Influence of the Redox Potential of the Primary Quinone Electron Acceptor on Photoinhibition in Photosystem II*

Christian Fufezan†*‡, Christine M. Gross‡*, Martin Sjödin†, A. William Rutherford§, Anja Krieger-Liszka†*, and Diana Kirilovsky‡

From the †Service de Bioénergétique, Département de Biologie, Joliot Curie, CNRS unite de recherche associé 2096, Commissariat à l’Energie Atomique Saclay, Gif-sur-Yvette 91191, France and the ‡Institut für Biologie II, Biochemie der Pflanzen, Universität Freiburg, Schänzlestrasse 1, Freiburg 79104, Germany

We report the characterization of the effects of the A249S mutation located within the binding pocket of the primary quinone electron acceptor, QA, in the D2 subunit of photosystem II in Thermosynechococcus elongatus. This mutation shifts the redox potential of QA by ~−60 mV. This mutant provides an opportunity to test the hypothesis, proposed earlier from herbicide-induced redox effects, that photoinhibition (light-induced damage of the photosynthetic apparatus) is modulated by the potential of QA. Thus the influence of the redox potential of QA on photoinhibition was investigated in vivo and in vitro. Compared with the wild-type, the A249S mutant showed an accelerated photoinhibition and an increase in singlet oxygen production. Measurements of thermoluminescence and of the fluorescence yield decay kinetics indicated that the charge-separated state involving QA was destabilized in the A249S mutant. These findings support the hypothesis that a decrease in the redox potential of QA causes an increase in singlet oxygen-mediated photoinhibition by favoring the back-reaction route that involves formation of the reaction center chlorophyll triplet. The kinetics of charge recombination are interpreted in terms of a dynamic structural heterogeneity in photosystem II that results in high and low potential forms of QA. The effect of the A249S mutation seems to reflect a shift in the structural equilibrium favoring the low potential form.

Photosystem II (PSII), the water/plastoquinone oxidoreductase, uses light energy to extract four electrons from water, producing oxygen (1–4). Each electron is transferred over a chain of redox cofactors to the terminal plastoquinone Qb, which accepts two electrons and two protons (5). The efficiency of PSII in converting light energy into a charge-separated state is remarkably high (5). The univalent photochemistry must interface with the four-electron chemistry occurring at the electron donor side with the two-electron chemistry at the acceptor side. This is achieved by different mechanisms. On the donor side, the so-called oxygen-evolving complex (OEC) accumulates four redox equivalents before it extracts four electrons from two water molecules. Accumulation is necessary, because the energy needed to extract the electrons one by one from water requires more driving force than visible light provides (4). During the catalytic cycle, the OEC exists in different oxidation states. These are designated S0, S1, S2, S3, and S4, where the subscript indicates the number of accumulated oxidation equivalents. The cycle is completed when the S4 state performs a 4-electron oxidation of water, with the OEC being returned to the most reduced of the so-called S states, S0. The OEC consists of four manganese ions, one calcium ion (3, 4, 6, 7), and probably one chloride ion (Ref. 8, but see also Ref. 9).

On the electron acceptor side, electron transfer involves two plastoquinones, the primary and secondary quinone acceptors (QA and QB). Although both are plastoquinones, their physical and chemical properties differ. QA is tightly bound and acts as a two-electron acceptor in bacterial reaction centers; QB is exchangeable with the plastoquinone pool in the membrane (12, 13). QA undergoes no observable protonation events during its lifetime (5). Qb, on the other hand, acts as a two-electron and two-proton acceptor with a stable semiquinone intermediate, Qb− (5, 10, 11). Although the semiquinone state, Qb−, is tightly bound, its quinone and quinol forms are exchangeable with the quinone pool in the membrane (12, 13).

PSII is known to be susceptible to damage under high light intensities, a process called photoinhibition. Under physiological conditions the D1 protein of PSII has the highest turnover rate of all the proteins in the cell (14). This is usually assumed to be the result of photodamage mediated by PSII photochemistry (Ref. 15; however, see also Ref. 16). It has been proposed that during charge recombination reactions, chlorophyll triplet-mediated, singlet oxygen (1O2) is produced and that this is the species responsible for the PSII damage (17–22).

Conditions that favor charge recombination reactions (e.g. either high light intensities or excitation by single turnover flashes (23)) induce a decrease in PSII activity. This is due to the fact that under photoinhibitory conditions the D1 protein is

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† To whom correspondence should be addressed (present address): Physics Dept., J419, City College of New York, 160 Convent Ave, New York, NY 10031. Tel.: 212-650-6872; Fax: 212-650-6940; E-mail: christian@fufezan.net.

‡ The abbreviations used are: PSII, photosystem II; P, primary electron donor; QA, secondary quinone acceptor; Qb, primary quinone acceptor; Qa, redox states of the charge accumulating part of the water-oxidizing enzyme; Pheo, phycophytin (primary electron acceptor in PSII); PbPheo, bacterial pheophytin (primary electron acceptor in bacterial reaction centers); TEMPO, 2,2,6,6-tetramethylpiperidine; TEMPO, 2,2,6,6-tetramethylpiperidine-1-oxyl; WT, T. elongatus strain that lacks the second gene coding for the D2 protein (ΔpsbD2 strain); OEC, oxygen-evolving complex; Chl, chlorophyll; MES, 4-morpholinethanesulfonic acid; DCMU, 13-(3,4-dichlorophenyl)-1,1-dimethyleurea.

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damaged and degraded faster than it is replaced (for review see Refs. 24 and 25).

Different classes of herbicides induce different rates of photoinhibition. Phenolic herbicides stimulate the process of photoinhibition relative to urea-type herbicides, and under some in vitro conditions urea herbicides appear to afford some protection against photoinhibition relative to the herbicide-free material (26–30). This was explained by the fact that the binding of herbicides influences the midpoint potential of the redox couple $Q_A/Q_A^-$ (31). The urea type herbicide DCMU raises the midpoint potential of $Q_A$ by 50 mV, whereas the phenolic herbicide bromoxynil lowers it by 45 mV (31). The relationship between the redox potential of $Q_A$ and photoinhibition was explained in terms of the following model (15, 31). The back-reaction between the radical pair $[P^+ Q_A^-]$ and the ground state $P$ occurs via two radical pathways: an indirect pathway involving the repopulation of the $[P^+ Pheo^-]$ radical pair and a direct pathway involving direct tunneling to the ground state (Refs. 15, 31, 32; see also Ref. 33). The yields of both pathways are modulated by the difference in the free energy between the $[P^+ Q_A^-]$ and $[P^+ Pheo^-]$ radical pairs (Refs. 15, 31, 32; see also Ref. 33). If this gap is small, i.e., in the presence of a phenolic herbicide, the back-reaction occurs with a higher yield via the indirect pathway forming a significant yield of $3P$. This $3P$ can then react with $O_2$ forming the toxic singlet oxygen species, $1O_2$. When the energy gap is increased, i.e., in the presence of DCMU, the probability of forming the $[P^+ Pheo^-]$ state is lowered, and the chances are higher that the $[P^+ Q_A^-]$ radical pair decays via the direct and safe route. EPR-spin trapping experiments supported this model, because the yield of $1O_2$ formation was significantly lower in the presence of DCMU than in the presence of bromoxynil (22).

To study further the influence of the redox potential of $Q_A$ on the rate of photoinhibition we attempted to change its midpoint potential without using herbicides, thereby avoiding any potential difficulties from possible secondary effects of these chemicals. Thus we created mutants with a single point mutation in the $Q_A$ binding pocket in Thermosynechococcus elongatus, the thermophilic cyanobacterium from which the structure of PSII was obtained by crystallographic methods (7, 34). One such mutant was used to study the influence of the midpoint potential of $Q_A$ on charge recombination reactions and on photoinhibition. The results provide independent support (in the absence of herbicides) for the relationship between the redox potential and photoinhibition, and hence for the charge recombination model. In addition this study gives new mechanistic insights concerning the presence of a dynamic structural heterogeneity around the $Q_A$ site and how this influences the redox potential.

**MATERIALS AND METHODS**

**Strain and Standard Culture Conditions—**T. elongatus cells were grown in a rotary shaker (120 rpm) at 45 °C under continuous illumination from fluorescent white lamps giving an intensity of ~80 microeinsteins m$^{-2}$ s$^{-1}$. Cells were grown in a DTN micro Einstein medium (35) in an enriched CO$_2$ atmosphere and in a 3-liter Erlenmeyer flask (1.5-liter culture). Cells were grown in the presence of spectinomycin (25 μg ml$^{-1}$) and streptomycin (10 μg ml$^{-1}$). Mutant strains were grown additionally in the presence of the antibiotic kanamycin (40 μg ml$^{-1}$).

**Plasmids, in Vitro Mutagenesis, and Transformation of T. elongatus Cells—**Site-directed mutations were performed using the Qiagen site-directed mutagenesis kit on the plasmid pUC18–43H (provided by Dr. M. Sugiuira, see Ref. 36). The pUC18-CP43H plasmid consists of the coding region for $psbD1$ and $psbC$ (gene product CP43), with a sequence coding for His$_6$ tag and the kanamycin resistance cassette. The construct is surrounded by the non-coding region of the $psbD1$ and $psbC$ to provide a higher target sequence for the homologous recombination. The primers used to insert the point mutation in the pUC18-CP43H plasmid that resulted in the A249S mutation were 5’-GAA GAC ACC TAC TCG ATG GTG ACG AGT AAC CCG TTT TGG AGC CAA-3’ and its complementary strand. The A249S mutation was introduced by changing GCG to AGT (bold). Additionally, a conservative point mutation was introduced into the third position of the codon for Arg-251, changing CGT to CGG (underlined). Together these modifications introduced a new AgeI restriction site, which was used for fast screening of potential successfully transformed clones. Amplification of the mutated pUC18-H43 plasmid was done in Escherichia coli. The plasmid was purified and used to transform a strain of T. elongatus lacking the $psbD2$ gene by electroporation (provided by Dr. M. Sugiuira, see Ref. 36). The $psbD2$ gene codes the second copy of D2. We will further refer to the $\Delta psbD2$ strain as WT’. The transformation was done as described in Kirilovsky et al. (37). Genomic DNA was isolated from T. elongatus cells essentially as described in Cai and Wolk (38).

**PSII Core Complexes Preparation—**PSII core complexes were prepared as described by Roncel et al. (35) with the modifications described in Kirilovsky et al. (37) except that the detergent concentration for the washing steps of the column was 0.03%. The preparations used in this work had an oxygen evolution activity of 2.2–3 mmol of O$_2$ (mg of Chl)$^{-1}$ h$^{-1}$.

**Oxygen Evolution Measurements—**Oxygen evolution was measured at 25 °C by polarography using a Clark-type oxygen electrode with saturating white light. Oxygen evolution in cells (10 μg of Chl ml$^{-1}$), thylakoid membranes (10 μg of Chl ml$^{-1}$), and PSII core complexes (5 μg of Chl ml$^{-1}$) was measured in 40 mM MES, pH 6.5, 15 mM MgCl$_2$, 15 mM CaCl$_2$, 10% (v/v) glycerol, 1 mM glycinebetaine, and in the presence of 0.5 mM 2,6-dichloro-p-benzoquinone (dissolved in ethanol) as electron acceptor.

**Thermoluminescence Measurements—**Thermoluminescence was measured with a customized apparatus as described in a previous study (39). Cells were centrifuged and resuspended in a 40 mM MES (pH 6.5) buffer containing 15 mM MgCl$_2$, 15 mM CaCl$_2$, 10% glycerol, at a Chl concentration of 100 μg ml$^{-1}$. After dark adaptation (15 min) the cells were frozen to ~80 °C in darkness and were maintained at that temperature for 30 min. After slow thawing, the cell suspension was incubated on ice in darkness. PSII complexes were measured at a concentration of 35 μg of Chl ml$^{-1}$. The particles were kept in complete darkness (for at least 30 min). To measure the thermoluminescence originating from the $S_2$–$Q_{B\ast}$ charge recombination (B-Band) (40) the samples were incubated for 5 min in the dark.
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at 40 °C and then flashed once or twice at 1 °C. To measure the thermoluminescence originating from the $S_2Q_{A^-}$ recombination (Q-Band), the dark-adapted samples were flashed once and incubated with either 20 μM DCMU or 100 μM bromoxynil or without any additions at 40 °C for 3 min. After this pre-treatment T. elongatus cells were cooled down to −5 °C and flashed. Although one flash should be sufficient to introduce the charge-separated state in most of the reaction centers two flashes were needed in T. elongatus to obtain the largest Q band. This may reflect a large concentration of $Q_{B^-}$ in darkness (41) that results in incomplete DCMU binding under the conditions of the experiment. For luminescence detection the samples were warmed at a constant rate (0.5 °C s$^{-1}$) from 1 °C or −5 °C to 80 °C. When TL was measured in PSII cores the sample concentration was adjusted based on the amplitude of stable EPR signal $\text{TyrD}^\ast$.

Singlet Oxygen Measurements—Spin-trapping assays were performed in PSII particles at a concentration of 10 μg of Chl ml$^{-1}$. Samples were illuminated for up to 60 min with 1.5 millieinstein m$^{-2}$ s$^{-1}$ red light in the presence of 10 mM 2,2,6,6-tetramethylpiperidine (TEMP), 4% (v/v) methanol, and buffer (40 mM MES, 15 mM MgCl$_2$, 15 mM CaCl$_2$, pH 6.5). If $\text{O}_2$ is produced, it can react with TEMP and forms thereby the stable nitroxyl radical TEMPO, the amount of which is linearly correlated to the unsaturated EPR signal. To compare the rate of singlet oxygen production in different preparations from different batches, the signal sizes were normalized. This was done by calculating a factor between the size of the dark signal and the signal after 30- and 60-min illumination, respectively. In different batches, the signal sizes were normalized. This was done by calculating a factor between the size of the dark signal and the signal after 30- and 60-min illumination, respectively. In control experiments, the EPR signal present immediately after mixing the spin trap with PSII did not change its amplitude upon incubation in the dark for 60 min. EPR signals in dark controls result from impurities in the spin trap. X-band spectra were recorded at room temperature with a Bruker ESP 200 spectrometer at 9.7-GHz microwave frequency, 63-milliwatt microwave power, 100-kHz modulation frequency, and a modulation amplitude of 2 Gauss.

Photoinhibition Assays in Vitro and in Vivo—Photoinhibition experiments in vivo were performed with a Chl concentration of 10 μg of Chl ml$^{-1}$. Cells were incubated at 30 °C in the presence of 34 μg ml$^{-1}$ chloramphenicol in a glass tube (3-cm diameter) while being stirred and with illumination from 3 Atralux spots of 150 watts (1 millieinstein m$^{-2}$ s$^{-1}$ each lamp). The effective light intensity encountered by each cell was diminished by approximately half due to the vessel used. For the photoinhibition experiments in vitro, PSII complexes were illuminated in a modulated fluorimeter (PAM; Walz, Effeltrich, Germany) adapted to a Hansatech oxygen electrode as previously described (42). All the experiments were carried out in a stirred cuvette of 1-cm diameter (32 °C) at a chlorophyll concentration of 3 μg of Chl ml$^{-1}$. The decrease of the yield of chlorophyll fluorescence was monitored in the modulated fluorimeter during illumination with white light at 1.5 millieinstein m$^{-2}$ s$^{-1}$. The decrease of the yield of chlorophyll fluorescence was measured with a PAM 101 fluorometer (Walz, Effeltrich, Germany) using a weak measuring light of 1.6 kHz.

Measurement of the $S_2Q_{A^-}$ Charge Recombination—The decrease of the maximal fluorescence yield in the seconds to minutes time range during photoinhibition was measured by using a modulated fluorimeter (PAM, Walz). The decay of the fluorescence yield due to $S_2Q_{A^-}$ charge recombination was measured using a double modulation fluorimeter (PSI Instruments, Brno, Czech Republic) in the 1-ms to 30-s time range in whole cells (5 μg of Chl ml$^{-1}$), in the presence of 20 μM DCMU (dissolved in ethanol). To follow the reoxidation of $Q_{A^-}$, a weak non-actinic probe flash was used. A small fraction of photons (~12% in closed centers) of the probing flash is re-emitted as fluorescence. The variable fluorescence yield was measured with the same fluorimeter.

Measurements of the decay of $S_2Q_{A^-}$ are not straightforward when measured as the decay of the fluorescence yield (44–46). It is known that the trapping efficiency of open reaction centers increases with the amount of closed reaction centers in the vicinity (44, 45). Consequently, a fluorescence probe pulse does not detect closed and opened reaction centers equally, but instead open centers are favored as a function of the fraction of closed centers according to Equation 1, where $c$ is the fraction of closed centers, $F(c)$ is the measured fluorescence yield, $J$ is the Joliot antenna connectivity factor (44), and $J + 1$ is the average number of visited reaction centers per exciton. From Equation 1 it is easily deducible that 1) the observed decay kinetics of the fluorescence yield (i.e. $F(c(t))$) is non-linearly dependent on the re-oxidation kinetics of $Q_{A^-}$ (i.e. $c(t)$) if $J > 0$ and 2) the $F(c(t))$ is equal to the kinetics of the re-oxidation of $Q_{A^-}$ (i.e. $c(t)$) if $J = 0$. It was shown by Cuni et al. (46) that, if a weak enough excitation flash is applied, which induces charge separation in only a small proportion of the reaction centers (<15%), no correction is needed, just as if $J = 0$. This was always the case under our conditions.

Under the conditions used, even with the lowest usable probe light intensity, the fluorescence yield probe flashes were only found to be non-actinic when a fluorescence probe pulse frequency of 5 Hz or less was used. However, with measuring pulses spaced at 200-ms intervals it is not possible to obtain a good measurement of the fast phase of decay. Therefore we had to use a different measuring protocol. In this protocol each sample was measured under four conditions resulting in four
sets of experiments. Each set had a time base shifted by 50 ms up to 2 s and the same time base from that point forward. The kinetic traces (usually three) of each set were averaged, and the sets were merged together resulting in: (a) a higher temporal resolution for the fast kinetic phase and (b) a better signal-to-noise ratio for the slow kinetic phase. This protocol, however, introduces an uneven weighting of the data points, because each of the points from 0 to 2 s after the flash originate from only one set, whereas each point from 2 to 30 s represents the average of all four sets. To compensate for this effect the points from 2 to 30 s were weighted appropriately, i.e. their weighting was doubled in the fit, because the number of experiments relates to the signal-to-noise ratio in a square root manner. All fits were done using the Levenberg-Marquardt algorithm (profit V. 6.0.6).

RESULTS

Site-directed Mutagenesis—The D2-A249S mutant was created in photosystem II of *T. elongatus.* The mutation was introduced by site-directed mutagenesis in a plasmid that contained a kanamycin-resistance cassette, the *psbD1* gene, the *psbC* gene containing a C-terminal His tag and the non-coding regions flanking the genes (36). The construct was introduced by electroporation into a strain in which the second copy of the gene coding for D2 (*psbD2*) was interrupted by an antibiotic cassette (*H9004* *psbD2* strain) (47). In the following we refer to the *H9004* *psbD2* strain as the “wild type” (WT). Complete segregation and homoplasticity of the mutant was tested by PCR analysis (data not shown). Because the double recombination could occur between the antibiotic cassette and the point mutation, not all kanamycin-resistant mutants contained the site-directed mutation. To select the mutant carrying the proper modified bases, amplified PCR fragments of 2.1 kb, obtained using the 5’-AGC GGA ACG GGG ATG GTT TGA CAT CCT CGA-3’ and 5’-GAT CTT GTT GAG GTC AAG ACC ATT GGG GC-3’ oligonucleotides as primers, were digested by *Agel* and *HinfI*. The digestion resulted in four and five fragments for the WT and the mutant, respectively. The two heavy fragments were sufficient to determine whether the mutation was present. Fig. 1 shows the 2% agarose gel loaded with the 1-kb marker (lane 1), left) and the digested PCR fragments of genomic DNA obtained from the wild type (WT) (lane 2) and the mutants A249S (lane 3) of *T. elongatus.* The digestion was done with *Agel* and *HinfI*. The mutation inserted also a new restriction site that leads to the difference in the restriction polymorphism. Shown is the region from 3000 to 500 bp.

![Figure 1. Agarose gel (2%) loaded with 1-kb marker (lane 1, left) and the digested PCR fragments of genomic DNA obtained from the wild type (WT) (lane 2) and the mutants A249S (lane 3) of *T. elongatus.*](image)

**FIGURE 1.** Agarose gel (2%) loaded with 1-kb marker (lane 1, left) and the digested PCR fragments of genomic DNA obtained from the wild type (WT) (lane 2) and the mutants A249S (lane 3) of *T. elongatus.* The digestion was done with *Agel* and *HinfI*. The mutation inserted also a new restriction site that leads to the difference in the restriction polymorphism. Shown is the region from 3000 to 500 bp.

**FIGURE 2.** Photoinhibition assays of *T. elongatus* in cells and in the presence of chloramphenicol (a) and isolated PSII particles without any addition (b), in the presence of 20 μM DCMU (c) and in the presence of 100 μM bromoxynil (d). Relative activity of PSII in vivo was measured by oxygen production in WT cells (circles) and in A249S mutant cells (triangles). In vitro (b–d) PSII activity was measured as a function of relative variable fluorescence in the WT (black trace) and A249S mutant (gray line).
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the wild type, or in general in the absence of a point mutation, shows the two fragments with sizes of 1.05 and 0.75 kb (lane 2), whereas the digestion of the A249S mutant PCR fragment results in a 1.05- and 0.6-kb fragment (lane 3). Sequencing of the PCR-amplified fragment confirmed the presence of the desired mutation (data not shown). These PCR analyses were regularly repeated to verify the genotype in the cells used for phenotype characterization. These mutants grow photoautotrophically with an identical growth rate to the wild type (data not shown).

**Influence of the A249S Mutation on Photoinhibition**—The sensitivity to strong light of the WT' and the A249S mutant was tested using whole cells and isolated PSII complexes. WT' and A249S mutant cells were exposed to high light intensities in the presence of chloramphenicol to prevent de novo protein synthesis to avoid recovery. Fig. 2a shows that the loss of oxygen-evolving activity in the A249S mutant (triangles) and in WT' (circles) cells. The loss of oxygen-evolving activity was faster in the A249S mutant compared with the WT', indicating that the mutant is more sensitive to photoinhibition. As expected, the loss of the variable fluorescence yield (Fig. 4). The variable fluorescence of PSII increased when

**Measurement of the Redox Potential of QA**—The midpoint potential of the redox couple QA/QA" was estimated by following chemically induced changes in the variable fluorescence yield (Fig. 4). The variable fluorescence of PSII increased when

**FIGURE 3. Singlet oxygen trapping assays of isolated PSII particles.** Typical EPR spectra of TEMPO are shown. TEMPO is generated as a stable radical after TEMP reacts with singlet oxygen. The control experiment (no PSII) (trace 1), the WT' (trace 2), and the A249S mutant (trace 3) after a 30-min illumination are shown.

**FIGURE 4. Redox titration curves of QA in purified PSII particles measured as fluorescence yield.** a, WT'; b, A249S mutant. Filled circles, oxidative titrations; open circles, reductive titrations. The curves shown represent one electron Nernst curves fitted to the data points. The midpoint potentials of the Nernst curves are 84 ± 24 mV (a) and 27 ± 20 mV (b) for the WT and the A249S mutant, respectively.

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$\text{QA}$ was reduced. This was achieved by adding dithionite. The oxidation of $\text{QA}^-$ seen as a decrease in fluorescence yield was achieved by the addition of ferricyanide. Even though it was difficult to obtain precise values for titration curves, a significant difference between the mutant and the WT' could be determined. The fitted one-electron Nernst curves showed a redox potential of $84 \pm 24$ mV for the WT' (Fig. 4a) and $27 \pm 20$ mV for the A249S mutant (Fig. 4b). The titration curves showed a shift of almost $60$ mV towards a more negative value for the redox potential of the A249S mutant in comparison to the WT'.

The value of the $E_{\text{m}}$ of $\text{QA}$ in WT' cells of $T. \text{elongatus}$ ($84 \pm 24$ mV) is $\approx 160$ mV more positive than that measured for $\text{QA}$ in PSII from spinach by the same technique (Krieger et al. 43). This could reflect a species difference and could be related to the fact that $T. \text{elongatus}$ is a thermophile. Another possible explanation however is that the sample could have lost its Mn cluster (and hence its Ca$^{2+}$) during the titration. In plant PSII a loss of the Ca$^{2+}$ leads to an up-shift of $150$ mV (43). These phenomena need to be looked at in detail and will be the subject of a future study. For the present work what is important is the demonstration that the mutation leads to down-shift in the potential of $\text{QA}$.

**Influence of the A249S Mutation on $S_2\text{QA}^-$ and $S_2\text{QB}^-$ Recombination:**

We used thermoluminescence to estimate whether the energy levels of the charge-separated states $S_2\text{QA}^-$ and $S_2\text{QB}^-$ were affected by the A249S mutation in comparison to the WT'. The energetics and the reduction state of photosystem II can easily be investigated by thermoluminescence (40). In this method, a charge-separated state is formed by a given number of single turnover flashes. The sample is then rapidly cooled to stabilize the charge-separated state. Light emission is recorded during the subsequent controlled heating of the sample. During the heating procedure, the system is given a defined thermal free energy that allows the charge-separated state to recombine if its activation energy is equal to or less than the thermal energy (for a review see e.g. Ref. 48).

Thermoluminescence data from cells were variable from one culture batch to another due to a difference in the dark-stable transmembrane electrochemical gradient, which induces a temperature down-shift of the B bands (49). Thus, the comparison between the mutants was done using cells that had been frozen and thawed. This treatment collapses the transmembrane proton gradient. In these treated cells the patterns of thermoluminescence data were reproducible, independent of the cell batch and similar to that obtained with cells treated with the ionophore nigericin (data not shown).

The thermoluminescence data are shown in Fig. 5 and Table 1. For clarity the WT' curves (circles) are shown with offset positioning. Fig. 5a shows a thermoluminescence curve obtained after two saturating flashes, the so-called B-band. This band is attributed to $S_2\text{QB}^-$ charge recombination (40). The peak maximum of the B bands obtained after two flashes was $54 \degree \text{C}$ and $60 \degree \text{C}$ in WT' (circles) and A249S mutant cells (triangles), respectively. A similar small up-shift of the peak maximum was observed after one excitation flash (Table 1), even though the emission was rather low. The weak band on the first flash is mainly ascribable to the high concentration of $\text{QB}^-$ present in the dark (41). Cells exhibit the so-called thermoluminescence Q-band, when the electron transfer between $\text{QA}$ and $\text{Q}_b$ is inhibited. This band is attributed to $S_2\text{QA}^-$ recombini-
nation (40). Fig. 5b shows the thermoluminescence bands of the WT’ (circles) and the A249S mutant (triangles) in vivo and in the presence of 20 μM DCMU. The peak temperature was at 27 °C in WT’ and 18 °C in A249S mutant cells.

Fig. 5 (c and d) shows the Q bands in isolated PSII complexes of the WT’ (circles) and of the A249S mutant (triangles) obtained in the presence of 20 μM DCMU or 100 μM bromoxynil, respectively. In the presence of DCMU (Fig. 5d) the thermoluminescence peak maxima were at 22 °C and 12 °C for the WT’ and the A249S mutant, respectively. In the presence of bromoxynil (Fig. 5d) the peak maxima for the Q-bands were at 10 °C and 5 °C for the WT’ and the A249S mutant, respectively. In addition to the S2QA− band, a small band was observed at ~65 °C. This so-called C-band is attributed to TyrD/QA− recombination, which is expected to occur in centers where S2QA− is present or where the water-splitting activity is non-functional (50).

Influence of the A249S Mutation on the Recombination Kinetics of S2QA− —The decay of the fluorescence yield after a saturating flash was used to monitor the re-oxidation of QA− by recombination with S2 in the presence of DCMU (which blocks electron transfer from QA− to Qb). Fig. 6 shows such a decay of the fluorescence yield (Φ) reflecting the S2QA− recombination at 30 °C obtained from the WT’ (circles) and from the A249S mutant (triangles). The kinetic trace was adequately described by the sum of two exponential decays with an offset (Equation 2),

$$\Phi(t) = A_0 + \sum_{i=1}^{2} A_i \cdot \exp(-k_i \cdot t)$$

where $A_0$ is the offset, $A_i$ is the amplitude of the $i$th exponential decay, and $k_i$ is its rate constant. The results of the mathematical descriptions of the kinetics trace at 30 °C are listed in Table 2. The fast ($i = 1$) and the slow ($i = 2$) exponential decays differ in the rate constant by approximately one order of magnitude. The difference between the kinetics seen with the A249S mutant and the WT’ were due to changes in the relative amplitudes of the fast and slow phase, whereas the rate constants of each phase remained similar (Table 2). In the A249S mutant the amplitude of the fast phase was larger than in the WT’ (47% versus 23%).

Fig. 7 shows the temperature dependence of the amplitudes of the fast phase (crosses) and the slow phase (diamonds) of the S2QA− decay in the wild-type (Fig. 7a) and in the A249S mutant (Fig. 7b). In the WT’ the slow phase dominates the recombination kinetics at low temperatures. The amplitude of the slow phase decreases with increasing temperature, and both phases participate equally at 50 °C. The amplitudes of the decay of the A249S mutant however also showed an interesting temperature dependence. Although at low temperatures the slow phase dominated as in the wild type, at higher temperature the switch to the faster phase was more marked, with it becoming dominant at the highest temperatures (>60%). The inversion point is at ~35 °C (Fig. 7b). The rate constants of the slow phase increased with the temperature in both strains.

The observed kinetic is a measurement of the equilibrium constant $K$, most probably between the [P7′ Pheo+] and [S2QA−] radical pairs. From its temperature dependence one can estimate the free energy gap between these radical pairs, provided that the decay rate of [P7′ Pheo+] to the ground state $P$, $k_{Pheo}$, is known (see below). However, the free enthalpy can be directly obtained from the van’t Hoff plot of $ln(k^a)$ versus $1/T$ or by fitting the data points to Equation 3,

$$k_{obs} = a \cdot \exp\left(-\frac{\Delta H^o \cdot i}{k_b T}\right)$$

where $k_{obs}$ is the observed rate constant and $\Delta H^o \cdot i$ is the free enthalpy for the fast ($i = 1$) or slow ($i = 2$) recombination phase/conformer and $k_b$ is the Boltzmann constant. Fig. 7 (c

### TABLE 1

| Thermoluminescence peak temperatures |
|--------------------------------------|
| Peak temperatures of the thermoluminescence bands shown in Fig. 2. |
|----------|----------|----------|
| B-Band, [S2QA−] | Q-Band, [S2QA−] |
| WT | A249S | WT | A249S |
|----------|----------|----------|----------|
| Cells | No addition (1 flash) | 57 | 62 | 27 | 18 |
| | No addition (2 flashes) | 54 | 60 | 22 | 12 |
| | + 20 μM DCMU | 27 | 18 | 10 | 5 |

### TABLE 2

| Fitted parameters for the fluorescence yield decay |
|--------------------------------------------------|
| Parameters ($A_i$ and $k_i$ (s−1)) were obtained after fitting a biexponential decay function to the fluorescence yield decay of the WT’ and the A249S mutant at 30 °C (Fig. 6). |
| $i = 0$ | $i = 1$ | $i = 2$ |
|----------|----------|----------|
| $A_i$ | $A_i$ | $A_i$ |
| $k_i$ | $k_i$ | $k_i$ |
| $\tau_{obs}$ | $\tau_{obs}$ | $\tau_{obs}$ |
|----------|----------|----------|
| WT’ | | |
| A249S | 0.2 | 47 | 1.87 | 0.37 | 51 | 0.28 | 2.47 |
| % | 76 | 0.26 | 2.65 |

**FIGURE 6. Fluorescence yield decay in T. elongatus cells due to QA− re-oxidation at 30 °C in the presence of 20 μM DCMU in the WT’ (circles) and in the A249S mutant (triangles).** Bi-exponential fits through the data points are shown, and their results are listed in Table 2. The residuals of the fits are shown below for the WT’ (dashed line) and the A249S mutant (solid line).
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FIGURE 7. Temperature dependence of the fitted parameters for the fluorescence yield decays in WT (left column, a and c) and in A249S mutant (right column, b and d) cells. Shown are the amplitudes (a and b) and the van’t Hoff plots (c and d) for the fast phase (crosses) and slow phase (diamonds).

FIGURE 8. Schematic view of the Qa binding pocket (pale gray) view from the top of PSII. For a clearer visualization the protein (darker gray) was sliced at the height of Qa. The alanine residue that was exchanged to serine makes up part of the Qa binding pocket. It is perpendicular to the top half of the Qa ring. The non-heme iron is shown.

and d) show the van’t Hoff plots (log $k_{obs}$ versus 1/$T$) for the wild type and the A249S mutant, respectively. The fast ($i = 1$) recombination phase is plotted as crosses, and the slow ($i = 2$) phase as diamonds. The lines through the data points represent the fit with Equation 3. The pre-exponential factor ($a$) is the ordinate and consists of the free entropy ($\Delta S^\circ$) and the decay rate of the intermediate $k_{pho\circ}$ ($a = k_{pho\circ} \exp(\Delta S^\circ/k_B)$).

The slow phase ($i = 2$, diamonds) of the WT (Fig. 7c) and the A249S mutant (Fig. 7d) show a standard free enthalpy ($\Delta H^\circ$) of 269 ± 66 meV ($a = 7566 ± 1900$) and 349 ± 74 meV ($a = 1.6 \times 10^5 ± 4.5 \times 10^5$), respectively. In contrast, the fast phases are difficult to resolve. The scatter in the values for the fast phase rate constants is due to their small amplitudes, especially at low temperatures.

**DISCUSSION**

Here we report a mutant in which the PSII reaction center protein D2 has been modified by a mutation in the binding site of the primary quinone electron acceptor Qα. This mutation D2-A249S shifts the redox potential of Qα to lower potential. The mutant has afforded us the opportunity to test how this change in redox potential of Qα influences 1) the charge recombination reactions and 2) the susceptibility of PSII to photodamage. The results strongly support the hypothesis that the back-reaction pathway and thence the amount of photodamage, is determined by the redox potential of Qα. Furthermore the data also support the idea that two conformers of PSII exist in a dynamic equilibrium in which the two states are distinguished by the potential of the Qα. The data presented here indicate that the mutation influences this equilibrium, favoring the low potential conformer.

The D2-A249S mutation was generated by site-directed mutagenesis in the psbD1 gene. The alanine residue, which was changed to serine, makes up part of the Qα binding pocket (Fig. 8) where it is perpendicular to the outer face of the Qα ring. Fig. 8 illustrates the Ala-249 residue and the pocket of Qα viewed from the top of PSII. For a clearer visualization the protein (darker gray) was sliced at the height of Qα, and the non-heme iron is shown as an additional point of reference.

The A249S mutation induced a down-shift in the midpoint potential of Qα by 60 mV. At the same time it resulted in altered charge recombination reactions. In vivo the S$_2$Qα$^-$ state showed a shorter halftime in the mutant (at 30 °C: 0.37 s (47%) and 2.47 s (51%)) than in the wild type (0.29 s (23%) and 2.65 s (76%)). A more detailed analysis of the recombination kinetics is presented below. Similarly the thermoluminescence arising mainly from S$_2$QB$^-$ charge recombination occurred at a lower temperature in the mutant (18 °C) than in the WT (27 °C). These effects on the charge recombination reactions are consistent with a situation in which the Qα/Qα$^-$ potential is down-shifted in the mutant by 57 mV. In the mutant the S$_2$Qα$^-$ recombination requires less energy, because the energy gap between [S$_2$Qα$^-$] and [P$^\circ$ Pheo$^\circ$] (and P$^\circ$) is smaller.

In contrast, the S$_2$Qb$^-$ recombination occurred at a higher temperature in the A249S mutant (60 °C) than in the wild type (54 °C). In principle the increased energy gap between [S$_2$Qb$^-$] and [P$^\circ$ Pheo$^\circ$] could occur from a small increase in the potential of Qb or from a decrease in the potential of Pheo. Such changes could occur as a secondary effect of the mutation.
The A249S mutant was more sensitive to light than was the wild type, and it produced more O₂ upon illumination than did the WT. These results provide strong independent support for the model of photoinhibition put forward by Krieger-Liszkay and Rutherford (15, 31, 32) in which the redox potential of QA modulates the back-reaction pathway and hence ³P-mediated singlet oxygen production.

This model (15, 31, 32) was based on the charge recombination occurring in purple bacterial reaction centers. In *Rhodobacter sphaeroides* the energy gap between [P⁺QA⁻] and [P⁺⁺BPho⁺⁻] is large enough (>400 mV) that the back-reaction is dominated by a slow direct recombination pathway, whereas in *Blastochloris viridis* the equivalent energy gap is smaller than 400 mV and a more rapid, activated back-reaction occurs via reformation of the [P⁺⁺BPho⁺⁻] radical pair (51–54).

Johnson *et al.* (32) invoked a comparable situation in PSII to explain the observations (32, 43, 55) that the potential of QA/QA⁻ was modulated by 150 mV depending on the presence of Ca²⁺ and the Mn²⁺ cluster. They showed that the potential of QA shifted from the high to the low potential form during the assembly of the oxygen-evolving enzyme (so-called photoactivation). This also demonstrated that the modulation of the redox potential of QA occurred under physiologically relevant conditions (32). It was suggested that the two types of pathway, occurring in the different bacterial species, corresponded to the two situations found in PSII: 1) a true back-reaction pathway in functional PSII and 2) a slower direct recombination pathway when Ca²⁺ (or both Ca²⁺ and Mn²⁺) were absent. Such drastic control of the charge recombination reactions was seen as being particularly important in PSII, because the true back-reaction pathway produces ³P (33, 56) at relatively high yield, and this state is known to be unquenched by carotenoid (57). Indeed it was shown that charge recombination in functional PSII gives a very high yield of photoinhibition presumably by ³P-mediated singlet oxygen formation (20).

Experimental support for the redox potential of QA⁻modulating photodamage came from studies of the PSII herbicide bromoxynil, where the redox potential of QA is shifted by ~50 mV, whereas in the presence of DCMU the redox potential is shifted by +45 mV (31). The herbicides thus modulate the energy gap between [P⁺⁺Pheo⁺] and [P⁺⁺⁺QA⁻] radical pairs, and consequently the yields of the charge recombination pathways are changed. When the redox potential of QA decreases and this energy gap is diminished (i.e. with bromoxynil), the indirect recombination via [P⁺⁺Pheo⁺] is expected to be favored resulting in increased formation of the ³P and hence increased production of singlet oxygen. Indeed bromoxynil (and related herbicides) were known to render plants and PSII hypersensitive to light compared with DCMU (26–30), and it was shown that this correlated to the production of singlet oxygen (22).

This model of the charge recombination pathways was also supported by work on mutants of *Synechocystis* with changes in the midpoint potential of Pheo (46, 48, 58) in which it was shown that the recombination rate depends on the free energy gap between the [P⁺⁺Pheo⁺] and the [S₂QA⁻] radical pairs. When the free energy gap was larger, direct electron transfer from QA⁻ to P⁺⁺ dominated.

The present study provides independent support for the role of the QA redox potential as a factor in controlling charge recombination photoinhibition. This is important because the earlier work was based on work using herbicides to modulate the QA potential and the herbicides could have been having additional effects relevant to photoinhibition. Having made this overall conclusion, which is an important result of the work, we shall now discuss further aspects of the results.

The destabilization of the charge-separated state [S₂QA⁻] in the A249S mutant was measured as acceleration in the fluorescence yield decay. At all measured temperatures between 20 °C and 50 °C, the decay of the fluorescence yield in the millisecond to second time range was faster in the A249S mutant than in the WT. The decay kinetics could be adequately fitted as a sum of two exponentials. Such biphasic recombination reactions have also been reported and studied in detail in the bacterial reaction center (54, 59–63) where the two phases have been distinguished spectroscopically (59, 60). The two phases were attributed to two different populations of reaction centers that exhibit different recombination rates (54, 59–63) and exist already prior to illumination (63). Although proteins are thought to occupy a large conformational space due to the Brownian motion (64), these two populations can be imagined as two local minima in the conformational space. Rappaport *et al.* observed two S₂QA⁻ recombination rates in *Synechocystis* (58) and *Chlamydomonas reinhardtii* (48) cells and proposed the existence of distinct forms of PSII reaction centers, as seen in the bacterial reaction centers (48). They interpreted their experiments with at least two populations that coexist prior to illumination (48). The two phases in the fluorescence yield decay kinetics in *T. elongatus* WT and A249S mutant cells reported here may also be assigned to two different populations of reaction centers as found in type II bacterial reaction centers (54, 59–63).

The two exponential phases differ in the rate of the fluorescence yield decay (i.e. recombination rates of S₂/3QA⁻) by approximately one order of magnitude. However, within our measuring sensitivity, the recombination rates themselves are not greatly influenced by the introduction of the A249S mutation. Instead the more rapid decay in the mutant is due to the increased proportion of the fast phase in the mutant compared with the wild type. This implies that the A249S mutation induced a change in the redox potential of QA, not because of a direct influence on the redox properties of the quinone but instead as a result of a change in the equilibrium between the two conformational states, favoring the state which decays more rapidly, i.e. the conformation in which QA⁻/QA⁻⁻ has a lower potential. Such a relationship between the redox potential and the ratio of conformers has been described before in the bacterial reaction center (65). Equally the influence of the herbicides on the Eₘ of QA was interpreted with a shift in the equilibrium between two conformational forms of the bacterial reaction center (65).

In the present work the binding of the phenolic herbicide bromoxynil destabilizes the S₂QA⁻ thermolaminescence band by the same amount in both the wild type and the mutant. Thus the mutation and the herbicide effects are additive. It is possible that both effects occur through an effect on the conformational
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equilibrium though we have no evidence for this in the present work.

The free enthalpies (\(\Delta H^*\)) given by the slope of the van’t Hoff plot for the \(S_2Q_A^*\) recombinations (Fig. 7, c and d) are much smaller than those reported for \(S_2Q_A^*\) for \(C. reinhardtii\) (625 meV (46) or 524 meV (66)). This might be due to the fact that in earlier work (46, 66) both values were obtained by describing the fluorescence decay yield with a single exponential. On the other hand, the authors obtained a free energy difference (\(\Delta G^*\)) between \([S_2Q_A^*]\) and \([P^+\text{Pheo}^*]\) of \(-600\) meV (58), which is in agreement with the \(\Delta G^*\) at 297 K obtained for the slow phase in the A2495 mutant and the WT’ assuming a \(k_{\text{pheo}}\) of \(1.6 \times 10^9\) s\(^{-1}\) as argued by Rappaport et al. (58). This difference in \(\Delta H^*\) could therefore be due to, for example, a different free entropy (\(\Delta S^*\)) contribution. An explanation for that could be that the reported free enthalpies are measured in mesophilic organisms, whereas our work was done on the thermophilic \(T. elongatus\). The difference could therefore be due to the higher stability of the proteins from \(T. elongatus\). One of the factors that determine protein stability is side-chain flexibility (67, 68). The more flexible the protein, the higher the entropic energy contribution, and the less stable it is. It could therefore be possible that the energetics in PSII from a thermophilic organism are less influenced by \(\Delta S^*\) due to greater conformational restrictions. That would result in a \(\Delta H^*\) closer to \(\Delta G^*\) in accordance with the finding here for PSII in \(T. elongatus\).

The results of this report suggest that PSII exists in at least two conformers that differ in their ability to withstand light-induced damage/photoinhibition due to a difference in the \(Q_A\) redox potential. By shifting the equilibrium between these two conformers the system has the possibility to adapt and to protect itself from photoinhibition. During the assembly of the Mn\(^{2+}\) complex, the situation where a \(Q_A\) modulation protection mechanism has been proposed (32), it may be worth looking for evidence of a shift in equilibrium of the conformers contributing to the redox effects observed.

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