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Site-directed mutagenesis in photosystem II of the cyanobacterium *Synechocystis* sp. PCC 6803: Donor D is a tyrosine residue in the D2 protein

(photosynthesis/protein engineering/oxygen evolution/electron paramagnetic resonance/electron transport)

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ABSTRACT **The chemical nature of electron donor(s) in photosystem II in photosynthetic membranes was analyzed by site-directed mutagenesis of the gene encoding the protein D2 of the photosystem II reaction center. Mutation of the Tyr-160 residue of the D2 protein into phenylalanine results in the disappearance of the electron paramagnetic resonance signal II_S originating from D^+, the oxidized form of the slow photosystem II electron donor D. Signal II_S is still present if a neighboring residue in D2, Met-159, is mutated into arginine. Both mutants have normal rereduction kinetics of the oxidized primary electron donor, $P680^+$, in octyl glucoside-extracted thylakoids, indicating that D is not directly involved in $P680^+$ reduction. However, overall photosystem II activity appears to be impaired in the Tyr-160–Phe mutant: photosystem II-dependent growth of this mutant is slowed down by a factor of 3–4, whereas photoheterotrophic growth rates in wild type and mutant are essentially identical. Binding studies of diuron, a photosystem II herbicide, show that there is no appreciable decrease in the number of photosystem II centers in the Tyr-160–Phe mutant. The decrease in photosystem II activity in this mutant may be interpreted to indicate a role of D in photoactivation, rather than one as an important redox intermediate in the photosynthetic electron-transport chain.**

The photosystem II (PSII) complex, localized in the thylakoid membrane, catalyzes the light-induced reduction of plastoquinone by water. The rates and efficiencies of the reactions in PSII depend on a delicate interaction between the PSII protein complex and cofactors/prosthetic groups that are bound to it. The PSII complex consists of six or more integral membrane polypeptides as well as several proteins extrinsic to the thylakoid membrane. Cofactors include chlorophyll a, pheophytin a, quinones, Mn, Fe, Cl^-, and Ca^{2+}. For detailed information regarding PSII structure and function, the reader is referred to recent reviews (1, 2).

A major impetus toward understanding the structure and function of PSII was the recognition of similarities between PSII and the photosynthetic reaction center from purple bacteria (3–7), for which high-resolution crystal structures have been determined (4, 8–11). According to current hypotheses (see ref. 12 and refs. therein), binding of P680, the primary electron donor of PSII, is shared between two integral membrane proteins, D1 and D2, just as the bacterial primary donor is bound virtually symmetrically between two subunits, L and M, of the reaction center complex. D1 is presumed to be homologous to L, and D2 to M (3–5). These conclusions have been corroborated by subfractionation and spectroscopic studies on PSII reaction centers (13–15) and site-directed mutagenesis of crucial D2 residues (16). However, at the electron donor side of the reaction center a functional similarity between purple bacteria and PSII is absent.

The PSII donor side appears to involve (*i*) Z, a rapid electron donor to photooxidized P680; (*ii*) D, a slow electron donor of as yet unknown function; and (*iii*) the Mn-containing oxygen-evolving complex (17). Until recently, Z and D were assumed to be quinols, since upon oxidation these intermediates gave rise to characteristic electron paramagnetic resonance (EPR) signals (signals II) resembling those of plastoquinol cation radicals (ref. 17 and refs. therein). However, plastoquinone extraction of PSII particles did not yield a proper stoichiometry to account for Z and D (18). Results of iodide-labeling experiments indicated that tyrosine residues in D1 and D2 were involved in electron donation to the reaction center (19, 20). The labeling data could be interpreted by identifying Z and D as tyrosine residues in D1 and D2, respectively.

Sequence analysis of the genes for the D1 and D2 proteins, *psbA* and *psbD*, respectively, indicated a tyrosine residue in the putative third membrane-spanning region at position 161 [amino acid numbering for spinach (5)] in both D1 and D2. According to D1/D2 folding models (5), these tyrosine residues are expected to be approximately as far from the inside of the membrane as the histidine residue is that presumably contributes to P680 binding (His-197 in cyanobacterial D2) (16). In addition, the high degree of symmetry in the vicinity of the Tyr-161 residues in D1 compared to D2 could account for the almost identical EPR spectra of Z^+ and D^+.

To test the hypothesis that an oxidized tyrosine residue in D2 could give rise to signal II, a site-directed mutation was introduced to change the Tyr-160 residue of D2 into phenylalanine in the cyanobacterium *Synechocystis* sp. PCC 6803. In this organism, the Tyr-160 residue of D2 is homologous to the Tyr-161 residue of spinach (21). Moreover, another D2 mutant was created in which a neighboring residue, Met-159, was replaced by arginine to test whether changes seen in the Tyr-160–Phe mutant are specific for residue 160.

MATERIALS AND METHODS

Site-Directed Mutagenesis of the *Synechocystis psbD-I* Gene. To induce a specific base change in the *psbD-I* gene from *Synechocystis* sp. PCC 6803, a 1.7-kilobase (kb) *Xba* I/*Eco*RI fragment containing *psbD-I* [except 201 base pairs (bp) at the 5′ end] and some of *psbC* was cloned into M13mp18 (22), single-stranded DNA was prepared (23), and complementary

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Abbreviations: Chl, chlorophyll; PSII, photosystem II; EPR, electron paramagnetic resonance.

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DNA was synthesized with a kinase-treated mutagenic oligonucleotide used as a primer, essentially by standard protocols (23, 24). For the Tyr-160–Phe mutation, the oligonucleotide used was 5′-TTTGATGTTCCCCTTG-3′ (the mutated base is underlined). For the Met-159–Arg mutation, 5′-TTTTTGAGGTACCCCT-3′ was used. The former mutation destroys a *Rsa* I site (recognition sequence, 5′-GTAC-3′) present in the wild type, whereas the latter mutation creates a *Kpn* I site (recognition sequence, 5′-GGTACC-3′) in the mutant. Mutants were selected by restriction digestion of the replicative form of the virus DNA, and the mutations were verified by sequencing using the dideoxynucleotide chain-terminator method (25). Subsequently, a 533-bp *Xba* I/*Nco* I *psbD-I* fragment containing the site-directed mutation was cut out. This fragment was ligated into a construction in pUC118 containing *psbD-I* (from the *Nco* I site onward), *psbC*, and the downstream region of *psbC* interrupted by a kanamycin-resistance cartridge [from Tn*903* (26)] at the *Xmn* I site ≈200 bp beyond the 3′ end of *psbC*. As a control, a wild-type *Xba* I/*Nco* I *psbD-I* fragment was ligated into this construct.

The ligated plasmids were used to transform a "double-deletion mutant" of *Synechocystis* sp. PCC 6803 (27), which lacks all but the 5′ 300 bp of *psbD-I*, all of *psbC*, and all but the 5′ and 3′ ends of *psbD-II*, the second *psbD* gene. Transformation was carried out as described (28), and kanamycin-resistant transformants were selected. The transformants obtained with the control construct (wild-type *psbD-I* and *psbC*) are referred to as wild type in this study, and although it lacks *psbD-II* and contains a kanamycin-resistance cartridge downstream of *psbC*, it is indistinguishable in growth, viability, and phenotype from the original *Synechocystis* sp. PCC 6803 wild-type strain (except for its kanamycin resistance).

Propagation of Cyanobacteria. Conditions for growth of *Synechocystis* sp. PCC 6803 have been described (28). For measuring growth rates, the strains were cultivated in BG-11 (29) medium, and at time 0, 75-ml cultures were started at an optical density of 0.08 at 730 nm [as measured with a Shimadzu (Kyoto, Japan) UV-160 spectrophotometer]. Where indicated, 5 mM glucose was added.

Thylakoid Preparation and Extraction. Cells were harvested and resuspended at 0.5 mg of chlorophyll (Chl) per ml in the isolation buffer containing 50 mM Mes, NaOH (pH 6.3), 25 mM $CaCl_2$, 0.4 M sucrose, and 10 mM NaCl. The cells were passed twice through a chilled French press at 20,000 psi (138 MPa). After centrifugation (4000 × *g*; 5 min), most thylakoids remained in the supernatant and were pelleted by another centrifugation step (50,000 × *g*; 15 min) and resuspended in the isolation buffer to 1 mg of Chl per ml. To obtain thylakoid extracts suitable for optical measurements, 35 mM octyl glucoside was added, and, after 10 min incubation on ice, the nonextracted fraction was pelleted (50,000 × *g*; 15 min). The supernatant was diluted with 3 vol of isolation buffer to well below the critical micelle concentration of octyl glucoside (which is ≈30 mM), and the thylakoid extract was pelleted (227,000 × *g*; 1 hr) and resuspended in isolation buffer at 0.3–1.0 mg of Chl per ml. This thylakoid extract is not active in oxygen evolution.

Herbicide Binding. To determine the diuron affinity and concentration of binding sites for this herbicide in wild type and mutant, various concentrations of [^{14}C]diuron (Amersham; 243 μCi/mg; 1 Ci = 37 GBq) were added to 1 ml of cyanobacterial cells (25–35 μg of Chl per ml) in 10 mM Tricine (pH 7.5). The mixtures were incubated for 90 min in the dark, cells were pelleted by a 4-min spin in a Beckman Microfuge (type E), and 0.8 ml of supernatant was taken out to mix with 10 ml of ACS scintillation fluid (Amersham) and to count in a scintillation counter.

EPR. Cells were washed, pelleted, and resuspended at a concentration of ≈2 mg of Chl per ml. They were then loaded into a quartz flat cell. EPR spectra were recorded at room temperature with a Bruker ESR 200D X-band spectrometer. Illumination of samples in the cavity was provided by an 800-W projector lamp; three Calflex filters and 2 cm of water were used to remove infrared radiation. Some experiments were performed on octyl glucoside-extracted thylakoids. In such preparations, a large, stable free radical (presumably $P700^+$) was sometimes seen. This was removable by the addition of sodium ascorbate. To remove signals from Mn^{2+}, 1 mM EDTA was added.

Absorption Changes at 820 nm. Rereduction kinetics of $P680^+$ were measured at 820 nm at room temperature with a flash-absorption spectrometer as described (30), except that the actinic flash was provided by a dye laser pumped by a frequency-doubled YAG laser (9 ns; broad band, ≈595 nm). Experimental conditions are described in the legend of Fig. 3.

RESULTS

Southern Hybridizations. To confirm that the site-directed mutations have been induced into the *psbD-I* gene in the cyanobacterial genome, restricted DNA from wild type and mutants was probed with radiolabeled fragments containing the region of the mutations (Fig. 1). The sizes of the observed fragments correspond to the values expected from the genome map of this area: there is an *Rsa* I site 304 bp upstream from the site of the mutation, as well as one 1.1 kb downstream of the codon for Tyr-160 (J. G. K. Williams, personal communication). Thus, in the wild type and Met-159–Arg mutant a 0.3-kb and a 1.1-kb fragment are expected to hybridize to the probe, whereas in the Tyr-160–Phe mutant the *Rsa* I site at the mutation has been deleted, and a 1.4-kb fragment results. The hybridization observed at 0.6 kb may reflect a DNA fragment that is rather similar to *psbD-I*; the same may be true for a 0.3-kb fragment that coelectrophoreses with the 0.3-kb fragment mentioned above. This is the

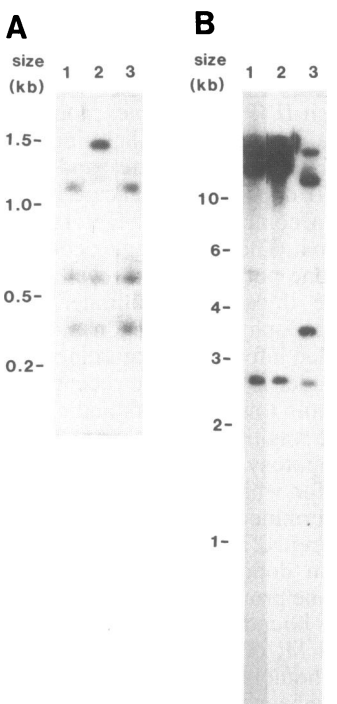

FIG. 1. Southern blot of *Rsa* I-digested (*A*) and *Kpn* I-digested (*B*) *Synechocystis* DNA from wild type (as defined in *Materials and Methods*) (lane 1), the Tyr-160–Phe mutant (lane 2), and the Met-159–Arg mutant (lane 3). (*A*) A nick-translated 533-bp *Xba* I/*Nco* I *psbD-I* fragment cloned in pUC120 was used as a probe. (*B*) A 5-kb *Kpn* I/*Eco*RI fragment containing *psbD-I* in pUC18 was used.

reason why a 0.3-kb fragment is visible in Fig. 1*A* (lane 2), which is fainter than that in lanes 1 and 3. Fig. 1*B* indicates that the Met-159–Arg mutation indeed induced an extra *Kpn* I restriction site at that site (see *Materials and Methods*), in addition to existing *Kpn* I sites 3.6 kb upstream from the base triplet encoding Met-159 (J. G. K. Williams, personal communication) and ≈12 kb downstream of this triplet (W.F.J.V. and J. G. K. Williams, unpublished data). Once again two extra bands are observed (one at 2.6 kb, and one fragment of >15 kb) that cannot yet be assigned. Since these extra bands are present in all three strains (wild type and mutants), they are unrelated to the subject of the present study.

The data in Fig. 1 indicate that the desired mutations indeed have been incorporated into the *Synechocystis* mutants.

EPR. In intact cells, the EPR signal from D^+, signal II_S, is known to be stable for many hours in darkness (31), whereas those from Z^+ and $P700^+$ disappear rapidly after turning off the light. It was found that signal II_S was absent in the Tyr-160–Phe mutant but was present in both wild type and the Met-159–Arg mutant, exhibiting its usual g value (g, ≈2.0046) and line shape (Fig. 2, solid lines). In the light, the spectrum is dominated by $P700^+$ (g, ≈2.0025) in all three samples. Since the minor structural change of a tyrosine to a phenylalanine residue causes the disappearance of the EPR signal, and the potentially more significant structural change of the neighboring methionine to arginine has only a relatively minor effect on its amplitude, it is concluded that signal II_S is specifically dependent on this tyrosine residue. It is not yet known why the amplitude of the EPR signal in the Met-159–Arg mutant is smaller and the shape is slightly different than in wild type.

In PSII-enriched thylakoid extracts prepared with octyl glucoside, similar results were obtained: no signal II_S could be detected in the Tyr-160–Phe mutant. The amplitude of signal II_S was somewhat decreased in the Met-159–Arg mutant compared to wild type.

Rereduction Kinetics of Oxidized P680. Since $P680^+$ is presumably directly reduced by Z (which may be the Tyr-161 of D1; see *Discussion*), and since D1 and D2 appear to be symmetrically arranged with regard to functional components, the effect of the mutations on electron donation to $P680^+$ in the microsecond range was measured. As shown in Fig. 3, no difference in the kinetics of $P680^+$ reduction after a flash was observed in octyl glucoside extracts of thylakoids from wild type and mutants ($t_{1/2}$, 7 μs). These extracts do not have a functional water-splitting complex. The half-times of reduction of $P680^+$ showed a similar pH dependence for the wild type and for the mutants, decreasing from ≈40 μs at pH 4.3 to ≈2 μs at pH 7.3 (data not shown). Such an effect is characteristic of Z donation to $P680^+$ in higher plants (30). This confirms that D^+ is not directly involved in electron transfer through PSII. Moreover, it indicates that $P680^+$ reduction kinetics are independent of D, at least in preparations that do not evolve oxygen. The smaller amplitude of the signal in the Met-159–Arg mutant is consistent with the EPR measurements described above.

Cyanobacterial Growth and Oxygen Evolution. As an indicator of overall PSII activity in mutants and wild type, cyanobacterial growth in liquid medium under photoautotrophic and photoheterotrophic conditions was measured. Under photoautotrophic conditions (PSII is required for growth), the Tyr-160–Phe mutant grows considerably slower than the wild type: the approximate doubling time of wild type is 12 hr, whereas that of the Tyr-160–Phe mutant is ≈40 hr (Fig. 4). However, under photoheterotrophic conditions, when PSII is not required for growth, wild type and mutant have similar growth curves (Fig. 4). This indicates that under our growth conditions the overall photosynthetic activity in the Tyr-160–Phe mutant is 3–4 times less than in wild type. Photoautotrophic growth of the Met-159–Arg mutant resembled that of the wild type (data not shown). We prefer to measure *in vivo* cyanobacterial growth curves rather than Hill reaction rates in intact cells to determine photosynthetic activity, since in our hands the electron transport rates measured in cells depend on age, condition, and possibly the energetic state of the culture. Hill reaction rates in the Tyr-160–Phe mutant varied between 20% and 120% of the rates of the wild-type cells depending on the experiment (data not shown).

Experiments were carried out by M. Picaud and A.-L. Etienne (Centre National de la Recherche Scientifique, Gif-sur-Yvette) to determine whether the decreased PSII-

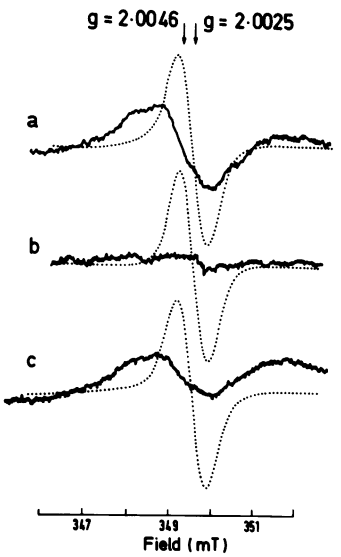

FIG. 2. Room temperature EPR spectra from *Synechocystis* cells (≈2 mg of Chl per ml) of wild type (spectrum a), Tyr-160–Phe (spectrum b), and Met-159–Arg (spectrum c) mutants. Solid lines were recorded in darkness ≈12 min after a 30-s illumination. Dotted lines were recorded under illumination at a 5 times lower gain. EPR conditions were as follows: microwave frequency, 9.80 GHz; microwave power, 20 mW (10 dB); modulation amplitude, 0.2 T.

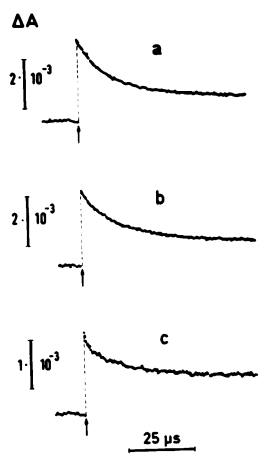

FIG. 3. Flash-induced absorption changes at 820 nm at room temperature in octyl glucoside extracts of thylakoids from *Synechocystis* wild type (spectrum a), Tyr-160–Phe (spectrum b), and Met-159–Arg (spectrum c) mutants. The extracts were suspended in 50 mM Mes/NaOH, pH 5.3/10 mM NaCl/25 mM $CaCl_2$/0.4 M sucrose to a Chl concentration of 50 μg/ml. $K_3Fe(CN)_6$ (5 mM) was added. The optical path lengths in the cuvette were 10 mm (measuring beam) and 4 mm (excitation flash). Averaged effect of 40 flashes.

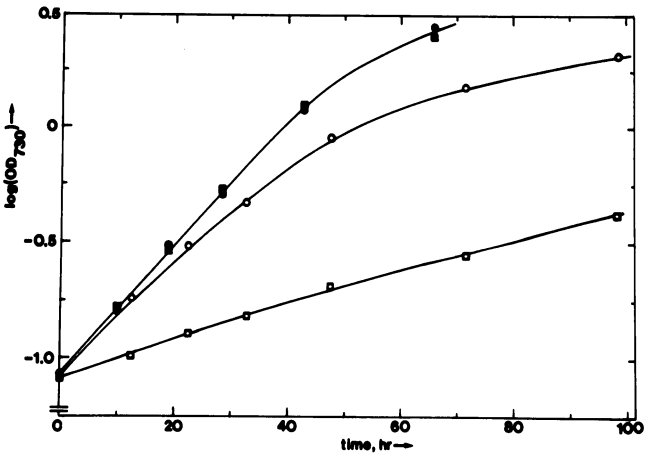

FIG. 4. Semilogarithmic growth curves of wild type (○, ●) and the Tyr-160–Phe mutant (□, ■) under photoautotrophic (open symbols) and photoheterotrophic (solid symbols) growth conditions. Cyanobacterial growth was measured as the optical density of the culture at 730 nm (OD_{730}). Appropriate dilutions were made in the cuvette to have the measured optical density not exceed 0.4.

dependent growth rates as found in the Tyr-160–Phe mutant were reflected in a change of the pattern of flash-induced oxygen evolution. The damping of the oxygen evolution pattern of mutant cells did not appreciably deviate from that in wild type (data not shown), indicating that the quantum yield of charge separation in PSII centers that are active in oxygen evolution is similar in wild type and the Tyr-160–Phe mutant.

Herbicide Binding. From Fig. 4 it is obvious that the Tyr-160–Phe mutant is impaired in PSII activity. Such an impairment can be due to, for example, a decrease in the number of PSII centers. To measure the total number of PSII centers in the mutants on a Chl basis, [^{14}C]diuron binding was monitored in wild type and mutants. The Tyr-160–Phe mutant and the Met-159–Arg mutant consistently showed a decrease (by a factor of 1.2–1.4) in the number of herbicide-binding sites on a Chl basis in comparison with wild type, but this difference is far too small to account for the decrease by a factor of 3 or 4 in growth rate of the Tyr-160–Phe mutant. This indicates that the lower photoautotrophic growth rate in this mutant is not due to a proportionally decreased PSII content. The diuron dissociation constant was 10 nM in wild type and both mutants.

DISCUSSION

The Molecular Identity of the EPR Signal II Components. It was previously assumed that signal II_S originated from a semiquinol radical cation (ref. 17 and refs. therein). The data presented here, however, indicate that the Tyr-160 residue of D2 is directly and specifically correlated with signal II_S. This result is best explained by the assignment of signal II_S to the D2 Tyr-160 radical. This conclusion has also been reached by Barry and Babcock (32). These authors showed that deuteration of plastoquinone does not affect signal II_S, whereas tyrosine deuteration had a large effect. Moreover, Debus *et al.* (33) have independently generated a D2 Tyr-160–Phe mutant, which is also observed to lack signal II_S.

On the basis of the similarity of the shape and *g* value of the EPR signals due to D^+ and Z^+, Z and D presumably are due to identical molecular species in virtually identical environments. We hypothesize that Z is the tyrosine residue in D1 that is symmetrical to Tyr-160 in D2. Indeed, it was this symmetry between the two tyrosine residues and their neighboring amino acid residues in D1 and D2 that led to the choice of D2 Tyr-160 as the target for mutagenesis. Arguments for D^+ and Z^+ being symmetrical tyrosine radicals in the PSII reaction center have been put forward (34). Evidence for the assignment of Z as the Tyr-161 in D1 includes (*i*) the observation that D1 is specifically labeled by $^{125}I^-$ under conditions where Z turns over (19, 20), and (*ii*) the localization of the iodination site at one of the two third helix tyrosine residues in D1 (42). It has been pointed out that both optical and EPR spectra of Z/Z^+ are fully compatible with the hypothesis of Z being a tyrosine residue that can undergo rapid redox reactions (35). Obviously, the involvement of tyrosine residues as substrates in redox reactions is unusual. However, it should be kept in mind that $P680^+$ is highly oxidizing and that at the PSII donor side extreme redox requirements need to be met.

It is startling, however, that the high symmetry of the Tyr-161/160 environments in D1 and D2 is paired with a dramatic difference in redox kinetics: Z/Z^+ undergoes rapid turnover, whereas D^+ is formed slowly [$t_{1/2}$ for formation of signal II_S is in the order of 1 s (31)] and is reduced even more slowly in darkness (minutes to hours). This may indicate that subtle differences in the structure of the reaction center environment have pronounced effects on the reaction rates of the tyrosine residues. This can be considered as another striking example of major functional differences between structurally symmetrical reaction center components, as has been observed in the bacterial reaction center complex.

The Function of D. From its slow kinetics it appears that the component giving rise to signal II_S is not on the "main line" of photosynthetic electron transport, and this work indicates that it does not have a role in influencing Z to $P680^+$ electron transfer. It has been shown that D is responsible for a slow reduction of the S_2 and S_3 states of the water-splitting system ($t_{1/2}$, ≈1 s) (31, 36, 37) and that D^+ can be reduced by S_0 even more slowly (38). However, such slow reactions should not significantly affect the PSII activity in the organism in continuous light. This presents an apparent paradox in that our data indicate that in the absence of Tyr-160 in D2 the number of PSII centers is virtually unchanged, but that the PSII-dependent growth rate is much lower in the Tyr-160–Phe mutant (Fig. 4). For this reason, alternatives for the function of D should be discussed.

It has been suggested that D^+ may play a role in photoactivation (38), the multiphoton process in which Mn is assembled in the oxygen-evolving system in the appropriate redox state, thus activating oxygen evolution in PSII (see ref. 39 and refs. therein). It is clear from the present results that D^+ does not play an obligatory role in this process. However, a decreased efficiency of photoactivation in the absence of D^+ could very well result in slower photosynthetic growth rates as observed in the Tyr-160–Phe mutant without changing appreciably the total number of PSII centers present in the thylakoid. If the absence of D^+ would decrease the efficiency of photoactivation, the number of PSII centers active in oxygen evolution would be lower in the Tyr-160–Phe mutant than in wild type (with the total number of PSII centers being the same in both strains), whereas the damping of the oscillation of the oxygen evolution flash pattern would be similar in wild type and mutant. These features are consistent with the properties observed in the Tyr-160–Phe mutant.

Since it has been shown that the extrinsic 33-, 24-, and 18-kDa proteins are not necessarily needed for Mn binding to PSII (40) (although the 33-kDa protein is involved in stabilization of Mn binding), it becomes tempting to speculate that D1 and D2 create part of the binding environment for the Mn cluster in the oxygen-evolving complex. Indeed, results obtained with a *Scenedesmus* mutant can be interpreted to indicate a direct effect of D1 on oxygen evolution (41). Further site-directed mutagenesis experiments seem to be appropriate to characterize and define the binding environ-

ment of the Mn cluster. This may finally lead to a structural elucidation of the oxygen-evolving complex in PSII.

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