Modified Ligands to \( F_A \) and \( F_B \) in Photosystem I

I. STRUCTURAL CONSTRAINTS FOR THE FORMATION OF IRON-SULFUR CLUSTERS IN FREE AND REBOUND PsaC*

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Cysteines 14, 21, 34, 51, or 58 in PsaC of photosystem I (PS I) were replaced with aspartic acid (C21D and C58D), serine (C14S, C34S, and C51S), and alanine (C14A, C34A, and C51A). When free in solution, the C34S and C34A holoproteins contained two \( S = \frac{1}{2} \) ground state [4Fe-4S] clusters; all other mutant proteins contained [3Fe-4S] clusters and [4Fe-4S] clusters; in addition, there was evidence in C14S, C51S, C14A, and C51A for high spin (\( S = \frac{3}{2} \)) [4Fe-4S] clusters, presumably in the modified site. These findings are consistent with the assignment of C14, C21, C51, and C58, but not C34, as ligands to \( F_A \) and \( F_B \). The [4Fe-4S] clusters in the unmodified sites in C14S, C51S, C14A, and C51A remained highly electronegative, with \( E_m \) values ranging from \(-495 \) to \(-575 \) mV. The [3Fe-4S] clusters in the modified sites were driven 400 to 450 mV more oxidizing than the native [4Fe-4S] clusters, with \( E_m \) values ranging from \(-98 mV \) to \(-171 mV \). A C14D/C51D double mutant contains [3Fe-4S] and \( S = \frac{1}{2} \) [4Fe-4S] clusters, showing that the 3Cys1Asp motif is also able to accommodate a low spin cubane. When C34S, C34A, C14S, C51S, C14A, and C51A were rebound to P700-F\(_X\) cores, electron transfer to \( F_A/F_B \) was regained, but functional reconstitution has not yet been achieved for C21D, C58D, or C14D/C51D. These data imply that PsaC requires two iron-sulfur clusters to refold, one of which must be a cubane. Since two [4Fe-4S] clusters are found in all reconstituted PS I complexes, the presence of two cubanes in free PsaC may be a necessary precondition for binding to P700-F\(_X\) cores.

PsaC in photosystem I (PS I)\(^1\) shares similarities with a number of soluble [2Fe-4S] ferredoxins in terms of molecular mass, amino acid sequence, and the presence of two CXXCXX-CXXCP iron-sulfur cluster binding motifs. The protein has greatest similarity to the amino acid sequence of Chromatium vinosum ferredoxin, for which no three-dimensional structure is available. However, there is enough sequence homology with the 54-amino acid [2Fe-4S] ferredoxin from Peptococcus aerogenes (1) and the first 58 residues of the 106-amino acid [3Fe-4S][4Fe-4S] ferredoxin from Azotobacter vinelandii (2) to make predictions of tertiary structure based on the known three-dimensional structure of these proteins (Fig. 1). Assuming that the overall folding of the Psac polypeptide backbone is analogous to these known structures, cysteines 11, 14, 17, and 58 should provide the ligands to one [4Fe-4S] cluster, and cysteines 21, 48, 51, and 54 should provide the ligands to the second [4Fe-4S] cluster. The two clusters in PsaC should also be related by a pseudo-2-fold axis of symmetry, so that cysteines 11, 14, 17, and 58 correlate with cysteines 48, 51, 54, and 21, respectively.

The sequences of naturally occurring ferredoxins suggest modifications that may be tolerated in PsaC. For example, several naturally occurring ferredoxins contain aspartic acid at the position analogous to cysteine 14 in PsaC. These proteins are capable of supporting a mixed spin (\( S = \frac{3}{2} \) and \( \frac{1}{2} \)) [4Fe-4S] cluster in the modified site (3, 4). Using site-directed mutagenesis, mutant PsaC proteins have been constructed with similar mutations (PsaC-C14D and PsaC-C51D), and the free proteins were found to contain [3Fe-4S] clusters at the modified sites and [4Fe-4S] clusters at the unmodified sites (5, 6). When rebound to PS I cores, the \( g \) values and reduction behavior of the [4Fe-4S] clusters in the unmodified sites indicated that \( F_B \) is ligated by residues 11, 14, 17, and 58 and that \( F_A \) is ligated by residues 21, 48, 51, and 54 (5). A ninth cysteine is located at position 34 in all PsaC proteins sequenced thus far, and based on the structural analogy with P. aerogenes, this residue is not predicted to be involved in ligation of either cluster.

The goal of this study is to determine the effect of cysteine replacements on the refolding of PsaC and on the rebinding of modified PsaC proteins to P700-F\(_X\) cores. Mutant PsaC proteins were constructed in which one of the cysteine ligands to an iron-sulfur cluster was changed to the charged amino acid aspartate (PsaC-C14D, previously reported in Ref. 6, and PsaC-C51D, in the accompanying paper (36)), the polar amino acid serine (PsaC-C14S and PsaC-C51S), or the neutral amino acid alanine (PsaC-C14A and PsaC-C51A). Modifications not previously known in ferredoxins (PsaC-C21D and PsaC-C58D) were also made to determine whether a [3Fe-4S] cluster could be accommodated when the proline-proximal cysteine in either
The similarity between the two proteins is highlighted by the x-ray crystal structure of the cyanobacterial PS I reaction center, which shows that the distance between the iron-sulfur clusters is 12 Å, identical to the intercluster distance in P. aerogenes ferredoxin. An extra 10 amino acids are required between the two iron-sulfur binding motifs, and an extra 14 amino acids are required on the C terminus for accurate alignment. The cysteines chosen for site-directed mutagenesis (C14D, C14S, C51D, C51S, C51A, and C58D) are identified; in addition, a double and triple mutant (C14D/C51D and C14D/C51D/C34S) were generated. Cysteine 34 lies in the loop region between cysteines 21 and 48. Iron-sulfur clusters \( F_A \) and \( F_B \) were identified (5) by their \( g \) values and response to illumination in C14D- and C51D-rebound PS I complexes.

### Materials and Methods

#### Engineering, Synthesis, and Purification of the Mutant PsaC Protein

All PsaC mutant proteins were produced from derivatives of the pET expression plasmid pET-36C, which contains the psaC gene isolated from Synechococcus sp. strain PCC 7002 (7). Site-directed mutagenesis was performed as described previously (5, 8). Escherichia coli BL2(DE3) harboring pET-36C and derivatives (psaC mutant psaC) were grown in NY2CM medium (9) except that magnesium sulfate was omitted. The growth medium for cells synthesizing PsaC proteins was supplemented with 6 mg ml\(^{-1}\) ferric ammonium citrate. Expression was performed as described previously (10) and was initiated by the addition of isopropyl-1-thio-D-galactopyranoside to a final concentration of 0.5 mM to the growth medium. After 1 h, 20 \( \mu \)g ml\(^{-1}\) rifampicin was added to the medium, and expression was continued for an additional period of 5–7 h. Cells were harvested by centrifugation and washed once with TS buffer (20 mM Tris-HCl, pH 8.0, and 10 mM NaCl). The cells were resuspended in TS buffer containing 2 mM dithiothreitol and 1 \( \mu \)g ml\(^{-1}\) DNase and disrupted by two passes through a French pressure cell at 18,000 p.s.i. at 4 °C. Inclusion bodies were collected by centrifugation of the whole-cell extract at 7600 \( \times \) g for 10 min at 4 °C. The inclusion bodies were solubilized with 7 M urea and 2 mM dithiothreitol in 50 mM Tris, pH 8.3, and 2 mM EGTA. The solubilized proteins were purified by gel exclusion chromatography over Sephadex G-75 and eluted with 2 mM dithiothreitol and 50 mM Tris-HCl, pH 8.3. Two protein bands were separated; the light brown band (6) was collected, analyzed by SDS-polyacrylamide gel electrophoresis and subjected to \( N \)-terminal amino acid sequencing to verify the presence of the amino acid substitution at the expected position.

Purified PsaC was refolded with iron-sulfur clusters as described (7) and purified by ultrafiltration in an Amicon column over a YM-5 membrane (Amicon, Beverly, MA) with 50 mM Tris-HCl, pH 8.3, and 0.1% \( \beta \)-mercaptoethanol. For electrochemistry, the PsaC holoproteins were desalted, and the \( \beta \)-mercaptoethanol was removed by gel filtration chromatography over a Sephadex G-25 (Pharmacia Biotech Inc.) column under anaerobic conditions in a Coy controlled environment chamber (Grass Lake, MI).

### Results

Substitutions in the Second Cysteine in the CXXCXX-CXXCCP Motif of PsaC: EPR Spectra of \([3F_4E_4S]\) and \([4F_4E_4S]\) Clusters in C14S, C51S, C14A, and C51A—The EPR spectra of free PsaC-C14S, PsaC-C51S, PsaC-C14A, and PsaC-C51A were found to be similar to those observed previously for the PsaC-C14D and PsAC-C51D mutant proteins (6). With no additions, the EPR spectra show a peak at \( g = 2.020 \) and a trough at \( g = 2.810 \).
1.998, characteristic of oxidized [3Fe-4S] clusters (Fig. 2, dotted lines), and the resonances disappear after mild chemical reduction with sodium dithionite at pH 8.0 ($E_m^{52} = -480$ mV). When the samples are more strongly reduced by the addition of sodium dithionite at pH 10.5 ($E_m^{52} = -630$ mV), the EPR spectra show an axial set of resonances with broad line shapes and a $g_{||}$ of 1.94, characteristic of reduced [4Fe-4S] clusters (Fig. 2, solid lines). The line shapes of the merged mid-field and high-field resonances of PsaC-C14S and PsaC-C14A (Fig. 2, A and C) are slightly broader than the corresponding features in PsaC-C51S and PsaC-C51A (Fig. 2, B and D), a pattern observed earlier for PsaC-C14D and PsaC-C51D (5). These features are consistent with the presence of an $S = \frac{1}{2}$ ground state [4Fe-4S]$^{2+}$ cluster, identified as $F_g$ in PS I-rebound PsaC-C14D, and by the presence of an $S = \frac{1}{2}$ ground state [4Fe-4S]$^{3+}$ cluster, identified as $F_g$ in PS I-rebound PsaC-C51D.

The temperature optimum of the [3Fe-4S] clusters in PsaC-C14S, PsaC-C14A, PsaC-C51S, and PsaC-C51A is 30 K (determined at 5 mW of microwave power). The temperature optimum of the [4Fe-4S] clusters in PsaC-C14S and PsaC-C51S are 12 and 9 K, and in PsaC-C14A and PsaC-C51A they are 12 and 15 K (determined at 20 mW of microwave power). The overall pattern is that the presence of a charged, polar, or hydrophobic amino acid result in small differences in the spin relaxation properties, as inferred from the temperature optimum and half-saturation parameter $P_{1/2}$ of the [3Fe-4S] clusters in the modified sites or in large changes in the relaxation properties of the [4Fe-4S] clusters in the unmodified sites (data not shown).

To demonstrate that the [3Fe-4S]$^{0}$ clusters are indeed present in these proteins (i.e. that chemical reduction does not result in their destruction), the four mutant proteins were analyzed by perpendicular and parallel mode EPR. A reduced [3Fe-4S]$^{0}$ is detectable because it is paramagnetic with a ground state spin $S = 2$, and can be observed in normal mode EPR as a single, asymmetric resonance at $g = 10 - 12$, which extends into zero field. Under mildly oxidizing conditions, we were unable to detect any significant resonances in PsaC-C14A, PsaC-C51A, PsaC-C14S, or PsaC-C51S between 0 and 100 mT (data not shown). When the samples were treated with sodium dithionite at pH 10.5 to reduce both the [3Fe-4S] and [4Fe-4S] clusters, the reduced [3Fe-4S]$^{0}$ clusters were easily observed around $g = 10 - 12$ at high microwave powers and at very low temperatures (Fig. 3, solid lines). The reduced PsaC-C14D (34) and PsaC-C51D (15) mutant proteins also show a single asymmetric resonance around $g = 10 - 12$, which tails toward the low field, with broadening into zero field. When analyzed by parallel mode EPR (Fig. 3, dotted lines), the resonances around $g = 10 - 12$ from the integer $S = 2$ [3Fe-4S]$^{0}$ clusters are enhanced and sharpened, and the resonances around $g = 2$ due to the half-integer [4Fe-4S]$^{1+}$ clusters have disappeared (data not shown).
Evidence for High Spin [4Fe-4S] Clusters in C14S, C51S, C14A, and C51A—

The strong asymmetric resonance at $g = 4.3$ present in PsaC-C14S and PsaC-C51S (Fig. 3, A and B) probably arises from $S = \frac{5}{2} d^3 Fe^{3+}$, which is not completely reduced by dithionite. Just downfield of this peak are a new set of broad resonances around $g = 5.5$, which are difficult to microwave-saturate. This rapidly relaxing spin system is attributed to the low field peak(s) of one or two of the Kramers doublets derived from an $S = \frac{3}{2} [4Fe-4S]$ cluster. The E/D values of high spin [4Fe-4S] clusters are likely to be close to the rhombic limit, with the consequence that the $g$ anisotropy is distributed over 3000 G; hence, the derivative of the low field turning point becomes a broad resonance in which a high concentration of cluster appears as a relatively small peak. Assuming an E/D value of $\frac{1}{3}$, the midfield resonances would be obscured by other resonances at $g = 2$, and the highfield resonances are difficult to observe (16). It is proposed that the resonance(s) around $g = 5.5$ arise from a high spin, mixed ligand [4Fe-4S] cluster resident in the modified sites of mutant PsaC-C14S and PsaC-C51S. Charged and polar side groups represented by PsaC-C14D (15), PsaC-C14D (accompanying paper), PsaC-C14S, and PsaC-C51S therefore appear capable of supporting a high spin [4Fe-4S] cluster.

This signal is also present, albeit at a reduced intensity, when the altered amino acid contains a hydrophobic side group such as alanine in PsaC-C14A and PsaC-C51A (Fig. 3, C and D). The intensity of the $g = 5.5$ resonances are considerably weaker in the alanine mutants, but the $g$ value, line shape, and temperature dependence are similar to the aspartate and serine mutants. The implication is that a ligand has been recruited from other than the replacement amino acid to occupy the fourth coordination site of the iron, a good candidate being water, hydroxide, or the free thiolate from $\beta$-mercaptoethanol present in the reconstitution mixture. The occurrence of high spin [4Fe-4S] clusters in the free PsaC proteins is relevant to the finding (see below) that mutant proteins that are rebound to the P700-FX core contain two [4Fe-4S] clusters.

Reduction Potentials of the [3Fe-4S] and [4Fe-4S] Clusters in C14S, C51S, C14A, and C51A—To further characterize the properties of the mutant proteins, a redox titration was performed on the [4Fe-4S]$^{2+/1}$ clusters of free wild-type PsaC and on the [3Fe-4S]$^{3+/2}$ and [4Fe-4S]$^{2+/1}$ clusters of free PsaC-C14S, PsaC-C14A, PsaC-C51S, and PsaC-C51A (Table I). Wild-type PsaC did not titrate with simple Nernstian behavior when the peak at 337 mT and the trough at 360 mT (see Fig. 5) were measured (the features around $g = 2.002$ were obscured by the

**Fig. 3.** Perpendicular (solid line) and parallel mode (dotted line) EPR studies of reduced [3Fe-4S] clusters in free PsaC-C14S (A), PsaC-C51S (B), PsaC-C14A (C), and PsaC-C51A (D) mutant proteins. The samples were reduced with sodium dithionite in 330 mM glycine buffer, pH 10.5, containing 0.67% $\beta$-mercaptoethanol. The resonance at 160.5 mT is probably due to a small amount of octahedrally coordinated iron that has remained in the oxidized state. The vertical axis shows signal intensity, with the reduced [3Fe-4S] cluster scaled arbitrarily to unity spin concentration; the comparison depicts relative intensity of the reduced, high spin [4Fe-4S] cluster. Spectrometer conditions were as follows: dual mode resonator, microwave power, 80 mW; microwave frequency, 9.647 GHz (perpendicular mode), 9.349 GHz (parallel mode); modulation amplitude, 10 G at 100 kHz; temperature, 4.2 K.
respectively. The replacement serine drives the potential of the 3Fe-4S cluster in the C34S and C34A—The backbone of PsaC can be modeled to overlay the backbone of P. aerogenes ferredoxin when additional amino acids are inserted in the loop region and added to the C terminus (19, 20). Cysteine 34 is located in the loop region but at a position that should not allow it to ligate an iron (see Fig. 1). To disallow iron ligation, a protein was constructed in which cysteine 34 was replaced by serine (PsaC-C34S). A conservative replacement amino acid was initially selected to minimize changes to the structure of the protein and to retain similar polarity should this residue be surface-located. The drawback is that serine has been shown to support iron-sulfur clusters in both 2Fe-S and 4Fe-S proteins. Based on precedent, PsaC-C34S to ligate an iron of a 4Fe-S cluster in PsaC, one would expect to find either a 3Fe-S cluster, as with PsaC-C14D and PsaC-C51D (6) and with DMSO reductase (21, 22); a 4Fe-S cluster retained but with altered EPR spectral properties, as shown with the mixed ligand [4Fe-4S] F4 cluster of PS (23, 24); or a 4Fe-S cluster missing, as shown with fumarate reductase (25).

When the iron-sulfur clusters were inserted into the purified PsaC-C34S apoprotein, the EPR spectrum showed no detectable [3Fe-4S] clusters under mildly oxidizing conditions (not shown). When the PsaC-C34S protein was reduced with sodium dithionite at pH 10.5 (Fig. 5A), the EPR spectrum showed a rhombic signal with broad line widths and with the characteristic splitting observed in proteins that contain two [4Fe-4S] clusters (26). Indeed, the g values and line widths are nearly identical to those of the [4Fe-4S] clusters representing FA and FB in free PsaC (Fig. 5B). The clusters in PsaC-C34S and in PsaC-C34S are similar but not quite identical relaxation properties, as depicted by the a slight change in line shape as the temperature is lowered from 30 to 5 K (data not shown). Cysteine 34 was also replaced with alanine (PsaC-C34A), an amino acid incapable of providing a ligand to an iron. The EPR spectrum of the holoprotein showed no [3Fe-4S] clusters in the oxidized state, but a rhombic spectrum in the reduced state. As with PsaC-C34S, the g values and line widths are nearly identical to those of the [4Fe-4S] clusters representing FA and FB in free PsaC (Fig. 5C). The EPR spectroscopic properties of the free PsaC-C34S and the PsaC-C34A proteins are consistent with the proposal that cysteine 34 does not ligate an iron. This assessment is supported by the wild-type EPR spectral properties of FA and FB in Photosystem I complexes reconstituted with PsaC-C34S and PsaC-C34A (see below).

Simultaneous Substitutions in the Second Cysteine of Both CXXCXXCXXCXXCP Motifs: Characterization of [3Fe-4S] and [4Fe-4S] clusters in C14D/S15D and C4D/S15D/C34A—A less likely explanation for the high spin [4Fe-4S] clusters in PsaC-C4X and PsaC-C51X (where X represents D, A, and S) is that they arise from the [4Fe-4S] clusters in the unmodified sites through changes induced by a non-cysteine amino acid in the modified sites. While it would be straightforward to address this issue were it possible to accurately count spins, this can be problematic in a protein that contains a mixture of high spin, low spin, and integer spin systems. In principle, it should be possible to oxidize chemically all of the [3Fe-4S] clusters in the modified site, to reduce chemically all of the [4Fe-4S] clusters in the unmodified site, and then to spin count the two $S = \frac{1}{2}$ systems. However, given that the distance between FA and FB is only 12 Å (27), there is a further complication that the reduced [3Fe-4S] and [4Fe-4S] clusters may interact magnetically. An alternative approach is to generate a double mutant.
in which Cys-14 and Cys-51 are both changed to aspartates, PsaC-C14D/C51D. The premise put to the test is that a [4Fe-4S] ferredoxin such as PsaC is refolded only when it contains at least one cubane cluster (the naive expectation is that this change would result in a protein that contains two [3Fe-4S] clusters). If the former expectation is borne out experimentally, then the double mutant would contain one or two [4Fe-4S] clusters, therewould be evidence that a mixed ligand site composed of the 3Cys1Asp motif in PsaC is able to support a cubane.

Fig. 6A shows the EPR spectrum of PsaC-C14D/C51D in the presence of sodium ascorbate. The presence of a peak at g = 2.02 and a trough at g = 1.98 indicates the presence of oxidized [3Fe-4S] clusters. When the sample is reduced with dithionite at pH 10.5, a rhombic EPR signal is observed with broad line widths in the g = 2 region that is virtually identical to the low spin [4Fe-4S] cluster seen in the single mutants. This could indicate the presence of one type of cluster located in the F_A site and another type of cluster located in the F_B site, or alternatively there may be no site differentiation. Independent of the issue of site distribution of the two types of clusters, a mixed ligand 3Cys1Asp site in PsaC is clearly capable of supporting a cubane. The only distinction is that the spin state of the [4Fe-4S] cluster in the double mutant is S = 1/2, while the spin state of the mixed ligand [4Fe-4S] clusters in the single mutants is S = 3/2. The likelihood is that the double mutant cannot support two [3Fe-4S] clusters; the implication is that the minimum requirement for a stable protein is the presence in PsaC of at least one cubane cluster.

One potential complication is that the nonligating cysteine in position 34 may have been recruited to function as the ligand to one of the two iron-sulfur clusters in the PsaC-C14D/C51D double mutant. A ligand rearrangement has been reported to occur in A. vinelandii ferredoxin I (AvFdI), where C20A is rearranged in the region of the [4Fe-4S] cluster to allow it to use the free cysteine 24 as a replacement ligand (28). A triple mutant of PsaC was therefore constructed to additionally substitute cysteine 34 with serine: PsaC-C14D/C51D/C34S. By invoking reasoning similar to that for the PsaC-C34S single mutant (see above), were this serine to be recruited to provide a ligand to an iron, the g values and line widths of the [4Fe-4S] cluster should be sufficiently different to distinguish it from the wild type. As shown in Fig. 6B, the EPR spectrum of the triple mutant in the presence of sodium ascorbate shows the presence of a [3Fe-4S] cluster and, after the addition of sodium dithionite at pH 10.5, the presence of an S = 3/2, [4Fe-4S] cluster. There are no significant differences in the g values, line shapes, temperature optima, or half-saturation parameters between the double and triple mutants (data not shown). These data verify that both high and low spin [4Fe-4S] clusters can be supported in a mixed ligand 3Cys1Asp environment in PsaC.

Substitutions in the Fourth Cysteine of the CXXCXXCXXCP motif proteins.
Motif: Characterization of [3Fe-4S] and [4Fe-4S] Clusters in C21D and C58D—There exist to our knowledge no naturally occurring or site-directed mutants of ferredoxins with non-cysteine ligands in the proline-proximal position of the CXX-CXXXCP iron-sulfur binding motif. If the analogy between PsaC and the ferredoxin of P. aerogenes holds, cysteines 58 and 21 should be located in the interior of PsaC (see Fig. 1), and their size and charge may be crucial in maintaining the three-dimensional structure. Alternatively, the fourth cysteine in each iron-sulfur binding motif also may not be as rigidly constrained by the presence of nearby, ligating cysteines in the CXXCXXX pattern, is the case in PsaC-C14D and PsaC-C51D. It was therefore of interest to determine whether a substitution of aspartic acid in this position would support the presence of a [3Fe-4S] cluster in this site.

The EPR spectra of the PsaC-C21D and PsaC-C58D mutant proteins are shown in Fig. 6, C and D. In the presence of sodium ascorbate, both proteins show a peak at \( g = 2.02 \) and a trough at \( g = 1.998 \), characteristic of an oxidized [3Fe-4S] cluster. When PsaC-C21D and PsaC-C58D were reduced with sodium dithionite at pH 10.5, the [3Fe-4S] clusters disappeared and a rhombic EPR signal with broad line widths appeared, which is characteristic of a reduced [4Fe-4S] cluster. The maximum signal intensity of the [4Fe-4S] clusters in PsaC-C21D and PsaC-C58D was achieved at 15 K when measured at 10 mW of power, and the half-saturation parameter \( P_{1/2} \) was also similar to [4Fe-4S] clusters in the other mutant proteins. These results indicate that it is possible to create a ferredoxin containing a [3Fe-4S] cluster by replacing the fourth as well as the second cysteine in each of the two CXXCXXX motifs. A corollary to this finding is that in addition to cysteines 14 and 51, cysteines 21 and 58 also provide ligands to the cubane clusters in PsaC. Since neither PsaC-C21D nor PsaC-C58D rebind to the P700-FX core under conditions appropriate for PsaC (shown in Fig. 7), the spectral and redox properties of these proteins were not studied further.

Rebinding of Mutant PsaC Proteins to P700-FX Cores—Fig. 7A shows the time course of the reduction of P700* after a saturating flash in PS I complexes after attempted reconstitution of P700-FX cores with PsaC-C34S, PsaC-C34A, PsaC-C21D, PsaC-C58D, and the double mutant PsaC-C14D/C51D. Under conditions appropriate for PsaC rebinding, only PsaC-C34S and PsaC-C34A were effective in restoring the 30-ms back reaction from \( F_{a}/F_{b} \); the other mutants retained the 1.2-ms back reaction from \( F_{b} \). The EPR spectra of PsaC-C34S-PS I and PsaC-C34A-PS I showed resonances primarily of \( F_{a} \) when frozen in darkness and illuminated at 15 K (data not shown) and an interaction-type spectrum similar to the wild-type control when frozen under continuous illumination. The dose similarity in the g values and line widths of \( F_{a} \) and \( F_{b} \) in the PsaC-C34S-PS I complex (Fig. 7B) and the PsaC-C34A-PS I complex (Fig. 7C) with the wild-type PsaC-reconstituted PS I complex (see Ref. 7) further supports the proposal that this cysteine does not participate in providing a ligand to an iron in PsaC. The analogy with P. aerogenes ferredoxin in terms of cysteine participation in ligand binding thus appears warranted.

When PsaC-C14A, PsaC-C14S, PsaC-C51A, and PsaC-C51S were combined with P700-FX cores and analyzed by room temperature optical kinetic spectroscopy, all four mutant reaction centers supported long lived charge separation to \( F_{a}/F_{b} \). Similarly, all reconstituted PS I complexes showed electron transfer to (the equivalent of) \( F_{a} \) and \( F_{b} \) when analyzed by low temperature EPR spectroscopy. The detailed electron transfer properties of these mutant reaction center complexes will be reported elsewhere.2

2 Jung, Y.-S., Vassiliev, I., Qiao, F., Bryant, D., and Golbeck, J. H., manuscript in preparation.
DISCUSSION

The EPR spectroscopic properties of the iron-sulfur clusters in free and PS I-rebound PsaC proteins were determined after the introduction of charged (aspartate), polar (serine), and hydrophobic (alanine) amino acids in the second position and after the introduction of a charged (aspartate) amino acid in the fourth position, of each CXXDXXCP motif (cf. Fig. 1). The following generalizations can be made when the second cysteine of the motif is changed. First, a [3Fe-4S] cluster can be found in the oxidized protein and is assumed to be resident in the modified site. The midpoint potentials of the [3Fe-4S] clusters are -400 mV more oxidizing than the wild-type [4Fe-4S] clusters, and the spin relaxation properties, inferred from the temperature dependence and half-saturation parameter, show differences among the three classes of amino acids. Second, a low-spin [4Fe-4S] cluster is found in the reduced protein and is assumed to be resident in the unmodified site. The replacement amino acids, which include those with charged side groups, polar groups, and a hydrophobic side group, show no consistent pattern in affecting the reduction potential of the cluster in the unmodified site (Table I). Third, high spin [4Fe-4S] clusters are tentatively identified in the PsaC-C14S, PsaC-C51S, PsaC-C14A, and PsaC-C51A reduced proteins and are assumed resident in the modified site. High spin [4Fe-4S] clusters have also been tentatively identified in free C14D-PsaC (15) and free C51D-PsaC (see accompanying paper (36)). One likely origin of the high spin (S = 5/2) [4Fe-4S] cluster is the conversion of a reduced [3Fe-4S] cluster as described previously in Peptococcus furiosus (3) and Desulfovibrio africanus ferredoxins (4), which contain similar CXXDXXCP motifs. Alternately, the [3Fe-4S] clusters may be derived from the loss of an iron from an oxidized [4Fe-4S] cluster, which may have been inserted in vitro as an intact cubane into the mixed-ligand site of the free PsaC protein. These are the limiting cases; there may well exist a dynamic equilibrium between the [3Fe-4S] and [4Fe-4S] clusters through insertion and loss of an iron controlled by redox potential. While the presence of a high spin [4Fe-4S] cluster was not surprising in the aspartate (or serine) mutants, it was not expected in the alanine mutants. The issue is whether the aspartate and/or serine oxygens provide ligands to the cubane iron as does aspartate in P. furiosus (29, 30), or whether water, hydroxide, or the thiolate from β-mercaptoethanol present in the reconstitution mixture, has contributed the fourth ligand. In this respect, it is interesting that only those mutant proteins that show evidence for a high spin [4Fe-4S] cluster in the mutant site are capable of recon-
stituting onto P700-Fx cores.

An additional new finding is that the PsaC-C14D/C51D double mutant does not contain two [3Fe-4S] clusters; rather it contains [3Fe-4S] and low spin [4Fe-4S] clusters in a nearly stoichiometric ratio (data not shown). In light of the above data on the single mutants, it is intriguing that two [4Fe-4S] clusters are not present in the PsaC-C14D/C51D double mutant. The presence of both types of clusters in the PsaC-C14D/C51D double mutant is in agreement with the premise that PsaC must contain at least one cubane cluster for stability. It is also interesting that an earlier attempt to introduce two [3Fe-4S] clusters into A. vinelandii Fd I also failed (28). This protein normally contains one [3Fe-4S] and one [4Fe-4S] cluster. When a cysteine ligand of the [4Fe-4S] cluster was altered, a nearby cysteine was recruited as a replacement ligand, resulting in a protein with structural modifications but still containing one [3Fe-4S] and one [4Fe-4S] cluster. In an attempt to minimize the chance that cysteine 34 provides a ligand to one of the clusters in PsaC-C14D/C51D, the triple mutant PsaC-C14D/C51D/C34S was constructed. This mutant does not contain a recruitable cysteine residue; hence, the [4Fe-4S] cluster must reside in a mixed ligand site. Nevertheless, serine cannot be dismissed as a potential ligand since it supports a [2Fe-2S] cluster with modified optical and EPR properties in a site-directed mutant of ferredoxin (31–33) and NADH-quinone oxidoreductase (34) and a low spin [4Fe-4S] cluster with modified EPR properties in a site-directed mutant of the interpolypeptide FX cluster in PS I (23, 24). However, the g values, line widths, temperature optimum, and P1/2 values of the PsaC-C14D/C51D/C34S mutant were identical to those of the PsaC-C14D/C51D double mutant, in agreement with the proposal that cysteine 34 is not a ligand to a cubane iron in this instance.

The detection of a high spin [4Fe-4S] cluster in the C14D and C51X (where X represents A, D, or S) single mutants provides a precedent for the presence of a [4Fe-4S] cluster in the C14D/C51D double mutant. The only significant difference is the S = ½ spin state of the [4Fe-4S] cluster in the single mutants and the S = ½ spin state of the [4Fe-4S] cluster in the double mutant. It should be noted that P. furiosus ferredoxin contains a single, mixed ligand [4Fe-4S] cluster that exists in both S = ½ (20%) and S = ½ (80%) ground states (3). The cross-over energy between the high and low spin states is likely to be small; instead, ethylene glycol and urea change the spin state in the A. vinelandii ferredoxin (35). One factor appears to be related to the degree of exposure to solvent, a consideration that may be relevant in the spin state changes that have been postulated to occur between the free and PS I-rebound PsaC-C51D (15).

In summary, these studies indicate that PsaC refolds only in the presence of two iron-sulfur clusters when cysteine is re-
placed in positions 14 and 51 by aspartate, serine, and alanine, and in positions 21 and 58 by aspartate. Since there was no occurrence of a mutant PsaC that contained only one [3Fe-4S] cluster, only one [4Fe-4S] cluster, or two [3Fe-4S] clusters, a corollary to the above premise is that a stable three-dimensional structure may require that two conditions be met: 1) two iron-sulfur clusters are present, and 2) one of the two clusters is a cubane. The inability to observe a [3Fe-4S] cluster in the photosynthesis, two points can be made. First, the important feature for electron transfer is that the mixed ligand, [4Fe-4S] clusters in reconstituted PsaC-C14D-PSI (34), PsaC-C14S-PSI, and PsaC-C51S-PSI, and PsaC-C51A-PSI complexes are capable of accepting electrons at both cryogenic and room temperatures. Second, a checkpoint for the assembly of PsaC onto PSI cores may be that the free PsaC protein must contain two [4Fe-4S] clusters.

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