Truncation of the D2 Protein in *Synechocystis* sp. PCC 6803: A Role of the C-Terminal Domain of D2 in Photosystem II Function and Stability†

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Received May 21, 1993; Revised Manuscript Received August 3, 1993®

ABSTRACT: Termination and deletion mutations were introduced near the C-terminal end of the D2 protein in the cyanobacterium *Synechocystis* sp. PCC 6803 in order to determine the role of the large hydrophilic C-terminal domain of D2 in the function and stability of photosystem II (PS II). The loss of 57 residues from the C-terminal end of D2 (most of the hydrophilic tail) resulted in the loss of D2 and PS II reaction centers from thylakoids. Truncation of 16, 15, 14, or 13 amino acid residues from the C-terminus of D2 resulted in a virtual disappearance of oxygen evolution, a loss of photoautotrophic growth, and a decrease in the number of PS II centers in thylakoids. The loss of 11 C-terminal amino acid residues led to a photoautotrophic mutant that grew at one-half the rate of the wild type under photoautotrophic conditions and that showed a progressive loss of oxygen evolution at high light intensity. Truncation of 9 residues from D2 led to a virtual loss of CP43, presumably because of interference of the mutation with the overlapping ribosome-binding site for *psb*C translation. To delete smaller portions of D2 and yet not interfere with *psb*C expression, various deletions were made between the tenth and twentieth amino acid residues from the C-terminal end of D2, resulting in the loss of 8, 7, 4, 3, and 2 residues. The deletion of 8 or 7 residues from within the C-terminal end of D2 resulted in photoautotrophic mutants. Surprisingly, the deletion of shorter fragments had more pronounced effects: deletion of 4 residues within the same domain gave rise to a mutant lacking D2 and PS II centers. A mutant with a deletion of 3 residues was an obligate photoheterotroph, containing functional PS II reaction centers but showing rapid photoinhibition. The deletion of 2 residues resulted in an obligate photoheterotrophic mutant with a 10-fold-reduced level of PS II centers. In the mutant lacking the 15 C-terminal residues of D2, fluorescence induction behavior indicated rapid inactivation at the donor side. In this and similar mutants, stable PS II-mediated electron transport between diphenylcarbazide and dichlorophenolindophenol could be observed at rates proportional to their PS II content. These results indicate that the primary effect of the mutations is on the oxygen-evolving complex. We conclude that domains near the C-terminal end of D2 have a role in oxygen evolution and contribute to determining the stability and activity of the PS II complex.

In thylakoid membranes of plants and cyanobacteria, photosystem II (PS II) is responsible for the series of light-driven redox reactions transferring electrons from water to the plastoquinone pool and resulting in the evolution of oxygen [reviewed by Andersson and Styring (1991), Vermaas and Ikeuchi (1991), and Erickson and Rochaix (1992)]. From the plastoquinone pool, electrons are transferred to other redox components involved in the photosynthetic process. The pathway of electron transfer in PS II is fairly well understood, but cofactor/protein interactions in the PS II complex are still rather unclear, even though such interactions are crucial to PS II activity.

One of the major challenges in PS II research is to understand the mechanism of oxygen evolution and to elucidate the protein regions involved in this process. Several PS II proteins have been shown to be directly or indirectly involved in water splitting and oxygen evolution. These proteins primarily include the reaction center proteins D1 (Nixon et al., 1992a; Nixon & Diner, 1992) and D2 (Vermaas et al., 1990a), but the chlorophyll-binding proteins CP43 (Vermaas et al., 1988; Rögner et al., 1991) and CP47 (Bricker, 1990) as well as the peripheral 33-kDa manganese-stabilizing protein (MSP) (Kuwabara et al., 1985), known as PS II-O, also appear to be involved in creating the environment of the water-splitting system. This 33-kDa protein is located on the lumenal side of the membrane and is involved in stabilizing the manganese that is necessary for the water-splitting process (Burnap & Sherman, 1991; Philbrick et al., 1991; Mayes et al., 1991). Additional evidence for involvement of the D1/D2 reaction center complex in water splitting comes from the analysis of a mutant, LF-1, of the green alga *Scenedesmus obliquus*. This mutant is defective in C-terminal processing of the D1 protein (Diner et al., 1988) and fails to functionally assemble the Mn cluster necessary for water splitting. The LF-1 mutant shows a reduction in the number of high-affinity sites for Mn binding (Seibert et al., 1989). In addition, a number of site-directed mutations introduced near the C-terminus of D1 greatly affect PS II activity [reviewed in Debus (1992), Nixon et al. (1992a)], and the C-terminal carboxylate group of mature D1 may serve as a ligand to Mn of the water-splitting system (Nixon et al., 1992b). Thus, these results indicate that the C-terminal end of D1 is involved in events related to oxygen evolution. On the basis of the apparent 2-fold symmetry in the PS II D1/D2 reaction center structure (Michel & Deisenhofer, 1988), we were interested to investigate whether the C-terminus of D2 is also involved in the

† This work has been supported by the National Science Foundation (DMB90-19248). This is publication no. 160 from the Arizona State University Center for the Study of Early Events in Photosynthesis. The center is funded by U.S. Department of Energy Grant DE-FG02-88ER13969 as part of the Plant Science Centers program of the U.S. Department of Agriculture, U.S. Department of Energy, and the National Science Foundation.

® Abstract published in *Advance ACS Abstracts,* September 15, 1993.

0006-2960/93/0432-11419$04.00/0

Table I: List of Mutagenic Oligonucleotide Primers Used in the Construction of the D2 Mutants

name	sequence of oligonucleotide
ET-9[a]	5′-GGAGAACTTACTCAGG-3′
ET-11	5′-CCTCCTCTTAGAAGATAA-3′
ET-13	5′-TCAGGGAATTAAAAGTTTT-3′
ET-14	5′-GGGAAGATTTAGTTTTC-3′
ET-15	5′-AAGATAAATTATTCATGG-3′
ET-16	5′-AAGTTTTAATGGGT-3′
ET-57	5′-CAAAGTCTTAGGCCC-3′
Δ(11–19)[b]	5′-GGGGAGAACCTCCTC/*GAG*/ATCTTGGGGAGCCAT-3′
Δ(11–15)	5′-GGGGAGAACCTCCTC/*GAG*/TTCATGGGGTTGATC-3′
Δ(11–14)	5′-GGGGAGAACCTCCTC/*GAG*/GTTTTCATGGGGTTG-3′
Δ(12–18)	5′-GGGGAGAACCTCCTCAGG//TTGATCTTGGGGAGC-3′
Δ(12–14)	5′-GAGAACCTCCTCCGG/*TCC*/GTTTTCATGGGGTTG-3′

[a] Altered bases have been underlined. Note that the sequence of the oligonucleotides is complementary to the coding sequence. [b] Slash marks indicate the area of deletion, and italicized letters represent a codon added to create a unique restriction site [Δ(12–14) contains an additional base change (underlined) that does not lead to a mutation in the protein].

formation of the water-splitting complex and possibly in the binding of Mn. As D1, the D2 protein has a long hydrophilic C-terminal stretch, which is presumed to be exposed to the lumenal side of the thylakoid; however, in contrast to D1, the D2 protein is not C-terminally processed.

We have developed an efficient method for directed mutagenesis of D2 in the transformable, photoheterotrophic cyanobacterium *Synechocystis* sp. PCC 6803; this organism can incorporate foreign DNA into its genome by homologous recombination (Williams, 1988). We have generated a *Synechocystis* mutant in which both copies of *psb*D, *psb*DI and *psb*DII, have been deleted (Vermaas et al., 1990b). Modified *psb*DI can be introduced into this mutant, and transformants carrying only mutated D2 will result. Using this system, we introduced premature stop codons into *psb*DI, resulting in the early termination of translation and thus in a truncated D2 protein. Similar mutagenesis techniques were used to delete amino acid residues within the C-terminus of D2, also resulting in a shortened D2 protein. From the results obtained, we conclude that, like D1, the C-terminus of D2 plays a prominent role in determining the protein environment of the oxygen-evolving complex.

MATERIALS AND METHODS

Growth Conditions and Transformation of Synechocystis sp. PCC 6803. The conditions for cell growth and transformation of *Synechocystis* sp. PCC 6803 were described previously (Vermaas et al., 1990a). Mutant strains were grown on BG-11 plates supplemented with 0.3% sodium thiosulfate, 10 mM TES (pH 8.0), 5 mM glucose, 25 μg/mL kanamycin, 25 μg/mL spectinomycin, and 20 μM atrazine. Wild type and mutants were grown in liquid culture in BG-11 with 5 mM glucose unless indicated otherwise.

Site-Directed Mutagenesis. The procedures used for mutagenesis were a combination of those described by Kunkel et al. (1987), Vandeyar et al. (1988), and Zoller and Smith (1983) and have been summarized by Vermaas et al. (1990b). The mutagenic oligonucleotides used to insert a premature stop codon or to delete amino acid codons in the *psb*DI gene sequence are listed in Table I. The mutants were identified by sequencing from single plaque isolates. The appropriate DNA region carrying the mutation was ligated into a plasmid containing regions up- and downstream of the *psb*DI/C operon. The resulting plasmid carries the entire *psb*DI/C operon (including the desired mutation), flanking up- and downstream regions, and a cartridge, conferring kanamycin resistance, downstream of *psb*C. Plasmids containing the desired mutations in *psb*DI were used to transform a double-deletion strain of *Synechocystis* sp. PCC 6803 that lacks *psb*DII and *psb*DI/C (Vermaas et al., 1990b). Double crossover between the plasmid and the genomic DNA in regions up- and downstream of the *psb*DI/C operon can occur, incorporating the mutated *psb*DI/C operon (along with the kanamycin resistance marker) into its proper location in the genome. The selection of cyanobacterial transformants is aided by a kanamycin resistance marker incorporated into the *psb*DI/C-containing plasmid, with which the cyanobacterial double-deletion mutant is transformed.

The region of the gene containing the mutation was amplified from the cyanobacterial genome using the polymerase chain reaction (PCR) to confirm that the correct mutation had been introduced into the cyanobacterial genome. Two oligonucleotides of 26 bases in length were used in the PCR reactions as primers for DNA polymerization. The resulting PCR product (2.0 kilobase pairs (kb)) was cleaned with a SpinBind DNA extraction cartridge (FMC) to remove PCR primers. The PCR product was then sequenced using the double-stranded DNA Cycle Sequencing System (Gibco/BRL, Bethesda MD) to confirm the presence of the desired mutation and the absence of inadvertently introduced secondary mutations.

In addition, if a mutant was an obligate photoheterotroph, this mutant was transformed with a small piece (~0.3 kb) of the wild-type *psb*DI/C operon covering the region of the mutation. Mutants could regain a wild-type (photoautotrophic) phenotype after this transformation, indicating that no secondary mutations affecting photosynthetic function were introduced in parts of the introduced *psb*DI/C construct that were not sequenced.

Thylakoid Preparation. Thylakoids were prepared from the wild-type and mutant strains of *Synechocystis* essentially as described in Yu and Vermaas (1990). Isolated thylakoids were resuspended in thylakoid buffer [50 mM HEPES/NaOH (pH 7.0), 5 mM $MgCl_2$, 50 mM $CaCl_2$, 5% (v/v) glycerol, and 0.5% (v/v) dimethyl sulfoxide (DMSO)] and were used immediately or frozen in liquid N_2 and stored at −70 °C.

Electron Transport Measurements. Oxygen evolution measurements on intact cells of wild type and mutants were performed on a Gilson Model KM oxygraph. Cells were diluted to 10 μg of chlorophyll mL^{-1} in 25 mM *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES)/NaOH (pH 7.0). The electron acceptor was 0.5 mM $K_3Fe(CN)_6$ with 0.1 mM 2,6-dimethyl-*p*-benzoquinone to mediate electron transfer between the intact cells and the nonpenetrating $K_3Fe(CN)_6$. A 150-W xenon arc lamp provided the actinic light. The light was filtered through 15

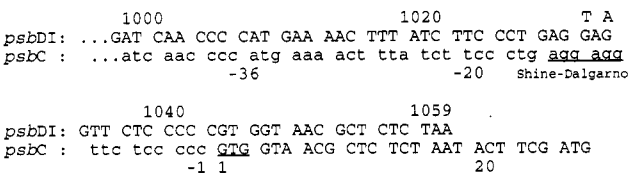

FIGURE 1: Overlapping sequence of the *psb*DI and *psb*C genes in *Synechocystis* 6803. Sequences in coding regions are capitalized. The start codon for *psb*C (GTG) is underlined. Also underlined is the Shine–Dalgarno region of the *psb*C gene. The two base changes that were made to create the ET-9 mutant are written above the *psb*DI sequence.

cm of water and through a Schott OG-570 filter before reaching the sample. The actinic light intensity was 1200 $\mu E/m^2/s$, unless indicated otherwise.

Dichlorophenolindophenol (DCPIP) reduction was measured at 605 nm using a Shimadzu UV-160 spectrophotometer. Thylakoid samples (10 μg of chlorophyll/mL) in thylakoid buffer were illuminated by a 150-W Xenon arc lamp with the light filtered through 15 cm of water and a Schott OG-570 filter. The actinic light intensity for these experiments was 7000 $\mu E/m^2/s$. Absorption measurements were taken every 30–60 s. Where indicated, thylakoids used for these measurements were incubated for 30 min in 0.8 M Tris (pH 8.0) on ice and were pelleted and resuspended in thylakoid buffer. DCPIP reduction in these Tris-washed thylakoids was measured in the presence of 0.5 mM diphenylcarbazide (DPC) as the artificial electron donor.

Fluorescence Induction. Chlorophyll *a* fluorescence yields were measured in intact cells as a function of illumination time using a commercial PAM fluorometer (Walz, Germany). Cells were resuspended at 10 μg of chlorophyll/mL in 25 mM HEPES (pH 7.0), 5 min before the start of the fluorescence measurement. Cells were dark-adapted for at least 30 min at room temperature prior to illumination.

Photosystem II Quantitation, Gel Electrophoresis, and Western Blotting. Methods used to quantitate PS II on a chlorophyll basis in whole cells using [^{14}C]diuron binding have been described (Vermaas et al., 1990a). SDS–polyacrylamide gel electrophoresis and Western blotting were carried out as in Vermaas et al. (1988). Antisera used were raised against spinach proteins (Vermaas et al., 1988).

RESULTS

Generation of Early Termination and Deletion Mutants. Twelve mutants were generated with mutations near the C-terminus of D2. Seven of these mutants were constructed by the introduction of a premature stop codon in the gene sequence (*psb*DI) of the D2 protein, causing the early termination of translation. The resulting mutants produce a truncated D2 protein that is missing a number of amino acid residues from the C-terminal end of the D2 protein. These mutants are referred to as ET-9, ET-11, ET-13, ET-14, ET-15, ET-16, and ET-57, with the number indicating the number of residues by which the mutant D2 protein has been truncated. ET is an abbreviation for early termination.

The 3′ end of *psb*DI overlaps with the 5′ end of *psb*C by 14 nucleotides (Figure 1; Carpenter et al., 1990). To avoid interference with *psb*C expression upon the introduction of stop codons close to the end of *psb*DI, we decided to study the effect of shortening D2 by introduction of deletions in *psb*DI upstream of the putative *psb*C ribosome-binding site. To this effect, amino acid residue deletions of various lengths were made between the tenth and twentieth amino acid residues from the C-terminal end of D2. The resulting mutants have D2 proteins that are eight (mutant Δ(11–19)), seven (mutant Δ(12–18)), four (mutant Δ(11–15)), three (mutant Δ(11–14)), or two (mutant Δ(12–14)) amino acid residues shorter than the wild type. The numbers indicated are the residue numbers (counted from the C-terminal end of the protein) that have been deleted in the D2 protein. In Δ(11–19), Δ(11–15), and Δ(11–14), a Leu residue was inserted at the site of the deletion, while in Δ(12–14) a Gly was added. In the *psb*DI gene sequences of the Δ(11–14), Δ(11–15), and Δ(11–18) mutants, unique restriction sites have been introduced at the site of the deletion; these restriction sites can be utilized for future mutagenesis. The amino acid sequence of the C-terminal hydrophilic domain of D2 in the mutants and wild type is shown in Figure 2.

Photoautotrophic Competence. In the absence of glucose, only three of the truncation and deletion mutants were able to grow photoautotrophically. These are ET-11, in which the D2 protein is missing eleven amino acid residues from the C-terminus, Δ(11–19), where D2 is eight amino acid residues shorter than in wild type, and Δ(12–18), where seven amino acid residues have been deleted between the eleventh and nineteenth amino acids from the C-terminus of D2. In all three mutants, the photoautotrophic doubling time is reduced by at least a factor of 2 as compared to that of the wild type (Table II).

Presence of D2 and CP43 in the Thylakoid Membrane. Antibodies raised against spinach D2 and CP43 were used in Western blots to check for the presence of D2 and CP43 proteins in the thylakoid membranes of mutants and wild type (Figures 3 and 4). As shown in Figure 3, the truncated D2 proteins ran at a slightly lower molecular weight due to the loss of amino acid residues. ET-57 and Δ(11–15) did not contain any detectable level of the D2 protein in their thylakoid membranes. The amount of immunoreaction between antisera and the D2 protein in thylakoids of the other mutants was decreased significantly. Thylakoids from the other early termination and deletion mutants (ET-16, ET-15, ET-14, ET-13, ET-11, ET-9, Δ(11–19), Δ(12–18), Δ(12–14), and Δ(11–14)) showed a significant level of immunoreaction with D2 antisera. We have not attempted to quantify the amount of D2, as the level of immunoreaction is not necessarily linear with the amount of antigen. As shown in Figure 4, most mutants showed rather normal levels of immunoreaction with CP43 antiserum. However, in ET-9 the CP43 protein appears to be virtually absent, while in the Δ(12–14) deletion mutant the immunoreaction between CP43 and the appropriate antiserum was visibly decreased. The possible reason for the loss of CP43 in ET-9 will be discussed in the Discussion section.

Quantitation of Photosystem II. To quantitate the number of PS II centers on a chlorophyll basis in wild type and mutants *in vivo*, binding assays were performed with different concentrations of radiolabeled diuron, a PS II-directed herbicide, using whole cyanobacterial cells. Such measurements provide information both on the number of chlorophylls per diuron-binding site and on the diuron dissociation constant. The results of diuron binding to wild type and mutant strains are summarized in Table III. The mutants ET-57, ET-9, and Δ(11–15) are impaired in their ability to bind diuron in that no significant atrazine-replaceable diuron binding could be detected. This suggests that no intact PS II centers are assembled stably in the thylakoid membranes of these mutants and is in agreement with the lack of D2 in ET-57 and Δ(11–15) and the lack of CP43 in ET-9. The other mutants do bind a significant amount of diuron, but all appear to have fewer PS II reaction centers on a chlorophyll basis than wild

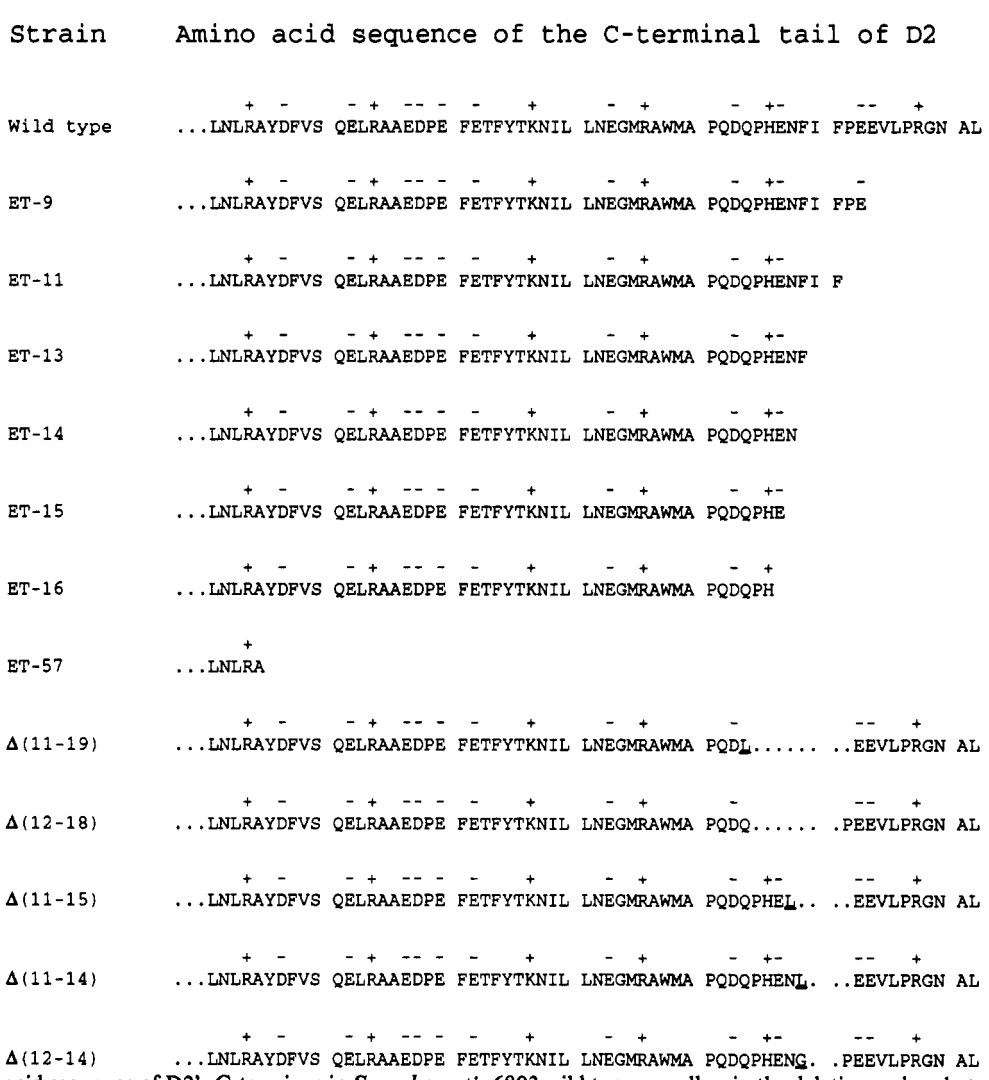

FIGURE 2: Amino acid sequence of D2's C-terminus in *Synechocystis* 6803 wild type as well as in the deletion and early termination mutants discussed in this article. Periods indicate the residues that were deleted, and the underlined letter is a residue added to create a unique restriction site in the gene sequence of *psb*DI.

Table II: Doubling Times of Wild Type and the Photoautotrophic D2 Mutants Grown in BG-11 without Glucose

strain	doubling time (h)
wild type	15
ET-11	30
Δ(11–19)	40
Δ(12–18)	36

type. The only photoautotrophic early termination mutant that is covered in this study (ET-11) has fewer PS II complexes than the wild type, but more than the other early termination mutants. Interestingly, it is the only mutant with an increased K_D for diuron (Table III). This increase in the dissociation constant is significant and reproducible; to illustrate this point, representative double-reciprocal plots of diuron binding to cells of wild type, ET-11, and ET-14 are presented in Figure 5. While ET-14 has a diuron affinity similar to that of wild type (the respective lines intersect near the *x*-axis), the diuron affinity of ET-11 (which is the negative reciprocal value of the intersect with the *x*-axis) is decreased by a factor of more than 2 as compared to wild type. This suggests that a mutation at the presumably lumenal-exposed C-terminus of D2 has a small effect on the herbicide-binding site located at the cytoplasmic (stromal) side of the thylakoid. Similar donor-side-induced modifications of acceptor–side properties have been found in a mutant lacking MSP (PS II-O) (Vass et al., 1992).

Although the loss of 16, 15, 14, or 13 amino acids from the C-terminus of D2 resulted in a loss of photoautotrophic growth, these mutants still have about 25–30% of the wild-type amount of PS II complexes present in the thylakoid membranes. The mutant with the two-residue deletion (Δ(12–14)) was found to have very few intact PS II complexes, as indicated by the high number of chlorophylls per herbicide-binding site (one PS II center per 8500 chlorophylls). As mentioned, Δ(11–15), carrying a deletion of four residues, completely lost diuron-binding sites, suggesting a complete destabilization of PS II. The deletion mutants Δ(11–19), Δ(12–18), and Δ(11–14), carrying deletions of eight, seven, and three amino acid residues, respectively, showed about one PS II reaction center per 1800 chlorophylls. However, the Δ(11–14) mutant, with three amino acid residues deleted, is unable to grow photoautotrophically even though it appears to have the same number of PS II reaction centers as the Δ(11–19) and Δ(12–18) mutants, which are photoautotrophic.

Photosystem II Electron Transport and Fluorescence Induction. Oxygen evolution measurements were used to determine whether the PS II complex in the various mutants was competent in water splitting. Whole cells were tested in the presence of ferricyanide and 2,6-dimethyl-*p*-benzoquinone

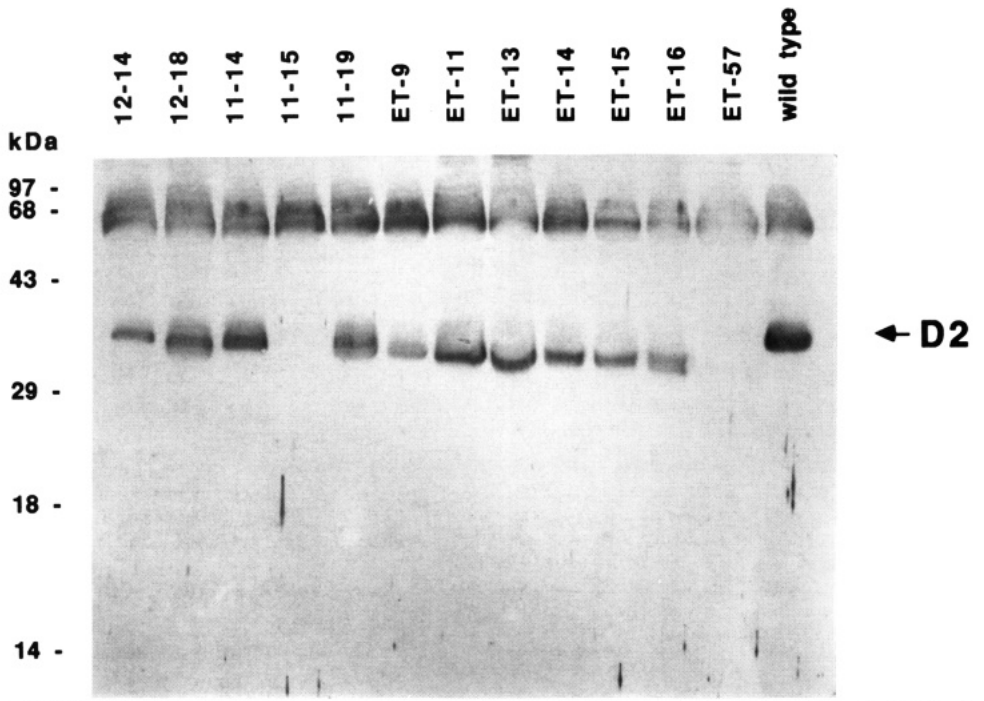

FIGURE 3: Western blot of thylakoid proteins from wild-type, early termination, and deletion mutants of *Synechocystis* 6803 probed with rabbit polyclonal antibodies raised against D2 from spinach. In lanes 1–5, thylakoid proteins from deletion strains have been loaded as indicated. The labels for the other lanes directly correspond to the names of the strains. Location and size of molecular weight markers have been indicated. The amount of material loaded per lane corresponded to 5 μg ofchlorophyll.

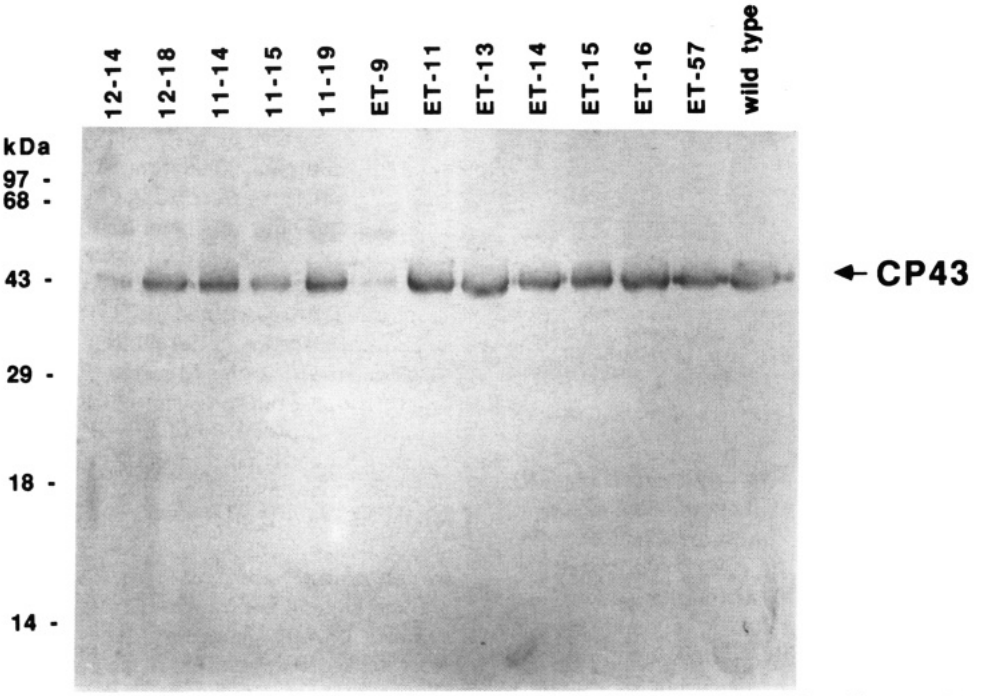

FIGURE 4: Western blot of thylakoid proteins from wild-type, early termination, and deletion mutants of *Synechocystis* 6803 probed with rabbit polyclonal antibodies raised against CP43 from spinach. In lanes 1–5, thylakoid proteins from deletion strains have been loaded as indicated. The labels for the other lanes directly correspond to the names of the strains. Location and size of molecular weight markers have been indicated. The amount of material loaded per lane corresponded to 5 μg of chlorophyll.

as electron acceptors. Oxygen evolution rates for wild-type and mutant strains are presented in Table III. Not surprisingly, the three mutants that lacked CP43 or D2 and were unable to bind diuron (ET-57, ET-9, and Δ(11–15)) were also unable to evolve oxygen in the presence of an artificial electron acceptor. For ET-16, ET-15, ET-14, ET-13, and Δ(12–14), only minimal rates of oxygen evolution were detected. Moreover, the rate of oxygen evolution by these five mutants declined rapidly during illumination; because of the small initial oxygen-evolving activity of these mutants, an accurate rate of inactivation is difficult to measure, but in all five mutants oxygen evolution activity was no longer detectable after 1 min of illumination. Comparison of the relative number of PS II complexes on a chlorophyll basis and the initial rate of oxygen evolution (Table III) shows that these rates in the ET-13, ET-14, ET-15, and ET-16 mutants are 5–20-fold lower than would be expected from the number of herbicide-binding sites, suggesting that a significant fraction of the PS II reaction

Table III: Number of Diuron Binding Sites, Diuron Affinity, and Initial Oxygen Evolution Rates in Wild Type and Mutant Strains of *Synechocystis* 6803

strain	chlorophylls per binding site[a]	diuron dissociation constant, K_D (nM)	O_2 evolution rate[b] (μmol of O_2/ mg of chlorophyll/h)
wild type	800	19	410
ET-57[c]	–	–	0
ET-16	2800	19	6
ET-15	2400	18	30
ET-14	2700	22	6
ET-13	2500	13	10
ET-11	1100	47	150
ET-9	–	–	0
Δ(11–19)	1800	21	330
Δ(11–15)	–	–	0
Δ(11–14)	1800	14	180
Δ(12–18)	1800	23	270
Δ(12–14)	8500	22	19

[a] Values are an average of several experiments. The K_D and chlorophyll/PS II ratios obtained for each mutant in different experiments were reproducible within 20%. [b] Light intensity: 1200 $\mu E/m^2/s$. Values are averaged from two measurements. [c] Dashes indicate that mutants did not yield measurable diuron binding, most likely signifying a disappearance of the PS II complex from the thylakoid.

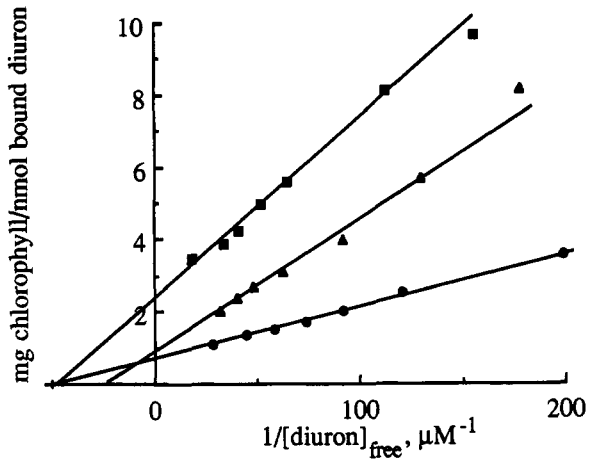

FIGURE 5: Double-reciprocal plot of [^{14}C]diuron binding to cells of wild type (●), ET-11 (▲), and ET-14 (■); 30 μg of chlorophyll/mL.

centers is inactivated at the time the light is turned on, or very shortly thereafter.

The photoautotrophic mutants Δ(12–18) and Δ(11–19) were able to evolve oxygen at a rate about 60–80% of that in wild type, while ET-11, also a photoautotroph, had an initial oxygen evolution rate of about 40% that of the wild-type strain; these rates correlate reasonably well with the photoautotrophic growth rates of the respective mutants. Steady-state oxygen evolution in ET-11 cells was sensitive to high light intensity: at 7000 $\mu E/m^2/s$, after 25 s the rate of oxygen evolution was one-half that of the initial rate, while at 1800 $\mu E/m^2/s$, after 95 s the steady-state oxygen evolution rate had decreased by 50% (not shown). In comparison, in wild-type cells, electron transport remained stable for the duration of the oxygen evolution measurement (2–3 min).

The one mutant included in this study that is an obligate photoheterotroph and yet shows a high initial rate of oxygen evolution is the Δ(11–14) mutant: while this mutant is an obligate photoheterotroph, it was able to evolve oxygen at an initial rate of about 50% compared to the wild-type strain. The reason for the obligate photoheterotrophic growth of this mutant may be that it is rapidly photoinhibited: even at just-saturating light intensity (1200 $\mu E/m^2/s$), after 60 s the

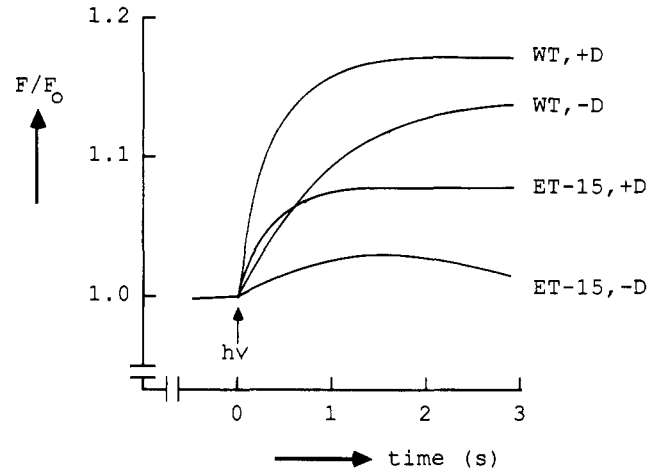

FIGURE 6: Fluorescence induction of wild type (wt) and ET-15 cells in the presence and absence of diuron (D). Actinic light was turned on at the time point indicated by the arrow.

oxygen-evolving activity of the cells is one-half the initial rate (not shown).

The early termination mutants ET-13, ET-14, ET-15, and ET-16 appear particularly interesting in that they contain a significant amount of PS II (as determined by herbicide binding), and yet have a very low initial rate of oxygen evolution. The phenotypes of these four early termination mutants also are strikingly similar. To determine the site of the lesion in electron transfer in an early termination mutant, two approaches were taken: (1) fluorescence induction was meaured in whole cells of wild type and one of the early termination mutants (ET-15), and (2) electron transport was measured with DCPIP as electron acceptor in the presence of DPC, which donates to Z^+, in thylakoids isolated from wild type and mutants. The results of fluorescence induction measurements are presented in Figure 6. While the wild type shows normal induction characteristics [fast rise in the presence of diuron, slower and incomplete rise when electron transport is uninterrupted and powerful electron sinks (PS I and respiration) are connected to PS II], in ET-15, in the absence of diuron, after an initial buildup of Q_A^- the fluorescence yield decreased to the F_o level. This suggests that, after a brief period of relatively normal PS II activity in the light, electron transport is gradually inhibited and Q_A does not continue to be reduced in the light at a pace exceeding that of plastoquinol oxidation. This would imply that in ET-15 whole-chain PS II electron transport is highly labile. This is in good agreement with the results of steady-state oxygen evolution: the "initial rate" (the rate observed in the first 20 s after the light is turned on) is less than expected from the number of available centers, and inactivation is rapid. The fluorescence trace of ET-15 in the presence of diuron resembles that seen in wild type, except that the amplitude of the variable fluorescence is decreased (compatible with the lower PS II/ chlorophyll ratio in ET-15).

To verify a donor-side inactivation in the early termination mutants, light-induced electron transfer to DCPIP was measured in wild-type and mutant thylakoids. To simplify the interpretation of the observations, Tris-washed thylakoids were utilized with DPC as electron donor (Table IV). The mutants tested showed a reduced amount of DCPIP reduction, which corresponds reasonably well to the reduced number of PS II reaction centers (compare Tables III and IV). The rate of DCPIP reduction was stable for several minutes (not shown). This implies that electron transport in these mutants is not inhibited between Z and the site of electron acceptance by

Table IV: DCPIP Reduction in Tris-Washed Thylakoids of Wild Type and Selected Early Termination Mutants of *Synechocystis* 6803

strain	DCPIP reduction in the presence of DPC (% of wild type)[a]
wild type	100
ET-15	42
ET-14	23

[a] Rate of DCPIP reduction in wild type was approximately 120 μequiv/mg of chlorophyll/h. The light intensity was 7000 $\mu E/m^2/s$, which may not be saturating for isolated thylakoids that have lost virtually all phycobilisomes.

DCPIP; thus, the site of initial inactivation is at the donor side before Z, i.e., at the water-splitting complex.

DISCUSSION

The Early Termination Mutants. This study implicates the C-terminal region of D2 in contributing to the environment of the water-splitting system. Of particular importance appears to be the region between 10 and 16 residues from the C-terminus. The loss of 16, 15, 14, or 13 amino acid residues from D2's C-terminus results in organisms that are unable to grow photoautotrophically. These four mutants appear to retain assembled PS II complexes in their thylakoid membranes, although approximately 3-fold fewer than the wild type. The loss of 16–13 amino acids from the C-terminal end of D2 appears to impair PS II function significantly as indicated by a very low initial rate of oxygen evolution (30 μmole of O_2/mg of chlorophyll/h or less) and decreased stability of activity. In the ET-13, ET-14, ET-15, and ET-16 mutants the number of PS II complexes is decreased, but the remaining centers by-and-large are not very active and stable in steady-state oxygen evolution. The loss of 16–13 C-terminal amino acids from D2 appears to interfere with electron transport and may also decrease the stability of the PS II complex (leading to a decreased PS II/chlorophyll ratio).

The results of fluorescence induction measurements on ET-15 cells suggest that PS II electron transport in this mutant is very rapidly inactivated in the light. The stability and quantity of electron transfer between Z and the site of electron acceptance by DCPIP indicate that events beyond and including Z appear normal. This implies that the primary site at which ET-15 is inhibited is the water-splitting complex.

The loss of 11 amino acid residues from the C-terminal tail of D2 gives rise to a photoautotrophic phenotype with a decreased ability to evolve oxygen. With fewer PS II complexes than the wild type (1100 versus 800 chlorophyll per binding site), it is still able to grow quite well photoautotrophically. Interestingly, it was the only mutant of the group studied here with a lower affinity for diuron. To verify that the effect on diuron affinity was specifically related to ET-11 and was not the result of a secondary mutation, the ET-11 mutant was generated in two separate mutagenesis experiments, and the effect on diuron affinity was found in both cases (not shown). This does not rigorously exclude the possibility of a frequent secondary mutation leading to altered diuron affinity, but makes it highly improbable.

A donor-side mutation leading to altered acceptor-side properties is not unparalleled. Also, the loss of PS II-O appears to affect acceptor-side characteristics (Vass et al., 1992). Conversely, in mutants affected primarily at the Q_B/herbicide-binding environment, effects on the water-splitting system have been implied [for example, see Kirilovsky et al. (1991)]. Thus, the acceptor and donor sides may be linked more closely than would be expected from their spatial separation.

The functional difference between the photoautotrophic ET-11 and the obligate photoheterotrophic ET-13, ET-14, ET-15, and ET-16 mutants is highly interesting. The two residues that are different between ET-11 and ET-13 are Ile and Phe (Figure 2); it is unlikely that the side groups of these residues have a direct functional role in the water-splitting complex. However, it is possible that the protein backbone in this area is important in providing the proper environment for the water-splitting system. Other possible reasons for the phenotypic differences between ET-11 and ET-13 will be discussed in more detail at the end of this section.

Perhaps not surprisingly, the loss of 57 amino acids (which is the majority of the hydrophilic tail) from the C-terminal end of D2 has a drastic effect on the function and stability of the PS II complex as shown by the loss of oxygen evolution and diuron-binding ability. With such a large number of amino acids missing from the C-terminus, the D2 protein appears unable either to insert into the thylakoid membrane or to help assemble a stable PS II complex in the membrane. The removal of nine C-terminal amino acids has a similarly drastic effect on PS II stability, but ET-9 differs from ET-57 in that ET-9 contains hardly any CP43 protein in its thylakoid membranes but retains some D2. Upon examination of the gene sequence of *psb*DI/C, it is clear that the codon change in the *psb*DI gene to terminate *psb*DI translation prematurely by nine codons also altered the Shine–Dalgarno region of *psb*C (Figure 1). Thus, the phenotype of the ET-9 mutant may be due primarily to a drastically decreased translation of CP43 rather than to the truncation of the D2 protein. Note that the phenotype of the ET-9 mutant (some D2, no CP43, and no herbicide binding) is strikingly similar to that of a mutant in which *psb*C is interrupted or deleted (Vermaas et al., 1988; Rögner et al., 1991).

In order to not interfere further with the expression of *psb*C, we decided to study the effects of decreasing the length of D2 by less than nine residues by introducing 2–8 residue deletions within the C-terminal tail of D2 in regions coded by parts of *psb*DI that do not overlap with the ribosome-binding site or the coding region of *psb*C. The results were rather unexpected. Mutants Δ(11–19) and Δ(12–18), with a loss of eight and seven residues, respectively, in the region between the tenth and twentieth amino acid residues from the C-terminus of D2, are photoautotrophic and show oxygen evolution at rates approximately 75% of those in the wild type. However, the deletion of fewer amino acid residues within the same domain leads to far more drastic effects: the mutants with four, three, or two residues deleted out from this domain [Δ(11–15), Δ(11–14), and Δ(12–14), respectively] are obligate photoheterotrophs, and the Δ(11–15) mutant even was unable to bind diuron and did not have D2 detectable in its thylakoid membranes. However, the Δ(11–15) mutant had normal amounts of CP43, excluding the possibility of a negative effect of this mutation on *psb*C expression. Its phenotype is similar to that of ET-57. It appears that with this Δ(11–15) deletion, D2 is unable to insert into the thylakoid membrane or help assemble a stable PS II complex. The area of the Δ(11–15) deletion is covered also in the photoautotrophic Δ(11–19) mutant. This implies that a further deletion of D2 in the obligate photoheterotrophic Δ(11–15) mutant would restore photosynthetic function.

The mutant with a two amino acid deletion, Δ(12–14), shows a decreased amount of the D2 and CP43 proteins in its thylakoid membranes, which is similar to that seen in ET-9. This Δ(12–14) mutant differs from ET-9 in that PS II complexes can be detected by diuron-binding assays albeit at

a severely decreased level (8500 chlorophylls per binding site). It also has a minimal ability to evolve oxygen, which goes along with the low number of PS II complexes. Because of the decreased amount of CP43 in this mutant and the marginal amounts of PS II reaction centers and oxygen evolution, a decrease (but not abolition) of *psb*C translation may occur in this mutant and be the primary cause for its phenotype. It is possible that the introduction of two additional GGA sequences just upstream of the *psb*C Shine–Dalgarno sequence in this mutant (see Table I) impairs proper recognition of the native ribosome-binding site.

The loss of three amino acids from the D2 protein (Δ(11–14)) results in a particularly interesting phenotype. The Δ(11–14) mutant is unable to grow photoautotrophically, yet it has close to the same number of reaction centers (1800 chlorophyll per binding site) as Δ(11–19) and Δ(12–18), which are photoautotrophic. Its initial oxygen evolution rate is about 50% that of the wild type, indicating that PS II complexes in the Δ(11–14) mutant initially are functional. The reason for its obligate photoheterotrophic behavior probably is that, at high light intensities, Δ(11–14) is rapidly photoinhibited. Other obligate photoheterotrophic D2 mutants with similar behavior have been reported: For example, in the D2 protein the change at Glu-69 to Gln, the mutation of Pro-161 to Leu, and the replacement of Gly-215 by Trp result in photoheterotrophic mutants with active oxygen evolution that is rapidly inactivated in the light (van der Bolt & Vermaas, 1992).

It is surprising that the smaller amino acid deletions have the most severe consequences for the PS II complex, especially in view of the fact that if the same amino acid residues are removed in the larger deletions a photoautotrophic phenotype results. This excludes the possibility of a critical function of particular amino acid residues that were deleted and suggests that the length and nature of the spacing between two residues may be important in determining the function of the PS II complex. Whether or not the effects of the mutations on PS II function are direct (i.e., are caused by direct perturbations of protein/cofactor interactions) or are mediated through other protein subunits, whose stability and/or function have been affected by the D2 mutations, cannot be determined at this moment. However, it should be kept in mind that the phenotypes observed here do not match, for example, the phenotype that results if PS II-O is absent (Burnap & Sherman, 1991; Mayes et al., 1991; Philbrick et al., 1991).

Even though no particular C-terminal residue of the D2 protein can be identified as the critical one for PS II function, several comments can be made regarding the apparent requirements of the C-terminal tail of the D2 protein. As is evident from the primary structure of the C-terminal domain in the various mutants as presented in Figure 2, the C-termini of the photoautotrophic ET-11 mutant and the obligate photoheterotrophic ET-13 mutant differ only by Ile and Phe residues. As the side groups of these residues are unlikely to interact specifically with any of the components of the water-splitting complex, the reason for obligate photoheterotrophy of ET-13 most probably is not to be sought in the deletion of side groups involved in, for example, Mn binding. At least two other options are available: In the first place, wild type and ET-11 differ from ET-13, ET-14, ET-15, and ET-16 in that the former two strains carry uncharged side chains at the four C-terminal residues, while the latter four strains have a smaller number of uncharged side chains in the four C-terminal residues. Thus, it is possible that a relatively hydrophobic C-terminus (not counting the C-terminal COO^- group) is required in D2 for proper function. Secondly, it is possible that a certain length of the C-terminal tail is required to reach the components with which it should interact; in that case, a loss of 11 residues is allowed without the loss of stable oxygen evolution, while a loss of 13 or more residues cannot be accommodated.

With either of these two possibilities (hydrophobicity or length), the results on the deletion mutants indicate that either hydrophobicity or length by itself is not the sole determinant of proper interaction with other components. With respect to the hydrophobicity of the C-terminus, all deletion mutants have the same sequence of the 10 C-terminal amino acid residues, and yet they have different photosynthetic properties. With respect to length, two mutants with small deletions, Δ(11–14) and Δ(11–15), do not appear to be affected in CP43 synthesis and yet are obligate photoheterotrophs.

The Δ(11–14) mutant is rapidly photoinactivated, while it contains the sequence Gln-Pro-His-Glu-Asn absent in the photoautotrophic Δ(11–19) mutant and lacks the sequence Phe-Ile-Phe-Pro present in the wild type (see Figure 2). A possible explanation for the results obtained on the Δ(11–14) and Δ(11–15) mutants is that the charge density in the region about 10 residues from the C-terminus is of importance in determining the proper folding of D2 and assembly of a functionally stable PS II complex. The Δ(11–15) mutant resembles ET-57 in that it does not contain a detectable amount of D2 in its thylakoids, but retains some CP43; as we think that D2 in the ET-57 mutant may not fold properly through the thylakoid membrane (the C-terminal hydrophilic tail is short), it is possible also that D2 in the Δ(11–15) mutant, where in the region about 10 residues from the C-terminus there are three Glu residues separated by one Leu, does not fold through the membrane properly. The Δ(11–14) mutant, with three negative charges separated by an Asn and a Leu about 10 residues from the C-terminus of D2, forms stable PS II centers, but functionally it appears to be unable to put together PS II complexes that can sustain photoautotrophic growth. Although further experimentation will be necessary to elucidate details regarding functionally and structurally important features of the C-terminus of D2, the results presented here clearly indicate the importance of the C-terminal domain of D2 in PS II activity.

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