Biochemistry

 Volume 31, Number 22 June 9, 1992

Accelerated Publications

Site-Directed Conversion of a Cysteine to Aspartate Leads to the Assembly of a [3Fe-4S] Cluster in PsaC of Photosystem I. The Photoreduction of F_A Is Independent of F_B[†]

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Received March 9, 1992; Revised Manuscript Received April 13, 1992

ABSTRACT: The terminal electron acceptors F_A and F_B exist as two [4Fe-4S] clusters located on the 8.9-kDa PsaC protein in photosystem I. We have used site-directed mutagenesis to produce a complementary pair of mutant PsaC proteins in which specific cysteine ligands to the [4Fe-4S] clusters were changed to aspartic acid residues. The mutant proteins, denoted C14D and C51D, were overproduced in *Escherichia coli*; the iron–sulfur clusters were inserted in vitro; and the reconstituted proteins were rebound to the P700–F_X core of *Synechococcus* sp. PCC 6301 in the presence of the PsaD protein. In complexes reconstituted with C51D a rhombic ESR spectrum with *g*-values of 2.063, 1.934, and 1.879 in the reduced state identifies the intact [4Fe-4S] cluster as F_B, while an intense axial spectrum with *g*-values of 2.020 and 1.997 in the oxidized state identifies the altered cluster in the aspartate site as a [3Fe-4S] cluster. The [3Fe-4S] cluster corresponding to F_A can be reduced chemically with dithionite and photochemically by illumination at room temperature but is not reduced by illumination at 15 K. With reconstituted C14D a rhombic ESR spectrum with *g*-values of 2.043, 1.942, and 1.853 in the reduced state identified the unaltered [4Fe-4S] cluster as F_A, while a complex spectrum with a g_z-value of 2.194 and an asymmetric $g_{x,y}$ set of resonances between 2.092 and 1.999 indicates an altered cluster of unknown identity in the site containing the aspartate ligand. The ESR signals arising from the altered cluster corresponding to F_B are not diminished by illumination at either room temperature or 15 K. Similar to the behavior of the control complex at 15 K, only 12% of the F_B cluster is photoreduced in the complex reconstituted with C51D, whereas about 73% of the F_A cluster is photoreduced in the complex reconstituted with C14D. On the basis of amino acid sequence similarities between PsaC and ferredoxins of known structure, we propose that the F_A cluster is liganded by cysteines 21, 48, 51, and 54 while the F_B cluster is liganded by cysteines 11, 14, 17, and 58. The ability to photoreduce F_A in the presence of a nonfunctional F_B indicates that the latter is not an obligatory intermediate in the pathway of electrons to F_A.

The photosystem I (PS I)[1] reaction center in cyanobacteria is a membrane-bound complex of 11 to 12 polypeptide subunits that functions as a light-driven oxidoreductase to catalyze the transport of electrons from reduced plastocyanin (or cytochrome c_{553}) to oxidized ferredoxin [or flavodoxin; for reviews see Golbeck and Bryant (1991), Chitnis and Nelson (1991), Golbeck (1992), Bryant (1992), and Sētif (1992)]. Following the absorption of a photon and migration of the exciton to the

[†] Published as Journal Series No. 9883 of the University of Nebraska Agricultural Research Division. This work was supported by National Science Foundation Grants DMB-9043333 to J.H.G. and DMB-8818997 to D.A.B.

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[1] Abbreviations: β-ME, β-mercaptoethanol; C14D, mutant *Synechococcus* sp. PCC 7002 PsaC protein with cysteine-14 replaced with aspartic acid; C51D, mutant *Synechococcus* sp. PCC 7002 PsaC protein with cysteine-51 replaced with aspartic acid; Chl, chlorophyll; DCPIP, 2,6-dichlorophenolindophenol; ESR, electron spin resonance; PAGE, polyacrylamide gel electrophoresis; PsaC, product of the *psaC* gene; PS I, photosystem I; SDS, sodium dodecyl sulfate.

0006-2960/92/0431-5093$03.00/0

trapping center, charge separation occurs between the primary electron donor, P700, and a monomeric Chl *a* denoted A_0. Subsequent electron-transfer steps include forward electron transport from A_0^- to phylloquinone (known spectroscopically as A_1) and rereduction of oxidized $P700^+$ by plastocyanin. Additional electron-transfer events involve three [4Fe-4S] clusters, denoted as F_X, F_A, and F_B, leading to the reduction of $NADP^+$ through the involvement of a soluble [2Fe-2S] ferredoxin and ferredoxin: $NADP^+$ oxidoreductase (Schluchter & Bryant, 1992).

A major unresolved question concerning the function of the PS I reaction center is the precise pathway(s) of electron flow through these terminal electron acceptors. In particular, the sequence of forward electron flow from F_X^- through F_B and F_A to ferredoxin remains unclear [see Golbeck and Bryant (1991)]. The redox potentials and low-temperature behavior after illumination imply a serial flow of electrons between F_B and F_A, as does the marked inhibition of the low-temperature photoreduction of F_A after selective chemical inactivation of F_B by diazonium compounds (Malkin, 1984). In contrast, the selective denaturation of F_B by urea–ferricyanide (Golbeck & Warden, 1982) and mercurials (Sakurai et al., 1991) hardly affects the room- and low-temperature photoreduction of F_A. There are also no reliable data on the physical identification of the F_A and F_B iron–sulfur clusters relative to the cysteine ligands within the PsaC protein. We decided, therefore, to employ a new strategy which involved genetically engineering altered iron–sulfur clusters into the F_A and F_B sites of the PsaC protein in PS I.

We recently established that chaotrope treatment of the P700–F_A/F_B complex of *Synechococcus* sp. PCC 6301 causes the release of the PsaC, PsaD, and PsaE proteins without affecting its chlorophyll content or electron transport to iron–sulfur center F_X (Parrett et al., 1989, 1990; Li et al., 1991a). Removal of the chaotrope and incubation of the released proteins in the presence of $FeCl_3$, Na_2S, and β-ME cause the reinsertion of the F_A and F_B [4Fe-4S] clusters into the PsaC protein, followed by the stoichiometric rebinding of PsaC, PsaD, and PsaE to the P700–F_X core (Li et al., 1991a). The high degree of efficiency (nearly 100%) of such reconstitutions suggested that in vitro reconstitutions with mutant PsaC proteins produced by recombinant DNA methods in *Escherichia coli* might be possible. Indeed, results with wild-type PsaC and PsaD proteins established the feasibility of the approach and established a role for the PsaD protein in orienting and stabilizing the PsaC–PS I core protein interaction (Zhao et al., 1990; Li et al., 1991b).

Since these studies indicated that the iron–sulfur clusters were essential for the rebinding of the PsaC protein to the P700–F_X core (Li et al., 1991a), we sought mutations which would alter the identity and function of the F_A and F_B clusters without grossly affecting the ability of PsaC to fold correctly. We reasoned that a [3Fe-4S] cluster would retain the three-dimensional structure of PsaC, a requirement that might be difficult to attain if we disallowed the formation of an iron–sulfur cluster entirely. [The first three cysteine ligands to a [4Fe-4S] cluster are derived from one CxxCxxCxxxCP binding motif; the fourth cysteine ligand is derived from the CxxCxxCxxxCP of the second binding motif in 2[4Fe-4S] proteins (Adman et al., 1973; Stout et al., 1988; Fukuyama et al., 1988; Stout, 1989).] We assumed that the requirement for the polypeptide to "wrap around" to provide the final cysteine ligand might be difficult if the second iron–sulfur cluster did not form. [In naturally occurring [4Fe-4S] proteins, a rigid α-helix is present which places the final CP in the correct position for ligation to the iron (Adman et al., 1973).]

The presence of an aspartic acid residue instead of a cysteine in naturally occurring ferredoxins of *Pyrococcus furiosus* (Conover et al., 1990) and *Desulfovibrio africanus* (George et al., 1989) is correlated with the occurrence of [3Fe-4S] clusters in these proteins. We reasoned that a similar change in PsaC might maintain the three-dimensional structure of the protein while altering the electron transport and spectroscopic properties of the iron–sulfur clusters. An examination of the protein sequences of the *P. furiosus* and *D. africanus* ferredoxins as well as the crystal structures of ferredoxins of *Peptococcus aerogenes* (Adman et al., 1973), *Azotobacter vinelandii* (Stout et al., 1988; Stout, 1989), *Desulfovibrio gigas* (Kissinger et al., 1991), and *Bacillus thermoproteolyticus* (Fukuyama et al., 1988) suggested that the second cysteine of each CxxCxxCxxxCP motif of PsaC should be changed to aspartic acid, since this position is altered in naturally occurring [7Fe-8S] ferredoxins and because these cysteines are most exposed at the surface of the protein at a bend region. We felt that the sequence homology between these proteins and PsaC is sufficiently strong, particularly around the iron–sulfur liganding regions, to make this a meaningful prediction. According to this logic, a bona-fide $[4Fe\text{-}4S]^{2+,1+}$ cluster should remain in the undisturbed F_A or F_B site; hence, one should be able to infer the identity of the cysteine ligands to that cluster. It was additionally anticipated that the properties of a $[3Fe\text{-}4S]^{1+,0}$ cluster might render that center incapable of forward electron transfer. In the present work a pair of complementary mutations affecting cysteine ligands to F_A and F_B have been constructed, and the effects of these mutations on PsaC structure and function have been analyzed.

Materials and Methods

Materials and E. coli Strains. Restriction enzymes, DNA-modifying enzymes, and T4 DNA ligase were purchased from Bethesda Research Laboratories (Gaithersburg, MD), New England BioLabs (Beverly, MA), or Boehringer-Mannheim Biochemicals (Indianapolis, IN) and were used according to the specifications of the manufacturers. The strain of *E. coli* used for routine genetic manipulations was strain DH5α (Bethesda Research Laboratories, Gaithersburg, MD). *E. coli* strain BL21 (DE3) was employed for high-level synthesis of proteins directed by the T7 RNA polymerase (Studier et al., 1990).

Site-Directed Mutagenesis, Plasmid Constructions, and Synthesis and Purification of PsaD and of the Mutant PsaC Proteins. Mutagenesis of the *psaC* gene of *Synechococcus* sp. PCC 7002 was performed by the method of Kunkel (1987) using appropriate synthetic oligonucleotides (24-mers) to prime second-strand synthesis. DNA sequence analysis was performed as previously described (Sanger et al., 1977) using [α-^{35}S]thio-dATP and Sequenase (U.S. Biochemicals, Cleveland, OH). The mutant *psaC* genes were recloned into plasmid pUC19 as *Xba*I–*Sst*I fragments and subsequently recloned into the *Xba*I and *Nde*I sites of plasmid pET-3a (Studier et al., 1990). Overproduction of the mutant PsaC proteins, denoted C14D and C51D, was accomplished in *E. coli* strain BL21 (DE3), and cell disruption and inclusion body purification were performed as previously described (Zhao et al., 1990; Li et al., 1991b). *E. coli* strains harboring plasmid pET-36C (encoding the *Synechococcus* sp. PCC 7002 *psaC* gene; Li et al., 1991b) and derivatives thereof encoding mutant proteins C14D and C51D or plasmid pET-3a/D (encoding the *Nostoc* sp. PCC 8009 *psaD* gene; Li et al., 1991b) were grown in medium NYZCM (Sambrook et al., 1989) except that magnesium sulfate was omitted. Protein overproduction, in-

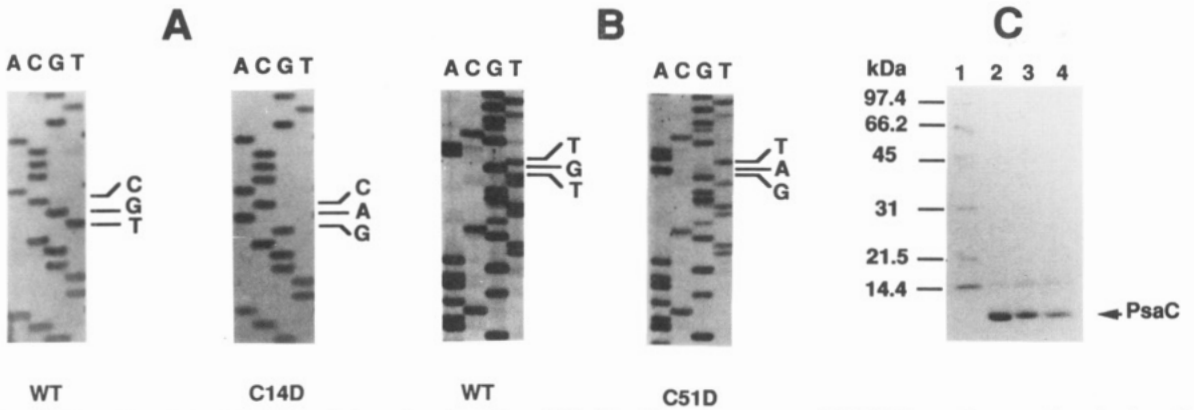

FIGURE 1: Confirmation of site-directed mutations at codons 14 and 51 of the *Synechococcus* sp. PCC 7002 *psaC* gene and production of mutant proteins in *E. coli*. Panel A: DNA sequence of the region surrounding codon 14 for the wild-type (WT) *psaC* gene of *Synechococcus* sp. PCC 7002 (Rhiel et al., 1992) and for the *psaC-C14D* (C14D) gene created by site-directed mutagenesis. Panel B: DNA sequence of the region surrounding codon 51 for the wild-type *psaC* gene of *Synechococcus* sp. PCC 7002 and for the *PsaC-C51D* gene created by site-directed mutagenesis. Panel C: SDS–PAGE analysis of inclusion bodies purified from *E. coli* strains overproducing the widl-type PsaC protein (lane 2), PsaC-C14D (lane 3), and PsaC-C51D (lane 4). Lane 1 contains molecular mass standards whose masses in kilodaltons are indicated at the left.

clusion body preparation, and purification of PsaD and PsaC proteins to electrophoretic homogeneity were performed as previously described (Li et al., 1991b).

Protein and Chlorophyll Analyses. N-Terminal amino acid sequencing of the C14D mutant protein was performed as previously described (Li et al., 1991b). Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was performed using the buffer system of Laemmli (1970) on slab gels containing a linear gradient of acrylamide [10–20% acrylamide (w/v), acrylamide–bis(acrylamide) = 30:0.8 (w/w)]. Protein concentrations were routinely determined using a dye-binding method (Bradford, 1976) after corrections based upon the quantitative analysis of acid-stable amino acid residues in the PsaC and PsaD proteins (Li et al., 1991b). Chl *a* was determined in 80% (v/v) acetone (Arnon, 1949).

Preparation of the P700–F_A/F_B Complex and the P700–F_X Core. The P700–F_A/F_B complex from *Synechococcus* sp. PCC 6301 was isolated as previously described (Li et al., 1991a,b). The F_A/F_B-containing PsaC protein, PsaD, and PsaE were dissociated from the P700–F_X core by treatment with 6.8 M urea at 20 °C. The reaction was monitored on real time by measuring the change from a 30-ms flash-induced transient derived from the $P700^+$ $[F_A/F_B]^-$ back-reaction to a 1.2-ms transient derived from the $P700^+$ F_X^- back-reaction. After the reaction had reached completion (~20 min), the solution was diluted 3-fold with Tris-HCl buffer (25 mM, pH 8.3); the low molecular mass polypeptides were subsequently removed by ultrafiltering and concentrating the sample three times with a YM-100 (Amicon, Beverly, MA) membrane in the presence of 0.1% Triton X-100 (Parrett et al., 1989).

Rebinding of the PsaC and PsaD Proteins to the Photosystem I Core Protein. The insertion of the iron–sulfur clusters and the rebinding of PsaC and the C14D and C51D mutant proteins to the P700–F_X core of *Synechococcus* sp. PCC 6301 were performed according to a published protocol (Parrett et al., 1990; Mehari et al., 1991; Li et al., 1991b). The PsaC and PsaD proteins were rebound to a P700–F_X core at a molar ratio of 10:10:1; the C51D and PsaD proteins were rebound to the P700–F_X core in the molar ratio 90:30:1; and the C14D and PsaD proteins were rebound to the P700–F_X core in the molar ratio 120:40:1. These ratios were determined experimentally to yield maximal restoration of long-lived ($t_{1/2}$ of ≥30 ms) charge separation within a 12-h incubation period when assayed by optical flash photolysis. The iron–sulfur reagents and unreacted low molecular mass proteins were subsequently removed by ultrafiltering the sample three times over a YM-100 membrane using 50 mM Tris-HCl buffer, pH 8.3, containing 0.04% Triton X-100. The reconstituted PS I complexes were concentrated to 1.0 mg mL^{-1} Chl *a* and stored at −80 °C.

Electron Spin Resonance Spectroscopy. Electron spin resonance (ESR) studies were performed with a Bruker ECS-106 X-band spectrometer. Cryogenic temperatures were maintained with an Oxford liquid helium cryostat and an Oxford ITC4 temperature controller. Microwave frequency was sampled during run time with a Hewlett-Packard 5340A frequency counter. The field calibration was checked by measuring the position of the $g = 2.0025$ resonance (ΔH = 7.2 G) derived from the $P700^+$ cation. Sample temperatures were monitored by a calibrated thermocouple situated beneath the 3-mm i.d. quartz sample tube and referenced to liquid nitrogen. Actinic illumination of the sample was provided by a 150-W xenon arc source filtered through 5 cm of water and passed through a "cold mirror" to remove the near-IR wavelengths.

Flash-Induced Absorption Changes. Flash-induced absorption transients were determined at 698 nm using a single-beam spectrophotometer described previously (Parrett et al., 1989). Illumination was provided by a 5-μs xenon flash (PTI Model 610, London, ON). The transient were captured with a Biomation Model 8100 digitizer interfaced to a Macintosh IIci computer for signal averaging, data storage, and data manipulation.

Results

Synthesis of the C14D and C51D Proteins in E. coli. Figure 1 documents the construction of the complementary cysteine-to-aspartic acid codon replacements in the *Synechococcus* sp. PCC 7002 *PsaC* gene and the production of the corresponding mutant proteins as inclusion bodies in *E. coli*. The DNA sequence of the region surrounding codon 14 for the wild-type (WT) *psaC* gene (Rhiel et al., 1992) and for the *PsaC-C14D* gene created by site-directed mutagenesis is shown in Figure 1A. The cysteine codon (TGC) at position 14 has been changed to an aspartic acid codon (GAC). The DNA seqeunce of the region surrounding codon 51 for the wild-type *psaC* gene of *Synechococcus* sp. PCC 7002 and for the mutant *psaC-C51D* created by site-directed mutagenesis is shown in Figure 1B. The cysteine codon (TGT) at position 51 has been changed to an aspartic acid codon (GAT). Figure 1C shows

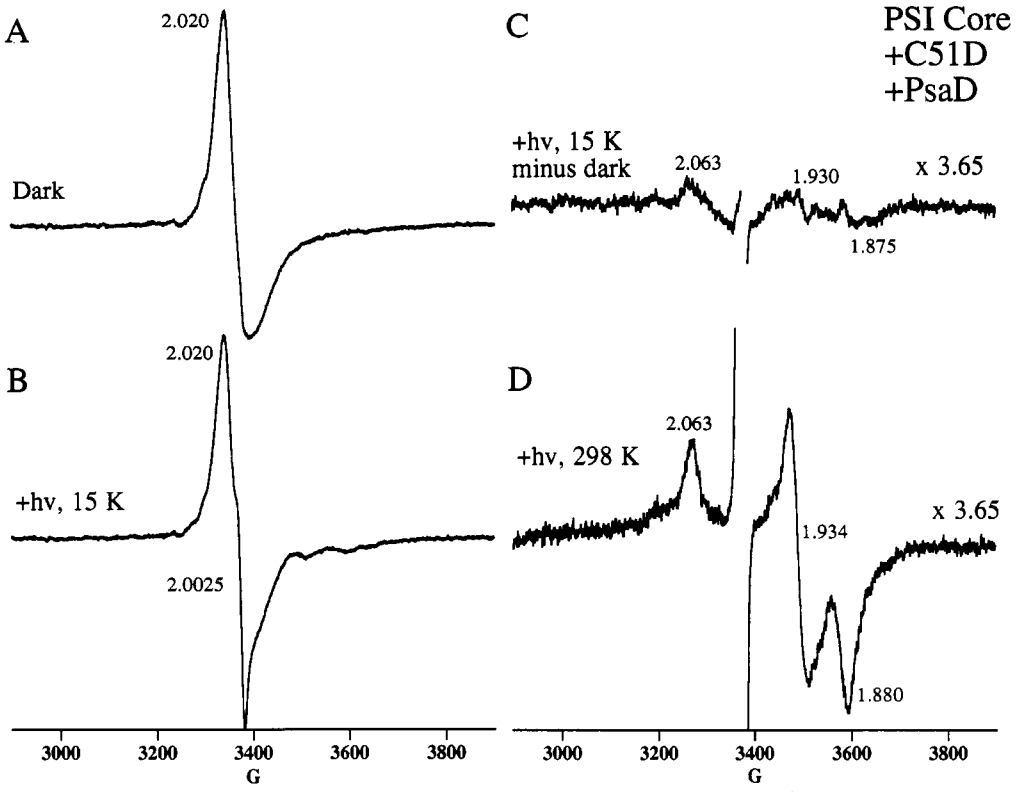

FIGURE 2: ESR spectra of the PS I complex reconstituted with the C51D mutant protein. Panel A: Reconstituted PS I complex frozen in darkness. Panel B: Reconstituted PS I complex frozen in darkness and illuminated at 15 K for 2 min. The spectrum was recorded in the dark. Panel C: Spectrum B minus spectrum A (the intense dark resonance of the $[3Fe\text{-}4S]^{1+}$ cluster is removed by subtraction in order to highlight the weaker $[4Fe\text{-}4S]^{1+}$ resonances). The spectrum has been expanded 3.65-fold. Panel D: Spectrum of the reconstituted PS I complex illuminated during freezing. The spectrum was recorded in the dark and expanded 3.65-fold. The *g*-values of the principal resonances are indicated in the figure. Spectrometer conditions: temperature, 19 K; microwave power, 20 mW; microwave frequency, 9.448 GHz; modulation amplitude, 20 G at 100 kHz.

the SDS–PAGE analysis of inclusion bodies purified from *E. coli* strains overproducing the wild-type PsaC protein (lane 2), C14D (lane 3), and C51D (lane 4). The wild-type and mutant proteins migrate with the expected molecular mass of 9 kDa. The N-terminal amino acid sequence of the purified C14D protein was determined to be SHSVKIYDTcIG-DTQcVRAcP (the lower-case c's indicate the presumed but undetectable cysteine residues). These data indicate that the N-terminal initiator methionine has been correctly removed [see Rhiel et al. (1992)] in *E. coli* and that the cysteine at position 14 has been correctly replaced by aspartic acid.

ESR Spectral Characterization of PS I Complexes Reconstituted with C14D and C51D. When the P700–F_A/F_B complex isolated from *Synechococcus* sp. PCC 6301 is frozen to 15 K and illuminated, only one electron is available in P700 for photoreduction and either F_A (g = 2.041, 1.940, and 1.851) or F_B (g = 2.064, 1.927, and 1.877) becomes photoreduced in any given reaction center [not shown; see Golbeck and Bryant (1991)]. When the sample is illuminated during freezing, both iron–sulfur clusters can be photoaccumulated in the reduced state, leading to the magnetically interacting spectrum of F_A^- (g = 2.041, 1.936, and 1.882) and F_B^- (g = 2.041, 1.918, and 1.882). The midfield resonance of F_A^- moves slightly upfield from g = 1.940 to g = 1.936 on reduction of F_B, and the midfield resonance of F_B^- moves a larger distance upfield from g = 1.927 to g = 1.918 upon reduction of F_A. This subtle but real difference will assume significance (see below) when attempts were made to correlate the photoreduction of the intact [4Fe-4S] cluster with either F_A or F_B in the mutant proteins.

In all higher plants (spinach, barley, tobacco), algae (*Chlamydomonas reinhardtii*), and cyanobacteria (*Synechococcus* sp. PCC 6301, *Synechococcus* sp. PCC 7002, *Synechococcus elongatis*, and *Synechocystis* sp. PCC 6803) studied to date, about 60% of F_A and 14% of F_B become photoreduced at 15 K when referenced to the levels of reduction obtained with dithionite or by photoaccumulation at 298 K. The reasons for the lack of quantitative reduction of the iron–sulfur clusters [$F_A^- + F_B^- \leq 0.74$ (P700)] and also for the fixed ratio of photochemically generated F_A^- and F_B^- are not known. The same pattern of photoreduction of F_A and F_B is observed when the P700–F_A/F_B complex is reconstituted from recombinant PsaC (derived from the *Synechococcus* sp. PCC 7002 *PsaC* gene), recombinant PsaD (derived from the *Nostoc* sp. PCC 8009 *psaD* gene), and the P700–F_X core isolated from *Synechococcus* sp. PCC 6301 (Li et al., 1991b).

When the PS I complex is reconstituted with PsaD and the C51D mutant protein and frozen in the dark, a strong axial resonance at g = 2.020 is present (Figure 2A) which is characteristic of an oxidized $[3Fe\text{-}4S]^{1+}$ cluster (Beinert & Thomson, 1983; Johnson et al., 1987; Conover et al., 1990; Knaff et al., 1991; Rothery & Weiner, 1991). When the reconstituted C51D–PS I complex is illuminated at 15 K (Figure 2B), the g = 2.020 resonance is not diminished (the difference spectrum is shown in Figure 2C), indicating that the $[3Fe\text{-}4S]^{1+}$ cluster does not function as an electron acceptor at low temperature, and a new set of weak, rhombic resonances appear at g = 2.063, 1.930, and 1.875 (Figure 2B; expanded in Figure 2C). When the reconstituted C51D–PS I complex is reduced with sodium dithionite at pH 8.3 (not shown) or frozen under continuous illumination, the g = 2.020 resonance derived from the $[3Fe\text{-}4S]^{1+}$ cluster disappears, consistent with its reduction to the $[3Fe\text{-}4S]^0$ state. Under these conditions, the [4Fe-4S[cluster with resonances at g = 2.063, 1.934, and

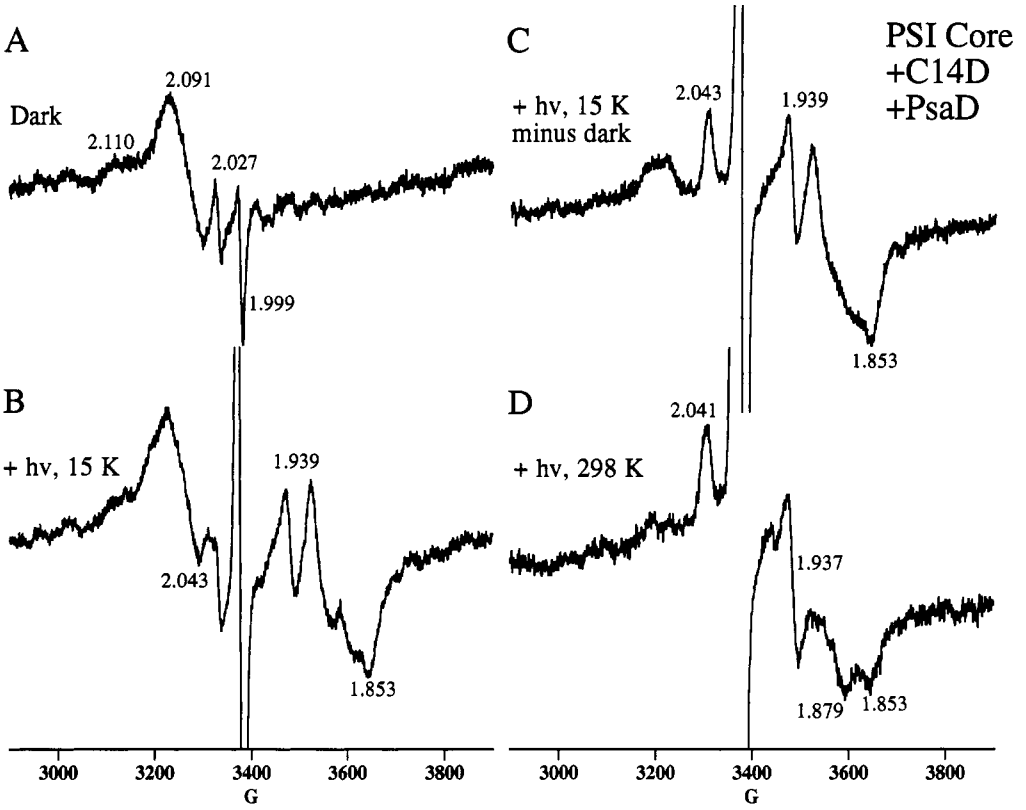

FIGURE 3: ESR spectra of the PS I complex reconstituted with the C14D mutant protein. Panel A: Reconstituted PS I complex frozen in darkness. Panel B: Reconstituted PS I complex frozen in darkness and illuminated at 15 K for 2 min. The spectrum was recorded in the dark. Panel C: Spectrum B minus spectrum A. Panel D: Spectrum of the reconstituted PS I complex illuminated during freezing minus spectrum A. The g-values of the principal resonances are indicated in the figure. Spectrometer conditions are identical to those for Figure 2.

1.880 becomes fully reduced (Figure 2D). Although the g = 1.934 resonance cannot be assigned with certainty, the g = 2.063 and g = 1.880 resonances are characteristic of F_B. The intensity of the resonances in Figure 2C,D indicates that 12% of the F_B cluster is photoreduced at 15 K; thus, the intact [4Fe-4S] cluster present in the C51D protein mimics the behavior of F_B in a control PS I complex. These results imply that the [3Fe-4S] cluster is located in the site incorporating aspartic acid residue 51 and that F_B is the intact, low-potential [4Fe-4S] cluster.

When the PS I complex is reconstituted with PsaD and the C14D mutant protein and frozen in the dark (Figure 3A), a weak, low-field feature at g = 2.110 is present along with a stronger feature at g = 2.091 (peak) and a group of sharp resonances at g = 2.027 (mid) and 1.999 (trough). These resonances do not disappear upon illumination of the reconstituted C14D–PS I complex at either room temperature or low temperature. When the reconstituted C14D–PS I complex is frozen in the dark and illuminated at 15 K, a new set of rhombic resonances appears in g = 2.043, 1.939, and 1.853 (Figure 3B). After subtraction of the dark spectrum (Figure 3C), an additional broad series of resonances can be seen around 3200 and 3600 G; it is not known if this represents photooxidation of the altered cluster or photoreduction of a new cluster. When the reconstituted C14D–PS I complex is illuminated during freezing, the resonance at g = 1.879 becomes distinct (Figure 3D) and the g = 2.041, 1.937, and 1.853 resonances become slightly larger. Although the g = 1.937 resonance cannot be assigned with certainty, the g = 2.041 and 1.853 resonances are characteristic of F_A. The existence of the additional resonance at g = 1.879 mimics the behavior of the high-field resonance of F_A^- in the presence of F_B^- in the wild-type PS I complex and indicates an interaction with another paramagnetic species (possibly the cluster in the F_B site). A comparison of the intensities of the resonances in Figure 3C,D indicates that 73% of the F_A cluster is photoreduced at 15 K; thus, the [4Fe-4S] cluster present in the C14D protein resembles the behavior of F_A in control samples. These results imply that the altered iron–sulfur cluster is located in the site incorporating aspartic acid residue 14 and that F_A is the intact, low-potential [4Fe-4S] cluster. Although the identity of the altered cluster is uncertain, the fact that it is photochemically inactive at 15 and 298 K allows electron transport studies of the intact F_A cluster in the absence of F_B.

Optical Characterization of the Reconstituted Photosystem I Complexes. The rebinding of the wild-type PsaC and PsaD proteins to the P700–F_X core is >90% complete within 60 min at a molar ratio of 10:20:1 (Li et al., 1991b). The time course of rebinding of the C14D mutant protein to the P700–F_X core is shown in Figure 4A. At a molar ratio of 120:40:1 of C14D:PsaD:P700–F_X core, the 1.2-ms back-reaction between $P700^+$ and F_X^- is only slowly replaced by a long-lived back-reaction indicative of the gradual replacement with one of the iron–sulfur clusters on C14D. There is no rebinding of C14D to the P700–F_X core in the absence of PsaD (data not shown). Therefore, in contrast to the reconstitution with wild-type PsaC, the reconstitution of the mutant PS I complex requires higher concentrations of C14D and PsaD and a longer period of incubation to reach ~80% completion. Since the ESR data have indicated that the altered cluster in the F_B site is photochemically inactive as an electron acceptor at 298 K, there is little question that the functional acceptor is the intact $[4Fe\text{-}4S]^{2+}$ cluster in the F_A site.

The kinetics of rebinding of the C51D mutant to the P700–F_X core is shown in Figure 4B. At a molar ratio of 90:30:1 of C51D:PsaD:P700–F_X core, the 1.2-ms back-reaction

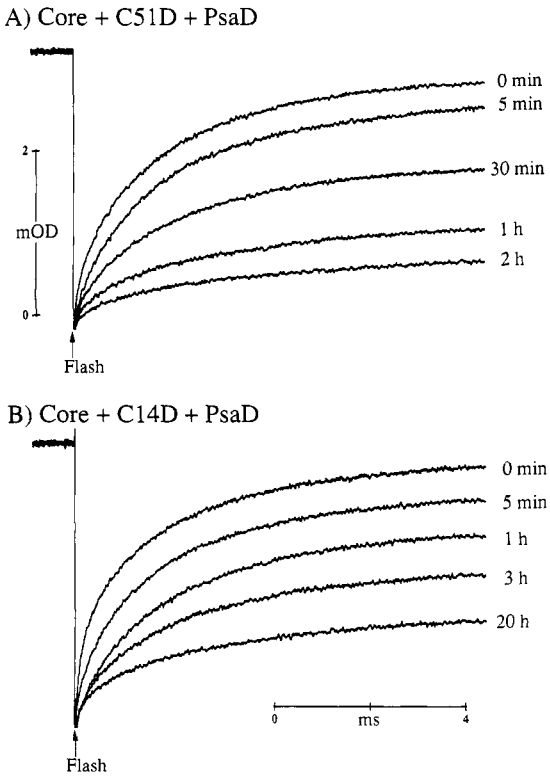

FIGURE 4: Kinetics of rebinding of the mutant proteins to the P700–F_X core. Panel A: Time course of the flash-induced absorption transient of P700 upon addition of the C51D and PsaD proteins to the *Synechococcus* sp. PCC 6301 P700–F_X core in a molar ratio of 90:30:1. Panel B: Time course of the flash-induced absorption transient of P700 upon addition of the C14D and PsaD proteins to the *Synechococcus* sp. PCC 6301 P700–F_X core at a molar ratio of 120:40:1. The samples were suspended in 25 mM Tris-HCl buffer, pH 8.3, containing 1 mM sodium ascorbate and 30 μM DCPIP. The traces represent an average of 10 flashes provided at a frequency of 0.1 Hz.

between $P700^+$ and F_X^- is also slowly replaced by a long-lived back-reaction which is indicative of replacement with a terminal iron–sulfur cluster on C51D. This reconstitution is more efficient than that with C14D but is still much less efficient than that obtained with wild-type PsaC. Similar to results obtained with C14D, there is no rebinding of C51D to the P700–F_X core in the absence of PsaD (data not shown). However, the interpretation of the PS I complex reconstituted with C51D is complicated by the ESR data which indicate that the $[3Fe\text{-}4S]^{1+}$ iron–sulfur cluster in the F_A site can accept electrons at 298 K. On the basis of these results, it is not possible to determine whether the acceptor in the room-temperature, optical flash experiments is the $[3Fe\text{-}4S]^{1+}$ cluster in the F_A site or the intact $[4Fe\text{-}4S]^{2+}$ cluster in the F_B site.

Discussion

On the basis of the data presented above and the structural similarity of PsaC to bacterial 2[4Fe-4S] ferredoxins (Oh-oka et al., 1988; Dunn & Gray, 1988), we propose that Cysteines 11, 14, 17, and 58 ligate the F_B cluster and that cysteines 21, 48, 51, and 54 ligate the F_a cluster (Figure 5). This reasoning is based upon (i) the nearly identical high-field and low-field g-values for F_A in both PsaC and C14D and for F_B in both PsaC and C51D, (ii) the similarity in the magnetic interaction observed in the downfield shift of the $g = 1.85$ resonance of F_A in both C14D and PsaC, and (iii) the extraordinary coincidence in the ability to photoreduce at cryogenic temperature only ~12% of F_B in PsaC and the C51D mutant but ~73% of F_A in PsaC and the C14D mutant. It should also be noted that the relative positions of the midfield resonances

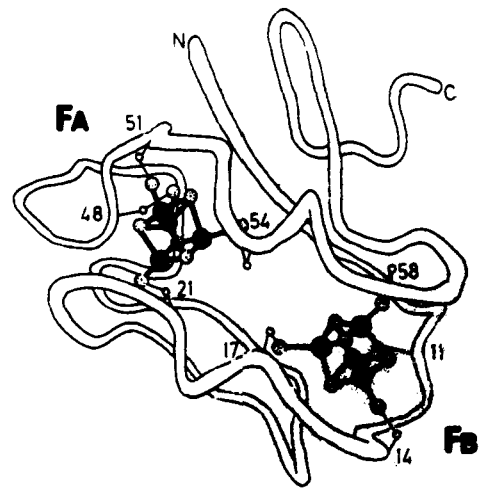

FIGURE 5: Predicted structure of the PsaC polypeptide showing the organization of the two [4Fe-4S] clusters. The model is adapted from Dunn and Gray (1988) and is based upon the structure of the *P. aerogenes* ferredoxin (Adman et al., 1973). The postulated cysteine ligands 21, 48, 51, and 54 to cluster F_A and cysteine ligands 11, 14, 17, and 58 to cluster F_B are indicated.

are correct. The $g = 1.939$ resonance in C14D is downfield of the $g = 1.934$ resonance in C51D and is similar to the position of the $g = 1.940$ resonance in (noninteracting) F_A^- relative to the $g = 1.927$ resonance of (noninteracting) F_B^- in the wild-type P700–F_A/F_B complex.

One important corollary of the results is that the F_A cluster can be photoreduced in the presence of a photochemically inactive F_B; this indicates that the latter is not an obligatory intermediate in the pathway of electrons to F_A. It is interesting that the percentages of low-temperature reduction of F_A and F_B in these mutants correspond to that seen in the wild type, implying that the structure and function of the bona-fide [4Fe-4S] cluster remain relatively undisturbed by the identity of the cluster of the other site. Within the framework of the currently accepted scheme of electron transport from $A_0 \rightarrow A_1 \rightarrow F_X \rightarrow [F_A/F_B]$, it is more difficult to explain the finding that there is no enhanced reduction of the F_B cluster at 15 K when the F_A cluster is altered and rendered nonfunctional. Nevertheless, a deeply satisfying interpretation of these findings is that the structural and functional properties of the intact iron–sulfur cluster in the mutant C14D and C51D proteins are strikingly similar to those of the wild-type PsaC protein. This makes the C14D and C51D proteins prime candidates for further studies of room-temperature electron flow from F_A or F_B to ferredoxin and ultimately to $NADP^+$.

Acknowledgments

We acknowledge Dr. Gautam Sarath for performing the amino acid sequence analysis and Mr. Kevin Macklin for excellent technical assistance.

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