Functional Characterization of Mutant Strains of the Cyanobacterium *Synechocystis* sp. PCC 6803 Lacking Short Domains within the Large, Lumen-Exposed Loop of the Chlorophyll Protein CP47 in Photosystem II†

Hermann M. Gleiter,‡,‖ Elisabeth Haag,‡ Jian-Ren Shen,§ Julian J. Eaton-Rye,⊥,# Yorinao Inoue,§ Wim F. J. Vermaas,⊥ and Gernot Renger*,‡

Max-Volmer Institute for Physical and Biophysical Chemistry, Technical University Berlin, 1000 Berlin 12, Germany, Solar Energy Research Group, The Institute of Physical and Chemical Research (RIKEN), Wako, Saitama, 351-01 Japan, and Department of Botany, Arizona State University, Tempe, Arizona 85287

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ABSTRACT: Several autotrophic mutant strains of *Synechocystis* sp. PCC 6803 carrying short deletions or a single-site mutation within the large, lumen-exposed loop (loop E) of the chlorophyll *a*-binding photosytem II core protein, CP47, are analyzed for their functional properties by measuring the flash-induced pattern of thermoluminescence, oxygen yield, and fluorescence quantum yield. A physiological and biochemical characterization of these mutant strains has been given in two previous reports [Eaton-Rye, J. J., & Vermaas, W. F. J. (1991) *Plant Mol. Biol. 17*, 1165–1177; Haag, E., Eaton-Rye, J. J., Renger, G., & Vermaas, S. F. J. (1993) *Biochemistry 32*, 4444–4454]. The results of the present study show that deletion of charged and conserved amino acids in a region roughly located between residues 370 and 390 decreases the binding affinity of the extrinsic PS II-O protein to photosystem II. Marked differences with PSII-O deletion mutants are observed with respect to Ca^{2+} requirement and the flash-induced pattern of oxygen evolution. Under conditions where a sufficient light activation is provided, the *psb*B mutants assayed in this study reveal normal S-state parameters and lifetimes. The results bear two basic implications: (i) the manganese involved in water oxidation can still be bound in a functionally normal or only slightly distorted manner, and (ii) the binding of the extrinsic PS II-O protein to photosystem II is impaired in mutants carrying a deletion in the domain between residues 370 and 390, but the presence of the PS II-O protein is still of functional relevance for the PS II complex, e.g., for maintenance of a high-affinity binding site for Ca^{2+} and/or involvement during the process of photoactivation.

In all oxygen-evolving photoautotrophic organisms, the key steps of light-induced water cleavage to dioxygen and chemically bound hydrogen take place within a multimeric pigment–protein complex referred to as photosystem II (PS II),[1] which is anisotropically integrated into the thylakoid membrane [for reviews, see Babcock (1987), Renger (1987), and Ikeuchi (1992)]. The overall process catalyzed by PS II comprises three types of reaction sequences: (a) photooxidation of a special Chl *a* component (P680) and subsequent stabilization of the primary charge separation by rapid electron transfer from the pheophytin anion ($Pheo^-$) to a specially bound plastoquinone (Q_A) [for a review, see Renger (1992)], (b) cooperation of four oxidizing redox equivalents with a manganese-containing unit giving rise to water oxidation into dioxygen under proton release [for reviews, see Rutherford et al. (1992), Debus (1992), and Renger (1993)], and (c) plastoquinone reduction to plastoquinol under proton uptake [for a review, see Crofts and Wraight (1983)]. On the basis of functional analogies (Rutherford, 1986) and structural homologies with reaction centers from purple bacteria (Trebst, 1986; Michel & Deisenhofer, 1988), all redox groups participating in reaction sequences a and c are inferred to be incorporated into a heterodimer of two polypeptides designated D1 and D2. On the other hand, only limited information exists concerning the protein matrix of the manganese-containing catalytic site of reaction sequence b. Among different polypeptides closely associated with the D1/D2 heterodimer, the chlorophyll a-binding protein CP47 (*psb*B gene product) is of special interest. This protein was originally thought to contain the photoactive pigment P680 (Nakatani et al., 1984). Later, a CP47/D1/D2 model was proposed for PS II (Renger, 1986). Although some assumptions on the location of the functional groups are no longer valid, the basic implication of this model is still worth consideration, i.e., the idea that CP47 not only is an essential part of the core antenna but also is of central relevance for a functionally competent PS II. Various lines of evidence indicate that CP47 is likely to be of structural importance:

(1) Deletion mutagenesis experiments reveal that CP47 plays a key role for the integrity of the PS II complex (Vermaas et al., 1986, 1988).

(2) Biochemical studies show that a stable charge separation cannot be achieved in isolated PS II subcomplexes which lack CP47. Extraction of CP47 by detergent and $LiClO_4$ treatment causes a loss of functional Q_A. On the other hand, biochemical removal of CP43 only marginally affects the photoreduction of Q_A (Yamagishi & Katoh, 1985; Akabori et al., 1988).

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* Correspondence address: Technical University, Berlin.
‡ Technical University, Berlin.
§ The Institute of Physical and Chemical Research, Wako.
‖ Present address: Department of Physiology, Phillips-University Marburg, Germany.
⊥ Arizona State University.
Present address: Department of Biochemistry, Otago University, Dunedin, New Zealand.

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[1] Abbreviations: Chl, chlorophyll; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PS II, photosystem II; SDS, sodium dodecyl sulfate.

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Recent reconstitution experiments reveal that functional reintegration of Q_A into the reaction center requires a CP47/D1/D2 complex and in addition the presence of several low molecular weight proteins and lipids in the reconstitution medium (Araga et al., 1993). These findings might suggest that CP47 is of structural relevance for stable Q_A binding but do not provide straightforward evidence for this possiblility. In contrast, CP43 is not required for a functional acceptor side, as shown by analysis of a *psb*C$^-$ mutant lacking CP43 (Rögner et al., 1991).

(3) With regard to its possible role as a constituent of a functionally competent water oxidase, CP47 appears to be an attractive candidate to contribute to stabilization and/or ligation of the tetranuclear manganese cluster. Of special interest are recent findings which indicate a close interaction with PS II-O, an extrinsic 33-kDa protein involved in regulation of water oxidation. Although the detailed function of PS II-O is still a matter of debate [for a review, see Debus (1992)], its close association with CP47 would imply a possible role of CP47 for the water-oxidizing system. In the past, several biochemical studies were performed in order to unravel the interaction between CP47 and the extrinsic PS II-O protein. The following interesting results were obtained: (i) extraction of PS II-O increases the accessibility of CP47 to proteases, labeling reagents, and/or antibodies against CP47 (Bricker & Frankel, 1987; Bricker et al., 1988; Frankel & Bricker, 1990), (ii) cross-linking experiments indicate a close proximity between PS II-O and CP47 (Enami et al., 1987, 1989, 1991; Bricker et al., 1988; Odom & Bricker, 1992), and (iii) limited proteolysis experiments suggest that the domain around K389 is important in binding of PS II-O and in maintaining oxygen evolving activity (Hayashi et al., 1993). All available data suggest that the N-terminus of PS II-O interacts with a domain between residues 364 and 440 of CP47 (Eaton-Rye & Murata, 1989; Frankel & Bricker, 1990; Odom & Bricker, 1992); this domain is located on the C-terminal half of a large hydrophilic loop (loop E) of CP47 which connects the putative transmembrane helices V and IV and is presumably exposed to the lumenal side [according to hydropathy analysis of the primary structure; see Vermaas et al. (1987) and Bricker (1990)]. The importance of loop E and specific basic residues of loop E for water oxidation have recently been also demonstrated by genetic investigations (Putnam-Evans & Bricker, 1992; Haag, 1993). Genetic engineering of the *psb*B gene, which encodes CP47, provides a powerful tool to analyze the role of CP47 by selective modifications of the protein. In a preceding study, oligonucleotide-directed mutagenesis was used in order to generate mutants of *Synechocystis* sp. PCC 6803 with deletions of 3–8 amino acid residues in conserved and charged regions of the long hydrophilic loop E. In addition, in one mutant the only histidine of this loop (H343) was changed to leucine (Haag et al., 1993).

In the present study these mutants were used in order to analyze structural changes of functional relevance by measuring the following properties: (a) abundance and binding of the PS II-O protein, (b) flash-induced thermoluminescence, (c) flash-induced oscillation pattern of oxygen evolution, and (d) flash-induced transient change of chlorophyll fluorescence quantum yield.

MATERIALS AND METHODS

Mutant Construction. Deletion of selected domains and mutation of H343 was performed employing a directed mutagenesis technique in the cyanobacterium *Synechocystis* sp. PCC 6803. The system used to manipulate the *psb*B gene of *Synechocystis* sp. PCC 6803 has been described previously (Eaton-Rye & Vermaas, 1991). Construction and verification of the 12 short deletions and the single-site mutation of H343 have been described in a preceding paper (Haag et al., 1993).

Cell Culture and Growth. Liquid cultures of control and mutant strains were cultivated at 30 °C in regular BG-11 medium (Rippka et al., 1979) supplemented with 5 mM glucose and 20 μg/mL kanamycin. During propagation on solid agar plates (BG-11 medium containing 1.5% w/v agar, 0.3% $Na_2S_2O_3$, 10 mM TES/NaOH, pH 8.2) 20 μM atrazine was additionally included in the medium in order to inhibit PS II activity and thus to reduce the probability of selection for revertants. The light intensity in the growth chamber was 60 $\mu E/m^2$ s.

Preparation of Thylakoids. Thylakoid membranes were isolated from cultures with an optical density of 0.7–0.9 at 730 nm according to Yu and Vermaas (1990) in the presence of protease inhibitors (2 mM ϵ-aminocaproic acid, 2 mM benzamidine, 1 mM phenylmethanesulfonyl fluoride). Thylakoid membranes were stored at –80 °C with an additional 10% glycerol.

Chlorophyll Analysis. Chlorophyll was extracted in 100% methanol from whole cells and in 80% acetone from isolated thylakoids. The Chl *a* concentration was determined according to MacKinney (1941).

Gel Electrophoresis and Immunoblot Analysis. SDS–polyacrylamide gel electrophoresis was performed according to Chua (1980) in the presence of 5 M urea. Samples were incubated in Laemmli buffer (Laemmli, 1970) containing 5% (v/v) β-mercaptoethanol for 30 min at room temperature prior to application. Immunoblot analysis was carried out as described previously (Vermaas et al., 1988) with antibodies raised against the PS II-O protein, kindly provided by Prof. B. Andersson.

Flash-Induced Thermoluminescence. Thermoluminescence was measured with a setup described in Rutherford et al. (1982). Samples were preilluminated with 500 saturating flashes and subsequently darkadapted for 5 min before measurement. Samples were excited by single saturating flashes from a Xenon flash lamp (FWHM $\approx$10 μs) for charge separation. The sample concentration was 100 μM Chl, and the heating rate was kept to 1 °C/s.

Flash-Induced Oxygen Evolution. The pattern of flash-induced oxygen yield induced by a train of short flashes from a Xenon lamp (FWHM $\approx$10 μs) was monitored with a Joliot-type electrode as described in Messinger and Renger (1990) with modifications by Gleiter (1993).

Flash-Induced Changes of Fluorescence Quantum Yield. Flash-induced transient changes of the relative fluorescence quantum yield were detected with a home-built instrument (Gleiter, 1988) which has been modified to detect fluorescence by single weak Xenon flashes. In order to minimize artifacts due to fluorescence from the phycobilisomes, the wavelength of the detecting flashes was in the blue region, i.e., the Soret band of chlorophyll. In this way a sufficient flash induced variable fluorescence could be achieved. Samples were preilluminated with 500 saturating flashes and subsequently dark-adapted for 5 min before measurement.

RESULTS

The CP47 mutants analyzed for their functional properties in this study are described with respect to their physiological and biochemical characteristics in a preceding paper (Haag et al., 1993). Only those mutants that exhibit at least some oxygen evolving capacity were analyzed in terms of their

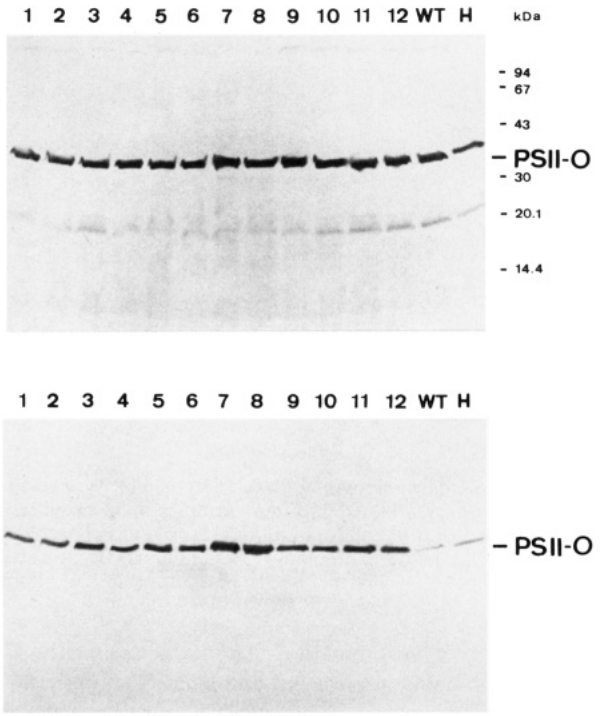

FIGURE 1: Immunoblots of proteins from wild-type and mutant thylakoids and supernatants probed with polyclonal antibodies raised against the extrinsic PS II-O protein. (A, top) thylakoids; (B, bottom) supernatants. Lanes: (1) mutant Δ1; (2) mutant Δ2; (3) mutant Δ3; (4) mutant Δ4; (5) mutant Δ5; (6) mutant Δ6; (7) mutant Δ7; (8) mutant Δ8; (9) mutant Δ9; (10) mutant Δ10; (11) mutant Δ11; (12) mutant Δ12; (WT) wild type; (H) mutant H343L.

functional properties. These include mutant strains Δ3 (K277-E283), Δ6 (G333-I336), Δ7 (K347-R352), Δ8 (A373-D380), Δ9 (V392-Q394), Δ10 (D416-F420), Δ11 (R422-E428), and H343L, where Δ indicates a short deletion in loop E, and the deleted amino acid residues are given in parentheses. In addition, the obligate photoheterotrophic mutant strains Δ1 (I265-F268), Δ2 (T271-K277), Δ4 (T304-L309), Δ5 (F311-N317), and Δ12 (D440-P447) were investigated only for the presence and binding of the extrinsic PS II-O protein.

Presence and Binding of the PS II-O Protein. Immunoblots of the PS II-O protein in thylakoids of the Δ-mutants and H343L as well as in wild-type thylakoids are shown in Figure 1A. The thylakoids were subjected to gel electrophoresis after undergoing one freeze/thaw cycle at a Chl concentration of 1 mg/mL. Subsequently, the thylakoids were diluted to 0.2 mg of Chl/mL, washed, and resuspended to the same Chl concentration in buffer A (5 mM $MgCl_2$, 10 mM $CaCl_2$, 0.5 M mannitol, 25 mM Hepes/NaOH, pH 7.0). Figure 1B shows the presence of PS II-O in aliquots of the corresponding supernatants. The material and experimental procedure applied in Figure 1A correspond to the protocol for the immunoblot analysis of the intrinsic PS II proteins presented in the preceding study (Haag et al., 1993). The data of Figure 1A reveal that, in contrast to the intrinsic proteins D1, D2 and CP47, the extrinsic PS II-O protein appears to be present in all mutant thylakoids at close to wild-type levels, irrespective of the presence of an assembled PS II complex. The results thus indicate that PS II-O is expressed to approximately the same extent in the photoheterotrophic mutant strains (Δ1, Δ2, Δ4, Δ5, and Δ12) as in the autotrophic mutant strains and the wild type. It further implies that PS II-O proteins that are not associated with PS II remain trapped in the lumen of undisrupted thylakoid vesicles or unspecifically associated with thylakoid components. This finding is in line with other

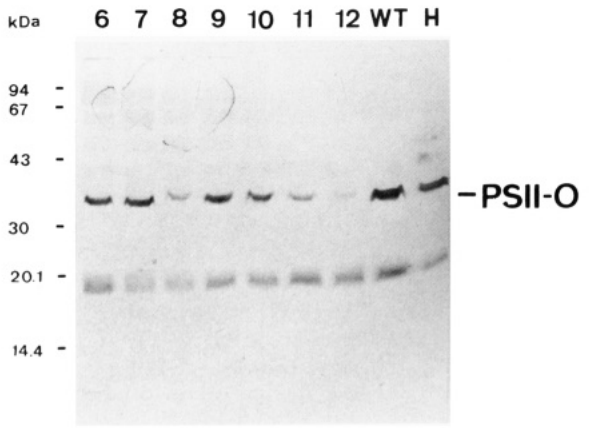

FIGURE 2: Immunoblots of proteins from wild-type and mutant thylakoids treated with 0.03% β-dodecyl maltoside and washed once. The blot was probed with polyclonal antibodies raised against the extrinsic PS II-O protein. Lanes: (6) mutant Δ6; (7) mutant Δ7; (8) mutant Δ8; (9) mutant Δ9; (10) mutant Δ10; (11) mutant Δ11; (12) mutant Δ12; (WT) wild type; (H) mutant H343L.

results (Nilsson et al., 1990; Ikeuchi et al., 1992; Mor et al., 1993). Figure 1B demonstrates that, in contrast to wild-type thylakoids, a considerable fraction of the PS II-O protein is released into the supernatant from all Δ-mutant thylakoids. Thus, at reduced levels or in the absence of an assembled PS II complex, some of the PS II-O proteins can be released into the supernatant, e.g., through statistically produced leakages of the membrane that could be formed during freeze/thaw cycles.

To correct for a nonspecific association of PS II-O with the membrane, thylakoids of the wild type and several mutant strains (at 0.1 mg/mL Chl) were treated with 0.03% β-dodecyl maltoside, washed in buffer A, and then subjected to gel electrophoresis. The results summarized in Figure 2 reveal that the amount of detectable PS II-O roughly parallels the abundance of assembled PS II centers as determined by herbicide binding (Haag et al., 1993), indicating that unspecific association of PS II-O has been eliminated. However, a closer inspection of the data shows that in some cases marked deviations from this parallel relation are observed. The most striking discrepancy is observed in thylakoids of mutant Δ8. An extremely low level of PS II-O was found after detergent treatment although the concentration of assembled PS II is reduced at most by a factor of 2 (Haag et al., 1993). Thus, in mutant Δ8 the PS II-O protein is either being bound only very weakly to assembled PS II centers or is associated unspecifically with the thylakoid membrane although assembled PS II centers are available in the membrane. At present, we cannot distinguish between these two possiblities. In any case, however, the result indicates that the binding affinity of PS II-O to an assembled PS II center is altered significantly in mutant Δ8. This result is especially interesting because the domain deleted in Δ8 is located within the region identified as antigenic determinant for the monoclonal antibody FAC2 which binds to CP47 only in the absence of the PS II-O protein and the manganese cluster of water oxidation.

In the two immunoblots probing for proteins of the thylakoid fraction (see Figure 1A and Figure 2), in addition to the PS II-O protein, a polypeptide with an apparent molecular mass of roughly 18–20 kDa is being detected by the antibody. Since this lower mass band appears in thylakoids of all mutants (including the photoheterotrophic ones), becomes enhanced after detergent treatment (compare Figure 1A with Figure 2), and is not present in the supernatant fraction (see Figure

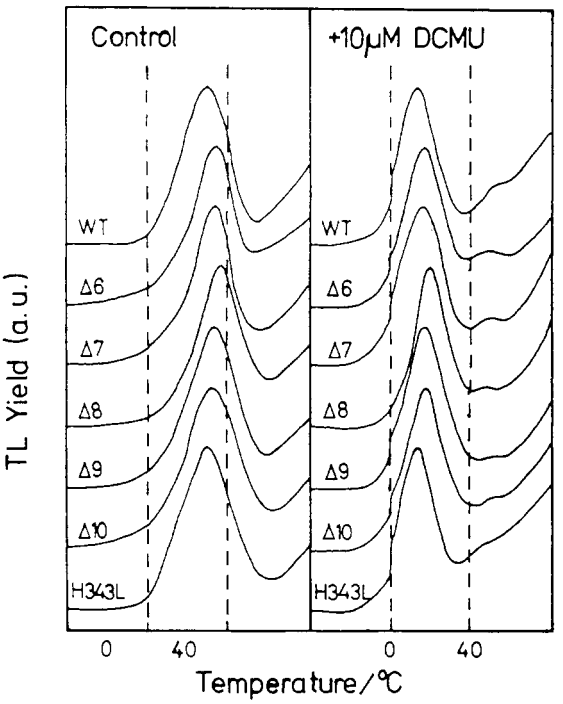

FIGURE 3: Normalized thermoluminescence glow curves recorded from cells of wild type (WT) and various *psb*B mutants from *Synechocystis* sp. PCC 6803 illuminated at 4 °C with one short saturating flash without or in the presence of 10 μM DCMU.

Table 1: Peak Temperature of Thermoluminescence Bands B and Q in Wild-Type and Various *psb*B Mutant Cells at pH 7.0

strain	TL peak temperature (°C)	
	B-band	Q-band
wild type	30	15
Δ6 (G333–I336)	33	18
Δ7 (K347–R352)	33	17
Δ8 (A373–D380)	37	20
Δ9 (V392–Q394)	33	18
Δ10 (D416–F420)	32	19
H343L	30	15

1B), we assign its presence to a cross-reaction of the antibody with some protein integrated into the thylakoid membrane irrespective of the presence of assembled PS II centers.

Thermoluminescence Measurements. The characteristic features of several thermoluminescence bands provide a useful tool to analyze the redox properties of the donor and acceptor side of PS II [for a recent review, see Vass and Inoue (1992)]. Figure 3 shows the normalized thermoluminescence of dark-adapted cells from wild type and a number of mutants which were illuminated with a single-turnover flash in the absence or presence of DCMU. Apart from effects on the signal heights due to the different PS II content and excitation energy trapping (data not shown), a shift of the peak emission temperature of these bands originating from the luminescent recombination of S_2 with Q_B^- or S_2 with Q_A^- (referred to as B-band and Q-band, respectively) is the most interesting phenomenon. The peak temperatures obtained are summarized in Table 1. In most deletion mutants the B- and Q-bands are up-shifted by about 1–3 °C, but this shift is more pronounced in mutant Δ8. Since the B-band emitted from samples illuminated with one flash originates from thermal detrapping of electrons and holes from Q_B^- and S_2, respectively, such an upshift (from 30 to 37 °C, see Table 1 and Figure 3) indicates a change of the energetic gap between the redox couples Q_B^-/Q_B and S_2/S_1 (Vass & Inoue, 1992). Correspondingly, the upshift of the Q-band which originates from thermal detrapping of electrons and holes from Q_A^- and S_2, respectively, indicates a change of the energetic gap between the redox couples Q_A^-/Q_A and S_2/S_1. The nearly parallel shift of both bands strongly indicates that in these mutants the redox potential of the S_2-state is slightly lowered compared to that of the wild type.

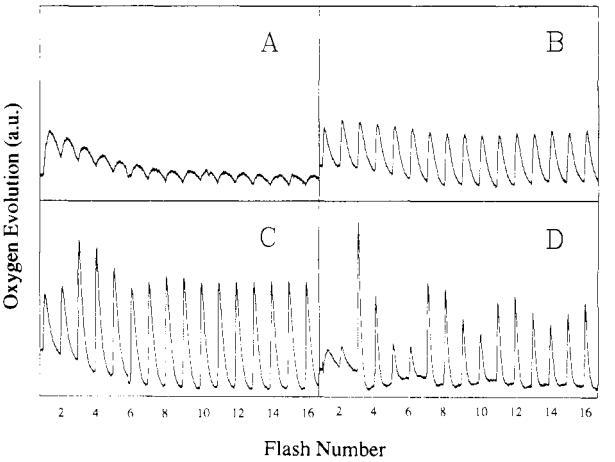

FIGURE 4: Polarographic signals detected by a Joliot-type electrode in thoroughly dark adapted (60 min) samples illuminated with a train of 16 flashes: (A) cells of mutant Δ12; (B) cells of mutant Δ8; (C) cells of mutant Δ8 additionally preilluminated for 30 s with a flash frequency of 3 Hz; (D) cells of mutant Δ8 additionally preilluminated for >2 min with a flash frequency of 3 Hz.

Flash-Induced Oxygen Evolution. In order to analyze possible effects on the functional properties of the water oxidase induced by deletion of selected charged domains in loop E or the exchange of H343 to leucine, the flash-induced O_2-oscillation pattern of dark-adapted cells was determined in wild type and mutants which exhibit autotrophic growth. Furthermore, the lifetimes of redox states S_2 and S_3 were determined [for a review on the methodology used, see Joliot and Kok (1975)]. For some mutants, for example Δ3, the signals were very small, and the error in these data therefore is large.

The O_2-oscillation patterns of cyanobacterial cells exhibit some striking differences to those of higher plant thylakoids. Excitation of dark-adapted cyanobacterial cells with a flash train leads to a signal pattern with a considerable polarographic response after the first flash. This observation corresponds with previous findings described for *Oscillatoria chalybea* and *Synechocystis sp.* (Bader et al., 1983; Cao et al., 1991; Vass et al., 1992). However, it is not certain whether the response caused by the first flash is related to oxygen formation or merely represents an electrochemical signal due to an unknown reaction at the electrode surface. To check whether the origin of this phenomenon is somehow related to PS II activity, analogous measurements were performed with cells of the obligate photoheterotrophic mutant strain Δ12 which completely lacks stably assembled PS II centers. The signal pattern induced by the flash train is shown in Figure 4A. Surprisingly, in these cells, a signal was not only observed after the first flash but also a small and long-lived signal appeared after each of the following flashes. These findings show that *Synechocystis* cells can give rise to flash-induced polarographic signals at the Joliot-type electrode that are entirely independent of PS II activity. Interestingly, a somewhat similar pattern was also observed for autotrophic cells after thorough dark-adaptation, although the rise and decay kinetics of the polarographic signal after one flash is slightly faster than in Figure 4A. A prominent pattern is

Table 2: Characterization of S-State Parameters and Lifetimes

	S-state parameters			S-state lifetimes (s)	
strain	$[S_1]$	α	β	S_2 (τ_f)	S_3 (τ_f)
wild type	0.70	0.13	0.06	36	12
Δ3 (K277–E283)	0.63	0.08	0.07	16	9
Δ6 (G333–I336)	0.69	0.10	0.07	29	11
Δ7 (K347–R352)	0.67	0.10	0.06	29	12
Δ8 (A373–D380)	0.79	0.14	0.04	50	16
Δ9 (V392–Q394)	0.67	0.13	0.08	38	11
Δ10 (D416–F420)	0.78	0.15	0.07	37	11
H343L	0.87	0.14	0.05	18	26

shown in Figure 4B for the case of mutant strain Δ8. The pattern is characterized by small signal amplitudes with unconventional slow rise and decay kinetics (compare to Figure 4D) and virtually no oscillation. Preillumination of these cells followed by a 10-min dark adaptation period led to improvement of both signal amplitude and oscillation. The pattern depicted in Figure 4C was obtained after preflashing for 30 s at 3 Hz frequency followed by a 10-min dark adaptation of Δ8 cells. An even longer period of preflashing led to the results shown in Figure 4D. Except for the signal after the first flash, the trace of Figure 4D closely resembles the normal oscillation pattern of the oxygen yield induced by a flash train in dark-adapted spinach thylakoids. A closer inspection of the data reveals that the signal due to the first flash markedly differs in shape from those caused by the subsequent flashes. Therefore, this signal cannot be considered as an unambiguous evidence for a long living S_3-state but probably originates from processes not related to normal oxygen evolution (as do those seen in Figure 4A and 4B). The drastic changes of the flash-induced patterns described in Figure 4B–D can be interpreted by the assumption that some physiological state exists in cells in darkness giving rise to an inhibition of electrode-detectable oxygen evolution by PS II. Similar phenomena were also observed in TL measurements (data not shown). A thorough analysis of the dark inactivation and the subsequent photoactivation pattern in wild-type and mutants cells will be presented in a forthcoming paper (H. M. Gleiter, E. Haag, J.-R. Shen, A. Seeliger, J. J. Eaton-Rye, W. F. J. Germaas, Y. Inoue, and G. Reuger, manuscript submitted).

In order to eliminate effects caused by the presumed physiological dark state(s), comparative studies on the characteristics of the water oxidase in mutant strains and wild type were performed in cells which were preilluminated until the oscillation patterns revealed a stable relative oxygen yield on the third and fourth flash. The oscillation patterns of light-treated cells can be satisfactorily described by using the conventional Kok-model assuming constant probabilities of misses (α) and double hits (β) throughout the flash sequence and apparent dark populations of S_0 and S_1. Results obtained by data fitting for mutant and wild-type cells are summarized in Table 2. The "Kok" parameters that characterize the S-state turnover are fairly similar in mutant and wild-type cells. The apparent dark population of centers in the S_1 state amounts to approximately 70–80%, and the miss factor roughly ranges from 0.1 to 0.15. Likewise, the values of β do not differ significantly. The latter finding indicates that the reoxidation kinetics of Q_A^- by $Q_B(Q_B^-)$ is not markedly affected in the mutants (Messinger et al., 1993). Direct evidence for this conclusion was gathered from fluorescence measurements (vide infra). In summary, the evaluation of the oscillation patterns of flash-induced oxygen yield reveals that all mutant strains are capable of a more or less normal S-state turnover.

Modifications of the extented loop E of CP47 could change the stability of the redox states S_2 and S_3. In order to address this point, the lifetimes of S_2 and S_3 were determined by the conventional method (Joliot & Kok, 1975): The oxygen yield of the third flash was measured as a function of the dark time between the first and second flash or between the second and third flash, respectively. Both S_2 and S_3 were found to decay with biphasic kinetics comprising about 50% slow and 50% fast phase. The lifetimes of the fast phases of the S_2 and S_3 decay are summarized in Table 2; the lifetimes of the slow phases are in the order of 4–6 min (data not shown). For the wild type, the S_2 and S_3 lifetimes were determined to be 36 s and 12 s, respectively, at 20 °C and in HEPES buffer at pH 7.0. Under these conditions the S_2 decay seems slightly slower compared to the 20 s lifetime determined by Nixon and Diner (1992) for wild-type cells in BG-11 medium. The S_2 lifetime of all Δ-mutants varied basically between 30 and 50 s, and the S_3 lifetime was in the range of 9–16 s, respectively (the values for mutant Δ3 will not be considered because of large experimental uncertainties due to small signals). The deviations from the values of the wild type were most pronounced in the single-site mutant H343L, although even in this case the lifetimes of the S states differed by at most a factor of 2. Interestingly, the ratio of $\tau_f(S_2)/\tau_f(S_3)$ decreases from about 3 in wild type to about 0.7 in mutant H343L. This phenomenon, however, could be due to secondary effects because the ratio of the rate constants of S_2 and S_3 decay is strongly pH-dependent, at least in thylakoids of higher plants (Renger et al., 1992), and thus is sensitive to any physiological differences between cell cultures, e.g., the light/dark pretreatment might have caused different lumenal pH conditions. Regardless of these details, the characterization of the "Kok" parameters and S_2/S_3 lifetimes leads to the general conclusion that the redox states of the manganese involved in water oxidation appear to remain almost unaffected by those mutations in loop E of CP47 that still permit photoautotrophic growth of the transformants.

Flash-Induced Change of Fluorescence Quantum Yield. In photosystem II, fluorescence quantum yield is correlated to the concentration of reduced Q_A^-. In contrast to higher plants, the mode of coupling between the entirely different antenna complexes and the reaction centers in the thylakoid membrane of cyanobacteria largely eliminates exciton transfer between different PS II units. Therefore, on the basis of an approximately linear correlation between fluorescence quantum yield and $[Q_A^-]$, the following properties were determined by measuring the flash-induced changes of the fluorescence quantum yield of cells: (i) the relative PS II concentration of the cell given by the maximum variable fluorescence induced by a single saturating flash normalized to the fluorescence level F_0 before the pulse, (ii) the kinetics of electron transport from Q_A^- to $Q_B(Q_B^-)$ as monitored by the time-resolved decay of the flash-induced change of fluorescence quantum yield, and (iii) the $Q_A^-S_2$ recombination kinetics reflected by the time-resolved decay of the variable fluorescence quantum yield after a single saturating flash in cells where the forward electron transport from Q_A^- to $Q_B(Q_B^-)$ is blocked by DCMU.

The maximum variable fluorescence was determined 50 μs after a saturating laser pulse (532 nm, FWHM = 10 ns) in cell suspensions containing 40 μM DCMU. A time of 50 μs is long enough to largely eliminate the quenching effect of $P680^+$ through fast reduction by Y_Z (van Best & Mathis, 1978; Renger et al., 1983; Brettel & Witt, 1983) and short enough to prevent Q_A^- reoxidation via recombination with S_2 (Bennoun, 1970; Renger & Weiss, 1982). Preincubation of cells with 300 μM benzoquinone and ferricyanide as described

Table 3: Characterization of Normalized Flash-Induced Variable Fluorescence, PS II Abundance, and Kinetics of Q_A^- Reoxidation and $Q_A^-S_2$ Recombination

strain	F_v/F_o	$(F_v/F_o)M$ / $(F_v/F_o)_{WT}$	$[PS\ II]_M$[a] / $[PS\ II]_{WT}$	$Q_A^- \rightarrow Q_B/Q_B^-$ (τ_f) (ms)	$Q_A^-S_2 \rightarrow Q_AS_1$ (τ_s) (s)
wild type	0.47	1.00	(1.00)	0.8	2.2
Δ3 (K277–E283)	0.13	0.28	(0.21)	nd	nd
Δ6 (G333–I336)	0.30	0.64	(0.59)	1.2	2.0
Δ7 (K347–R352)	0.27	0.57	(0.67)	1.2	1.7
Δ8 (A373–D380)	0.24	0.51	(0.73)	1.2	2.0
Δ9 (V392–Q394)	0.32	0.68	(0.84)	0.8	3.5
Δ10 (D416–F420)	0.11	0.23	(0.36)	nd	nd
Δ11 (R422–E428)	0.07	0.15	(0.19)	nd	nd
H343L	0.34	0.72	(0.55)	nd	2.6

[a] The values for PS II abundance, [PS II], given in brackets represent the content normalized to that of the wild type as determined by [^{14}C]diuron binding. The averages of the values given in Table II of Haag et al. (1993) were used for this estimation.

by Nixon and Diner (1992) did not significantly increase the maximum variable fluorescence. Likewise, the flash-induced fluorescence quantum yield was not enhanced by addition of 20 mM hydroxylamine. Nevertheless, the measured value of the normalized (to F_0) variable fluorescence, F_v/F_0, of 0.47 for wild-type cells is in line with data reported by other groups (Philbrick et al., 1991; Nixon & Diner, 1992). Although the normalized maximum of the flash-induced variable fluorescence is influenced by various factors (e.g., properties of energy trapping and transfer and charge separation), it can be used as a measure of the content of photoreduceable Q_A. Therefore, the normalized value $(F_v/F_0)_M/(F_v/F_0)_{WT}$ should provide an approximate measure of the relative amount of photochemically intact PS II complexes in mutant (M) thylakoids compared with the wild type (WT). The results obtained are summarized in Table 3. A comparison with data obtained from herbicide-binding assays (Haag et al., 1993) reveals a reasonably good correspondence. This correlation independently confirms our previous results and is in support of the assumption of a linear correlation between maximum fluorescence quantum yield and the amount of photoreducable Q_A in wild type and all mutants investigated. Furthermore, it suggests that the assembled PS II centers in all mutants investigated are functionally competent entities.

As CP47 was reported to be important for the binding and/or stabilization of Q_A (Yamagishi & Katoh, 1985; Akabori et al., 1988; Araga et al., 1993), structural modifications of this protein at the lumen side could allosterically affect the acceptor side. In order to check for this possibility, the kinetics of Q_A^- reoxidation by $Q_B(Q_B^-)$ were determined in cells of the wild type and mutants by monitoring the decay of the flash-induced change of the relative fluorescence quantum yield. In general, the decay of wild type and all mutant cells investigated exhibits an at least biphasic kinetics with a fast phase characterized by a lifetime of the order of 1 ms (see Table 3) and an extent of 60–80%. This finding is indicative of a basically intact acceptor side. It strongly supports the conclusion drawn from the invariance of the double hit probabilities (vide supra). The lifetimes of the slower phase are of the order of milliseconds (30–400 ms, data not shown).

The interaction between the acceptor and donor side can be tested by measuring the Q_A^- reoxidation kinetics in the presence of DCMU which blocks the electron transfer to $Q_B(Q_B^-)$. Under this condition, Q_A^- becomes reoxidized by S_2 simultaneously formed by flash excitation. Typical relaxation kinetics of the flash-induced fluorescence change in DCMU-treated cells of the wild type and mutant Δ8 are

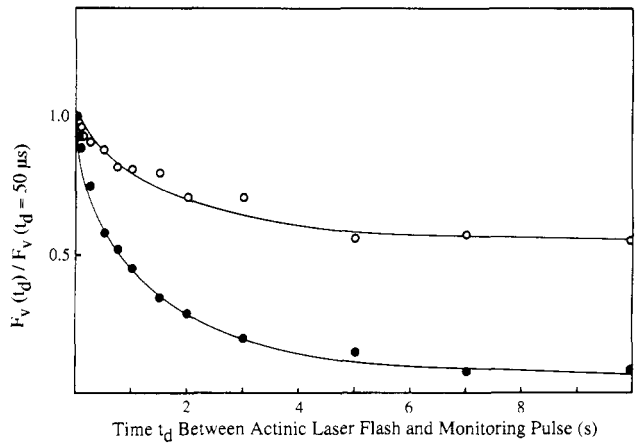

FIGURE 5: Normalized flash-induced change of fluorescence quantum yield as a function of time after the actinic flash in DCMU-treated cells of wild type (solid circles) and mutant Δ8 (open circles) from *Synechocystis* sp. PCC 6803.

shown in Figure 5. The decay kinetics in wild-type cells are dominated by a 2-s component typical for the reoxidation of Q_A^- by S_2 (Bennoun, 1970; Renger & Weiss, 1982). Similar values were obtained for the mutant cells as is shown by the data compiled in Table 3. Differences, however, are observed for the relative extent of these kinetics. In addition to this dominating component in the kinetics, a fast phase with a 40–300-ms lifetime is observed comprising an amplitude of up to 30% of the total decay (data not shown). A similar biphasicity of the Q_A recovery has been previously observed in DCMU blocked spinach thylakoids using the entirely different approach of electrochromic absorption changes (Renger & Wolff, 1975). Surprisingly, marked deviations from this general pattern were found in mutant Δ8 where about 60% of the flash induced fluorescence yield relaxes rather slowly as shown in Figure 5. This could be indicative for some changes in the properties of S_2.

DISCUSSION

In a recent study the structural and functional role of CP47 was analyzed in genetically engineered mutants lacking a short sequence of 3–8 amino acids in different regions of the large hydrophilic loop (Haag et al., 1993), which is thought to be exposed to the lumen side (Vermaas et al., 1987; Bricker, 1990). It was found that in certain regions, especially those close to the putative transmembrane helices V and VI, such short deletions appear to prevent the assembly of a stable PS II complex and completely suppress photoautotrophic growth. On the other hand, in other parts of the loop deletions of the same length caused only comparatively minor effects in terms of PS II assembly and photoautotrophic growth. Among the latter type, mutants with deletions in the domain of amino acid residues 360–400 are of special interest because the region between E364 and D440 was inferred to be involved in binding of the PS II-O protein (Odom & Bricker, 1992) and mutation of the basic residue pair within this region was found to affect water oxidation (Putnam-Evans & Bricker, 1992). Furthermore, removal of this extrinsic protein and manganese release were found to be necessary to permit the interaction with the monoclonal antibody FAC2, whose antigenic determinant is in the region between P360 and S391 of loop E (Bricker & Frankel, 1987; Frankel & Bricker, 1990). The results of the present study reveal that the strength of interaction between the membrane-integral PS II complex and the extrinsic PS II-O protein is remarkably reduced in mutant Δ8 compared with the wild type. This finding confirms the key role of

CP47, and particularly the region around A373–D380 (deleted in mutant Δ8) of loop E for a stable binding of the regulatory PS II-O protein. In order to corroborate this conclusion, immunoblot experiments were also performed with a Δ-mutant lacking the adjacent sequence R384–V392 previously described by Eaton-Rye and Vermaas (1991). A similar weakening of the interaction with the PS II-O protein was observed (data not shown).

Phenomenologically, a resemblance between the two mutants carrying a short deletion in the region between A373 and V392 of loop E, Δ8 and Δ(R384–V392), and *psb*O⁻ mutants is also reflected by similarly modified TL properties: Although in most mutants the B- and Q-bands [reflecting formation of the excited singlet state $^1P680^*$ by $Q_A^-S_2$ recombination of $Q_B^-S_2$ and $Q_A^-S_2$, respectively; see Vass and Inoue (1992)] are shifted to higher temperatures by about 2–3 °C, this up-shift is more pronounced in Δ8 and Δ(R384–V392) (data not shown). Qualitatively similar effects have been reported for *psb*O⁻ mutants with an up-shift of the Q-band of 10–15 °C (Burnap et al., 1992a,b; Vass et al., 1992). However, there is no consensus on the properties of the B-band in *psb*O⁻ mutants. In two studies a 8–9 °C temperature increase in the position of the B-band is shown (Burnap et al., 1992a,b), whereas a temperature decrease of 5 °C is described in another report (Vass et al., 1992). Therefore, unambiguous conclusions cannot be gathered from a comparison with these data. In any event, the thermoluminescence data presented in this study suggest that a structural modification of CP47 at the lumen side by direct changes in certain domains of loop E or by elimination of the interaction with the PS II-O protein affects the free energy of the hole trapped in S_2. This interpretation resembles that discussed for changes observed in the TL-properties of thylakoids after removal of the PS II-O protein (Inoue, 1987; Vass et al., 1987a,b) and of mutants lacking the PS II-O protein (Burnap et al., 1992a,b; Vass et al., 1992).

However, it should be emphasized in this context that the *psb*B mutants analyzed in this study, including Δ8 and Δ-(R384–V392), have a phenotype different from that observed for *psb*O⁻ mutants which have been described in several recent reports (Burnap & Sherman, 1990; Philbrick et al., 1991; Bockolt et al., 1991; Mayes et al., 1991; Burnap et al., 1992a,b; Vass et al. 1992). There seems to be consensus that photoautotrophic growth of *psb*O⁻ mutants depends on the presence of Ca^{2+}. In contrast, none of the deletion mutants of loop E which retain their capability of photoautotrophic growth exhibited a similar demand of Ca^{2+} (Haag et al., 1993). Therefore, based on the Ca^{2+} requirement, the functional properties of these mutants cannot be considered simply as a consequence of lacking the PS II-O protein bound to CP47.

This conclusion appears to be supported by oxygen yield measurements. An inspection of oscillation patterns reported for the oxygen yield induced by a flash train in dark-adapted samples reveals a marked damping in the *psb*O⁻ mutants compared with the wild type (Burnap et al., 1992a,b; Vass et al., 1992). An analogous phenomenon was not found in fully light activated mutant Δ8. The data in Figure 4 clearly show that in Δ8 cells an almost normally pronounced oscillation pattern can be obtained under suitable experimental conditions (the signal induced by the first flash has a different shape and will not be considered here). In this respect, it has to be emphasized that in all cell types of *Synechocystis* sp. PCC 6803 analyzed in this study a light pretreatment is required in order to elicit the normal pronounced period four oscillation (see Figure 4). The light activation could be related either to a limitation on the acceptor side, for instance due to an extremely reduced level of the plastoquinone pool, and/or to a nonfunctional donor side, e.g., due to incomplete assembly of the manganese cluster. However, the former possibility is unlikely since the maximum variable fluorescence remains unaffected by addition of the oxidizing reagents ferricyanide and benzoquinone. The latter case would imply some sort of "photoactivation" of the manganese cluster. Also, an instantaneous use of evolved oxygen by the respiratory chain cannot be excluded. A thorough analysis of this phenomenon is beyond the scope of this study and will be presented elsewhere (H. M. Gleiter, E. Haag, J.-R. Shen, A. Seeliger, J. J. Eaton-Rye, W. F. J. Vermaas, Y. Inoue, and G. Reuger, manuscript submitted). However, regardless of the underlying mechanism, this light-induced change of the overall physiological state in cyanobacteria has to be taken into account for all studies of the PS II reaction pattern. Therefore, a straightforward comparison with the results reported for *psb*O⁻ mutant is impossible unless the physiological state is defined where these measurements were performed.

Although thermoluminescence measurements mainly show effects of the presented mutations on the donor side of photosystem II, changes at the acceptor side of PS II owing to conformational changes cannot be excluded. Comparative measurements of the transient flash-induced fluorescence quantum yield in wild-type and mutant cells, however, reveal that the kinetics of Q_A^- reoxidation by $Q_B(Q_B^-)$ are not markedly affected by the deletions in loop E (see Table 3). On the other hand, striking differences emerge for mutant Δ8 in the presence of DCMU as reflected by the results in Figure 5. In wild-type cells the relaxation is dominated by a 2-s kinetics typical for $Q_A^-S_2$ recombination. A minor fraction with a much faster decay of the flash-induced fluorescence quantum yield could reflect PS II complexes lacking a functionally competent water oxidase. Kinetics of the order of hundred milliseconds are in line with a recombination between Q_A^- and Y_Z^{ox} as shown for Tris-washed spinach thylakoids (Weiss & Renger, 1984).

In DCMU-blocked cells of mutant Δ8, a very slow Q_A^- reoxidation dominates the overall decay at the expense of faster kinetics. This phenomenon could either be explained by a fast S_2 reduction by a competitive electron donor slightly exceeding the rate of $Q_A^-S_2$ recombination. Since a kinetic component of this type was not observed in the oxygen yield measurements, the presumed fast S_2 decay could be due to effects of DCMU on the donor side. A possible interaction of DCMU with the donor side has been reported previously (Bouges-Bocquet et al., 1973; Renger, 1973). The fast S_2 decay could therefore escape detection by oxygen yield measurements performed in the absence of DCMU. Alternatively, a heterogeneity of PS II could originate from incomplete activation of mutant Δ8 by the applied activating preillumination. In this case one has to assume the presence of an additional donor species, most likely nonfunctional Mn^{2+}, which prevents Q_A^- reoxidation via recombination with Y_Z^{ox}. If the time course of the dark inactivating processes in mutant Δ8 is of the same order as the light activation processes, one would always observe a certain amount of inactivated PS II. This latter possibility implies that, at least in mutants Δ8, the observed dark inactivation of oxygen evolution is mainly due to inactivation of the water-oxidizing system, i.e., the manganese cluster (Haag, 1993). However, an additional PS II inactivation via dark reduction of the acceptor side cannot be excluded. Since centers with a deactivated water-oxidizing system cannot be observed by flash-induced oxygen yield measurements or thermoluminescence, such centers may have escaped detection in these measurements.

In summary, the results of this study provide strong evidence for a close interaction between CP47 and PS II-O, in correspondence with conclusions drawn from other lines of evidence (Enami et al., 1987, 1989, 1991; Bricker et al., 1988; Eaton-Rye & Murata, 1989; Frankel & Bricker, 1990; Odom & Bricker, 1992). It is shown that deletions of only few amino acids in the region between residues 370 and 390 of the lumen-exposed loop E of CP47 give rise to structural modifications which lead to loss of a tight binding of PS II-O. The *psb*B mutants Δ8 and Δ(R384–V392) resemble to some extent those carrying a deletion of the PS II-O protein. This includes a similar susceptibility toward photoinhibition (Haag et al., 1993) and a somewhat similar pattern of up-shifted TL bands. Furthermore, Δ8 is supposed to show a faster inactivation of the oxygen-evolving enzyme than the wild type and all other mutant strains investigated in this study, which may be due to less effective shielding or stabilization of the manganese cluster by the PS II-O protein. Alternatively, the manganese cluster involved in water oxidation might be slightly distorted and/or destabilized such that the functional properties are changed, e.g., reactivity with DCMU or some other unknown component(s). It should be emphasized that the analysis of *psb*B mutants presented in this study leads to the interesting conclusion that the presence of PS II-O per se is of functional relevance, irrespective of its binding strength to PS II. This idea is supported by significant phenotypical differences between *psb*B Δ-mutants with disturbed PS II-O binding and *psb*O$^-$ mutants lacking PS II-O. The former do not require elevated Ca^{2+} levels for growth and are, at least after an appropriate pretreatment, capable of a "normal" O_2 oscillation pattern. This could indicate that PS II-O, even in a weakly bound form, is of structural importance for a Ca^{2+} binding site and/or plays an important role during the process of photoactivation. In this respect, the *psb*B mutants characterized in this study provide a suitable complement to the *psb*O$^-$ mutants for functional studies of the PS II donor side.

NOTE ADDED IN PROOF

Recently, a model of possible pathways of inactivation/activation processes of the water oxidizing complex in WT and in the mutants has been proposed (Haag, 1993). Detailed studies on the inactivation/activation phenomena will be presented in a forthcoming paper.

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