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Glu-69 of the D2 Protein in Photosystem II Is a Potential Ligand to Mn Involved in Photosynthetic Oxygen Evolution†

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ABSTRACT: To probe the involvement of amino acid residues of the D2 protein in the water-splitting process in photosystem II, site-directed mutagenesis was applied to identify D2 residues that might contribute to binding the Mn cluster involved in oxygen evolution. Mutation of Glu-69 to Gln or Val in D2 of the cyanobacterium *Synechocystis* sp. PCC 6803 was found to lead to a loss of photoautotrophic growth. However, in cells of the Gln mutant (E69Q) a significant Hill reaction rate could be observed upon the start of illumination, but the oxygen evolution rate declined with a half-time of approximately 1 min. Addition of 1 mM Mn^{2+} stabilized oxygen evolution in E69Q thylakoids. Other divalent cations were ineffective in specific stabilization. When the water-splitting system was bypassed, the rate of electron transport remained stable during illumination, indicating that the inactivation of oxygen evolution is localized in the water-splitting complex. We interpret these observations to indicate that Glu-69 is a Mn ligand and that the loss of oxygen evolution in the E69Q mutant upon turnover of PS II is initiated by changes in the Mn cluster, possibly leading to Mn release from the water-splitting complex. The addition of exogenous Mn to E69Q thylakoids may help to keep the Mn cluster active for a longer time, perhaps by providing Mn to rebind in the cluster after release of one Mn and before the Mn cluster had disintegrated. The Glu-69 residue is conserved in D2 of higher plants, and Glu and Asp residues are present in D1 at locations potentially homologous to that in D2; such D1 residues may also be involved in providing a proper binding environment to the Mn cluster.

Oxygen evolution is one of the most intriguing "black boxes" in the process of photosynthesis. One molecule of oxygen is evolved upon every four single-electron turnovers of the photosystem II (PS II) complex, with an oxidizing equivalent accumulating in the oxygen-evolving system with each turnover of PS II, until sufficient equivalents have accumulated to oxidize 2 molecules of water [reviewed by Babcock (1987) and Rutherford (1989)].

The oxidizing equivalents are stored in the oxygen-evolving complex, and a (presumably tetranuclear) Mn cluster plays a major role in accumulating these equivalents. It is unknown with which proteins of the PS II complex the Mn cluster primarily interacts, but there are a number of observations suggesting that the reaction center protein D1 and perhaps also D2 are involved in Mn binding. It has been shown that at least part of Mn binding is impaired in a mutant that does not process the C-terminal end of D1 (Metz et al., 1986; Diner et al., 1988a; Seibert et al., 1988), thus suggesting that the C-terminal end of D1 interacts, directly or indirectly, with the Mn cluster. Also, the realization that the donors D and Z (which interact with the Mn cluster) are Tyr residues in D2 and D1, respectively (Debus et al., 1988a,b; Vermaas et al., 1988a; Metz et al., 1989), has enhanced the notion that the Mn cluster may be closely associated with D1 and D2. On the basis of the sensitivity of the magnetic relaxation kinetics of EPR signals associated with D and Z to the presence of Mn it has been estimated that the distance between Z and the Mn cluster is considerably shorter than that between D and Mn (Babcock, 1987), possibly implying that the interaction of the Mn cluster is stronger with D1 than with D2. However, it should be kept in mind that the calculations on which these estimates are based are dependent on the (unknown) magnetic properties of the medium between the various paramagnetic species and that the actual distances could be significantly different from these estimates. Also, as was discussed by Rutherford (1989), the average redox state of the Mn cluster was not identical for the measurements of the D^+/Mn vs

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Z^+/Mn interactions, thus complicating the comparison further.

Mononuclear Mn ions have a coordination preference of 5–6, depending on the Mn valence [reviewed by Pecararo (1988)]. Assuming that in the tetranuclear Mn cluster the coordination preference of each Mn is similar to that of mononuclear Mn, a total of 20–24 ligands to the Mn cluster would be expected. Part of this large number of ligands presumably is provided by the protein environment. From EXAFS measurements on PS II particles in S_1 and S_2 states it was concluded that nitrogens and/or oxygens can serve as ligands, with distances between these atoms and the Mn being 1.8 and 2.0 Å (Yachandra et al., 1987). However, this information is not very helpful in determining potential Mn ligands in the PS II reaction center proteins, since not only are Glu, Gln, Asp, Asn, and His residues ligand candidates but also the participation of amino acid residues such as Ser and Thr or of the polypeptide backbone cannot be excluded.

Since at least some of the Mn ligands are likely to be located on D1 and/or D2, and since we have developed an efficient system for site-directed mutagenesis in D2 using the cyanobacterium *Synechocystis* sp. PCC 6803 (Vermaas et al., 1990), we set out to mutate conserved residues in D2 that, according to a topographical D2 model as proposed by Trebst (1986), are located on the lumenal side of the thylakoid membrane. We report here on the use of site-directed mutagenesis to change the *psbDI* codon for Glu-69 in D2 to code for Gln and Val. The mutant gene was then introduced into a deletion mutant of *Synechocystis* sp. PCC 6803 lacking both of its *psbD* genes, *psbDI* and *psbDII*. The resulting cyanobacterial mutants lack stable PS II activity and appear impaired in the oxygen-evolving system.

Materials and Methods

Growth conditions and transformation protocols of *Synechocystis* sp. PCC 6803 have been described by Vermaas et al. (1987, 1989). Wild type and mutants were propagated in the presence of 5 mM glucose, unless noted otherwise.

Many aspects of the method used for site-directed mutagenesis have been described in detail (Vermaas et al., 1990). To introduce site-directed mutations in *psbDI*, oligonucleotide-directed mutagenesis was applied by using an M13 mp19 template containing an *Eco*RV/*Eco*RI *psbDI/C* fragment from *Synechocystis* sp. PCC 6803. The mutagenic oligonucleotides used for mutation of the Glu-69 residue were 5′ GTTAGCTCCTACTAGATAGGA 3′ and 5′ TAGCTCCCTGTAGATAGG 3′ for mutation to Val and Gln, respectively, and are complementary (except at the underlined bases corresponding to the mutations to be introduced) to the appropriate *psbDI* region cloned in M13. In the method described by Vermaas et al. (1990), an independent mutant enrichment procedure as developed by Vandeyar et al. (1988) was included. However, to obtain the mutants analyzed in this paper, this enrichment procedure was not used.

After identification of desired mutants by sequencing of the M13 mp19 construct from single plaque isolates, the region containing the mutation was cut out and inserted into an appropriate plasmid containing regions up- and downstream of the *psbDI/C* operon. The resulting plasmid contains the entire and uninterrupted *psbDI/C* operon, with a change at the codon for Glu-69, flanking regions up- and downstream, and a DNA fragment conferring kanamycin resistance just downstream of *psbC*.

The plasmids containing a site-directed mutation in *psbDI* were used to transform the *psbDI/C*- and *psbDII*-lacking double-deletion strain of *Synechocystis* sp. PCC 6803 (Vermaas et al., 1990). Incorporation of the *psbDI/C* region of the plasmid carrying the site-directed mutation into its proper location in the genome of the double-deletion mutant can occur by double crossover between genome and plasmid in regions up- and downstream of the *psbDI/C* operon. A kanamycin-resistance marker, cotransforming the *psbDI/C* operon, is included for selection of cyanobacterial transformants.

To ascertain that the correct mutation had been introduced into the cyanobacterium, the region containing the mutation was amplified from the cyanobacterial genome by using the polymerase chain reaction (PCR) with *Taq* DNA polymerase (Saiki et al., 1988). For PCR of the Glu-69 mutants, two 26-mers were used as primers for DNA polymerization. One, 5′ GCCTCCTTTAGAAATTCTTGCCTTTG 3′, hybridizes to the complementary strand upstream of *psbDI*, just upstream of an *Nhe*I restriction site. The other oligonucleotide, with the sequence 5′ GAACCCCTGCAGGAACAAAATGAACC 3′, is complementary to a region in the middle of the *psbDI* coding strand, except for a single mismatch at the underlined nucleotide. This mismatch is necessary for the design of a *Pst*I restriction site (recognition sequence: 5′ CTGCAG 3′). Twenty-five PCR cycles (each 55 s at 92 °C for denaturation followed by 3 min at 65 °C for hybridization and chain extension) in reaction medium as recommended by Stratagene (La Jolla, CA) were sufficient to generate several micrograms of the amplified 0.5-kbp DNA fragment in between the hybridization sites of the oligonucleotides. The *Nhe*I/*Pst*I fragment containing the site-directed mutation was cloned into M13 mp19, and the DNA was sequenced to confirm the presence of the desired mutation and the absence of other mutations within this fragment.

For the preparation of thylakoids from the wild-type and mutant *Synechocystis* strains, a procedure modified from that described by Burnap et al. (1989) was used. Ten liters of late-exponential phase cells grown in BG11 with 5 mM glucose was harvested by centrifugation and washed in 50 mM sodium phosphate (pH 7.0). The cells were resuspended into 100 mL of thylakoid buffer containing 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). The suspension was incubated on ice for 1–2 h to allow equilibration, and after centrifugation, the cells were resuspended into approximately 30 mL of the same buffer and mixed with an equal volume of 0.1-mm glass beads. Cells were broken in a Braun homogenizer by three 30-s bursts at the maximum speed setting. Cell debris, glass beads, and unbroken cells were pelleted by centrifugation at low speed (1000*g*; 4 min), and the supernatant was centrifuged again at 15000*g* for 15 min. The pellet, containing larger thylakoid fragments, was resuspended in thylakoid buffer, and, after chlorophyll determination, was frozen in liquid N_2 and stored at −85 °C.

Oxygen evolution measurements on wild-type and mutant thylakoids and cells were performed on a Gilson oxygraph, Model KM. Actinic light was provided by an Oriel xenon arc lamp (Model 6143). The light was filtered through 15 cm of water and through a Schott OG-570 filter before reaching the sample. The incident light intensity was 7000 μE m^{-2} s^{-1} and was saturating for a maximal rate of electron transfer with 0.5 mM $K_3Fe(CN)_6$ as electron acceptor. For electron transport measurements in intact cells, 0.1 mM 2,6-dimethyl-*p*-benzoquinone (DMQ) was added to mediate electron transfer between thylakoids and the nonpenetrating $K_3Fe(CN)_6$. In addition to the electron acceptors, the medium for oxygen evolution measurements in intact cells contained only 25 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES)/NaOH, pH 7.2. Electron transport in isolated

thylakoids was measured in thylakoid buffer supplemented with electron acceptors. For the oxygen evolution stability measurements as a function of Mn^{2+} concentration, the buffer contained 50 mM HEPES/NaOH (pH 7.0), 35 mM $MgCl_2$, 5% (v/v) glycerol, 0.5% (v/v) DMSO, and 5 mM $CaCl_2$. For electron transport stability measurements as a function of Ca^{2+} concentration, the same buffer was used except that 5 mM $CaCl_2$ was replaced by 1 mm $MnCl_2$.

Dichlorophenolindophenol (DCPIP) reduction was measured at 590 nm in a Cary 219 spectrophotometer equipped for side illumination. The actinic light was filtered through a RG645 filter (Schott), and the photodetector was shielded from scattered actinic light by a 4-96 filter (Corning). The actinic light (800 $\mu E\ m^{-2}\ s^{-1}$ at maximum light output) illuminated the entire sample, and no stirrer was used in the cuvette.

Quantitation of PS II on a chlorophyll basis in whole cells was done by [^{14}C]diuron binding. Various concentrations (5–100 nM) of radiolabeled diuron (243 μCi/mg; Amersham) were added to 1-mL samples containing cells at 25 μg of chlorophyll/mL in 25 mM HEPES/NaOH, pH 7.2. To correct for the amount of diuron bound to cells at sites other than PS II, control experiments were performed in which diuron binding was measured in the presence of 20 μM unlabeled atrazine. After incubation for 15 min at room temperature in dim light, cells were pelleted by centrifugation in a microcentrifuge, and 0.8 mL of the supernatant was mixed with scintillation cocktail and counted in the scintillation counter. The amount of PS II bound diuron was calculated for each of the concentrations of added diuron by subtracting the amount of free diuron in samples without unlabeled atrazine from that in samples with 20 μM atrazine.

Methods used for SDS–polyacrylamide gel electrophoresis and Western blotting have been described by Vermaas et al. (1988b).

Results

Photoautotrophic Competence. Two D2 mutants were constructed with a mutation at the Glu-69 residue: E69Q and E69V, with Glu-69 altered to Gln and Val, respectively. Under photoautotrophic conditions (in the absence of glucose) the two mutants were unable to grow, whereas under conditions in which PS II activity is not required (in the presence of glucose) growth of E69Q and E69V is comparable to that of wild type (Figure 1). According to a topographical model of D2 (Trebst, 1986) residue 69 is located on the lumenal side of the thylakoid, between the first and second putative transmembrane helices. Under photoheterotrophic conditions (in the presence of glucose), the growth rates of E69Q and E69V were similar to those observed for wild type, indicating that the site of the lesion in these mutants indeed is located in PS II.

To ascertain that the correct mutant had been introduced into the cyanobacterium, a 0.6-kbp region containing the mutation was amplified from the cyanobacterial genome by using the polymerase chain reaction (PCR) and was sequenced after the fragment containing the site-directed mutation was cloned into M13 mp19. The presence of the desired mutation was confirmed, and no other mutations within 200 bp to either side were detected.

To exclude the possibility of an undesired mutation elsewhere in the genome contributing to the phenotype of the E69Q and E69V mutants, these mutants were transformed with a 0.34-kbp *Taq*I/*Taq*I fragment of *psbDI* wild-type DNA spanning the E69 codon. Upon transformation of the mutants with this fragment, photoautotrophic transformants were

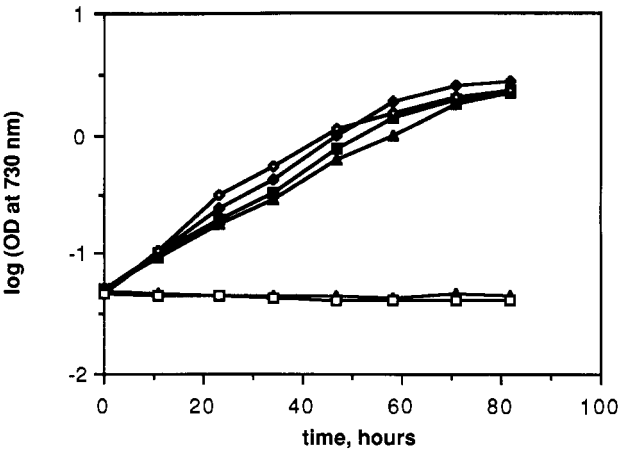

FIGURE 1: Growth curve of wild type (◇, ◆), E69Q (△, ▲), and E69V (□, ■) in BG-11 in the absence (open symbols) and presence (solid symbols) of 5 mM glucose. Data points of E69Q and E69V grown in the absence of glucose overlap on several occasions. The logarithm of the optical density (cell scattering) at 730 nm has been plotted on the *Y* axis to introduce linearity in the initial stage of growth.

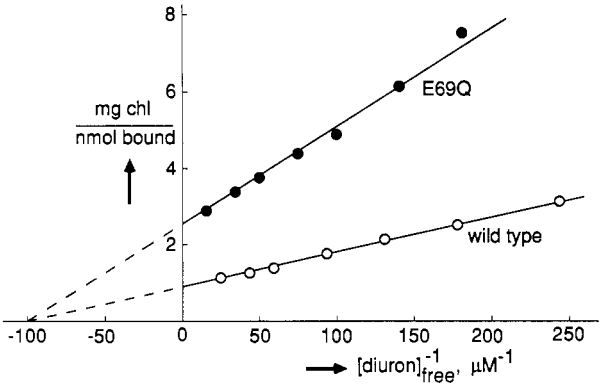

FIGURE 2: Double-reciprocal plot of [^{14}C]diuron binding to wild-type (open circles) and E69Q (solid circles) cells; 25 μg of chlorophyll/mL.

generated, indicating that the mutation causing the obligate photoheterotrophic phenotype was located in the region between the two *Taq*I restriction sites. Thus, the phenotype observed for E69Q and E69V is due exclusively to the mutation at residue 69 of D2.

Quantitation of PS II. We utilized binding assays with different concentrations of radiolabeled diuron (DCMU; a PS II directed herbicide) to whole cyanobacterial cells as a rapid and accurate method to quantitate the number of PS II centers on a chlorophyll basis in wild type and mutants in vivo. Double-reciprocal plots of diuron binding to wild-type and E69Q cells grown under identical conditions (in the presence of glucose) are shown in Figure 2. E69Q appears to have significantly fewer PS II reaction centers on a chlorophyll basis than wild type (1 PS II center per 1000 chlorophylls in wild type, as compared to 1 per 2800 in E69Q, calculated from the intercept with the *Y* axis and by taking the molecular weight of chlorophyll *a* to be 894); cells from the two strains were grown under identical conditions. The diuron binding sites in E69Q appear to be normal in that the K_D (the negative inverse of the intercept with the *X* axis) is approximately equal to that of the wild type (10 nM). The E69V mutant is dramatically impaired in its ability to bind diuron; no significant atrazine-replaceable diuron binding is observed (now shown). These observations imply that the E69Q mutant can form relatively intact PS II centers in the thylakoid, whereas E69V does not.

PS II Electron Transport. As described in a previous section, neither E69Q nor E69V grew photoautotrophically;

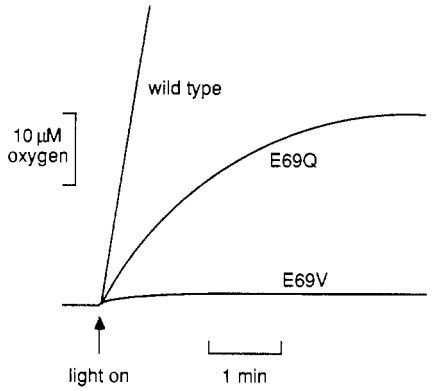

FIGURE 3: Oxygen concentration in wild-type, E69Q, and E69V cell suspensions as a function of time; 15 μg of chlorophyll/mL. The initial rates of oxygen evolution were 240 and 68 μmol of O_2 (mg of chl)$^{-1}$ h^{-1} for wild type and E69Q, respectively. The oxygen evolution rate in wild type remained stable for at least 2 min, until the electron acceptor became depleted.

the medium needed to be supplemented with glucose to support growth. Therefore, it was expected that these mutants would not evolve oxygen in the presence of artificial PS II electron acceptors, as had been shown for another obligate photoheterotrophic mutant (Vermaas et al., 1987). However, E69Q cells were found to be capable of oxygen evolution in the presence of ferricyanide and 2,6-dimethyl-*p*-benzoquinone as electron acceptors at reasonable initial rates (Figure 3). However, oxygen evolution in E69Q appeared to be quite unstable and was inhibited fully within minutes after the start of illumination (Figure 3). E69V did not show appreciable oxygen evolution under any experimental condition applied (including prolonged dark incubation before the oxygen evolution measurement).

To investigate whether, after inactivation in the light, the oxygen-evolving capability of E69Q could be restored, "photoinhibited" E69Q cells were incubated in complete darkness or in dim light for up to 20 min. The percentage of reactivation was variable between different experiments and low (maximally 20% of the original activity). We have not yet been able to define conditions under which an almost full recovery of oxygen-evolving capacity can be obtained in E69Q within 20–30 min after exposure to high-intensity light.

Site of Electron Transport Inhibition. The results obtained with E69Q suggested that this mutant was capable of assembling an active PS II reaction center, with electron transport from water to plastoquinone, but that electron transport was quickly inhibited in strong light, at a rate far exceeding the reactivation of PS II. To determine the location of this inhibition, electron transport with dichlorophenolindophenol (DCPIP) as acceptor was measured in thylakoids isolated from wild type and mutant with and without an artificial donor, diphenylcarbazide (DPC), which circumvents the water-splitting system and is thought to donate to Z^+. Table I indicates that in E69Q the electron transport rate with DCPIP as electron acceptor and water as electron donor is light sensitive, whereas in the presence of DPC electron transport remains relatively stable. It should be pointed out that the rate of inactivation of DCPIP reduction in the absence of DPC (Table I) is slower than that of oxygen evolution (Figure 3). This most likely is due to a difference in light intensity (800 vs 7000 μE m^{-2} s^{-1}). At just-saturating light intensity (about 1000 μE m^{-2} s^{-1}) the inactivation rate of the ferricyanide/DMQ Hill reaction as measured by oxygen evolution is also significantly slowed as compared to the inactivation rate at 7000 μE m^{-2} s^{-1} (unpublished results). Nonetheless, it is

Table I: PS II Electron Transport in Wild Type and E69Q[a]

strain	DPC	electron transport rate at time t after start of illumination [μequiv (mg of chlorophyll)$^{-1}$ h^{-1}]		
		t = 10 s	t = 2 min	t = 5 min
wild type	−	348	330	322
wild type	+	367	352	347
E69Q	−	121	101	85
E69Q	+	155	151	150

[a] Electron transport was measured in thylakoids from wild type and E69Q. The medium consisted of thylakoid buffer (see Materials and Methods) with 100 μM DCPIP. Where indicated, 0.5 mM DPC was added. DCPIP reduction was followed spectrophotometrically at 590 nm. The rate of electron flow was measured at time t after the beginning of illumination (800 μE m^{-2} s^{-1}). 4 μg of chlorophyll/mL. For measuring the 5-min time point in wild-type thylakoids, DCPIP was added again after 2.5 min to avoid an electron-acceptor-imposed limitation on the electron-transfer rate. The results are average of three independent experiments.

Table II: Stability of Oxygen Evolution in Thylakoids from Wild Type and Site-Directed D2 Mutants[a]

Mn^{2+}				Ca^{2+}			
	$t_{1/2}$ (s)				$t_{1/2}$ (s)		
$[MnCl_2]$ (mM)	wild type	E69Q	P161L	$[Ca(NO_3)_2]$ (mM)	wild type	E69Q	P161L
0	190	25	28	0	134	48	34
1	168	55	32	1	158	64	43
5	98	38	30	5	160	54	39
10	64	34	26	10	140	40	32

[a] Half-time of oxygen evolution rate in thylakoids from wild type, E69Q, and P161L as a function of manganese and calcium ion concentrations. The medium contained 25 mM HEPES/NaOH, pH 7.0, 10 mM NaCl, 35 mM $MgCl_2$, 5% (v/v) glycerol, and 0.5% (v/v) DMSO. For experiments in which the Mn^{2+} concentration was varied, 5 mM $CaCl_2$ was added. For the Ca^{2+} experiments, 1 mM $MnCl_2$ was added to all samples. Light intensity: 7000 μE m^{-2} s^{-1}. Values shown are averages of three independent experiments; relative electron transport rates were reproducible between two experiments within 15%.

obvious that the inhibition of PS II electron transport in E69Q in the light originates from an inhibition of the oxygen-evolving system; in contrast to electron transport in the absence of an artificial electron donor, electron transfer in the presence of DPC is not significantly inhibited upon light treatment.

An interesting question to address was whether ionic conditions could influence the inactivation rate of the oxygen-evolving system in E69Q. Since from the data presented in Table I an inactivation of the water-splitting complex appears to be the primary cause of the progressive inhibition of electron transport in the light, the inactivation rate of oxygen evolution in the light was monitored at different concentrations of ions known to interact with the water-splitting complex (Mn^{2+}, Ca^{2+}, and Cl^-). It was observed that 1 mM Mn^{2+} could significantly stabilize oxygen evolution in E69Q thylakoids in the light (Table II). This stabilization was observed independent of the presence or absence of Ca^{2+} (not shown). Mn^{2+} at 0.5 mM was found to lead to less stabilization in E69Q thylakoids than 1 mM Mn^{2+} (not shown), indicating that the optimum stabilization is obtained at about 1 mM Mn^{2+}. A small stabilizing effect of Ca^{2+} was observed (regardless of the presence or absence of Mn^{2+}), but this effect was not specific for E69Q and was also found in wild type. Note that for the Mn^{2+} and Ca^{2+} experiments 35 mM $MgCl_2$ was added to the assay medium to rule out any nonspecific divalent cation effects. No Cl^- effects on oxygen evolution stability were observed (not shown). To verify that the Mn^{2+} effect is specific for E69Q and does not occur in other mutants with similar

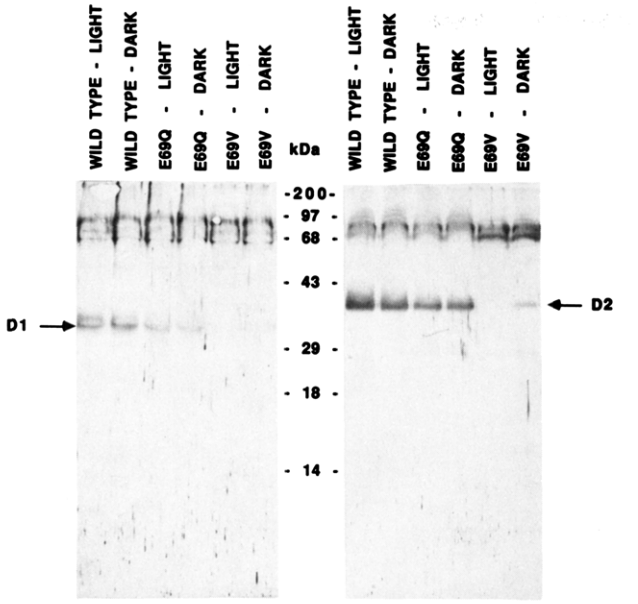

FIGURE 4: Immunoreaction of antisera against D1 and D2 with their respective antigens in thylakoids from wild type, E69Q, and E69V. Cells were grown in the presence of glucose and were incubated in room light or in darkness at room temperature for 2 h before thylakoid isolation.

phenotype, Mn^{2+} and Ca^{2+} effects on electron transport stability of thylakoids of the mutant P161L were measured (Table II). In this mutant, the Pro-161 residue next to Y_D (Tyr-160) in D2 is mutated to Leu, leading to a loss of photoautotrophic growth; as in E69Q, this mutant shows unstable oxygen evolution in the light (unpublished results). As shown in Table II, oxygen evolution in P161L cannot be stabilized by addition of Mn^{2+}, thus indicating that the Mn^{2+}-induced stabilization of oxygen evolution as seen in E69Q thylakoids is not a general phenomenon for mutants impaired at the donor side.

Presence of D2 and D1 in the Thylakoid. To probe for the physical presence of the reaction center proteins in E69Q and E69V, Western blots of thylakoid proteins, isolated from wild type and these two mutants after a 2-h preincubation in room light or in darkness at room temperature, were treated with antisera raised against spinach D1 and D2 proteins. The results of immunodetection are shown in Figure 4. A reasonable amount of crossreactivity between antisera and D1 and D2 is detectable in thylakoids from E69Q, whereas D1 and D2 are present at a much lower level in E69V thylakoids. In E69V thylakoids isolated from dark-incubated cells the amounts of D2 and, not so obvious in Figure 4, D1 are significantly higher than in thylakoids from light-incubated cells. Thus, the PS II reaction center complex in E69V appears unstable in the light, whereas in darkness a small amount of PS II can accumulate in the thylakoid. Whether or not the PS II reaction center present in E69V is functionally active remains unresolved; no oxygen evolution can be detected upon illumination of these cells, but that does not preclude the existence of several redox turnovers of the PS II reaction center before inhibition occurs. Note that Figure 4 shows a prominent D2 band at about 68 kDa in E69V that is virtually absent in E69Q and wild type. The 68-kDa band may represent a dimeric form of D2 in the thylakoid. We have not been able to affect the protein gel pattern recognized by D2 antisera by increasing the detergent concentration.

Since oxygen evolution was found to be unstable in E69Q, the presence of D1 and D2 was monitored after various times of illumination to investigate whether loss of oxygen evolution was linked to the loss of one of the reaction center proteins.

Table III: Diuron Binding in Wild Type and E69Q as a Function of Illumination Time[a]

strain	light (min)	chlorophyll/ binding site	%	K_D (nM)
wild type	0	825	100	12
	1	925	89	13
	3	1100	75	13
	5	1300	67	14
E69Q	0	1900	100	13
	1	2500	76	13
	3	3450	55	14
	5	4500	42	15

[a] Light intensity: 7000 $\mu E\ m^{-2}\ s^{-1}$.

The results are presented in Figure 5. There is no significant breakdown of either D1 or D2 even after 5 min of illumination, when there is no detectable amount of oxygen evolution left. In parallel, [^{14}C]diuron binding to illuminated and nonilluminated wild-type and E69Q cells was measured (Table III). A 5-min illumination leads to a somewhat greater loss of herbicide binding sites in the mutant than in wild type, but it does not compare with the complete loss of oxygen evolution observed within the same time period. Thus, in E69Q inactivation of oxygen evolution is not caused by degradation of one of the reaction center proteins but, on the basis of the Mn^{2+}-induced stabilization of oxygen evolution, could be attributed to a functional change in (or perhaps even loss of) part or all of the Mn cluster.

Discussion

From the results reported here, it is obvious that Glu-69 of D2 fulfills an important role in oxygen evolution. Upon mutation of Glu to Gln, the oxygen-evolving complex is destabilized in the light: oxygen evolution is rapidly inactivated, whereas PS II activity in the presence of an artificial donor remains stable. Upon mutation of Glu to Val, a complete inactivation of the PS II complex appears to occur, apparently initiated by events in the oxygen-evolving complex. In the E69V mutant, the structural integrity of the PS II complex is very sensitive to light, and even incubation in room light is sufficient for a complete loss of the reaction center proteins.

An important question to be raised is whether the light-dependent inactivation of oxygen evolution as observed here is related to the complex phenomenon of photoinhibition in wild-type systems, the light-dependent PS II inactivation observed in vivo and in vitro. There are few if any indications that photoinhibition in wild-type PS II is related primarily to inactivation of the water-splitting system, even though light is known to play a crucial role in ligation of Mn and activation of the water-splitting complex (Radmer & Cheniae, 1977). Nonetheless, the results presented in this paper indicate that in the Gln-69 mutant primarily the water-splitting process is inactivated in the light, thus suggesting that—at least in this case—photoinhibition may be primarily caused by donor-side events rather than by changes in the PS II acceptor side or at the reaction center. It should be pointed out, however, that an inhibition of electron transport at the donor side by itself is not sufficient to lead to an efficient photoinhibition and degradation of primarily the D1 protein. Upon mutation of Z, the Tyr-161 residue in D1, electron transport at the donor side was blocked (Debus et al., 1988b; Metz et al., 1989), but a reasonable amount of PS II remained in the thylakoid as evidenced by the amplitudes of absorbance and fluorescence changes (Metz et al., 1989). This suggests that factors more directly related to oxygen evolution, such as Mn binding, have an important function in stabilizing the PS II complex in vivo.

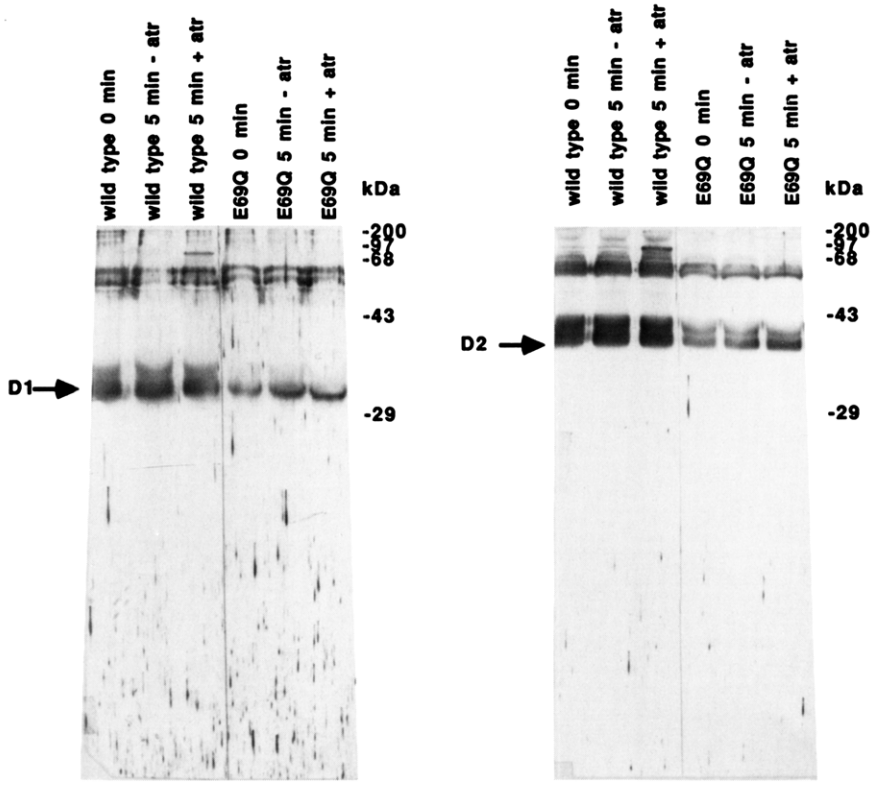

FIGURE 5: Immunological detection of D1 and D2 in thylakoids isolated from wild type and E69Q exposed to high light intensity. Cells were grown in the presence of glucose, and before thylakoid isolation, the cell sample of each strain was divided into three parts. One part was kept in darkness (0 min), one part was illuminated with strong light (7000 μE m^{-2} s^{-1}) in the presence of electron aceptor for 5 min in conditions identical with those used in Figure 3 (5 min − atr), and the third part was also illuminated, but 20 μM atrazine was added before the start of the illumination (5 min + atr).

However, a role of the Mn cluster in stabilizing PS II could be argued to be in apparent contradiction with data obtained with a *Scenedesmus* D1 processing mutant, in which a reasonably normal amount of PS II reaction center was detected in the membrane, even though the Mn content of thylakoids was decreased by 60% in this mutant [see Diner et al. (1988a) and Seibert et al. (1989) for a discussion of PS II properties of this mutant]. The *Scenedesmus* results would argue that stable PS II assembly is possible without binding the full Mn complement, whereas the results presented in this paper are interpreted to indicate a Mn-binding requirement for PS II stability. This could indicate that not all four Mn are required for PS II stability.

A direct role of Mn binding in stable assembly of the PS II complex is supported by the analysis of a *Chlamydomonas* mutant devoid of the manganese-stabilizing protein (OEE-1) (Mayfield et al., 1987). In this mutant, which presumably lacks stable Mn binding to the oxygen-evolving complex, drastically decreased amounts of the PS II core complex proteins were found to accumulate in the thylakoid membrane (Mayfield et al., 1987). We interpret our data to indicate that binding of Mn interacting with E69 is required for stable assembly of PS II and that in E69V Mn binding may be possible (although perhaps with a changed affinity) in darkness, but is rapidly released upon turnover of the oxygen-evolving system in the light. We hypothesize that in E69Q Mn can bind to the oxygen-evolving system, but Mn can be released upon one or more of the S-state transitions or while the system is in a certain S state.

It should be pointed out that there is not a linear relationship between electron-transfer rates and the rate of photoinhibition in E69Q: the stability of electron transport in thylakoids from this mutant (but not wild type) is much higher upon illumination with a light intensity just saturating electron transfer than is observed at a 5–10-fold higher intensity (W. Vermaas and G. Shen, unpublished results). This accounts for the observation that the rate of inhibition of the DCPIP Hill reaction at almost saturating light intensity (Table I) is far slower than that of oxygen evolution at 10-fold higher light intensity (Figure 3). The increased rate of inactivation at supersaturating light intensity can be indicative of a rate limitation at the level of water splitting and oxygen evolution at high light intensity in this mutant, causing the accumulation of "electron holes" at the donor side, which in turn could trigger inactivation of the oxygen-evolving system in E69Q. Indeed, a rate limitation at the level of oxygen evolution has been implied recently (Plijter et al.,1988).

Note that the inhibition kinetics of oxygen evolution in E69Q upon illumination require that, on average, several thousand turnovers of the PS II reaction center can be successfully completed before inhibition occurs. Thus, the rate or probability of Mn release upon each transition or turnover in E69Q is relatively low. Once inhibition occurs, however, repair appears a rather slow process in intact E69Q cells (after a 20-min incubation in the dark or in dim light, not much reactivation has occurred).

The stabilizing effect of exogenous Mn on oxygen evolution in E69Q leads us to believe that there is a direct interaction between the negatively charged Glu residue and a Mn of the cluster. We hypothesize that Glu-69 provides one or two ligands to one of the Mn and interacts electrostatically with the positively charged Mn, whereas Gln can provide ligands but cannot have a strong electrostatic interaction with Mn, thus destabilizing one of the Mn in the cluster, perhaps particularly in one of its higher valence states. Our results are consistent with the notion that binding of exogenous Mn to the cluster from which one Mn had been released can occur before the cluster further disintegrates, resulting in stabilization

of oxygen evolution. A similar phenomenon where a PS II cofactor could be rebound and restore functional activity when added shortly after extraction, but not after a longer time after extraction, was observed in the case of Q_A (Diner et al., 1988b). A Val residue at position 69 in D2 presumably is not able to provide ligands to the Mn and destabilizes binding of at least one Mn in the cluster to a point where no significant burst of oxygen evolution can be observed, not even in the presence of exogenous Mn.

In E69Q, the loss of PS II activity does not appear to be correlated directly with the amount of structurally intact PS II reaction center complexes in the membrane. After a 5-min illumination of E69Q cells, causing oxygen evolution to be abolished virtually entirely, there is only a moderate decrease in the number of diuron-binding sites in PS II on a chlorophyll basis (reflecting the relative amount of rather intact PS II reaction centers) and no significant decrease of the amount of D1 and D2 in the thylakoid membrane. Thus, in this mutant, reaction center degradation is not the cause of photoinhibition, but rather an effect. This correlates with photoinhibition effects in wild-type systems: the degradation of D1 lags behind the inactivation of oxygen evolution (Arntz & Trebst, 1986; Virgin et al., 1988), even though in many cases the difference in kinetics is not very distinct [reviewed by Kyle (1987)].

It would be interesting to investigate whether the possible release of one Mn from the cluster also leads to the release of other Mn from the cluster. Unfortunately, it has not yet been possible to reliably quantitate Mn^{2+} associated with oxygen evolution in thylakoids from E69Q and wild type before and after light treatment. Preliminary results have indicated that the Mn content of *Synechocystis* sp. PCC 6803 thylakoids greatly varies with the preparation. Also, the use of purified oxygen-evolving PS II preparations is not expected to lead to less ambiguous results, since the oxygen evolution rate of the best *Synechocystis* sp. PCC 6803 preparation available to date (Burnap et al., 1989) is indicative of the presence of an intact oxygen-evolving system in not more than 20–25% of its PS II centers, thus making quantitative conclusions with respect to Mn^{2+}/P680 stoichiometries ambiguous. However, even if a correlation between Mn release and oxygen evolution could be shown in this system, it would remain difficult to prove that Mn release is the primary event leading to inactivation of oxygen evolution.

The Glu-69 residue in D2 is conserved throughout evolution: it is present in green algae (Rochaix et al., 1984) and higher plants (Alt et al., 1984; Holschuh et al., 1984), although in plants it is residue number 70 (due to an extra amino acid residue near the N terminus in plants as compared to cyanobacteria). In D1 there are a number of negatively charged conserved residues (Glu-65, Asp-59, and Asp-61) in this region that may be functionally homologous to Glu-69 in D2. However, the homology between D1 and D2 is not extensive in this area. Site-directed mutagenesis of this area in D1 will be necessary to probe functional homologies of residues in D1 with the Glu-69 residue of D2.

Even though it has been tacitly assumed in this discussion that the apparent twofold symmetry in PS II may extend to components of the oxygen-evolving system, it should be kept in mind that it is possible that the Mn cluster is not symmetrical with respect to D1 and D2; indeed, the difference in distance between D/Mn and Z/Mn as estimated from paramagnetic interactions could be used to argue that the Mn cluster could interact more with D1 than with D2. In one of the possible models put forward for the molecular organization of the Mn cluster, three Mn interact closely with each other, with the four one showing a little less interaction with the other three (Rutherford, 1989). In such a model the fourth Mn could be the one interacting with Glu-69 while the other three interact mainly with D1.

Obviously, a concerted creation of site-directed mutants with altered D1, D2, and perhaps other proteins will be necessary to identify the ligands to the Mn involved in water splitting. Nonetheless, Glu-69 in D2 is the first potential Mn ligand to be identified on the basis of experimental data rather than of theoretical hypotheses, and once again site-directed mutagenesis has been proven to be a valuable tool for the study of PS II structure and function.

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Registry No. Glu, 56-86-0; Mn, 7439-96-5; oxygen, 7782-44-7.

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Oxygen Gradients in Mitochondria Examined with Delayed Luminescence from Excited-State Triplet Probes†

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ABSTRACT: Phosphorescent probes are described that are quenchable by dioxygen and that partition into membranes. These probes are derivatives of porphyrin, in which the central metal, either zinc or palladium, induces intersystem crossing to enhance the triplet yield. The location of the probe in a suspension of membranes depends upon the charge distribution of side groups on the porphyrins. Probes that partition into the membrane are sensitive to phase transitions in lecithin artificial membranes. In the mitochondria these membrane probes are within transfer distance from tryptophans in membrane proteins. Although absolute concentrations of oxygen in membranes cannot be determined by this method, by comparing the oxygen dependence of a probe in the aqueous phase with that in the membrane phase under actively respiring and inhibited conditions, it is possible to examine the extent of O_2 depletion at the mitochondrial surface. We show that at oxygen tensions of 0.2 μM and higher these gradients are insignificant at usual oxygen consumption rates of mitochondria.

Oxygen-consuming reactions in cells are localized in mitochondria and microsomes. It follows that there should be a drop in O_2 tension at the membrane surface of these organelles, the size of which will be determined by the rate of oxygen utilization by the relevant reactions and the rate that it is replenished via diffusion. Opinions on the magnitudes of such gradients differ sharply. Experiments from some laboratories [Gayeski & Honig, 1986; Wittenberg & Wittenberg, 1985; Wilson et al., 1988; for a review see Wittenberg and Wittenberg (1989)] indicate that the pressure drop surrounding isolated mitochondria is very small, less than 0.2 Torr (0.32 μM), whereas other studies (Jones, 1986; Tamura et al., 1978; Williamson & Rich, 1983) suggest very steep gradients, as high as several Torr, in cardiac myocytes. This apparent discrepancy could be resolved by direct measurement of $[O_2]$ in the medium surrounding the mitochondrion and in the interior of the mitochondrial membrane. In this work we have used hydrophobic molecules with long-lived delayed luminescence (delayed fluorescence and phosphorescence) to monitor $[O_2]$ in the lipid bilayer whereas water-soluble probes were employed to determine O_2 tension in the bulk phase of the medium.

Because delayed luminescence is quenched only by those O_2 molecules that collide with the sensor during its excited-state lifetime, i.e., those in the immediate vicinity of the probe, local oxygen levels can be sensitively reported. By using two probes in different locations with distinguishable emission properties, differences in oxygen concentration in the two respective sites can be monitored. The purpose of this study was to design the appropriate probes, to examine their O_2-sensing properties, and to use them to measure the O_2 drop across the mitochondrial membrane. By comparing the O_2 dependence of the two sensors under actively respiring and inhibited conditions, it was possible to examine the significance of an O_2 depletion at the mitochondrial surface at tensions of 0.2 μM and higher.

Experimental Procedures

Materials. Chemicals were obtained as follows: coproporphyrin and mesoporphyrin derivatives from Porphyrin

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