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Active photosynthesis in cyanobacterial mutants with directed modifications in the ligands for two iron–sulfur clusters in the PsaC protein of photosystem I

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The PsaC protein of the Photosystem I (PSI) complex in thylakoid membranes coordinates two [4Fe–4S] clusters, F_A and F_B. Although it is known that PsaC participates in electron transfer to ferredoxin, the pathway of electrons through this protein is unknown. To elucidate the roles of F_A and F_B, we created two site-directed mutant strains of the cyanobacterium *Anabaena variabilis* ATCC 29413. In one mutant, cysteine 13, a ligand for F_B was replaced by an aspartic acid (C13D); in the other mutant, cysteine 50, a ligand for F_A was modified similarly (C50D). Low-temperature electron paramagnetic resonance studies demonstrated that the C50D mutant has a normal F_B center and a modified F_A center. In contrast, the C13D strain has normal F_A, but failed to reveal any signal from F_B. Room-temperature optical studies showed that C13D has only one functional electron acceptor in PsaC, whereas two such acceptors are functional in the C50D and wild-type strains. Although both mutants grow under photoautotrophic conditions, the rate of PSI-mediated electron transfer in C13D under low light levels is about half that of C50D or wild type. These data show that (i) F_B is not essential for the assembly of the PsaC protein in PSI and (ii) F_B is not absolutely required for electron transfer from the PSI reaction center to ferredoxin.

Keywords: cyanobacteria/directed mutagenesis/iron–sulfur centers/photosynthesis/photosystem I

Introduction

One of the earliest events in photosynthesis is the conversion of solar energy to chemical energy by specialized membrane-bound pigment–protein complexes known as reaction centers. In oxygenic photosynthetic organisms, two reaction centers, Photosystem II (PSII) and Photosystem I (PSI), operate in series to transfer electrons from donor to acceptor molecules. PSI mediates light-induced electron transfer from plastocyanin or cytochrome *c*553 to ferredoxin (reviewed in Pakrasi, 1995). The core of the PSI complex is a heterodimer composed of the PsaA and PsaB proteins, and contains four redox cofactors that participate in intraprotein electron transfer: a primary electron donor P700 (a dimer of chlorophyll *a*), a primary acceptor A_0 (a monomeric chlorophyll *a*), an intermediate electron acceptor A_1 (a phylloquinone) and an iron–sulfur center F_X (Krauss *et al.*, 1993; Fromme *et al.*, 1994). The reduction of ferredoxin is mediated by PsaC, a 9 kDa polypeptide located on the stromal side of PSI, which coordinates two iron–sulfur centers, F_A and F_B (Golbeck, 1992). Biochemical and genetic studies have shown that the PsaC protein is required for the binding of the PsaD and PsaE polypeptides to the PSI complex (Golbeck, 1992; Mannan *et al.*, 1994).

The iron–sulfur centers in PSI are [4Fe–4S] clusters and are among the strongest reductants known in any biological system. The F_A and F_B clusters were first characterized by a low-temperature electron paramagnetic resonance (EPR) technique. Malkin and Bearden (1971) identified a ferredoxin-like bound iron–sulfur protein involved in the primary photochemical reaction of PSI. Illumination at liquid helium temperatures resulted in the photoreduction of F_A with EPR *g*-values of 2.05, 1.94 and 1.86. Further studies revealed the presence of a second iron–sulfur center, F_B, with EPR *g*-values of 2.07, 1.92 and 1.89, which can be photoreduced in the presence of a reduced F_A center (Bearden and Malkin, 1972; Evans *et al.*, 1972). When both F_A and F_B are reduced, the high- and low-field values of F_A and F_B merge to produce a composite spectrum at g = 2.05, 1.94, 1.92 and 1.89. Recent crystal structure data of a cyanobacterial PSI complex show that these two centers are located within 12 Å of each other (Krauss *et al.*, 1993; Fromme *et al.*, 1994).

Although it is known that the PsaC protein mediates electron transfer from F_X to ferredoxin, the path of electrons through PsaC is unknown. A linear and a branched scheme are currently being considered. Judged solely by the equilibrium midpoint potentials of F_A (–530 mV) and F_B (–580 mV) (Ke *et al.*, 1973; Heathcote *et al.*, 1978), a serial pathway of electrons from F_X to F_B and then to F_A is favored. This view is supported by the finding that there is a marked inhibition of the low-temperature photoreduction of F_A after selective chemical inactivation of F_B by diazonium compounds (Malkin, 1984). However, the observation that a selective destruction of F_B by urea–ferricyanide (Golbeck and Warden, 1982) or mercurials (Sakurai *et al.*, 1991; He and Malkin, 1994) does not noticeably affect the room- and low-temperature photoreduction of F_A challenges the F_B to F_A pathway. Furthermore, it has been discovered that electron transfer pathways exist at low temperatures that are not representative of the pathways operating at physiological temperatures. For example, the extraction of A_1 from PSI reaction center particles does not inhibit the photoreduction of the iron–sulfur centers at cryogenic temperatures but does inhibit room-temperature electron transfer through PSI (Setif *et al.*, 1987).

Using an *in vitro* system in which mutant PsaC proteins overproduced in *Escherichia coli* are reconstituted into isolated PSI core complexes, Zhao *et al.* (1992) found that F_B is not an obligatory intermediate in the pathway of electrons to F_A. However, as discussed below, a recent analysis of the same experimental system has led to significantly different conclusions (Yu *et al.*, 1995). Although such *in vitro* reconstitution experiments have provided important information regarding the identification of specific amino acid residues in the PsaC protein that act as ligands for F_A and F_B, they have not elucidated the path of the electrons in the PsaC protein under *in vivo* conditions. In particular, the question remains open as to whether both of these iron–sulfur clusters are obligate electron transport intermediates during photosynthesis.

To understand the roles of the F_A and F_B clusters in the assembly of the PSI complex and in mediating electron transfer from F_X to ferredoxin, we used site-directed mutagenesis to create mutants in each of which a cysteine ligand in the PsaC protein was changed to an aspartic acid. Our experimental system for the 'reverse genetic' analysis of various PSI proteins is based on *Anabaena variabilis* ATCC 29413 (henceforth called *Anabaena* 29413). This filamentous cyanobacterium is capable of dark heterotrophic growth and maintains a fully developed and functionally active photosynthetic apparatus under such conditions (Pakrasi, 1995). Our analysis of site-specific mutant strains of *Anabaena* 29413 demonstrates that in the absence of an F_B cluster, a functional PSI reaction center is assembled and the cells can grow photoautotrophically. Moreover, we show that electron transfer through PSI to $NADP^+$ can occur in the absence of a functional F_B cluster.

Results

Creation of targeted mutations in the psaC gene

Our objective was to specifically modify cysteine residues in the PsaC protein that serve as ligands for the iron–sulfur clusters F_A and F_B in *Anabaena* 29413. The first step was to generate a *psaC* deletion strain to serve as a host for the introduction of mutated versions of the *psaC* gene. The top panel in Figure 1 represents a 3.5 kb *Ssp*I fragment of the chromosomal DNA containing the coding region of the *psaC* gene (Mannan and Pakrasi, 1992). A synthetic oligonucleotide was used to generate an *Nco*I site at the beginning of this coding region. Next, the region between the newly created *Nco*I site and an *Eco*RI site immediately downstream of the *psaC* coding sequence was replaced by an *aadA* cassette which confers resistance to spectinomycin (Sp) and streptomycin (Sm) (Goldschmidt-Clermont, 1991). The DNA insert in this recombinant plasmid was transferred to the shuttle vector pRL271 to create a donor plasmid pSL677 (Figure 1, middle panel), which was then used in conjugation experiments to generate T677, a Sp^r/Sm^r derivative strain of *Anabaena* 29413. Southern blotting and PCR analysis of the chromosomal DNA of the T677 cells showed that the *psaC* gene was replaced by the *aadA* cassette (data not shown). The biochemical and physiological properties of this Δ*psaC* strain (data not shown) are similar to those of T398, a previously characterized strain in which the *psaC* gene was interrupted by a neomycin-resistance (Nm^r)

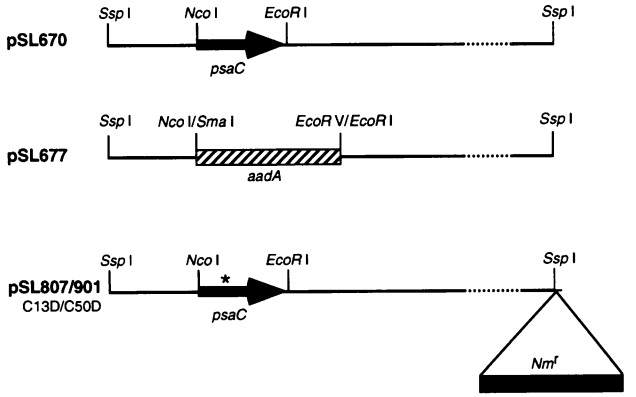

Fig. 1. A scheme for the site-directed mutagenesis of the *psaC* gene in *Anabaena* 29413. The top panel depicts a 3.5 kb *Ssp*I fragment of *Anabaena* 29413 genomic DNA cloned in plasmid pSL670. The solid black arrow represents the coding region (243 bases) of the *psaC* gene. The middle panel represents the pSL677 plasmid construct in which the *psaC* gene has been replaced by an *aadA* cassette, conferring resistance to Sp and Sm. This plasmid was used to generate a *psaC* deletion strain, T677. *In vitro* mutagenized plasmids (pSL807 and pSL901) were engineered with a Nm^r cassette inserted downstream of the *psaC* gene for the introduction of site-specific mutations in the *psaC* gene. The asterisk represents a site-specific mutation in the *psaC* gene. The dotted lines represent a region of genomic DNA not shown here for clarity.

cassette (Mannan *et al.*, 1991). The strategy for the creation of site-specific mutations in the *psaC* gene is illustrated in the bottom panel of Figure 1. An Nm^r cartridge was introduced downstream of the *psaC* gene in a pRL271-based donor plasmid. After the introduction of site-specific mutations in the *psaC* gene, such plasmids (e.g. pSL807 and pSL901) were conjugated into the Δ*psaC* strain. The presence of the desired mutations was confirmed by thermal cycle sequencing of the PCR amplification products obtained from the chromosomal DNA of the mutant strains (data not shown). Because most cyanobacterial PSI-deficient mutants are light sensitive and suffer photodamage even under low-intensity growth light (Pakrasi, 1995), the Nm^r exconjugants were maintained in the dark.

Choice of cysteine 13 and cysteine 50 in the PsaC protein as the targets for site-specific mutagenesis

All PsaC proteins characterized to date contain two conserved CxxCxxCxxxC motifs that combine to provide eight cysteine ligands for the F_A and F_B clusters (Golbeck, 1992). Using a combined site-directed mutagenesis and *in vitro* reconstitution approach, Zhao *et al.* (1992) identified the specific cysteine ligands for F_A and F_B. In the PsaC protein in *Anabaena* 29413 (Mannan and Pakrasi, 1992), the F_A cluster is predicted to be coordinated by cysteines 20, 47, 50 and 53, while the F_B cluster is predicted to be coordinated by cysteines 10, 13, 16 and 57.

A guide for replacing cysteine ligands in [4Fe–4S] clusters is provided by the naturally occurring ferredoxins of *Desulfovibrio africanus* (George *et al.*, 1989) and *Pyrococcus furiosus* (Conover *et al.*, 1990). Each of these organisms contains a mixed ligand [4Fe–4S] center in which an aspartic acid is present in place of the second cysteine residue in the CxxCxxCxxxC motif. With the goal of modifying the iron–sulfur centers without significant impact on the tertiary structure of the PsaC protein, we

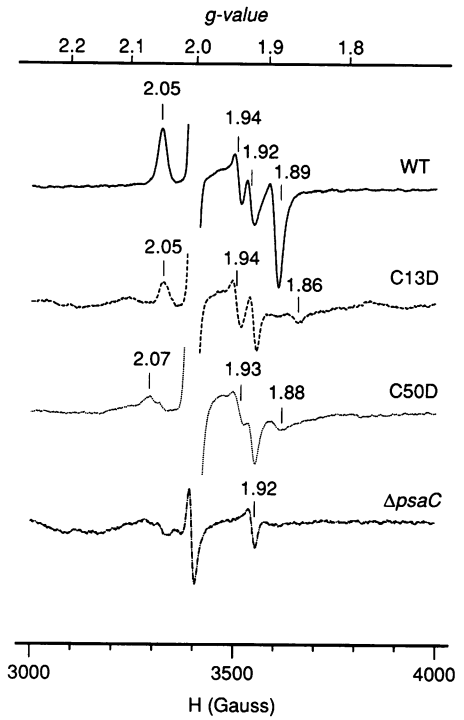

Fig. 2. EPR spectra of thylakoid membranes measured at low temperature under reducing conditions for samples isolated from *Anabaena* 29413 wild-type, C13D, C50D and Δ*psaC* strains. Chl concentrations: wild-type, 1.4 mg/ml; C13D, 0.95 mg/ml; C50D, 1.5 mg/ml; Δ*psaC*, 1.3 mg/ml. Spectra of wild-type, C13D and Δ*psaC* strains were recorded at 18 K and of C50D at 25 K. Spectra of C13D and Δ*psaC* were multiplied by a factor of 2. Spectrometer settings: gain, 2×10^5; modulation, 12.5 Gauss at 100 kHz; microwave frequency, 9.440 GHz; microwave power, 20 mW.

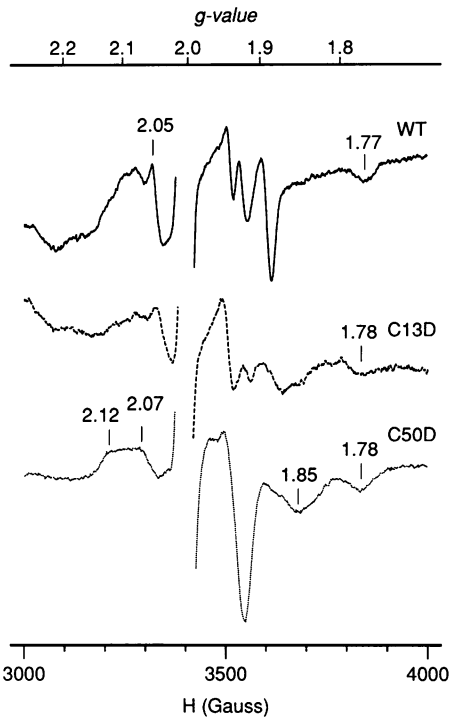

Fig. 3. EPR spectra of thylakoid membranes measured at 12 K under reducing conditions for samples isolated from wild-type, C13D and C50D cells. Samples were frozen in light in the presence of sodium dithionite and 0.1 mM PMS. Chl concentrations and instrument settings were similar to those described in the legend to Figure 2.

chose to follow this pattern by replacing the second cysteine residue in each of the four-cysteine motifs in the PsaC protein by an aspartic acid residue. Two mutant strains were created in the PsaC protein of *Anabaena* 29413: one with the C13D mutation (T807) and the other with the C50D mutation (T901).

EPR analysis of the mutant strains

Thylakoid membranes from dark-grown cultures of wild-type and mutant strains of *Anabaena* 29413 were characterized by a low-temperature EPR analysis to determine the effects of the site-directed mutations on the iron–sulfur clusters in PSI. When thylakoid samples were frozen in the presence of a strong reductant, such as sodium dithionite, under actinic illumination, the reduced iron–sulfur centers in PSI photoaccumulated. In the case of wild-type samples (solid line in Figure 2), a typical interacting spectrum for F_A^- and F_B^- with *g*-values of 2.05, 1.94, 1.92 and 1.89 was observed (Aasa *et al.*, 1981), indicating that both F_A and F_B were reduced. Although the F_X center might have been reduced, it was not detected at this temperature because of its fast spin relaxation (see below). Similar measurements using membranes from the deletion strain (Δ*psaC*) revealed an axial signal of a [4Fe–4S] center with *g*-values of 2.05 and 1.92. This signal is attributed to a reduced NADH/NADPH dehydrogenase (NDH) and has been observed in a variety of cyanobacterial species (Sandmann and Malkin, 1983). The signal from NDH was also observed in the spectra of samples frozen with ascorbate in the dark, but not in the light-minus dark spectrum (data not shown), indicating that it is unrelated to low-temperature photochemistry in PSI. The EPR spectrum of Δ*psaC* confirms the absence of the F_A and F_B iron–sulfur clusters in this strain.

The EPR signal shown in Figure 2 for the C13D mutant reveals a normal rhombic, non-interacting F_A signal with *g*-values of 2.05, 1.94 and 1.86 (Aasa *et al.*, 1981), in addition to the NDH signal. There was no evidence of a normal F_B signal in this mutant. On the other hand, the C50D mutant showed a normal, non-interacting F_B signal, with *g*-values of 2.07, 1.93 and 1.88, and no evidence of a normal F_A signal. It is noteworthy that the EPR characteristics of F_A in C13D and F_B in C50D samples were not affected by the respective cysteine to aspartic acid mutations. Furthermore, these data confirm the assignment of C13 as a ligand for F_B and C50 as a ligand for F_A.

To gain a clearer view of the effects of the targeted mutations on the formation and properties of the F_A and F_B clusters, and to establish the presence of F_X, the EPR spectra of samples frozen under illumination with added sodium dithionite and *N*-methyl phenazonium methosulfate (PMS) were recorded at 12 K. As shown in Figure 3, the F_X signal at $g = 1.77$–1.78 was observed in the wild-type, C13D and C50D samples. In addition, we observed a set of new resonances in the C50D sample, with *g*-values of 2.12, 1.93 and 1.85. The maximum amplitude of this signal was observed at 9 K (data not shown). We believe that this signal originates from a modified F_A cluster with mixed ligands, similar to the recently described F_B' cluster (Yu *et al.*, 1995). Presumably, the ligand modification of cysteine to aspartate altered the spin relaxation properties

of the F_A cluster in our C50D mutant, so that the optimal temperature required to observe the corresponding EPR signal decreased. It is noteworthy that in contrast to the *in vitro* study of Yu *et al.* (1995), our *in vivo* analysis of the C50D mutant showed that the modified signal from F_A and the signal from F_B had distinct *g*-values, with no evidence for any spin–spin interaction between the two centers. No comparable EPR signal was observed in the C13D sample (Figure 3), indicating the absence of a mixed-ligand F_B'-type center in this mutant strain.

As mentioned above, in naturally occurring ferredoxins in which the cysteine residue in the second position of the four-cysteine motif has been replaced by an aspartate residue, the corresponding [4Fe–4S] cluster has mixed ligands. The fact that it can be converted into a [3Fe–4S] cluster (George *et al.*, 1989; Conover *et al.*, 1990) raises the possibility that a [3Fe–4S] center may exist in the C13D or C50D cells. In the reduced state, such a [3Fe–4S] cluster does not have an EPR signal in the $g = 2$ region because of its integral spin quantum ($S = 2$). However, when oxidized, it exhibits an intense axial-shaped signal centered around $g = 2.01$ (Beinert and Thomson, 1983). Low-temperature EPR spectra of the C13D and C50D samples recorded under oxidizing conditions failed to reveal a signal attributable to a [3Fe–4S] cluster (data not shown).

Optical spectroscopic analysis

To determine the total number of functional iron–sulfur centers in the cysteine replacement mutants, we used a multiple flash technique developed by Mathis and co-workers (e.g. Sakurai *et al.*, 1991) and subsequently employed in other laboratories (He and Malkin, 1994; Yu *et al.*, 1995). The key to this technique is to determine the number of functional electron acceptors in PsaC by giving three closely timed actinic flashes. The success of the experiment depends on the fact that the back reaction between F_X and $P700^+$ is fast (half-time <500 μs), while the back reaction between F_A or F_B and $P700^+$ is slow (half-time >50 ms) (e.g. Mannan *et al.*, 1991). We measured the back reaction between F_A/F_B and $P700^+$ in the wild-type and mutant strains after a single turnover flash, and observed half-times of 50–70 ms in wild type and 80 ms in both the C13D and C50D mutants (data not shown). Using redox mediators, conditions in the triple-flash experiments were controlled so that only the back reaction from F_X was detectable. This was accomplished by adding the mediator PMS at a concentration that reduces $P700^+$ more rapidly than the F_A/F_B back reaction but slower than the F_X^- $P700^+$ back reaction (Sakurai *et al.*, 1991). Under these conditions, electron transfer from P700 to F_A or F_B is stabilized because $P700^+$ is reduced rapidly by PMS (half-time ~2 ms), but electron transfer to F_X gives a rapid back reaction with a half-time of 200–400 μs. Figure 4A shows the result of such an experiment for wild-type membranes. After the first flash, one of the two [4Fe–4S] clusters associated with the PsaC protein was reduced by an electron from P700, followed by the re-reduction of $P700^+$ by PMS ($t_{1/2}$ = 1.8 ms). After the second flash, the second [4Fe–4S] cluster in PsaC was reduced, followed by the re-reduction of $P700^+$ by PMS (66% of the kinetics for the absorbance change exhibited a $t_{1/2}$ = 2.3 ms). After the third flash, F_X was

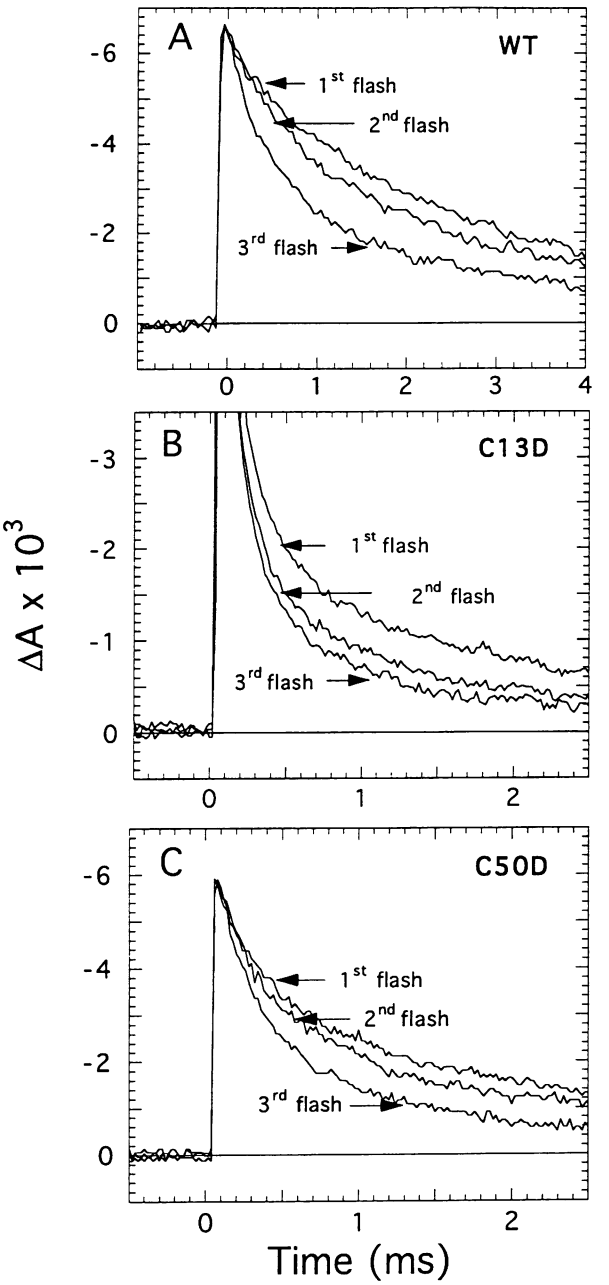

Fig. 4. Kinetics of absorption changes at 705 nm due to P700 induced by three single turnover flashes. Measurements were made at 23°C using thylakoid membranes isolated from *Anabaena* 29413 cells. Chl concentrations: (**A**) wild-type, 14 μM; (**B**) C13D, 17 μM; (**C**) C50D, 11 μM. The kinetic traces are superimposed for ease of comparison. Further details are given in the text.

reduced. Because charge recombination between F_X^- and $P700^+$ is much faster than the electron donation from PMS, the majority of the $P700^+$ re-reduction after the third flash exhibited fast kinetics with a $t_{1/2}$ = 290 μs. The kinetic traces shown in Figure 4A are similar to the results of other workers, demonstrating that both F_A and F_B can accept electrons from F_X in wild-type cells. In contrast, a similar analysis of thylakoid membranes isolated from the C13D mutant (Figure 4B) showed a rapid re-reduction of $P700^+$ after the second flash. Figure 4B shows that 70% of the absorbance decrease after the second flash had $t_{1/2}$ = 370 μs, suggesting that in this

Table I. Growth and photosynthetic electron transport rates, Chl/P700 ratio and PSI polypeptide profiles of wild-type and *psaC* mutant strains of *Anabaena* 29413

	Wild-type	Δ*psaC*	C13D	C50D
Electron transport rates				
H_2O to $NaHCO_3^-$[a]	175 ± 13	0	176 ± 16	164 ± 10
DCIP/Asc to MV[a]	1002 ± 40	950 ± 27	1105 ± 35	1060 ± 40
DCIP/Asc to $NADP^+$[b]	74 ± 8	0	72 ± 5	72 ± 5
Chl/P700 (mol/mol)[c]	131 ± 5	129 ± 4	138 ± 2	128 ± 7
PsaC, PsaD and PsaE polypeptides[d]	+	–	+	+
Doubling time (h)				
Dark	38 ± 3	36 ± 3	37 ± 4	35 ± 5
Light	36 ± 3	NG	62 ± 5	40 ± 5

Asc, sodium ascorbate; MV, methyl viologen; NG, no growth. Each value is the mean ± SD of at least four independent measurements.
[a]Determined by polarography and expressed as μmol of oxygen per mg Chl per h.
[b]Determined by spectrophotometric means in the presence of 3.2 μM spinach ferredoxin and expressed as μmol of $NADP^+$ reduced per mg Chl per h.
[c]Determined from chemical difference spectra.
[d]Western blot analysis with specific antibodies was performed to detect each of these three polypeptides in isolated thylakoid membranes. See text for further details.

mutant only one electron acceptor is present after F_X. The slight differences between the traces induced by the second and third flashes may be caused by a small but variable amount of F_A oxidation which occurs after each flash. A similar effect was observed by Sakurai *et al.* (1991). Thylakoid membranes from the C50D mutant showed a pattern similar to that of the wild-type cells (Figure 4C), indicating that both F_B and the modified F_A centers are functional in this strain.

Impacts of the C13D and C50D mutations in the PsaC protein on the form and function of PSI

When grown in dark, wild-type *Anabaena* 29413 cells assemble a photosynthetically competent thylakoid system (Mannan and Pakrasi, 1993). As shown in Table I, whole-chain photosynthetic electron transfer did not occur in the Δ*psaC* strain. In contrast, the C13D and C50D cells could mediate light-induced whole-chain electron transfer at rates comparable with that in the wild-type cells, suggesting that in these two mutant strains the PSI complex is assembled and is functionally competent.

Chemical difference spectra of membrane samples indicated that the chlorophyll (Chl)/P700 ratios are similar in the wild-type and mutant cells (Table I). Thus, the mutations in the *psaC* gene did not affect the biogenesis and stability of the PSI reaction center core complex. With methyl viologen (1 mM) as the electron acceptor, significant rates of electron transfer were observed in thylakoid samples from the deletion as well as the C13D and C50D mutant strains. However, with $NADP^+$ as the electron acceptor, electron transfer through the PSI complex was detected in the wild-type, C13D and C50D mutant strains, but not in the Δ*psaC* strain. The difference in the results from these two experiments lies in the fact that methyl viologen can accept electrons directly from F_X, whereas $NADP^+$ accepts electrons from ferredoxin which, in turn, can accept them only from the F_A/F_B clusters (Fujii *et al.*, 1990). Thus, the ability to reduce $NADP^+$ provides evidence for the presence of functionally active F_A/F_B cluster(s) in the C13D and C50D mutant strains.

Western blot analysis of thylakoid membrane preparations from the C13D and C50D strains showed the presence of PsaC, PsaD and PsaE, three stromal polypeptides, also suggesting that the PSI complex is assembled normally in these mutant strains. All of these polypeptides are absent from the thylakoid membranes from the Δ*psaC* cells (Table I) as well as from the T398 *psaC* insertion mutant strain (Mannan *et al.*, 1994).

Table I also shows the growth rates of these strains under photoautotrophic and dark heterotrophic growth conditions. Under heterotrophic conditions, the Δ*psaC*, C13D, C50D and the wild-type strains showed comparable growth rates. However, under 50 $\mu E.m^{-2}\ s^{-1}$ of illumination, the doubling times of the wild-type and C50D strains were comparable, while the C13D strain exhibited a significantly decreased growth rate. As shown in Figure 5, the relative quantum efficiency of PSI-mediated electron transfer was significantly lower in the C13D mutant, while those of the C50D mutant and the wild-type cells were similar. This lower quantum yield may account for the decreased growth rate of the C13D strain under photoautotrophic conditions.

Discussion

The PsaC protein serves to transfer electrons from F_X, a [4Fe–4S] center located in the core of PSI, to the soluble carrier ferredoxin. PsaC contains two [4Fe–4S] centers, F_A and F_B, each coordinated by four cysteine residues. To elucidate the roles of F_A and F_B in the reduction of ferredoxin, we used directed mutagenesis to replace specific cysteine ligands in the PsaC protein in *Anabaena* 29413. The F_A center, which is coordinated by cysteine residues at positions 20, 47, 50 and 53, was modified by replacing cysteine 50 with an aspartic acid, creating a C50D mutant strain. A low-temperature EPR analysis indicates that the F_A center in this mutant strain is significantly different from that in the wild-type strain. The modified EPR spectrum is consistent with a mixed ligand [4Fe–4S] center, with aspartic acid 50, water or OH^- possibly replacing the missing cysteine ligand

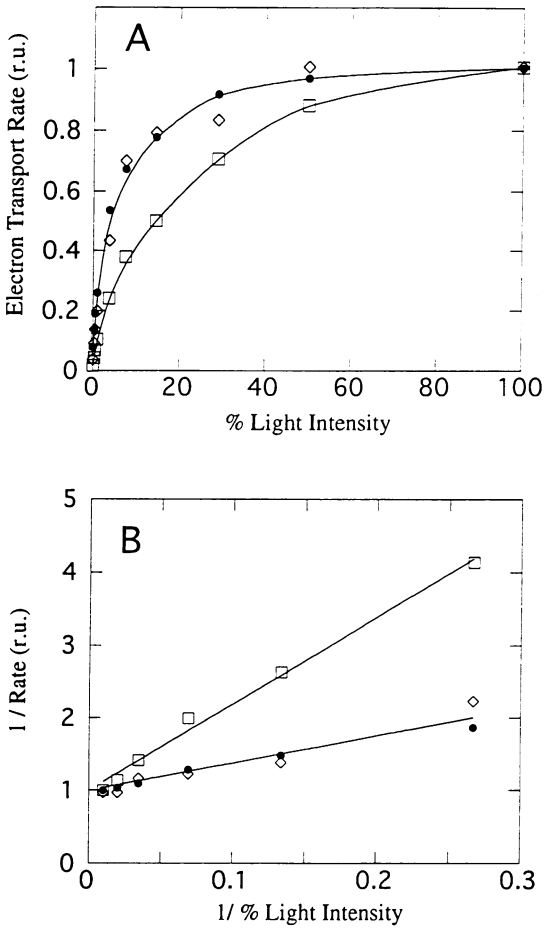

Fig. 5. (**A**) The rates of PSI-mediated electron transport as a function of light intensity for thylakoid membranes isolated from *Anabaena* 29413 wild-type (●), C13D (□) and C50D (◇) strains. Normalized rates of electron transport are shown. The light saturated rates are given in Table I. 100% light intensity corresponded to 3500 $\mu E.m^{-2} s^{-1}$. Other experimental conditions are described in Materials and methods. (**B**) Double reciprocal plots of the data shown in (A).

(George *et al.*, 1989; Conover *et al.*, 1990). In comparison, the F_B center in the C50D mutant exhibits a normal EPR signal, with no indication of an interaction between the F_A and F_B centers. Room-temperature optical spectroscopy demonstrates that F_B, as well as the modified F_A center, can accept electrons from F_X. *In vivo* measurements of steady state light-induced electron transport showed that the C50D mutant can reduce carbon dioxide at the same rate as wild-type cells. These data, together with measurements of $NADP^+$ reduction using isolated thylakoid membranes, show that despite the replacement of cysteine 50 by aspartic acid, electron transfer through the PsaC protein to ferredoxin is efficient and rapid. Furthermore, the C50D mutant cells grow photoautotrophically at rates comparable with wild-type cells.

The F_B center, which is coordinated by cysteine residues at positions 10, 13, 16 and 57 of the PsaC protein, was modified in a manner similar to that of F_A. In particular, cysteine 13 was replaced by an aspartic acid. In this mutant, low-temperature EPR analysis showed that the normal signal caused by F_B is absent, and that, unlike C50D, no modified signal from a mixed ligand [4Fe–4S] center could be detected. Furthermore, any signal from a [3Fe–4S] center could not be detected. However, these data cannot exclude the possibility of an anomalous signal that would escape EPR detection under our experimental conditions. Room-temperature optical spectroscopy demonstrated that only one iron–sulfur center in the PsaC protein of the C13D mutant is functional. Because F_A gives a normal EPR signal in C13D, we conclude that this mutant strain lacks a functional F_B center. Interestingly, measurements of steady state light-induced electron transport in whole cells under saturating light demonstrated that the C13D mutant can reduce carbon dioxide at the same rate as wild-type cells and that isolated thylakoid membranes can reduce $NADP^+$. These data show that a functional F_B center is not absolutely necessary for the light-induced electron transfer from F_X to ferredoxin. This is also demonstrated clearly by the fact that the C13D mutant cells can grow well photoautotrophically.

The results presented here based on the C13D and C50D mutants of *Anabaena* 29413 differ from those of Yu *et al.* (1995) based on an *in vitro* system. Earlier reconstitution studies by the same research group suggested that a C14D mutation (equivalent to our C13D modification) in the PsaC protein leads to the formation of a [3Fe–4S] cluster (Zhao *et al.*, 1992). Another observation in this earlier study was that F_A can be photoreduced in the absence of F_B. However, using an improved reconstitution protocol and refined spectroscopic analysis, Yu and co-workers (1995) have recently concluded that PSI centers reconstituted with this mutant PsaC protein contain a modified F_B center (F_B') that is functionally active. In addition, in these studies, F_A and F_B' exhibited spin–spin interactions. In contrast, in our study the EPR signals of F_A in the C13D mutant did not indicate the presence of another interacting spin (Figure 2). The basis for such contrasting observations may lie in the use of an *in vitro* reconstitution system (Yu *et al.*, 1995) as opposed to *in vivo* mutagenesis (our study) to generate targeted mutations in the PsaC protein.

A significant observation in our investigation is the ability of the PsaC protein to maintain its structural and functional integrity despite the absence of one of its associated [4Fe–4S] clusters. Thylakoid membranes from the C13D mutant can reduce $NADP^+$, indicating that the conformation of the PsaC polypeptide as well as that of the PSI complex is not altered significantly in this mutant. A comparison of the tertiary structure of a monocluster ferredoxin from *Bacillus thermoproteolyticus* (Fukuyama *et al.*, 1988) with that of a 2[4Fe–4S] ferredoxin from *Peptococcus aerogenes* (Adman *et al.*, 1973) reveals that the absence of an iron–sulfur cluster in the *B.thermoproteolyticus* ferredoxin has been compensated for by the creation of a rigid α-helix in its place. It is interesting to note that the domain of the PsaC protein between residues 10 and 20 has the ability to form an α-helix (Hayashida *et al.*, 1987). Future analysis of the C13D mutant should elucidate whether such an α-helix is present in the PsaC protein in this mutant.

The most interesting finding in our study is that PSI centers in a mutant that appears to lack an F_B cluster and in a mutant that has a significantly altered F_A cluster are functional. However, this observation is not universal, as shown in a recent study in which mutants of the green alga *Chlamydomonas reinhardtii* with site-specific modifications in the PsaC protein were analyzed for the

assembly and function of the PSI complex (Takahashi *et al.*, 1992). When cysteine 14 in *Chlamydomonas* PsaC (equivalent to C13 in our study) was modified to an aspartic acid residue, the steady state level of the PSI complex was severely diminished. Moreover, the mutant strain could not grow photoautotrophically. It was observed earlier that insertional inactivation of the *psaC* gene destabilizes PSI in *Chlamydomonas*, whereas the PSI core complex assembles normally in a similar mutant strain of *Anabaena* 29413 (Pakrasi, 1995). Such differences suggest that the details of biogenesis of the PSI complex may be distinctly different between cyanobacteria and *Chlamydomonas*, a eukaryote.

The observation that the C13D mutant strain can grow photoautotrophically, even though it lacks a detectable F_B center, indicates that F_A is sufficient for electron transfer from F_X to ferredoxin. Recent X-ray structural analyses of PSI crystals show that the two [4Fe–4S] centers in the PsaC protein are respectively 15 Å and 22 Å away from the F_X center (Krauss *et al.*, 1993). However, the identities of the F_A and F_B centers in the crystal are not known. Although speculative, the data presented in our study support the notion that the center nearest to F_X is F_A. In addition, although the exact binding site of ferredoxin on the PsaC protein has not been determined, Fromme *et al.* (1994) have postulated that the PsaC protein may have two alternate binding sites for ferredoxin. In this scenario, electrons from F_A can reach ferredoxin bound closer to it without passing through F_B. In any case, we have demonstrated that if a physiological pathway of electron transfer through F_B exists then it is dispensable, and the only consequence of a non-functional F_B center is a decrease in the relative quantum yield of PSI.

Materials and methods

Materials

All chemicals used were of reagent grade and obtained from Sigma Chemical Co. Enzymes for recombinant DNA work were purchased from New England Biolabs; Klentaq DNA polymerase used in PCRs was acquired from Dr W.Barnes, Washington University; [α-^{32}P]dCTP (3000 Ci/mmol) used for the radioactive labeling of DNA fragments, [α-^{35}S]ATP (>1000 Ci /mmol) used for nucleotide sequencing and [γ-^{32}P]ATP used to label oligonucleotide primers for thermocycle sequencing were obtained from Amersham.

Cyanobacterial strains and growth conditions

The culture medium and growth conditions for *Anabaena* 29413 strains were as described previously (Mannan *et al.*, 1991). During autotrophic growth, cultures were illuminated at 50 $\mu E.m^{-2}\ s^{-1}$ of white light. Stock solutions of Nm, Sp, Sm and fructose were filter-sterilized and added at final concentrations of 50 μg/ml, 25 μg/ml, 25 μg/ml and 5 mM, respectively. Cell growth was determined by measuring optical scattering at 730 nm on a DW2000 spectrophotometer (SLM Aminco).

DNA manipulations

Chromosomal DNA from *Anabaena* 29413 cells was isolated according to Maldener *et al.* (1991). Southern hybridization and other routine DNA manipulations were performed essentially as described in Sambrook *et al.* (1989). Site-directed mutagenesis was carried out as described in Pakrasi *et al.* (1991). To replace Cys13 in the PsaC protein by an Asp residue (C13D), we used the following oligonucleotide: 5′-GCA-GGCCCGGACACATTGAGTATCGCCAAT-3′. Another oligonucleotide with the sequence 5′-GGGCAAGCTGTTTCACAGCGCTTGTCGCCT-AGCAGTCTTC-3′ was used to replace Cys50 by an Asp residue (C50D). Each of these oligonucleotides also introduced a silent change, leading to the creation of either a *Hae*III site (for the C13D mutation) or a *Hae*II site (for the C50D mutation), that was subsequently used in screening for the presence of the desired mutations. Site-directed mutations were introduced into *Anabaena* 29413 cells via conjugation-mediated DNA transfer from *E.coli* cells (Elhai and Wolk, 1988). The segregation of such mutations was ultimately verified by thermal cycle sequencing (Krishnan *et al.*, 1991) of the *psaC* gene in PCR amplification products from chromosomal DNA of the C13D and C50D mutant cells.

Isolation of thylakoid membranes, estimation of pigment compositions, protein electrophoresis and immunodetection

The isolation of thylakoid membranes from cyanobacterial cells, the fractionation of proteins on SDS–polyacrylamide gels and the immunodetection of proteins on Western blots were performed as described previously (Mannan and Pakrasi, 1993). The concentrations of chlorophyll in the isolated thylakoid membranes and the Chl/P700 ratio were determined as described earlier (Mannan *et al.*, 1991).

EPR spectroscopy

Thylakoid membrane samples in HEPES buffer (pH 8.0) were reduced by the addition of a few crystals of sodium dithionite, and frozen to 77 K under continuous actinic illumination. EPR measurements were carried out on an ER-200tt X-band spectrometer (Bruker) equipped with an EPR-900 continuous flow cryostat (Oxford Instruments) for low-temperature studies. The sample temperature was monitored with a thermocouple below the sample tube and measured by an ITC4 temperature controller (Oxford Instruments). Instrument settings are indicated in the figure legends.

Transient optical absorption spectroscopy

Room-temperature flash-induced absorption transients from P700 were measured using a laboratory-built time-resolved single-beam spectrophotometer essentially as described previously (Mannan *et al.*, 1991). Thylakoid samples were suspended in a reaction mixture containing 50 mM Tris–Cl (pH 8.3), 3.4 mM sodium ascorbate and 100 μM 2,6-dichlorophenolindophenol (DCIP). Prior to illumination, 100 μM PMS were added to the sample, followed by the addition of sodium dithionite (from a stock solution suspended in 100 mM Tris–Cl, pH 9.0), to a final concentration of 3 mg/ml. After 10 s, the sample was exposed to a single train of three actinic flashes given 50 ms apart. Each actinic flash had a half-peak width of 6 μs. A fresh sample was used for each measurement.

Measurement of the rates of electron transfer reactions

A Clark-type oxygen electrode was used to measure the rates of photosynthetic electron transfer reactions at 30°C under saturating red light (CS 2-63, Corning). Filaments of exponentially growing cultures were collected by centrifugation at 3000 *g* for 5 min at room temperature, and then suspended in 20 mM Tris–Cl (pH 7.0). Whole-chain electron transport in intact cells was measured in the presence of 10 mM $NaHCO_3$. The rates of PSI-mediated electron transport using isolated thylakoid membranes were measured in a reaction mixture containing 50 mM HEPES (pH 7.5), 10 mM NaCl, 5 mM $MgCl_2$, 1 mM KCN, 20 μM DCMU, 10 μg/ml superoxide dismutase, 0.5 mM sodium ascorbate, 1 mM DCIP, 1 mM methyl viologen and thylakoid samples equivalent to 6.6 μg Chl/ml. Rates of $NADP^+$ photoreduction were measured at 340 nm with actinic cross-illumination on a DW2000 spectrophotometer. The sample contained thylakoid membranes equivalent to 10 μg Chl/ml in a reaction mixture containing 50 mM Tris–Cl (pH 7.8), 0.3 M sucrose, 10 mM NaCl, 5 mM $MgCl_2$, 5 mM sodium ascorbate, 0.1 mM DCIP, 10 mM $NADP^+$, saturating amounts of spinach ferredoxin–$NADP^+$–oxidoreductase and spinach ferredoxin.

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