The PsbS Protein Controls the Organization of the Photosystem II Antenna in Higher Plant Thylakoid Membranes

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The PsbS subunit of photosystem II (PSII) plays a key role in nonphotochemical quenching (NPQ), the major photoprotective regulatory mechanism in higher plant thylakoid membranes, but its mechanism of action is unknown. Here we describe direct evidence that PsbS controls the organization of PSII and its light harvesting system (LHCII). The changes in chlorophyll fluorescence amplitude associated with the Mg$^{2+}$-dependent restacking of thylakoid membranes were measured in thylakoids prepared from wild-type plants, a PsbS-deficient mutant and a PsbS overexpressor. The Mg$^{2+}$ requirement and sigmoidicity of the titration curves for the fluorescence rise were negatively correlated with the level of PsbS. Using a range of PsbS mutants, this effect of PsbS was shown not to depend upon its efficacy in controlling NPQ, but to be related only to protein concentration. Electron microscopy and fluorescence spectroscopy showed that this effect was because of enhancement of the Mg$^{2+}$-dependent re-association of PSII and LHCII by PsbS, rather than an effect on stacking per se. In the presence of PsbS the LHCII-PSII complex was also more readily removed from thylakoid membranes by detergent, and the level of PsbS protein correlated with the amplitude of the psi-type CD signal originating from features of LHCII-PSII organization. It is proposed that PsbS regulates the interaction between LHCII and PSII in the grana membranes, explaining how it acts as a pH-dependent trigger of the conformational changes within the PSII light harvesting system that result in NPQ.

Light harvesting antennae in plant photosynthesis increase the rate at which absorbed photons excite the photosynthetic reaction centers. In the case of photosystem II (PSII) in higher plants, light harvesting is carried out by a complex assembly of chlorophyll protein antenna complexes (LHCII) located in the grana membranes of the chloroplast (1). The main components are trimeric LHCIIb and monomeric CP24, CP26, and CP29. These are associated together with the dimeric PSII reaction center core complexes to form LHCII-PSII supercomplexes (2). The minimal unit of the supercomplex comprises two cores (C$_2$) and two strongly bound LHCIIb trimers (S$_3$), together with two copies of CP26 and CP29, referred to as the C$_2$S$_3$ LHCII-PSII supercomplex (3). Two further trimers bound with moderate strength (M$_3$) together with CP24 make up the most predominant supercomplex, C$_2$S$_3$M$_3$. LHCII-PSII supercomplexes are segregated from photosystem I, associating together in the lateral plane of the thylakoid membrane, sometimes forming highly ordered semi-crystalline domains. Transverse associations between the outer stromal surfaces of these proteins result in membrane appression and the characteristic grana stack. Although this highly conserved macro-organization of the PSII antenna has been rationalized in terms of efficient capture and utilization of light (4), it can be argued that it is the enabled structural and functional flexibility that is probably more important. Changes in interaction between subunits of the macrostructure allow the regulation of photosynthetic light harvesting (5).

The PSII antenna also contains a number of proteins of relatively low abundance, which have been implicated as playing roles in the regulatory processes (6). One of these proteins, PsbS, has been shown to play a key role in nonphotochemical quenching (NPQ). NPQ describes the processes of energy dissipation, induced under excess light conditions that provide photoprotection of the thylakoid membrane from the potentially damaging effects of the unwanted absorbed light energy (7, 8). The main component of NPQ is a feedback regulatory mechanism that is induced in response to the build up of the thylakoid ΔpH and is frequently referred to as qE (9). Mutants of Arabidopsis deficient in PsbS show greatly inhibited levels of qE (10), and overexpression of the psbS gene results in an enhancement of the maximum extent of qE (11). However, the mechanism of action of PsbS has not been determined. Muta tion of glutamate residues on the putative lumen-facing surface of the protein inhibits qE formation suggesting that PsbS is involved in sensing the ΔpH (12). It is further proposed that PsbS provides the site of quenching; binding of zeaxanthin stimulated by protonation followed by interaction with a chlorophyll-protein complex that allows chlorophyll-carotenoid energy transfer and energy dissipation (13). Alternatively, it has been suggested that PsbS acts by modulating a quenching process that is intrinsic to a chlorophyll-protein complex (14).
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Evidence to support this suggestion comes from the observation that the stimulation of qE by increased levels of PsbS does not require zeaxanthin (16). It is known that complexes such as LHCII can switch between conformational states with different extents of energy dissipation both in vitro and in vivo (17, 18). An indirect modulating role for PsbS, which may still include pH sensing and/or zeaxanthin binding, implies that it controls this conformational switching in LHCII.

Previous work has suggested that NPQ is influenced by the organization of the light harvesting antenna system in the grana membranes of the chloroplast. Thus, depletion of the minor complex CP24 resulted in a partial inhibition of qE and a disruption of the C3S3M2 LHCII/PSII macro-organization (19). Similarly, qE is also partially inhibited in lutenin-deficient mutants, which display a decreased stability of the trimeric LHCII structure that is such an intrinsic part of the macro-organization (20). Although alternative explanations of these data can be offered, the question is nevertheless raised whether the function of PsbS could be to provide dynamic regulation of this macrostructure, controlling interactions between LHCII components, modulating their change in conformation. There is already some circumstantial evidence to support this view. In one study, PsbS is shown to associate either with LHCII or the PSII core, depending upon pretreatments, which lead to the monomerization of PsbS dimers (21). In later work, PsbS was found to be widely distributed in the membrane and associated with a number of different complexes (22). It was also found that the PsbS-deficient npq4 mutant displays alterations in PSI function that are not related to NPQ (23). Here, a new direct approach has been taken to test whether PsbS has a role in the organization of the PSII antenna. We show that PsbS specifically controls the association between LHCII and the PSII core.

EXPERIMENTAL PROCEDURES

Arabidopsis thaliana cv Columbia and mutant lines derived from it (npq4-1, npq4-5, npq4-6, npq4-7, E122Q, E226Q, and the PsbS overexpresser L17) were grown for 8 to 9 weeks in Conviron plant growth rooms with a 10-h photoperiod and a day/night temperature of 22/15 °C. Thylakoid membranes were isolated from leaves, plants were dark-adapted for 1 h as described by Ruban et al. (25) and data analyzed as in (19). Fast protein liquid chromatography (FLPC) was performed on detergent-solubilized thylakoid membranes as described by Ruban et al. (24).

For electron microscopy, thylakoid membrane samples were fixed in 3% glutaraldehyde phosphate buffer and stained in uranyl acetate. Electron microscopy was performed on a FEI Tecnai transmission electron microscope operating at 80 kV.

RESULTS

Re-organization of LHCII-PSII during Thylakoid Re-stacking

Is Regulated by the Level of PsbS—In the presence of sufficient [Mg2+] isolated thylakoid membranes exhibit a high PSII fluorescence yield. Reduction in [Mg2+] results in a dramatic decrease of fluorescence, which is associated with the disruption of the LHCII-PSII macrostructure and eventual unstacking of the grana membranes (26, 27). Subsequent addition of Mg2+ causes an increase in fluorescence as PSII is re-organized and grana stacks return. These changes were used as an assay system to probe the effect of PsbS on the organization of PSII, specifically to explore its role in LHCII-PSII interaction. Thylakoid membranes from wt, npq4-1 (deficient in PsbS), and L17 (overexpression of PsbS) plants were resuspended in a medium free of bivalent cations. Fig. 1A shows the decrease of fluorescence yield during the measurement using chloroplasts from wt plants. At a minimum fluorescence level (stage 1) MgCl2 was added back in steps of 0.1 mM, resulting in Mg2+-titration curves for the fluorescence yield increase. At a final concentration of 1.2 mM MgCl2 the fluorescence level reached its maximum, which was about 85% of that of the control thylakoid membranes.

Fig. 1B shows the plots of the relative fluorescence change against the MgCl2 concentration in wt, npq4-1, and L17 thylakoids. Although each of the samples showed MgCl2-dependent fluorescence increases, the titration curves were strikingly different. The npq4-1 PsbS-deficient mutant showed a higher requirement for MgCl2 for the restoration of fluorescence than the wt, whereas the L17 PsbS overexpresser needed less. The shapes of the titration curves of the three genotypes were conspicuously different; thus, the titration curve of npq4-1 was strongly sigmoidal, whereas in L17 the sigmoidicity was greatly reduced. The data were fitted to the following equation: $Y = Y_{\text{max}} C^n / (C_0^n + C^n)$, where $Y = Fm'/Fm$, normalized to the range of 0–1, $Y_{\text{max}}$ is the theoretical maximum of $Y$, $C = MgCl_2$ concentration, $C_0 = MgCl_2$ concentration at $C = 0.5 Y_{\text{max}}$, and $n$ is sigmoidicity parameter. The sigmoidicity parameters were 4.28 ± 0.09, 2.58 ± 0.13, and 1.64 ± 0.004 and $C_0$ values 0.655 ± 0.020, 0.460 ± 0.016, and 0.321 ± 0.019 (means ± S.E.) for the titration curves of npq4-1, wt, and L17, respectively. These data are clearly dependent on the amount of expressed PsbS; the
greater the amount of this protein the less sigmoidal the response is and the higher the Mg\(^{2+}\) requirement.

In these three lines the extent of $q_E$ is dependent on the amount of PsbS (10, 11). The question arises about the structural requirements of the effect of PsbS on Mg\(^{2+}\)-dependent re-organization of PSII and its function in $q_E$. To answer this question, different $npq$ mutants were studied in which PsbS had point mutations. Mutations in the putative transmembrane helices lead to the partial loss of function of PsbS resulting in reduced $q_E$ (28). Three different mutants were studied: $npq4-5$, $npq4-6$, and $npq4-7$, where the mutations are S104L, A97T, and P185L, respectively. All three lines had highly reduced $q_E$ (Fig. 2A), and their expression levels of PsbS were also lower than that of the wild-type plants (B). The function of PsbS in $q_E$ can also be altered by mutation of two glutamate residues on the lumenal side in positions 122 and 226 (12). A single glutamate to glutamine change in either of these sites (E122Q, E226Q) resulted in a reduction of $q_E$ (Fig. 2A). A double mutant carrying mutations in both positions (E122Q,E226Q) had a highly reduced $q_E$, similar to the PsbS-deficient $npq4-1$ mutant. These mutants were generated by expression of site-directed mutant genes and consequently were predicted to have high levels of PsbS protein; in fact, the mutants had 3–5 times more PsbS protein than the $wt$. Comparing Fig. 2, A and B, it can clearly be observed that the level of $q_E$ in these mutated lines did not correlate with the expression level of PsbS. Fig. 2C shows the lack of any correlation between $q_E$ and the sigmoidicity parameters obtained from the titration curves. In complete contrast, there was a clear correlation between the expression level of PsbS and the sigmoidicity parameter (Fig. 2D) irrespective of its functionality in $q_E$. The level of the PsbS protein per se appears to determine the differences in kinetics of the Mg\(^{2+}\)-induced fluorescence increase in the $wt$ plants, the $L17$ overexpresser, and all the $npq4$ mutants. The structural requirements of PsbS for these two roles are clearly distinct. It should be pointed out that all mutants displayed the same ratio of variable to maximum fluorescence ($F_v/F_m$) (data not shown) indicating that they all had the same intrinsic efficiency of light harvesting.

\textbf{PsbS Controls the Re-association of LHCCI and PSII rather than Grana Stacking per se—Mg\(^{2+}\)-dependent re-organization of the thylakoid membrane during re-stacking is composed of at least two processes: the changes in interaction between PSII complexes and PSI modulating the spillover of excitation energy and the association of LHCCI to PSII core complexes (27, 29, 30). The summation of both processes gives rise to the increase in fluorescence and the formation of grana stacks.}
Experiments were carried out to determine which of these events was controlled by the level of PsbS in the thylakoid membrane. Fig. 3, A–C shows the electron micrographs of wt thylakoids in different stacking states. The micrograph of control membranes at 5 mM MgCl₂ (Fig. 3A) clearly shows grana stacks (arrows). In the absence of MgCl₂ (Fig. 3B) long unstacked lamellae can be observed (position 1 in Fig. 1A), whereas after titration with 1.2 mM MgCl₂ newly organized grana stacks can be observed (position 2 in Fig. 1A) (Fig. 3C). Samples were taken of thylakoids of L17 and npq4-1 at 0.4 mM MgCl₂ (position 3 in Fig. 1B) at which point there was a maximum difference in fluorescence, the former being at about 70% of its maximum, whereas for the latter the increase was only 10%. As expected, in the npq4-1 thylakoids there was no evidence of restacking (Fig. 3D). However, it was found that there were also no detectable stacks in the L17 thylakoids (Fig. 3E). Thus the PsbS-dependent fluorescence increase was not associated with restacking; rather it must be because of regulation of the association of LHClII and PSII.

Fluorescence spectroscopy was used to test this hypothesis for the role of PsbS. Fig. 4 shows the 77 K fluorescence emission spectra of unstacked (u) and stacked (s) thylakoid membranes from L17 (A) and npq4-1 (B). The four main bands appearing in the emission spectra are the LHClII band (680-nm shoulder), PSII bands (at 685 and 693 nm), and a PSI band (at 732 nm). Compared with the stacked thylakoids, in the membranes lacking Mg³⁺ ions (position 1 in Fig. 1A), the PSI bands decreased relative to the emission at 732 nm. The same features were found in both L17 and npq4-1. However, at 0.4 mM MgCl₂ (m), whereas in L17 there was a significant increase in PSII fluorescence; in npq4-1 the spectrum was almost identical to that of the unstacked membranes. The excitation spectra for PSI fluorescence showed that the absorption cross-section of PSI was larger in the unstacked membranes than the restacked ones, in both samples (Fig. 4, C and D). The difference spectra, stacked-minus-unstacked normalized at the position of the long wavelength PSI absorption (705–715 nm region) have bands at 650, 660, 670, and 676 nm, characteristic of PSI (see the close resemblance between the stacked-minus-unstacked spectrum and the excitation spectrum of a PSI “BBY” preparation), indicating increased energy transfer from PSII to PSI. However, at 0.4 mM MgCl₂ for both L17 and npq4-1 the excitation spectra were almost identical to those of the unstacked membranes, i.e. the PsbS-dependent increase in PSI fluorescence was not accompanied by a decrease in PSI cross-section. The excitation spectra for PSII fluorescence (693-nm band) show a decrease in cross-section in the unstacked thylakoids compared with the restacked ones in both L17 and npq4-1 (Fig. 4, E and F). At 0.4
mm the spectrum resembles the stacked spectrum for L17 but the unstacked spectrum for npq4-1. The difference spectrum, 0.4 mm-minus-unstacked, has no detectable features for npq4-1, but for L17 it has bands at 433, 441, 472, 486, 495, and 511 nm, all of which are characteristic of LHCII (31), i.e. the PsbS-dependent increase in PSII fluorescence is accompanied by an increase in energy transfer from LHCII. It is concluded that PsbS stimulates the Mg$^{2+}$-dependent association of LHCII to PSII and not the dissociation of PSII from PSI.

**FIGURE 5.** Circular dichroism spectroscopy of npq4-1, Col-0, and L17 leaves. A, CD spectra of leaves of npq4-1 (I), wt (2), and L17 (3) at room temperature. The spectra are averages of more than 20 independent measurements. B, difference CD spectra in the red region (620–730 nm). Solid line, wt-minus-npq4-1; dashed line, L17-minus-npq4-1. Spectra were first normalized on the difference between CD at 620 nm minus CD at 650 nm, the amplitude of the chlorophyll b band.

Changes in PsbS Level Are Associated with Altered Organization of LHCII-PSII in Grana Membranes—CD spectroscopy was used to investigate the macro-organization of the thylakoid membranes in vivo (19, 32). Excitonic CD bands arising from the short range interactions of the chromophores at (~) 653 nm, (+) 435 nm, and (+) 448 nm represent the smaller peaks in the spectrum (Fig. 5A). The large bands at around (+) 685–690 nm and (+) 500 nm are the so-called psi-type bands originating from the long-range interaction of the chromophores in large, chiral macrodomains (33). A smaller negative band at around 670 nm has the same origin. These CD bands are sensitive to the macro-organization of the thylakoid membrane (19, 34). Qualitatively, the spectra of npq4-1, wt and L17 are very similar (Fig. 5A) but there are small but significant differences, particularly with respect to this negative band at 670 nm. The wt-minus-npq4-1 spectrum shows that the wt exhibits a larger psi-type CD bands than the mutant, with peaks at (+) 685 nm and (~) 670 nm (Fig. 5B). In the spectrum of L17 the psi-type bands are larger than in the wt. Thus, a difference spectrum L17-minus-npq4-1 shows further enhancement, especially of the 670-nm band. The amplitude of the difference CD$_{685nm}$ - CD$_{670nm}$ is 4.29 ± 0.11, 4.97 ± 0.11, and 5.66 ± 0.18 for npq4-1, wt, and L17, respectively (± S.E.; the differences between npq4-1 and wt and between L17 and wt are significant with >99.9% confidence level by t test, n = 54 and 65, respectively). Clearly, the amplitude of the psi-type CD bands correlates with the amount of PsbS in the membrane.

The results of solubilization of grana membranes by mild detergent treatment have previously been used to assess the stability of the domains of LHCII-PSII in the grana membrane (35). Isolated stacked thylakoids were solubilized with 0.7% α-DM and the resultant macromolecular complexes separated by gel filtration using fast protein liquid chromatography (Fig. 6A). Several fractions were separated, the identities of which have been previously documented (24, 35). The first fraction contains the largest particles, which consist of PSII-enriched membrane fragments (I). This is followed in the gel filtration profile by the LHCII-PSII supercomplexes (II). In the npq4-1 mutant the ratio between the amplitudes of peak I and peak II is higher than that of the wt, whereas in the L17 PsbS overexpressor this ratio is lower than the wt. These differences were also observed when 1.0% α-DM was used for solubilization (Fig. 6B). There was a strong shoulder arising from peak I in npq4-1, but in the wt this shoulder was much weaker and in L17 not detectable. Thus the increase in level of PsbS correlated with the increase in extent to which LHCII-PSII supercomplexes were removed from the grana membranes.

**DISCUSSION**

The PsbS protein is an essential component of the regulatory machinery that allows the thylakoid membrane to dynamically respond to changes in the rate of light absorption by the reversible switching between the light harvesting and light dissipation modes of the PSII antenna. However, its mechanism of action has not been established. Previous work has focused on the notion that PsbS is the site of energy dissipation by a mechanism, which involves direct interaction of PsbS-bound zeaxanthin with antenna chlorophyll (12, 13). The main thrust of the experimental support for this model is the evidence that zeaxanthin is a direct quencher of excitation energy (36) and that rapidly reversible NPQ is inhibited when PsbS is absent (10). Here we have provided support for an alternative explanation for the mode of action of PsbS; i.e. it controls interactions between the components of the PSII light harvesting antenna in
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The influence of PsbS on the organization of PSII persists in the stacked membranes. The amplitude of the psi-type CD bands correlated with the amount of PsbS protein. This CD signal has been correlated with Mg$^{2+}$-dependent grana stacking (34) and with the accumulation of LHCII during chloroplast development (32). More specifically, it was recently shown to be associated with the presence of the C$_2$S$_2$M$_2$ macrostructure of the thylakoid membrane (19). The differences between L17, wt, and npq4-1 are only 7–8% of the CD signal. Clearly, in the absence of PsbS there is not a large-scale alteration in grana organization. Rather, it is suggested that the molecular interactions giving rise to the CD signal are enhanced by PsbS. Evidence to support differences in interactions in grana membranes also came from the observation of the differing sensitivity of the thylakoid membranes to solubilization by detergent. In the L17 membranes, PSII supercomplexes are more easily released from the membranes. This organizing function of PsbS does not depend upon the protein being active in qE. Point mutations and site-directed mutations in PsbS, which eliminate or reduce qE have no effect on this process. Although it could be argued that this indicates that these structural effects of PsbS are secondary ones, not related to NPQ, with the primary effect of PsbS being as providing the quenching site, we would argue oppositely. Thus, the ΔpH-dependent action of PsbS can be depicted as acting upon the structure of PSII antenna domain, triggering a change in LHCII interactions (Fig. 7). There is increasing evidence that qE proceeds via conformational change in the subunits of the PSII antenna (17, 18), and that interactions between these subunits are an integral part of the process (5, 15, 19). We therefore propose that protonation of PsbS drives the concerted conformational change in LHCII and thus the formation of qE. The two functional modes of PsbS function in Fig. 7 are hence both reflections of its LHCII-organizing properties.

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