

## Deregulation of Electron Flow within Photosystem II in the Absence of the PsbJ Protein\*

Received for publication, March 5, 2001, and in revised form, June 26, 2001  
Published, JBC Papers in Press, August 23, 2001, DOI 10.1074/jbc.M102007200

Ralf E. Regel<sup>‡§</sup>, Natalia B. Ivleva<sup>§¶\*\*</sup>, Hagit Zer<sup>‡‡</sup>, Jörg Meurer<sup>‡</sup>, Sergey V. Shestakov<sup>¶</sup>,  
Reinhold G. Herrmann<sup>‡</sup>, Himadri B. Pakrasi<sup>¶</sup>, and Itzhak Ohad<sup>‡‡§§</sup>

From the <sup>‡</sup>Botanisches Institute, Ludwig-Maximilians-University, Menzinger Str. 67, D-80638 München, Germany, the <sup>¶</sup>Department of Biology, Washington University, St. Louis, Missouri 63130, the <sup>||</sup>Department of Genetics, Moscow State University, Moscow 119899, Russia, and the <sup>‡‡</sup>Department of Biological Chemistry, The Hebrew University of Jerusalem, Jerusalem 91904, Israel

The photosystem II (PSII) complex of photosynthetic oxygen evolving membranes comprises a number of small proteins whose functions remain unknown. Here we report that the low molecular weight protein encoded by the *psbJ* gene is an intrinsic component of the PSII complex. Fluorescence kinetics, oxygen flash yield, and thermoluminescence measurements indicate that inactivation of the *psbJ* gene in *Synechocystis* 6803 cells and tobacco chloroplasts lowers PSII-mediated oxygen evolution activity and increases the lifetime of the reduced primary acceptor  $Q_A^-$  (more than a 100-fold in the tobacco  $\Delta psbJ$  mutant). The decay of the oxidized  $S_{2,3}$  states of the oxygen-evolving complex is considerably accelerated, and the oscillations of the  $Q_B^-/S_{2,3}$  recombination with the number of exciting flashes are damped. Thus, PSII can be assembled in the absence of PsbJ. However, the forward electron flow from  $Q_A^-$  to plastoquinone and back electron flow to the oxidized Mn cluster of the donor side are deregulated in the absence of PsbJ, thereby affecting the efficiency of PSII electron flow following the charge separation process.

The photosystem II complex (PSII)<sup>1</sup> of the thylakoid membrane is involved in the photochemical process resulting in water oxidation, oxygen evolution, and reduction of plastoquinone. PSII comprises a core complex formed by a few proteins binding the ligands required for primary photochemistry and electron transfer and the chlorophyll-binding antennae as well as a number of low molecular weight proteins whose functions have not yet been identified (1, 2). The process of light-induced charge separation and reduction of a quinone acceptor, similar to that performed by PSII, is also carried out by photosynthetic

bacteria that do not evolve oxygen and perform cyclic electron flow around the bacterial photochemical reaction center (RC) (3). While in both cases a small number of proteins binding the appropriate ligands are capable of light-driven charge separation and electron transfer, the linear electron flow performed by PSII using water as a source of electrons is more complex and may require regulatory steps that are not demanded by the cyclic electron flow of the bacterial RC. Thus it is plausible that proteins of PSII, besides those acting as energy-transferring antennae (4), may play some role in the regulation of the forward and backward electron flow within the PSII core complex. Recombination of the primary charge-separated pair due to back electron flow may lead to the generation of singlet oxygen considered to be the cause of PSII oxidative stress and turnover of its core proteins (5, 6). Indeed cytochrome  $b_{559}$ , an essential component of the PSII core complex is considered to play such a regulatory role by diverting electrons from the reducing side of PSII and channeling them to the oxidized donor side of the complex (7). This protein is missing from the RC complex of purple anoxygenic photosynthetic bacteria. The *psbE* and *psbF* genes encoding the  $\alpha$  and  $\beta$  subunits of cytochrome  $b_{559}$ , respectively, are with a few exceptions members of the *psbEFLJ* operon (8), suggesting that the products of the remaining genes of this operon, *psbL* and *psbJ*, may be involved in regulatory processes as well. *Synechocystis* 6803 cells in which *psbL* has been inactivated do not have a functional PSII despite the fact that components of the complex are present (9). Recently, inactivation of each of the genes in the *psbEFLJ* gene cluster in tobacco plastids was successfully performed.<sup>2</sup> The  $\Delta psbL$  tobacco mutant lacked functional PSII, while the  $\Delta psbJ$  mutant had impaired but detectable PSII activity. In the present study, functional analysis of both the cyanobacterial and tobacco  $\Delta psbJ$ -less mutants demonstrate that the *psbJ*-encoded protein plays a regulatory role in the electron flow within the PSII complex.

### MATERIALS AND METHODS

**Transformation of Tobacco Chloroplasts**—A 3,675-base pair *SalI*/*Eco47III* fragment of the plastid chromosome of *Nicotiana tabacum* cv. Petit Havana, nucleotide positions 65,310–68,985 (GenBank<sup>TM</sup>/EBI data bank accession number Z00044), containing the entire *psbEFLJ* operon was inserted into *SmaI*/*SalI* double-digested Bluescript SK-vector and cloned in *E. coli* XL1-Blue (Stratagene, Heidelberg, Germany). A terminator-less, chimeric *aadA* gene conferring spectinomycin resistance to chloroplast mutants (10) was fused to the homologous *psbA* promoter. This construct was integrated into *psbJ* modified by site-specific mutagenesis to generate an artificial *PstI* restriction site at the 5'-end of the structural gene using the synthetic oligonucleotide

<sup>2</sup> R. E. Regel, N. B. Ivleva, H. Zer, J. Meurer, S. V. Shestakov, R. G. Herrmann, H. B. Pakrasi, and I. Ohad, manuscript in preparation.

\* This work was supported in part by Deutsche Forschungsgemeinschaft Grants SFB 184 (to R. G. H. and I. O.) and the Fonds der Chemischen Industrie (to R. G. H.), the National Institutes of Health Grant GM 45797 and the U. S. Department of Energy (to H. B. P.), the German-Israeli foundation (GIF), and the Israeli Science Foundation administered by the Israel Academy of Sciences (to I. O.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Both authors contributed equally to this work.

\*\* Supported in part by a training grant from the Monsanto Co. to the Plant Biology Program at Washington Univ.

§§ To whom correspondence should be addressed. Tel.: 972-2-658-5423; Fax: 972-2-658-6448; Email: ohad@vms.huji.ac.il.

<sup>1</sup> The abbreviations used are: PSII, photosystem II; RC, reaction center; DCBQ, 2,6-dichloro-*p*-benzoquinone; Chl, chlorophyll; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; TL, thermoluminescence signal; LHCI, light harvesting protein complex associated with PSII; PSI, photosystem I; PQ, plastoquinone; PQH<sub>2</sub>, plastoquinol.

5'-GGGGTAAATGGCCGATACTGCAGGAAGGATTCCTC-3'. This operon-conform insertion interrupts the gene N-terminally between the two threonine residues at positions 4 and 5. The *aadA* cassette with terminator was also placed into an *EcoRV* site located 3' in the untranslated region of the operon (nucleotide position 66,053). This site turned out to be insertion neutral. These plants are referred to throughout this article as RV5 control plants. Transformation of *N. tabacum* chloroplasts, selection, and culture of transformed material and assessment of the homoplasmic state were performed essentially as described in De Santis-Maciossek *et al.* (11). The material was grown for 4–5 weeks in 12 h dark/12 h light cycles at 10  $\mu\text{mol}$  of photons  $\text{m}^{-2} \text{s}^{-1}$  and 25 °C on agar-solidified medium (12) containing 2% sucrose and 500  $\mu\text{g}/\text{ml}$  spectinomycin under selective conditions. As opposed to the leaves of the wild type control plants that continued to grow and maintained PSII activity most of the  $\Delta\text{psbJ}$  leaves gradually lost photosynthetic activity and senesced within several weeks. The material chosen for analysis was homoplasmic and fairly green when grown in dim light. A detailed description of the *psbE* operon mutants will be presented elsewhere.<sup>2</sup>

**Cyanobacterial Cells—***Synechocystis* 6803 wild type, the strain in which *psbJ* was inactivated (13) (T203 mutant; referred to throughout this article as the *psbJ*-stop mutant) and the CP47 histidine-tagged HT-3 cells (14) were grown at 30 °C in BG11 medium (15). For the growth of *psbJ*-stop cells, the medium was supplemented with 5  $\mu\text{g}$   $\text{ml}^{-1}$  chloramphenicol. The liquid cultures were grown under continuous fluorescent white light (50  $\mu\text{mol}$  of photons  $\text{m}^{-2} \text{s}^{-1}$ ) with vigorous bubbling with filtered room air.

**Measurement of Photosynthetic Activity: Oxygen Evolution—**PSII activity of cyanobacterial cells or isolated tobacco thylakoids using DCBQ and potassium ferricyanide as artificial electron acceptors were measured using a Clark-type oxygen electrode as described (16, 17).

**Flash-induced Oxygen Yield—**Flash-induced oxygen yield of cyanobacterial cells was measured after dark adaptation for 7 min using a home-built, bare-platinum, Joliot-type electrode as described (18). For the estimation of the rates of decay of the  $S_2$  or  $S_3$  states, the cells were exposed to 1 or 2 single turnover exciting flashes (EG&G, LS-11340–4, 6  $\mu\text{s}$  duration), respectively, followed by additional flashes after increasing time intervals, and the oxygen yield at the third flash was measured.

**Chlorophyll *a* Fluorescence Induction Kinetics—**Chlorophyll *a* fluorescence induction kinetics of the tobacco wild type and mutant plant leaves was measured using a Pulse Modulated Fluorometer (PAM-101, Waltz, Germany) (19). Leaves were dark-adapted for 15–30 min prior to the measurements. The light intensity of the modulated measuring beam (1, 6 kHz) was 0.5  $\mu\text{mol}$  of photons  $\text{m}^{-2} \text{s}^{-1}$ . The intensity of the saturating light pulse (1 s) used for the measurement of the  $F_m$  level at the leaf surface was 3,000  $\mu\text{mol}$  of photons  $\text{m}^{-2} \text{s}^{-1}$ . Red actinic light (650 nm, 60  $\mu\text{mol}$  of photons  $\text{m}^{-2} \text{s}^{-1}$ ) was used for the measurements of fluorescence quenching. Variable fluorescence of tobacco leaves ( $F_v/F_m$ ), was calculated as  $(F_m - F_0)/F_m$ . Fluorescence quenching was measured as  $F_v'/F_v$  in which  $F_v'$  is defined as the variable fluorescence emitted by the leaves after exposure to actinic light (650 nm, 30–50  $\mu\text{mol}$  of photons  $\text{m}^{-2} \text{s}^{-1}$ ). Fluorescence emission from cyanobacterial cells was measured as described by Meentemeyer *et al.* (18) with a FL-100 dual-modulation kinetic fluorometer (Photo Systems International, Brno, Czech Republic) (20) using 3  $\mu\text{s}$  exciting flashes, thus permitting a better resolution of the cyanobacterial fluorescence kinetics. Cells suspensions in BG-11 medium (2  $\mu\text{g}$  Chl  $\text{ml}^{-1}$ ) were dark-adapted for 2 min prior to the fluorescence measurements.

**Thylakoid Membranes—**Thylakoid membranes were obtained by centrifugation of tobacco leaf homogenates according to Zer *et al.* (21). For preparation of thylakoids, 8–12 leaves that exhibited variable fluorescence in the range of 0.3–0.4 for the  $\Delta\text{psbJ}$  mutant and >0.60 for the wild type plants were collected from four to six individual plants.

**PSII Preparations—**PSII preparations exhibiting high rates of oxygen evolution were obtained from the CP47 histidine-tagged HT-3 *Synechocystis* 6803 mutant cells as described by Bricker *et al.*, (14).

**Thermoluminescence Measurements—**Charge recombination of  $Q_B^-/S_{2,3}$  states of the manganese electron donor cluster or of the  $Q_A^-/S_2$  state in presence of DCMU (10  $\mu\text{M}$ ) were measured as described (5, 22). The emission due to the recombination of a mixed population of  $Q_B^-/S_2$  or  $Q_B^-/S_3$  states generated by a single flash in the absence of DCMU is not resolved into two distinct TL signals (23). For TL signal measurements, tobacco thylakoids (5–10  $\mu\text{g}$  Chl  $\text{ml}^{-1}$ ) were suspended in buffer containing 20 mM Tris-HCl, pH 7.4, 5 mM  $\text{MgCl}_2$ , 20 mM NaCl, 200 mM phenylmethylsulfonyl fluoride, 1 mM benzamide, and 100 mM sorbitol. For TL measurements of cyanobacteria, cells were suspended in fresh BG11 medium (200  $\mu\text{g}$  Chl  $\text{ml}^{-1}$ ) and dark-adapted at room tempera-



**FIG. 1. Localization of PsbJ in the PSII-complex of *Synechocystis* 6803 thylakoid membranes.** Thylakoids membrane proteins of the wild type and *psbJ*-stop cells and a purified His-tagged PSII-core preparation from *Synechocystis* 6803 HT-3 strain were resolved by denaturing SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane, and immunodecorated with anti-PsbJ antibodies. A, membranes from wild type cells; B, membranes from *psbJ*-stop cells; C, His-tagged PSII core preparation from HT-3 cells. Lanes A and B had 15  $\mu\text{g}$  and lane C had 7  $\mu\text{g}$  of Chl-containing material.

ture for 2 h followed by storage on ice until use (24). Samples (10–15  $\mu\text{g}$  of Chl) were dark adapted on the thermoluminescence apparatus stage at 20 °C for 3 min and then cooled to –10 °C and excited by one or multiple single-turnover light flashes (Xenon arc discharge lamp, EG&G, 0.05 microfarads capacitor, charged at 1000 v) as indicated followed by rapid cooling to –30 °C. The samples were then heated at a constant rate (0.5 °C  $\text{s}^{-1}$ ), and light emission *versus* temperature was measured by photon counting. The decay time course of the  $Q_B^-/S_{2,3}$  charge separated state was measured at 20 °C. The samples were exposed to two consecutive exciting flashes (0.3 Hz) and further incubated in darkness for times as indicated before rapid freezing to –30 °C and activating the heating and photon counting process. Freezing time from 20 °C to 0 °C was < 3 s, and the data were corrected for this value.

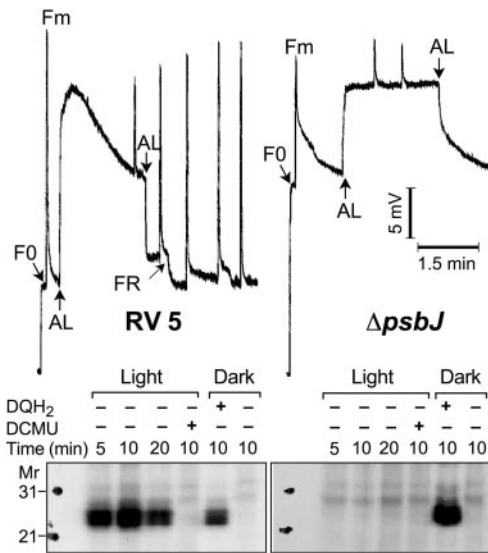
**Phosphorylation Assay—**Redox-dependent LHCII phosphorylation of wild type and  $\Delta\text{psbJ}$  tobacco thylakoids was carried out as described (21). The phosphorylation mixture (100  $\mu\text{l}$ ) contained 50 mM Tris-HCl, pH 8.0, 10 mM NaCl, 10 mM  $\text{MgCl}_2$ , 0.2 mM ATP (25  $\mu\text{Ci}$  [ $\gamma$ - $^{32}\text{P}$ ]ATP), 10 mM NaF and thylakoid membranes equivalent to 20  $\mu\text{g}$  of chlorophyll. All incubations were carried out in Eppendorf tubes at 25 °C and in darkness or under room light as indicated. Activation of the protein kinase in darkness by duroquinol (1 mM) was as described (21). Phosphorylation was terminated by addition of denaturing SDS/polyacrylamide gel electrophoresis sample buffer, and the membrane polypeptides were resolved as described (25). The extent of phosphorylation was assayed by autoradiography.

**Measurements of Chlorophyll, PSI Activity, and Immunodetection of PsbJ—**Chlorophyll concentration was measured in methanolic extracts of cyanobacterial cells (26) and in 80% acetone extracts for tobacco thylakoids. Photosystem I activity in intact leaves was estimated by measuring absorption changes related to  $P_{700}$  oxidoreduction using the PAM-101-dedicated PSI attachment. Immunodetection of PsbJ on Western blots was carried out as described using monospecific polyclonal  $\alpha$ -PsbJ antibodies (13).

## RESULTS

**Localization of PsbJ in PSII—**We have previously shown that PsbJ is a membrane-associated protein in *Synechocystis* 6803 (13). To ascertain that the changes in PSII activity described in this work are due to the loss of a PSII-intrinsic component, we have tested the presence of the PsbJ protein in oxygen evolving PSII preparations. The data presented in Fig. 1 demonstrate that PsbJ is a component of the PSII complex in *Synechocystis* 6803 and is absent in the *psbJ*-stop cyanobacterial mutant cells. PsbJ was also detected in the PSII crystals of *Synechococcus elongatus* (2). Since this gene is present in the chloroplast genome of higher plants, and its inactivation drastically affects PSII electron flow in tobacco thylakoids (see below), we infer that PsbJ is also a component of the higher plant PSII.

**Loss of PsbJ Affects the Oxygen Evolution Activity and the Kinetics of  $Q_A^-$  Oxidation by Forward Electron Flow—**Oxygen evolution was impaired in both the  $\Delta\text{psbJ}$  and *psbJ*-stop mutants. The  $\Delta\text{psbJ}$  thylakoids evolved 20–40  $\mu\text{mol}$  of oxygen  $\text{Chl}^{-1} \text{h}^{-1}$  (mg) when measured with DCBQ as an electron acceptor as compared with 120  $\mu\text{mol}$  of oxygen (mg)  $\text{Chl}^{-1} \text{h}^{-1}$  for the control thylakoids. However, the impairment of oxygen evolution activity on a chlorophyll basis in the cyanobacterial *psbJ*-stop mutant was less pronounced allowing slow autotrophic growth and compatible at least partially with a lower PSII/PSI ratio in these cells (13). The  $F_v/F_m$  values were in the range of 0.74–0.76 for the control RV5 tobacco leaves and 0.37 ( $\pm$  0.1) for the  $\Delta\text{psbJ}$  mutant. The variations in the oxygen evolution activity and of the  $F_v/F_m$  fluorescence parameter of the tobacco mutant were due to increasing senescence with the time of growth of the plants in the plastic containers. The  $\Delta\text{psbJ}$  mutant of tobacco exhibited  $P_{700}$  oxidoreduction activity as measured by the PAM-101 attachment. However, the rate of  $P_{700}$  reduction in the mutant was considerably slower as compared with that of the control plants indicating that the reduction of the PQ pool is impaired



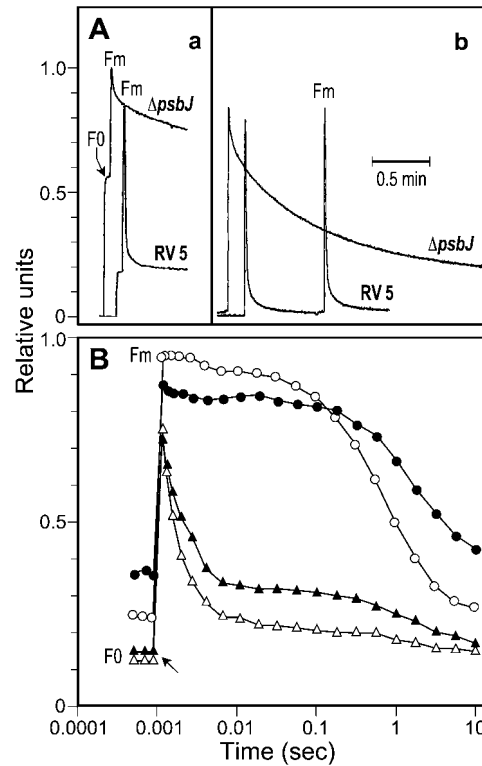
**FIG. 2. Fluorescence kinetics, fluorescence quenching and LHCII phosphorylation.** Upper panel, fluorescence kinetics of tobacco control RV5 (left side traces) and  $\Delta psbJ$  mutant leaves (right side traces) indicating absence of fluorescence quenching and slow oxidation of  $Q_A^-$  in the mutant leaves. Lower panel, LHCII phosphorylation in tobacco control and  $\Delta psbJ$  thylakoids; upward and downward arrows, saturating light pulse on/off, respectively; AL, actinic light; FR, far red light (710 nm).

in the mutant plants (not shown). Fluorescence quenching ( $F_v'/F_v$ ) in the control tobacco leaves after 2–3 min exposure to red actinic light was about 0.3. LHCII phosphorylation in the RV5 thylakoids was light activated and inhibited by DCMU. The  $\Delta psbJ$  mutant did not exhibit fluorescence quenching activity and the redox-dependent LHCII phosphorylation in isolated thylakoids (21) was not activated by light (Fig. 2). However, redox-dependent protein kinase activity could be elicited in the  $\Delta psbJ$  thylakoids by addition of duroquinol. These results can be explained by a low rate of PQ reduction in the mutant. The ratio  $PQH_2/PQ$  induced by white light illumination used in the phosphorylation assay could be low due to the activation of PSI and plastoquinol oxidation by ambient oxygen and thus may not be sufficient to activate the redox-dependent LHCII kinase. Addition of far red light (710 nm,  $20 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) slightly accelerated the oxidation of  $Q_A^-$  in the wild type (Fig. 2) as well as in the  $\Delta psbJ$  leaves (not shown) indicating that quinone/quinol exchange at the  $Q_B$  site occurs also in the mutant.

In both the tobacco and cyanobacterial mutants, the oxidation of  $Q_A^-$  as measured by fluorescence kinetics was slower as compared with that of the wild type (Figs. 2 and 3). The oxidation of  $Q_A^-$  in the tobacco  $\Delta psbJ$  plants exhibits an initial relatively fast phase accounting in various leaves from 10–40% of the signal followed by an extremely slow phase ( $t_{1/2}$  ranging between 20–60 s) (Figs. 2 and 3A). The fluorescence decay in the absence of DCMU is due mostly to forward electron flow from  $Q_A^-$  to  $Q_B$ . The above results indicate that this path of electron flow is significantly hindered in the mutant. Inhibition of electron flow to  $Q_B$  by DCMU and acceptance of electrons from  $Q_A^-$  by DCBQ indicates that binding of ligands at the  $Q_B$  site and thus,  $PQ/PQH_2$  exchange is not affected and can not explain the above observed changes in the fluorescence decay. One should note that DCBQ bound at the  $Q_B$  site accepts electrons from  $Q_A^-$ . Since the rate of electron flow to DCBQ in the tobacco mutant thylakoids was significantly slower than that in the wild type thylakoids, it is reasonable to conclude that the mutation affects the donation of electrons from  $Q_A^-$  to the natural  $Q_B$  quinone acceptor as it does to the artificial electron acceptor, DCBQ.

Measurements of the decay time of  $Q_A^-$  in the cyanobacterial *psbJ*-stop cells showed a slower rate as compared with that of the wild type cells. However, the difference between the mutant and the wild type cells was significantly smaller than that in the tobacco plants and could not be accurately resolved by the PAM 101 fluorometer. This difference was, however, clearly resolved when the oxidation of  $Q_A^-$  was measured using a dual modulation kinetic fluorometer that has a time resolution in the range of 10  $\mu\text{s}$ . The oxidation of  $Q_A^-$  was about 40% slower in the cyanobacterial mutant cells as compared with that of the wild type cells (Fig. 3B).

#### Alteration of Back Electron Flow in the *PsbJ*-less PSII of Cyanobac-



**FIG. 3. Fluorescence decay kinetics in tobacco leaves (A) and *Synechocystis* 6803 cells (B).** Note the high level of  $F_0$  in the  $\Delta psbJ$  leaves in A, a; A, b, traces normalized to the  $F_0$  level demonstrating the slow fluorescence decay in the  $\Delta psbJ$  tobacco mutant; B, triangles, traces in the absence of DCMU; circles, traces in the presence of DCMU; open and closed symbols, wild type and *psbJ*-stop cells, respectively.

*terial Cells*—Back electron flow from the acceptor to the donor side of PSII was measured by several methods. The decay of  $Q_A^-$  by back electron flow in the presence of DCMU as estimated by fluorescence kinetics showed a doubling of the oxidation time in the *psbJ*-stop cyanobacterial cells ( $t_{1/2}$  1.2 s as compared with 0.5 s for the wild type cells) (Fig. 3B). The most striking difference between the *psbJ*-stop and wild type strains of *Synechocystis* 6803 cells was found in the decay of the  $S_3$  state *via* back electron flow from the acceptor side as evidenced by measurements of oxygen flash-yield ( $t_{1/2}$  about 3 s and 60 s, respectively) (Fig. 4A). The decay of the TL signal resulting from the charge recombination of the  $Q_B^-/S_{2,3}$  state was also significantly faster in the *psbJ*-stop mutant as compared with the wild type cells (5.6 s and 22 s, respectively) (Fig. 4B). The maximal photon emission due to charge recombination of  $Q_B^-/S_{2,3}$  was shifted to a slightly lower temperature for the cyanobacterial mutant as compared with the wild type cells (26 °C and 30 °C, respectively), while the temperature of the maximal emission resulting from the recombination of  $Q_A^-/S_2$  in the presence of DCMU was slightly increased in the mutant relative to the wild type cells (11 °C and 9 °C, respectively). This is in agreement with the increase in the  $Q_A^-$  oxidation time in the presence of DCMU by the same route as measured by fluorescence kinetics (Fig. 3B). The intensity of both the  $Q_A$  and  $Q_B$  thermoluminescence emission bands of the *psbJ*-stop cells on a Chl basis was significantly lower as compared with that of the wild type cells (Fig. 5A) and amounts to about 11 and 25% of the intensity of wild type TL signals for the  $Q_B^-/S_{2,3}$  and the  $Q_A^-/S_2$  recombination, respectively. The oscillations of the TL signals intensity with the number of exciting flashes showed a pattern of maximal emission following excitation by two and six consecutive flashes for the mutant and wild type cyanobacterial cells. However, the signal from the mutant was slightly damped (Fig. 5C).

*Alteration of the Back Electron Flow in the *PsbJ*-less PSII of Tobacco Thylakoids*—The TL signal resulting from the recombination of the  $Q_B^-/S_{2,3}$  states was downshifted by about 10 °C in the  $\Delta psbJ$  tobacco thylakoids relative to that of the control RV5 membranes, while the emission maximum due to the recombination of  $Q_A^-/S_2$  state in the presence of DCMU was upshifted by about 4 °C (Fig. 5B). The TL signals intensity of the tobacco  $\Delta psbJ$  mutant did not oscillate with the number of exciting flashes (Fig. 5D). The decay of the  $Q_B^-/S_{2,3}$  states at

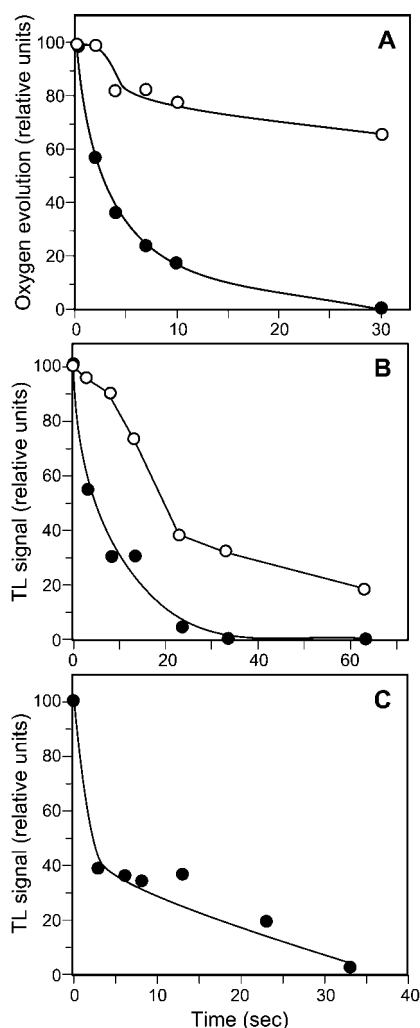


FIG. 4. Decay of the S states due to back electron flow. A, decay of the  $S_3$  state in *Synechocystis* 6803 cells measured by oxygen flash yield; B, decay of the  $Q_B^-/S_{2,3}$  states measured by TL in *Synechocystis* 6803 thylakoids; C, decay of the  $Q_B^-/S_{2,3}$  states measured by TL in tobacco  $\Delta psbJ$  mutant thylakoids. Open circles, wild type; closed circles, mutant, respectively. The data shown are typical of several experiments. The variation between different cell or thylakoid batches was within 15%.

20 °C as estimated by TL measurements in the thylakoids of  $\Delta psbJ$  tobacco mutant was basically similar to that of the *psbJ*-stop cyanobacterial mutant. However, the shape of the curve clearly indicates presence of two major phases, an initial fast phase accounting for about 60% of the signal with a  $t_{1/2}$  roughly estimated to be less than 2 s and a second slow phase extending up to 33 s with an estimated  $t_{1/2}$  of about 18 s (Fig. 4B). The decay of the above TL signal in thylakoids of the RV5 tobacco thylakoids exhibited a  $t_{1/2}$  of about 25 s (not shown) as also reported for higher plant thylakoids (27). The slow phase of the TL signal decay in the  $\Delta psbJ$  tobacco mutant could explain the slow phase of fluorescence decay due to the oxidation of  $Q_A^-$  in the absence of DCMU observed in intact leaves (Figs. 2 and 3).

#### DISCUSSION

The findings presented in this work demonstrate that functional PSII can be assembled in absence of PsbJ. However, the electron flow within the PsbJ-less PSII complex is deregulated and characterized by a slower oxidation of the primary electron acceptor quinone  $Q_A^-$  via forward electron flow and a faster reduction of the oxidized S states. While the changes of fluorescence decay, oxygen flash yield and charge recombination detected by TL, are common to both, the cyanobacterial and tobacco mutants, the extents of these changes are more accen-

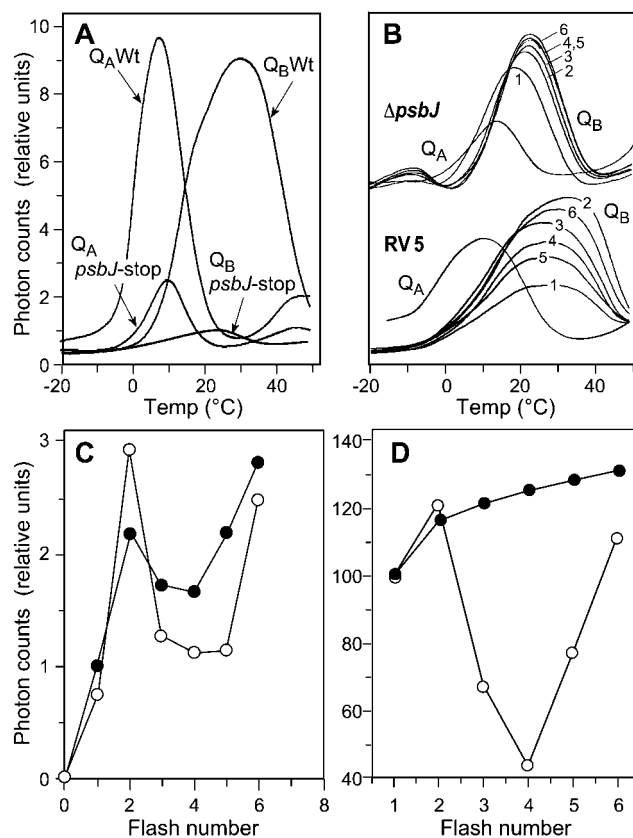


FIG. 5. Thermoluminescence signals from *Synechocystis* 6803 cells and tobacco thylakoids. A, thermoluminescence signals due to recombination of  $Q_A^-/S_2$  and  $Q_B^-/S_{2,3}$  states ( $Q_A$  and  $Q_B$  traces, respectively) in *Synechocystis* 6803. B, thermoluminescence signal due to recombination of  $Q_A^-/S_2$  ( $Q_A$  trace) and oscillation of the  $Q_B^-/S_{2,3}$  signal with flash number ( $Q_B$  traces) in tobacco thylakoids. The shifts in the emission peak temperature as a function of the flash number is due to alternating changes in  $S_2/S_3$  ratio with the number of flashes from 1 to 6. C and D, oscillation of TL intensities with the number of flashes calculated from data similar to those shown in B for *Synechocystis* cells (C) and tobacco thylakoids (D), respectively. The data are representative of several experiments, and the variation between different batches of cyanobacterial cultures or tobacco thylakoids preparations was within 15%. Wt, wild type *Synechocystis* 6803 cells; RV5, control tobacco thylakoids.

tuated in the tobacco mutant as compared with the cyanobacterial mutant.

**Electron Flow within PSII**—The major difference between the *psbJ*-stop cyanobacterial and  $\Delta psbJ$  tobacco mutants appears in the oxidation of  $Q_A^-$  via forward electron flow to  $Q_B$ . This process is slower by about 40% in the *psbJ*-stop cells as compared with a slowing by almost 2 orders of magnitude in the tobacco  $\Delta psbJ$  mutant. This large increase in the life time of  $Q_A^-$  explains the suppression of the TL signal oscillations with the number of exciting flashes in the tobacco thylakoids as compared with the small TL oscillation damping effect observed in the cyanobacterial cells (Fig. 5). Thus the  $Q_A^-/Q_B$  electron flow and the two-electron gating at the  $Q_B$  site that is responsible for the oscillation of the back reaction are hindered. This interpretation is in agreement with the high  $F_0$  level of the  $\Delta psbJ$  mutant as measured with the PAM-101 fluorometer and the temperature downshift of about 10–12 °C in the TL emission peak resulting from  $Q_B^-/S_{2,3}$  charge recombination. The TL signal measured in the absence of DCMU in the mutants is actually a result of the recombination of  $Q_A^-$ ,  $Q_B^-/S_2$ ,  $S_3$  mixed populations in which the ratio  $Q_A^-/Q_B^-$  is higher than in the wild type thylakoids. Back electron flow from  $Q_A^-$  occurs at a temperature lower than that of the  $Q_B^-/S_{2,3}$  recombination (5).

Since the TL signal due to charge recombination in the absence of DCMU does not resolve the contributions of back electron flow from  $Q_A^-$  from that from  $Q_B^-$ , the photon emission peak is broader and is shifted to a temperature lower than that of the wild type thylakoids. Accordingly, the decay of the TL signal resulting from this recombination is also considerably faster (Fig. 5).

The back electron flow due to the  $Q_B^-/S_{2,3}$  charge recombination in the cyanobacterial *psbJ*-stop cells as measured by thermoluminescence shows a small downshift of the TL signal emission temperature ( $\sim 4-5^\circ\text{C}$ ) and a marginal upshift of the TL signal ( $< 2^\circ\text{C}$ ) for the recombination of the  $Q_A^-/S_2$  state in presence of DCMU. This is in agreement with the small degree of damping of the TL signal oscillation with the number of exciting flashes that maintains a normal 2/6 pattern (5, 27) in the wild type cells. However, the extent of the TL signal on a chlorophyll basis as compared with that of the wild type cyanobacterial cells is extremely low. Considering that the PSII/PSI ratio in this mutant cells on a chlorophyll basis is about 0.4 (13), one would expect a proportional loss of the TL signal intensity. The lower TL emission of the *psbJ*-stop cells as compared with the wild type strain observed in this work indicates that in a large fraction of the PSII population back electron flow and reduction of the  $S_{2,3}$  states occurs via a different route that may not involve the recombination of the primary charge-separated state and the related photon emission. Back electron flow measured by the oxygen flash yield technique in the *psbJ*-stop cells in the absence of DCMU indeed shows a very fast decay of the  $S_3$  and  $S_2$  states as compared with that in the wild type cells with a lifetime compatible with that measured by the TL method in the absence of DCMU. These results indicate that the electron donor for this process could be  $Q_A^-$ . However, the pathway of electron transfer possibly involves an intermediate electron transfer carrier reducing  $S_2$  and  $S_3$ , yet avoiding the recombination of the primary charge-separated pair. The pathway for this route of back electron flow could include cytochrome  $b_{559}$  directly (28) or in combination with  $\text{Chl}_z$  (29) and possibly via a carotenoid cation radical (6, 30).

**Oxygen Evolution**—The interpretation of the experimental results presented above is supported by the oxygen evolution measurements. The rate of electron flow in the thylakoids of the tobacco mutant using DCBQ as an electron acceptor that binds to the  $Q_B$  site and accepts electrons from  $Q_A^-$  was significantly lower than that of the wild type thylakoids. The reduction of PQ was even slower as indicated by the extremely slow oxidation of  $Q_A^-$  in the absence of artificial electron acceptors. These results possibly indicate that the loss of PsbJ induces a change in the redox potential of the  $Q_A^-/Q_A$  pair relative to DCBQ bound at the  $Q_B$  site that is more negative than that relative to PQ bound at this site. Binding of ligands at the  $Q_B$  site has been reported to affect the redox potential of  $Q_A$  (31). The fact that the  $Q_A^-/Q_A$  potential can be significantly altered following interactions of ligands with the  $Q_B$  site of the D1 protein in proximity to the  $Q_A$  site points toward the possibility that the  $Q_A$  potential may be influenced by interactions between the D2 protein harboring the  $Q_A$  binding site and adjacent proteins.

The possibility that the absence of PsbJ induces changes in the  $Q_B$  site affecting the rate of PQ/PQH<sub>2</sub> turnover at the  $Q_B$  site could also explain the slow oxidation of  $Q_A^-$  in the absence of DCMU. However, changes in the PQ turnover at the  $Q_B$  site can not explain the observed changes in the lifetime of the S states, nor those of the shifts in the TL signal temperature for the recombination of both the  $Q_B^-/S_{2,3}$  and  $Q_A^-/S_2$  states.

The question arises as to the difference in the extent of PSII

activity induced by the loss of PsbJ between tobacco and cyanobacteria. *A priori* in view of the similarity of the activity of PSII between higher plants and cyanobacteria, one would not expect such a difference. However, one should consider that PSII in plants differs in terms of its polypeptide composition from that of cyanobacteria. The 17- and 23-kDa oxygen evolution-enhancing polypeptides as well as the tightly bound light-harvesting complexes encoded by the *lhcb3,4* genes are present in plant PSII but are absent from the cyanobacterial PSII. It is possible that the PSII complexes differ in their plasticity at both structural and functional levels. The degree of PSII alteration by inactivation of PSII related genes between cyanobacteria and higher plants has been reported to differ, possibly indicating a less stringent control of PSII activity in the cyanobacterial cells as compared with the eukaryotic PSII (28, 32). This may be related to the fact that in the cyanobacterial thylakoids the redox state of the PQ pool, and thus back electron flow, is affected by both the light-driven as well as the respiratory pathways.

**Mechanism**—The phenomena described in this work may be explained assuming that the  $Q_A$  potential of the mutants is more positive relative to that of the wild type PSII. The upshift of the TL peak emission temperature by about  $4^\circ\text{C}$  for the recombination of  $Q_A^-/S_2$  state in the presence of DCMU and the shorter lifetime of the S states are in agreement with this suggestion. Changes observed in these parameters are considered as being related to changes in the redox potentials of the above electron donors/acceptors (29, 33–35). The experimental evidence in support of the above-offered hypothesis is circumstantial. Additional experiments including measurements of the  $Q_A^-/Q_A$  potential of the mutants need to be performed to further elucidate the mechanism of the observed changes in the electron flow within PSII induced by the absence of PsbJ. The highly hydrophobic PsbJ protein with a single transmembrane helix is not known to participate in the binding of any electron carrier ligand. Recently it was proposed that the interaction between cytochrome  $b_{559}$  and  $Q_A$  is involved in the setting of the redox potential of both these PSII components (30, 36). The absence of PsbJ may affect the interaction of additional proteins in the PSII complex that may be involved in this process. Alternatively, PsbJ may interact directly with and affect the proteins involved in the electron transfer process. A close interaction between cytochrome  $b_{559}$  and the D2 protein that forms the  $Q_A$  binding niche has been suggested (6, 35, 37) and supported by the recently reported x-ray crystal structure of PSII at  $3.8\text{ \AA}$  resolution (2). It is possible that an association of PsbJ with the above-mentioned two proteins regulates the interaction between these electron carriers, thus affecting the electron flow path. Among the unidentified transmembrane  $\alpha$ -helices resolved by x-ray analysis of the *S. elongatus* PSII (2), one is located in close vicinity to cytochrome  $b_{559}$  and may belong to PsbJ. Further work using cross-linking methods may provide support for this hypothesis. While it is established that cytochrome  $b_{559}$  may assume a high or low potential in response to factors affecting the protein environment (29, 31), no such change has been reported to occur for the  $Q_A$  site of functional PSII. Possibly, the H subunit of the bacterial RC may play a similar role in the regulation of electron flow via  $Q_A$  (38). Based on the results presented in this work it is conceivable that PsbJ functions in conjunction with the other components encoded by the *psbEFLJ* operon in the regulation of the electron traffic within PSII and thus plays an important role in the efficiency of forward electron flow following the charge separation process.

**Acknowledgments**—We thank Dr. Yasuhiro Kashino for the cyanobacterial PSII preparation, Dr. Terry Bricker for the HT-3 strain of

*Synechocystis* 6803, and Martina Reymers for her skilful technical assistance.

## REFERENCES

1. Debus, R. J. (2000) *Met. Ions. Biol. Syst.* **37**, 657–711
2. Zouni, A., Witt, H.-T., Kern, J., Fromme, P., Krauss, N., Saenger, W., and Orth, P. (2001) *Nature* **409**, 739–743
3. Okamura, M. Y., Paddock, M. L., Graige, M. S., and Feher, G. (2000) *Biochim. Biophys. Acta* **1458**, 148–163
4. Barber, J. (1998) *Biochim. Biophys. Acta* **1365**, 269–277
5. Keren, N., Gong, H., and Ohad, I. (1995) *J. Biol. Chem.* **270**, 806–814
6. Hanley, J., Deligiannakis, Y., Pascal, A., Faller, P., and Rutherford, A. W. (1999) *Biochemistry* **38**, 8189–8195
7. Whitmarsh, J., and Pakrasi, H. B. (1996) *Oxygenic Photosynthesis: The Light Reactions* (Ort, D. R., and Yocum, C. F., eds), pp. 249–264, Kluwer Academic Publishers, Dordrecht, The Netherlands
8. Mor, T. S., Ohad, I., Hirschberg, J., and Pakrasi, H. B. (1995) *Mol. Gen. Genet.* **246**, 600–604
9. Anbudurai, P. R., and Pakrasi, H. B. (1993) *Z Naturforsch. [C]* **48**, 267–274
10. Koop, H. U., Steinmuller, K., Wagner, H., Rossler, C., Eibl, C., and Sacher, L. (1996) *Planta* **199**, 193–201
11. De Santis-MacJossek, G., Kofer, W., Bock, A., Schoch, S., Maier, R. M., Wanner, G., Rudiger, W., Koop, H. U., and Herrmann, R. G. (1999) *Plant J.* **18**, 477–489
12. Murashige, T., and Skoog, F. A. (1962) *Physiol. Plant.* **15**, 473–479
13. Lind, L. K., Shukla, V. K., Nyhus, K. J., and Pakrasi, H. B. (1993) *J. Biol. Chem.* **268**, 1575–1579
14. Bricker, T. M., Morvant, J., Masri, N., Sutton, H. M., and Frankel, L. K. (1998) *Biochim. Biophys. Acta* **1409**, 50–57
15. Allen, M. M. (1968) *J. Phycol.* **4**, 1–4
16. Mannan, R. M., and Pakrasi, H. B. (1993) *Plant Physiol.* **103**, 971–977
17. Tal, S., Keren, N., Hirschberg, J., and Ohad, I. (1999) *J. Photochem. Photobiol. B Biol.* **48**, 120–126
18. Meetam, M., Keren, N., Ohad, I., and Pakrasi, H. B. (1999) *Plant Physiol.* **121**, 1267–1272
19. Schreiber, U., and Krieger, A. (1996) *FEBS Lett.* **397**, 131–135
20. Trtilek, M., Kramer, D. M., Koblizek, M., and Nedbal, L. (1997) *J. Luminescence* **72**, 597–599
21. Zer, H., Vink, M., Keren, N., Dilly-Hartwig, H. G., Paulsen, H., Herrmann, R. G., Andersson, B., and Ohad, I. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 8277–8282
22. Zer, H., Prasil, O., and Ohad, I. (1994) *J. Biol. Chem.* **269**, 17670–17676
23. Rutherford, A. W., Crofts, A. R., and Inoue, Y. (1982) *Biochim. Biophys. Acta* **682**, 457–465
24. Carpenter, S. D., Ohad, I., and Vermaas, W. F. (1993) *Biochim. Biophys. Acta* **1144**, 204–212
25. Laemmli, U. K. (1970) *Nature* **227**, 680–685
26. Lichtenthaler, H. K. (1987) *Methods Enzymol.* **148**, 350–383
27. Keren, N., Berg, A., van Kan, P. J. M., Levanon, H., and Ohad, I. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 1579–1584
28. Barber, J., and De Las Rivas, J. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 10942–10946
29. Stewart, D. H., and Brudvig, G. W. (1998) *Biochim. Biophys. Acta* **1367**, 63–87
30. Vrettos, J. S., Stewart, D. H., dePaula, J., and Brudvig, G. W. (1999) *J. Phys. Chem.* **103**, 6403–6406
31. Krieger, A., and Rutherford, A. W. (1997) *Biochim. Biophys. Acta* **1319**, 91–98
32. Pakrasi, H. B. (1995) *Annu. Rev. Genet.* **29**, 755–776
33. Vass, I., and Govindjee. (1996) *Photosynth. Res.* **48**, 117–126
34. Vass, I., Horwath, G., Herczeg, T., and Demeter, S. (1981) *Biochim. Biophys. Acta* **634**, 140–152
35. Minagawa, J., Narusaka, Y., Inoue, Y., and Satoh, K. (1999) *Biochemistry* **38**, 770–775
36. Mizusawa, N., Yamashita, T., and Miyao, M. (1999) *Biochim. Biophys. Acta.* **1410**, 273–286
37. Shukla, V. K., Stanbekova, G. E., Shestakov, S. V., and Pakrasi, H. B. (1992) *Mol. Microbiol.* **6**, 947–956
38. Debus, R. J., Feher, G., and Okamura, M. Y. (1986) *Biochemistry* **25**, 2276–2287