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The Eight-amino Acid Internal Loop of PSI-C Mediates Association of Low Molecular Mass Iron-Sulfur Proteins with the P700-F_X Core in Photosystem I*

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The PSI-C subunit of photosystem I (PS I) shows similarity to soluble 2[4Fe–4S] ferredoxins. PSI-C contains an eight residue internal loop and a 15 residue C-terminal extension which are absent in the ferredoxins. The eight-residue loop has been shown to interact with PSI-A/PSI-B (Naver, H., Scott, M. P., Golbeck, J. H., Møller, B. L., and Scheller, H. V. (1996) *J. Biol. Chem.* 271, 8996–9001). Four mutant proteins were constructed. Two were modified barley PSI-C proteins, one lacking the loop and the C terminus (PSI-C_{core}) and one where the loop replace the C-terminal extension (PSI-$C_{core}L_{c\text{-}term}$). Two were modified *Clostridium pasteurianum* ferredoxins, one with the loop of barley PSI-C and one with both the loop and the C terminus of PSI-C. Wild-type proteins and the mutants were used to reconstitute barley P700-F_X cores lacking PSI-C, -D, and-E. Western blotting showed that PSI-$C_{core}L_{c\text{-}term}$ binds to PS I, whereas PSI-C_{core} does not. Without PSI-D the PSI-$C_{core}L_{c\text{-}term}$ mutant accepts electrons from F_X in contrast to PSI-C mutants without the loop. Flash photolysis of P700-F_X cores reconstituted with *C. pasteurianum* ferredoxin showed that only the ferredoxin mutants with the loop accepted electrons from F_X. From this, it is concluded that the loop of PSI-C is necessary and sufficient for the association between PS I and PSI-C, and that the loop is functional as an interaction domain even when positioned at the C terminus of PSI-C or on a low molecular mass, soluble ferredoxin.

The photosystem I (PS I)[1] reaction center complex mediates electron transport from plastocyanin to ferredoxin in oxygenic photosynthesis. PS I contains the primary electron donor P700 (a Chl *a* dimer) and the electron acceptors A_0 (Chl *a*), A_1 (phylloquinone), and three [4Fe–4S] centers F_X, F_A, and F_B (1–3). The terminal electron acceptors F_A and F_B are bound to the PSI-C subunit (4, 5) while the remaining electron acceptors are bound to the PSI-A/PSI-B heterodimer (6, 7). Little is known about the nature of the interaction between PSI-C and PSI-A/B. PSI-C interacts with two other extrinsic proteins located at the stromal side of PS I, PSI-D, and PSI-E. In the absence of PSI-D, PSI-C can still interact with PS I and accept electrons, but stable binding of PSI-C is possible only when PSI-D is present (8, 9). Furthermore, PSI-D is required for the docking of ferredoxin (10). The presence or absence of PSI-E does not influence binding of PSI-C or electron transport to F_A and F_B. PSI-E is required for optimal rates of ferredoxin reduction but is not essential (8, 11, 12).

The amino acid sequence of PSI-C is highly conserved among species and contains two C*XXCXXCXXX*CP motifs which are characteristic of 2[4Fe–4S] ferredoxins. The eight conserved cysteine residues are ligands to the two [4Fe–4S] clusters in PSI-C and ferredoxin and the conservation of the cysteine motifs shows that the folding of PSI-C is similar to that of low molecular mass, soluble iron-sulfur proteins whose three-dimensional structures have been solved (*e.g. Peptostreptococcus asaccharolyticus* ferredoxin) (13, 14). Apart from the cysteine motifs, PSI-C and the ferredoxins show only little sequence similarity. Nevertheless, alignment analysis clearly reveals two domains present only in PSI-C, an internal segment of eight residues and a C-terminal addition of 15 residues. The published models of PSI-C show the eight additional residues forming a loop extending from the known structure of *P. asaccharolyticus* ferredoxin (5, 15–17). In a previous study, we reported that the eight-residue loop and the C-terminal addition play a role in the interaction of PSI-C with PSI-A/B and PSI-D, respectively (18). These data have been supported by data of Rodday *et al.* (19), who identified the two negatively charged residues Glu-26 and Asp-31 of the loop as important for the PSI-C-PSI-A/B interaction. In the present study, we show that the eight-residue loop is necessary and sufficient for the functional association of *Clostridium pasteurianum* ferredoxin with P700-F_X cores. The C-terminal domain mediates interaction with PSI-D but is not sufficient for stable binding to the cores.

Using a modification of the method of Parrett *et al.* (20), we showed that urea treatment of barley PS I complexes followed by a combined detergent, salt, and urea wash leads to specific dissociation of the PSI-C, -D, and -E subunits without affecting the remaining polypeptides (8). The resulting P700-F_X core can

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[1] The abbreviations used are: PS I, photosystem I; Chl, chlorophyll; Cl.Fd, *C. pasteurianum* ferredoxin; Cl.FdL, *C. pasteurianum* ferredoxin with residue 26–33 of PSI-C from barley inserted at residue 23; Cl.FdLC, *C. pasteurianum* ferredoxin with residue 26–33 of PSI-C from barley inserted at residue 23 and with the 10 C-terminal residues of PSI-C applied as a C-terminal extension; FNR, ferredoxin:$NADP^+$ oxidoreductase, PSI-CΔC, PSI-C from *Synechococcus* sp. 6301 lacking the 10 C-terminal residues; PSI-C, PSI-C from barley; PSI-CΔL, PSI-C from barley lacking residues 26–33; PSI-C_{core}, PSI-C from barley lacking the 10 C-terminal residues and residue 26–33; PSI-$C_{core}L_{c\text{-}term}$, PSI-C from barley where residue 26–33 has been moved to the C terminus so that they replace the original 10 C-terminal residues.

```
                      1         10        20        30        40
Barley PSI-C          MHSVKIYDTCIGCTQCVRACPTDVLEMIPWDGCKAKQIAS
PSI-CΔC               MHSVKIYDTCIGCTQCVRACPLDVLEMVPWDGCKAGQIAA
PSI-CΔL               MHSVKIYDTCIGCTQCVRACPTDVL--------KAKQIAS
PSI-C_core L_C-term.  MHSVKIYDTCIGCTQCVRACPTDVL--------KAKQIAS
PSI-C_core            MHSVKIYDTCIGCTQCVRACPTDVL--------KAKQIAS
Cl.Fd                   AYKIADSCVSCGACASECPVNAI--------SQGDSIF
Cl.FdL              GIPMAYKIADSCVSCGACASECPVNAIEMVPWDGCSQGDSIF
Cl.FdLC             GIPMAYKIADSCVSCGACASECPVNAIEMVPWDGCSQGDSIF

                           50        60        70        80
Barley PSI-C          APRTEDCVGCKRCESACPTDFLSVRVYLGPETTRSMALSY
PSI-CΔC               SPRTEDCVGCKRCETACPTDFLSIRVYLGA
PSI-CΔL               APRTEDCVGCKRCESACPTDFLSVRVYLGPETTRSMALSY
PSI-C_core L_C-term.  APRTEDCVGCKRCESACPTDFLSVRVEMIPWDGC
PSI-C_core            APRTEDCVGCKRCESACPTDFLSVRVYLGPE
Cl.Fd                 VIDADTCIDCGNCANVCPVGAPVQE
Cl.FdL                VIDADTCIDCGNCANVCPVGAPVQE
Cl.FdLC               VIDADTCIDCGNCANVCPVGAPVQEETTRSMALSY
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FIG. 1. **Amino acid sequences of mutant and wild-type PSI-C (16) and *C. pasteurianum* ferredoxins (24).** The inserted loop sequence is shown and the applied C terminus is *underlined*. The conserved cysteines in the C*XXC*XX*C*XXX*C*P motifs are shown enlarged in *italics*. Authentic barley PSI-C has serine as the N-terminal residue rather than the methionine present in the recombinant protein.

be used for reconstitution by addition of *Escherichia coli*-expressed PSI-C and -D in the presence of the reagents Na_2S, $FeCl_3$, and 2-mercaptoethanol, which serve to rebuild the FeS clusters of PSI-C (8, 9, 21). In this work, we report the specificity of the loop for interaction of PSI-C with the PSI-A/B heterodimer by producing four modified proteins. Two were derived from PSI-C, one lacking both the loop and the C-terminal extension, and one in which the loop replaced the residues in the C-terminal extension. Two were derived from soluble *C. pasteurianum* ferredoxin, one in which the eight-residue loop from barley PSI-C was inserted between the two C*XXC*XX*C*XXX*CP motifs and one which contained both the loop and the C-terminal extension at positions analogous to their locations in PSI-C. Using the *in vitro* reconstitution system, we established that the eight-residue internal loop of PSI-C is sufficient for interaction with the PS I complex.

EXPERIMENTAL PROCEDURES

Plasmid Construction and Protein Isolation—The production of the PSI-C expression clone from barley, PSI-CΔL and PSI-CΔC has been described previously (8, 18). PSI-CΔC was derived from *Synechococcus* sp. PCC 6301 *psaC* (22). However, previous studies have shown that barley PSI-C and *Synechococcus* sp. PCC 6301 PSI-C behave identically in reconstitution experiments when using a barley P700-F_X core (18). The expression clone for *C. pasteurianum* ferredoxin (23) was a gift from Dr. A. Wedd, University of Melbourne, Australia. The amino acid sequences of the PSI-C mutants are shown in Fig. 1 where the amino acid sequence of the ferredoxin from *C. pasteurianum* is compared with that of PSI-C from barley. To produce barley PSI-C lacking both the loop and the C-terminal domain, appropriate oligonucleotides were used as primers in a polymerase chain reaction with *psaCΔL* as template to yield one fragment. The $psaC_{core}$ gene was cloned into an *Nco*I and *Bam*HI digested Pet3d expression vector (Novagen, Stockholm, Sweden). PSI-C with the loop at the C terminus was made by a polymerase chain reaction where oligonucleotides were used as primers with *psaCΔL* as template and the resulting $psaC_{core}L_{c\text{-}term}$ gene was inserted into Pet3d. To produce the *C. pasteurianum* ferredoxin (Cl.Fd) with the loop inserted, four oligonucleotides containing the loop from PSI-C, were used with the *Cl.Fd* gene in two polymerase chain reactions to yield the two products: the 5′ end of the gene with the whole loop and the 3′ end with the whole loop. The two products which overlap in the loop region were used together in an overlap extension polymerase chain reaction yielding one product, the *Cl.FdL* gene. This being flanked by *Bam*HI and *Sal*I sites was cloned into the *Bam*HI and *Sal*I sites of the pGex-5x-3 vector (Pharmacia, Sollentuna, Sweden). A polymerase chain reaction in which the 3′ oligo encoded the 10 C-terminal residues of PSI-C in addition to the 3′ end of *Cl.Fd* was used to produce the *Cl.FdLC* gene, which was inserted into the *Bam*HI and *Sal*I sites of the pGex-5x-3 vector. The phrase "the PSI-C polypeptides" is used for the total of PSI-C, PSI-CΔL, PSI-$C_{core}L_{c\text{-}term}$, PSI-$C_{core}$, and PSI-CΔC. The phrase "the clostridial ferredoxin polypeptides" is used for the total of Cl.Fd, Cl.FdL, and Cl.FdLC. The PSI-D expression clone from barley was made as described by Scott *et al.* (25).

Overexpression of the proteins was carried out in *E. coli* strain BL21, plysS (DE3). Purification of the PSI-C polypeptides was performed as previously reported (25, 26). The Cl.FdL and Cl.FdLC were each expressed as proteins fused to glutathione S-transferase with a factor Xa cleavage site between the fused proteins. The Cl.FdL and Cl.FdLC were purified by allowing the fusion proteins to bind to a glutathione-Sepharose matrix, incubating the matrix with factor Xa, and eluting the Cl.FdL and Cl.FdLC from the matrix after cleavage as described elsewhere (27).

Sequence determination of the plasmid constructs by the dideoxy reaction method (28) gave the expected sequences of the $psaC_{core}$, $psaC_{core}L_{c\text{-}term}$, *Cl.FdL* gene and *Cl.FdLC* constructs (data not shown). Barley plastocyanin, ferredoxin, and ferredoxin:$NADP^+$ oxidoreductase (FNR) were isolated as described previously (29–31). Cl.Fd was obtained from Sigma.

Mass Spectroscopy—Matrix-assisted laser desorption ionization time-of-flight mass spectra of Cl.FdL and Cl.FdLC were recorded on a TofSpec E spectrometer (Micromass, Manchester, UK). Targets were prepared by loading 1 μl of a mixture of equal volumes sample and matrix solution (saturated sinapinic acid in 0.1% trifluoroacetic acid/MeCN, 6:4). In order to remove phosphate impurities, the air-dried target was washed with 1.5 μl of water and again allowed to dry. Bovine insulin and cytochrome *c* were added as internal standards.

Preparation of the P700-F_X Core—The PS I complex lacking PSI-C, PSI-D, and PSI-E from barley (*Hordeum vulgare* cv. Svalöfs Bonus) was isolated by a modification of the procedure of Andersen *et al.* (32) as described previously (18).

Rebinding of PSI-C, Cl.Fd, and PSI-D to the P700-F_X Core—Reconstitutions were performed in 2-ml reaction volumes containing 10 μg Chl/ml with a molar ratio of 20 PSI-C proteins or clostridial ferredoxin proteins per P700 according to Parrett *et al.* (33) and Naver *et al.* (8, 18). When present, PSI-D was also used in a molar ratio of 20 per P700. The molar ratios of the PS I subunits to P700 were determined by comparing the intensity of Coomassie Brilliant Blue staining of SDS gels with a dilution series of isolated subunits and known amounts of PS I. Molar ratios of the clostridial ferredoxin proteins were determined spectroscopically after rebuilding the iron-sulfur clusters using the extinction coefficient, $\epsilon_{400\ nm} = 31.6\ mM^{-1}\ cm^{-1}$ determined for Cl.Fd. After incubation for 16 h at 4 °C the reconstituted complexes were used for the first set of analyses. Subsequently, the samples were washed by ultrafiltration five times over a YM 100 membrane (Amicon) using 50 mM Tris (pH 8.3), 0.1% Triton X-100 and used for EPR, $NADP^+$ photoreduction or flash photolysis analysis.

EPR Spectroscopy—EPR spectra were acquired as described previously (18). Illumination in the EPR cavity was performed by directing the beam of a 150-watt xenon arc lamp to the frozen sample through the slotted opening in the resonator. Photoaccumulation of electrons in the terminal acceptors was accomplished by passing the thawed sample through the focused beam of a 300-watt tungsten lamp into a container with liquid nitrogen over the course of 1 min.

Flash-induced Absorption Change—Flash-induced absorption transients at 834 nm were measured in a reaction mixture of 200–400 μl containing 50 mM Tris (pH 8.3), 2 mM sodium ascorbate, 60 μM 2,6-dichlorophenolindophenol, and 10–20 μg of Chl/ml using the setup described previously (18, 34). Reconstitution is expressed as the percentage of the total absorbance change at 834 nm that decayed with the long time constant (>30 ms) characteristic of the $P700^+$ $[F_A/F_B]^-$ back reaction.

$NADP^+$ Photoreduction Measurements—$NADP^+$ photoreduction activity was determined from the absorbance change at 340 nm as described previously (18). $NADP^+$ photoreduction measurements of the samples containing the clostridial ferredoxin proteins were carried out under strictly anaerobic conditions maintained using the glucose oxidase/catalase system as described by Kjaer and Scheller (35).

Additional Analytical Procedures—Chlorophyll was determined according to Arnon (36). SDS-polyacrylamide gel electrophoresis was carried out in 8 to 25% gradient gels according to Fling and Gregerson (37). Prior to electrophoresis, the reconstituted PS I complexes were pelleted by ultracentrifugation. Western blot analysis was carried out by transferring electrophoresed proteins to nitrocellulose membranes followed by incubation with monospecific rabbit antibodies (32) and visualized using a secondary antibody conjugated with alkaline phosphatase (DAKO, Copenhagen, Denmark).

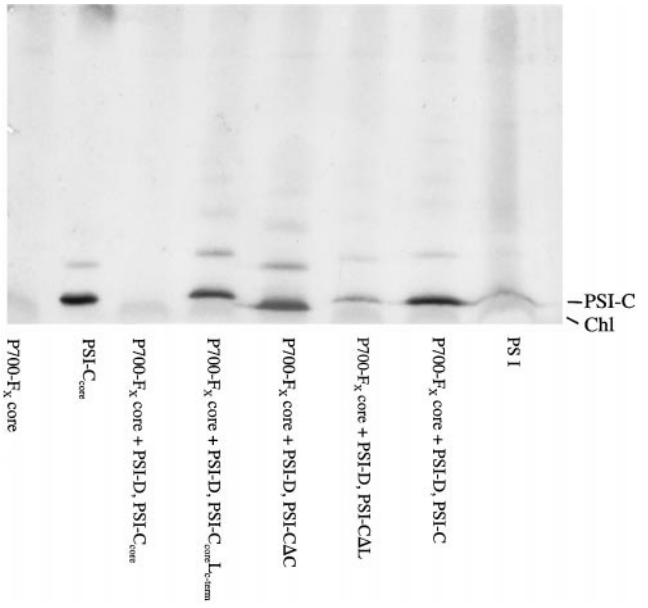

FIG. 2. **Western blot analysis of the P700-F_X core after reconstitution with the PSI-C polypeptides.** The samples were pelleted by ultracentrifugation, and each was applied to a lane of the gel. The blots were incubated with polyclonal antibodies raised against the PSI-C subunit. *Chl* indicates the position of pigments released from PS I. The PS I preparation before the urea treatment, the P700-F_X core before reconstitution, and the isolated PSI-C_{Core} protein were included for reference.

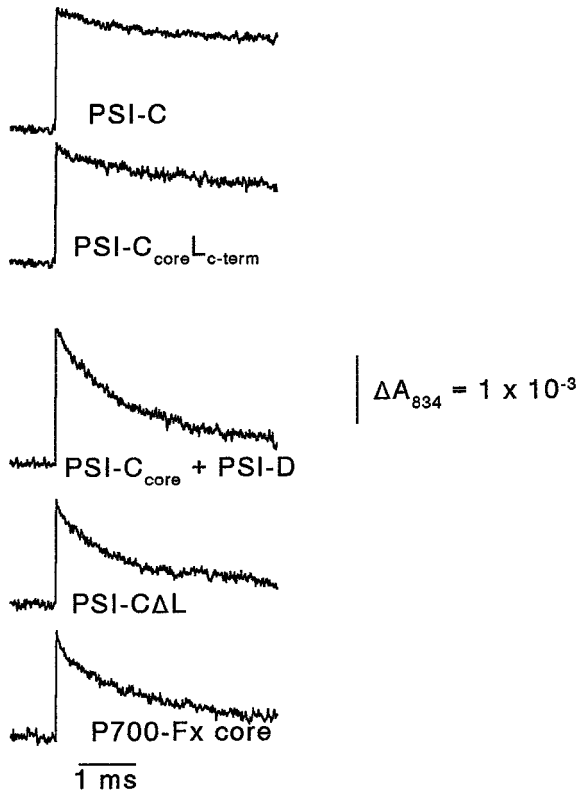

FIG. 3. **Flash-induced absorption changes of PS I reconstituted with PSI-C polypeptides.** The transients represent the primary data for one of the experiments summarized in Table I. The P700-F_X core reconstituted with PSI-C, PSI-$C_{core}L_{c\text{-}term}$, PSI-CΔL in the absence of PSI-D, and with PSI-C_{core} in the presence of PSI-D is shown.

RESULTS

Characterization of the Rebinding of PSI-C to the P700-F_X Core by Western Blot Analysis—Treatment with urea leads to dissociation of the PSI-C subunit from barley PS I. Reconstitution using the PSI-C polypeptides and PSI-D resulted in rebinding of each of PSI-C, PSI-CΔL, PSI-CΔC, and PSI-$C_{core}L_{c\text{-}term}$ as shown by Western blot analysis of the washed complexes (Fig. 2). The PSI-C_{core} did not bind to the P700-F_X core. As expected the PSI-CΔC and the PSI-C_{core} migrate faster in SDS-polyacrylamide gel electrophoresis than PSI-C. The PSI-CΔL and PSI-$C_{core}L_{c\text{-}term}$ migrate the same as the full-length PSI-C; however, the amino acid sequence of PSI-CΔL has been confirmed by protein sequencing (18). The band around 18 kDa seen in some lanes probably represents a small amount of PSI-C dimers present due to insufficient solubilization.

Flash-induced Absorbance Changes of P700-F_X Cores Reconstituted with the PSI-C Polypeptides—The back reactions from the PS I electron acceptors were measured by flash induced absorbance changes at 834 nm after addition of each of the PSI-C polypeptides to P700-F_X cores in the presence and absence of PSI-D. In a P700-F_X core where F_A and F_B are absent, the charge recombination between F_X^- and $P700^+$ will proceed with a time constant of approximately 1 ms (38). In contrast, when iron-sulfur clusters F_A and F_B are functional, the forward electron transfer from F_X^- to $[F_A/F_B]$ is so efficient that no back reaction from F_X^- can take place and only the much slower (>30 ms) back reaction from $[F_A/F_B]^-$ is observed. Thus the degree of functional reconstitution can be determined by observing the replacement of the ≈1-ms back reaction of $P700^+ F_X^-$ with the slower back reaction $P700^+[F_A/F_B]^-$ Addition of PSI-CΔL and PSI-$C_{core}L_{c\text{-}term}$ to P700-F_X cores in the absence of PSI-D resulted in 12 and 60% recovery of the slow phase, respectively (Fig. 3, Table I), indicating the importance of the loop sequence for electron transport. In contrast, when PSI-D was present reconstitution with PSI-CΔL resulted in a substantial recovery of the slow phase (52%) while reconstitution with PSI-$C_{core}L_{c\text{-}term}$ resulted in an improved recovery of the slow phase (73%). The PSI-C_{core} did not show any restoration with the P700-F_X core in the presence or absence of PSI-D. To assay the stability of the associations, the reaction mixtures were washed with 0.1% Triton X-100 over an ultrafiltration membrane. As expected from earlier studies with wild-type PSI-C (39), none of the associations was stable in the absence of PSI-D (Table I). In the presence of PSI-D the associations were stable, although a small decrease in the slow phase was observed with all the PSI-C proteins except the wild-type protein.

$NADP^+$ photoreduction activity was also determined in the washed samples after supplementation with barley ferredoxin, plastocyanin, and FNR. The P700-F_X core reconstituted with

TABLE I
Extent of back reaction from F_A/F_B in reconstituted PS I determined by flash photolysis

Polypeptides added to the P700-F_X core during reconstitution	Extent of backreaction from F_A/F_B in percent			
	No wash		After wash	
	Mean ± S.D.	*n*	Mean ± SD	*n*
	%		%	
None	17 ± 10	7	22 ± 3	2
PSI-D	21 ± 2	2	23	1
PSI-C − PSI-D	88 ± 12	40	84 ± 1	2
PSI-C_{core} + PSI-D	18 ± 8	3	ND[a]	
PSI-CΔC + PSI-D	82 ± 10	21	53 ± 2	4
PSI-CΔL − PSI-D	52 ± 6	13	38 ± 4	2
PSI-$C_{core}L_{c\text{-}term}$ + PSI-D	73 ± 8	4	57 ± 7	2
PSI-C	64 ± 5	5	23	1
PSI-CΔC	85 ± 3	5	24	1
PSI-CΔL	12 ± 10	3	ND	
PSI-$C_{core}L_{c\text{-}term}$	60 ± 3	4	32 ± 2	2

[a] ND, not determined.

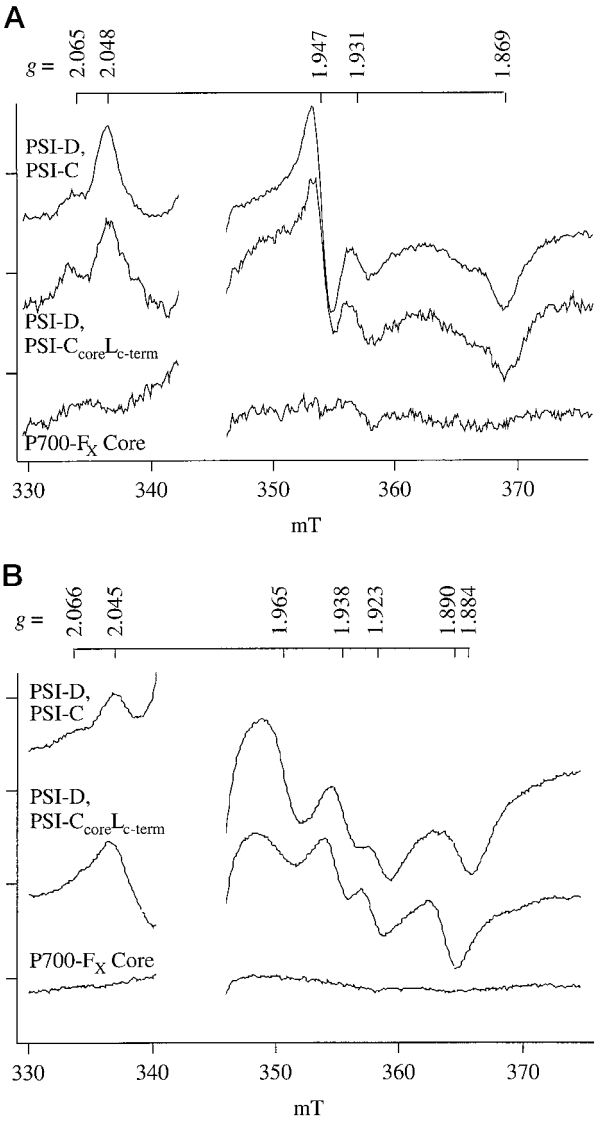

FIG. 4. **EPR analysis of a P700-F_X core rebuilt with PSI-C and PSI-$C_{core}L_{c\text{-}term}$.** *A*, spectrum after illumination of a dark-frozen sample at 15 K. *B*, spectrum after photoaccumulation at room temperature and freezing in the light.

PSI-$C_{core}L_{c\text{-}term}$ and PSI-D had no activity. The activity after addition of wild-type PSI-C and PSI-D to P700-F_X cores was 51 μmol of NADPH mg Chl^{-1} h^{-1}; the P700-F_X cores alone showed no detectable $NADP^+$ reduction.

EPR Analysis of the PS I Complexes Reconstituted with PSI-D and PSI-$C_{core}L_{c\text{-}term}$—Iron-sulfur clusters can be reconstituted in all of the PSI-C polypeptides as shown by their absorbance spectra (26) (data not shown). Having found that PSI-$C_{core}L_{c\text{-}term}$ binds to PS I and accepts electrons from F_X, the next task was to determine if the EPR properties of F_A and F_B were different from those of wild-type PSI-C. The reconstituted PS I complex was illuminated at 15 K in the EPR cavity to promote one electron to the acceptor system. The resulting spectrum showed that the field positions, line widths and ratios of F_B (g = 2.065 and 1.931) and F_A (g = 2.048, 1.947, and 1.869) were identical to that of a P700-F_X core reconstituted with PSI-C and PSI-D (Fig. 4*A*). The reconstituted PS I complex was next frozen during illumination, a protocol which allows two or more electrons to accumulate in the electron acceptor system. The resulting interaction spectrum was similar to that of a wild-type PSI-C reconstitution, containing the features characteristic of magnetic coupling between F_A and F_B (Fig. 4*B*).

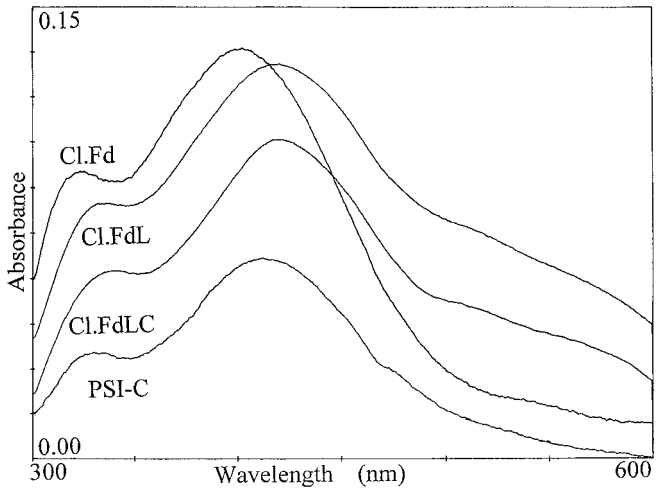

FIG. 5. **Difference spectra of Cl.Fd, Cl.FdL, Cl.FdLC, and PSI-C.** The iron-sulfur clusters of PSI-C, Cl.FdL, and Cl.FDLC were reconstituted according to Parrett *et al.* (33), and the samples were desalted anaerobically. Difference spectra of the proteins were recorded as described previously (26).

Reconstitution of the P700-F_X Core Using the Clostridial Ferredoxin Proteins—The pure clostridial ferredoxin proteins were obtained after cleavage of the fusion proteins by factor Xa. The amount of holo protein obtained after rebuilding the iron-sulfur clusters was determined by UV-visible spectroscopy using $\epsilon_{400\ nm}$ = 31.6 mM^{-1} cm^{-1}. The UV-visible difference spectra of mutant proteins Cl.FdL and Cl.FdLC resembles that of PSI-C (Fig. 5). The molecular masses of Cl.FdL and Cl.FdLC were determined by matrix-assisted laser desorption ionization time-of-flight spectrometry to 6808.8 and 7947.4 Da, respectively. These values are within 0.3–0.6 mass units of the calculated molecular masses of 6806.4 and 7946.7 Da. It is therefore not likely that any secondary modification has taken place during the expression of the proteins.

When the wild-type Cl.Fd was used in reconstitution experiments, there was no recovery of the slow phase in the presence or absence of PSI-D (Fig. 6, Table II). In the absence of PSI-D, Cl.FdL and Cl.FdLC did interact with the P700-F_X core as indicated by the recovery of the slow phase (71 and 59%, respectively). In the presence of PSI-D, the Cl.FdL protein showed the same degree of interaction (73% slow phase). However, when PSI-D was present the Cl.FdLC protein showed a stronger association (84% slow phase) confirming that the C-terminal domain interacts with PSI-D (18).

To investigate stability of the association, the reaction mixtures were washed as described above. In the presence of PSI-D the slow phase was lost by washing showing that the association of the *Clostridium* ferredoxin with the PS I core is not as strong as the associations of the PSI-C proteins.

$NADP^+$ photoreduction activity was also measured on each of the reaction mixtures. Before measuring, the samples were purified using a desalting column. Each sample retained its activity after purification as determined by flash photolysis (data not shown). However, none of the clostridial ferredoxin-containing samples were able to sustain $NADP^+$ reduction.

DISCUSSION

The removal of both the loop and the C-terminal extension from PSI-C led to a protein (PSI-C_{core}) unable to bind to PS I or accept electrons from F_X. Thus, to obtain a functional PSI-C it is essential that either the loop domain and/or the C-terminal domain is present. PSI-CΔL is unable to accept electrons from F_X when PSI-D is not present. However, when the C-terminal extension of this mutant was replaced with the loop sequence,

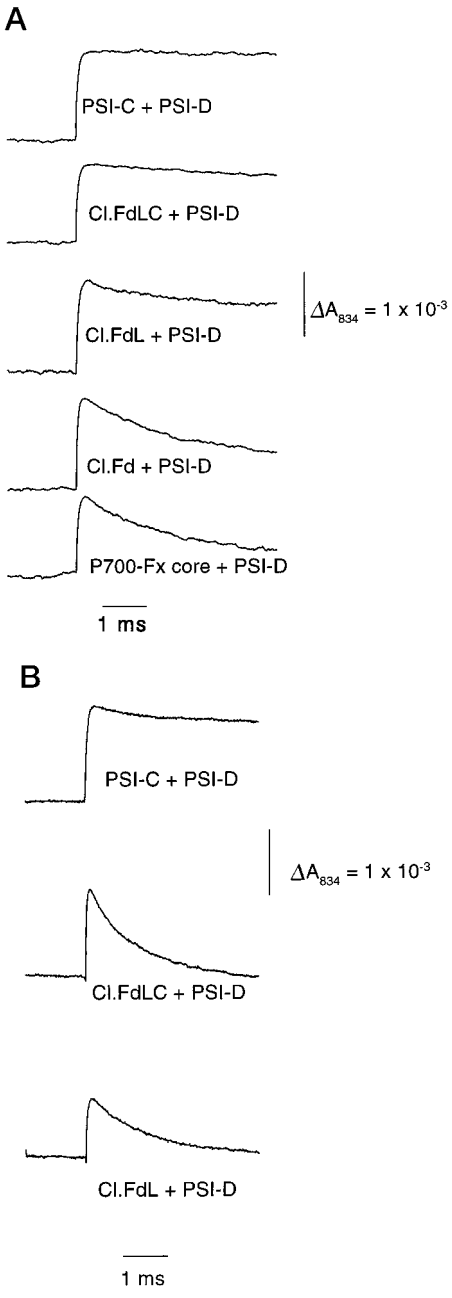

FIG. 6. **Flash-induced absorption changes of PS I reconstituted with clostridial ferredoxin proteins.** The transients represent the primary data for one of the experiments summarized in Table II. *A*, reconstitution of the P700-F_X core with each of the clostridial ferredoxin proteins and PSI-D. The reconstitution with PSI-C is shown for comparison. *B*, the Cl.FdL + PSI-D and Cl.FdLC + PSI-D samples analyzed in *panel A* after washing by ultrafiltration.

TABLE II
Extent of slow back reaction from terminal acceptors in reconstituted PS I determined by flash photolysis

Polypeptides added to the P700-F_X core during reconstitution	Extent of slow back reaction in percent			
	No wash		After wash	
	Mean ± S.D.	*n*	Mean ± S.D.	*n*
	%		%	
None	17 ± 10	7	22 ± 3	2
PSI-D	21 ± 2	2	23	1
Cl.Fd. + PSI-D	13 ± 3	4	ND[a]	
Cl.Fd.L + PSI-D	73 ± 2	3	22 ± 5	2
Cl.Fd.LC + PSI-D	84 ± 4	5	20 ± 2	2
Cl.Fd.L	71 ± 2	3	ND	
Cl.Fd.LC	59 ± 3	6	ND	

[a] ND, not determined.

the resulting protein (PSI-$C_{core}L_{c\text{-}term}$) is able to accept electrons from F_X, even when PSI-D is not present. Therefore the interaction between PSI-C and PSI-A/B must depend only on the presence of the loop sequence, independent of its position in the protein. Since the orientation of the functional PSI-C protein has been limited to two possibilities (14), and since the loop and C terminus of PSI-C have been predicted to extend from opposite sides of the molecule (5, 15–17) it is possible that the PS I-PSI-$C_{core}L_{c\text{-}term}$ interaction involves an inverted orientation of the PSI-$C_{core}L_{c\text{-}term}$ compared with the wild type PSI-C. The EPR measurements would seem to contradict this notion since the spectra of the P700-F_X core reconstituted with wild type PSI-C and with PSI-$C_{core}L_{c\text{-}term}$ are almost identical. We can envision two mechanisms to explain these results. Illumination of PS I at cryogenic temperatures result in reduction of F_A in 76% and F_B in 24% of the complexes (40). This distribution is similar to that calculated assuming an equilibrium between F_B and F_A with redox midpoint potentials at −580 mV and −520 mV, respectively (41). If the reduction potentials of F_A and F_B are not altered by misbinding to PS I (40), and if the electron equilibrates between the two acceptors at low temperature, then the same charge distribution should be expected even if the mutated PSI-C protein were oriented differently than the wild-type. Alternatively, the resonances of the F_A and F_B clusters in the mutant protein may be transposed. Studies of unbound mutant PSI-C proteins show that the EPR resonances of F_A and F_B are virtually indistinguishable in terms of g values and line widths (36). When the mutant PSI-C proteins are bound to PS I, the F_A and F_B resonances narrow and become differentiated with respect to their principal g values (42, 43). Thus, the final determinants of the g tensors of F_A and F_B may be ultimately related to protein:protein interactions with PSI-A/B and PSI-D (41). It is important to note that the PS I complex reconstituted with the PSI-$C_{core}L_{c\text{-}term}$ does not support $NADP^+$ reduction. Apparently, those determinants which confer binding and/or electron transfer properties from F_A/F_B to ferredoxin are more stringent than those which confer forward electron transfer from F_X to F_A/F_B. EPR analysis after chemical reduction in the dark showed that the reduction potential of PSI-$C_{core}L_{c\text{-}term}$ is not significantly higher than that of the wild-type PSI-C (data not shown). Thus, the inability to reduce ferredoxin efficiently is a consequence of improper docking of ferredoxin rather than of a change in the equilibrium of the redox reaction. The analysis of the two mutants PSI-C_{core} and PSI-$C_{core}L_{c\text{-}term}$ therefore lead to the following conclusions: 1) the loop residues of PSI-C interact specifically with PSI-A/B even when it is moved to the C terminus of the PSI-C and 2) The binding of PSI-C to PS I is carried out by the two domains, the loop and the C terminus since deletion of both results in no interaction between the mutant and PS I.

The Cl.Fd protein is unable to bind to PS I or accept electrons from F_X, however when the loop residues of PSI-C were added, the resulting protein Cl.FdL was able to accept electrons from F_X. This result shows that the Cl.FdL and Cl.FdLC are in fact associated with PS I although weakly. If the proteins merely function as soluble electron acceptors and the electron transfer is a diffusion limited process, it is unlikely that the second-order rate constant would be high enough with submicromolar concentrations of these proteins to out-compete the back reaction from F_X. The PS I complexes reconstituted with Cl.FdL or Cl.FdLC were not able to support $NADP^+$ reduction. Most likely the Cl.Fd proteins do not interact properly with barley ferredoxin. One residue of PSI-C, namely lysine in position 34,

has been identified as essential for the PSI-C-ferredoxin interaction (44). Cl.Fd does not have a lysine or any equivalent around position 34. Another factor may be that Cl.Fd does not have sufficiently low midpoint potential to reduce ferredoxin with sufficient efficiency to out-compete the back reaction from F_X. Cl.Fd has a redox potential of −420 mV (45) which is similar to that of plant [2Fe–2S] ferredoxin (−401 mV) (46), while F_A has a redox potential of −520 mV (41).

Cl.Fd is a far more acidic protein than PSI-C, but the addition of the eight residues of the loop in PSI-C to Cl.Fd is apparently sufficient to allow the protein (Cl.FdL) to interact with PS I. The addition of the C terminus of PSI-C to Cl.FdL produced a protein Cl.FdLC which gives higher recovery of the slow rereduction in reconstitution experiments than Cl.FdL, but only when PSI-D was present. Cl.FdL appears to interact with the P700-F_X core to the same extent in the presence and absence of PSI-D. A similar result was obtained previously for the PSI-CΔC mutant (18) (Table I), and these results support the earlier conclusion that the C-terminal extension of PSI-C provides an interaction site for PSI-D. The Cl.FdLC protein accepted electrons from F_X significantly more efficiently than the Cl.FdL protein electrons from F_X and, by inference, interact with PSI-D. However, the association of Cl.FdLC was not sufficiently stable to survive a washing step. Reconstitution with PSI-$C_{core}L_{c\text{-}term}$ and PSI-CΔC both show a higher degree of stability in the presence of PSI-D despite the lack of the C terminus. These results show that PSI-C contains residues apart from the C terminus which are also important for the interaction with PSI-D. Further site-specific mutagenesis will be required to identify these sites of interaction with PSI-D. Contrary to this result, the loop affects binding of PSI-C and Cl.Fd in a very similar manner. Thus, residues of PSI-C other than the eight residue internal loop appear to be of little consequence for the binding to the P700-F_X core itself. Recent data of Rodday *et al.* (19) reveal that not only the two acidic residues of the loop (residues 26 and 31) but also the aspartic acid residue 8 are important for the interaction. However, this aspartic acid is conserved among soluble 2[4Fe–4S] ferredoxins and thus also present in the *Clostridium* ferredoxin (Fig. 1).

The interaction between the loop and the PSI-A/B heterodimer is most simply understood if PSI-C is placed on PSI-A/B with the iron-sulfur cluster F_B proximal to F_X as previously proposed, since in this orientation the loop would face the membrane (1, 14, 47, 48). In contrast, the inability for electron transfer to occur to ferredoxin in F_B-deficient PS I complexes and the resumption of electron transfer to ferredoxin in F_B-reconstituted PS I complexes favor the opposite orientation of PSI-C with F_A proximal to F_X (49–51) (see also discussion in Brettel (48)). In this orientation the loop would seem to extend from a position on PSI-C facing the stroma. However, we note that helices *j*/*j′* and *k*/*k′* in the F_X-binding region of PSI-A and PSI-B are connected with loops of about 40 amino acid residues (52). These loops are poorly resolved in the electron density map and could extend a considerable distance away from the membrane and contact the PSI-C loop in both suggested orientations of PSI-C. The resolution of the issue of PSI-C orientation ultimately awaits an x-ray crystal structure at a higher level of resolution.

In conclusion, the eight-residue loop of PSI-C is essential for the interaction of PSI-C and PS I. The domain is functional in mediating protein-protein association both when placed as the C terminus of the PSI-C or when inserted into *Clostridium* ferredoxin between the two C*XX*C*XX*C*XXX*CP motifs.

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The Eight-amino Acid Internal Loop of PSI-C Mediates Association of Low Molecular Mass Iron-Sulfur Proteins with the P700-F_X Core in Photosystem I

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