Alterations of the Oxygen-Evolving Apparatus in a ^{448}Arg → ^{448}S Mutant in the CP47 Protein of Photosystem II under Normal and Low Chloride Conditions†

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ABSTRACT: We have shown previously that a mutant which contained the alteration ^{448}R → ^{448}S (R448S) in the CP47 protein of photosystem II exhibited a defect in its ability to grow and assemble functional photosystem II reaction centers under chloride-limiting conditions [Wu, J., Masri, N., Lee, W., Frankel, L. K., and Bricker, T. M. (1999) *Plant Mol. Biol. 39*, 381−386]. In this paper we have examined the function of the oxygen-evolving complex under chloride-sufficient (480 μM) and chloride-limiting (<20 μM) conditions. When placed under chloride-limiting conditions, both the control strain K3 and R448S cells exhibit a loss of steady-state oxygen evolution, with $t_{1/2}$ of 16 and 17 min, respectively. Upon the addition of chloride, both recover their oxygen-evolving capacity relatively rapidly. However, R448S exhibits a much slower reactivation of oxygen evolution than does K3 ($t_{1/2}$ of 308 and 50 s, respectively). This may indicate a defect at the low-affinity, rapidly exchanging chloride-binding site [Lindberg, K., and Andréasson, L.-E. (1996) *Biochemistry 35*, 14259−14267]. Additionally, alterations in the distribution of S states and S-state lifetimes were observed. Under chloride-sufficient conditions, the R448S mutant exhibits a significant increase in the proportion of reaction centers in the S_3 state and a greatly increased lifetime of the S_3 state. Under chloride-limiting conditions, the proportion of reaction centers in both the S_2 and S_3 states increases significantly, and there is a marked increase in the lifetime of the S_2 state. These alterations are not observed in the control strain K3. Our observations support the hypothesis that ^{448}R of CP47 may participate in the formation of the binding domain for chloride in photosystem II and/or in the functional interaction with the 33 kDa protein with the photosystem.

In higher plants and cyanobacteria at least six intrinsic proteins appear to be required for oxygen evolution by photosystem II (PS II)[1] (*1*−*3*). These are CP47, CP43, the D1 and D2 proteins, and the α and β subunits of cytochrome b_{559}. Insertional inactivation or deletion of the genes for these components results in the complete loss of oxygen evolution activity. Additionally, a number of low molecular mass components appear to be associated with PS II (*4*, *5*), although the functions of these proteins remain obscure. While PS II complexes containing only these components can evolve oxygen, they do so at low rates (about 25−40% of control), are extremely susceptible to photoinactivation, and require high, nonphysiological levels of calcium and chloride for maximal activity (*1*, *3*). In higher plants three extrinsic proteins, with apparent molecular masses of 33, 24, and 17 kDa, are required for high rates of oxygen evolution at physiological inorganic cofactor concentrations. The 33 kDa component has been termed the manganese-stabilizing protein due to its stabilization of the manganese cluster during exposure to low chloride concentrations or to exogenous reductants. In cyanobacteria, only the 33 kDa component is present, with the functions of the 23 and 17 kDa proteins possibly being provided by cytochrome c_{550} and a 12 kDa protein (*6*). These three extrinsic components apparently interact with intrinsic membrane proteins and possibly with each other to yield fully functional oxygen-evolving complexes.

Chloride is absolutely required for the oxygen-evolving process and appears to play both structural and functional roles within the photosystem. It is clear that, in PS II membranes which are depleted of the 33 kDa extrinsic protein, two of the four manganese associated with the oxygen-evolving site are lost at low (<100 mM) chloride concentrations (*7*). These are termed the chloride-sensitive manganese. The labile manganese first become paramagnetically uncoupled from the manganese cluster and are observable as *protein-bound* Mn(II) by Q-band EPR and then are lost to the bulk media (*8*). Thus, chloride is required for manganese cluster stability. Similar results have also been obtained for thylakoid membranes isolated from the ΔpsbO mutant in *Synechocystis* (*9*). Additionally, in membranes which lack the 33 kDa protein, chloride is absolutely required for photoactivation (*10*). Functionally, chloride depletion of PS II results in the inactivation of the oxygen-evolving complex. Readdition of a number of anions (order of effectiveness:

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[1] Abbreviations: Chl, chlorophyll; CMC, critical micelle concentration; DCBQ, 2,6-dichloro-*p*-benzoquinone; DCMU, dichloromethylurea; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; LiDS−PAGE, lithium dodecyl sulfate−polyacrylamide gel electrophoresis; PS II, photosystem II; S_{0-4} states, oxidizing states of the oxygen-evolving complex; TMBZ, 3,3′,5,5′-tetramethylbenzidine; XANES, X-ray absorbance near edge structure.

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chloride > bromide > iodide > nitrate) (*11*) restores oxygen evolution activity. It has been proposed that chloride binds directly to manganese or to a site very close to the manganese, affecting the redox properties of the complex (*12*, *13*). A charge-neutralizing role for chloride has also been proposed (*14*, *15*). It is also at least theoretically possible that chloride acts as a bridging ligand between manganese and a positively charged amino acid residue (B. Hales, personal communication). While there is some controversy concerning the number of chloride ions associated with the photosystem, recent studies in higher plants indicate that a single chloride ion is required at the oxygen-evolving site (*16*). Lindberg and Andréasson (*17*) have presented a one-site, two-state model for chloride's interaction with the photosystem in spinach PS II membranes. In this model, the binding of chloride can occur either at a low-affinity (K_d = 0.5 mM), rapidly exchanging ($t_{1/2}$ < 15 s) site or at a high-affinity (K_d = 20 μM), slowly exchanging ($t_{1/2}$ = 1 h) site. The low-affinity site can then be converted over time to the high-affinity configuration.

Depletion of chloride from PS II membranes which possess the 33 kDa protein leads to a stabilization of the S_2 and S_3 states and a retardation of the $S_3 \rightarrow [S_4] \rightarrow S_0$ transition (*18*). Ono et al. (*19*), examining XANES edge shifts, found that shifts to higher energies were arrested at the S_2 state under chloride-depleted conditions. Lubbers et al. (*20*) found that proton release was blocked beyond the S_2 state in chloride-depleted samples. Additionally, chloride depletion leads to a diminishment of the S_2 multiline signal and an increase of the g = 4.1 signal associated with the S_2 state. Readdition of chloride leads to a reconstitution of the S_2 multiline signal (at the expense of the g = 4.1 form) (*21*). Recent studies have indicated that the chloride binds at or near the manganese cluster in both the higher [S_2 and S_3 (*22*)] and lower [S_0 and S_1 (*23*)] S states. Interestingly, the chloride has an apparent higher affinity in the lower S states (*23*).

We have previously suggested that the 33 kDa protein forms a "sequestered domain" for chloride and, in concert with the intrinsic proteins of the photosystem, acts as a diffusional barrier which maintains chloride in the vicinity of the manganese cluster (*24*). Additionally, in our laboratory we have identified specific amino acid residues in the CP47 protein (^{448}R and ^{321}K), the alteration of which drastically modifies the ability of mutant *Synechocystis* cells to carry out PS II function and/or assembly under low chloride conditions (*25*−*27*). Since CP47 contributes a major binding domain for the 33 kDa protein to PS II, we hypothesize that these two components (CP47 and the 33 kDa protein) contribute to the formation of the chloride-sequestering domain of the photosystem.

In this paper, we have examined the characteristics of the oxygen-evolving apparatus of the mutant R448S of the CP47 protein. Steady-state oxygen evolution capability was examined during chloride depletion and reconstitution. Additionally, S-state distributions, S-state parameters, and the lifetimes of the S_2 and S_3 states were examined under both chloride-sufficient and chloride-limiting conditions. Our findings indicate that both the wild type and the R448S mutant lose the ability to evolve oxygen at similar rates during chloride depletion. However, during chloride reconstitution the mutant recovers its ability to evolve oxygen much more slowly than does wild type. Under chloride-sufficient conditions, the principal effect of the introduction of the ^{448}R → ^{448}S mutation was an increase in the S_3-state lifetime with an alteration of the S-state distributions in favor of the S_3 state. Under chloride-limiting conditions we observed an increase in both the S_2 and S_3 lifetimes, with a concomitant increase in the number of PS II reaction centers in the S_2 and S_3 states. These alterations are not observed in the control strain K3 even under chloride-limiting conditions. These findings are consistent with the hypothesis that ^{448}R in the CP47 protein contributes to the chloride-binding domain in PS II and/or disrupts the functional interaction of the 33 kDa protein with PS II.

MATERIALS AND METHODS

The control and mutant strain of *Synechocystis* sp. PCC 6803 were grown in liquid BG-11 media containing 5 mM glucose and 20 μM DCMU at 30 °C with air bubbling and continuous illumination at a light intensity of 50 μmol of photons·m^{-2}·s^{-1}. The construction, isolation, and preliminary characterization of the cyanobacterial strains used in these studies have been previously described (*25*, *27*, *28*). After 4 days of growth, cells were harvested by centrifugation (5 min at 10000*g*) and washed twice either with fresh BG-11 media containing normal concentrations of chloride (480 μM) or, for experiments under chloride-limiting conditions, with low chloride BG-11 media which contained less than 20 μM chloride. In the latter media, salts which normally contained chloride were replaced with their counterparts containing either nitrate or sulfate. The chloride concentration was measured by use of a chloride-specific electrode. The cells were then resuspended in the appropriate BG-11 media to a Chl concentration of about 100 μg of Chl/mL and incubated at room temperature under 5 μmol of photons·m^{-2}·s^{-1} illumination with rocking until use. For S-state distributions and S_2- and S_3-state lifetime measurements, cells were incubated for 2 h in either BG-11 media or low chloride BG-11 media. Chl was measured by the method of Lichtenthaler (*29*) in 100% MeOH.

For the chloride depletion experiment, steady-state oxygen evolution activity was measured by oxygen polarography using a Hansatech oxygen electrode. Cells were suspended in low chloride BG-11 media at a Chl concentration of 10 μg/mL and incubated at 30 °C at 50 μmol of photons·m^{-2}·s^{-1}. After incubation for various lengths of time, 1 mL aliquots were removed, brought to 1 mM DCBQ, and assayed for oxygen-evolving activity. Oxygen evolution was measured at a light intensity of 3000 μmol of photons·m^{-2}·s^{-1} of copper sulfate-filtered white light at 25 °C. After 4 h incubation under chloride-limiting conditions the cells were then brought to 5 mM NaCl and at various times were examined for oxygen-evolving activity as described above.

Flash oxygen yield measurements were made on a bare platinum electrode (Artesian Scientific Co., Urbana, IL). Flashes were supplied by an integrated, computer-controlled xenon flash lamp (20 μs width at half-height). For the measurements of S-state distributions and Kok parameters, cells were pelleted and applied to the electrode as a thin paste. The cells were then incubated for 10 min in the dark, the electrode was polarized at 0.73 V for 20 s, and a series of 16 saturating flashes was applied. Data points were collected at 500 μs intervals during the duration of the flash train. For

simplicity, the data were analyzed using a four-step, homogeneous model (*33*).

For lifetime measurements of the S_2 and S_3 states, cells were harvested as described above, incubated for 10 min in the dark, given a single saturating flash, incubated another 10 min, given either a single flash (S_2 measurements) or two flashes at a 300 ms interval (S_3 measurements), and then incubated in the dark for a variable length of time. Finally, the sample was given a train of 50 saturating flashes, during which data were collected. Regardless of the length of the last dark incubation, the electrode was polarized for 20 s before data collection commenced. The oxygen yield on either the first flash (S_3 measurement) or second flash (S_2 measurement) of the 50 flash series was normalized to the average yield obtained on the 47th through 50th flashes. The very first saturating flash and the second dark incubation were included to poise the maximum number of PS II centers in the S_1 state prior to subsequent flashes. The data were analyzed by plotting the normalized oxygen yield of the first flash of the series for the S_3 measurement (or the second flash of the series for the S_2 measurement) vs delay time.

To evaluate the ability of the cyanobacterial thylakoids to bind the 33 kDa protein, thylakoids were treated as previously described (*30*) with some modification. Briefly, thylakoid membranes were isolated from wild type and R448S cells by vortexing (three times for 2 min each) with glass beads in 50 mM Hepes−NaOH, pH 7.1, 5 mM $CaCl_2$, 10 mM $MgCl_2$, and 1 M sucrose at 4 °C. The supernatant was removed, and the glass beads were washed three times with 50 mM Hepes−NaOH, pH 7.1, 5 mM $CaCl_2$, 10 mM $MgCl_2$, and 1 M sucrose. After the original supernatant and the washes were pooled, residual glass beads were removed by centrifugation at 1000*g* for 5 min. The supernatant was then centrifuged at 6000*g* for 5 min to remove unbroken cells. The supernatant was collected and centrifuged at 35000*g* for 40 min to collect the thylakoid membranes. The membranes were suspended at a Chl concentration of 200 μg/mL in 50 mM Hepes−NaOH, pH 7.1, 5 mM $CaCl_2$, 10 mM $MgCl_2$, and 1 M sucrose and then treated with 0.04% dodecyl β-D-maltoside. This detergent treatment, which is below the CMC for dodecyl β-D-maltoside, permeabilizes the membranes and releases weakly bound membrane proteins from the membranes (*30*, *31*). After centrifugation at 35000*g* for 40 min, both the pellet and supernatant were collected. The pellet was then washed once with 50 mM Hepes−NaOH, pH 7.1, 5 mM $CaCl_2$, 10 mM $MgCl_2$, 0.04% dodecyl β-D-maltoside, and 1 M sucrose, and then the pellet and the original supernatant were analyzed by LiDS−PAGE. Electrophoresis, Western blotting, and immunological detection of the 33 kDa protein were performed as previously described (*25*). Staining for cytochromes with TMBZ was performed as described by Guikema and Sherman (*32*).

RESULTS AND DISCUSSION

We have shown previously that alterations at position ^{448}R in the CP47 protein of PS II produce mutants which exhibit marked changes in the ability to grow photoautotrophically, assemble functional PS II reaction centers, and evolve oxygen in low chloride (<20 μM) environments (*25*, *27*). R448S assembles 30−40% of the PS II centers observed in control strains and exhibits oxygen evolution rates about 30−40% of that observed in the control when grown under chloride-sufficient conditions (480 μM). Under chloride-limiting conditions (<20 μM) photoautotrophic growth is abolished, and no oxygen evolution is observed (*27*). We have previously interpreted these data as supporting the hypothesis that ^{448}R in CP47 is involved in the formation of the binding domain for chloride in PS II.

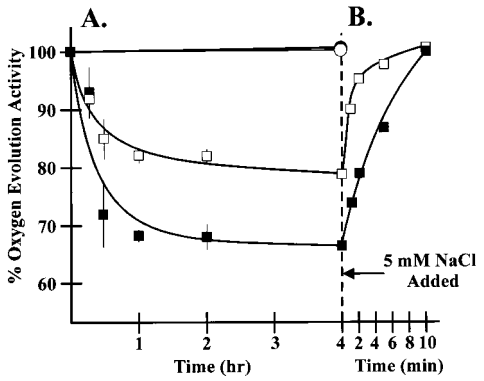

FIGURE 1: Effects of chloride depletion and reconstitution on the oxygen-evolving activity of control and R448S cells. (A) Cells of the control strain K3 (open symbols) and the mutant R448S (closed symbols) were grown in chloride-sufficient BG-11 media (480 μM chloride) and then washed and resuspended in chloride-limiting BG-11 media (<20 μM chloride). The rates of steady-state oxygen evolution were measured at various times. (B) After 4 h incubation in chloride-limiting BG-11, the cultures were brought to 5 mM NaCl, and the oxygen evolution rates were measured at various times. Note the different time scales for (A) and (B). Error bars equal ±1.0 SE ($n = 3$). Some of the error bars are smaller than the symbols and are not shown.

Figure 1A illustrates a chloride depletion experiment in which the loss of steady-state oxygen evolution was followed over time after control (K3) and R448S cells were placed in a chloride-depleted environment. Both cell types lose oxygen-evolving activity relatively slowly, with K3 exhibiting a $t_{1/2}$ of 16 ± 5 min and R448S a $t_{1/2}$ of 17 ± 7 min. The K3 strain lost about 20% of its activity, and R448S lost about 35% of its activity over a 4 h time course. During the reconstitution phase of the experiment (Figure 1B), oxygen evolution activity was restored after the addition of 5 mM NaCl. The control strain, K3, recovered activity very rapidly with a $t_{1/2}$ of 50 ± 4 s while R448S recovered its activity much more slowly, with a $t_{1/2}$ of 308 ± 116 s. It should also be noted that the reconstitution of the R448S strain is clearly not complete after 10 min even though 100% of the initial activity (activity prior to chloride depletion) was restored. Extrapolation of the exponential fit indicates that about 115% of the initial activity would be recovered after full chloride reconstitution. This may indicate that 15% of the oxygen-evolving PS II centers in the R448S strain are inactive under standard growth conditions (480 μM chloride).

These results are qualitatively very similar to those observed for PS II membranes placed in a low chloride environment (*17*). These authors observed that, after extensive washing and dialysis (24 h) to remove chloride, PS II membranes lost about 65% of their control activity. This activity was rapidly restored upon addition of chloride. They presented a model in which chloride is normally present at a high-affinity ($K_d = 20$ μM), slowly exchanging ($t_{1/2} = 1$ h) site. During reconstitution, chloride binds to a low-affinity ($K_d = 0.5$ mM), rapidly exchanging ($t_{1/2} = <15$ s) site. The

low-affinity site then converts over time to the high-affinity state.

Our results appear to indicate that the mutant R448S exhibits a defect in its ability to bind chloride at the low-affinity, rapidly exchanging site. One must be very cautious, however, in the interpretation of our results. We have not performed the detailed studies analogous to those described in Lindberg and Andréasson (*17*) since we cannot directly measure the K_d of the chloride-binding site in an in vivo experiment. Chloride transport proteins may be present in the cell membrane and/or thylakoid membrane which could affect the local concentration of chloride at the oxygen-evolving site. Additionally, the $t_{1/2}$ which we observe for reconstitution of chloride in the oxygen-evolving site in vivo is significantly longer than that observed for PS II membranes (50 vs <15 s) (*17*) while the $t_{1/2}$ which we observe for chloride depletion is significantly shorter (16 vs 60 min) than these authors observed. Either the kinetics of chloride binding and release at the rapidly exchanging and slowly exchanging sites are different in *Synechocystis* or barriers exist (cell membrane and/or intact thylakoid) which prevent the rapid equilibration of exogenously added chloride with the lumenal chloride pool. Obviously, an in vitro measure of the affinity of R448S (and wild type *Synechocystis* PS II) for chloride would be most welcomed. We are currently introducing the ^{448}Arg → ^{448}S mutation into a His-tagged PS II background (*34*) so that this direct measure of chloride affinity can be performed.

It is also possible that differences in the experimental conditions between this study and that of Lindberg and Andréasson (*17*) could account for the differences which we observe in the $t_{1/2}$ values for chloride depletion and binding. Lindberg and Andréasson (*17*) carried out their chloride depletion and reconstitution experiments in the dark while our experiments were carried out under 50 μmol of photons·m^{-2}·s^{-1} light. In the dark, most of the PS II reaction centers would be in the S_0 and S_1 states while in the light a mixture of all the S states would be present. Since chloride binds more tightly to PS II in the lower S states (*22*, *23*), it would be expected that under the conditions employed by Lindberg and Andréasson (*17*), a slower loss of chloride would occur during chloride depletion and a more rapid binding would be observed during chloride reconstitution. In any event, the slower rate of reconstitution which we observe is consistent with the hypothesis that a defect in chloride binding and/or sequestration does exist in the R448S mutant.

One possible explanation of the phenotype of R448S would be a disruption of the interaction between CP47 and the 33 kDa protein. We have hypothesized that the 33 kDa protein forms a sequestered domain which maintains the chloride associated with PS II in close proximity to the oxygen-evolving site (*24*). Additionally, we have shown previously that arginyl residues in the large extrinsic loop of CP47 form a binding domain for the 33 kDa protein. Specifically, replacement of ^{384,385}Arg with either glycyl residues or glutamyl residues leads to a marked decrease in binding of the 33 kDa protein to PS II (*30*). Disruption of the 33 kDa protein−CP47 interaction, thus, could give rise to a phenotype similar to that observed in the mutant R448S. To test the hypothesis that R448S exhibits a weaker binding of the 33 kDa protein to thylakoids than does the control strain K3, detergent-permeabilized thylakoid membranes were examined by LiDS−PAGE as we have described previously (*30*). In this experiment the amount of 33 kDa protein released to the supernatant after treatment of the thylakoid membrane at a sub-CMC concentration of dodecyl β-D-maltoside (0.04%) was monitored immunologically. Figure 2 illustrates the result of this analysis. No difference was observed between K3 and R448S. Essentially all of the 33 kDa protein remained associated with the detergent-permeabilized membrane pellet. As a positive control, the partitioning of cytochrome c_{553} was examined by LiDS−PAGE followed by staining with TMBZ (*32*). This cytochrome is a lumenally localized electron carrier between the cytochrome b_6/f complex and photosystem I, and it only transiently and relatively weakly associates with the thylakoid membrane. As expected, the majority of this protein is observed to partition in the supernatant fraction after membrane permeabilization. Additionally, a negative control was included. The major PS I chlorophyll−protein was found to partition strongly into the pellet fraction following membrane permeabilization and was observed as a green band on an unstained polyacrylamide gel. Localization of this protein into the pellet fraction was expected since the chlorophyll-binding subunits of PS I are intrinsic membrane protein components

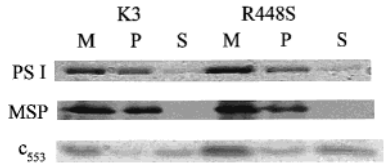

FIGURE 2: Analysis of the partitioning of the 33 kDa protein, cytochrome c_{553}, and a PS I chlorophyll−protein in dodecyl β-D-maltoside-permeabilized thylakoid membranes. The 33 kDa protein was detected immunologically, cytochrome c_{553} was detected by staining with TMBZ, and the PS I chlorophyll−protein was observed as a green band on unstained polyacrylamide gels. Key: M, thylakoid membranes; P, pelleted membranes after permeabilization with 0.04% dodecyl β-D-maltoside; S, supernatant obtained after permeabilization. For K3, 3 μg of Chl of either membranes or permeabilized membranes were loaded or an equivalent volume of the supernatant fraction. For R448S, 6 μg of Chl were loaded. This was required to partially correct for the lower number of PS II reaction centers present in the R448S strain (*25*).

The results obtained here for the 33 kDa protein are in marked contrast to what we have previously observed for the CP47 mutants RR384385EE and RR384385GG and several mutants bearing site-directed alterations in the 33 kDa protein (*30*) which affect the binding of this component to the photosystem. In all of these cases, the association between the 33 kDa protein and the thylakoid membranes was compromised, and significant amounts of the 33 kDa protein were found in the supernatant fraction. Our results with R448S indicate that there exists no gross *structural alteration* in the association of the 33 kDa protein with PS II. It should be noted that any alteration in the *function* of the 33 kDa protein would not be detected in this experiment (see below).

As noted previously, while R448S produces fewer PS II reaction centers than does the control strain, the rate of oxygen evolution per reaction center is very similar (*27*). Mutants bearing lesions at ^{448}Arg, however, photoinactivate much more rapidly than does the K3 strain, with a $t_{1/2} = 3$

Table 1: S-State Characteristics of the Control Strain K3 and the Mutant R448S under Chloride-Sufficient (480 μM) Conditions[a]

	S-state distributions				S-state parameters				S-state lifetimes (s)	
strain	S_0	S_1	S_2	S_3	α	β	γ	δ	$S_2\,\tau$	$S_3\,\tau$
K3	38.2 ± 0.5	57.0 ± 0.2	1.4 ± 0.3	3.3 ± 0.2	12.7 ± 0.3	83.5 ± 0.3	2.1 ± 0.2	2.0 ± 0.2	44 ± 15	13 ± 5
R448S	35.8 ± 0.6	55.1 ± 0.4	3.0 ±0.3	6.0 ± 0.4*	8.3 ± 0.4*	85.3 ± 0.3	3.9 ± 0.1*	0.8 ± 0.2	62 ± 12	113 ± 20*

[a] Cells were grown photoheterotrophically in chloride-sufficient media. S-state distributions and parameters were analyzed using a four-state, homogeneous model (*33*). α is the percent of misses, β is the percent of single hits, γ is the percent of double hits, and δ is the percent of deactivations. The results shown are averages of 8−10 experiments (in the case of S-state distributions and parameters) or 3 experiments (S-state lifetimes). For simplicity, the decay of the S_2 and S_3 states was fit to a single-exponential decay model. Results are shown ± 1.0 standard error. An asterisk indicates that the differences between R448S and K3 are significant at the 0.95 confidence level using Student's *t*-test.

Table 2: S-State Characteristics of the Control Strain K3 and the Mutant R448S under Chloride-Depleted (<20 μM) Conditions[a]

	S-state distributions				S-state parameters				S-state lifetimes (s)	
strain	S_0	S_1	S_2	S_3	α	β	γ	δ	$S_2\,\tau$	$S_3\,\tau$
K3	39.6 ± 0.6	57.2 ± 0.3	0.9 ± 0.3	2.0 ± 0.1	9.7 ± 0.1	86.5 ± 0.1	2.7 ± 0.2	1.0 ± 0.1	29 ± 11	30 ± 10
R448S	36.0 ± 0.2	52.8 ± 0.5*	5.8 ±0.3*	5.4 ± 0.3*	7.4 ± 0.1*	87.0 ± 0.1	4.1 ± 0.1*	0.8 ± 0.1	115 ± 25*	184 ± 32*

[a] Cells were grown photoheterotrophically in chloride-sufficient media. After harvesting, they were washed twice with and resuspended in chloride-depleted BG-11 media. Calculations of the S-state distributions etc. are as for Table 1. The results shown are averages of 6 experiments (in the case of S-state distributions and parameters) or 3 experiments (S-state lifetimes). Results are shown ± 1.0 standard error. An asterisk indicates that the differences between R448S and K3 are significant at the 0.95 confidence level using Student's *t*-test.

vs 6.5 min, respectively (*25*). This indicates that an alteration in PS II function, nevertheless, exists in this mutant. To assess the nature of the defect in PS II in R448S, the characteristics of the S-state transitions which give rise to oxygen evolution were examined by flash oxygen yield polarography. The oxygen signals were analyzed using a four-state homogeneous model implemented by eigenvector analysis (*33*). Table 1 shows the distribution of S states, the S-state parameters, and S-state lifetimes for the control strain K3 and R448S, which had been grown photoheterotrophically under chloride-sufficient conditions. A number of differences are evident upon comparison of R448S with the control strain. First, the proportion of PS II centers in the S_3 state is significantly increased in the mutant R448S. Second, the mutant exhibits significantly fewer misses (α) and significantly more double hits (γ) than does the control strain. Both of these results would be consistent with an increase in the S_3 lifetime in the mutant cell line. This was observed to be the case. While the S_2-state lifetimes for both the mutant and control strain are similar, the observed S_3 lifetime is 9-fold longer in the mutant.

When placed under chloride-limiting conditions (20 μM for 2 h), defects in the R448S strain become even more pronounced (Table 2). Significant increases in the number of PS II reaction centers in both the S_2 and S_3 states were observed with a concomitant decrease in the number of reaction centers in S_1. Little additional change was evident in the S_3 lifetime of R448S under these chloride-limiting conditions; however, a 2.5−4-fold increase in the S_2-state lifetime was observed. Since the S_3 state decays to S_2 and S_2 decays to S_1, alterations in the decay rates of these states would be additive and would act to dramatically increase the lifetime of the higher S states in R448S under chloride-limiting conditions. It should be noted that the K3 strain exhibited only minor alterations in both S-state distributions and S-state parameters upon transfer to a chloride-limiting environment.

Under chloride-limiting conditions, the $S_2 \rightarrow S_3$ and $S_3 \rightarrow [S_4] \rightarrow S_0$ state transitions are inhibited (*18*). Under these conditions, electron transfer to Y_Z^+ would be significantly slowed during these S-state advancements. A slowing in Y_Z^+ reduction has been suggested (*35*) to lead to an accumulation of oxidizing-side radicals (such as Y_Z^+ and Chl^+) which could damage the PS II reaction center and lead to photoinactivation. Indeed, in short-term experiments at high light intensity, mutants at position ^{448}R photoinactivate at much greater rates than do control strains (*25*). Our working hypothesis is that an increased rate of photoinactivation is present in these mutants even at the relatively low photon fluence rates used during the growth of these *Synechocystis* cultures (50 μmol of photons·m^{-2}·s^{-1}). An increased rate of photoinactivation during culture growth would lead to lower steady-state concentrations of PS II reaction centers. This was observed to be the case in the mutant R448G which, under chloride-sufficient conditions, accumulates about 55% of the reaction centers observed in K3. Under chloride-limiting conditions the effect of the mutation becomes more extreme, with essentially no PS II reaction centers being stably assembled (*25*).

Other investigators have also noted an increased sensitivity to chloride-limiting environments in mutants bearing site-directed modifications in the large extrinsic loop E of CP47 (^{257}W−^{450}W). Clarke and Eaton-Rye (*36*) reported that the mutant F363R was unable to grow photoautotrophically under chloride-limiting conditions. Additionally, the genetic removal of either the 33 kDa protein or cytochrome c_{550} in the F363R background led to a loss of photoautotrophy even under chloride-sufficient conditions. Putnam-Evans and Bricker (*26*) characterized the mutant K321G, which exhibited high rates of photoinactivation under chloride-sufficient conditions. Under chloride-limiting conditions, the ability to grow photoautotrophically was lost. Tichy and Vermaas (*37*) used combinational mutagenesis to replace the eight amino acids deleted in the obligate photoheterotrophic deletion mutant Δ(D440−P447) (*38*). After screening for the restoration of photoautotrophic growth, they recovered 20 combinational mutants. None of the replaced amino acid residues was found to be critical for function. All of the combinational mutants, however, exhibited high rates of photoinactivation, and many showed up to a 1000-fold increase in the chloride

requirement for photoautotrophic growth. These authors speculated that the phenotype observed was not a consequence of altered binding of the extrinsic proteins associated with the photosystem. The proximity of these mutations to ^{448}Arg should be noted. ^{448}Arg also appears to be quite susceptible to mutation. Using random mutagenesis, four independent mutations were recovered at this position including ^{448}R → ^{448}K, ^{448}R → ^{448}Q, ^{448}R → ^{448}W, and ^{448}R → ^{448}S (*27*). None of these mutants exhibits photoautotrophic growth or significant levels of PS II assembly (during photoheterotrophic growth) under chloride-limiting conditions. Clarke and Eaton-Rye (*39*) have also determined that small deletions in the small lumenally exposed loop C (^{162}W−^{197}G) can also affect the chloride requirement for photoautotrophic growth.

In PS II membranes, chloride depletion led to a 20-fold stabilization of the S_2 lifetime with no concomitant stabilization of the S_3 state (*18*). Additionally, these workers demonstrated that in this system chloride was required for the $S_2 \rightarrow S_3$ and $S_3 \rightarrow [S_4] \rightarrow S_0$ transitions. It seems clear that, in PS II membranes, significantly more efficient removal of chloride is possible than in intact cells. Under the conditions employed in our study, we observed no major effects on the S_2 or S_3 decay lifetimes in the control strain under our chloride-limiting conditions (minor alterations which were not statistically significant were observed). This indicates that the control strain K3 is able to maintain significant chloride in the vicinity of the oxygen-evolving site even under the chloride-limiting conditions employed in this study. This correlates well with the modest loss of oxygen-evolving activity observed in this strain.

The functional characteristics of PS II membranes which have had the 33 kDa protein removed by salt washing and the observed phenotype of ΔpsbO mutants are both remarkably similar to the characteristics of chloride-depleted PS II membranes (for an in-depth discussion, see ref *24*). The principal difference that has been observed is an increased S_3 lifetime in the 33 kDa protein-depleted systems (*40*−*42*), which is not observed in chloride-depleted systems (*18*). Our observation of a significantly increased S_3 lifetime in the R448S mutant may indicate a disruption of a *functional* interaction between the 33 kDa protein and CP 47 which is present even under chloride-sufficient conditions. Clearly, however, the binding of the 33 kDa protein and PS II does not seem to be significantly altered in this mutant (Figure 1).

Thus, two principal defects appear to be present in the R448S strain. First, the binding of chloride to the low-affinity, rapidly exchanging site seems to be impaired. This is evidenced by a 6-fold retardation in the rate of restoration of maximal oxygen evolution rates after chloride depletion. We hypothesize that the observed phenotype is a result of a disruption in the normal chloride-sequestering mechanism operational in the photosystem. Our obvious assumption is that the manipulation of the chloride concentration in the media leads to a modulation in the chloride concentration in the vicinity of PS II. Clearly, the regulation of the ionic environment in vivo is complex, and it is at least theoretically possible that the manipulation of the media chloride concentration could affect other lumenal ionic components, which could lead to the alterations which we have observed. We feel, however, that this is unlikely.

Second, the S_3 state appears to be significantly more stable in the mutant than in the control strain. The decay of the S_3 state is nearly 10-fold slower than in the control strain K3. Such an alteration in S_3-state lifetime is not observed as a result of chloride depletion (*18*) alone. This result has been observed, however, in a number of experiments in which the 33 kDa protein has been removed from the photosystem (*40*−*42*). Since we have demonstrated that the 33 kDa protein is structurally retained in the R448S mutant, we speculate that there may be a disruption in the functional association of the 33 kDa protein with PS II in this mutant.

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