Reconstitution of electron transport in photosystem I with PsaC and PsaD proteins expressed in *Escherichia coli*

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A fusion protein, denoted PsaC1, which contains an amino-terminal extension of five amino acids (MEHSM...) and is derived from an in vitro modified form of the *psaC* gene of *Synechococcus* sp. PCC 7002, has been over-expressed in *Escherichia coli*. The product of the *psaD* gene of *Nostoc* sp. PCC 8009 has similarly been over-expressed. The PsaC1 and PsaD proteins can be combined with the photosystem I core protein of *Synechococcus* sp. PCC 6301 to reconstitute electron transport from P700 to the terminal F_A/F_B acceptors. Reconstitution was found to be absolutely dependent on reinsertion of the iron-sulfur clusters in the PsaC1 apoprotein and on the presence of the PsaD protein. This implies that the PsaC1 holoprotein does not bind solely to the PsaA/PsaB heterodimer but rather that its interaction with these proteins is mediated through the PsaD protein.

P-700; Iron-sulfur centers F_A/F_B; Photosystem I reconstitution; PsaC expression in *E. coli*; F_A/F_B iron sulfur reinsertion; PsaD expression in *E. coli*

1. INTRODUCTION

The photosystem I complex of cyanobacteria and higher plants is a membrane-bound oxidoreductase which catalyzes the light-driven transport of electrons from reduced plastocyanin or cytochrome c_{553} to soluble ferredoxin or flavodoxin (for reviews, see [1-2]). The complex is comprised of 11 and 12 polypeptides in cyanobacteria and higher plants, respectively; about 100 chlorophyll *a* molecules; 10-15 β-carotene molecules; two molecules of phylloquinone (vitamin K_1); and three [4Fe-4S] centers known as F_X, F_A, and F_B. The core antenna chlorophyll *a* molecules, P700, A_0, A_1, and the F_X center are bound to the products of the *psaA* and *psaB* genes which each encode polypeptides of approximately 83 kDa, and the *psaC* gene product is a protein of 8.9 kDa which binds the F_A/F_B iron-sulfur centers. Golbeck and coworkers [3,4] have found that the photosystem I core protein, which contains P700, A_0, A_1 and F_X, can be prepared in a fully functional state by treating a photosystem I complex with chaotropic agents (e.g. 6.8 M urea). The photosystem I core protein is recovered in the retentate following ultrafiltration over a YM-100 membrane, and the low molecular mass proteins, including the PsaC, PsaD, PsaE, and possibly PsaG polypeptides, are recovered in the YM-100 filtrate ([5], N. Li, J.H. Golbeck, D.A. Bryant and G. Frank, unpublished results). Although the terminal iron-sulfur clusters are destroyed by the chaotrope treatment, the F_A/F_B iron-sulfur clusters can be reconstituted in the presence of $FeCl_3$, Na_2S and β-mercaptoethanol or DTT to yield a PsaC protein completely functional in accepting electrons from P700 [5]. The reconstituted PsaC protein could be rebound onto photosystem I core protein: moreover, it seemed likely that the PsaD and PsaE polypeptides were also rebound [5].

These observations suggested that it might be possible to produce the *psaC*, *psaD*, and *psaE* gene products in *Escherichia coli* and reconstitute electron transport to the F_A/F_B centers by reinserting the iron-sulfur clusters in the presence of the photosystem I core protein. In this communication, we report the overexpression in *E. coli* of a PsaC fusion protein and a PsaD protein and demonstrate that these proteins can be combined with photosystem I core particles of *Synechococcus* sp. PCC 6301 to reconstitute electron transport to the terminal electron acceptors.

2. MATERIALS AND METHODS

The strains of *Escherichia coli* used in this study were strains DH5α (Bethesda Research Laboratories, Gaithersburg, MD), BL21, and BL21(DE3) [6]. The *psaC* gene from *Synechococcus* sp. PCC 7002 and the *psaD* gene from *Nostoc* sp. PCC 8009 were cloned in plasmid vector pUC19 [7] and their nucleotide sequences were determined ([8], E. Rhiel, V.L. Stirewalt and D.A. Bryant, manuscript in preparation). The construction of an expression vector for the *psaC* gene is

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Abbreviations: Tris, tris(hydroxymethyl)-aminomethane; DTT, DL-dithiothreitol; DCPIP, 2,6-dichlorophenolindophenol; IPTG, isopropyl β-D-thiogalactopyranoside; MES, 2-[*N*-morpholino)-ethanesulfonic acid

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shown in Fig. 1. A 436 bp *Hpa*I–*Nco*I fragment containing the *psaC* gene was made blunt-ended by treatment with Mung bean nuclease and inserted into the *Hin*cII site of pUC19 to generate plasmid pCHN2. An *Nco*I linker (6-mer) was then inserted into the *Hin*cII site of plasmid pCHN2 yielding plasmid pCHN2N. This plasmid contained a variant *psaC* gene, designated *psaC1*, carrying a four-codon extension at its 5′ end. The *Nco*I-*Bam*HI fragment of pCHN2N was cloned into plasmid pKK480-3 (Dr J. Brosius, Mount Sinai School of

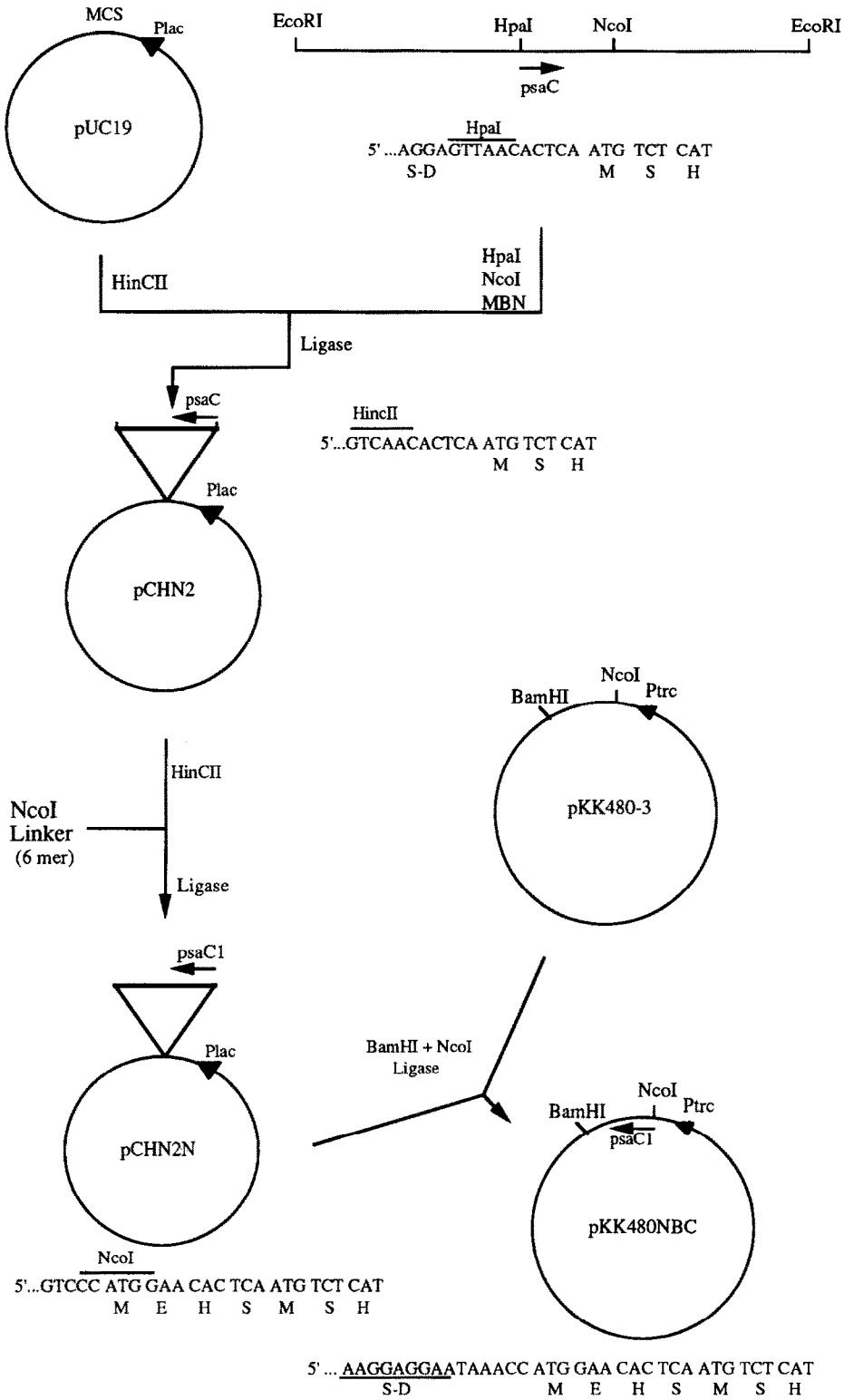

Fig. 1. Diagram showing the construction of plasmids leading to plasmid pKK480NBC for the expression of the PsaC1 protein in *E. coli*. The sequences shown for each step in the construction indicate the positions of restriction sites, start codons, and/or Shine-Dalgarno (S-D)-type sequences. MBN, Mung bean nuclease. For additional details, see section 2.

Medicine, New York, personal communication) by replacing its *NcoI-BamHI* fragment. In the resultant plasmid pKK480NBC, the *psaC1* gene is expressed under the control of the P_{trc} promoter. The expression of the *psaC1* gene in *E. coli* strain BL21 was carried out according to the method described [9] except that medium M9ZB [6] supplemented with 50 mM ferric ammonium citrate was used. Expression was continued for 8 h after the addition of 0.5 mM IPTG. Whole-cell extracts were prepared as described [10] except that 2 mM DTT was added to the cell suspension. Inclusion bodies of the PsaC1 polypeptide were obtained by centrifugation of the whole-cell extract at 7650 × g for 5 min at 4°C. The pellet was washed once with TS buffer containing 20 mM Tris-HCl, pH 8.0, and 10 mM NaCl, and resuspended in TS buffer.

Complete details concerning the construction of an expression plasmid and expression of the *psaD* gene product will be published elsewhere (J. Zhao and D.A. Bryant, in preparation). Briefly, the *psaD* gene of *Nostoc* sp. PCC 8009 was inserted into plasmid pET-3a [6] to generate plasmid pET-3a/D. The *psaD* gene product was overexpressed as described [6] and was isolated as inclusion bodies as described above except that growth medium NYZCM [11] was used. Solubilization and refolding of the protein were carried out as described [12] except that 7.0 M urea was used. The solubilized and refolded protein was absorbed to CM-Sepharose CL-6B and the column was developed with a linear gradient of NaCl (50 to 1000 mM NaCl) prepared in 50 mM Tris-HCl buffer, pH 8.0. Fractions containing the PsaD protein were pooled and concentrated by ultrafiltration.

The Photosystem I core protein was isolated from a *Synechococcus* sp. PCC 6301 [4]. Reconstitution of the F_A/F_B iron sulfur clusters and rebinding of the Photosystem I core protein were performed according to the following protocol: (i) a solution of 50 mM Tris-HCl, pH 8.3, containing the Photosystem I core protein at 5 $\mu g \cdot ml^{-1}$ chlorophyll *a*, was purged with oxygen-free nitrogen in an anaerobic cuvette; (ii) after 2 h, DTT was added through a septum to a final concentration of 0.01%; (iii) the PsaC1 apoprotein was added at an 80-fold molar excess (relative to P700); (iv) after 10 min an aliquot of 30 mM $FeCl_3$ was slowly added to a final concentration of 0.15 mM; (v) after 5 min an aliquot of 30 mM Na_2S was slowly added to a final concentration of 0.15 mM. Where indicated the PsaD protein was added simultaneously with the PsaC1 apoprotein at molar ratios indicated in the text.

Polypeptides were analyzed on polyacrylamide gels (10–20% linear gradients; acrylamide : bis-acrylamide ratio = 30 : 0.8) in the presence of sodium dodecyl sulfate. Protein samples were dialyzed into water and aliquots were subjected to automated Edman degradation at the University of Nebraska Protein Core Facility. Flash-induced absorption changes were determined at 698 nm as described previously [5].

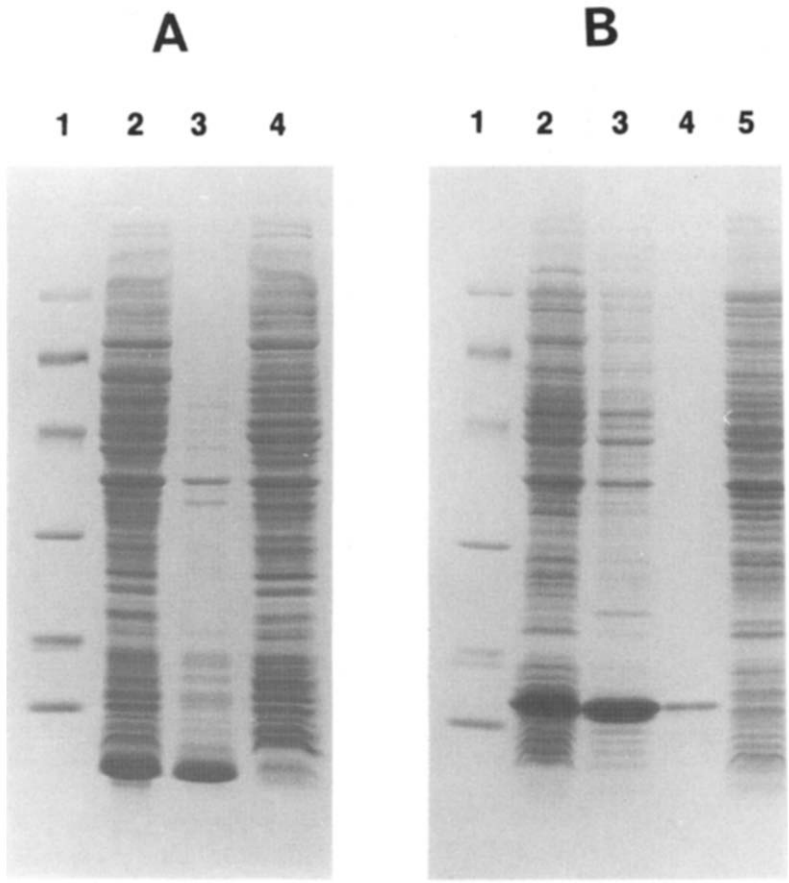

Fig. 2. Polyacrylamide gel electrophoresis of proteins produced in *E. coli* (A) (Lane 1) Molecular mass markers: phosphorylase B, 97.4 kDa; bovine serum albumin, 66.2 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa; soybean trypsin inhibitor, 21.5 kDa; lysozyme, 14.4 kDa (Lane 2) Whole-cell extract of *E. coli* strain BL21 harboring plasmid pKK480NBC eight hours after induction with IPGT. (Lane 3) Purified inclusion bodies of the PsaC1 protein from *E. coli* strain BL21 harboring plasmid pKK480NBC. (Lane 4) Whole-cell extract of *E. coli* strain BL21 harboring plasmid pKK480NBC in the absence of IPTG. (B) (Lane 1) Molecular mass markers as for lane 1, Panel A. (Lane 2) Whole-cell extract of *E. coli* strain BL21 harboring plasmid pET-3a/D five hours after induction with IPTG and four hours after the addition of rifampicin. (Lane 3) Purified inclusion bodies of the PsaD protein from *E. coli* strain BL21 harboring plasmid pET-3a/D. (Lane 4) Purified PsaD protein after chromatography on CM-Sepharose CL-6B. (Lane 5) Whole-cell extract of *E. coli* strain BL21 harboring plasmid pET-3a.

The sample was repetitively flashed with a xenon flashlamp (PRA Model 6100E) at a frequency of 0.3 Hz for a total of 16 averages.

3. RESULTS

Fig. 1 shows the manipulations employed to construct plasmid pKK480NBC for over-expression of the PsaC1 protein. Induction of PsaC1 expression by the addition of IPTG resulted in the accumulation of the polypeptide as inclusion bodies representing as much as about 20% of the total cellular protein (Fig. 2, Panel A, lane 2). The inclusion of additional iron in the medium seemed to enhance the stability of the protein and greatly increased the total yield of protein (data not shown). The *E. coli* cells expressing the *psaC1* gene and the isolated inclusion bodies were brown in color, suggesting that a substantial amount of iron was chelated by the protein. When analyzed by polyacrylamide gel electrophoresis, the inclusion bodies were observed to

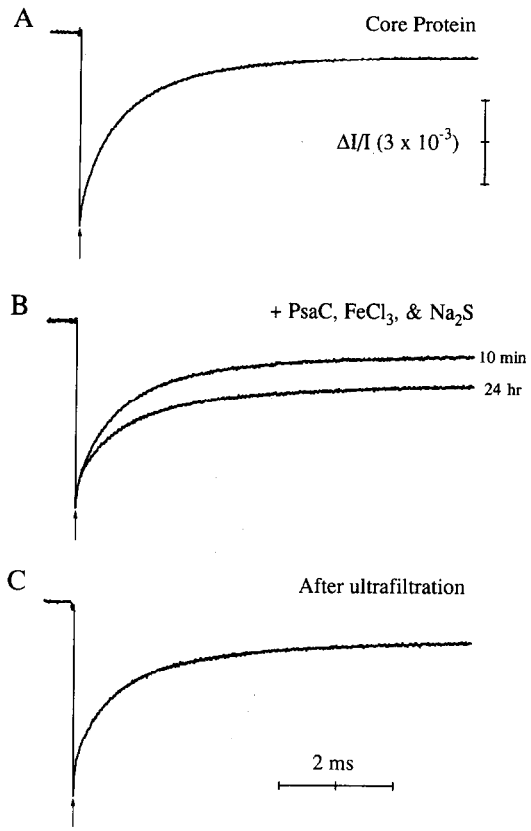

Fig. 3. Flash-induced absorption transient of P700 after addition of the PsaC1 protein to a photosystem I core protein. (A) Absorption transient at 698 nm in the *Synechococcus* sp. PCC 6301 photosystem I core protein. (B) Absorption transient 10 min and 24 h after addition of 150 μM $FeCl_3$, 150 μM Na_2S, 0.01% DTT, and the PsaC1 fusion protein in a molar ratio of 80 PsaC1 proteins per photosystem I core protein. (C) Absorption transient after ultrafiltration with 4 vols of 50 mM Tris-HCl buffer, pH 8.3, containing 0.04% Triton X-100 over a YM-100 membrane. All measurements were performed at 5 $\mu g \cdot ml^{-1}$ chlorophyll *a* in 50 mM Tris-HCl buffer, pH 8.3, containing 1.7 mM ascorbate and 0.033 mM DCPIP under anaerobic conditions.

be highly purified (Fig. 2A, lane 3). N-Terminal amino acid sequence analysis of the total inclusion body protein revealed a single sequence which matched precisely that expected from the construction (see Fig. 1): MEHSMSHSVK... Since the PsaC proteins of *Synechococcus* sp. PCC 6301 (N. Li, J.H. Golbeck, D.A. Bryant and G. Frank, unpublished results) and *Synechococcus* sp. PCC 7002 (E. Rhiel and D.A. Bryant, unpublished results) have the N-terminal sequence SHSVK..., the PsaC1 protein produced in *E. coli* contains an N-terminal extension of five amino acids as compared to authentic *psaC* gene products.

E. coli cells harboring plasmid pET-3a/D accumulated large amounts (approximately 20% of the total cellular protein) of the PsaD protein as inclusion bodies when expression of the T7 RNA polymerase was triggered by the addition of IPTG (Fig. 2B, lane 2). The purified inclusion bodies (Fig. 2B, lane 3) could be solubilized by treatment with 7.0 M urea and Psa purified to electrophoretic homogeneity by chromatography on CM-Sepharose CL-6B (Fig. 2B, lane 4). N-Terminal amino acid sequence analysis of the purified PsaD protein revealed a single sequence, AEQLSGKTPL..., which was identical to that determined for the protein isolated from the photosystem I complex of this cyanobacterium [8,13].

Fig. 3 shows the flash-induced absorption transient at 698 nm before and after addition of the PsaC1 protein to a *Synechococcus* sp. PCC 6301 photosystem I core protein. The flash-induced absorption change in the core preparation decays with a half-time of 1.2 ms, which is diagnostic of the back-reaction between $P700^+$ and F_X^- in the absence of the terminal iron-sulfur centers, F_A/F_B (Fig. 3A). When the PsaC1 protein is added to the photosystem I core protein at a molar ratio of 80:1 in the presence of $FeCl_3$, Na_2S, and DTT and incubated for 10 min, there is little change in the kinetics of the $P700^+$ transient (Fig. 3B). After 24 h of incubation there is a slightly greater suppression of the 1.2 ms back-reaction (Fig. 3B), but when this sample is washed by ultrafiltration over a YM-100 membrane, the amount of the slow phase diminishes (Fig. 3C). The iron-sulfur reconstitution reagents alone in the absence of the PsaC1 apoprotein had only a small effect on the $P700^+F_X^-$ back-reaction kinetics at these concentrations (data similar to 10 min in Fig. 3B).

One feature of this attempted reconstitution is that the PsaC1 protein has been added to a *Synechococcus* sp. PCC 6301 core protein in the absence of the PsaD and PsaE polypeptides from photosystem I. The rebinding experiments reported earlier [5] were performed by adding back a mixture of the polypeptides that were removed from the photosystem I complex by chaotropic agents - the PsaC, PsaD and PsaE polypeptides - in the presence of the iron-sulfur reconstitution reagents. These studies showed nearly complete reconstitution of electron flow to F_A/F_B after 60 min of

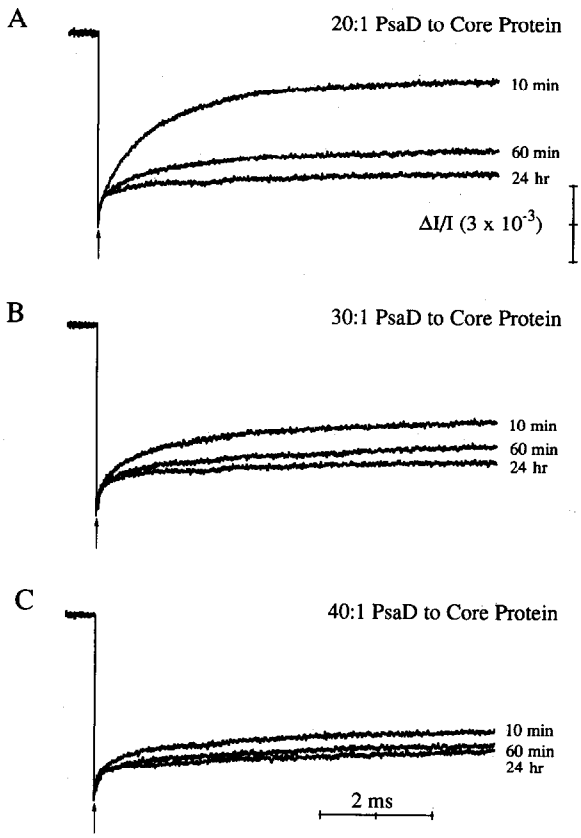

Fig. 4. Time course of the flash-induced absorption transient of P700 upon addition of the PsaC1 and PsaD proteins in molar ratios of 80 PsaC1 proteins to (A) 20 PsaD, (B) 30 PsaD, and (C) 40 PsaD proteins per photosystem I core protein. The reconstitution mixtures contained 150 μM $FeCl_3$, 150 μM Na_2S, 0.01% DTT. All measurements were performed at 5 $\mu g \cdot ml^{-1}$ chlorophyll *a* in 50 mM Tris-HCl buffer, pH 8.3, containing 1.7 mM ascorbate and 0.033 mM DCPIP under anaerobic conditions with no further purification.

incubation with a 20:1 molar ratio of *Synechococcus* sp. PCC 6301 PsaC apoprotein to photosystem I core protein [5]. The failure of the PsaC1 polypeptide to reconstitute electron flow to F_A/F_B raised the possibility that another low molecular mass polypeptide may be necessary for the rebinding of PsaC1 to the Photosystem I core protein. Fig. 4 shows the effect of the addition of the PsaD protein to the reaction mixture which contains the PsaC1 protein, the iron-sulfur reconstitution cofactors, and the *Synechococcus* sp. PCC 6301 core protein. The flash-induced P700 absorption transient is shown after 10 min, 1 h, and 24 h incubation at a constant molar ratio of 80:1 for the PsaC1 protein to Photosystem I core protein. At a molar ratio of 20:1 PsaD to core protein, the 1.2 ms $P700^+F_X^-$ transient is replaced in 24 h with long-lived optical transient. Measurements at a slower timescale indicate a half-time of 40 ms (not shown), which is diagnostic of the $P700^+$ $[F_A/F_B]^-$ back-reaction. When the molar ratio is increased to 30:1, a shorter period of incubation time produces the same effect, and when the molar ratio is 40:1, the effect is observed within a few minutes. Clearly, the higher the molar ratio of PsaD protein to photosystem I core protein, the faster the replacement of the 1.2-ms, $P700^+$ F_X^- back-reaction with the 40-ms $P700^+$ $[F_A/F_B]^-$ back-reaction.

When the PsaC1, PsaD, and photosystem I core proteins are incubated at molar ratios of 80:20:1 for 24 h with $FeCl_3$, Na_2S, and DTT and then washed by ultrafiltration over a YM-100 membrane, the 1.2 ms, $P700^+$ F_X^- back-reaction (Fig. 5A) continues to be replaced with a long-lived transient (Fig. 5B). This additional purification step, which should remove all inorganic and organic cofactors and all unbound polypeptides with a mass < 100 kDa, indicates that the PsaC1 holoprotein or a binary complex of the PsaC1 and PsaD proteins are not simply serving as a soluble electron acceptor from F_X^-. The replacement of a 1.2-

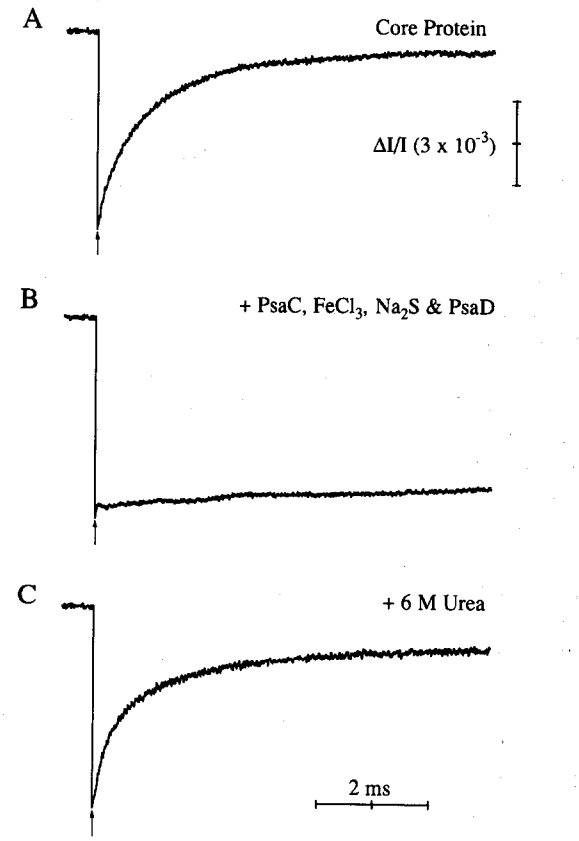

Fig. 5. Flash-induced absorption transient of P700 in the photosystem I core protein and in the reconstituted photosystem I complex. (A) Absorption transient at 698 nm in the *Synechococcus* sp. PCC 6301 photosystem I core protein. (B) Absorption transient of the reconstituted photosystem I complex after washing over a YM-100 ultrafiltration membrane. The reaction mixture was incubated for 24 h with 150 μM $FeCl_3$, 150 μM Na_2S, 0.01% DTT, PsaC1 protein, PsaD protein, and photosystem I core protein at a molar ratio of 80:20:1. (C) Absorption transient 30 min after addition of 6 M urea to the reconstituted photosystem I complex. All measurements were performed at 5 $\mu g \cdot ml^{-1}$ chlorophyll *a* in 50 mM Tris-HCl buffer, pH 8.3, containing 1.7 mM ascorbate and 0.033 mM DCPIP under anaerobic conditions.

ms optical transient with a long-lived transient is identical to that found when the authentic PsaC polypeptide (and other low molecular mass polypeptides released with chaotropes from the photosystem I complex of *Synechococcus* sp. PCC 6301) are rebound to a *Synechococcus* sp. PCC 6301 photosystem I core protein in the presence of $FeCl_3$, Na_2S, and β-mercaptoethanol [5]. When the reconstituted photosystem I sample from Fig. 5B is treated with 6 M urea, the long-lived transient due to the $P700^+$ $[F_A/F_B]^-$ back-reaction becomes gradually replaced with a 1.2 ms transient characteristic of the $P700^+$ F_X^- back-reaction. As shown in Fig. 5C, the reaction is complete in about 30 min. This behavior is identical to that found with either a native *Synechococcus* sp. PCC 6301 photosystem I complex or with a photosystem I complex that has been reconstituted from the *Synechococcus* sp. PCC 6301 core protein and the low molecular mass polypeptides released earlier by chaotrope treatment [5].

4. DISCUSSION

We have shown that the PsaC1 fusion protein, a genetically engineered form of the PsaC protein of *Synechococcus* sp. PCC 7002, is capable of functioning as a terminal acceptor of electrons from P700 when the F_A/F_B iron-sulfur clusters are reinserted. Moreover, the presence of the PsaD protein appears to be an absolute requirement for the binding of the PsaC1 protein to a photosystem I core protein. We suggest that the PsaC1 and PsaD proteins form a ternary complex with the PsaA/PsaB heterodimer which is competent in electron flow from P700 to the terminal iron-sulfur clusters. Studies with the authentic PsaC protein will be needed to clarify this point, and attempts to produce such a protein in *E. coli* are underway.

The system described in this communication may be superior to in vivo mutagenesis systems for studying certain aspects of photosystem I. Interposon mutagenesis of the *psaA* and *psaB* genes of *Synechococcus* sp. PCC 7002 failed (J. Zhou and D.A. Bryant, unpublished observations), presumably because photosystem I function in this cyanobacterium is required for viability. Similar problems, as well as undesired secondary mutations, could arise during attempts to probe the structural and functional properties of the low molecular mass polypeptides of the photosystem I complex by site-directed mutagenesis. Expression of mutant proteins in *E. coli*, combined with in vitro reconstitution of these proteins onto photosystem I cores, provides a mechanism to study protein alterations that could severely perturb electron transport and thereby prevent viability in the cyanobacterium. The present study demonstrates the feasibility of this approach and has suggested a new role for the PsaD protein in photosystem I.

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