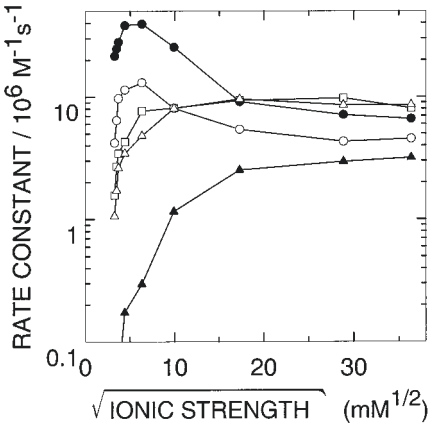

FIG. 7. **Salt dependence of the rate of the bimolecular reaction between cytochrome c_6 and PSI.** *Open symbols,* second-order rate constants for cyt c_6 from *S. elongatus; closed symbols,* second-order rate constants for cyt c_6 from *C. reinhardtii*. For conditions and kinetic deconvolution see Fig. 5 and text. For PSI carrying the hybrid PsaF (strain H53), the two kinetic components with relative amplitudes of 30 and 70% are indicated by *circles* and *triangles*, respectively. For PSI from wild type *S. elongatus* the rate of the monophasic decay is given (*squares*).The ionic strength was adjusted by varying the concentration of $MgCl_2$.

from wild type *S. elongatus* and the mutant strains FK2 and H53 induced by a laser flash in the presence of 10 μM cytochrome c_6 from *S. elongatus* (*A*), and 10 μM cytochrome c_6 (*B*) or 10 μM plastocyanin from *C. reinhardtii* (*C*). In the presence of *S. elongatus* cytochrome c_6 electron transfer is monophasic for photosystem I from wild type *S. elongatus* with a half-life of ~20 ms (Fig. 5*A*), and at first glance, the introduction of the hybrid PsaF in PSI from H53 causes only a small increase of the apparent rate of $P700^+$ reduction (see below for a more detailed analysis). Cytochrome c_6 and plastocyanin from *C. reinhardtii,* however, react much slower with PSI isolated from wild type *S. elongatus* or the *psaF* deletion strain FK2, and the kinetic traces can be fitted to monophasic decays (Fig. 5, *B* and *C*). For *C. reinhardtii* cytochrome c_6, electron transfer to the cyanobacterial PSI is about four times slower ($t_{½}$ ~ 80 ms) than with the cyanobacterial cytochrome, and the electron transfer from plastocyanin shows a half-life of ~1 s, which is ~50 times slower than for cytochrome c_6 from *S. elongatus*. In contrast, the electron transfer to the chimeric PSI from *S. elongatus* strain H53 by cytochrome c_6 and plastocyanin from *C. reinhardtii* shows a faster and a slower component, accounting for about 30 and 70% of the total amplitude, respectively (Fig. 5, *B* and *C*). The rates of both phases depend on the donor concentrations, indicating second-order processes. The fast component of these biphasic decays has a half-life of 2.1 and 6.5 ms for cytochrome c_6 and plastocyanin (at 10 μM each), respectively, whereas the slow kinetic component with both electron transfer donors is about 2 orders of magnitude slower than the fast phase and similar to those observed for PSI from wild type *S. elongatus*. A similar ratio of 30–70% between the fast and slow phase is also observed for $P700^+$ reduction in photosynthetic membranes of the mutant strain H53 (not shown). This suggests that only one-third of the PSI reaction centers have incorporated a functional chimeric PsaF, due perhaps to a decelerated expression of the modified gene in *S. elongatus*, as a likely result of the high G/C codon bias of *C. reinhardtii* or due to an imperfect translocation, assembly, or stability of the hybrid PsaF subunit in the cyanobacteria.

Fast first-order kinetic components that are indicative of electron transfer reactions within a preformed complex between cytochrome c_6 and PSI are directly observable only at high concentrations of cytochrome c_6 and plastocyanin for PSI from *C. reinhardtii* (19). For PSI from strain H53, the kinetic traces observed at high cytochrome c_6 concentrations can be deconvoluted into three components (Fig. 6*C*) as follows: first, a fast component with a constant half-life of 4 μs is resolved, similar to those found for intramolecular electron transfer between cytochrome c_6 and PSI from *C. reinhardtii* within a stable, preformed complex (19); second, an intermediate component ($t_{½}$ = 154 μs) with a half-time that decreases with increasing concentration of reduced donor protein typically for second-order reactions between soluble reactants (see also Fig. 5). The amplitudes of the first- and second-order phases contribute to about 15 and 25% of the entire signal, respectively. The third, very slow component ($t_{½}$ = 70 ms) with an amplitude of ~65% of the total signal is attributed to PSI lacking a chimeric PsaF. In contrast, the absorbance transients of $P700^+$ reduction in H53 PSI show only two kinetic components at plastocyanin concentrations up to 500 μM that are both concentration-dependent (Fig. 6*B*). The slower component ($t_{½}$ = 49 ms at 300 μM plastocyanin) most likely reflects the reduction of PSI complexes without a functional hybrid PsaF and plastocyanin, whereas the faster component ($t_{½}$ = 96 μs at 300 μM plastocyanin) is attributed to the reaction of plastocyanin with PSI containing the chimeric PsaF. No fast first-order component can be detected with plastocyanin (Fig. 6*B*). Thus, the presence of the hybrid PsaF subunit in the cyanobacterial PSI is not sufficient to generate a tight complex between plastocyanin and PSI although its presence is sufficient for complex formation with cytochrome c_6 from *C. reinhardtii*.

Whereas the introduction of the hybrid PsaF into the cyanobacterial PSI has a strong effect on the binding of the algal donors, the effects observed with the cyanobacterial cytochrome c_6 are minor (see Fig. 5). However, upon analyzing the transient signals in more detail, it appears that although the kinetics of $P700^+$ reduction by *S. elongatus* cytochrome c_6 are monophasic for PSI from wild type *S. elongatus* (see *lower panel* of residuals in Fig. 5*A*) and the *psaF* deletion mutant (not shown), they are biphasic in the presence of the hybrid PsaF (*upper panel* of residuals in Fig. 5*A*). Therefore, in order to analyze a possible interaction between the hybrid PsaF and the cyanobacterial cyt c_6 in more detail, $P700^+$ reduction was monitored under a wide range of salt concentrations. Fig. 7 shows the effect of the ionic strength on the rate of the kinetic components for cytochrome c_6 from *S. elongatus* (*open symbols*) and *C. reinhardtii* (*closed symbols*). At low ionic strength (<1 mM $MgCl_2$) the kinetics of $P700^+$ reduction by *S. elongatus* cyto-

chrome c_6 are monophasic for PSI from wild type *S. elongatus* (*open rectangles*; $t_{1/2}$ ~50 ms) and the *psaF* deletion mutant ($t_{1/2}$ ~50 ms, not shown) and biphasic in the presence of the hybrid PsaF, displaying two kinetic components with half-lives of ~17 and ~70 ms (*open circles* and *open triangles* in Fig. 7). The amplitude ratio of these two components is similar to the one found for PSI from *S. elongatus* H53 in the presence of the algal donors. At ~30 mM $MgCl_2$ the kinetics become almost monophasic, and at higher salt concentrations deviations from monophasic behavior are again detected. These observations are best rationalized when different salt dependences for the rates of the two kinetic components are assumed. For the determination of individual rate constants, the amplitude ratio for the two components was therefore kept fixed at a ratio of 30:70 during the final curve-fitting analysis of the data (Fig. 7). The component with a relative amplitude of 30% (*open circles*) shows optimum rates (1.5×10^7 M^{-1} s^{-1}) at ~3–10 mM divalent ions, *i.e.* at an ionic strength that is close to the optimal salt concentrations found for the faster phase of $P700^+$ reduction of the hybrid PSI by *C. reinhardtii* cytochrome c_6 (4×10^7 M^{-1} s^{-1} at 3–10 mM $MgCl_2$). In addition, the general shape of the salt dependence of this component is very similar for the algal and the cyanobacterial cytochrome and reminiscent of the one observed for *C. reinhardtii* photosystem I (19). On the other hand, the salt dependence of the slower phase of $P700^+$ reduction of PSI from *S. elongatus* H53 by *S. elongatus* cyt c_6 (*open triangles*, 70% relative amplitude) is very similar to the one observed for PSI from the wild type (*open rectangles*) and the *psaF*-deletion strain FK2 (not shown), and those observed for the slow phase in the presence of *C. reinhardtii* cytochrome c_6 (*closed triangles*) is similar to the one observed for PSI from the *C. reinhardtii psaF*-deletion strain b3f (19). Thus, the salt dependences of the slow phases (70% relative amplitude) corroborate the attribution of these phases to the reduction of those PSI complexes from strain H53 that lack a functional hybrid PsaF subunit. The existence of a new, faster kinetic component for $P700^+$ reduction of the hybrid PSI by cytochrome c_6 from *S. elongatus* with a salt dependence reminiscent of the one observed for *C. reinhardtii* cyt c_6 shows that the cyanobacterial cytochrome is affected by the introduction of a eukaryotic PsaF. However, the difference between the rate of the faster and slower components is rather small (factor <4) in comparison to those observed between the corresponding two phases for cytochrome c_6 from *C. reinhardtii* (factor ~200) which is fully adapted to use the eukaryotic PsaF subunit efficiently.

PSI from S. elongatus H53 Cross-links to Plastocyanin and Cytochrome c_6—The interactions of isolated PSI particles from *C. reinhardtii*, *S. elongatus* wild type, and strain H53 with plastocyanin and cytochrome c_6 from *C. reinhardtii* and *S. elongatus* were examined by immunoblot analysis of cross-linked complexes using *C. reinhardtii* PsaF antibodies (Fig. 8). Cross-linking products between plastocyanin or the two cytochromes and PsaF from PSI from *C. reinhardtii* with molecular masses of 29 and 28.5 kDa were detected, respectively (Fig. 8, *left part*). Essentially the same cross-linking pattern was obtained with PSI from *S. elongatus* H53 (Fig. 8, *center*). The cross-linking products observed for the hybrid PsaF from strain H53 were very similar with respect to molecular masses and intensity to the corresponding products in *C. reinhardtii*. However, no cross-linking was obtained when the electron donors were incubated with PSI particles from wild type *S. elongatus* (Fig. 8, *right panel*). Thus, the N-terminal part of *C. reinhardtii* PsaF is necessary and sufficient for cross-linking plastocyanin and cytochrome c_6 from *C. reinhardtii* and *S. elongatus* to photosystem I. However, for *S. elongatus* cytochrome c_6, the efficiency of cross-linking to PSI from *C. reinhardtii* or *S. elongatus* H53 is low in comparison with plastocyanin or cytochrome c_6 from *C. reinhardtii*.

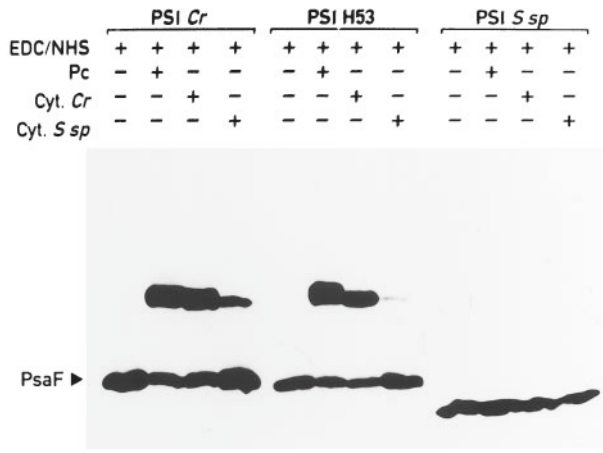

FIG. 8. **Immunoblot analysis of cross-linked photosystem I complexes from *C. reinhardtii* (*Cr*, *left panel*), *S. elongatus* strain H53 (*central panel*), and *S. elongatus* wild type (*S sp*, *right panel*) probed with anti-*C. reinhardtii*-PsaF antibodies.** For each type of PSI, cross-linked complexes generated in the absence of donor protein (*lanes 1*) and in the presence of either plastocyanin (*Pc*, *lanes 2*) or cytochrome c_6 from *C. reinhardtii* (*Cyt. Cr*, *lane 3*) or cytochrome c_6 from *S. elongatus* (*Cyt. S sp*, *lane 4*) were analyzed. Samples corresponding to 2 μg of chlorophyll were loaded on each lane, fractionated by SDS- polyacrylamide gel electrophoresis, electrotransferred to nitrocellulose membrane, and probed with antibodies against *C. reinhardtii*

DISCUSSION

Comparison of the PsaF subunits of PSI from eukaryotic and prokaryotic photosynthetic organisms has revealed that the former contain a basic region near their N-terminal end which is absent in cyanobacteria. This region has been postulated to form an amphipathic helix whose positively charged face interacts electrostatically with acidic patches of plastocyanin (26). Studies by site-directed mutagenesis of the *psaF* gene from *C. reinhardtii* have confirmed this hypothesis and have further shown that this region forms a recognition site for the binding of plastocyanin and cytochrome c_6. In particular, Lys-16 to Lys-23 of PsaF appears to play a crucial role in the electrostatic interaction with both electron donor proteins (37).

In this study we have used a complementary approach to study the function of the N-terminal region of the eukaryotic PsaF by inserting this sequence into a cyanobacterial PsaF protein. We have thereby shown that it is possible to express a chimeric algal-cyanobacterial PsaF protein in a *psaF*-deficient strain of *S. elongatus* and to incorporate it into its PSI complex. This report thus demonstrates that site-directed mutagenesis of non-essential photosynthetic genes can be performed in *S. elongatus*, the cyanobacterium from which the crystallographic structure of PSI was determined (11, 12). The organization of the *psaF*/*psaJ* operon of the *S. elongatus* strain analyzed in this work corresponds to a combination of a eukaryotic *psaF* with a cyanobacterial *psaJ* gene that is very similar to the *psaF*/*psaJ* operon of *Cyanophora paradoxa* and *P. purpurea* (38, 39) indicating that the basic N-terminal domain of PsaF was already present very early in the evolution of algae and land plants (1) (see Fig. 2).

The introduction of the N-terminal basic patch of PsaF from *C. reinhardtii* into the cyanobacterial subunit clearly improves the binding of plastocyanin or cytochrome c_6 from *C. reinhardtii* to cyanobacterial PSI. The electron transfer rates are increased by 2 and 3 orders of magnitude, and the second-order rate constants (k_2 = 4.0 and 1.7×10^7 M^{-1} s^{-1} for cytochrome

c_6 and plastocyanin, respectively) are similar to the values found for electron transfer to PSI from wild type *C. reinhardtii* (19). In addition, a fast first-order electron transfer occurs between cytochrome c_6 from *C. reinhardtii* and the hybrid PSI indicating that the N-terminal domain of the eukaryotic PsaF subunit is required and sufficient for complex formation between cytochrome c_6 and PSI. Finally, the electron donors from *C. reinhardtii* can be cross-linked almost equally well to PSI complexes from *S. elongatus* H53 and *C. reinhardtii*, demonstrating that the basic N-terminal part of PsaF which is unique to eukaryotes is important for binding of the electron donors from *C. reinhardtii* and even from cyanobacteria. Surprisingly, however, no fast first-order kinetic component is detected for $P700^+$ reduction by *C. reinhardtii* plastocyanin, indicating that although the electron transfer to the cyanobacterial PSI is drastically improved by the N-terminal domain of PsaF, its interaction cannot be stabilized and thus cannot lead to the formation of an intermolecular electron transfer complex. This indicates that, besides the N-terminal region of PsaF, additional structural differences must exist between PSI from *S. elongatus* and *C. reinhardtii* which affect the docking of plastocyanin to PSI. Site-directed mutagenesis of plastocyanin (26, 40–42) suggests that the binding of plastocyanin to PSI involves long range electrostatic interactions between PsaF and plastocyanin and a docking mechanism which brings the flat hydrophobic surface of plastocyanin in close contact to the PSI core proteins. The absence of complex formation with plastocyanin could indicate that the electrostatic interaction with PsaF is incompletely restored in the chimeric PSI due to a slight misalignment of the hybrid PsaF within PSI. Alternatively, a second recognition site is different in PSI from cyanobacteria, most likely the hydrophobic contact surface on the PsaA or PsaB subunits. However, gross structural alterations of the hybrid PsaF are not likely since the intermolecular electron transfer with a half-life of 4 μs observed in the complex with algal cytochrome c_6 is the same as within the eukaryotic complex. This is indicative of a similar orientation within both electron transfer complexes, since the half-life of electron transfer is very sensitive to changes in distance between two redox partners (43).

The new N-terminal domain of PsaF in the hybrid photosystem I also influences the interaction with cytochrome c_6 from *S. elongatus*, but the effects are apparently more subtle. In contrast to its algal counterpart, this protein reacts very efficiently with *S. elongatus* PSI regardless of the presence of a cyanobacterial type PsaF (Fig. 5). However, the salt dependence of the faster of the two second-order kinetic components observed for $P700^+$ reduction of the hybrid PSI by cytochrome c_6 from *S. elongatus* qualitatively resembles the one observed for cytochrome c_6 from *C. reinhardtii* (Fig. 7), and the cyanobacterial protein cross-links to the eukaryotic PsaF subunit, although less efficiently than its algal counterpart (Fig. 8). In addition, the presence of the eukaryotic PsaF subunit results in a 4-fold rate increase in the rate of $P700^+$ reduction by *S. elongatus* cytochrome c_6 at optimal salt concentrations. This suggests that the introduction of the new basic domain on PsaF during evolution resulted in a small immediate improvement of the reaction between cyanobacterial cytochrome c_6 and PSI. However, the rates observed for cytochrome c_6 from *C. reinhardtii* are still 4–5 times faster than those of the cyanobacterial cytochrome, a clear indication that during the evolution from cyanobacteria to algae further structural changes must have been introduced into cytochrome c_6 in order to develop an efficient interaction with the basic domain of PsaF. In this context, the slow $P700^+$ reduction rates that are observed with cytochrome c_6 in the PsaF-less complex from *C. reinhardtii* but not from cyanobacteria also show that during evolution the PsaF subunit has become an essential component in the binding of cytochrome to PSI.

This essential role of the N-terminal domain of eukaryotic PsaF is even more pronounced in the case of the binding of plastocyanin to PSI in *C. reinhardtii* (19). The extremely poor rates of electron transfer between plastocyanin and the cyanobacterial PSI (10 times slower than with algal cyt c_6) indicates that, during evolution, the eukaryotic plastocyanin most likely lost essential structural elements required for the recognition of cyanobacterial PSI. These are still present to some extent in algal cytochromes. With regard to its binding mechanism to PSI, cytochrome c_6 from *C. reinhardtii* thus appears to represent an evolutionary intermediate between cytochrome c_6 from *S. elongatus* and plastocyanin from *C. reinhardtii*, since it interacts efficiently with the positively charged patch of a eukaryotic PsaF and still partially retains the ability to interact with the recognition site for the periplasmatic electron donors of a cyanobacterial PSI. In this context, it is noteworthy that cyanobacterial cytochromes contain a single arginine residue at the otherwise hydrophobic surface that contacts PSI. This residue is conserved in algal cytochromes and in cyanobacterial plastocyanin but is absent in eukaryotic plastocyanin and may thus play an important role in the interaction between PSI and the periplasmic electron donors in cyanobacteria.

Taken together, our data indicate that during the evolution from cyanobacteria to algae and land plants, the reaction pathway from the periplasmic electron donor proteins to photosystem I changed and required structural changes in both PSI and cytochrome c_6 or plastocyanin. For PSI, these were (i) the introduction of the positively charged N-terminal recognition site of PsaF to bind plastocyanin and cytochrome efficiently and to locate them in the vicinity to P700, and (ii) structural change(s) that most likely occurred on the PSI subunits PsaA or PsaB. In addition, changes were required on cytochrome c_6 and plastocyanin in order to adapt to the new mechanism. For cytochrome c_6 from *C. reinhardtii*, the presence of an algal type PsaF subunit suffices to establish tight complex formation with PSI, whereas for plastocyanin both changes are required. In this respect, the algal cytochrome c_6 appears to be an evolutionary intermediate between a cyanobacterial cytochrome c_6 and an algal/land plant plastocyanin.

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