Regulation of Photosynthetic Light Harvesting Involves Intrathylakoid Lumen pH Sensing by the PsbS Protein*

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The abbreviations used are: A, antheraxanthin; Chl, chlorophyll; DCCD, N,N'-dicyclohexylcarbodiimide; LED, light-emitting diode; NPQ, nonphotochemical quenching of chlorophyll fluorescence; PSII, photosystem II; qE, rapidly inducible, pH- and xanthophyll-dependent component of NPQ; V, violaxanthin; Z, zeaxanthin.
Summary: The biochemical, biophysical, and physiological properties of the PsbS protein were studied in relation to mutations of two symmetry-related, lumen-exposed glutamate residues, E122 and E226. These two glutamates are targets for protonation during lumen acidification in excess light. Mutation of PsbS did not affect xanthophyll cycle pigment conversion or pool size. Plants containing PsbS mutations of both glutamates did not have any rapidly inducible nonphotochemical quenching (qE) and had similar chlorophyll fluorescence lifetime components as npq4-1, a psbS deletion mutant. The double mutant also lacked a characteristic leaf absorbance change at 535 nm (ΔA₅₃₅), and PsbS from these plants did not bind dicyclohexylcarbodiimide (DCCD), a known inhibitor of qE. Mutation of only one of the glutamates had intermediate effects on qE, chlorophyll fluorescence lifetime component amplitudes, DCCD binding, and ΔA₅₃₅. Little if any differences were observed comparing the two single mutants, suggesting that the glutamates are chemically and functionally equivalent. Based on these results a bifacial model for the functional interaction of PsbS with photosystem II is proposed. Further, based on the extent of qE inhibition in the mutants, photochemical and nonphotochemical quenching processes of photosystem II were associated with distinct chlorophyll fluorescence lifetime distribution components.
INTRODUCTION

In conditions of excess light, photosynthetic light harvesting is regulated by a feedback de-excitation mechanism termed energy-dependent quenching (qE), which increases thermal dissipation of excess absorbed light energy in photosystem II (PSII). The qE mechanism is triggered by conditions that limit photosynthetic carbon fixation and result in increased acidification of the chloroplast thylakoid lumen (1-4). The thermal dissipation of excess excitation energy is most commonly measured and referred to as nonphotochemical quenching (NPQ) of PSII chlorophyll (Chl) a fluorescence. Although there are several components of NPQ, in higher plants qE can account for the major part of NPQ and is characterized by its relatively fast induction and relaxation kinetics, on a physiological time scale of seconds to minutes. The decrease in the intensity of Chl fluorescence is the result of the decrease in the electronic excited state lifetime of Chl caused by an increased thermal dissipation rate constant (5).

The rapid response of the qE process is chemically associated with changes in the trans-thylakoid membrane pH gradient (ΔpH). The ΔpH change has at least two functions in qE. First, it activates the violaxanthin de-epoxidase (VDE) that converts violaxanthin (V) to antheraxanthin (A) and zeaxanthin (Z) (6). A and/or Z are essential elements of qE (7-9). Second, the lower pH in the lumen results in protonation of PSII proteins, including the 22 kD PSII subunit, PsbS, which plays a key role in qE (10). When both pH-induced changes occur together it is believed that Chls in PSII can transfer their excess energy to Z, which can return to the ground state via thermal decay (7,11,12).

The pH-sensing mechanism of the PsbS protein is influenced by two pairs of symmetrically arranged glutamate residues, each located within or close to the two lumen-exposed loops of the protein (13). Dicyclohexylcarbodiimide (DCCD), a well known inhibitor of
qE (14-16) is a carboxylate-modifying agent (17) that binds to PsbS (18). Although it was suggested that the DCCD binding site is in the luminal loops of PsbS, the exact binding site has not been determined. Importantly, site-directed mutagenesis experiments indicated that two of the PsbS glutamates, E122 and E226, are necessary for the function of PsbS (13).

In this paper we used single and double mutations of PsbS (E122Q/E226Q) to make a detailed biochemical and biophysical analysis of the role of these two glutamates in pH sensing and DCCD binding. We probed the role of the E122 and E226 residues by monitoring the changes in the PSII Chl $a$ fluorescence lifetime distributions, intensities, and photochemical efficiencies. Additionally, we also monitored the absorbance change at 535 nm ($\Delta A_{535}$) (19) that is obligatorily associated with qE (10,20,21). The $\Delta A_{535}$ has been suggested to reflect a red shift in the absorption spectrum of Z that occurs upon binding to PsbS (22,23). From these measurements we formulated a model of the influence of the glutamate mutants on the fractional quenching of the populations of PSII (24) that is consistent with the symmetrical structure of the PsbS protein, the DCCD binding stoichiometry, and the stoichiometry of the xanthophyll components on a per PSII unit basis.
EXPERIMENTAL PROCEDURES

Plant materials and growth conditions—Arabidopsis thaliana (Columbia strain) was used in all experiments. npq4-1 is a psbS deletion mutant (10). Site-directed mutagenesis of psbS and transformation protocols were the same as described previously (13). In the T1 generation, lines with single insertions were selected, and homozygous lines were confirmed in the T3 generation. All plants were grown in a growth chamber with a light intensity of 120 µmol photons m⁻² s⁻¹ and a photoperiod of 10 h light (22°C)/14 h dark (20°C).

DNA and immunoblotting—psbS DNA gel blotting and PsbS protein immunoblotting analyses were performed as described previously (13), except that genomic DNA was digested with Spe I.

NPQ measurement—Room temperature Chl fluorescence was measured on attached rosette leaves with a commercial fluorometer (FMS2, Hansatech, King’s Lynn, UK). Plants were dark-adapted overnight before the measurement. After measuring Fₘ during a 1.0-s saturating pulse of 10,000 µmol photons m⁻² s⁻¹, actinic light of 1200 µmol photons m⁻² s⁻¹ was switched on for 10 min. A saturating 1.0-s pulse of 10,000 µmol photons m⁻² s⁻¹ was triggered every 60 s to determine Fₘ’. NPQ was calculated as (Fₘ-Fₘ’)/Fₘ’.

Crosslinking and immunoblot analysis—Fresh thylakoids were extracted from mature rosette leaves (24). Thylakoids containing 50 nmol Chl were diluted in 1 ml reaction buffer (50 mM HEPES pH 8, 0.1 M sucrose, 10 mM NaCl, 10 mM KCl, 5 mM MgCl₂, 1 mM KH₂PO₄, 30 mM sodium ascorbate, 50 µM methyl viologen, 0.3 mM ATP) and stirred in the dark or under qE-inducing illumination. After 10 min, dithiobis(succinimidylpropionate) dissolved in dimethylsulfoxide was added to a final concentration of 0.1 mM and stirred for 10 min. Crosslinking was terminated with the addition of Tris-HCl (pH 8) to 20 mM and stirring for an
additional 10 min. The crosslinked thylakoids were centrifuged for 2 min at 15300 g and resuspended with protein solubilization buffer without reductant for analysis by SDS-PAGE. For immunoblotting of crosslinked samples, 5 nmol Chl was loaded in each lane.

**Xanthophyll cycle pigment analysis**—For zeaxanthin formation experiments, leaf discs were sampled from overnight dark-adapted plants as controls, sampled at noon in the growth chamber (low light conditions), and sampled at noon, floated on water, and then treated at 1600 \( \mu \text{mol photons m}^{-2} \text{s}^{-1} \) for 30 min (high light-treated). All samples were rapidly frozen in liquid nitrogen and stored at \(-80^\circ\text{C}\) until analysis. Pigments were measured by HPLC (25), and the mean \( \pm \) SD was calculated (n=6-9).

**Leaf absorbance measurements**—Leaf absorbance changes were measured using the Non-Focusing Optics Spectrophotometer (NoFOSpec) (26), but modified as described (27) to allow semi-simultaneous measurements of absorbance changes at four different wavelengths. This was accomplished by aiming four separate banks of light-emitting diodes (LEDs, HLMP-CM15, Agilent Technologies, Santa Clara, CA), each filtered through a separate 5 nm bandpass interference filter (Omega Optical, Brattleboro, VT), into the entrance of a compound parabolic concentrator. Each bank of LEDs was filtered with a separate interference filter, at 500, 520, 535 and 545 nm each with a 5 nm bandpass (full width at half height). The photodiode detector was protected from direct actinic light by a Schott BG-18 filter. Current from the photodiode was converted to a voltage by an operational amplifier, and the resulting signal was AC-filtered to remove background signals, and sampled by a 16-bit analog-to-digital converter on a personal computer data acquisition card (DAS16/16-AO, Measurement Computing, Middleboro, MA). Timing pulses were generated by digital circuitry (PC card D24/CTR 3, Measurement Computing, Middleboro, MA) controlled by software developed in-house. The duration of the
probe pulses was set at 10 µs. Actinic illumination was provided by a set of 12 red LEDs (HLMP-EG08-X1000, Agilent Technologies, Santa Clara, CA) and controlled by the timing circuitry. Measuring pulses at each wavelength were given in sequence at 1-100 ms intervals, depending upon experiment.

Chl a fluorescence changes were also measured with the NoFOSpec instrument using the 525 nm measuring pulse to excite Chls, while protecting the detector with a Schott RG-9 filter (28).

Leaves were cut from plants dark-ad适应ed overnight, and the leaf petiole was wrapped in a small piece of moist cotton. The Fm was first recorded by giving 800 ms white light of 30,000 µmol photons m⁻² s⁻¹. After 30 s in the dark, a red actinic light of 1300 µmol photons m⁻² s⁻¹ was switched on for 215 s, and the leaf absorption at 535 nm was recorded. After allowing relaxation of qE in the dark for 110 s, leaf absorption at 535 nm was recorded again, and the ∆A₅₃₅ was calculated. After finishing the 535 nm measurement, the same actinic light was switched on again for 2 min, and then a white light pulse (800 ms, 30,000 µmol photons m⁻² s⁻¹) was applied to measure the Fm’.

**DCCD binding analysis**—¹⁴C-DCCD labeling was conducted on thylakoids prepared as described previously (29) at two different pH conditions, pH 7.8 and pH 5, for 3 h at room temperature. For labeling at pH 7.8, thylakoid samples containing 30 µg of Chl were labeled in buffer T2 (5 mM Tricine pH 7.8, 50 mM sorbitol, 10 mM EDTA). For labeling at pH 5, thylakoid samples were labeled in citrate buffer (30 mM NaCitrate pH 5, 50 mM sorbitol, 10 mM EDTA). After mixing thylakoids with buffer, 7.5 µl of 100 µM ¹⁴C-DCCD (0.5 µCi) in ethanol was added to make a final total volume of 100 µl. For gel analysis, a first dimension separation using a 12% acrylamide Tris-Sulfate gel with 6 M urea was used (30). After staining
the gel with Coomassie blue, the region corresponding to PsbS and light-harvesting complex proteins was cut out and equilibrated in Tris-HCl buffer pH 6.8 with 2% (w/v) SDS for 25 min, then transferred to the same buffer containing 60% glycerol for another 10 min. The gel slice was then loaded for second dimension separation by SDS-PAGE on a 10-16% gel without urea (31). The labeling intensities of PsbS mutants with respect to the wild type were acquired from the dried gels with the Instant Imager (Packard) instruments and calculated from the average of three independent measurements after correcting for the quantity of PsbS on the SDS-PAGE.

Room temperature Chl fluorescence lifetime measurement—Leaf samples were dark-adapted for 12 h at room temperature prior to detachment at the petiole and complete vacuum infiltration of the intercellular airspace with 0.35 M glucose (30 min in dimmed room light at room temperature) in a 5 ml disposable syringe. Infiltrated leaves were gently pressed using cotton wool into a front surface configuration free of air bubbles in a low-fluorescence plastic-quartz cuvette submerged in 0.35 M glucose solution. The samples were placed in an aluminum block-type sample holder that could be temperature controlled using circulating water baths. After dark-adaptation for 10 min at 20°C the samples were illuminated, and Chl a fluorescence was monitored using the fiber-optic probe of a Chl fluorometer (PAM 101-103, Heinz-Walz Instruments Ltd, Effeltrich, Germany). Experiments were initiated at 20°C with the low-intensity modulated measuring beam (1.6 KHz, ≈0.25 µmol photons m⁻² s⁻¹, 440 nm) to measure the PSII fluorescence intensity (interference filter 685 nm, 10 nm bandpass) under conditions of maximal photochemistry (F₀). After 30 s Fₘ was measured during a saturating 2-s pulse of white light (13,600 µmol photons m⁻² s⁻¹, Walz DT-Cyan filter), and then a strong actinic white light (1250 µmol photons m⁻² s⁻¹, Walz DT-Cyan filter) was applied to induce photosynthetic electron transport, thylakoid lumen acidification, V de-epoxidation, and PsbS protonation. Saturating
pulses were applied every 60 s to measure $F_m'$. After 10 min the sample temperature was reduced to 3°C (requiring ~3 min after switching water baths), while the actinic light was continued for a total of 15 min. All illumination was extinguished, and the sample was then rapidly turned to face the fiber-optic probe of the fluorescence lifetime instrument to illuminate the sample (140 $\mu$mol photons m$^{-2}$ s$^{-1}$, 635 nm) for 5 min before initiating the fluorescence lifetime determination. Fluorescence lifetime determinations were made under conditions of light- and low temperature (3°C)-saturated lumen acidification (32) to maintain maximal levels of V de-epoxidation and PsbS protonation while the PSII redox state was approximately 60-80% reduced, depending on the level of energy dissipation (33). The fluorescence intensity conditions during the lifetime acquisition corresponded to those defined as $F_s$ (34).

The PSII Chl $a$ fluorescence lifetimes were determined using a multifrequency phase-modulation fluorimeter (Model K2-004, ISS Instruments, Urbana, IL, USA) using a red laser diode (peak emission 635 nm) for excitation and a red-sensitive microchannelplate photomultiplier tube (Hamamatsu Photonics, Hamamatsu, Japan R3809U-50) for emission detection. Excitation and emission were applied and measured, respectively, from the front surface of the sample using a bifurcated quartz fiber-optic probe with the single terminus facing the sample. The excitation diode-laser intensity was attenuated to 140 $\mu$mol photons m$^{-2}$ s$^{-1}$ at 30 MHz to avoid photobleaching during measurements, which normally required 10 min to complete. The reference and sample signals were detected at each modulation frequency using high-transmittance (>80%) narrow waveband (12 nm halfwidth) interference filters (Corion Inc, Franklin MA USA) centered at 645 nm and 689 nm, respectively. An automatic rotating filterwheel exchanged the sample and reference positions such that the phase and modulation signals were repeatedly determined using 4-6 cycles at each modulation frequency until standard
errors were reduced below 0.04 degrees for the phase angle shift and 0.001 for the demodulation ratios.

*Global Analysis of the Phase and Modulation Fluorescence Lifetime Data*—The phase angle shift and demodulation ratio data sets for all samples were analyzed globally by fitting to a multimodal Lorentzian distribution model with the $\tau$-center value and width of each fractional component being linked together and all amplitudes free-floating. Similar to the analysis described previously (33), a minimum of five positive and one negative amplitude components were necessary to fit the data from all sample types globally. The goodness of fit for the data set, which included 62 free fitting parameters and 404 data points, was judged on the following criteria: the residual error distribution was randomly and normally distributed around 0 after minimizing the reduced chi-square to a value of 2.0223 using frequency-independent standard deviation values $\sigma_{\text{phase shift}} = 0.25^\circ$ and $\sigma_{\text{demodulation}} = 0.005$ (35). Global analysis was performed on a program assembled with Excel 2002, Visual Basic (Microsoft) and Large-Scale GRG Solver Engine (Frontline Systems, Inc., Incline Village, NV, USA). The program was evaluated by using statistical reference datasets provided by the National Institute of Standards and Technology (http://math.nist.gov).
RESULTS

Effect of glutamate mutations on PsbS function and qE—Glutamate residues in the lumenal loops of PsbS were changed to glutamines by site-directed mutagenesis to eliminate H⁺-binding capacity while minimizing alteration of the protein structure (13). The E122Q and E226Q mutations were made individually and pairwise and expressed in npq4-1 Arabidopsis plants that lack endogenous wild-type PsbS. As a control, npq4-1 plants were transformed with the wild-type psbS gene. For each single mutant (npq4-E122Q and npq4-E226Q), the double mutant (npq4-E122QE226Q), and the control (npq4-1 + psbS), two to three independent lines carrying single insertions were selected (Fig. 1A).

Immunoblot analysis showed that PsbS protein levels in all transgenic lines were 3- to 5-fold higher than in wild-type plants (Fig. 1). Immunoblotting was conducted on washed thylakoid membranes, demonstrating that the PsbS protein was inserted in the membrane in all cases. Furthermore, protein crosslinking experiments with isolated thylakoids showed no differences in PsbS crosslinking patterns (data not shown), indicating that the glutamate mutations of PsbS and enhanced protein expression level do not affect the physical association of PsbS within the thylakoid membranes or its interaction with other proteins.

Figure 2 shows the NPQ induction traces in the transgenic lines, the wild type, and npq4-1. Compared with the npq4-1 + psbS lines, which had higher levels of NPQ than the wild type due to overexpression of PsbS (33), the single mutant lines had about one-third as much NPQ. Most of the NPQ was rapidly reversible (qE). There was no significant difference between the single mutant lines npq4-E122Q and npq4-E226Q. The NPQ level in the npq4-E122QE226Q double mutant lines was indistinguishable from that of npq4-1, which lacks qE, indicating that the double mutant PsbS was nonfunctional.
Xanthophyll cycle pigment composition and $\Delta A_{535}$—There were no significant differences in either the total xanthophyll cycle pigment pool sizes or V de-epoxidation states in high light (1700 $\mu$mol photons m$^{-2}$ s$^{-1}$) in any of the different lines sampled at the same conditions (data not shown). The xanthophyll cycle pool size was 26.5±0.8, 28.2±0.9, and 29.6±1.2 mmol/mol Chl $a$ in the three sample conditions, and the respective de-epoxidation states were 0.053±0.010 in overnight dark-adapted leaves, 0.068±0.010 in leaves sampled at noon under growth light conditions, and 0.649±0.010 in leaves treated for 30 min with light that was ten times higher than the growth light. Thus, we conclude that neither the amount of PsbS in the thylakoid membrane, as shown before (33,36), nor the mutations of the PsbS protein influence the xanthophyll cycle pool size or de-epoxidation in high light.

Figure 3A shows that $\Delta A_{535}$ was linearly correlated with the extent of NPQ in each sample. There was a significant x-intercept value of about 0.36, indicating that a small component of the total NPQ is independent of $\Delta A_{535}$ and PsbS. This component is attributable to $qI$, a component of NPQ that is related to photoinhibitory damage to PSII and associated with a slowly reversing component of $F_m$ quenching that is observed in the absence of PsbS, as discussed in more detail below. We further analyzed the linear relationship between $\Delta A_{535}$ and NPQ by consolidating the variations of the sampling in each measurement and plotting the lines for the mean values of NPQ and $\Delta A_{535}$ from the wild type, npq4-1, npq4-1 + psbS, npq4-E122Q, npq4-E226Q, and npq4-E122QE226Q lines (Fig. 3B). This analysis increased the significance of the slope and intercept values (Table 1). There were no significant differences between npq4-1 and the double mutant or between the npq4-E122Q and npq4-E226Q single mutants.
DCCD binding—DCCD binds to carboxylate residues in hydrophobic environments (17,37). It is known for its ability to inhibit qE (14-16), which suggests that carboxylate residues are involved in the process. PsbS purified from spinach or Arabidopsis and PsbS expressed in E. coli bind DCCD at pH 7.5. Three acidic residues in each of the two lumen-exposed loops were suggested to be the possible binding sites (18). Fig. 4 shows that, in thylakoids isolated from wild-type Arabidopsis, DCCD bound to PsbS at pH 5. However, no binding of DCCD was detected at pH 7.8 (data not shown). DCCD binding was only 45±10% and 50±12% of the control in the npq4-E122Q and npq4-E226Q single mutants, respectively, and binding was undetectable in the npq4-E122Q-E226Q double mutant (Fig. 4). These results indicate that DCCD can equally bind to E122 and E226 and that mutation of one of the glutamates does not affect the chemical characteristics of the other in a cooperative manner.

Chl a fluorescence lifetimes—We further analyzed the Chl a fluorescence lifetime distributions in these mutants. Figure 5A shows the phase shift and demodulation ratio data as a function of modulation frequency (symbols) and the globally fit model (lines), with the residual errors in the lower panel. The patterns of the phase angle shifts and higher demodulation ratios indicate that the npq4-1 + psbS overexpression lines have the fastest decay times (lowest phase angles and highest demodulation ratios), followed by the wild type, the two single mutants npq4-E122Q and npq4-E226Q, the double mutant, and finally the npq4-1. The residual error plot indicates the model exhibits no remarkable systematic deviations and a random distribution around the mean = 0 for both the phase angle shifts and demodulation ratios.

Figure 5B and Table 2, respectively, show and tabulate the lifetime-weighted fractional intensity distributions as a function of the fluorescence lifetime and their integral contributions to the average lifetime for the five positive amplitude components (c1, c3-c6) and one negative
amplitude component (c2) used in the global analysis. There were two main positive fraction components, namely c6 and c4, in the npq4-1 and npq4-E122QE226Q double mutants that both lacked PsbS function. There were two positive components (c3 and c5) that correlated with PsbS function in all the other lines. The negative component c2 (centered ~140 ps) was weakest in the npq4-1 mutant that lacked PsbS and in the wild type that has only 20% of the PsbS protein level of the other mutant lines (Fig. 1). The other lines all exhibited larger c2 components. This observation suggests the negative c2 component was possibly more influenced by the PsbS protein level as opposed to the PsbS functional activity. The most rapid c1 component (<20 ps) was present in all PsbS-protein containing lines, and lowest in npq4-1. Consistent with previous results (33), c1 was stronger in the npq4-1 + psbS lines than in the wild type but appeared to be independent of the PsbS function, because it was similarly resolved in the single and double mutants, which have roughly the same levels of PsbS protein as the npq4-1 + psbS.

With respect to the effect of PsbS on PSII photochemical efficiency, the npq4-1 and npq4-E122QE226Q double mutant lifetime distributions were predominated by c6 with the longest, broadest lifetime (~1100 ps), attributed to fluorescence from closed PSII traps (Fm), lesser contributions of the c4 component (~300 ps) that is likely mainly associated with open PSII trap (Fo) emission. The npq4-1 showed significantly higher amplitudes of c6 (and less c4) than the double mutant. The larger c4 component in the double mutant compared to the npq4-1 coincided with a larger negative c2 component. The two single mutants both exhibited nearly identical patterns for the distribution components with levels of c6 roughly 50% of those exhibited in the double mutant and also reduced levels of c4. The two single mutants also exhibited the two narrow components, c5 centered at approximately 525 ps and c3 at approximately 220 ps, that were clearly dependent on PsbS function and most likely originated
from closed and open PSII traps, respectively. The wild type exhibited about 10% of the c6 level exhibited in the npq4-1 and was predominated by the c5 component. The npq4-1 + psbS was, similar to the wild type + psbS lines reported earlier (33), dominated by the c1, c3, and c5 components indicating the strongest energy dissipation and highest degree of PSII trap opening (largest c3 contribution), leading to the fastest average decay time in all lines compared in Figure 5A. The npq4-1 + psbS exhibited less than 3% of the c6 level in the double mutant, indicating the PsbS functionality may have been nearly saturated. It was clear, however, that the npq4-1 + psbS line had slightly less PsbS activity than the wild type + psbS lines described previously (33). The wild type + psbS lines with higher PsbS protein levels exhibited larger relative c1:c3 ratios than the npq4-1 + psbS line analyzed here, leading to about a 56 ps (16%) faster average lifetime in the former samples.

Figure 6 compares the intensity ratios measured with a PAM Chl fluorimeter during (Fm' / Fm) and after (Fm' / Fm) maximal levels of light-induced fluorescence quenching, along with the calculated average lifetime <τ> values under the Fs conditions from the experiments described in Figure 5. The <τ> at Fs correlated strongly with the Fm' / Fm values in all lines. The npq4-1 and double mutant exhibited close parallels, with both the largest <τ> values (1.132 ns and 0.957 ns) and Fm' / Fm values (0.54 and 0.52). The single mutants exhibited slightly higher <τ> and Fm' / Fm values (0.665 ns and 0.39) than the wild type (0.577 ns and 0.34) and the npq4-1 + psbS line showed the lowest values (0.386 ns and 0.23). Both the npq4-1 and the double mutant exhibited approximately 60% recovery of the original Fm, the two single mutants each exhibited about 65%, while the wild type and npq4-1 + psbS showed approximately 70 and 73%, respectively.
DISCUSSION

PsbS senses the ΔpH through two symmetrically arranged, lumen-exposed glutamates, E122 and E226—Unlike typical light-harvesting complex proteins, PsbS has four rather than three transmembrane helices. Protein sequence analysis showed high similarity between helix I and helix III and also between helix II and helix IV (38,39), reflecting the symmetrical topology of the PsbS protein (Fig. 7). The two lumen-exposed loops (40) are also highly similar, with E122 and E226 located in the middle of each loop. Mutating one or the other of these two glutamates only partly inhibits the PsbS function in qE, whereas the npq4-E122QE226Q double mutant totally disrupts the PsbS function (Fig. 2). These results are consistent with the suggestion that glutamates E122 and E226 of PsbS serve as the pH sensors for qE (13).

The function of E122 and E226 was further confirmed by investigation of DCCD binding. DCCD is a protein modifying reagent which covalently binds to carboxylate residues involved in reversible protonation in hydrophobic environments (17). DCCD has previously been shown to act as a specific inhibitor of qE (14-16), thus leading to the search for target sites using 14C-DCCD. Binding sites have been identified in Lhcb proteins (41), namely CP26 (42) and CP29 (43), but evidence for these proteins being the functional binding site(s) of this qE inhibitor was lacking. Our results show that the single mutations E122Q and E226Q each reduce the level of DCCD binding to PsbS by approximately 50% and in the same way decrease the amplitude of qE. These finding imply that PsbS, rather than CP26 or CP29, is the target site for qE inhibition by DCCD. Moreover, since the double mutant is not labeled, E122 and E226 are the only DCCD binding sites in PsbS, implying that they have an essential role in PsbS function. It should be noticed that purified PsbS, either extracted from thylakoids or recombinant from E. coli, binds DCCD at pH 7.5, but low pH is required for labeling of PsbS in thylakoid membranes,
suggesting that either the conformation of PsbS is different in the two environments or that interactions with neighbor subunits in PSII supercomplexes prevents exposure of lumenal glutamate residues until the pH is decreased.

It is interesting to note that the two glutamate residues are responsible each for 50% of both DCCD binding and control of qE activity. This suggests that, whatever the mechanism is for the activity of PsbS in qE, the two halves of the two-fold symmetrical molecule are acting independently. Recent work (12) showed that in the presence of PsbS, a 10 ps spectral component is present that can be interpreted as a Chl $a$ to Z energy transfer, and Z binding to PsbS has also been reported (23). On this basis, the qE quenching can be tentatively understood in terms of the protonation of each glutamate leading to binding of two Z molecules, which are capable of accepting and dissipating energy from singlet excited Chl $a$. Thus, PsbS can be viewed as a thylakoid sensor of excess light. In the presence of a low thylakoid lumen pH, protonation of PsbS results in thermal dissipation of excess absorbed light energy, i.e., a feedback downregulation of photosynthetic light harvesting by the $\Delta$H.

In addition to influencing qE, the E122Q and E226Q mutations also inhibit the $\Delta A_{535}$ leaf absorbance change (Fig. 3). It was found early on that $\Delta A_{535}$ is invariably associated with qE (20,21). Here we report a single linear relationship exists between NPQ and $\Delta A_{535}$ even when taking into consideration two different factors, namely different levels of PsbS protein and different amino acid substitutions in the PsbS protein (Fig. 3). It was hypothesized that $\Delta A_{535}$ is due to a red absorption shift of Z upon binding to PsbS (22). The linear relationship between $\Delta A_{535}$ and NPQ suggests that one of two Z-binding sites is affected in each single mutant (E122Q or E226Q), whereas Z binding might be eliminated completely in the double mutant (E122QE226Q).
Interpreting the fluorescence lifetime distribution components with respect to the glutamate mutants, PsbS protein level, and PSII function—As illustrated in Figure 8A, our current biochemical model of PsbS function postulates that PsbS associated with PSII may exist in one of three states depending on the lumen acidity and the concentration of Z (and/or A). The W state is defined as a PsbS with its critical glutamate residues (E122 and E226) in an unprotonated state. W converts reversibly to X upon protonation of the glutamates as determined by their $pK_a$ value. The protonated state X is hypothesized to be an activated state for potential binding of Z. Upon binding of Z, the PsbS switches from the X to the Y state, which is the fully functional energy dissipation mode.

Consistent with our recent papers (33,44), we have found it necessary to expand our model to six states to account for the opening/closing (oxidation/reduction) of the PSII reaction centers. In order to simplify the model parameterization and interpretation, our experiments were designed to yield both saturating light-induced lumen pH and Z concentration conditions, thereby reducing to a minimum both the W (by saturated protonation) and X states (by saturated xanthophyll binding). Practically, we have also concluded that we are unable to resolve the W and X components in either the open or closed PSII states, most likely because they are likely to differ by less than 20% between the open and closed W or X components. Therefore, our empirical global model linking scheme assumes the $W_c+X_c$ and $W_o+X_o$ components to be indistinguishable, yielding a simplified four component scheme (Fig. 8B). As mentioned in the results, we postulate the rapid c1 and negative c2 components to be more strongly related to the PsbS protein level than PsbS function per se, because these components were resolved in the double and both single mutants. Hence, we do not consider them to be associated directly with a particular state of PSII in the above scheme. We propose that extremely high PsbS
concentrations may have indirect effects on the photochemical activity of the PSII core antenna and possibly alter energy transfer pathways among Chl complexes, perhaps between the antenna and core. With respect to Figure 8B, the (1100 ps) c6 component of our lifetime distribution model would be assigned to the combined \([W_c+X_c]\) states. We emphasize that the assignment of c6 to the W state is supported by the fact that the c6 contribution is maximal in the \(npq4-1\), very similar to \(npq4-1\) in the double mutant, reduced by \(~50\%\) in both single mutants (E122Q and E226Q) compared to the double mutant, reduced by \(>80\%\) in the wild type and down to less than \(3\%\) in the \(npq4-1 + psbS\). The c4 (300 ps) component appears consistent with emission from the \(W_o+X_o\) state PsbS conformations with open PSII traps. However, as indicated above it is unlikely that the \(W_o\) state would be a significant component because the key PsbS glutamate residues were likely titrated to virtually complete protonation. Likewise, we feel it is logical to assign the c3 (220 ps) and c5 (525 ps) states to the open \(Y_o\) and closed \(Y_c\) PSII centers, respectively. A main tenet of the model outlined in Figure 8B, that is consistent with many steady-state spectroscopy experiments, is that increasing the population of the Y state acts synergistically to yield a higher relative fraction of open/oxidized PSII traps \(Y_o\) with enhanced levels of energy dissipation. The increased rate constant of dissipation of excess absorbed light in the open and closed PSII traps reduces the ‘excitation pressure’ on the PSII reaction center Chls leading to a higher population of open PSII traps (10,33,44).

The c6 mode was approximately 600 ps shorter than observed in thylakoid experiments (\(W=1740\) ps). This observation is consistent with the larger decrease of the \(F_m\) (30-40\%) following the illumination treatments in these leaf experiments (1250 \(\mu\)mol photons m\(^{-2}\) s\(^{-1}\) for 20 min) as opposed to 20-25\% observed in isolated thylakoids which were treated with lower (500 \(\mu\)mol photons m\(^{-2}\) s\(^{-1}\)) light intensities (36). We note as mentioned before that in thylakoids we
measured an additional lumen pH-induced 16% decrease in the lifetime center of the W mode tentatively attributed to protonation and/or conformational changes of other PSII proteins besides PsbS, which was absent in the npq4-1 mutant. Previously, we reported that the W mode center was decreased during aerobic photoinhibition in thylakoids largely independent of the width or fraction of the distribution (45). So both things considered, we conclude that the ~50% decrease in the W mode in the Fm' condition compared to W in the Fm state in these leaf experiments is attributed to both photoinhibitory damage caused by the strong light and protonation/conformational changes in other PSII proteins besides PsbS. Further we consider that all lifetime modes may be partially attenuated by PSII photochemical activity through kinetic processes that are beyond the resolution of our instrument (i.e., 5-10 ps).

Concluding Remarks

One of the key points of the model described above is the implications of the lifetime distribution fractional intensities on the structure-function of PsbS. In our view the simplest explanation for the similarities of the two single mutants (E122Q and E226Q) is that they have equivalent effects because of the bilateral symmetry of the PsbS protein structure. The data indicate, as suggested in Figure 9, that each single mutation elicits a ~50% inhibition effect that becomes 100% in the double mutant. Hence, we propose that PsbS has two equivalent functional sites, one on each side, that are likely associated with pH-activated binding of the xanthophylls. It is thus suggested that PsbS associates with its binding site to attach itself to the PSII holocomplex (likely in or near the core antenna proteins) in one of two facial orientations with a 50% probability. And further, according to this model, two Z per PsbS will be the saturation amount for qE.
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### TABLE 1

*Statistical analysis of the results in Figure 3*

|                  | Slope     | P value     | Intercept   | P value     | Adjusted R² |
|------------------|-----------|-------------|-------------|-------------|-------------|
| Data from Fig. 3A| 8.249±0.373 | 2.916E-19   | -3.024±0.591 | 2.029E-05   | 0.9438      |
| Data from Fig. 3B| 8.288±0.216 | 2.752E-06   | -2.997±0.299 | 5.56E-04    | 0.997       |
**TABLE 2**

*Fluorescence lifetime distribution component parameters*

Data represent the integral lifetime-weighted fractional intensity contributions of each component in units of ps as the mean of three measurements with separate leaf samples. Confidence intervals were defined by standard deviations of less than 10% of the average lifetime values for each mutant. Component distributions were as identified in Figure 5 under steady-state PSII fluorescence conditions as defined previously (34).

| Strain                  | c1  | c2  | c3  | c4  | c5  | c6  | <τ>  |
|-------------------------|-----|-----|-----|-----|-----|-----|------|
| Wild type               | 48  | -72 | 15  | 0   | 472 | 115 | 578  |
| npq4-1                  | 8   | -20 | 0   | 49  | 0   | 1104| 1141 |
| npq4-1 + psbS           | 104 | -244| 248 | 0   | 255 | 22  | 385  |
| npq4-E122Q              | 87  | -244| 158 | 46  | 310 | 288 | 645  |
| npq4-E226Q              | 59  | -200| 167 | 42  | 242 | 374 | 684  |
| npq4-E122QE226Q         | 111 | -317| 0   | 346 | 0   | 818 | 958  |
Figure Legends

FIG. 1. DNA gel blot analysis and protein immunoblot analysis of Arabidopsis wild-type and PsbS mutant plants. Shown here are the wild type, three lines of npq4-1 + psbS, three E122Q mutant lines (npq4-E122Q), two E226Q mutant lines (npq4-E226Q), two double mutant lines (npq4-E122QE226Q), and npq4-1. For DNA gel blotting 10 µg of genomic DNA was digested with Spe I prior to loading. For immunoblots 1.0 nmol Chl was loaded in each lane, and PsbS and D1 proteins were detected using specific antibodies.

FIG. 2. Induction and relaxation of NPQ. Measurements were conducted on two separate batches of dark-adapted plants with 3 to 5 replications each. NPQ was measured during 10 min of illumination with high light (1200 µmol photons m^{-2} s^{-1}), followed by relaxation in the dark for 5 min. All data points were plotted as the mean ± SE (n=8).

FIG. 3. Linear relationship between ΔΔA_{535} and NPQ. A, data from several individual leaves of each genotype. B, same data except that each point represents the mean ± SE for each genotype.

FIG. 4. 14C-DCCD binding to PsbS at pH 5.0. After separating thylakoid proteins by two-dimensional PAGE, the gel was stained with Coomassie blue and then dried, and the radioactivity was detected with the Instant Imager (Packard). Thylakoids from plants carrying different mutations affecting PsbS were used for the labeling. The circles on the gel panel indicate other unidentified proteins labeled by 14C-DCCD in all thylakoids.
FIG. 5. Multifrequency phase and modulation time-resolved Chl α fluorescence data and simulated model interpretation from leaves of wild-type Arabidopsis and PsbS mutants. A, phase (angle) shifts and demodulation ratios as a function of modulation frequency for each type of leaf under conditions of saturating lumen acidity and subsaturating PSII photochemistry. The lower panel plots the residual errors for the phase shift (phase res) and demodulation ratios (demod res). B, lifetime-weighted fractional intensity distribution (fτ(τ)) as a function of the fluorescence lifetime, τ. The five positive (c1, c3-c6) and one negative (c2) amplitude modes in the global model solution are indicated. Integration of the fτ(τ) distribution for each sample yields the average lifetime <τ>.

FIG. 6. Comparison of steady-state PSII Chl α fluorescence ratios during and average fluorescence lifetimes from leaves of wild-type Arabidopsis and PsbS mutants. Fm'/Fm represents the steady-state Chl α fluorescence ratio under conditions of qE. <τ> is the average lifetime from fluorescence lifetime measurements. Fm/Fm represents the steady-state Chl α fluorescence ratio after relaxation of qE.

FIG. 7. Topology of PsbS. Symmetrically arranged amino acid residues in the first half (dark shading) and second half (gray shading) of the protein are highlighted. The two glutamates discussed in this paper are denoted by square symbols and numbered.

FIG. 8. Biochemical scheme of the lumen pH- and Z-dependent effects on the PsbS protein with respect to energy dissipation and reaction center photochemistry in PSII. A, model scheme with H⁺ and Z binding steps (24). B, interpretation of the PSII fluorescence lifetime
distribution components with respect to the combined influence of energy dissipation and reaction center photochemistry. \( W_o (W_c), X_o (X_c), \) and \( Y_o (Y_c) \) represent PSII in the \( W, X, \) and \( Y \) states (defined in panel A) with the redox state of the primary PSII electron acceptor \( Q_A \) in an oxidized (reduced) state. The lifetime components \( c_3-c_6 \) were as defined in Fig. 5.

**FIG. 9.** Model explaining the effect of PsbS glutamate mutants on \( H^+ \) and \( Z \) binding and proposed interactions between PsbS and PSII. The glutamate to glutamine mutations of PsbS are represented by the \(-\text{CONH}_2\) moieties, and their lack of function in \( qE \) is represented by the black ovals (putative xanthophyll binding sites) to which they are attached. The \(-\text{COO}^-\) and \(-\text{COOH}\) moieties represent the charged and protonated glutamates, respectively. Upon protonation, a xanthophyll binding site is generated, as indicated by the change from the gray (\(-\text{COO}^-\)) to the white ovals (\(-\text{COOH}\)). The presence of a xanthophyll molecule (\( Z, A, \) or possibly lutein) occupying a protonated binding site is shown in the \( Y \) state. In the double mutant \( npq4-E112Q/E226Q \) shown here and in \( npq4-1 \) (not shown), protonation of PsbS does not occur, and it is assumed that \([W]=1\). In the single mutants \( npq4-E122Q \) or \( npq4-E226Q \), two possible interactions of symmetrical PsbS with PSII are shown, one of which is a functional interaction. In this case, \([W]\) would be 0.5. For \( npq4-1 + psbS \) (and in the wild type, not shown), either possible interaction orientation of PsbS with PSII is functional.
Li et al., Fig. 1

Li et al., Fig. 1

D1

PsbS

DNA blot

wild type
npq4-1+psbS #2
npq4-1+psbS #3
npq4-1+psbS #9
npq4-E122Q #1
npq4-E122Q #7A
npq4-E122Q #7B
npq4-E226Q #2
npq4-E226Q #3
npq4-E122QE226Q #4
npq4-E122QE226Q #9
npq4-1

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Li et al., Fig. 2

Li et al., Fig. 2
Li et al., Fig. 3

A

\[ Y = 8.2496X - 3.0241 \]
\[ R^2 = 0.9458 \]

B

\[ Y = 8.2884X - 2.997 \]
\[ R^2 = 0.9973 \]
Li et al., Fig. 4
Li et al., Fig. 5

![Graph A](image)

**A**

- **Phase shift, degrees**
  - wild type
  - npq4-1+psbS
  - npq4-E122Q
  - npq4-E226Q
  - npq4-E122QE226Q
  - npq4-1

- **Demodulation ratio**

**B**

- Lifetime-weighted fraction, f(τ)

**Legend**

- c1
- c2
- c3
- c4
- c5
- c6

**Axes**

- Frequency, MHz
- Lifetime, ns
Li et al., Fig. 6
Li et al., Fig. 7
Li et al., Fig. 8

A

\[
\text{model} \quad W \xrightarrow{pK_a} X \xrightarrow{K_a} Y
\]

\[
\text{H}^+ \quad X \quad \text{Z}
\]

B

\[
C_6(1100 \text{ ps}) \quad C_5(525 \text{ ps})
\]

\[
W_C + X_C \leftrightarrow Y_C
\]

\[
W_O + X_O \leftrightarrow Y_O
\]

\[
C_4(300 \text{ ps}) \quad C_3(220 \text{ ps})
\]
Li et al., Fig. 9

Model: $W \xrightarrow{pKa} X \xrightarrow{K_a} Y$

- npq4-E122Q/E226Q

- npq4-E122Q or npq4-E226Q

- npq4-1+psbS
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