Site-Directed Mutagenesis of the CP47 Protein of Photosystem II: Alteration of the Basic Residue ^{448}R to ^{448}G Prevents the Assembly of Functional Photosystem II Centers under Chloride-Limiting Conditions†

Cindy Putnam-Evans‡ and Terry M. Bricker*,§

Department of Biology, East Carolina University, Greenville, North Carolina 27858, and Department of Botany, Louisiana State University, Baton Rouge, Louisiana 70803

Received April 19, 1994; Revised Manuscript Received May 31, 1994®

ABSTRACT: The *psb*B gene encodes the intrinsic chlorophyll protein CP47 (CPa-1), a component of photosystem II in higher plants, algae, and cyanobacteria. Oligonucleotide-directed mutagenesis has been used to introduce mutations into a segment of the *psb*B gene which encodes the large extrinsic loop E of CP47 in the cyanobacterium *Synechocystis* sp. PCC 6803. One mutation, R448G, produced a strain with impaired photosystem II activity. When grown in standard BG-11 media (480 μM chloride), this strain grew photoautotrophically at about 50% the rate of control strains and exhibited 63% of the control photosystem II activity. Quantum yield measurement at low light intensities indicated that this mutant had 55% of the fully functional photosystem II centers contained in control strains of *Synechocystis*. Upon exposure to high light intensities, the mutant strain exhibited a 2.2-fold increase in the rate of photoinactivation. When grown in BG-11 which was depleted in chloride (20 μM chloride), the mutant strain exhibited dramatically altered characteristics. Little or no growth was observed in the mutant while the control strains grew at nearly normal rates. Growth rates of the mutant strain could be restored by the addition of 480 μM bromide to the chloride-deficient BG-11 media. In the presence of glucose, the mutant and control strains grew at comparable rates under either chloride-sufficient or chloride-limiting conditions. Analysis of the mutant cell line grown in the absence of chloride and in the presence of glucose indicated that it exhibited essentially no capacity for oxygen evolution. [^{14}C]Atrazine binding experiments indicated that the mutant assembled 75% fewer photosystem II centers than it is capable of assembling in the presence of chloride. Immunological analysis of a number of photosystem II proteins in this mutant indicated that CP43 and the 33-kDa extrinsic manganese-stabilizing protein were present in normal quantities but that CP47 and D1 were present in significantly lower amounts. These results indicate that the mutation R448G in the CP47 protein affects photosystem II assembly and/or stability under chloride-limiting conditions. This is the first mutant identified in any system which exhibits an altered chloride requirement for photosystem II.

Photosystem II (PS II)[1] is a multisubunit thylakoid membrane protein complex which catalyzes the light-driven oxidation of water to molecular oxygen and the reduction of plastoquinone to plastoquinol. This complex consists of both intrinsic and extrinsic protein subunits. Intrinsic polypeptides with apparent molecular masses of 49 (CP47), 45 (CP43), 34 (D1), 32 (D2), 9 and 4.5 (α and β subunits of cytochrome b_{559}), and 4 kDa (*psb*I gene product) appear to form the minimum complex capable of photosynthetic oxygen evolution (Burnap & Sherman, 1991; Bricker, 1991). In higher plants, three extrinsic protein components with apparent molecular masses of 33, 24, and 17 kDa are associated with the oxygen-evolving complex and enhance oxygen evolution rates under normal physiological conditions. Removal of the 24- and 17-kDa components by salt-washing (usually 1.0 M NaCl) dramatically lowers the oxygen-evolving capacity of PS II vesicles (Akerlund et al., 1982) and PS II membranes (Kuwabara & Murata, 1982). Much of the lost activity can be recovered by reconstitution with the 24- and 17-kDa proteins (Akerlund et al., 1982) or by the addition of moderate concentrations of calcium (Ghanotakis et al., 1984) and chloride (Andersson et al., 1984). These proteins are assumed to play a role in the regulation of calcium and chloride concentrations within the PS II complex. In cyanobacteria, no proteins homologous to the 24- and 17-kDa proteins have been unequivocally identified [however, see Shen and Inoue (1993) for possible analogous proteins]; only the 33-kDa, manganese-stabilizing protein is present. The oxygen-evolving complex also requires the presence of four manganese, one or two calcium, and one (Lindberg et al., 1993) to several (Theg & Homann, 1982; Sinclar, 1984) chloride ions [see Debus (1992) for a review] for oxygen evolution activity.

Chloride appears to play an important, though poorly understood, role in PS II. It is required for normal steady-state oxygen evolution (Kelley & Izawa, 1978) and for S-state advancement (Itoh et al., 1984; Theg et al., 1984). Two models have been proposed for the function of chloride in PS II. Sandusky and Yocum (1983, 1986) have suggested that chloride acts as a bridging ligand within the manganese cluster at the active site of PS II. Homann (1987) has suggested that the binding of chloride leads to conformational changes in the proteins which bind the manganese cluster, thus indirectly influencing the structure of the PS II active site. Recent

† This work was sponsored by USDA-NRICGP Grant 91-37036-6350 to T.M.B.

* To whom correspondence should be addressed.

‡ East Carolina University.

§ Louisiana State University.

® Abstract published in *Advance ACS Abstracts,* July 15, 1994.

[1] Abbreviations: bp, base pairs; chl, chlorophyll; DCBQ, 2,6 dichloro-*p*-benzoquinone; DTSP, dithiobis(succinimidyl propionate); EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-(2-ethanesulfonic acid); kb, kilobase; NHS-biotin, *N*-hydroxysuccinimidobiotin; PCR, polymerase chain reaction; PS II, photosystem II; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; TRIS, tris(hydroxymethyl)aminomethane.

0006-2960/94/0433-10770$04.50/0

EXAFS experiments have suggested that at least one chloride may act as a terminal ligand in the manganese cluster (Yachandra et al., 1993). Proteins which bind functional chloride within PS II have not yet been identified.

The chlorophyll protein CP47, a component of the interior antenna for PS II, is an integral membrane protein which is predicted to contain six membrane-spanning α-helical domains (Vermaas et al., 1987; Bricker, 1990). In addition to its role in light-harvesting, a variety of lines of evidence suggest that the large extrinsic loop E of this protein, ^{257}W–^{450}W (Bricker, 1990), structurally interacts with the oxygen-evolving complex of PS II. Experiments utilizing monoclonal antibodies have demonstrated that the removal of the chloride-independent manganese from PS II membranes leads to a conformational change which exposes a domain of loop E (^{360}P–^{391}S) to a specific monoclonal antibody (Bricker & Frankel, 1987; Frankel & Bricker, 1989). Protein cross-linking data have documented the formation of cross-linked products between the extrinsic loop E of CP47 and the 33-kDa extrinsic protein with DTSP (the domain ^{418}K–^{438}K on CP47) (Enami et al., 1987; Bricker et al., 1988; Queirolo, 1992) and EDC (the domain ^{364}E–^{440}D on CP47) (Bricker et al., 1988; Enami et al., 1991; Odom & Bricker, 1992). In the absence of the 33-kDa extrinsic protein, two domains in loop E are labeled with the amino group-specific labeling reagent NHS-biotin (^{304}K–^{321}K and ^{389}K–^{419}K) (Frankel & Bricker, 1992). Finally, the loss of oxygen evolution following trypsin digestion of PS II membranes is correlated with the cleavage of CP47 at ^{389}K (Hayashi et al., 1993).

CP47 is encoded by the *psb*B gene. Insertional mutagenesis (Vermaas et al., 1987) or deletion (Eaton-Rye & Vermaas, 1991a) of the *psb*B gene in the cyanobacterium *Synechocystis* 6803 leads to a PS II$^-$ phenotype, and it has been hypothesized that CP47 is required for PS II assembly (Vermaas et al., 1988). Eaton-Rye and Vermaas (1991b) have produced a number of mutations in the conserved histidines which are located near the membrane surface in transmembrane helixes II and VI. One of these, H466Q, grows slower than wild-type, has a 5-fold higher chl/PS II ratio, and exhibits a greatly reduced 695 nm 77 K fluorescence emission peak. These authors concluded that ^{466}His is required for the stable incorporation of CP47 into the photosynthetic membrane. A number of short deletion mutations have also been produced in the large extrinsic loop. While some of these short deletions have no apparent effect on PS II function, others have mild to severe deleterious consequences (Eaton-Rye & Vermaas, 1991a; Haag et al., 1993). The interpretation of the nature of the functional lesion in such mutants, however, within the protein's structural and functional framework is difficult.

In our laboratory, we have been systematically modifying all of the conserved charged residues in the lumenally exposed loops of CP47. A number of mutants in the large extrinsic loop E have been produced which exhibit lesions in PS II function. The mutant RR384385GG exhibits a 50% loss of steady-state oxygen evolution which is directly related to a defect in the stability of the oxygen-evolving complex (Putnam-Evans & Bricker, 1992). R384G and R385G exhibit a loss of about 25% of control PS II activity while the simultaneous replacement of these residues with aspartate (RR384385EE) leads to a phenotype which appears to be identical to that of RR384385GG (Putnam-Evans and Bricker, unpublished observations). Thus, the positive charge density in this vicinity of the large extrinsic loop of CP47 is required for optimal PS II activity. Our current working hypothesis is that this mutation leads to a destabilization of the manganese cluster in PS II. This may be a result of a decreased ability of PS II to bind the 33-kDa manganese-stabilizing protein. The manganese-stabilizing protein appears to be easily lost from PS II particles made from this mutant (Putnam-Evans and Bricker, unpublished data).

In this paper, we examine a second site-directed mutation in the large extrinsic loop E of CP47 which affects PS II function. The alteration of ^{448}R to ^{448}G leads to a significant loss of PS II activity. Under chloride-sufficient conditions, the mutant R448G exhibits about a 40% loss of oxygen-evolving activity which is related to about a 50–60% decrease in the number of functional PS II centers. Under chloride-limiting conditions, however, the mutant can no longer grow photoautotrophically, and essentially all oxygen-evolving capacity is lost. This loss is related to a large decrease in the number of functional PS II centers. This mutant is the first identified in any PS II protein which affects the chloride requirement for PS II.

MATERIALS AND METHODS

Growth Conditions. Wild-type and mutant *Synechocystis* sp. PCC 6803 were grown in liquid BG-11 media (Rippka, 1979) at light intensities of 20 μmol of photons·(m^2)$^{-1}$·s^{-1} either on a rotary shaker or, for large cultures, in 15 L polycarbonate carboys at 28 °C. The *psb*B partial deletion strain (Del-1) was grown in BG-11 media containing 5 mM glucose. Cultures on plates were maintained in BG-11 supplemented with 1.5% agar, 0.3% sodium thiosulfate, 10 mM TES/KOH, pH 8.2, and 5 mM glucose (where appropriate). For the [^{14}C]atrazine binding experiments and preparation of thylakoid membranes, control and mutant cells were grown in 15 L carboys in BG-11 media supplemented with 0.3% sodium thiosulfate, 5 mM glucose, and appropriate antibiotics. Antibiotics were added to the media at a final concentration of 10 μg/mL.

For growth under chloride-limiting conditions, added chloride was rigorously excluded from both the macronutrient and micronutrient components of the BG-11 media. Calcium nitrate, cobalt nitrate, and manganese sulfate replaced their respective chloride salts. These changes lead to insignificant changes in the total nitrate and sulfate composition of the media. Additionally, polycarbonate flasks and carboys were used to prevent leaching of chloride from glass. The chloride concentration in the growth media was measured with a chloride-specific electrode. Growth of the cells in the presence of bromide was carried out by the addition of 480 μM sodium bromide to the chloride-deficient growth media.

Site-Directed Mutagenesis. Restriction digests, cloning, growth, and transformation of bacterial strains, and isolation of DNA fragments, were performed according to standard procedures (Maniatis et al., 1982). Phagemid isolations were performed using disposable anion-exchange columns (Qiagen Inc.). Site-directed mutagenesis was performed by the method of Kunkel et al. (1985). The plasmids used in this procedure, the transformation procedures which were used for *Synechocystis*, and a description of the recipient *psb*B deletion strain (DEL-1) and the control strain K3 have been described elsewhere (Putnam-Evans & Bricker, 1992).

PCR and DNA Sequencing. To confirm the mutations, genomic DNA was isolated from putative mutants from cell lysates according to the procedure of Williams (1988) except that the cesium chloride ultracentrifugation steps were omitted. Oligonucleotides flanking the *Kpn*I/*Kpn*I fragment of the *psb*B gene were used to amplify this region from the genomic DNA of each mutant using the polymerase chain reaction. The thermal cycling routine consisted of the following steps

performed on 100 μL reactions: 1 min denaturation at 93 °C, 45 s annealing at 65 °C, and 2 min elongation at 72 °C, for a total of 20 cycles. The PCR products were directly cloned into the plasmid pCR-3000 (InVitrogen), and the plasmids were sequenced by the double-stranded Sequenase (USB) method.

Oxygen Evolution Assays. PS II activity was measured by O_2 polarography using a Hansatech oxygen electrode. Cells were assayed in BG-11 media with 1 mM DCBQ added as an electron acceptor. The light intensity in these experiments was 3000 μmol of photons·$(m^2)^{-1}$·s^{-1} of copper sulfate-filtered white light at 25 °C. For the quantum yield experiments, the light intensity was varied between 25 and 100 μmol of photons·$(m^2)^{-1}$·s^{-1}. Light intensity was measured with a spectroradiometer (Li-Cor, Inc.) equipped with a quantum probe. For the photoinactivation experiments, cells were incubated in BG-11 media at a chl concentration of 10 μg/mL at 4000 μmol of photons·$(m^2)^{-1}$·s^{-1} at 25 °C. At various times, aliquots were removed, 1 mM DCBQ was added, and the samples were assayed for oxygen-evolving activity as described previously. The chl concentration in all of these oxygen evolution assays was 10 μg/mL. In whole cells and thylakoid membranes, chl was measured in methanol by the method of Lichtenthaler (1987).

[^{14}C]Atrazine binding assays were performed essentially as described by Tommos et al. (1993). Various concentrations of labeled atrazine (Sigma Chemical Co.) were added to cells (200 μg of chl/mL) which were suspended in 25 mM HEPES–NaOH, pH 7.5. After incubation for 30 min in the dark, the cell suspension was centrifuged for 5 min at 12000*g* and 800 μL of the supernatant removed for scintillation counting. The measured binding was corrected for nonspecific atrazine binding by the addition of 20 μM DCMU to duplicate samples.

Electrophoresis and Immunological Analysis. Thylakoid membranes were isolated by the method described by van der Bolt and Vermaas (1992) with the exception that the breaking buffer consisted of 50 mM HEPES–NaOH, pH 7.0, 5 mM $MgCl_2$, 5 mM $CaCl_2$, and 1 M sucrose (Yu & Vermaas, 1991). Electrophoresis, "Western" blotting, and antibody probing were performed as previously described (Bricker et al., 1988). The anti-manganese-stabilizing protein antibody used in this study was produced by immunization of mice with manganese-stabilizing protein purified by the procedure of Kuwabara et al. (1985). This purified protein also cross-reacts with a monoclonal antibody, FCC4, which recognizes the manganese-stabilizing protein (Frankel & Bricker, 1990). The anti-CP47 and anti-CP43 antibodies used were gifts from Dr. N.-H. Chua and were originally raised against the purified peptides "5" and "6" from *Chlamydomonas*, respectively (Chua & Bloomberg, 1979). The anti-D1 antibody was supplied by Dr. A. Trebst and was produced by immunization with a β-galactosidase–*psb*A fusion protein.

RESULTS

Verification of the Mutant Genotype. For the mutant described in this paper (R448G), a mutagenic oligonucleotide primer with the sequence 5′-TTCCGCACCAGTCCC-GGAGGTTGGTTTACCTTT-3′ was used to convert an arginyl residue at position 448 to a glycyl residue in the plasmid pTZ18K3 (Putnam-Evans & Bricker, 1992) using the Kunkle procedure (1985). After sequencing of the plasmid to verify the introduced alteration, this plasmid was used to transform the partial deletion strain DEL-1 (Putnam-Evans & Bricker, 1992). Transformants were screened for the loss of spectinomycin resistance and the acquisition of kanamycin resistance. After several rounds of streaking, mutant *Synechocystis* colonies were isolated, their genomic DNA was extracted, and the 1400 base pair *Kpn*I/*Kpn*I fragment containing the introduced mutation was amplified by PCR. The amplified fragment was cloned into the pCR-3000 vector (In Vitrogen Co.). The resultant transformants were pooled, with Figure 1 showing the result of DNA sequencing performed in the immediate vicinity of the introduced alteration. The arginine codon CGG was changed to GGA, which encodes glycine, thus demonstrating that R448G is the intended mutant. Additionally, the entire *Kpn*I/*Kpn*I fragment was sequenced, and no other mutations were found to be present in this region.

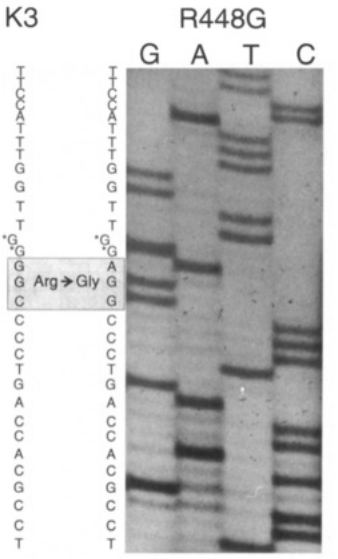

FIGURE 1: Sequence of the mutant R448G genomic DNA. PCR was used to amplify the *Kpn*I/*Kpn*I fragment of the *psb*B gene from genomic DNA isolated from the site-directed mutant. The amplified fragments were cloned into the plasmid pCR-3000 (In Vitrogen) and sequenced using the Sequenase protocol for double-stranded DNA. The R448G sequence is shown to the right. The nucleotide sequence of K3 is shown to the left. The shaded box indicates the altered codon. The two G's in the sequences which are labeled with asterisks were a compression which was resolved with dITP (data not shown).

Cell Growth. Figure 2 shows the results of a number of growth experiments performed with K3 and R448G cells. K3 is a control strain of *Synechocystis* which contains a kanamycin resistance cartridge in the 3′-flanking region of the *psb*B gene (Putnam-Evans & Bricker, 1992). This strain is identical to the mutant strain described in this paper except that it lacks the site-directed alteration. In Figure 2A, the autotrophic growth of these cell lines was compared under chloride-sufficient (480 μM) and chloride-limiting (20 μM) conditions. K3 cells grew at similar maximum rates under both conditions. Under chloride-limiting conditions, these cells do, however, exhibit a longer lag phase. The growth of R448G cells differs dramatically from that of K3. Under chloride-sufficient conditions, the mutant cells exhibited a maximum growth rate which was somewhat less than that of K3 cells and exhibited a longer lag phase than K3. The addition of high amounts of chloride (960 μM or 1.4 mM) to the mutant cells did not increase the rate of growth (or oxygen evolution capacity) to levels higher than that observed under chloride-sufficient conditions (480 μM) (data not shown). Under chloride-limiting conditions, however, no photoautotrophic growth was observed.

In Figure 2B, the photoheterotrophic growth characteristics of these cell lines under chloride-sufficient and chloride-limiting conditions were documented. The cells were grown in the presence of 5 mM glucose. Under these conditions, all

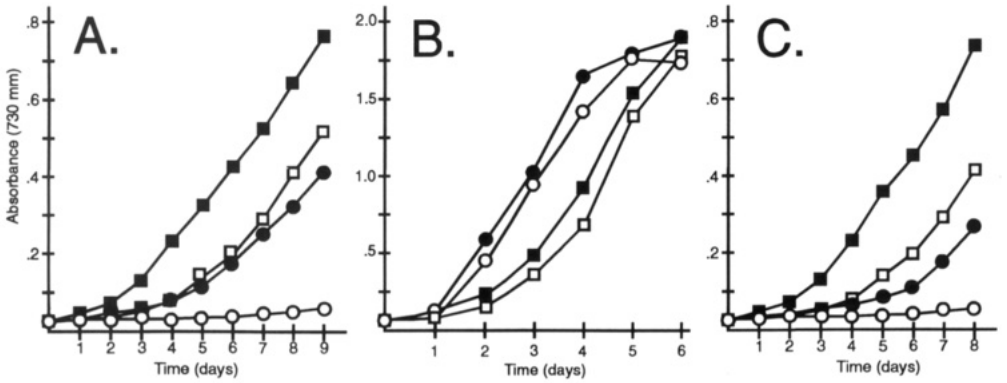

FIGURE 2: Growth characteristics of the control strain K3 and the mutant R448G under various conditions. (A) Photoautotrophic growth of K3 cells (squares) and R448G cells (circles) under chloride-sufficient (480 μM, filled symbols) and chloride-limiting (20 μM, open symbols) conditions. (B) Photoheterotrophic growth in 5 mM glucose of K3 cells (squares) and R448G cells (circles) under chloride-sufficient (filled symbols) and chloride-limiting (open symbols) conditions. (C) Photoautotrophic growth of K3 cells (squares) and R448G cells (circles) in the presence of 480 μM bromide (filled symbols) and under chloride-limiting (open symbols) conditions. These are the averages of three independent experiments. Standard deviations of these data averaged 10–15% for each data point. Please note that the absorbance axis for cells grown in glucose (B) is different from that in (A) and (C).

of the cell lines grew at nearly identical maximum rates, although the mutant cells did exhibit a shorter lag phase. The significance of this observation is unclear at this time. No significant growth rate differences were observed for either K3 cells or R448G cells grown under either chloride-sufficient and chloride-limiting conditions. The photoheterotrophic growth of *Synechocystis* in the absence of functional PS II is well documented (Williams, 1988). These results demonstrate that the lesion induced by the site-directed mutation is associated with the chloride requirement of PS II.

In Figure 2C, the growth of these cell lines in the presence or absence of 480 μM bromide and in the absence of glucose was examined. In the presence of bromide, K3 cells exhibited essentially normal growth, with the increased lag phase observed under chloride-limiting conditions being abolished. R448G cells also exhibited significant growth in the presence of bromide, although at levels somewhat less than observed in the presence of chloride. This experiment demonstrates that bromide can replace chloride in supporting photoautotrophic growth in the K3 and mutant cells. It is well-known that bromide can functionally replace chloride in supporting oxygen evolution in isolated thylakoids (Kelley & Izawa, 1978) and PS II membranes (Sandusky & Yocum, 1984). Additionally, cyanobacteria have been grown on bromide-containing media to replace chloride with bromide at the oxygen-evolving site (Yachandra et al., 1993).

PS II Characterization. Figure 3 illustrates the steady-state oxygen evolution rates observed for K3 and R448G cells which were grown in the presence of glucose and in the presence and absence of chloride or bromide. Control oxygen evolution rates for K3 and R448G cells were 402 and 254 μmol·(mg of chl)$^{-1}$·h^{-1}, respectively. K3 cells exhibited no significant differences in oxygen evolution rates under any of the tested growth conditions. Oxygen evolution under chloride-limiting conditions was nearly 95% that observed under chloride-sufficient conditions. Cells grown in the presence of bromide (480 μM) exhibited similar high rates of oxygen evolution. A dramatically different pattern was observed for the R448G cells. Under chloride-sufficient conditions or in the presence of bromide (480 μM), relatively high rates of oxygen evolution were observed. Under chloride-limiting conditions, however, no oxygen evolution was observed within the limits of detection of our oxygen polarograph [about 25 μmol·(mg of chl)$^{-1}$·h^{-1}]. These results clearly demonstrate that the PS II in the mutant strain is severely damaged and lacks the ability to evolve oxygen when grown under chloride-limiting conditions. Additionally,

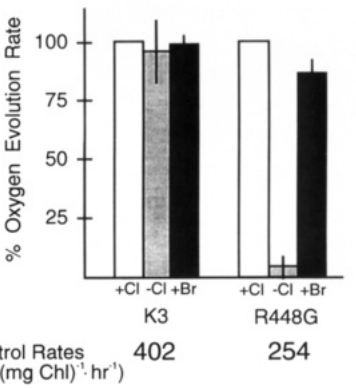

FIGURE 3: Oxygen evolution (water to DCBQ) of the control strain K3 and the mutant R448G grown in the presence of glucose and under anion-sufficient and anion-deficient conditions. Oxygen evolution rates were measured from cells grown under chloride-sufficient (480 μM chloride, labeled +Cl) and chloride-limiting (20 μM chloride, labeled –Cl) conditions and in the presence of 480 μM bromide (labeled +Br). The control (100%) rates observed under chloride-sufficient conditions are shown below. These data are the average of three experiments; the error bars are plus and minus 1.0 standard deviation.

Table 1: [^{14}C]Atrazine Binding to the Control Strain K3 and Mutant R448G Cells Grown in the Presence of Glucose and at High (480 μM) and Low (20 μM) Chloride Concentrations

cell type	chloride concn (μM)	chl/bound atrazine	k_d (nM)
K3	480	800	240
K3	20	1200	220
R448G	480	1500	250
R448G	20	6000	240

bromide can replace chloride in supporting oxygen evolution in the mutant cells. It should be noted that the addition of millimolar quantities of chloride during the oxygen evolution assays to cells grown under chloride-limiting conditions does not restore oxygen-evolving activity.

The binding of [^{14}C]atrazine to K3 and R448G cells was used to provide an estimate of the number of assembled PS II centers present in cell lines grown in the presence of glucose under chloride-sufficient and chloride-limiting conditions. The results of these experiments are shown in Table 1. R448G cells grown under chloride-sufficient conditions contain about half the number of PS II centers found in K3. This value is consistent with the steady-state oxygen evolution rate and quantum yield measurements (see below) observed for this

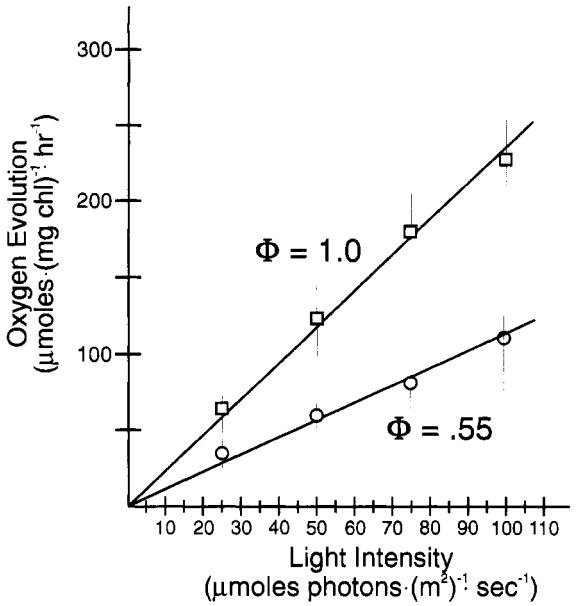

FIGURE 4: Results of quantum yield experiments for the control strain K3 and the mutant R448G cells grown in the presence of glucose and under chloride-sufficient conditions are shown. Oxygen evolution (water to DCBQ) was measured under limiting light conditions. Analysis of the first-order rate constants for K3 and R448G suggests that there are approximately 45% fewer active PS II centers in the mutant than in K3. The relative quantum yields are shown. These data are the result of two experiments. Error bars = 1 standard deviation. Open squares, K3; open circles, R448G.

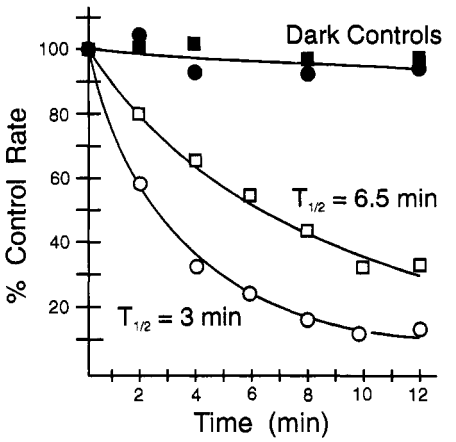

FIGURE 5: Photoinactivation experiment performed on the control strain K3 and the mutant R448G cells at 4000 μmol of photons·$(m^2)^{-1}$·s^{-1}. Closed squares, K3 cells, no photoinactivating light; closed circles, R448G cells, no photoinactivating light; open squares, K3 cells plus photoinactivating light; open circles, R448G cells plus photoinactivating light. The data shown are the average of three experiments. The standard deviations for these data points averaged 5–10%.

mutant. Under chloride-limiting conditions, K3 cells appear to assemble 66% of the functional PS II centers assembled under chloride-sufficient conditions. This is a relatively small difference. R448G cells, however, assemble only about 25% of the PS II centers assembled under chloride-sufficient conditions. It should additionally be pointed out that the value obtained for R448G cells is at or near the limit of detection of this assay system in our hands. The deletion strain DEL-1, which does not evolve oxygen and which contains no functional PS II centers, yields similar values (data not shown).

Figure 4 shows the result of a quantum yield experiment performed on K3 and R448 cells grown in the presence of glucose and under chloride-sufficient conditions. Measurement of oxygen evolution rates at limiting light intensities suggests that the mutant possesses about 55% of the fully functional PS II centers found in the control strain K3. Similar experiments cannot, of course, be performed under chloride-limiting conditions since essentially no oxygen evolution is observed in the mutant. The results of the quantum yield, [^{14}C]atrazine binding, and steady-state oxygen evolution experiments suggest that under chloride-sufficient conditions R448G contains 50–60% of the PS II centers found in K3. It could be hypothesized that the PS II centers which do assemble are normal with respect to PS II function. This, however, is not the case. Figure 5 demonstrates that the PS II centers which do assemble in R448G are damaged. In this photoinactivation experiment, K3 and R448G cells which were grown in the presence of glucose and under chloride-sufficient conditions were exposed to 4000 μmol of photons·$(m^2)^{-1}$·s^{-1} of white light at 25 °C. At various time intervals, samples were taken and assayed for their ability to evolve oxygen using DCBQ as an electron acceptor. K3 cells exhibited a $T_{1/2}$ for photoinactivation of 6.5 min. R448G cells photoinactivated much faster, exhibiting a $T_{1/2}$ for photoinactivation of 3 min. Similar results have been obtained for a variety of oxidizing-side mutations (Philbrick et al., 1991; van der Bolt & Vermaas, 1992). It is hypothesized that long-lived oxidizing species such as $TyrZ^+$ and/or P_{680}^+ accumulate in these mutants in the absence of a competent oxygen-evolving complex. The presence of these highly oxidized species is hypothesized to photodamage the reaction center proteins. Additionally, biochemical treatments of PS II preparations which lead to oxidizing-side electron transport limitations (TRIS treatment, exposure to low chloride conditions, etc.) also lead to increased rates of photoinactivation *in vitro* [for a review, see Aro et al. (1993)].

In Figure 6, the results from an immunoblot experiment are shown. Thylakoid membrane proteins were resolved by LiDS–PAGE, electroblotted to PVDF membranes, and either stained with Coomassie blue or blocked and then probed with a number of different antibody reagents. No differences were observed for any of the examined proteins from K3 cells grown either under chloride-sufficient or under chloride-limiting conditions. R448G, however, when grown under chloride-limiting conditions, exhibited decreases in the steady-state levels of CP47 and D1. These results suggest that the assembly and/or stability of the PS II reaction center is affected in the mutant under chloride-limiting conditions.

DISCUSSION

The mutant R448G, while exhibiting a moderate alteration in PS II function when grown under chloride-sufficient conditions, is severely impaired when grown under chloride-limiting conditions. It should be noted that another mutant which we have produced at ^{448}Arg, R448S, exhibits a phenotype that is apparently identical to that observed for R448G (Wu and Bricker, unpublished observations). A number of hypotheses can be presented to explain these results. Structurally, it appears that chloride is required *in vitro* for manganese cluster stability (Miyao & Murata, 1984) and the presence of this anion significantly enhances assembly of the manganese cluster during photoactivation (Tamura & Chenaie, 1989). It is a reasonable assumption that chloride functions in a similar manner *in vivo*. An alteration in the ability of chloride to bind to PS II may lead to a relative destabilization of the manganese cluster. Under chloride-sufficient conditions, this destabilization would lead to an increased susceptibility to photoinactivation at high light intensities since, in

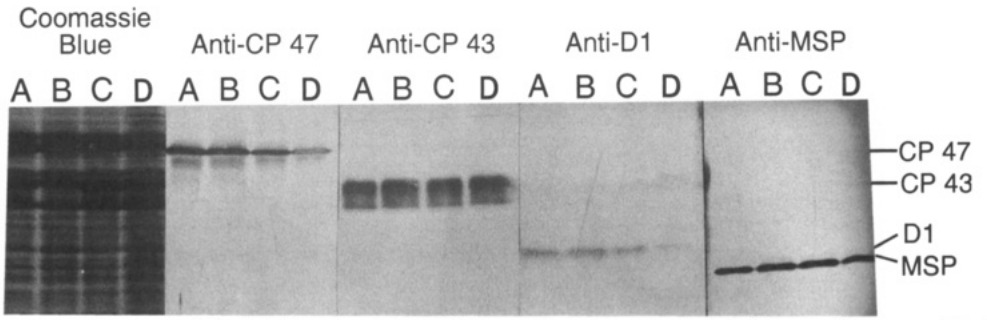

FIGURE 6: "Western" blots of the control strain K3 and mutant R448G membrane proteins isolated from cells grown with glucose and under chloride-sufficient and chloride-limiting conditions. Thylakoid membranes were isolated as described under Materials and Methods; K3 and R448G membranes were separated by LiDS–PAGE (12.5–20% acrylamide gradient), electroblotted onto PVDF membranes, and, after blocking and washing, probed with various primary antibodies. The protein bands were visualized by incubation with the appropriate peroxidase-conjugated secondary antibody and color development with 4-chloro-1-naphthol + H_2O_2. Lane A, K3 proteins, chloride-sufficient growth conditions; lane B, K3 proteins, chloride-limiting growth conditions; lane C, R448G proteins, chloride-sufficient growth conditions; lane D, R448G proteins, chloride-limiting growth conditions. The primary antibody is indicated above.

those centers which lack a fully functional oxygen-evolving complex, $TyrZ^+$ could not be reduced. This would lead to an accumulation of the oxidizing-side radicals, $TyrZ^+$ and P_{680}^+. Accumulation of these radicals would lead to increased photodamage of the reaction center proteins and a decrease in the number of functional PS II centers (Aro et al., 1993). These effects would be expected to be greatly magnified at low chloride concentrations and could lead to photoinactivation of essentially all of the PS II centers even at the low light intensities [20 μmol of photons·$(m^2)^{-1}$·s^{-1}] at which these cells are normally grown. A similar phenomenon has been observed in ^{190}H mutants in the D1 protein of *Chlamydomonas reinhardtii* (Roffey et al., 1994).

Chloride has also been shown to be required for S-state advancement. Chloride-depleted PS II is blocked at the $S_2 \Rightarrow S_3$ transition (Ono et al., 1986, 1987; Rozsa & Demeter, 1987) and/or the $S_3 \Rightarrow [S_4] \Rightarrow S_0$ transitions (Sinclar, 1984; Homann et al., 1986). In either case, alteration of the binding of chloride to PS II could affect advancement of these higher S-states. This would also be expected to lead to the accumulation of oxidizing-side radicals and to a similar cascade of photoinactivation events as described above. Again, these effects would be greatly exacerbated under chloride-limiting conditions. It should be noted that these two mechanisms, destabilization of the manganese cluster and alteration of S-state advancement kinetics, are not necessarily mutually exclusive and may both operate to yield the observed phenotype of R448G. Experiments are currently underway which may allow us to discriminate between these different mechanisms.

It is unclear, at this time, if ^{448}Arg is a chloride binding site or if the ^{448}Arg $\Rightarrow$ ^{448}G mutation induces a conformational change in CP47 which disrupts a distant chloride binding site(s). This ambiguity exists for virtually all site-directed mutations which have been produced in PS II (and in all other systems which lack a crystal structure). Some precedence does exist for arginyl residues acting as chloride ligands. Alcohol dehydrogenase, carboxypeptidase A, hemoglobin, halorhodopsin, and the erythrocyte band 3 anion transport protein all appear to utilize arginyl residues in a chloride binding capacity [reviewed by Coleman (1990)].

As noted previously, two major hypotheses have been presented concerning the role of chloride in PS II. Sandusky and Yocum (1984, 1986) have suggested that chloride acts as a bridging ligand within the manganese cluster. This hypothesis was based mainly on the observation that chloride interacts in a purely competitive manner with ammonia, protecting PS II from inactivation by the amine. Alternatively, Homann (1986) has suggested that this protective effect of chloride is indirect and involves charge-screening mechanisms. Since it appears that there may be a number of different binding sites for chloride within PS II [see Coleman (1990) for a review], these two hypotheses may not be mutually exclusive. If ^{448}Arg acts as a chloride binding site, however, a charge-screening role for the anion, at least at this site, would be supported.

CONCLUSIONS

The results presented in this paper strengthen the hypothesis that CP47 interacts with the oxygen-evolving site of PS II and suggest that this protein may have a role in chloride binding. Alteration of ^{448}Arg to ^{448}Gly (or ^{448}Ser) leads to a loss of photoautotrophic growth, oxygen evolution, and functional PS II centers under chloride-limiting conditions. Under chloride-sufficient conditions, the mutant exhibits a decreased V_{max} for oxygen evolution and an enhanced photoinactivation rate which is consistent with oxidizing-side electron donation deficiency.

ACKNOWLEDGMENT

Special thanks to Ms. Laurie K. Frankel for her help in preparing the manuscript.

REFERENCES

Akerlund, H.-E., Jannson, C., & Andersson, B. (1982) *Biochim. Biophys. Acta 681*, 1–10.
Andersson, B., Critchley, C., Ryrie, I., & Jannson, C. (1984) *FEBS Lett. 168*, 113–117.
Aro, E.-M., Virgin, I., & Andersson, B. (1993) *Biochim. Biophys. Acta 1143*, 113–134.
Bricker, T. M. (1990) *Photosynth. Res. 24*, 1–13.
Bricker, T. M. (1992) *Biochemistry 31*, 4623–4628.
Bricker, T. M., & Frankel, L. K. (1987) *Arch. Biochem. Biophys. 256*, 295–301.
Bricker, T. M., Odom, W. R., & Queirolo, C. B. (1988) *FEBS Lett. 231*, 111–117.
Burnap, R. L., & Sherman, L. A. (1991) *Biochemistry 30*, 440–446.
Chua, N.-H., & Blomberg, F. (1979) *J. Biol. Chem. 254*, 215–223.
Coleman, W. J. (1990) *Photosynth. Res. 23*, 1–27.
Debus, R. J. (1992) *Biochim. Biophys. Acta 1102*, 269–352.
Eaton-Rye, J. J., & Vermaas, W. F. J. (1991a) *Plant Mol. Biol. 17*, 1165–1177.
Eaton-Rye, J. J., & Vermaas, W. F. J. (1991b) *Plant. Physiol.* s96, 115.
Enami, I., Satoh, K., & Katoh, S. (1987) *FEBS Lett. 226*, 161–165.

Enami, I., Kaneko, M., Kitamura, N., Koike, H., Sinoike, K., Inoue, Y., & Katoh, S. (1991) *Biochim. Biophys. Acta 1060*, 224–232.
Frankel, L. K., & Bricker, T. M. (1989) *FEBS Lett. 257*, 279–282.
Frankel, L. K., & Bricker, T. M. (1990) in *Current Research in Photosynthesis* (Batcheffsky, M., Ed.) Vol. I, pp 825–828, Kluwer Academic Press, Dordrecht, The Netherlands.
Frankel, L. K., & Bricker, T. M. (1992) *Biochemistry 31*, 11059–11064.
Ghanotakis, D. F., Babcock, G. T., & Yocum, C. F. (1984) *FEBS Lett. 167*, 127–130.
Haag, E., Eaton-Rye, J. J., Renger, G., & Vermaas, W. F. J. (1993) *Biochemistry 32*, 4444–4454.
Hayashi, H., Fujimura, Y., Mohanty, P. S., & Murata, N. (1993) *Photosynth. Res. 36*, 35–42.
Homann, P. H. (1986) *Photosynth. Res. 10*, 497–503.
Homann, P. H. (1987) *J. Bioenerg. Biomembr. 19*, 105–123.
Homann, P. H., Gleiter, H., Ono, T., & Inoue, Y. (1986) *Biochim. Biophys. Acta* 850, 10–20.
Itoh, S., Yerkes, C. T., Koike, H., Robinson, H. H., & Crofts, A. R. (1984) *Biochim. Biophys. Acta 766*, 612–622.
Kelly, P. M., & Izawa, S. (1978) *Biochim. Biophys. Acta 502*, 198–210.
Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 488–492.
Kuwabara, T., & Murata, N. (1982) *Plant Cell Physiol. 24*, 741–747.
Kuwabara, T., Miyao, M., Murata, T., & Murata, N. (1985) *Biochim. Biophys. Acta 806*, 283–289.
Lichtenthaler, H. K. (1987) *Methods Enzymol. 148*, 350–382.
Lindberg, K., Vanngard, T., & Andreasson, L.-E. (1993) *Photosynth. Res. 8*, 401–408.
Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning—A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Miyano, M., & Murata, N. (1984) *FEBS Lett. 168*, 281–286.
Odom, W. R., & Bricker, T. M. (1992) *Biochemistry 31*, 5616–5620.
Ono, T., Zimmermann, J. L., Inoue, Y., & Rutherford, A. W. (1986) *Biocheim. Biophys. Acta 851*, 193–201.
Philbrick, J. B., Diner, B. A., & Zilinskas, B. A. (1991) *J. Biol. Chem. 266*, 13370–13376.
Putnam-Evans, C., & Bricker, T. M. (1992) *Biochemistry 31*, 11482–11488.
Queirolo, C. B. (1992) Dissertation, Louisiana State University.
Rippka, R., Derulles, J., Waterbury, J. B., Herdman, M., & Stanier, R. Y. (1979) *J. Gen. Microbiol. 111*, 1–61.
Roffey, R. A., van Wijk, K. J., Sayre, R. T., & Styring, S. (1994) *J. Biol. Chem. 269*, 5115–5121.
Rozsa, Z., & Demeter, S. (1987) *Photochem. Photobiol. 45*, 425–428.
Sandusky, P. O., & Yocum, C. F. (1983) *FEBS Lett. 162*, 339–343.
Sandusky, P. O., & Yocum, C. F. (1982) *Biochim. Biophys. Acta 849*, 85–93.
Shen, J.-R., & Inoue, Y. (1993) *Biochemistry 32*, 1825–1832.
Sinclair, J. (1984) *Biochim. Biophys. Acta 764*, 247–252.
Tamura, N., & Cheniae, G. (1987) *Biochim. Biophys. Acta 890*, 179–194.
Theg, S. M., Jursinic, P. A., & Homann, P. H. (1984) *Biochim. Biophys. Acta 766*, 636–646.
Tommos, C., Davidsson, L., Svensson, B., Madsen, C., Vermaas, W., & Styring, S. (1993) *Biochemistry 32*, 5436–5441.
van der Bolt, F., & Vermaas, W. (1992) *Biochim. Biophys. Acta 1098*, 247–254.
Vermaas, W. F. J., Williams, J. G. K., & Arntzen, C. J. (1987) *Plant Mol. Biol. 8*, 317–326.
Vermaas, W. F. J., Ikeuchi, M., & Inoue, Y. (1988) *Photosynth. Res. 17*, 97–113.
Williams, J. G. K. (1988) *Methods Enzymol. 167*, 766–778.
Yachandra, V. K., DeRose, V. J., Latimer, M. J., Mukerji, I., Sauer, K., & Klein, M. P. (1993) *Science 260*, 675–679.