Reconstitution of Barley Photosystem I with Modified PSI-C Allows Identification of Domains Interacting with PSI-D and PSI-A/B*

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The PSI-C subunit of photosystem I shows similarity to soluble 2[4Fe-4S] ferredoxins. Alignment analysis clearly shows that PSI-C contains an 8-residue internal loop and a 15-residue C-terminal extension that are absent in the ferredoxins. The remaining residues in PSI-C are likely to be folded in a way similar to the soluble 2[4Fe-4S] ferredoxins. Two modified PSI-C subunits lacking either the 8-residue loop or 10 residues of the C terminus were expressed in Escherichia coli and used to reconstitute a barley P700-Fx core prepared to specifically lack PSI-C, PSI-D, and PSI-E. As shown by EPR spectroscopy, the modified proteins carry two 2[4Fe-4S] clusters with characteristics similar to those of native PSI-C. Western blot analysis of the reconstituted photosystem I complexes showed that the modified PSI-C proteins bind to the P700-Fx core. Flash photolysis revealed that in photosystem I complexes reconstituted in the presence of PSI-D with the C-terminally deleted PSI-C, the Fx/Fb back-reaction was less efficiently restored than with wild-type PSI-C. The loop-deleted PSI-C was even less efficient. We attribute these differences to altered binding properties of the modified proteins. Comparison of reconstitutions performed in the presence and absence of PSI-D shows that the loop-deleted PSI-C is unable to bind without PSI-D, whereas the C-terminally deleted PSI-C binds only weakly with PSI-D. These results imply that the internal loop of PSI-C interacts with the PSI-A/B heterodimer and that the C terminus of PSI-C interacts with PSI-D.

The photosystem I (PS I) reaction center complex mediates electron transport from plastocyanin to ferredoxin in oxidogenic photosynthesis. PS I contains the primary electron donor P700 (a Chl a dimer) and the electron acceptors A0 (Chl a), A1 (phylloquinone), and three 2[4Fe-4S] centers Fx, Fx, and Fb (1-3). The terminal acceptors Fx and Fb 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). A major unresolved question is the precise pathway of electron flow through the terminal acceptors Fx and Fb. Fx/Fb has been shown to be essential for electron transfer to ferredoxin but not for low temperature photo reduction of Fx, suggesting Fb to be the final acceptor in the electron transfer chain (8-10).

The amino acid sequence of PSI-C is highly conserved among species and contains a repeated CXXCXXXCP motif that is characteristic of 2[4Fe-4S] proteins. Alignment analysis of these proteins shows that, apart from an internal segment of 8 residues and 15 residues in the C terminus that are not present in the ferredoxins, PSI-C is similar to the bacterial 2[4Fe-4S] ferredoxins (5, 11, 12). The folding of PSI-C must be very similar to that of the soluble ferredoxins, indicating that the 8 internal residues form an extra loop. Since the ferredoxins are soluble and the PSI-C polypeptide is membrane-bound, the additional amino acids in the loop and in the C terminus may be responsible for the binding of PSI-C to the P700-Fx core (12). EPR data obtained with PS I crystals and comparison with the known structure of Peptococcus aerogenes ferredoxin allow the orientation of PSI-C within the PS I complex to be limited to two possibilities by positioning the two clusters and two highly conserved helices along a fixed axis (13). Ligands for Fx are provided by Cys20, Cys47, Cys50, and Cys53, while Cys10, Cys13, Cys16 and Cys57 provide ligands for Fb (14). The positions of two 2[4Fe-4S] clusters within the complex are known from the crystal structure of PS I (15), but which cluster represents Fx and which represents Fb cannot be distinguished at 6-Å resolution. From alignment with P. aerogenes ferredoxin, the internal loop and the C terminus are predicted to extend from opposite sides of PSI-C. Therefore, determination of the orientation of the loop and the C terminus relative to PS I would yield further information on the positions of Fx and Fb.

We characterized the interaction and binding of PSI-C within the PS I complex by producing two PSI-C deletion mutants that lack either the 8-residue internal loop or 10 residues of the C terminus. Using a modification of the method of Parrett et al. (16), we recently established that urea treatment of barley PS I followed by a combined detergent, salt, and urea wash leads to specific dissociation of the PSI-C-PSI-D and PSI-E subunits without affecting the remaining polypeptides (17). The resulting P700-Fx complex can be used for reconstitution by the addition of Escherichia coli expressed PSI-C and PSI-D in the presence of the reagents Na2S, FeCl3, and 2-mercaptoethanol, which serve to rebuild the FeS clusters of PSI-C (17-19). Using this reconstitution system, we show that the PSI-C protein lacking the internal loop interacts inefficiently with the P700-Fx core, while the protein lacking the C terminus interacts efficiently with PSI-D.

EXPERIMENTAL PROCEDURES

Plasmid Construction and Protein Isolation—The PSI-D expression clone from barley was made as described by Scott et al. (20) using the cDNA clone of Kjaerulf and Oikkels (21). The PSI-C expression clone from barley has been described previously (17). The design of the two
P. aerogenes

Barley PSI-CHor

Synecochlorella PSU 3001 PSI-CHor

Barley PSI-CHor

P. aerogenes

Barley PSI-CHor

Synecochlorella PSU 3001 PSI-CHor

Barley PSI-CHor

P. aerogenes

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Synecochlorella PSU 3001 PSI-CHor

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Synecochlorella PSU 3001 PSI-CHor

Barley PSI-CHor

P. aerogenes
reconstituted PS I complexes. The complex reconstituted with PSI-CSynΔC was illuminated at 15 K in the EPR cavity, and the resulting spectrum was nearly identical to that of PS I reconstituted with PSI-CHor (Fig. 3). Features of FA were apparent at the midpoint in the spectrum, while the features of FB were found to be absent. The two clusters are reduced in the same ratio in PSI-CSynΔC as they are in wild-type PSI-CHor. From this, we conclude that the C-terminal region is not required for electron transfer from P700 to Fb/Fa.

Similar experiments were performed with complexes reconstituted with PSI-CHorΔL (Fig. 4). When illuminated at 15 K, the low-field peaks of Fα and Fβ were clearly visible but small in amplitude, while the mid-field and high-field features were not well resolved. We next froze the sample during illumination, conditions allowing two or more electrons to accumulate in the electron acceptor system. The resulting spectrum had peaks with much increased amplitude and was very similar to that of the wild-type PSI-CHor. The time course of the flash-induced absorption changes was determined at various molar ratios of PSI-C to P700 in all experiments. The extent of reconstitution was determined by fitting the traces to biphasic exponential decay curves containing phases with time constants of ~1 ms and ~16 ms. The fraction constituting the slow decay phase was calculated in percentage of the total absorbance change.

Flash-induced Absorption Changes—The back-reaction of the PSI complex was studied after incubation of the P700-Fα core with purified PSI-D and one of the PSI-C polypeptides (PSI-CHorΔC/PSI-CSynΔC/PSI-CHorΔL) in the presence of FeCl3, Na2S2O3 and 2-mercaptoethanol. The back-reaction was observed to be an Fβ/Fa back-reaction after a 5-min incubation at room temperature (data not shown) when each of the PSI-C polypeptides was present in excess (see below), but all samples were left for 16 h at 5°C before the measurements. The reconstituted PS I complexes were characterized by flash-induced absorption changes at 834 nm in the millisecond time range. The extent of the reconstitution was monitored at room temperature by calculating the absorbance change from the 1-ms back-reaction from Fβ/Fa to P700 versus the slower back-reaction from [Fβ/Fa]. The time course of the flash-induced absorption changes was determined at various molar ratios of PSI-C to P700 (Fig. 5). In all experiments, PSI-D was added in a molar ratio of 20 (PSI-D/P700). With the wild-type PSI-CHor, the reconstitution was 77% complete at a PSI-C:P700 ratio of 4. Higher ratios of PSI-C to P700 did not result in more complete reconstitution. PSI-CSynΔC was saturating at a molar ratio of 7.5 (PSI-CSynΔC:P700) and led to the same level of reconstitution as the full-length PSI-CHor. PSI-CHorΔL was saturating at a molar ratio of 10 (PSI-CHorΔL:P700), but the loop deletion led to less complete restoration of the PS I complex (55%) than PSI-CHor. Thus, greater amounts of PSI-CSynΔC and PSI-CHorΔL than PSI-CHor are required in the reconstitution reaction relative to P700. This could be a result of a different folding...
Binding of PSI-C to Photosystem I

The PSI I complex was reconstituted using molar ratios of 20:20:1 (PSI-C:PSI-D:P700). The PSI I complex reconstituted with PSI-D and PSI-C<sub>Hor</sub>, PSI-C<sub>Syn</sub>, or PSI-C<sub>Syn</sub>, ΔC resulted in 81, 84, and 82% recovery of the slow decay phase, respectively (Fig. 6A, Table I). Rebuilding with PSI-C<sub>Hor</sub>, ΔL and PSI-D resulted in an incomplete reconstitution, with only 52% recovery of the PSI I activity (Fig. 6A, Table I). To assay stability, the reconstituted complexes were washed with 0.1% Triton X-100 over an ultrafiltration membrane. The PSI I complex reconstituted with PSI-C<sub>Hor</sub> and PSI-C<sub>Syn</sub> remained stable, showing 84 and 88% reconstitution, respectively, whereas the PSI I complex reconstituted with PSI-C<sub>Syn</sub>, ΔC was unstable as shown by a reduction in the magnitude of the slow Δτ<sub>BA</sub> decay from 82 to 53% (Fig. 6B, Table I). The PSI I complex reconstituted with PSI-C<sub>Hor</sub>, ΔL was also affected by the wash (52% before and 38% after). It should be noted that mock reconstitution of the P700-F<sub>X</sub> core in the absence of added PSI-C resulted in about 17% recovery of a slow absorbance decay. This background value has not been subtracted from the data presented in Table I. Subtraction of the background value would obviously accentuate the differences reported.

Rebuilding the PSI I complex with the PSI-C<sub>Hor</sub> and PSI-C<sub>Syn</sub> in the absence of PSI-D resulted in retention of 64 and 66% of the slow decay phases, respectively (Fig. 6C, Table I), which is less than when PSI-D was included for reconstitution (88 and 84%). The PSI-C<sub>Syn</sub>, ΔC had an enhanced ability to bind to the P700-F<sub>X</sub> core, retaining 85% of the slow phase. PSI-C<sub>Syn</sub>, ΔL did not bind to the P700-F<sub>X</sub> core in the absence of PSI-D. The [F<sub>α</sub>/F<sub>β</sub>]<sup>−1</sup> P700<sup>−</sup> back-reaction in all the PSI I complexes reconstituted without PSI-D was greatly reduced by washing (Fig. 6D, Table I), indicating that PSI-C was removed by this procedure.

NADP<sup>+</sup> Photoreduction Measurements—The extent to which complexes provided with PSI-C<sub>Hor</sub>, PSI-C<sub>Syn</sub>, or PSI-C<sub>Syn</sub>, ΔC and PSI-D were reconstituted as measured by NADP<sup>+</sup> photoreduction after the wash protocol described above corresponded to that measured by flash analysis (Table II). PSI-C<sub>Hor</sub>, ΔL was not able to support NADP<sup>+</sup> reduction after the wash. However, when the reconstituting agents were removed using a desalting column, the sample exhibited NADP<sup>+</sup>-reducing activity in correspondence with the flash analysis. Flash analysis of a P700-F<sub>X</sub> core reconstituted with PSI-C<sub>Hor</sub>, ΔL and PSI-D confirmed that purification of the sample using a desalting column did not alter the extent of F<sub>α</sub>/F<sub>β</sub> back-reaction as compared with the unwashed samples (data not shown). Complexes reconstituted in the absence of PSI-D and washed by ultrafiltration were unable to mediate NADP<sup>+</sup> reduction (data not shown).

DISCUSSION

Deletion of neither the internal B-residue loop nor the 10 C-terminal residues of PSI-C affected the iron-sulfur clusters F<sub>x</sub> and F<sub>β</sub> in the unbound proteins. Therefore, any differences in reconstitution efficiency exhibited by these proteins can be attributed to altered binding efficiency of the modified proteins PSI-C<sub>Syn</sub>, ΔC and PSI-C<sub>Hor</sub>, ΔL to the P700-F<sub>X</sub> core. PSI-C<sub>Hor</sub>, ΔL is a highly conserved subunit, and only nine amino acid residues are different between PSI-C from barley and Synechococcus sp. PCC 6301 (Fig. 1). In all experiments, the wild-type proteins PSI-C<sub>Hor</sub> and PSI-C<sub>Syn</sub> have given identical results.

Western blot (Fig. 2) and EPR analysis (Figs. 3 and 4) show that the P700-F<sub>X</sub> core does not contain detectable amounts of PSI-C. Nevertheless, the core exhibited 17% recovery of the slow phase of the flash-induced absorbance change at 834 nm upon mock reconstitution. This suggests that a small fraction of the slow back-reaction is due to the presence of reconstitution reagents that may function as electron acceptors. This would be in agreement with the previous observation that electron transport to NADP<sup>+</sup> cannot be accomplished in the presence of the reagents employed for F<sub>α</sub>/F<sub>β</sub> reconstitution (17).

EPR analysis at 15 K of the P700-F<sub>X</sub> core reconstituted with the PSI-C<sub>Hor</sub>, ΔL showed features of F<sub>x</sub> and F<sub>β</sub>, but the electron transport was inefficient at 15 K (Fig. 4). However, when the sample was subjected to photoaccumulation, the spectrum was
nearly identical to that of PS I reconstituted with PSI-CHor and contained resonances typical of an interaction spectrum of FA and FB. This is in contrast to the PSI-CsynAC reconstitution, which gives a spectrum similar to wild-type reconstitution when illuminated at 15 K.

In order to measure NADP⁺ reduction, it is essential to remove the reconstitution reagents, and this was generally accomplished by ultrafiltration. However, in the case of PSI-CsynΔC significant loss of reconstitution efficiency occurred as determined by flash photolysis, and no NADP⁺ reduction could be detected. When a milder desalting procedure was employed, there was no impairment of the reconstitution, and NADP⁺ reduction was detected. This shows that PSI-CsynΔC does bind to PS I in the presence of PSI-D, but the binding is weak. The measured NADP⁺-reducing rates are low compared with native PS I but correspond well to previously observed values in reconstitution experiments (17). Washing in the presence of Triton X-100 causes a loss of activity possibly due to loss of PS I-F and thereby an inefficient interaction with plastocyanin (33, 34). This effect of Triton washing is also seen with native PS I (17). In all cases, the extent of NADP⁺ reduction mirrored the extent of reconstitution observed by flash analysis. Therefore, the data indicate that PS I reconstituted with the mutant PSI-C polypeptides functions identically to wild-type proteins in ferredoxin reduction. Further flash experiments to determine the kinetic constants of ferredoxin reduction will be needed to address this question in detail.

The C-terminally truncated PSI-CsynAC restored the [F₆/F₇] P700- back-reaction nearly as well as PSI-CHor and PSI-Csyn, but the complex was unstable. When the PS I complex was washed intensively, the recovery of reconstituted PS I decreased to 53%. Using PSI-CHor and PSI-Csyn the reconstituted PS I complexes were resistant to the wash procedure. Thus the C terminus is not essential for correct positioning of PSI-C on the P700-F₇ core, but it is important for the stability of the resulting PS I complex. In reactions with and without PSI-D, the reconstitution behavior of PSI-CsynAC was similar, in contrast to the significant effect of PSI-D observed with wild-type PSI-C. This suggests that the C-terminal domain that was removed in PSI-CsynAC includes the major site of interaction with PSI-D. PSI-CsynΔC, PSI-Csyn, and PSI-CsynAC were almost completely removed by washing of the PS I complexreconstituted in the absence of PSI-D. This is in agreement with previous results showing PSI-D as essential for the stabilization of PSI-C in PS I (17, 19). However, since the activity of the PS I complex reconstituted with PSI-CsynAC in the presence of PSI-D was not entirely destroyed by the extensive wash, there must be some additional sites of interaction with PSI-D that remain on PSI-CsynAC.

Rebuilding the PS I complex with the loop deletion mutant in the presence of PSI-D was achieved, but it was incomplete. Furthermore, while the other proteins tested interacted weakly with PS I in the absence of PSI-D, PSI-CsynΔC was unable to interact functionally with PS I under these conditions. This suggests that the PSI-D interaction provides the only functional link between the PS I complex and PSI-CsynΔC. The 8-residue loop, which was deleted in this mutant, must therefore contain sites of interaction between PS I-C and the P700-F₇ core. The sites of interaction between PSI-C and the PS I polypeptides have been shown to be limited to PSI-A, PSI-B, PSI-D, and PSI-E (35, 36) in the native PS I complex. The loop was shown not to be involved in binding of PSI-D, and reconstitution can be performed completely in the absence of PSI-E (this paper and Refs. 17 and 19); therefore, the binding activity of the loop must involve interaction with PSI-A and PSI-B. Studies of Roddial et al. (36, 37) suggest that arginine residues in the conserved domain in PSI-A and PSI-B that binds F₇ are important for the interaction with PSI-C. The 8-residue internal loop contains two negative charges that may be responsible for the specific interaction with the arginines. To interact with PSI-A and PSI-B and most likely with residues very close to the ligands of F₇, we propose that the loop of PSI-C must face the thylakoid membrane, whereas the C terminus providing the binding site for PSI-D is likely to be oriented toward the stroma.

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