Functional Characterization of *Synechocystis* sp. PCC 6803 Δ*psb*U and Δ*psb*V Mutants Reveals Important Roles of Cytochrome *c*-550 in Cyanobacterial Oxygen Evolution†

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ABSTRACT: The functions of cytochrome *c*-550 and a 12 kDa protein in cyanobacterial oxygen evolution were studied with directed deletion mutants Δ*psb*V and Δ*psb*U of *Synechocystis* sp. PCC 6803, and the following results were obtained. (1) In contrast to the Δ*psb*U mutant which is capable of autotrophic growth in the absence of Ca^{2+} or Cl^- at a reduced rate, the Δ*psb*V mutant lacking cytochrome *c*-550 could not grow at all without Ca^{2+} or Cl^-. (2) The Δ*psb*V mutant had a significantly reduced thermoluminescence emission intensity and flash oxygen yield, whereas the Δ*psb*U mutant showed slight decreases in thermoluminescence intensity and flash oxygen yield, indicating corresponding decreases in the concentrations of O_2-evolving centers in these mutants. (3) The Δ*psb*V and Δ*psb*U mutants exhibited elevated peak temperature for the thermoluminescence B- and Q-bands indicative of more stable S_2 states. (4) The rise time of the O_2 signal during the $S_3-[S_4]-S_0$ transition was increased slightly in the Δ*psb*V mutant but not in the Δ*psb*U mutant. (5) The oxygen evolution was inactivated in the dark rapidly in the Δ*psb*V mutant with a half-time of 28 min, but this did not happen in the Δ*psb*U mutant. (6) Photoactivation of the oxygen-evolving complex after removal of the manganese cluster by hydroxylamine showed a higher quantum yield in the Δ*psb*V mutant than in the Δ*psb*U mutant or wild type. Taken together, these results indicated that cytochrome *c*-550 plays a substantial role in maintaining the stability and function of the manganese cluster in algal photosystem II, whereas the 12 kDa protein plays primarily a regulatory role in maintaining normal S-state transitions. These functional features of cytochrome *c*-550 and the 12 kDa protein were compared with those of the 23 and 17 kDa proteins in higher plant photosystem II and of the 33 kDa protein in both algal and plant photosystem II.

Photosynthetic oxygen evolution in cyanobacterial, algal, and green plant cells is catalyzed by a tetranuclear Mn-cluster ligated to one or several polypeptides of the photosystem II (PSII)[1] complex. In order to evolve one molecule of oxygen, two molecules of H_2O must be decomposed with the concomitant extraction of four electrons and four protons. This occurs by sequential one-electron extraction reactions driven by photochemical charge separation within the PSII reaction center. Successive turnovers of the reaction center lead to the sequential formation of progressively more oxidized states of the oxygen-evolving complex, designated S_0-S_4. Among these, the S_4 state is unstable and has not been experimentally detected, whereas the S_1 state is the stable, predominant state of the PSII complex after dark-adaptation (for reviews, see ref *1*, *2*).

The PSII complex consists of more than 10 membrane-spanning polypeptides including the reaction center subunits D1 and D2, the chl-binding antenna subunits CP43 and CP47, and several low molecular weight polypeptides (*3*). Due to the lack of high-quality crystals, the structure of the PSII complex has not been elucidated. Likewise, the subunits responsible for binding of the Mn-cluster have not been unambiguously identified, although the reaction center polypeptides D1 and D2 are considered to be the most probable candidates, and indeed several residues of D2 and in particular of D1 have been suggested as possible ligands to Mn from site-directed mutagenesis studies (*1*, *4*, *5*). In addition to the membrane-spanning proteins, three extrinsic proteins are associated with PSII and are required for optimum activity of the oxygen-evolving complex (see *6* for review). Among the three extrinsic proteins, the 33 kDa protein encoded by the *psb*O gene is ubiquitous in PSII from cyanobacteria to higher plants and plays the most important role in maintaining the stability and function of the Mn-cluster. Removal of the 33 kDa protein from isolated PSII of higher plants results in a significant decrease in oxygen evolution and destabilization of the Mn cluster. The effects of removal of the 33 kDa protein include a stabilization of the S_2 state (*7*), a delay in oxygen release during the $S_3-[S_4]-S_0$ transition (*8*), an increase in the requirement for Ca^{2+} and Cl^- of oxygen evolution (*9*−*12*), release of two out of

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[1] Abbreviations: chl, chlorophyll; cyt, cytochrome; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; HA, hydroxylamine; HN buffer, 10 mM Hepes and 20 mM NaCl (pH 7.0); PSII, photosystem II.

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the four Mn atoms under low Cl^- concentrations (*9*), and an increased accessibility to exogenous reductants of the Mn-cluster (*13*). Most of these effects have also been observed in cyanobacterial mutants lacking the 33 kDa extrinsic protein (*14*−*17*), suggesting a similar function of the 33 kDa protein between cyanobacterial and higher plant PSII. In addition, the cyanobacterial mutant cells lacking the 33 kDa protein have been found to lose their oxygen evolution very rapidly in the dark (*18*, *19*), a consequence probably resulting from an increased accessibility of the Mn-cluster in the absence of the 33 kDa protein to endogenous reductants accumulated in the cells incubated in the dark.

In addition to the 33 kDa protein, two other extrinsic proteins are associated with PSII and play some regulatory roles in oxygen evolution. In green algal and higher plant PSII, these two proteins are the 23 and 17 kDa proteins encoded by *psb*P and *psb*Q nuclear genes, respectively (*1*, *6*, *20*), whereas in PSII from cyanobacteria (*21*−*24*), red alga (*25*), and probably diatoms (*26*, *27*), they are cyt *c*-550 and a 12 kDa protein encoded by *psb*V and *psb*U genes. Cyt *c*-550 and the 12 kDa protein are apparently different from the 23 and 17 kDa proteins in terms of their primary sequences, but they seem to share some common binding and functional features. For example, removal of any of these proteins causes a decrease in oxygen evolution under physiological conditions, and the binding of both the 12 and 17 kDa proteins requires the presence of other extrinsic proteins (20, 28). There do exist, however, differences in the binding and functional features of these two sets of proteins; the most distinct differences are that removal of the 23 kDa protein from plant PSII creates a dramatic increase in the requirement for Ca^{2+} and removal of the 17 kDa protein increases the requirement for Cl^- of oxygen evolution (*1*, *6*, *20*), whereas removal of cyt *c*-550 and the 12 kDa protein from isolated cyanobacterial PSII only slightly increases the requirement for Ca^{2+} (*28*), and that cyt *c*-550 alone can bind and function independent of the 33 kDa protein (*28*, *29*). In contrast, the 23 kDa protein cannot bind to PSII in the absence of the 33 kDa protein (*20*). The latter difference perhaps accounts for the difference observed in the phenotypes of two *psb*O gene knockout mutants, one being the green alga *Chlamydomonas reinhardtii* (*30*) and the other one being the cyanobacterium *Synechocystis* sp. PCC 6803 (*15*, *16*, *31*, *32*). The *Chlamydomonas* Δ*psb*O mutant is unable to grow photoautotrophically presumably because of the lack of binding and functioning of the 23 kDa protein, whereas the *Synechocystis* Δ*psb*O mutant is able to grow autotrophically as a result of the binding and functioning of cyt *c*-550, as further deletion of the *psb*V gene encoding cyt *c*-550 from the *psb*O-deletion mutant resulted in an obligate photohetrotrophic mutant (*29*). These observations suggest a more independent and, possibly, more direct role of cyt *c*-550 in cyanobacterial oxygen evolution as compared with the 23 and 17 kDa proteins in green algal and higher plant PSII. In the present study, we examined the oxygen-evolving system of Δ*psb*U and Δ*psb*V mutants of *Synechocystis* sp. PCC 6803 lacking the 12 kDa protein and cyt *c*-550, respectively, by means of flash-induced thermoluminescence emission and oxygen evolution. The results showed that while deletion of the 12 kDa protein resulted in relatively small impairments of PSII function, deletion of cyt *c*-550 caused large alterations of the H_2O oxidation complex. The most significant features found for the cyt *c*-550-deletion mutant are its lack of growth in the absence of added Ca^{2+} or Cl^- in the growth medium, and its rapid deactivation of oxygen evolution in the dark which can be photoactivated upon light illumination with a very high efficiency.

MATERIALS AND METHODS

Synechocystis sp. PCC 6803 wild-type cells were grown in BG11 medium at 30 °C at a light intensity of 30 μEinsteins m^{-2} s^{-1}. For mutant cells, 5 mM glucose was supplemented to the BG11 medium. The Δ*psb*U and Δ*psb*V mutant strains lacking coding regions for the 12 kDa protein and cyt *c*-550, respectively, were constructed and characterized as described previously (*23*, *24*). To determine the growth rate of wild-type and mutant strains in the absence of Ca^{2+} or Cl^-, 0.24 mM $CaCl_2$ in the original BG11 medium was replaced by either 0.48 mM NaCl or 0.24 mM $Ca(NO_3)_2$. *Synechocystis* cells grown in the normal BG11 medium were harvested and washed twice with distilled water, and then transferred to the medium lacking added Ca^{2+} or Cl^-. Growth curves of wild-type and mutant strains were recorded by measuring the light scattering of cells at 730 nm.

For measurements of thermoluminescence and flash oxygen yield, *Synechocystis* wild-type and mutant cells were harvested, washed once with HN buffer (10 mM Hepes, 20 mM NaCl, pH 7.0) by centrifugation at 1000*g* for 5 min at room temperature, and then resuspended in the same buffer at a chl concentration of 0.5 mg/mL. Before measurements, the harvested cells were kept shaking with a rotary shaker at room temperature under weak room light. For thermoluminescence measurements, cells were diluted to 100 μg of chl/mL, dark-adapted at room temperature in the absence or presence of 20 μM DCMU, and then illuminated with single-turnover flashes at 0 °C for samples without DCMU or with continuous light for 30 s at −5 °C for samples supplemented with DCMU. Unless otherwise indicated, the time for dark adaptation prior to flash illumination was 5 min. Thermoluminescence glow curves were recorded as described in ref *33*, with a heating rate of 1.0 °C/s. Flash O_2 yields were measured with a bare platinum electrode as described in (*19*). Cells of wild-type and mutant strains containing 5 μg of chl were deposited onto the surface of the electrode by centrifugation at 5000*g* for 5 min at 25 °C in the dark followed by illumination with actinic, single-turnover xenon flashes. The oxygen signals generated were digitized and recorded by a computer, which also allowed the postmeasuring treatment of the data (*19*).

For determination of dark-deactivation, harvested cells were kept shaking at room temperature in complete darkness in HN buffer in the absence or presence of 5 mM glucose at 0.5 mg of chl/mL, and then an aliquot of cells was taken for thermoluminescence or flash O_2 yield measurements at the designated times of incubation. Extraction of PSII Mn was carried out by treating the cells with 1 mM HA (hydroxylamine) at 0.5 mg of chl/mL for 10 min, and washed twice with HN buffer (*19*, *34*). The HA-treated cells were resuspended in HN buffer supplemented with 50 μM $MnCl_2$ and 1 mM $CaCl_2$, collected onto the surface of the electrode, and then subjected to photoactivation by illumination with various numbers of the xenon flashes as described in (*19*).

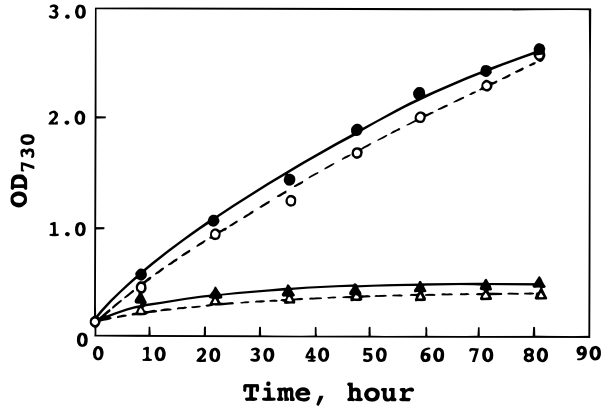

FIGURE 1: Growth curves of *Synechocystis* sp. PCC 6803 wild-type and mutant strains in BG11 depleted of either Ca^{2+} (open symbols) or Cl^- (closed symbols) at 30 °C. The cell densities were determined by measuring light scattering at 730 nm. (○) Wild type; (△) Δ*psb*V mutant.

RESULTS

Growth in the Absence of Ca^{2+} or Cl^-. Figure 1 shows the growth curves of *Synechocystis* sp. PCC 6803 wild-type and Δ*psb*V mutant cells lacking cyt *c*-550 in BG11 in the absence of Ca^{2+} or Cl^-. The Δ*psb*V mutant virtually could not grow in the absence of either added Ca^{2+} or Cl^-. This is similar to the Δ*psb*O mutant which also cannot grow in the absence of Ca^{2+} or Cl^- (data not shown; see ref *15*, *18*). In contrast, the wild-type cells were able to grow in the absence of either added Ca^{2+} or Cl^- at a rate slightly lower than that in normal BG11 (see also *15*, *24*), and the Δ*psb*U mutant lacking the 12 kDa protein grew more slowly in the absence of Ca^{2+} or Cl^- than that of the wild-type strain (*24*). These results suggest that cyt *c*-550 functions in maintaining the affinity of PSII for Ca^{2+} and Cl^-, whereas the 12 kDa protein functions in modulating the affinity of PSII for Ca^{2+} and Cl^- but is not absolutely required for their functioning in cyanobacterial oxygen evolution.

Characterization of the S-State Transitions. S-state transitions in wild-type and mutant cells were characterized by PSII thermoluminescence measured in either the absence or the presence of DCMU. As Figure 2 shows, the intensities of the thermoluminescence emission, following either one, two, or three flashes in the absence of DCMU, were remarkably lower in the Δ*psb*V mutant than those from the wild type, indicating a significantly lower activity of the oxygen-evolving complex in the Δ*psb*V mutant. This may be caused by either a significantly lower efficiency of the S-state transitions or a decreased number of reaction centers active in S-state transitions. In addition, the peak temperature of the B-band resulting from $S_2Q_B^-$ charge recombination (*35*) was upshifted from 24−26 °C in the wild-type strain to 30−32 °C in the Δ*psb*V mutant strain. Similarly, the peak temperature of the Q-band, resulting from charge recombination of $S_2Q_A^-$ (*35*), was upshifted from 16−18 °C in the wild-type strain to 24−26 °C in the Δ*psb*V mutant strain. These suggest a stabilization of the S_2 state upon deletion of cyt *c*-550. Comparable upshifts of the B- and Q-bands (each upshifted 4 °C), however, have been observed in the Δ*psb*U mutant (*24*), suggesting that the stabilizing effect was mainly caused by loss of the 12 kDa protein, as this protein cannot bind and function in the absence of cyt *c*-550. The

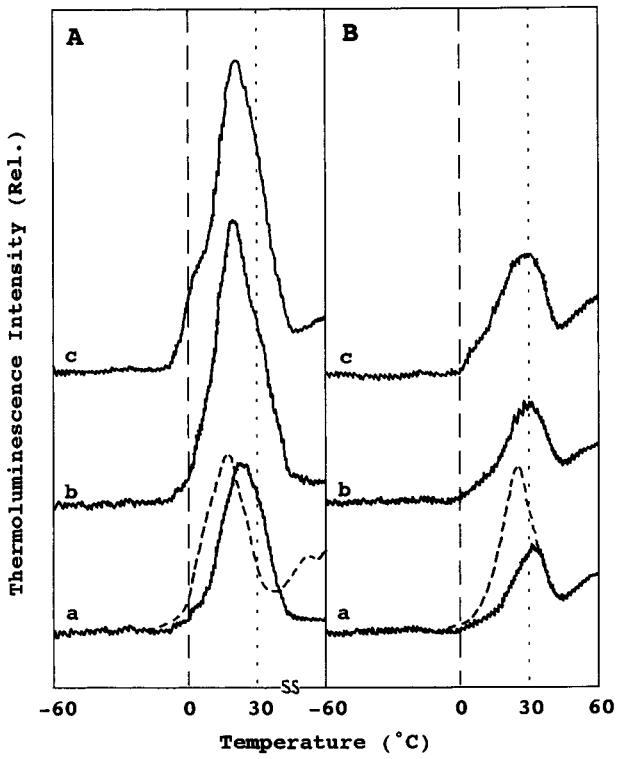

FIGURE 2: Thermoluminescence glow curves of wild-type and mutant cells of *Synechocystis* sp. PCC 6803 in BG11. Cells suspended in BG11 at 100 μg of chl/mL were dark-adapted for 5 min at room temperature, illuminated at 0 °C for one (traces a), two (traces b), or three (traces c) saturating flashes, and immediately transferred to liquid nitrogen for the thermoluminescence measurement. The dashed lines on traces a are the glow curves measured in the presence of 20 μM DCMU and after continuous illumination for 30 s at −5 °C. (A) Wild type; (B) Δ*psb*V mutant.

major effect of cyt *c*-550 deletion, therefore, is the decrease of the B-band intensity.

In addition to the peak temperature, a relative increase in the intensity of the Q-band, but not the B-band, was observed in both the Δ*psb*V (Figure 2) and Δ*psb*U mutants (*24*). This may be due to that the same S_2 modification in the two mutants caused a larger effect on the thermoluminescence intensity of the $S_2Q_A^-$ recombination than that of the $S_2Q_B^-$ recombination, as the former charge pair had a higher potential difference than the latter one (*35*). In addition, the Q-band was measured following 30 s continuous illumination whereas the B-band was measured with one flash illumination; this would also give rise to a slight increase in the Q-band intensity in the two mutants because of their increased miss factors for S-state transitions (see below).

Figure 3 depicts typical oscillation patterns of oxygen evolution induced by a train of single-turnover flashes. While both the Δ*psb*U and Δ*psb*V mutants were capable of oxygen evolution with a characteristic period four oscillation, their yields were apparently lower than that of the wild-type cells: The oxygen yield from the Δ*psb*U mutant was typically about 60−80% whereas the yield from the Δ*psb*V mutant was approximately 20−50% as compared with that of the wild-type cells. The activity of the Δ*psb*V strain exhibits considerable variation in response to the age of the culture and light conditions (unpublished observations). Analysis of the oscillation patterns (*36*) revealed that the wild-type cells had miss and double hit factors of 9.1% and

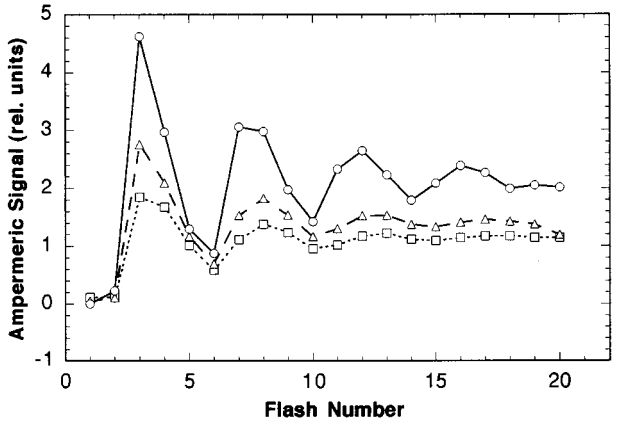

FIGURE 3: Oscillation patterns of flash-induced oxygen yields of *Synechocystis* sp. PCC 6803 wild-type and mutant strains measured by a bare platinum oxygen electrode. The cells of wild type and mutants containing the same amount of chl (5 μg) were harvested and suspended in HN buffer and pelleted onto the surface of the electrode by a brief centrifugation for 5 min in the dark, followed by illumination with a train of 20 flashes given at 4 Hz. (○) Wild type; (△) Δ*psb*U mutant; (□) Δ*psb*V mutant.

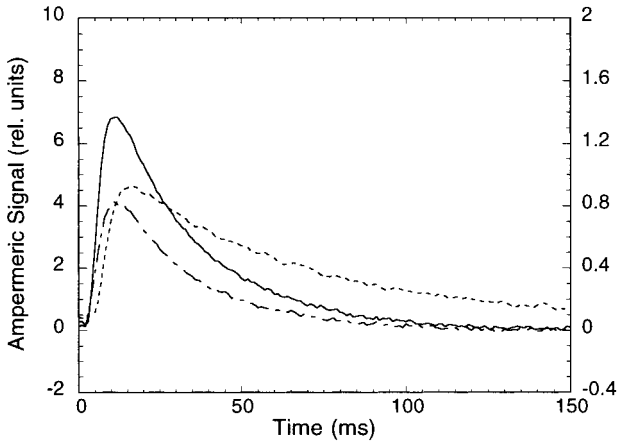

FIGURE 4: Time-resolved O_2 release signals from the bare platinum oxygen electrode from the S_3 state. Cells of *Synechocystis* sp. PCC 6803 were collected onto the surface of the electrode as in Figure 3, and then subjected to illumination with a train of 20 flashes at 4 Hz. The O_2 signal produced by the third flash illumination was amplified and displayed. (—) Wild type; (– -) Δ*psb*U mutant; (---) Δ*psb*V mutant. The left side *y*-axis represents the signal intensity for the wild-type and Δ*psb*U mutant cells, and the right side *y*-axis represents the signal intensity for the Δ*psb*V mutant cells.

3.0%, respectively, whereas the Δ*psb*U and Δ*psb*V mutants showed miss and double hit factors of 15.9%, 2.5% and 15.3%, 3.6%, respectively. Thus, both mutants produce significant increases in the miss factor, which is apparent in the faster damping of the oscillation pattern shown in Figure 3. However, since the absence of cyt *c*-550 results in a loss of the ability of the 12 kDa protein to bind, the observed increase in the miss probability is most probably caused by deletion of the 12 kDa protein but not cyt *c*-550.

Deletion of the 33 kDa protein retarded the oxygen release from the S_3 state (*14*). In order to examine whether a similar effect occurred in the Δ*psb*U or Δ*psb*V mutants, we measured the kinetics of the oxygen release signal from the S_3 state using the bare platinum electrode. As Figure 4 shows, the peak of the oxygen signal for both the wild-type and Δ*psb*U strains was located at 10−12 ms immediately following the third flash, similar to that observed for the *Synechocystis* wild-type cells previously (*14*). This indicates

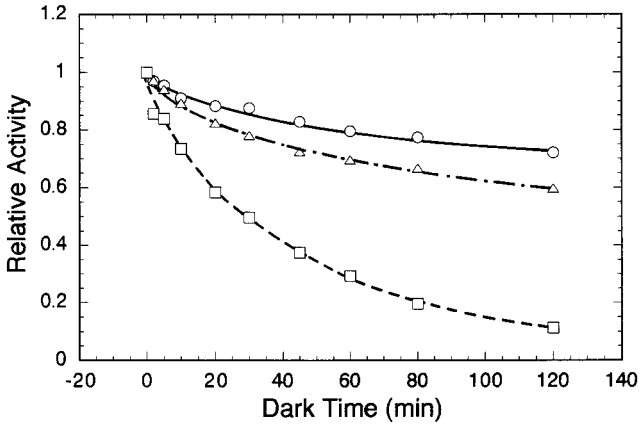

FIGURE 5: Time courses of dark-deactivation of oxygen evolution of *Synechocystis* sp. PCC 6803 wild-type (○), Δ*psb*U mutant (△) and Δ*psb*V mutant (□) cells. Cells were suspended in HN buffer and collected onto the surface of the bare platinum electrode by centrifugation, and then incubated in complete darkness. To measure the O_2-evolving activity, a train of 20 flashes at 4 Hz was given at the designated time points, and the averages of O_2 signal intensities from flashes number 14 to 17 of each measurement were taken as relative activities, with the activity after 5 min centrifugation but before incubation in the dark as a control.

that deletion of the 12 kDa protein did not give any effect on oxygen release kinetics during the $S_3-[S_4]-S_0$ transition. The peak of the oxygen signal for the Δ*psb*V mutant reproducibly increased slightly to 14−15 ms, whereas its decay kinetics were very similar to those of the wild-type or Δ*psb*U mutant strains. This suggests that deletion of cyt *c*-550 slightly retarded the $S_3-[S_4]-S_0$ transition. The effect caused by cyt *c*-550 deletion, however, was much smaller than that caused by the 33 kDa protein deletion (*14*) with respect to the $S_3-[S_4]-S_0$ transition.

Dark-Deactivation. Upon dark-incubation, the Δ*psb*V mutant cells were found to lose thermoluminescence emission rapidly (data not shown). This suggests a rapid dark-deactivation of the oxygen-evolving system in the absence of cyt *c*-550. This was proved by measuring flash-induced oxygen evolution incubated in the dark on the bare platinum oxygen electrode (Figure 5). The wild-type cells showed a slight decrease of 25−30% in its activity after 2 h dark-incubation on the surface of the electrode, and the Δ*psb*U mutant cells showed a decrease of 30−40% in its activity following the same period of dark-incubation. This indicates that deletion of the 12 kDa protein had a very small effect on the dark-stability of the oxygen-evolving complex. Moreover, addition of 5 mM glucose during the dark-incubation did not give rise to any significant differences in the extent of dark-deactivation of both the wild-type and the Δ*psb*U strains (data not shown). In contrast, the activity of the Δ*psb*V strain decreased by 90% after 2 h of dark-incubation (Figure 5), and this decrease became somewhat more larger in the presence of 5 mM glucose (not shown). The half-decay times estimated for this mutant in the absence or presence of glucose are 28 and 17 min, respectively. These results indicate that deletion of cyt *c*-550 resulted in a much more unstable oxygen-evolving complex in the dark.

Photoactivation. Dark-inactivated oxygen evolution in the Δ*psb*V mutant cells was found to be reactivated by light illumination. Figure 6 depicts the results of such photoactivation experiments in which cells incubated in the dark for

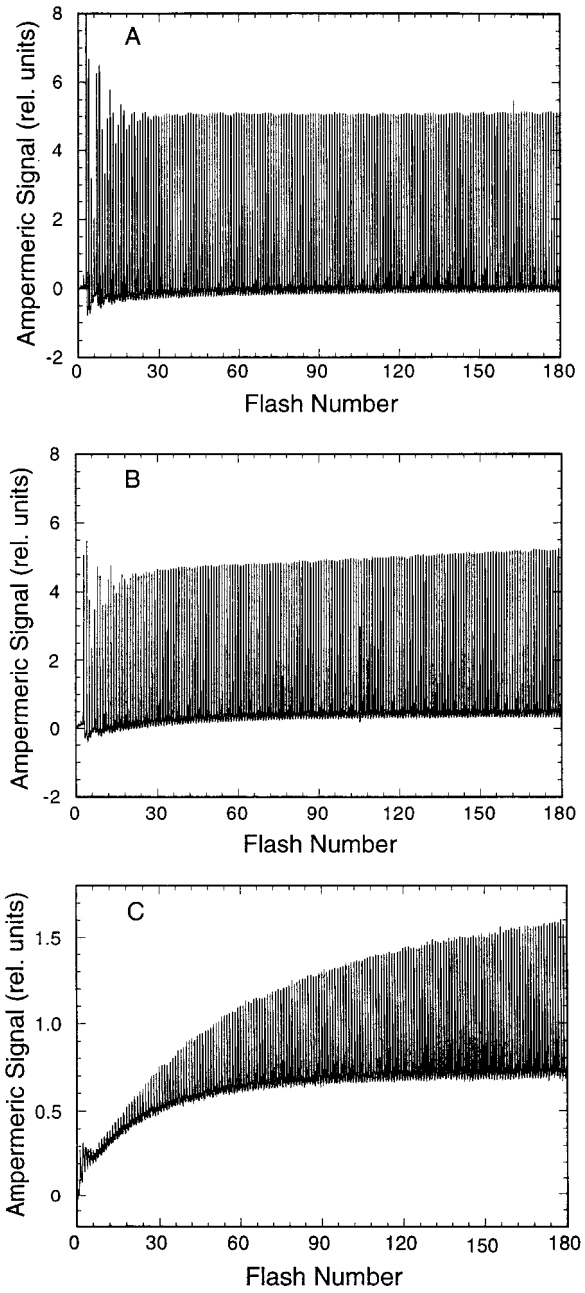

FIGURE 6: Flash-induced oxygen signals after 6 h dark-incubation. Cells of *Synechocystis* sp. PCC 6803 wild type (A), Δ*psb*U mutant (B), and Δ*psb*V mutant (C) were incubated in the dark with shaking on a rotary shaker in HN buffer at room temperature for 6 h and pelleted onto the surface of the bare platinum electrode by centrifugation in the dark, and then oxygen signals were measured by illumination with a train of 180 flashes given at 3 Hz.

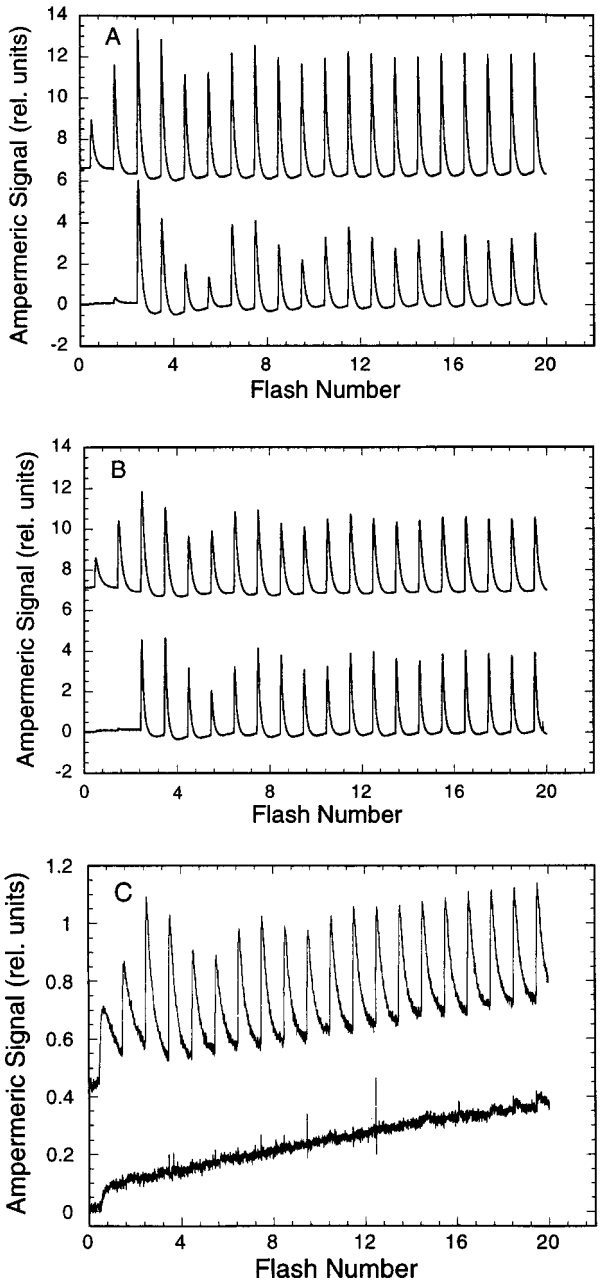

FIGURE 7: Flash-induced oxygen signals after 24 h dark-incubation. Cells of *Synechocystis* sp. PCC 6803 wild type (A), Δ*psb*U mutant (B), and Δ*psb*V mutant (C) were incubated as in Figure 6 for 24 h in the dark and pelleted onto the surface of the bare platinum electrode, followed by measurement of oxygen signals with 20 flashes given at 4 Hz. Lower traces, measured immediately after 24 h dark-incubation; upper traces, measured after illumination with 500 preflashes given at 4 Hz.

6 h were illuminated with a sequence of 180 flashes given at 3 Hz. The wild-type cells showed significant oxygen signals already in the first several flashes, and their intensities increased only slightly upon further flash illumination. A similar situation was observed with the Δ*psb*U mutant, indicating that even after 6 h incubation in the dark, only a small loss of oxygen evolution occurred with the wild-type and Δ*psb*U mutant cells, which was restored upon flash illumination. In contrast, the oxygen signals from the Δ*psb*V mutant were extremely low during the first several flashes, which increased gradually with the increase in the flash number. This not only demonstrated the severe dark-inactivation in this mutant as has been described in the previous section, but also suggested an apparent photoactivation of oxygen evolution upon flash illumination.

The photoactivation in the Δ*psb*V mutant was more clearly seen in Figure 7, in which the activities of cells incubated in the dark for 24 h were measured with a train of 20 flashes given at 4 Hz without any preflash illumination (lower traces in Figure 7) or with 500 preflash illumination (upper traces). Here again, the wild-type and Δ*psb*U mutant cells retained their activities significantly after 24 h dark incubation, and only a small increase in the activities was seen upon 500 preflash illumination. In contrast, the Δ*psb*V mutant showed no oxygen release at all after 24 h dark-incubation when no

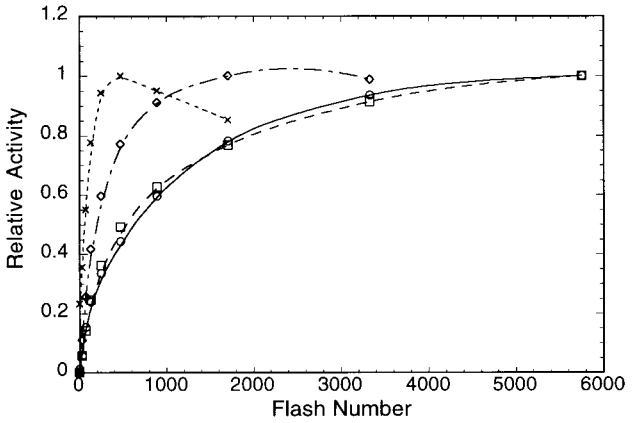

FIGURE 8: Flash number dependencies of photoactivation of *Synechocystis* sp. PCC 6803 wild-type and mutant cells. Cells which had been inactivated either by HA-treatment or by dark-incubation were pelleted onto the surface of the bare platinum electrode, subjected to illumination with different numbers of flashes given at 4 Hz, and the oxygen signals were measured by a train of 20 flashes at 4 Hz. The signal intensities from flashes number 14 to 17 were averaged and plotted as the relative extent of photoactivation, with the maximum photoactivation of 100% determined with 5700 preflash illumination for the wild-type and Δ*psb*U mutant strains, and 1700 preflash illumination for the Δ*psb*V mutant cells treated with HA, or 470 preflash illumination for the Δ*psb*V mutant cells without HA-treatment but after 6 h dark-incubation. (○) HA-treated wild-type cells; (□) HA-treated Δ*psb*U mutant; (◇), HA-treated Δ*psb*V mutant; (×) Δ*psb*V mutant cells without HA-treatment but after 6 h dark-incubation.

preflash illumination was provided. After 500 preflash illumination, the oxygen signals appeared, although their intensities were not very large due to the inherent low activity of the mutant cells and also probably due to an irreversible inactivation resulted from the prolonged dark-incubation (note the difference in the scales of *y*-axis between panels A, B, and C). This is consistent with the results described in the previous section, and thus clearly indicates photoactivation of the oxygen-evolving system in the Δ*psb*V mutant cells by preflash illumination.

In order to examine the photoactivation efficiency, the wild-type and mutant cells were treated with HA to extract PSII Mn, and then photoactivated by different numbers of preflashes given at 4 Hz in the presence of $MnCl_2$ and $CaCl_2$. The results obtained (Figure 8) demonstrate that, while the wild-type and Δ*psb*U mutant cells showed a similar preflash number dependence for their photoactivation which increased gradually with the increase of preflash numbers even above 5700 preflashes, the Δ*psb*V mutant exhibited a significantly different preflash number dependence, with a much lower flash number of 1700 for saturation. This suggests a significantly higher efficiency of photoactivation in the Δ*psb*V mutant than in the wild type or the Δ*psb*U mutant. An even higher efficiency of photoactivation was seen for the Δ*psb*V mutant after 6 h dark-inactivation but without HA treatment; in this case, the photoactivation reached its saturation level with only 470 preflashes.

DISCUSSION

Functional Implications of Cyt c-550 and the 12 kDa Protein. Our previous studies have revealed a significant loss (ca. 60%) of oxygen evolution upon deletion of cyt *c*-550 which can partly be attributed to a destabilization of the PSII complex in vivo (*23*). The present study now reveals the following important defects in the cyanobacterial oxygen-evolving system upon deletion of cyt *c*-550: (a) a complete loss of photoautotrophic growth in the absence of Ca^{2+} or Cl^-; (b) a rapid dark-deactivation of oxygen evolution; (c) a slight retardation in O_2 release from the S_3 state; (d) a slight upshift in the peak temperature of the thermoluminescence B- and Q-bands concomitant with an increase in the Q-band intensity. While effect (d) was also observed in the Δ*psb*U mutant (*24*) and thus should be attributed to the loss of binding of the 12 kDa protein in the absence of cyt *c*-550 (*28*), the former three effects appeared only in the Δ*psb*V mutant and thus are caused by the loss of cyt *c*-550.

The loss of autotrophic growth in the absence of Ca^{2+} or Cl^- suggests that the Δ*psb*V mutant has a dramatically decreased affinity for Ca^{2+} and Cl^-. Thus, one of the important functions of cyt *c*-550 is to maintain the affinity for Ca^{2+} and Cl^- of algal PSII required for normal oxygen evolution. This somewhat contradicts our previous in vitro observations that the absence of cyt *c*-550 did not inhibit the oxygen evolution completely even in the absence of Ca^{2+} (*28*). The activity observed in isolated PSII lacking cyt *c*-550, however, was very low even in the presence of a high concentration of Cl^- but without Ca^{2+} (13.5% of control PSII supplemented with $CaCl_2$) (*28*), which may not be high enough to support the photoautotrophic growth of intact cells. This resembles the case observed for the Δ*psb*O mutant which also cannot grow in the absence of Ca^{2+} (*15*), but isolated PSII depleted of the 33 kDa protein still retained some oxygen-evolving activity in the absence of Ca^{2+} but with a suitable high concentration of Cl^- (*9*−*12*).

Another important functional consequence of cyt *c*-550 deletion is the significantly increased instability of the oxygen-evolving complex, as suggested from the rapid dark-deactivation of oxygen evolution in the Δ*psb*V mutant. Two possibilities may be considered as responsible for this increased dark-instability. First, the binding of the Mn-cluster may be weakened upon depletion of cyt *c*-550 to be prone to disassemble during the dark-incubation. Second, binding of cyt *c*-550 may shield the Mn-cluster from access to bulk solution, and loss of the cyt results in an increased accessibility of the Mn-cluster to chemicals such as reductants which may accumulate in the cells incubated in the dark. Part or all of the four Mn atoms may then dissociate from their binding sites upon attack by the bulk reductants. Currently, we favor the second possibility based on the following two observations: First, the extent of dark-deactivation of oxygen evolution in the Δ*psb*V mutant became even larger in the presence of glucose during incubation, which is a rich source of endogenous reductants by serving as a respiratory substrate. Second, the photoactivation efficiency was much higher in the Δ*psb*V mutant than that in the wild type or Δ*psb*U mutant. The best explanation for this is that cyt *c*-550 binds to a site close to the Mn-cluster; loss of this cyt permits the Mn to go in and out of its site more easily, which may thus result in the rapid dark-inactivation and high efficiency of photoactivation in the mutant cells. A similar, rapid dark-inactivation of oxygen evolution has been observed in the Δ*psb*O mutant (*18*, *19*) and several mutants of CP47 that carry short deletions in its large extrinsic E-loop exposed to the lumenal side (*37*, *38*). Moreover, the Δ*psb*O mutant (*19*) and one of the CP47

mutants [(Δ(A373−D380)] (*38*) also showed a higher photoactivation efficiency as we observed for the Δ*psb*V mutant in the present study.

In contrast to cyt *c*-550, the damages caused by deletion of the 12 kDa protein were rather small. These include a moderate decrease of growth in the absence of Ca^{2+} or Cl^- (*24*), an increase in the miss factor for S-state transition, an upshift of the peak temperature of thermoluminescence for B- and Q-bands (*24*), and an increase in the Q-band intensity (*24*). These suggest a role for the 12 kDa protein in maintaining the maximum affinity of PSII for Ca^{2+} and Cl^-, and a possible modification in the S_2 state upon loss of the 12 kDa protein. This may be responsible for the observed increase in the miss factor for S-state transitions.

Comparison between the Functions of Cyt c-550 and the 12 kDa Protein with Those of the 23 and 17 kDa Proteins. Cyt *c*-550 in cyanobacterial and some eukaryotic alga resembles the 23 kDa protein in green algal and higher plant PSII in terms of their roles in maintaining the affinity for Ca^{2+} of PSII (*20* and this study), and their requirements for binding of the third extrinsic protein, the 12 kDa protein in cyanobacteria (*28*) and the 17 kDa protein in higher plants (*20*). The present study, however, revealed several roles of cyt *c*-550 that are not found for the 23 kDa protein; e.g., cyt *c*-550 maintains the PSII affinity for Cl^- and is required for the normal O_2 release kinetics from the S_3 state, its loss causes a rapid deactivation of cells kept in the dark, and the deactivated Mn-cluster can be photoactivated with a much higher efficiency in the absence of cyt *c*-550. As discussed above, the latter two effects suggest a close association of the cyt with the Mn-cluster. These suggest that cyt *c*-550 has a more important and direct role in oxygen evolution in cyanobacterial PSII than the 23 kDa protein in higher plant PSII.

As to the 12 kDa protein in cyanobacteria and the 17 kDa protein in higher plants, some minor differences can be pointed out between the functions of the two proteins. Loss of the 12 kDa protein resulted in a slight modification of the S_2 state and a slight decrease in the affinity of PSII for both Ca^{2+} and Cl^- (*24*), whereas no such modification of the S_2 state and only a decrease in the PSII affinity for Cl^- occurred upon removal of the 17 kDa protein (*20*). Despite these differences, the two proteins share some apparent similarities in that both of them cannot bind to PSII in the absence of the other two extrinsic proteins, and that loss of them caused relatively smaller decreases in oxygen evolution than those of the other extrinsic proteins.

Comparison between the Functions of Cyt c-550 and the 33 kDa Protein. The functional consequences caused by cyt *c*-550 deletion revealed in the present study showed some striking similarities with those caused by deletion of the 33 kDa protein. That is, both the Δ*psb*V (this study) and Δ*psb*O (*15*) mutants cannot grow in the absence of Ca^{2+} or Cl^-, both mutants showed a rapid dark-deactivation of oxygen evolution with a similar time course (*18*, *19*, and this study), and photoactivation of the Mn-cluster had a higher efficiency in the absence of either cyt *c*-550 (this study) or the 33 kDa protein (*19*, *39*). These suggest that both cyt *c*-550 and the 33 kDa protein function in maintaining the high affinity of PSII to Ca^{2+} and Cl^-, and are located close to the Mn-cluster to protect the Mn-cluster from attack by bulk reductants. In addition, a retardation of O_2 release from the S_3 state previously observed for the Δ*psb*O mutant (*14*) appears to be occurring in the Δ*psb*V mutant, although the extent of decrease in the rate of O_2 release caused by deletion of cyt *c*-550 is much smaller than that caused by the 33 kDa protein. The functioning of cyt *c*-550 in cyanobacterial oxygen evolution, however, is not dependent on that of the 33 kDa protein and vice versa, as both components can bind to PSII independently and both are required for the normal growth of *Synechocystis* sp. PCC 6803 (*28*, *29*). In addition, the deletion of cyt *c*-550 has been shown to cause a destabilization of the PSII complex which was not observed in the Δ*psb*O mutant. These results imply that the intriguing, low-potential cyt *c*-550 plays important roles in cyanobacterial oxygen evolution that are compatible with but independent of those of the well-characterized extrinsic 33 kDa protein.

REFERENCES

1. Debus, R. J. (1992) *Biochim. Biophys. Acta 1102*, 269−352.
2. Britt, D. (1996) in *Oxygenic Photosynthesis: The Light Reactions* (Ort, D. R., and Yocum, C. F., Eds.) pp 137−164, Kluwer Academic Publishers, The Netherlands.
3. Bricker, T. M., and Ghanotakis, D. F. (1996) in *Oxygenic Photosynthesis: The Light Reactions* (Ort, D. R., and Yocum, C. F., Eds.) pp 113−136, Kluwer Academic Publishers, The Netherlands.
4. Chu, H.-A., Nguyen, A. P., and Debus, R. J. (1995) *Biochemistry 34*, 5839−5858.
5. Chu, H.-A., Nguyen, A. P., and Debus, R. J. (1995) *Biochemistry 34*, 5859−5882.
6. Seidler, A. (1996) *Biochim. Biophys. Acta 1277*, 36−60.
7. Vass, I., Ono, T., and Inoue, Y. (1987) *Biochim. Biophys. Acta 892*, 224−235.
8. Miyao, M., Murata, N., Lavorel, J., Maison-Peteri, B., Boussac, A., and Etienne, A.-L. (1987) *Biochim. Biophys. Acta 890*, 151−159.
9. Ono, T., and Inoue, Y. (1984) *FEBS Lett. 168*, 281−286.
10. Miyao, M., and Murata, N. (1984) *FEBS Lett. 170*, 350−354.
11. Kuwabara, T., Miyao, M., Murata, T., and Murata, N. (1985) *Biochim. Biophys. Acta 806*, 283−289.
12. Bricker, T. M. (1992) *Biochemistry 31*, 4623−4628.
13. Tamura, N., Inoué, H., Inoue, Y., and Cheniae, G. M. (1990) *Plant Cell Physiol. 31*, 469−477.
14. Burnap, R. L., Shen, J.-R., Jursinic, P. A., Inoue, Y., and Sherman, L. A. (1992) *Biochemistry 31*, 7404−7410.
15. Philbrick, J. B., Diner, B. A., and Zilinskas, B. A. (1991) *J. Biol. Chem. 266*, 13370−13376.
16. Bockholt, R., Mesepohl, B., and Pistorius, E. K. (1991) *FEBS Lett. 294*, 59−63.
17. Vass, I., Cool, K. M., Deàk, Z., Mayes, S. R., and Barber, J. (1992) *Biochim. Biophys. Acta 1102*, 195−201.
18. Engels, D. H., Lott, A., Schmid, G. H., and Pistorious, E. K. (1994) *Photosynth. Res. 42*, 227−244.
19. Burnap, R. L., Qian, M., and Pierce, C. (1996) *Biochemistry 35*, 874−882.
20. Miyao, M., and Murata, N. (1987) in *Topics in Photosynthesis* (Kyle, D. J., Osmond, C. B., and Arntzen, C. J., Eds.) Vol. 9, pp 289−307, Elsevier, Amsterdam.
21. Shen, J.-R., Ikeuchi, M., and Inoue, Y. (1992) *FEBS Lett. 301*, 145−149.
22. Shen, J.-R., and Inoue, Y. (1993) *J. Biol. Chem. 268*, 20408−20413.
23. Shen, J.-R., Vermaas, W. F. J., and Inoue, Y. (1995) *J. Biol. Chem. 270*, 6901−6907.
24. Shen, J.-R., Ikeuchi, M., and Inoue, Y. (1997) *J. Biol. Chem. 272*, 17821−17826.
25. Enami, I., Murayama, H., Ohta, H., Kamo, M., Nakazato, K., and Shen, J.-R. (1995) *Biochim. Biophys. Acta 1232*, 208−216.

26. Shimazaki, K., Takamiya, K., and Nishimura, M. (1978) *J. Biochem. 83*, 1631−1638.
27. Kowallik, K. V., Stoebe, B., Schaffran, I., Kroth-Pancic, P., and Freier, U. (1996) *GeneBank/EMBL/DDBJ data bases*, Accession No. Z67753.
28. Shen, J.-R., and Inoue, Y. (1993) *Biochemistry 32*, 1825−1832.
29. Shen, J.-R., Burnap, R. L., and Inoue, Y. (1995) *Biochemistry 34*, 12661−12668.
30. Mayfield, S. P., Bennoun, P., and Rochaix, J.-D. (1987) *EMBO J. 6*, 313−318.
31. Burnap, R. L., and Sherman, L. A. (1991) *Biochemistry 30*, 440−446.
32. Mayes, S. R., Cook, K. M., Self, S. J., Zhang, Z., and Barber, J. (1991) *Biochim. Biophys. Acta 1060*, 1−12.
33. Ono, T., and Inoue, Y. (1986) *Biochim. Biophys. Acta 850*, 380−389.
34. Cheniae, G. M., and Martin, I. F. (1972) *Plant Physiol. 50*, 87−94.
35. Inoue, Y. (1996) in *Biophysical Techniques in Photosynthesis* (Amesz, J., and Hoff, A. J., Eds.) pp 93−107, Kluwer Academic Publishers, The Netherlands.
36. Meunier, P. C., Burnap, R. L., and Sherman, L. A. (1995) *Photosynth. Res. 45*, 31−40.
37. Gleiter, H. M., Haag, E., Shen, J.-R., Eaton-Rye, J. J., Inoue, Y., Vermaas, W. F. J., and Renger, G. (1994) *Biochemistry 33*, 12063−12071.
38. Gleiter, H. M., Haag, E., Shen, J.-R., Eaton-Rye, J. J., Seeliger, A. G., Inoue, Y., Vermaas, W. F. J., and Renger, G. (1995) *Biochemistry 34*, 6847−6856.
39. Chu, H.-A., Nguyen, A. P., and Debus, R. J. (1994) *Biochemistry 33*, 6150−6157.

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