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Polypeptide composition of the Photosystem I complex and the Photosystem I core protein from *Synechococcus* sp. PCC 6301 *

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The polypeptide composition of the Photosystem I complex from *Synechococcus* sp. PCC 6301 was determined by sodium-dodecyl sulfate polyacrylamide gel electrophoresis and N-terminal amino acid sequencing. The PsaA, PsaB, PsaC, PsaD, PsaE, PsaF, PsaK and PsaL proteins, as well as three polypeptides with apparent masses less than 8 kDa and small amounts of the 12.6 kDa GlnB (P_{II}) protein, were present in the Photosystem I complex. No proteins homologous to the PsaG and PsaH subunits of eukaryotic Photosystem I complexes were detected. When the Photosystem I complex was treated with 6.8 M urea and ultrafiltered using a 100 kDa cutoff membrane, the resulting Photosystem I core protein was found to be depleted of the PsaC, PsaD and PsaE proteins. The filtrate contained these missing proteins, along with five proteolytically-cleaved polypeptides with apparent masses of less than 16 kDa and with N-termini identical to that of the PsaD protein. The PsaF and PsaL proteins, along with the three < 8 kDa polypeptides, were not released from the Photosystem I complex to any significant extent, but low-abundance polypeptides with N-termini identical to those of PsaF and PsaL were found in the filtrate with apparent masses slightly smaller than those found in the native Photosystem I complex. When the filtrate was incubated with $FeCl_3$, Na_2S and β-mercaptoethanol in the presence of the isolated Photosystem I core protein, the PsaC, PsaD and PsaE proteins were rebound to reconstitute a Photosystem I complex functional in light-induced electron flow from P700 to F_A/F_B. In the absence of the iron-sulfur reconstitution agents, there was little rebinding of the PsaC, PsaD or PsaE proteins to the Photosystem I core protein. No rebinding of the truncated PsaD polypeptides occurred, either in the presence or absence of the iron-sulfur reagents. The reconstitution of the F_A/F_B iron-sulfur clusters thus appears to be a necessary precondition for rebinding of the PsaC, PsaD and PsaE proteins to the Photosystem I core protein.

Introduction

The Photosystem I core complex in cyanobacteria and higher plants is comprised of twelve known polypeptides, labeled PsaA to PsaL (for reviews, see Refs. 1, 2). The core antenna chlorophyll *a* molecules and electron transport components P700, A_0, A_1 and F_X are located on the PsaA and PsaB proteins and function in light-harvesting, primary charge separation and secondary charge stabilization. The PsaC protein binds the F_A and F_B iron-sulfur centers which serve as tertiary electron acceptors [3–7]. The roles of the PsaD–PsaL proteins in Photosystem I are only imperfectly understood. Cross-linking studies suggest that the PsaD protein interacts with soluble ferredoxin and may be responsible for the docking of this protein onto the stromal (reducing) side of the Photosystem I complex [8–10]. The PsaD protein has also been implicated in the binding of a PsaC fusion protein to the Photosystem I core protein [11]. Cross-linking studies suggest that the PsaF protein is located on the lumenal (oxidiz-

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Abbreviations: Chl, chlorophyll; CP1, chlorophyll protein 1; PVDF, poly(vinylidene difluoride); DCPIP, dichlorophenolindophenol; DTT, dithiothreitol; β-ME, β-mercaptoethanol; LHC I, light-harvesting chlorophyll 1.

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ing) side of the Photosystem I complex and interacts with plastocyanin or cytochrome *c*-553 [10,12,13]. The PsaE protein is believed to lie on the stromal side of the Photosystem I complex, but its function is unknown [14–18]. The PsaG and PsaH proteins are also believed to occur on the stromal side of the Photosystem I complex, but have only been identified in eukaryotes [19–22]. The PsaI, PsaJ and PsaK proteins are small, membrane-intrinsic polypeptides whose roles in Photosystem I assembly and function are not understood [22–26]. A recently identified, membrane-intrinsic protein denoted PsaL has been identified and characterized in barley [27] and, by implication, in cyanobacterial [24,28,29] Photosystem I preparations. Finally, a polypeptide with an apparent mass of 4.8 kDa has been identified in cyanobacterial Photosystem I complexes from *Synechococcus vulcanus* and *Anabaena variabilis* ATCC 29413 (Ref. 24; Nyhus, K., Ikeuchi, M., Inoue, Y. and Pakrasi, H., personal communication), but the gene encoding this subunit has not yet been characterized.

Previously, our laboratory had defined Photosystem I subthylakoid preparations primarily on their spectroscopic characteristics: the 'Photosystem I complex' contains components P700 through F_A/F_B; the 'Photosystem I core protein' contains components P700 through F_X; and the 'SDS-CP1 particle' contains only P700 and A_0 (see Refs. 30–34). With the recent advances in high resolution gel systems for very-low-molecular-mass polypeptides, and the availability of amino acid sequencers sufficiently sensitive to analyze proteins purified by preparative SDS-PAGE, we decided to reanalyze the polypeptide composition of the the Photosystem I complex and the Photosystem I core protein from *Synechococcus* sp. PCC 6301. In particular, we wanted to reexamine our earlier assertion that the P700- and F_X-containing Photosystem I core protein from *Synechococcus* sp. PCC 6301 was devoid of low-molecular-mass polypeptides [30,32]. We also wanted to identify the polypeptides with apparent masses of 16.4 and 8.9 kDa that had rebound to the Photosystem I core protein upon addition of the F_A/F_B-reconstituted PsaC protein [33]. This study was aided by the availability of amino acid sequences for what is considered to be the entire complement of Photosystem I polypeptides from cyanobacteria, green algae and higher plants (for compiled list, see Refs. 2 and 27).

Materials and Methods

The Photosystem I complex from *Synechococcus* sp. PCC 6301 was isolated by treating the thylakoid membranes with 1% Triton X-100 followed by two sucrose-density ultracentrifugation steps, the first in the presence of 0.1% Triton X-100 and the second in its absence [30,32]. The Photosystem I core protein was isolated from a *Synechococcus* sp. PCC 6301 Photosystem I complex with 6.8 M urea [30,32]. The low-molecular-mass polypeptides were recovered and concentrated by ultrafiltration over a YM-2 membrane. Reconstitution of the F_A/F_B iron-sulfur clusters and rebinding of the low-molecular-mass polypeptides to the Photosystem I core protein were performed according to the protocol outlined in Refs. 33, 34.

Polypeptides were analyzed on $120 \times 100 \times 1.5$ mm polyacrylamide slab gels (16.5% T, 6% C separating gel; 4% T, 3% C stacking gel; 10% T, 3% C spacer gel) according to the method of Schägger and Von Jagow [35]. The samples were incubated in 62.5 mM Tris buffer (pH 6.8), 2% SDS, 10% glycerol and 5% β-ME for 3 min at 90 °C. The sample load was equivalent to 12.5 μg chlorophyll. Electrophoresis was carried out at room temperature at a constant current of 30 mA for 8–10 h. Gels were stained with 0.125% Coomassie brilliant blue and destained first with 50% methanol and 10% acetic acid and then with 5% methanol and 7% acetic acid. The gels were dried with Bio-Gel Wrap (Biodesign product No. G101) over an incandescent lamp for 3–4 h. The transparent gel was scanned with an LKB 2202 Laser Densitometer and digitized with a Keithley voltmeter (Model 195A) and a Macintosh Plus computer. Electrophoresis of the preparative gels was performed as described above except that after 1 h of staining and destaining, the gel was soaked with distilled water. Single stained bands corresponding to the individual polypeptides were excised with a razor blade. The bands were stored at −80 °C until electroelution for amino-acid sequencing.

Gel fragments were soaked for 3–4 h in 25 mM Tris/192 mM glycine buffer (pH 8.3), containing 0.1% SDS at 37 °C prior to electrophoretic transfer to PVDF (Immobilon-P, Millipore Corp., Bedford, MA) membrane circles with a Bio-Rad Model 422 Electro-Eluter (Richmond, CA). Two 4 mm circles of PVDF membrane were placed inside M_r 3500 cut-off dialysis caps in the elution apparatus, and proteins were eluted overnight at 100–200 V with 25 mM Tris 192 mM glycine electrode buffer (containing no SDS) (pH 8.3) at room temperature. Filters carrying eluted proteins were washed twice with 10 ml of 100% methanol and four times with 10 ml of 20% (v/v) methanol/water. Filters carrying eluted proteins were stored at −20 °C until subjected to amino-acid sequencing.

Amino-acid sequences were determined with a Knauer model 810 sequencer (Dr. Ing. H. Knauer, Berlin) whose main feature is a cross-flow reaction cell which directly accommodates specially shaped pieces of PVDF membrane carrying the sample. In order to place the 4-mm disks from the electroelution procedure in the reactor, two 4-mm holes were punched into an original PVDF profile and the disks were inserted into these holes, thus completely filling the space in the

reaction cell with a layer of PVDF. Phenylthiohydantoin adducts were automatically injected into an HPLC system for identification. The original elution gradient of the HPLC was replaced by an isocratic procedure which has been described previously [36]. Protein samples ranging from approx. 25 to 250 pmol were successfully sequenced for 20 to 40 cycles using the methods described above. For example, the P_{II} (GlnB) polypeptide was successfully sequenced in one experiment for 24 cycles from an initial yield of about 15 pmol (the protein was subsequently resequenced for 40 cycles). Sequences of up to 40 to 50 residues have been obtained when initial yields were in the range of 200 pmol.

EPR spectra and flash-induced absorption transients were measured as described previously [32]. Chlorophyll *a* was determined in 80% acetone [37]. Protein concentration was determined by the method of Bradford [38].

Results

The Photosystem I complex

The spectroscopic characteristics of the *Synechococcus* sp. PCC 6301 Photosystem I complex are shown in Fig. 1 (reviewed in Ref. 39). The room-temperature, flash-induced absorption transient shows a 30 ms half-time, which corresponds to the $P700^+[F_A/F_B]^-$ back-reaction (Fig. 1A), and the low temperature EPR spectrum shows the characteristic resonances of F_A^- and F_B^- at $g = 2.05$, 1.94, 1.92 and 1.89 (Fig. 1B). The polypeptide composition of the Photosystem I complex after SDS-PAGE is shown in Fig. 1C.

The Photosystem I complex resolves into a broad, chlorophyll-containing band which constitutes the PsaA/PsaB heterodimer and ten low-molecular-mass proteins (Table I). The N-terminal sequence of the polypeptide at 16.4 kDa identifies this protein as PsaD (Table II; [10]). The sequence determined directly differs at two positions (amino acids 14 and 23) from that deduced from the gene sequence presented by Wynn et al. [10], but is identical to that determined by Alhadeff et al. [40] by amino acid sequence analysis. The reason for this discrepancy is not known. The shoulder at 15.8 kDa represents a distinct protein whose N-terminal sequence (Tables I and II) corresponds most closely to the 18 kDa, PsaL protein from barley [27] rather than to the 10.8 kDa, PsaG protein from spinach [19]. It is likely that sequences previously identified as PsaG from *Synechococcus* sp. PCC 7002 and *Synechococcus vulcanus* also correspond to PsaL. This issue is complicated by the observation that some intermolecular homology is observed between some PsaG and PsaL polypeptides [27], a situation similar to that observed at

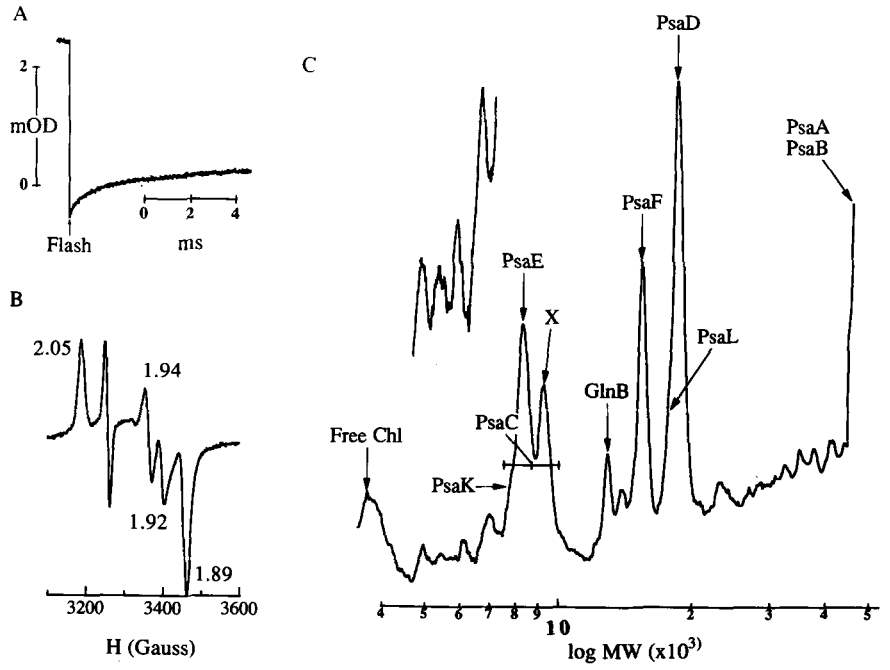

Fig. 1. Polypeptide composition and spectroscopic properties of the Photosystem I complex. The complex was isolated from *Synechococcus* sp. PCC 6301 membranes with Triton X- 100, followed by ultracentrifugation in a sucrose gradient. (A) Flash-induced absorption transient of P700. Measurements at 698 nm were performed on 5 μg Chl/ml in 50 mM Tris buffer (pH 8.3), containing 1.7 mM ascorbate and 33 μM DCPIP. (B) EPR spectrum of the F_A/F_B iron-sulfur clusters. The sample was suspended in 50 mM Tris buffer (pH 8.3), containing 1 mM sodium ascorbate and 0.3 mM DCPIP at 125 μg Chl/ml and illuminated during freezing. Spectrometer conditions: temperature, 19 K; microwave power, 20 mW; microwave frequency, 9.128 GHz; receiver gain, $5.0 \cdot 10^3$; modulation amplitude, 10 G at 100 kHz. (C) Laser densitometric tracing of the polypeptides present in the Photosystem I complex after separation by SDS-PAGE. The identities of the proteins were determined by N-terminal amino acid sequencing. The elevated inset shows the region from 4.5 to 8 kDa amplified in software.

TABLE I

N-terminal amino-acid sequences of Synechococcus sp. PCC 6301 Photosystem I polypeptides (see Fig. 1C)

Residues shown in parentheses are tentative assignments; slashes refer to ambiguity between two possible amino acids.

Apparent mass (kDa)	Gene	Protein	N-terminal amino-acid sequence
64	*psaA*	PsaA [a]	n.d. [b]
64	*psaB*	PsaB [a]	n.d.
16.4	*psaD*	PsaD	AETLTGKTPVFGGSTGGLLKSAET
15.8	*psaL*	PsaL	AQDVIANGG(T)AEIGN(L)A(SDK)N
14.7	*psaF*	PsaF	DVAGLTPTSESPRFIQRAEAAATPQAKAR
12.6	*glnB*	GlnB (P_{II})	MKKIEAIIRPFKLDEVKIALVNAGIVGMTVSEVRGFGRQK
9.3		'X'	(A)G(G/A)V(T/G)MIETLGFPAVVEAADAMVKAARVTLVG
8.9	*psaC*	PsaC	SHSVKIYDT(C)IG(C)TQ(C)VR
8.4	*psaE*	PsaE	AIARGDKVRILRPESYWF
8.2	*psaK*	PsaK	(Partial Sequence Only)
7.6 [c]	*psaJ*	PsaJ	n.d.
6.5 [c]		'4.8-kDa'	n.d.
4.7 [c]	*psaI*	PsaI	n.d.

[a] Assigned from previous studies.
[b] n.d., not determined.
[c] The PsaJ, '4.8 kDa' protein, and PsaI proteins are tentatively assigned based upon similar results obtained with the cyanobacteria *Synechococcus vulcanus* [24] and *Anabaena* sp. ATCC 29413 (Nyhus, K., Ikeuchi, M., Inoue, Y., and Pakrasi, H. personal communication).

the N-terminal regions of PsaD and PsaE in barley [41]. The PsaL protein of *Synechococcus vulcanus*, on the other hand, shows a high degree of homology with those of barley and *Synechococcus* sp. PCC 6301 (Table II).

The polypeptide at 14.7 kDa has an N-terminal

TABLE II

Comparisons of N-terminal amino-acid sequences for Photosystem I polypeptides

Sequences which begin with a methionine residue in parentheses (M) have been confirmed by sequence analyses of the corresponding genes; other parentheses indicate tentatively identified residues.

Protein	Organism	Sequence	References
PsaD	*Synechococcus* sp. PCC 6301	(M)AETLTGKTPVFGGSTGGLLKSAET	this work; 10, 41
PsaD	*Nostoc* sp. PCC 8009	(M)AEQLSGKTPLFAGSTGGLLTKANV	47
PsaD	*Synechocoystis* sp. PCC 6803	(M)TE-LSGQPPKFGGSTGGLLSKANR	54
PsaD	*Synechococcus vulcanus*	TT-LTGQPPLYGGSTGGLLSADT-	24
PsaL	*Synechococcus* sp. PCC 6301	AQDVIANGG(T)AEIGN(L)A(SDK)N	this work
PsaL	*Synechococcus* sp. PCC 7002	MDIIQHGG D PQVGN L A TPI NASAFIKAKI	28
PsaL	*Synechococcus vulcanus*	AEELVKPYNG D PFVGH L S TP	24
PsaL	*Anabaena* sp. ATCC 29413	EVVFPAGR D PQWGN L E TPV NASPLVKKF	unpublished results [a]
PsaF	*Synechococcus* sp. PCC 6301	DVAGLTPTSESPRFIQRAEAAATPQAKAR	this work
PsaF	*Synechococcus vulcanus*	DVAGLVPAKDSPAFQKRAAAAVNTTAD	24
PsaF	*Cyanophora paradoxa*	DVAGLIPCSQSDAFERRLKNTTQRLENRL	unpublished results [b]
GlnB	*Synechococcus* sp. PCC 6301	MKKIEAIIRPFKLDEVKIALVNAGIVGMTVSEVRGFGRQK	this work; 44
GlnB	*Escherichia coli*	MKKIDAIIKPFKLDDVRERLAEVGITGMTVTEVKGFGRQK	55
GlnB	*Klebsiella pneumoniae*	MKKIDAIIKPFKLDDVREALAEVGITGMTVTEVKGFGRQK	56
PsaC	*Synechococcus* sp. PCC 6301	SHSVKIYDT(C)IG(C)TQ(C)VR	this work
PsaC	*Synechococcus* sp. PCC 7002	(M)SHSVKIYDT C IG C TQ C VRA	47
PsaC	*Synechococcus vulcanus*	(M)AHTVKIYDT C IG C TQ C VRA	24, 57
PsaC	*Nostoc* sp. PCC 8009	(M)SHTVKIYDT C IG C TQ C VRA	47
PsaE	*Synechococcus* sp. PCC 6301	(M)AIARGDKVRILRPESYWF	this work; 41, 47
PsaE	*Synechococcus* sp. PCC 7002	(M)AIERGSKVKILRKESYWY	47
PsaE	*Synechocystis* sp. PCC 6803	(M)ALNRGDKVSIKRTESYWY	17
PsaE	*Nostoc* sp. PCC 8009	(M)-VQRGSKVRILRPESYWF	47

[a] Nyhus, K., Ikeuchi, M., Inoue, Y. and Pakrasi, H., personal communication.
[b] Bryant, D.A. and Stirewalt, V.L., unpublished observations.

sequence (Table I) that is homologous to the PsaF protein from spinach [19], *Synechococcus vulcanus* [24] and *Cyanophora paradoxa* (Table II). The PsaF protein is highly variable in Photosystem I preparations from *Synechococcus* sp. PCC 6301 and other organisms (see Ref. 2 and Fig. 5 in Ref. 33). It is usually present when the isolation of the Photosystem I complex is performed with minimal exposure to Triton X-100, and is frequently lost when the complex is exposed to 0.2% to 1% Triton X-100 followed by sucrose density-gradient ultracentrifugation or ion-exchange chromatography [42]. Indeed, the ability of detergents to extract the PsaF protein has led to difficulties in identifying its presence, and therefore its role, in Photosystem I reactions (for discussion, see Refs. 2 and 43). We find that recentrifugation in the absence of Triton X-100 (see Materials and Methods) is critical to the retention of the PsaF protein on the *Synechococcus* sp. PCC 6301 complex.

The N-terminal sequence of the low-abundance polypeptide at 12.6 kDa (Table I) is identical to the previously reported sequence of the *Synechococcus* sp. PCC 6301 P_{II} (GlnB) protein, which is the product of the *gln B* gene [44]. The association of the GlnB protein with the Photosystem I complex was unexpected, but its presence may be related to a postulated role in State 1–State 2 transitions and energy-spillover between Photosystem II and Photosystem I [44]. Alternatively, it may have co-purified with the Photosystem I complex as a contaminant due to the application of the mild Triton X-100 treatment. The low abundance of this polypeptide in the Photosystem I preparation and its absence in other well-characterized preparations, including those isolated with a higher concentration of Triton X-100, suggests that this protein is probably not a legitimate subunit of the complex.

The doublet in the 8 to 10 kDa region contains four distinct polypeptides. The 9.3 kDa band, identified as 'X', represents a previously uncharacterized protein. This protein is similar in sequence to the putative products of two open reading frames which occur upstream from the *rbcLS* operon in *Synechococcus* sp. PCC 6301 [45] and 7942 [46] and which have been implicated in carboxysome assembly. Its stoichiometry, unlike that of the known Photosystem I polypeptides, varies from isolate to isolate, and it is absent when the Photosystem I complex is isolated and purified with 0.2 to 1% Triton X-100. Hence, this protein appears to be a co-purifying contaminant, and is not an integral component of the Photosystem I complex. This result is supported by the observation that, unlike PsaC, PsaD and PsaE, the protein does not rebind to the core protein. The N-terminal sequence of the 8.4 kDa polypeptide (Table I) corresponds to that of the PsaE protein in *Synechococcus* sp. PCC 6301 [40,47]. The N-terminal sequence of the polypeptide that occurs as a shoulder the low-molecular-mass side of PsaE is identified, tentatively, as the PsaK protein. Although we were not able to obtain an unambiguous sequence, a number of characteristic residues at positions 12 through 19 could be determined which are in homologous positions with other cyanobacterial PsaK proteins [24]. All gel slices in this region contained a polypeptide whose N-terminal sequence (Table I) is identical to that of the PsaC protein in *Synechococcus* sp. PCC 7002 (see Table II and Ref. 47). We believe that neither peak corresponds precisely to the PsaC protein, which most likely migrates as a diffuse band at 8.9 kDa that underlies both peaks [48].

The Schägger and Von Jagow gel system [35] resolves three very-low-molecular-mass polypeptides at 7.6, 6.5 and 4.7 kDa in the *Synechococcus* sp. PCC 6301 Photosystem I complex. Although the N-terminal sequences of these proteins were not determined, the pattern of polypeptides is very similar to those identified in the cyanobacteria *Synechococcus vulcanus* [24] and *Anabaena variabilis* ATCC 29413 (Nyhus, K., Ikeuchi, M., Inoue, Y. and Pakrasi, H., personal communication). Hence, it is probable that these bands correspond to the PsaJ, '4.8 kDa' and PsaI proteins, respectively. Because stain intensity is protein-specific, we are not able to determine whether these proteins are present in stoichiometric amounts in the *Synechococcus* sp. PCC 6301 Photosystem I complex.

A protein homologous to the PsaH protein of spinach was not found in *Synechococcus* sp. PCC 6301, nor has such a protein been reported in *Synechococcus* sp. PCC 7002 [28], *Synechococcus vulcanus* [24], or *Anabaena variabilis* ATCC 29413 (Nyhus, K., Ikeuchi, M., Inoue, Y. and Pakrasi, H., personal communication). The absence of this protein correlates positively with the suggestion that it could be involved in organization or assembly of the LHC I proteins onto Photosystem I [21]. Similarly, a protein with substantial homology to PsaG was not identified in *Synechococcus* sp. PCC 6301. PsaG proteins have been identified in spinach [19], barley [27], and *Chlamydomonas reinhardtii* [18], but have not yet been found in Photosystem I preparations from prokaryotes.

The YM-100 retentate: the Photosystem I core protein

The spectroscopic characteristics of the *Synechococcus* sp. PCC 6301 Photosystem I core protein are shown in Fig. 2. The room-temperature flash-induced absorption transient shows a 1.2 ms half-time, which corresponds to the $P700^+$ F_X^- backreaction (Fig. 2A), and the low-temperature EPR spectrum shows the characteristic, broad resonances of F_X^- at $g = 2.05$, 1.86 and 1.76 (Fig. 2B). The polypeptide composition of the Photosystem I core protein after SDS-PAGE is shown in Fig. 2C.

The Photosystem I core protein resolves into a

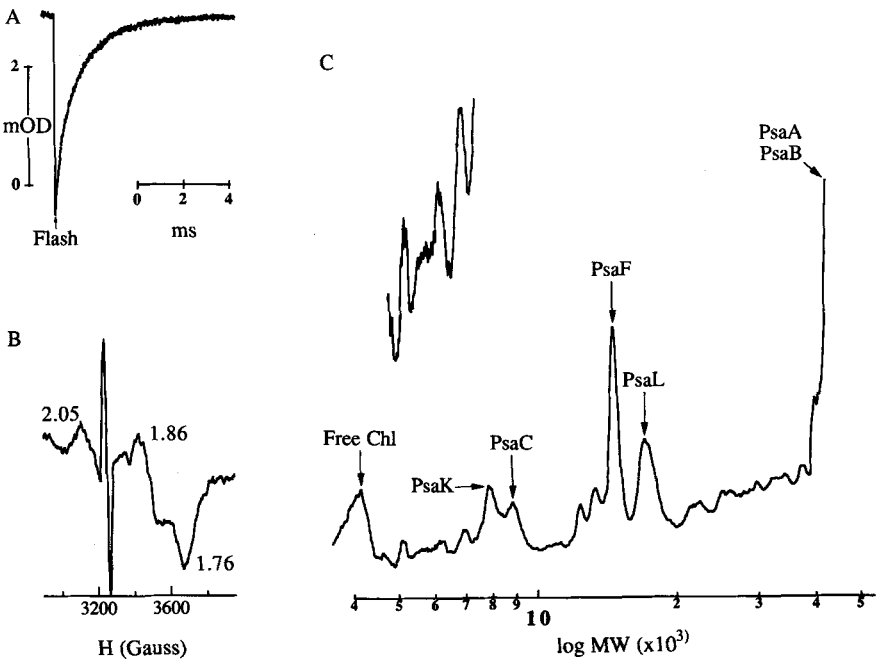

Fig. 2. Polypeptide composition and spectroscopic properties of the Photosystem I core protein. The core protein was isolated from the *Synechococcus* sp. PCC 6301 Photosystem I complex with 6.8 M urea for 15 min, followed by dialysis and ultracentrifugation in a sucrose gradient. (A) Flash-induced absorption transient of P700 (see Fig. 1A, legend). (B) EPR spectrum of the F_X iron-sulfur cluster. The sample was suspended in 50 mM Tris buffer (pH 8.3) containing 1 mM sodium ascorbate and 0.3 mM DCPIP at 500 μg Chl/ml and illuminated during freezing. The resonances were resolved by subtracting the light-off from the light-on spectrum. Spectrometer conditions were the same as in Fig. 1. (C) Laser densitometric tracing of the polypeptides present in the Photosystem I core protein after separation by SDS-PAGE (see Fig. 1C, legend). The elevated inset shows the region from 4.5 to 8 kDa amplified in software.

broad, chlorophyll-containing band which constitutes the PsaA/PsaB heterodimer and a subset of the low-molecular-mass proteins (Table I). As shown by N-terminal amino-acid sequencing, the PsaD protein at 16.4 kDa is completely absent. The PsaL protein at 15.8 kDa is retained, and is now shown to correspond to a rather diffuse zone which stains weakly with Coomassie blue. The PsaL protein is also retained in the Photosystem I core protein obtained by chaotrope treatment of the *Synechococcus* sp. PCC 7002 Photosystem I complex (data not shown). These observations are consistent with the assignment of the PsaL protein as a hydrophobic protein. Likewise, most of the PsaF protein is retained (compare Figs. 1C and 2C), and we suspect that this polypeptide is anchored into the membrane by its C-terminus (see below) which binds it tightly to the Photosystem I core. Since chaotropic agents remove peripheral membrane proteins, the presence of PsaL and PsaF in the Photosystem I core protein is expected: both contain hydrophobic domains that might span the thylakoid membrane [18,19,27].

The PsaE protein at 8.4 kDa is completely absent in the Photosystem I core protein, as is the 'X' protein at 9.3 kDa. The PsaK protein can now be clearly seen at 8.2 kDa. Since PsaK is found in CP1, an SDS preparation which only shows primary photochemistry between P700 and A_0 [26], its presence in the Photosystem I core protein was not surprising. Although the band at 8.9 kDa has been identified as a very small amount of remaining PsaC protein, the loss of area under the 8.4 to 9.5 kDa protein doublet generally agrees with the spectroscopic data (Fig. 2A and 2B) which indicates that the PsaC protein is depleted. Because the PsaC protein tends to run as a poorly-staining, diffuse band, it is difficult to quantify from the electrophoresis data alone.

The *Synechococcus* sp. PCC 6301 Photosystem I core protein contains three very-low-molecular-mass polypeptides with apparent masses of 7.6, 6.5 and 4.7 kDa. Their presence is consistent with the assessment that the PsaJ, the '4.8'-kDa' and PsaI proteins are highly hydrophobic and membrane-spanning.

The YM-100 filtrate: the low-molecular-mass polypeptides

All of the proteins missing in the Photosystem I core protein are recovered in the YM-100 filtrate. The PsaD, PsaC, PsaE and GlnB (P_{II}) proteins are found at positions in the electrophoretogram which correspond to their molecular masses as observed in the holocomplex (Fig. 3A; also see Fig. 1C). There are three additional polypeptides present which were found by amino acid sequencing to have N-termini identical to that of the PsaD protein when the YM-100 filtrate is assayed shortly after its recovery. After 90 min of exposure to chaotropic agents, the polypeptide pattern

changes to show a diminished amount of the intact PsaD protein at 16.4 kDa and at least five distinct cleavage products of the PsaD protein (Fig. 3B). The cleaved PsaD polypeptides in the YM-100 filtrate occur initially with their N-termini intact; hence the proteinase-sensitive sites must be exposed first at the C-terminus of the protein. These results suggest that a protease has copurified with the Photosystem I complex which is effective in cleaving the PsaD protein after it is released by chaotropes. This protease does not appear to cleave PsaD when it is bound to the Photosystem I complex, but does when it is dissociated and free in solution. Under the conditions employed to remove the PsaD protein, it is possible that the protein is substantially unfolded by the chaotrope treatment.

A low-abundance polypeptide with an N-terminus identical to the PsaF protein was found in the YM-100 filtrate at a mass approx. 1 kDa less than that found in the Photosystem I complex. Proteolytic cleavage near the C-terminus might release a small amount of this

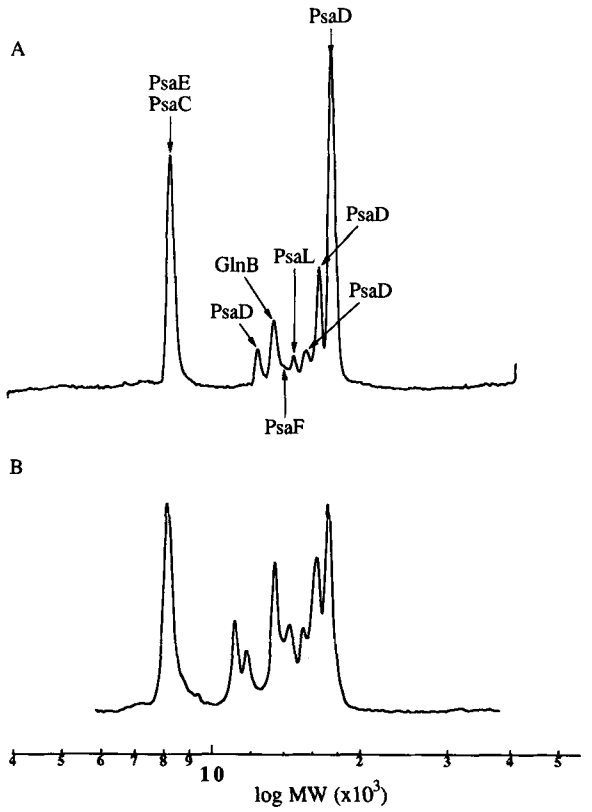

Fig. 3. Polypeptide composition of the low molecular mass polypeptides released from the Photosystem I complex by chaotropic agents. The released, low-molecular-mass polypeptides were removed from the Photosystem I core protein by ultrafiltration over a YM-100 membrane and concentrated over a YM-2 membrane. (A) Laser densitometric tracing of the polypeptides present in the YM-100 filtrate after SDS-PAGE. The Photosystem I complex was exposed to the 6.8 M urea for 15 min. (B) Same as (A) except that the Photosystem I complex was exposed to the 6.8 M urea for 90 min. The identities of the proteins were determined by N-terminal amino-acid sequencing.

polypeptide from the Photosystem I core. This observation is consistent with the suggestion that the C-terminus of the PsaF protein is hydrophobic and anchors the protein in the thylakoid membane [18,19]. A low-abundance polypeptide with an N-terminus identical to the PsaL protein is also found in the YM-100 filtrate at a mass about 2 kDa less than that found in the Photosystem I complex. We suspect that this relatively hydrophobic protein might also be cleaved near the position where the polypeptide enters the thylakoid membrane. Hydropathy analyses indicate that the barley PsaL protein probably has a membrane-spanning helix very near the C-terminus of the protein [27].

The reconstituted Photosystem I complex

The spectroscopic characteristics of the reconstituted *Synechococcus* sp. PCC 6301 Photosystem I core protein are shown in Fig. 4. The room-temperature, flash-induced absorption change shows a transition of the 1.2 ms, $P700^+$ F_X^- backreaction to a long-lived, $P700^+$ $[F_A/F_B]^-$ backreaction (Fig. 4A), and the low-temperature ESR spectrum shows the restoration of the $g = 2.05$, 1.94, 1.92 and 1.89 resonances of F_A^- and F_B^- (Fig. 4B). In the absence of $FeCl_3$ and Na_2S, most of the flash-induced absorption change continues to represent the 1.2 ms optical transient characteristic of the $P700^+$ F_X^- backreaction (Fig. 5A), and the low-temperature EPR spectrum remains largely that of F_X^-, with g-values of 2.05, 1.86 and 1.76 (Fig. 5B). The low-molecular-mass polypeptides that are rebound to the Photosystem I core protein under conditions of added $FeCl_3$, Na_2S and β-ME are shown in Fig. 4C.

N-terminal amino acid sequencing of the 16.4, 8.9 and 8.4 kDa polypeptides indicate that the PsaD, PsaC and PsaE proteins have rebound to the Photosystem I core protein concurrently with the reinsertion of the F_A/F_B iron-sulfur clusters. The stain intensity on SDS-PAGE of the PsaD protein matches that of PsaD in the control Photosystem I complex, indicating quantitative rebinding. Only the intact PsaD polypeptide, and not the proteolytic cleavage fragments derived from the PsaD protein, are competent to rebind to the Photosystem I core protein. Since the proteinase cleaves near the C-terminus, this region might be involved in binding to the Photosystem I complex or this region may be required for proper folding of the PsaD protein.

As in the control Photosystem I complex (Fig. 1C), the PsaC protein runs as a diffuse zone and is seen here as the broad shoulder at the high-molecular-mass side on the PsaE protein. The ratio of the PsaE protein to the PsaC protein (estimated to be approx. 1:1 to 2:1) was identical to that for the control Photosystem I complex. The sequencing analyses also showed that the 14.9 kDa polypeptide contains the retained PsaF protein (there is a slight loss encountered at each purifica-

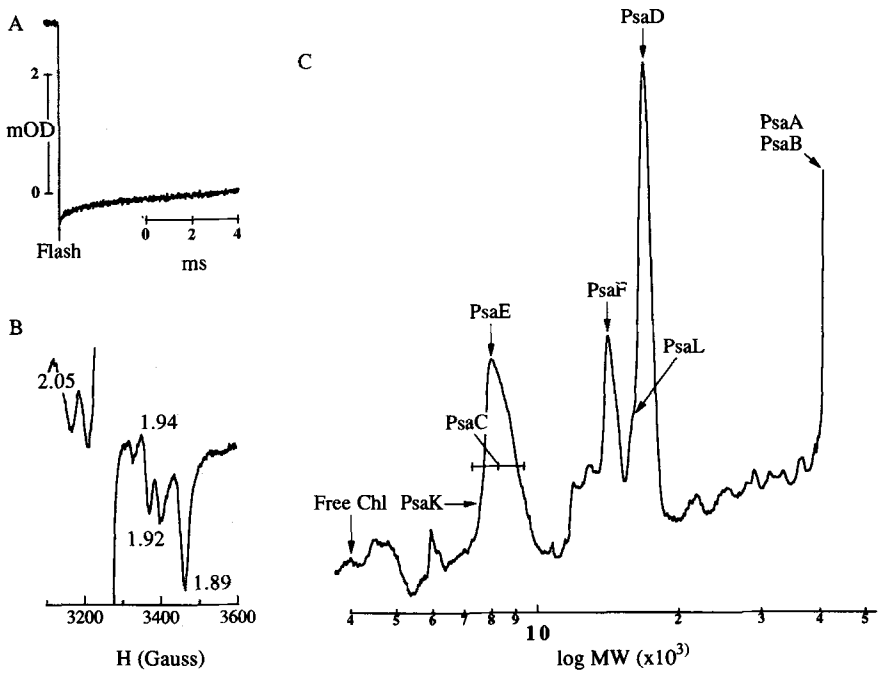

Fig. 4. Polypeptide composition and spectroscopic properties of the reconstituted Photosystem I complex. The reconstitution was performed by incubating the low-molecular-mass proteins in the YM-100 filtrate (Fig. 3) with the Photosystem I core protein (Fig. 2) in the presence of $FeCl_3$, Na_2S and β-ME for 20 h, followed by dialysis, ultrafiltration and ultracentrifugation in a sucrose gradient. (A) Flash-induced absorption transient of P700 (see Fig. 1A, legend). (B) ESR spectrum of the F_A/F_B iron-sulfur clusters (see Fig. 1B legend). (C) Laser densitometric tracing of the polypeptides present in the reconstituted Photosystem I complex after separation by SDS-PAGE. The identities of the proteins were determined by N-terminal amino acid sequencing. The presence of Triton X-100 in the reaction mixture interfered with the separation of the 4.5 to 8 kDa polypeptides.

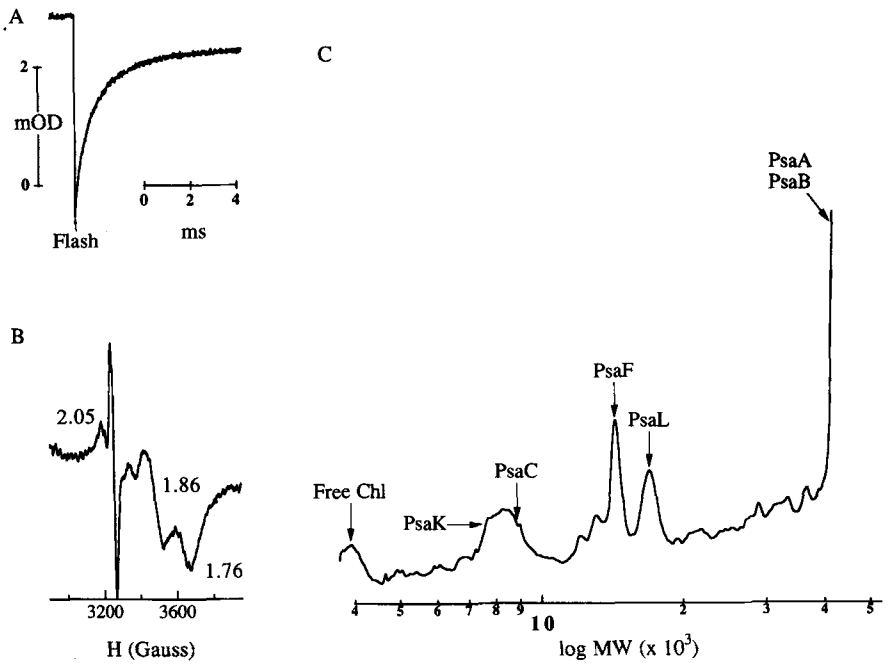

Fig. 5. Polypeptide composition and spectroscopic properties of an attempted Photosystem I reconstitution in the absence of iron-sulfur agents. The attempted reconstitution was performed by adding the low molecular mass proteins in the YM-100 filtrate (Fig. 3) to the Photosystem I core protein (Fig. 2) in the absence of $FeCl_3$, Na_2S, and β-ME for 20 h, followed by dialysis, ultrafiltration and ultracentrifugation in a sucrose gradient. (A) Flash-induced absorption transient of P700 (see Fig. 1A, legend). (B) ESR spectrum of the F_X iron-sulfur cluster (see Fig. 2B, legend). (C) Laser densitometric tracing of the polypeptides present in the reconstituted Photosystem I complex after separation by SDS-PAGE (see Fig. 1C, legend).

tion step; cf. Figs. 1, 2, 4 and 5), and that the 16.4 kDa polypeptide includes the retained PsaL protein as a minor (about 10%) component due to its migration as a diffuse zone (see above). In contrast, there is little rebinding of either the PsaC, PsaD or PsaE proteins to the Photosystem I core protein in the absence of $FeCl_3$, Na_2S and β-ME (Fig. 5C). The further addition of $FeCl_3$, Na_2S and β-ME to this preparation did not alter the flash-induced absorption changes or the ESR spectrum.

Discussion

These results show that the *Synechococcus* sp. PCC 6301 Photosystem I core protein represents a unique Photosystem I preparation in which the three hydrophilic, stromal proteins – PsaC, PsaD and PsaE – have been removed from the hydrophobic Photosystem I core polypeptides. The gel depicted in Fig. 5A of Ref. 33 showed the presence of five low-molecular-mass polypeptides at 17.2, 16.4, 13.4, 8.9 and 7.5 kDa in the Photosystem I complex from *Synechococcus* sp. PCC 6301. Based upon correlation with the electrophoretic patterns shown here, these polypeptides corresponded to the PsaL, PsaD, PsaF, PsaC and PsaE polypeptides. The additional polypeptides in the YM-100 filtrate represent PsaD degradation products; hence, most of the polypeptides in the electrophoretogram depicted in Fig. 5C from Ref. 33 are not unresolved Photosystem I proteins. In retrospect, our previously published electrophoresis data for the *Synechococcus* sp. PCC 6301 Photosystem I core protein [30,32] showed a nearly complete absence of low-molecular-mass polypeptides because the PsaF protein had been removed in the purification scheme by Triton X-100, and the PsaL protein ran as a weakly-staining, diffuse zone. In the gel system used in Ref. 33, PsaL migrated more slowly than PsaD, and a close examination of Fig. 5B shows a weakly-stained band at 17.2 kDa which probably corresponds to PsaL. This gel system had insufficient resolution to detect or resolve the very low-molecular-mass proteins between 2 and 8 kDa which exhibit very weak staining with Coomassie blue.

The simultaneous rebinding of the PsaC, PsaD and PsaE proteins indicates a close physical relationship among these proteins and the Photosystem I core. Oh-oka et al. [3] have shown that three proteins are butanol-extractable from spinach Photosystem I complexes; they have been identified as PsaC, PsaD and PsaE by N-terminal amino acid sequencing. Additionally, in spinach thylakoid membranes 19 and 14 kDa proteins are labeled with 2,4,6-trinitrobenzene sulfonate and are sensitive to pronase digestion [49], indicating that they are located on the stromal side of the membrane. These proteins are most likely PsaD and PsaE, respectively (the higher plant PsaE protein is larger than that in cyanobacteria, and the apparent molecular mass of this protein on SDS-PAGE is frequently considerably larger than indicated from the gene sequence). Cross-linking studies, employing the homobifunctional agent dithiobis(succinimidyl propionate), with Photosystem I particles isolated from *Synechococcus elongatus* have shown the formation of conjugates of the trio of 10, 11 and 18 kDa proteins [50]. By analogy to the work described here and to results discussed above, these three proteins are most likely PsaE, PsaC and PsaD, respectively. Examination of the spinach Photosystem I complex by alkaline and chaotropic ion treatments, tryptic digestion and cross-linking shows a close association of the 9, 14 and 19 kDa proteins [51]. These molecular masses are consistent with their identification as PsaC, PsaE and PsaD, respectively. The absence of a thylakoid transit sequence is also in concert with the assessment that the PsaC, PsaD and PsaE proteins are located on the stromal side of the thylakoid membrane [15,16,21]. A structure compatible with these data would place the PsaC protein in contact with the PsaA/PsaB heterodimer and overlaid with the PsaD and PsaE proteins. The shielding of PsaC by PsaD and PsaE would explain the failure of the PsaC protein to be labeled by 2,4,6-trinitrobenzene sulfonate and would be consistent with its insensitivity to proteinase digestion as well [49]. Additional support for this model comes from the recent observation that stable rebinding of a PsaC1 fusion protein to the Photosystem I core requires the presence of the PsaD protein [11]. This model is in excellent agreement with a recent model developed from structural analyses by electron microscopy of ordered arrays of cyanobacterial Photosystem I complexes [52]. Accordingly, a critical event in the dissociation of the three proteins might be the denaturation of the F_A/F_B iron-sulfur clusters in the PsaC protein. Since an iron-sulfur protein has no stable three-dimensional structure in the absence of the clusters (Markley, J., personal communication), the oxidative denaturation of the PsaC protein would lead to its unfolding, which in turn would lead to a destabilization of the complex comprised of the PsaA/PsaB heterodimer and the PsaC, PsaD and PsaE proteins.

The co-binding of the PsaC, PsaD and PsaE proteins immediately suggests a mechanism for the self-assembly of these proteins onto the Photosystem I core during development. Our earlier work [33] showed that addition of $FeCl_3$, Na_2S and β-ME to a mixture of the F_X apoprotein (the Photosystem I core protein after oxidative denaturation of F_X) and F_A/F_B apoprotein resulted in full recovery of electron flow from P700 to the terminal iron-sulfur clusters. The reconstitution of the iron-sulfur clusters was accompanied by quantitative rebinding of a 16.4 kDa and an 8.9 kDa polypeptide to the Photosystem I core protein. The restoration

of electron flow to F_A/F_B therefore presupposes that the F_X cluster was reinserted into the Photosystem I core protein prior to the rebinding of the PsaC, PsaD and PsaE proteins. We later found that the PsaC holoprotein does not rebind to the Photosystem I core protein after F_X has been oxidatively denatured, even in the presence of PsaD and PsaE (unpublished results). Together, these observations indicate that the F_X iron-sulfur cluster must be present before the binding of PsaC, PsaD and PsaE can occur. This could represent a mechanism to prevent the premature assembly of an F_X-deficient Photosystem I complex and could additionally provide a mechanism for regulation, if such clusters can be reversibly formed and dismantled as has been suggested to occur in mitochondrial electron transport proteins [53]. Accordingly, the assembly of the Photosystem I complex would be a multi-step process, potentiated by the assembly of the F_X iron-sulfur cluster. During the latter stages of assembly, the PsaC, PsaD and PsaE proteins would diffuse to the thylakoid-bound Photosystem I core protein and become irreversibly bound through the insertion of the F_A/F_B iron-sulfur clusters in the PsaC apoprotein.

Conclusions

We have identified the polypeptide components of Photosystem I preparations that had been defined previously using spectroscopic criteria.

The Photosystem I complex

The Photosystem I complex shows electron transport from P700 to F_A/F_B. The polypeptide composition includes the PsaA/PsaB heterodimer, PsaC, PsaD, PsaE, PsaF, PsaK, PsaL and three < 8-kDa polypeptides. By analogy with data for *Synechococcus vulcanus* and *Anabaena* sp. ATCC 29413, the three small polypeptides are likely to include PsaJ, the '4. 8 kDa' protein and PsaI (in order of descending apparent mass).

The Photosystem I core protein

The Photosystem I core protein shows electron transport from P700 to F_X. The polypeptide composition includes the PsaA/PsaB heterodimer, PsaF, PsaL, PsaK and three < 8-kDa polypeptides (probably PsaJ, the '4.8-kDa' protein, and PsaI). The hydrophilic PsaC, PsaD and PsaE proteins are dissociated from the hydrophobic Photosystem I core with chaotropes, and they can be recovered after ultrafiltration through a YM-100 membrane.

The reconstituted Photosystem I complex

The reconstituted Photosystem I complex shows restored electron transport from P700 to F_A/F_B. The polypeptide data indicate that in the presence of $FeCl_3$, Na_2S and β-ME, the PsaC, PsaD and PsaE proteins rebind to the Photosystem I core protein. There is little or no rebinding of PsaC, PsaD or PsaE in the absence of the iron-sulfur reconstitution agents.

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