THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 25, Issue of June 21, pp. 22750–22758, 2002

Printed in U.S.A.

Biochemical Properties of the PsbS Subunit of Photosystem II Either Purified from Chloroplast or Recombinant*

Received for publication, January 22, 2002, and in revised form, April 3, 2002
Published, JBC Papers in Press, April 4, 2002, DOI 10.1074/jbc.M200604200

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The biochemical properties of PsbS protein, a nuclear-encoded Photosystem II subunit involved in the high energy quenching of chlorophyll fluorescence, have been studied using preparations purified from chloroplasts or obtained by overexpression in bacteria. Despite the homology with chlorophyll *a*/*b*/xanthophyll-binding proteins of the Lhc family, native PsbS protein does not show any detectable ability to bind chlorophylls or carotenoids in conditions in which Lhc proteins maintain full pigment binding. The recombinant protein, when refolded *in vitro* in the presence of purified pigments, neither binds chlorophylls nor xanthophylls, differently from the homologous proteins LHCII, CP26, and CP29 that refold into stable pigment-binding complexes. Thus, it is concluded that if PsbS is a pigment-binding protein *in vivo*, the binding mechanism must be different from that present in other Lhc proteins. Primary sequence analysis provides evidence for homology of PsbS helices I and III with the central 2-fold symmetric core of chlorophyll *a*/*b*-binding proteins. Moreover, a structural homology owed to the presence of acidic residues in each of the two lumen-exposed loops is found with the dicyclohexylcarbodiimide/Ca^{2+}-binding domain of CP29. Consistently, both native and recombinant PsbS proteins showed [^{14}C]dicyclohexylcarbodiimide binding, thus supporting a functional basis for its homology with CP29 on the lumen-exposed loops. This domain is suggested to be involved in sensing low luminal pH.

Photosystem II (PSII)[1] of higher plants is a multisubunit membrane complex composed of many polypeptides that are encoded by the chloroplast or nuclear genes. Chloroplast gene products are located in the core complex where electron transport reactions are catalyzed, whereas the surrounding light-harvesting system is composed of nuclear-encoded chlorophyll *a*/*b*/xanthophyll proteins belonging to the Lhc family (1). Lhc proteins of PSII include Lhcb1–3 gene products that form heterotrimeric complexes (LHCII) located peripherally in the PSII·LHCII supercomplex and Lhcb4–6 proteins, which form a layer of monomeric subunits located between the core complex and trimeric LHCII. Besides the role of harvesting light and transferring excitation energy to the PSII reaction center (RC), Lhc proteins are involved in regulative mechanisms aimed at both the optimization of excitation energy distribution between PSI and PSII and the protection of the PSII RC from photoinhibition when absorbed light exceeds the electron transport capacity of the chloroplast. The former mechanisms acting at moderate to low light intensity include the reversible phosphorylation of LHCII, which leads to the detachment of phospho-LHCII from PSII and its migration to stroma membranes where it transfers energy to PSI (2). The mechanisms of protection from photoinhibition are elicited at high light intensity and include the reversible phosphorylation of Lhcb4 (CP29) (3) and the xanthophyll cycle-dependent non-photochemical energy quenching (NPQ). In excess light conditions, low luminal pH activates the enzyme violaxanthin de-epoxidase leading to the formation of zeaxanthin (4), which is found either free in the lipid phase (5) or bound to the Lhcb proteins (6, 7). Concomitantly, a strong quenching of chlorophyll fluorescence takes place, leading to the dissipation of up to 75% Chl*a* S1-excited states into heat (8). Fluorescence quenching is dependent on protonation events on the luminal side of thylakoid membranes, and it is inhibited by dicyclohexylcarbodiimide (DCCD), a protein-modifying agent that covalently binds to acidic residues in hydrophobic environment (9). DCCD binding sites have been localized on the lumen-exposed domains of Lhcb4 (10) and Lhcb5 (11).

Recently, a Lhc-like polypeptide has been shown to be essential for NPQ. The *npq-4 Arabidopsis thaliana* mutant deleted in the *psbS* nuclear gene is defective in qE. However, mutant plants are competent in violaxanthin de-epoxidation (12). Determination of the role of PsbS in qE appears to be an important step in the elucidation of the functional organization of PSII. Nevertheless, information on the biochemical properties of this protein is limited. Primary sequence analysis suggests four transmembrane helices and a strong homology with Chl*a*/*b*/xanthophyll-binding proteins at least in the helix A/B domains (13). A point of major interest is the pigment binding properties of PsbS. Chlorophyll and xanthophyll binding to PsbS was previously reported (14), thus suggesting together with its requirement for qE that this protein rather than CP29 or CP26 (6) is the site of ΔpH and xanthophyll-dependent excitation quenching (12). In this work, we have studied the biochemical properties of PsbS either purified from thylakoid membranes or overexpressed in *Escherichia coli* and refolded *in vitro* in the presence of pigments. Our results suggest that PsbS does not bind pigments, or the binding mechanism is very

* This work was supported by the grants from MURST-COFIN 2000 Program and the CNR Target Program in Biotechnology (to R. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: PSII, photosystem II; PSI, photosystem I; α(β)-DM, *n*-dodecyl-(α)β-D-maltopyranoside; LHCII, light harvesting complex of photosystem II; CP29, CP26, CP24, chlorophyll protein of 29, 26, 24 kDa, respectively; DCCD, dicyclohexylcarbodiimide; Lhc, light-harvesting complex; Chl, chlorophyll; NPQ, non-photochemical (energy) quenching; qE, energy-dependent quenching; RC, reaction center; MES, 4-morpholineethanesulfonic acid; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; IEF, isoelectric focusing.

different from that involved in pigment binding in other Lhc proteins. We found that both native and recombinant proteins bind DCCD, suggesting that its function may be the transduction of low luminal pH signal into a conformational change of neighbor chlorophyll-binding proteins where the quenching process could occur.

EXPERIMENTAL PROCEDURES

Isolation of PsbS from Spinach Thylakoids—Purification of native PsbS protein from spinach was performed according to Kim *et al.* (13) with minor modifications. After solubilization of G&Y PSII preparation (15) and centrifugation to recover solubilized material, the 22-kDa protein was purified by cation exchange chromatography (Source 15S, Amersham Biosciences) using a BIO-LOGIC fast protein liquid chromatography system (Bio-Rad). The column was preequilibrated with 20 mM MES, pH 6.0, 0.2 mM $CaCl_2$, 400 mM sucrose, and 0.3% β-DM. Most of the green material eluted in the void volume. PsbS was eluted with 1 M NaCl in the presence of 0.3% β-DM. Alternatively, PsbS was purified by flat-bed preparative IEF (16) with the exception that 0.1% β-DM rather than 0.06% was incorporated in the slurry.

BBY Particles and Thylakoid Membrane Preparation and Solubilization—BBY particles and thylakoids were prepared according to Berthold *et al.* (17) and Bassi *et al.* (18), respectively. Membranes corresponding to 500 μg of Chl were washed with 5 mM EDTA and then solubilized in 1 ml of 0.6% dodecyl-α-D-maltoside (α-DM), 10 mM Hepes, pH 7.5, by vortexing for 1 min. The solubilized samples were centrifuged at 15,000 × *g* for 10 min to eliminate unsolubilized material and then fractionated by ultracentrifugation in a 0.1–1 M sucrose gradient or 15–40% glycerol gradient containing 0.06% α-DM and 10 mM Hepes, pH 7.5, for 3.5–6.5 h at 480,000 × *g* in SW 60 rotor at 4 °C.

Sucrose/Lipids Gradient Ultracentrifugation—The PsbS fraction from fast protein liquid chromatography was concentrated, added to a mixture of 25 mg/ml PG:PC:PA (7:3:1 ratio, L-α-phosphatidyl-DL-glycerol, dipalmitoyl, L-α-phosphatidylcholine, L-α-phosphatidic acid, dipalmitoyl) and loaded on a sucrose gradient (0.1–1 M) containing 1 M NaCl, 0.2 mM $CaCl_2$, and 0.3% β-DM. The sample was ultracentrifuged for 15 h at 40,000 rpm in a SW 40 Beckman rotor.

Overexpression of PsbS cDNA in E. coli—A full-length cDNA coding for PsbS protein was obtained from the Arabidopsis Biological Resource Center, DNA Stock Center, The Ohio State University (Columbus, OH) (clone 137M5T7, GenBank™ accession number T45632). The coding region for the putative mature polypeptide was amplified by PCR (forward primer, TCGAGATCTGCAGCTCCTAAAAAGGTTGAG; reverse primer, AGCTTCGAATTCTTAGCTTTCTTCACCATC). The amplified region was cloned in the pBAD/His B (Invitrogen) expression vector containing a sequence encoding six histidines at the 5′ end of the polylinker. The recombinant protein was expressed in *E. coli* strain Top10 and purified from inclusion bodies with a nickel column (Chelating-Sepharose Fast Flow, Amersham Biosciences).

In Vitro Reconstitution of PsbS Protein—Recombinant PsbS was refolded *in vitro* in the presence of purified pigments (chlorophyll *a*, chlorophyll *b*, and carotenoids) as described previously (19). LHCII, CP29, and CP26 recombinant apoproteins were subjected to the same procedure as positive controls for reconstitution.

Pigment Analysis—Pigment analysis was performed by HPLC on a reversed-phase C_{18} bondclone column using a Beckman (Gold 126) system equipped with an 168-element diode array detection system (20).

Spectroscopic Measurements—Absorption spectra were measured on an Aminco DW 2000 spectrophotometer, and fluorescence emission spectra were measured on a Jasco spectrofluorimeter model FP 777. CD spectra were obtained with a Jasco J-600 spectropolarimeter as previously reported (21).

Dicyclohexylcarbodiimide Labeling—[^{14}C]DCCD from Amersham Biosciences (54 μCi/μmol in toluene) was dried under N_2 atmosphere and dissolved at 5 mM in absolute ethanol. Incubation of the samples was performed in 2 mM Tricine/NaOH, pH 7.5, containing 5 mM $MgCl_2$, 10 mM NaCl, and 0.06% β-DM. The final ethanol concentration was <1% by volume (DCCD <50 μM). Samples were incubated 10 min at room temperature and then concentrated for SDS-PAGE analysis on a 12% acrylamide gel. Radioactivity was revealed with a Packard imager either directly from the gel or after transferring the proteins to a nitrocellulose filter.

Electrophoresis and Immunoblotting—SDS-PAGE was performed using the Tris-Tricine buffer system (22) and the Tris sulfate buffer system (23). Non-denaturing PAGE using Deriphat-160 was performed according to Santini *et al.* (24). Immunoblotting was performed as reported previously (25) using a polyclonal antibody obtained in rabbit.

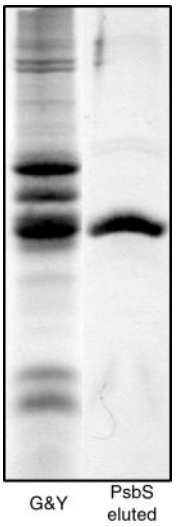

FIG. 1. **Purification of PsbS by cation exchange chromatography.** Coomassie Blue-stained SDS-PAGE of G&Y PSII preparation (*left*) and of the PsbS-enriched fraction eluted from the cation exchange chromatography (*right*).

RESULTS

Purification of PsbS from Thylakoid Membranes—To clarify whether or not PsbS is a pigment-binding protein, we have isolated this protein by several methods upon solubilization of the thylakoid membranes with mild detergents that were shown to preserve pigment binding to Lhc proteins homologous to PsbS.

In a first set of experiments, a PSII preparation enriched in PsbS (13, 15) was extracted with 0.3% β-DM, and the solubilized material was fractionated by cation exchange chromatography. PsbS efficiently bound to the column. Eluted fractions containing PsbS (Fig. 1) were pale green and after concentration were loaded into a sucrose gradient. Upon ultracentrifugation, the gradient was fractionated, and each fraction was assayed by SDS-PAGE and immunoblotting. PsbS was found in the bottom few fractions as a pellet, whereas the green material migrated at lower sucrose concentrations (Fig. 2). No pigments were associated with the pellet fraction, whereas the green material in the upper part of the gradient was associated with low levels of contamination by CP43 as detected by SDS-PAGE.

In an alternative approach, β-DM-solubilized G&Y particles were fractionated by flat-bed IEF in the pH range of 4–7 . After focusing overnight, the plate (21-cm long) was fractionated into 1-cm fractions, which were loaded into econo-run columns, and proteins therein were eluted with 50 mM Hepes, pH 7.6, + 0.1% β-DM. The distribution of PsbS was assayed by SDS-PAGE and immunoblotting. PsbS was found as a broad band (pI = 6.0–6.5) at ~1 cm from the application point (data not shown). Again, the PsbS-containing fractions were run in a sucrose gradient where the protein migrated in fractions 16–20 from the top out of 20. In the gradient, a pale green band extended through fractions 13–16. Absorption spectra of fraction 16 containing PsbS and fraction 15 without PsbS were essentially identical, thus suggesting that PsbS was not binding pigments (data not shown).

Although the data presented above did not suggest that PsbS may bind pigments, in these experiments the aggregation of the protein occurred at some extent. Lhc proteins analyzed thus far assume their final folding upon interaction with pigments, whereas the apoprotein form requires strong solubilizing conditions to maintain solubility. 2% SDS is required in order to solubilize Lhcb1 apoprotein, whereas upon refolding in

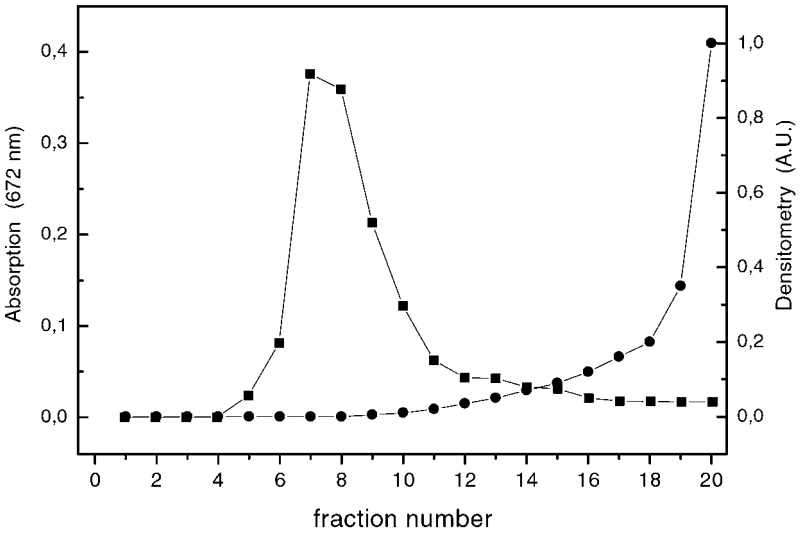

FIG. 2. **Fractionation of PsbS from cation exchange chromatography by sucrose gradient ultracentrifugation.** Distribution of PsbS polypeptide (●) and chlorophylls (■) on the sucrose gradient following ultracentrifugation. Each of the 20 fractions was analyzed by SDS-PAGE and densitometry for PsbS detection and by absorption spectroscopy (672 nm) for pigment detection.

the presence of pigments, Lhc pigment-protein complexes are soluble in dilute solutions of mild detergents (20). Therefore, the hypothesis could be considered that most of the PsbS protein as purified by chromatography or by IEF represented an aggregated and pigmentless form while it could possibly bind pigments in a non-aggregated form. To check this possibility, fractions from cation exchange chromatography were ultracentrifuged into a gradient (0.1–1 M sucrose, 0.3% β-DM, 1 M NaCl, 0.2 mM $CaCl_2$, 20 mM MES, pH 6.0) containing lipids and detergent in mixed micelles. This procedure was shown to avoid aggregation of highly hydrophobic membrane proteins, thus conserving their structure (26). Upon ultracentrifugation, the gradient was fractionated into 26 fractions (Fig. 3), each analyzed for the presence of PsbS and pigments. PsbS was found in fractions 7–10 without contamination from other proteins as assessed by SDS-PAGE (data not shown), thus showing that it was indeed possible to purify PsbS in a non-aggregated form while pale-green material migrated in fractions 2–4 and 19–20. Fractions 7–10 were analyzed by absorption and circular dichroism spectroscopy but did not reveal any optical activity in the visible range. Fractions 2–4 and 19–20 were analyzed by SDS-PAGE and did not contain polypeptides. Absorption and fluorescence spectra suggested that the *upper bands* contained free pigments, whereas the *lower band* contained aggregated pigments (data not shown).

An additional search for conditions leading to aggregation *versus* solubilization of PsbS was performed on a PSII preparation (G&Y) (15). Among other conditions, solubilization with 0.5% β-DM, 0.5% β-DM + 0.6% Zwittergent, 1% OGP + 0.1% SDS followed by centrifugation at 40,000 × *g* for 30 min yielded besides a green pellet containing unsolubilized green material, a clear supernatant containing PsbS. When 2 mM $CaCl_2$ was added during solubilization, PsbS was severely depleted in the supernatant and was instead found in the pellet (Fig. 4*A*). In an attempt to verify pigment association with PsbS, supernatants were analyzed by non-denaturing Deriphat PAGE (Fig. 4*B*). Although distinct green band patterns were obtained from extracts with different detergents, no difference was detected between samples obtained in the presence or in the absence of Ca^{2+}, thereby containing very different levels of PsbS. When gel lanes were excised and a second dimension separation by denaturing SDS-PAGE was applied, PsbS was found in all cases as a long trail extending from apparent molecular mass of 20–200 kDa of the first dimension, whereas other thylakoid proteins mostly appeared as well defined spots (Fig. 4*C*). It is worth noting that a 9-kDa polypeptide (Fig. 4C, *asterisk*) shows

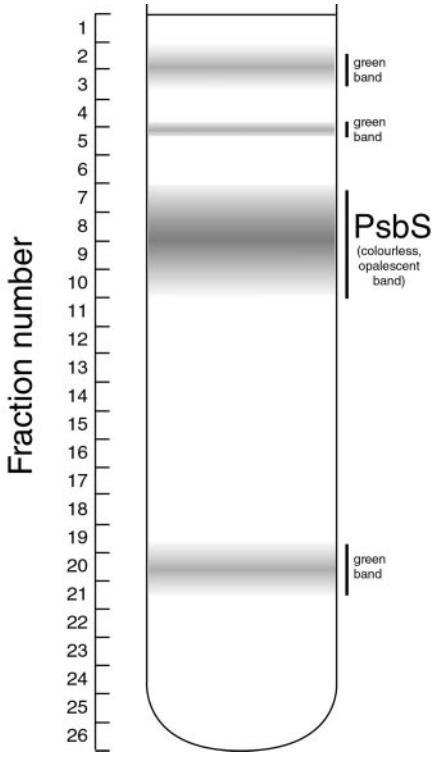

FIG. 3. **Detergent/lipids sucrose density gradient profiles of PsbS fraction.** Upon ultracentrifugation, the gradient was fractionated into 26 fractions and analyzed for the presence of PsbS and pigments. PsbS was found in a non-aggregated form in colorless opalescent fractions 7–10 without contamination, whereas pale-green material in fractions 2–4 and 19–20 contained free or aggregated pigments, respectively.

the same distribution as PsbS in the two-dimensional gel, thus strongly suggesting the two polypeptides form a complex in the first dimension.

Recombinant Proteins—Lhc proteins can be refolded *in vitro* in the presence of purified pigments yielding pigment-protein complexes indistinguishable from those purified from thylakoid membranes (20, 27, 28). In the attempt of further verifying the possibility of PsbS being a pigment-binding protein, we overexpressed PsbS from *A. thaliana* in *E. coli* in the form of the mature protein lacking the transit peptide with a six-histidine tail at the N terminus to assist in purification. Inclusion bodies of PsbS, purified from *E. coli* transformed with the DNA en-

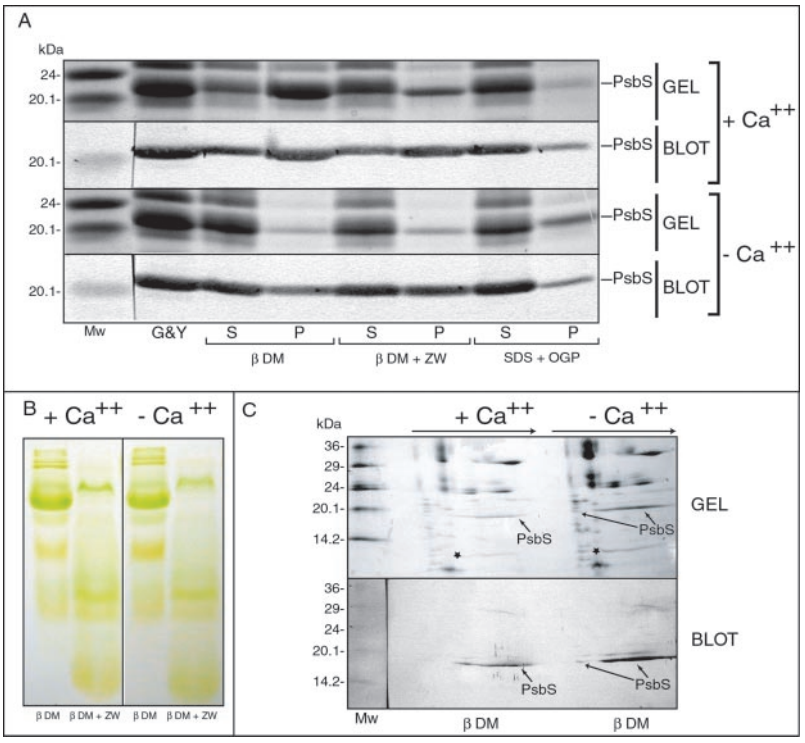

FIG. 4. **Solubilization of G&Y preparation.** *A*, Coomassie Blue-stained SDS-PAGE of solubilized G&Y PSII preparation (*GEL*) and immunoblotting of the same gel probed with antibodies against PsbS (*BLOT*). Different conditions of solubilization were tested (β-DM = 0.5% β-DM; β-DM + ZW = 0.5% β-DM + 0.6% Zwittergent; SDS + OGP (*n*-octyl-β-D-glucopyranoside) = 0.1% SDS + 1% OGP) in presence of $CaCl_2$ (+ Ca^{2+}) or not (−Ca^{2+}). *S*, supernatant; *P*, pellet of a 15,000 × *g* centrifugation. *B*, non-denaturing Deriphat PAGE of the supernatants of two different solubilizations of G&Y preparation in the presence or absence of Ca^{2+} showing no difference among samples containing different amounts of PsbS. *C*, two-dimensional separation of the proteins present in the gel lanes of β-DM-solubilized G&Y (*panel B*) by SDS-PAGE (Coomassie Blue-stained GEL) and immunoblotting of the same gel probed with antibodies against PsbS (BLOT). The *arrows* above the gels show the migration direction in the first dimension. PsbS was found in all cases as a long stream extending from 20 to 200 kDa, whereas the other thylakoid proteins mostly appeared as well defined spots. A 9-kDa polypeptide (*asterisk*) shows the same distribution as PsbS in the two-dimensional gel thus suggesting that the two polypeptides form a complex in the first dimension.

coding the putative mature protein cloned in pBAD/His vector, were solubilized in SDS-urea and loaded into a Ni^{2+}-Sepharose column (Amersham Biosciences). Upon washing unbound material, it was possible to elute recombinant PsbS with 0.1 M imidazole without any contamination. The protein, analyzed by SDS-PAGE, exhibited an apparent MW of 24 kDa, slightly higher than the corresponding protein in thylakoid membranes consistent with its six-histidine extension and spacer sequence (see Materials and Methods). PsbS bound to Ni^{2+} column was subjected to the reconstitution according to described procedures (19). A control experiment was also performed by using the homologous protein Lhcb1. Upon washing the excess pigments from the column, PsbS (and Lhcb1) proteins were eluted with imidazole and loaded into a sucrose gradient. Ultracentrifugation yielded a sharp green band in the case of Lhcb1, whereas the tube loaded with PsbS showed diffuse green material but not defined bands. Fractionation of the gradient and detection of PsbS showed that most of the protein was in the pellet, whereas a minor fraction migrated in the lower part of the gradient where no pigments were detected (Fig. 5*A*).

An additional experiment was performed to verify the possibility that PsbS could interact with other Lhcb proteins during folding. A mixture of PsbS, CP29 and CP26 in equimolar amounts was refolded *in vitro* in the presence of lipids following the procedure described by Giuffra *et al.* (20). Because PsbS had a His tail, whereas CP26 and CP29 did not, an establishment of stable interactions with CP26 and/or CP29 would have been detected by the formation of complexes retained in the Ni^{2+} column. This procedure yielded well reconstituted CP26 and CP29 in the flow-through of the Ni^{2+} column and unpigmented material in the imidazole-eluted material. After sucrose gradient ultracentrifugation, PsbS was found in the lower fractions in a non-pigmented form (Fig. 5*B*).

Accumulation of PsbS in Reaction Center and Antenna Mutants—The results described above strongly suggest that PsbS, despite its homology with Lhc proteins, is not a pigment-binding protein. Its activity of quenching Chl fluorescence (12) must therefore be obtained by an indirect mechanism. One possibility is that PsbS could perform its physiological function by interacting with neighbor PSII Chl-binding proteins.

To obtain information on the localization of PsbS within the PSII supramolecular complex, we have used two different approaches. First, we checked the accumulation of the protein in a series of barley mutants affected in either PSI, PSII, or Lhc proteins. Second, we evaluated the presence of PsbS in a series of PSII-LHCII supercomplexes with different antenna size.

Mutations affecting accumulation of one subunit of PSI, PSII, Cyt b6/f, or ATPase complexes prevents accumulation of the others subunits of the same complex (29). Therefore, we reasoned that if PsbS is a unique PSII subunit, its accumulation would be affected by mutations, preventing the accumulation of PSII core complex. On the contrary, Lhc proteins accumulates independently from each other (30). Fig. 6*A* shows the results of probing with α-PsbS antibodies a series of barley mutants affected in PSI, PSII, or Lhc proteins accumulation. Clearly, it appears that this subunit is present in all the genotypes analyzed, thus suggesting that PsbS belong to the antenna system rather than to PSII core. Further screening of six chlorina mutants of barley (31) showed that although in each mutant the level of several Lhc polypeptides was severely af-

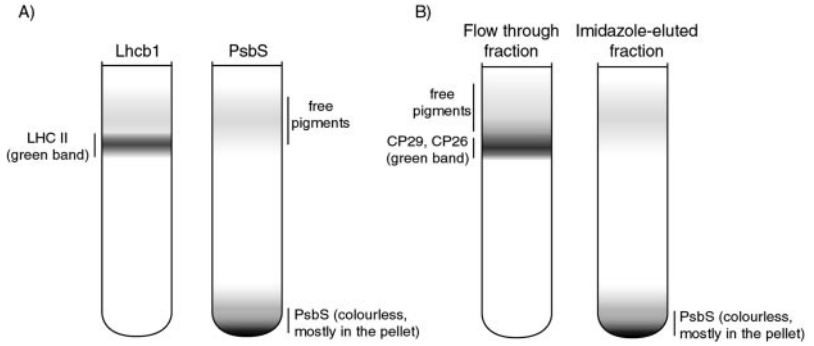

FIG. 5. **Sucrose density gradient profiles of *in vitro* reconstitution.** *A*, the reconstitution protocol was effective in the case of Lhcb1 giving a sharp *green band* in the gradient (*left*), whereas in the case of PsbS (*right*), a diffuse band of free pigments was present at the top of the gradient. The PsbS protein was found in the unpigmented pellet. *B*, sucrose gradients showing the results of co-reconstituting experiment of PsbS with CP29 and CP26. The recombinant PsbS protein, presenting a six-histidine tail, was bound efficiently to the Nickel column and eluted with 0.1 M imidazole without pigments, whereas reconstituted CP29 and CP26 were found in the flow-through fraction.

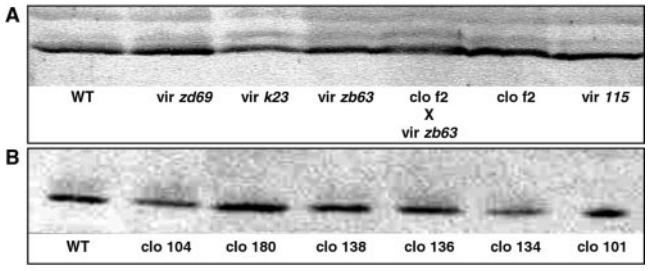

FIG. 6. **Accumulation of PsbS in reaction center and antenna mutants.** *A*, immunoblotting with antibodies against PsbS of barley mutants affected in either PSI (*vir k23*, *vir zb63*), PSII (*vir zd69*, *vir k23*, *vir 115*), or Lhc complexes (*clo f2*). *B*, immunoblotting of chlorina mutants of barley showed that although the level of more than one Lhc protein in each mutant was severely decreased, PsbS accumulation was very similar to wild-type level (each lane was loaded with thylakoid membranes corresponding to 5 μg of Chl).

fected, PsbS accumulation was very similar to wild-type levels. This effect was also observed in the mutants affected in the Lhcb subunits enriched in the G&Y preparation, *i.e.* CP29 and/or CP26 (Fig. 6*B*).

Occurrence of PsbS in Supramolecular PSII·LHCII Complexes of Different Antenna Content—It was previously reported that PsbS was lost during the preparation of PSII supercomplexes (32, 33). However, we found that when the α isomer rather than the β isomer of DM was used during solubilization of BBY particles, PsbS was retained (Fig. 7*A*). Moreover, when PSII supramolecular complexes with different antenna proteins content were analyzed, we found that the PsbS content decreased less with respect to Lhcb proteins in supercomplexes when antenna size was decreased. This finding suggests a stronger association between PsbS and the inner part of the supercomplex, either PSII core or CP29/CP26 (see Table I). Additional support to the idea of an association with PSII core can be obtained from the analysis of Fig. 4*C* in which a two-dimensional analysis of G&Y preparation (where most of LHCII was removed from PSII membranes while CP29 and CP26 were retained together with PSII core) was shown. Immunoblotting of two-dimensional gels showed that PsbS was retained in fractions resolved in the upper region of the green gel of Fig. 4*B* containing PSII core together with CP29 and CP26 and lacking most of LHCII. Although most of PsbS migrated as a smeared band in the medium to the low molecular weight range (*right*) of the gel (Fig. 4*C*), the Coomassie Blue stain and immunoblotting of the second dimension of the green gel clearly showed the association of PsbS to high molecular weight PSII core complexes from which LHCII was removed. When thylakoids or PSII membranes were fractionated by sucrose gradient ultracentrifugation upon solubilization with α-DM, PsbS was found not only in the lowest part of the gradient containing PSII·LHCII supercomplexes but also in fractions containing PSII core complex and the CP29·CP24·LHCII complex (34) as revealed by immunoblotting (data not shown). This finding could either be explained by a diffuse distribution of PsbS through the gradient because of aggregation similar to its appearance in the green gels of Fig. 4*C* or to a genuine association to other PSII components. To check this point, we harvested 14 fractions from the bottom of a gradient tube, and each fraction was analyzed for pigment composition, absorption spectroscopy, SDS-PAGE, and immunoblotting with PsbS antibodies (Fig. 7*B*). It clearly appears that PsbS is located in the bottom (pellet) fraction containing the PSII·LHCII complex and also into two bands at lower density, one corresponding to monomeric antenna proteins and the second corresponding to overlapping fractions containing PSII core and CP29·CP24·LHCII supercomplex.

Detection of Proton Active Residues in PsbS—The formation of qE is obligatorily dependent on the presence of a proton gradient across the thylakoid membrane (35). The triggering of NPQ is blocked by the protein-modifying agent DCCD, which binds to acidic residues in hydrophobic environment (36). The recent finding that PsbS is essential for NPQ (12), together with the presence of six acidic residues exposed on the luminal side of the membrane, suggests that protonation might be a step in PsbS function. To test this hypothesis, we have treated PsbS either extracted from G&Y particles or produced by overexpression in *E. coli* and subjected to refolding in the Ni^{2+} column to [^{14}C]DCCD in conditions leading to specific labeling (10). Fig. 8 shows the SDS-PAGE analysis of G&Y particles mildly solubilized with 0.5% α-DM and centrifuged to eliminate unsolubilized material and shows the analysis of the recombinant PsbS preparation. Notice that recombinant PsbS shows a somewhat higher apparent MW with respect to native PsbS in G&Y particles, consistent with its His tail and spacer extension. The position in the gel of native PsbS is indicated by an *arrow* on the left side of the gel as detected by immunoblotting (data not shown). Recombinant CP29 was also included as a positive control of DCCD binding (10). Both CP29 and PsbS were labeled by DCCD, and bands with mobility in the gel corresponding to native CP29 and PsbS were also detectable in the autoradiogram of solubilized G&Y particles, although less clearly because of the higher background.

DISCUSSION

To gain information on the role of PsbS in thermal dissipation of excitation energy (12), its ability of binding pigments is particularly interesting. If PsbS is a Chl-binding protein, a

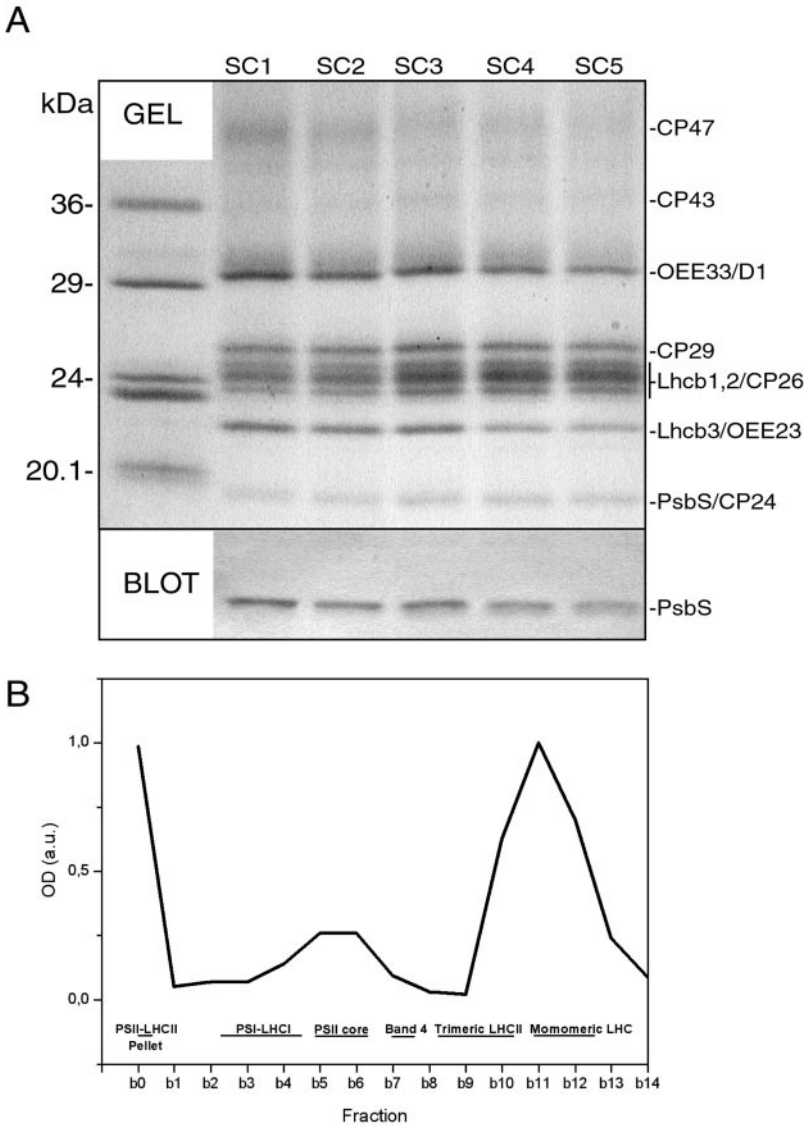

FIG. 7. **Occurrence of PsbS in different preparations.** *A*, SDS-PAGE and immunoblotting of different PSII·LHCII supercomplexes isolated by sucrose gradient ultracentrifugation of α-DM-solubilized PSII membranes. The Chl*a*/*b* and PsbS/Lhcb protein ratios are indicated in Table I. *SC*, supercomplex. *B*, distribution of PsbS in fractions from glycerol gradient ultracentrifugation of thylakoid membranes solubilized with 0.6% α-DM. Fractions are numbered from the bottom of the gradient (*b0* is the pellet). The PsbS content in each fraction was assayed by densitometry of the filter following a SDS-PAGE and immunoblotting. The localization of different thylakoid complexes is indicated as determined by SDS-PAGE, immunoblotting, and absorption spectroscopy (data not shown). *Band 4* is the CP29·CP24·LHCII supercomplex.

TABLE I
Characteristics of PSII · LHCII supramolecular complexes purified by sucrose gradient ultracentrifugation of α-DM solubilized PSII membranes

Fractions are numbered according to their mobility from the top to the bottom of the sucrose gradient. The PsbS content in each supercomplex is calculated by densitometry of the immunoblotting (not from the Coomassie-Blue-stained SDS-PAGE attributed to the superimposition of PsbS with CP24). The PsbS/Lhcb protein ratio is normalized to one for the higher molecular weight supercomplex (SC5). SC, supercomplex.

	Chl*a*/*b* ratio	PsbS/Lhcb proteins ratio
SC1	4.3	2.25
SC2	3.3	1.75
SC3	2.7	1.60
SC4	2.3	1.41
SC5	2.1	1

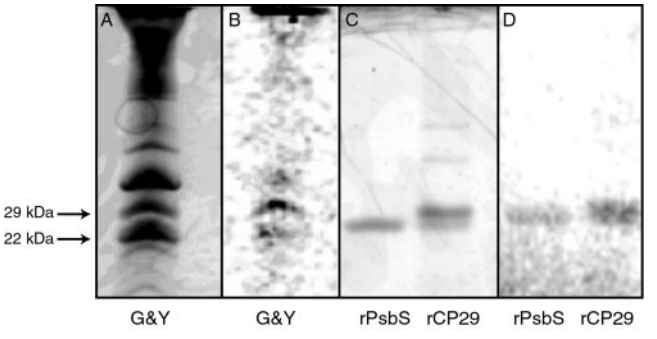

FIG. 8. **[^{14}C]DCCD binding to PsbS.** *A*, Coomassie Blue-stained SDS-PAGE of [^{14}C]DCCD-labeled G&Y preparation. *B*, autoradiogram. *C*, Coomassie Blue-stained SDS-PAGE of [^{14}C]DCCD-labeled recombinant PsbS (*rPsbS*) and CP29 refolded *in vitro*. *D*, autoradiogram of the gel. Notice that recombinant PsbS shows a somewhat higher apparent MW with respect to native PsbS in G&Y particles consistent with its His-tail and spacer extension.

quenching center could be formed within the protein itself and the excitation energy could be transferred from the surrounding Lhc and/or PSII core Chl-binding proteins through pigment-pigment interactions, similar to those involved in excitation energy transfer to PSII RC for catalysis of charge separation. If PsbS is not a pigment-binding protein, the alternative mechanisms of quenching should be considered, *e.g.* the possibility that PsbS could act as a signal transduction component able to undergo conformational changes upon protonation on its lumen-exposed domains and transfer conformational information to neighbor Chl-binding proteins where the actual quencher would be formed.

PsbS Does Not Bind Pigments Tightly—We have investigated the possibility that PsbS, isolated from either thylakoid membranes or from overexpression in *E. coli* and subsequent *in vitro* refolding, binds pigments similarly to other members of

the Lhc protein family. In a first approach, PsbS was purified either by cation exchange chromatography or by preparative flat-bed IEF followed by sucrose gradient ultracentrifugation in the presence of mild detergents to purify thylakoid membrane proteins. In both cases, the first purification step yielded a preparation containing small amount of pigments; however, further fractionation by sucrose gradient ultracentrifugation separated pigments and proteins into different fractions. In the few cases in which PsbS-containing fractions were green, the presence of pigments was due to contamination by PSII core subunits or by free pigments as judged by absorption and CD spectra.

Because the purification procedures induced partial aggregation of PsbS, the possibility could be considered that aggregation was secondary to a denaturation process proceeding through the loss of pigments and exposure of hydrophobic protein domains. Thus, we explored conditions in which PsbS could be recovered in unaggregated form. Ultracentrifugation in a detergent/lipid sucrose gradient prevented aggregation, but no pigment binding was detected. Similar results were obtained by solubilizing G&Y particles by a combination of detergents. The addition of Ca^{2+} lead to extensive aggregation of PsbS, whereas the same detergents without Ca^{2+} were effective in solubilizing PsbS. In solubilized G&Y, PsbS is a major component because its abundance is similar to CP29 as judged from Coomassie Blue stained gel. If PsbS was a pigment-binding protein, the differences would have been detected in the green band pattern obtained by Deriphat PAGE when comparing gel lanes loaded with supernatant fractions enriched or depleted in PsbS (plus Ca^{2+} *versus* $-Ca^{2+}$). Nevertheless, no differences in either the mobility or the relative intensity of green bands could be detected not only by visual inspection but also by densitometry (data not shown), thus strongly suggesting that no pigments were bound to PsbS extracted from thylakoids.

Since the first report of *in vitro* refolding of Lhcb proteins (27), the procedure has been improved to obtain pigment proteins indistinguishable from their counterparts purified from membranes and extended to all the members of the protein family including Lhcb1, CP29 (20), CP26 (37), CP24 (38), Lhca1, and Lhca4 (39). In some instances, owing to the relative harshness of the purification procedure and lability of pigment binding, recombinant proteins were shown to be more stable than the same protein purified from thylakoids. This was the case of CP24. The purified protein was reported earlier to bind five Chls/polypeptide (40), whereas the recombinant protein refolded *in vitro* has been shown to bind 10 Chls/polypeptide (38). PsbS was expressed in *E. coli* and subjected to the *in vitro* refolding procedure in the presence of purified pigments (19) in conditions that are effective in producing pigment proteins with Lhcb1. Nevertheless, no pigments were found to be bound to this protein.

An additional experiment was performed to investigate the possibility of PsbS binding its hypothetical pigment complement in cooperation with neighbor Lhc proteins CP29 and CP26. *In vitro* reconstituted Lhc proteins can form both homo and hetero oligomers (39, 41), inducing modification in their spectroscopic properties. PsbS did not establish interactions with other Lhcb proteins stable enough to be detected by our methods.

Following *in vitro* refolding, PsbS behaves similarly to the protein purified from membranes as judged by its recovering in sucrose gradient, partially in a detergent soluble form in the lower part of the gradient and partially in the pellet. It is worth noticing that rPsbS acquired its DCCD binding capacity only if submitted to the refolding procedure. No labeling was obtained with solubilized inclusion bodies, suggesting that the refolding procedure was actually effective with PsbS, although this does not imply pigment binding. This view is supported by the report on another DCCD-binding protein, CP29 (10), which belongs to Lhc family. In this case, refolding *in vitro* could be easily followed by pigment binding which appeared together with DCCD binding (10). This finding suggests that the recombinant protein assumes a conformation similar to that in thylakoids, even in the absence of bound chlorophyll and/or carotenoids.

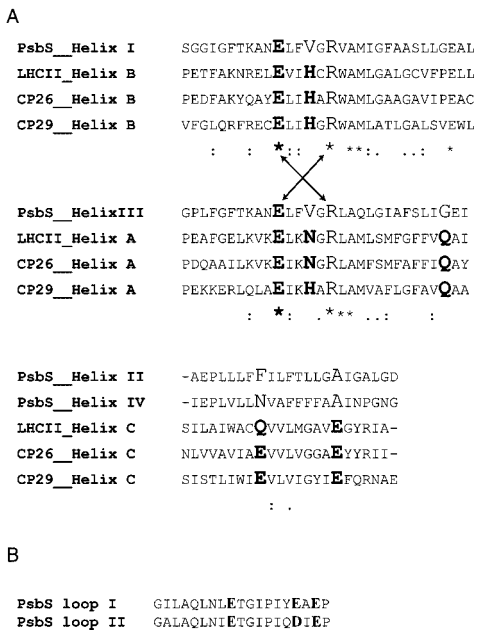

FIG. 9. **Sequence and structural homology of PsbS.** *A*, sequence alignment of transmembrane regions of PsbS, LHCII, CP29, and CP26. The *first* and the *third helices* of PsbS display significant homologies with the correspondent Lhc regions including conservation of the Glu/Arg ionic pairs (*underlined*) (ionic pair interaction is indicated by *arrows*) involved in the stabilization of the central helix cross-domain. Six residues shown to bind chlorophyll (*bold*) in Lhc proteins are not conserved. *, identity; **:**, strong homology; ., weak homology. *B*, sequences of the two luminal loops of PsbS. The presence of three acidic residues similar as in the Ca^{2+}/DCCD-binding loop of CP29 may suggest similar function.

Pigment binding to PsbS was previously hypothesized on the basis of its homology with Lhc proteins (13, 42) followed by experimental report of both Chl*a* and Chl*b* binding (14). Our findings contrast with this report. Not only were we unable to show pigment binding by using both the same techniques described in Funk *et al.* (14) and other methods as well, the use of *in vitro* refolding, a procedure that was effective in reconstituting pigment binding in many Lhc proteins (actually in all Lhcb and Lhca proteins so far described), was ineffective.

Fig. 9*A* shows the partial alignment of PsbS sequence with the transmembrane regions of CP29, CP26, and LHCII. The first and the third helices display significant homology. The Glu/Arg ionic pairs involved in the stabilization of the central cross in the structure of LHCII and in the binding of two chlorophylls are conserved. However, six residues shown to bind chlorophyll in both CP29 (43) and LHCII (21) are not conserved. Also, four carotenoid binding sequences identified in the extrinsic loops of LHCII at each side of the transmembrane helices (44) are not conserved in PsbS.

We conclude that PsbS is either not a pigment-binding protein or its interaction with pigments is of a different nature with respect to that exhibited by the Lhca1–4 and Lhcb1–6 gene products. We cannot exclude that an interaction with pigments exists in the thylakoid membrane; however, if this is

the case, the interaction must be much weaker and of a different nature with respect to that present in the other Lhc proteins. In particular, although Lhc proteins depend on pigment binding for their folding, PsbS seems not to do so because DCCD binding was reconstituted even in the absence of pigments. Our results are consistent with the previous finding that PsbS is stable in etiolated leaves (45), which do not contain chlorophyll. The previous report of pigment binding to PsbS differs from our present results only with respect to the extent of co-purification of pigments with the PsbS protein, whereas many of the results in Funk *et al.* (14) are consistent with the fact that pigments are not bound to the protein. In particular there was no evidence for Chl*b* to Chl*a* singlet energy transfer, no circular dichroism spectrum (14), and no triplet energy transfer.[2] We suggest that the contamination of the PsbS protein preparation by pigments was caused by a modification introduced by Funk *et al.* (14) in the purification method, consisting in the omission of the sucrose gradient ultracentrifugation step suggested by the original report of the IEF technique (16). As a result, free pigments in the detergent solution were not separated from the co-eluted PsbS protein. An application of the sucrose gradient ultracentrifugation step resulted into the separation of a free-pigment band from the PsbS-containing fractions.

DCCD Binding Supports the Proton-sensing Role for PsbS—Pigment binding is not the only function of Lhc proteins. Members of this protein family may have either additional/alternative functions besides to chlorophyll *a/b* and carotenoid binding. In this context, the recent result that CP29 is a Ca^{2+}-binding protein (46) is of particular relevance. It was found that DCCD displaces Ca^{2+} from its binding site, and yet Chl binding was not affected (46), suggesting that pigment binding and Ca^{2+}/DCCD-binding domains were at least in part distinct in CP29. Acidic residues binding Ca^{2+} in CP29 have been localized in the luminal loop of CP29. This domain shows three acidic residues, a characteristic shared with the two luminal loops of PsbS (homologous to each other), thus suggesting similar function (Fig. 9*B*). Although we did not perform $^{45}Ca^{2+}$-binding experiments, the finding of DCCD binding by PsbS is a strong indication that this protein has two functional domains similar to those characterized as DCCD/Ca^{2+} binding in CP29. Thus, PsbS appears to be a Lhc-type protein specialized in the function catalyzed by the Ca^{2+}/DCCD-binding domain.

The function of DCCD binding domains of Lhc proteins is not completely clear. It has been proposed that reversible protonation of acidic residues induces conformational changes triggering NPQ (11). Consistently, DCCD has been shown to inhibit the Δ535-nm absorption signal associated with a pH-induced conformational change (47, 48). The 535-nm absorption change is missing in the *Arabidopsis thaliana npq-4* mutant, and yet CP26 and CP29 are not affected in this mutant (12). This finding suggests that the conformational changes within the thylakoid membrane associated with NPQ are induced by protonation of PsbS.

Our finding that PsbS does not stably bind pigments implies that quenching must be induced through an indirect mechanism. One possibility is that pigments are reversibly bound to PsbS and that the quenching function is catalyzed by the hypothetical pigment binding form present in the thylakoids but not resistant to purification. An alternative possibility is that one or more chlorophyll-binding proteins are involved other than PsbS where actual quenching of Chl-excited states can occur. In this context, it is relevant to determine the identity of the nearest neighbors to PsbS. The extraction of G&Y membranes with mild detergents preserve in part the interaction of PsbS with PSII supramolecular complex as detected by two-dimensional Deriphat PAGE (Fig. 4*C*). Previous determinations showed low levels of PsbS in these preparations obtained with β-DM (32, 33, 50, 51), suggesting that this subunit is not firmly bound to other PSII components and can be easily removed. However, our results obtained by using the α isomer of DM show that not only is PsbS a component of the PSII·LHCII supercomplex, its concentration increases when PSII core to LHCII ratio is higher. On the other hand, PsbS accumulated at normal levels in PSII RC mutants, thus suggesting it is not a genuine PSII core subunit but rather an antenna component. In fact, Lhc mutants did not show pleiotropic effects, thus allowing accumulation of Lhc complexes independently from each other (30). Because PsbS is enriched in the G&Y preparation containing PSII core and the inner layer of Lhcb proteins, namely CP29 and in part CP26, our data are thus consistent with the localization of PsbS outside of PSII core complex, possibly in a region where PSII core and CP29 interact. It cannot be excluded that PsbS has more than one possible location within the PSII·LHCII supercomplex.

Lifetime fluorescence analysis of CP29, CP26, CP24, and LHCII (52–54) showed that Lhc proteins in detergent solution are found in two different conformations characterized by either low or high fluorescence yield, the quenched conformation being favored by zeaxanthin binding to xanthophyll site L2 (53, 54). PsbS could possibly act by detecting low luminal pH through lumen-exposed DCCD-binding domains and undergo conformational change. Although PsbS did not bind pigments *in vitro*, it is well possible that newly formed zeaxanthin might bind to PsbS and contribute to the stabilization of a new conformation that is transferred to neighbor chlorophyll proteins. Recent work by Resonance Raman Spectroscopy supports this possibility (55). These proteins in turn could be induced to change their conformation into a quenched state. Quenching could be amplified through the establishment of new protein-protein interactions within the lipid membrane (49) as recently shown in a reconstituted system (54).

In this paper, we show that the PsbS protein binds the NPQ inhibitor DCCD, and that it either does not bind pigments or the binding is much less stable than in the case of other members of the Lhc protein family. Moreover, pigment binding is not indispensable for the refolding of the protein *in vitro*. This information is important for the understanding of the role of PsbS on the mechanism of NPQ. Further studies are needed to test the different hypotheses consistent with these findings and particularly if PsbS itself catalyzes the ^{1}Chl*a**-quenching reaction. Alternatively, PsbS could be a component of a signal transduction device sensing low luminal pH, thus inducing a conformational change in chlorophyll-binding proteins to a quenching state as recently suggested (54).

Acknowledgments—We thank Roberto Barbato (University of Padua) for the kind gift of the anti-PsbS antibody, Dr. Jan Dekker (Vrjie University, Amsterdam) for helpful discussions and for sharing unpublished results, and Alexandre Galiotto for technical collaboration.

REFERENCES

1. Jansson, S. (1999) *Trends Plant Sci.* **4,** 236–240
2. Allen, J. F. (1992) *Biochim. Biophys. Acta* **1098,** 275–335
3. Bergantino, E., Dainese, P., Cerovic, Z., Sechi, S., and Bassi, R. (1995) *J. Biol. Chem.* **270,** 8474–8481
4. Yamamoto, H. Y., Nakayama, T. O. M., and Chichester, C. O. (1962) *Arch. Biochem. Biophys.* **97,** 168–173
5. Havaux, M., and Niyogi, K. K. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 8762–8767
6. Bassi, R., Pineau, B., Dainese, P., and Marquardt, J. (1993) *Eur. J. Biochem.* **212,** 297–303
7. Ruban, A. V., Young, A. J., Pascal, A. A., and Horton, P. (1994) *Plant Physiol.* (*Bethesda*) **104,** 227–234
8. Demmig-Adams, B., and Adams, W. W. (1992) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **43,** 599–626

[2] Dekker, J., personal communication.

9. Ruban, A. V., Walters, R. G., and Horton, P. (1992) *FEBS Lett.* **309,** 175–179
10. Pesaresi, P., Sandona, D., Giuffra, E., and Bassi, R. (1997) *FEBS Lett.* **402,** 151–156
11. Walters, R. G., Ruban, A. V., and Horton, P. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 14204–14209
12. Li, X. P., Bjorkman, O., Shih, C., Grossman, A. R., Rosenquist, M., Jansson, S., and Niyogi, K. K. (2000) *Nature* **403,** 391–395
13. Kim, S., Sandusky, P., Bowlby, N. R., Aebersold, R., Green, B. R., Vlahakis, S., Yocum, C. F., and Pichersky, E. (1992) *FEBS Lett.* **314,** 67–71
14. Funk, C., Schröder, W. P., Napiwotzki, A., Tjus, S. E., Renger, G., and Andersson, B. (1995) *Biochemistry* **34,** 11133–11141
15. Ghanotakis, D. F., Babcock, G. T., and Yocum, C. F. (1984) *Biochim. Biophys. Acta* **765,** 388–398
16. Dainese, P., Hoyer-hansen, G., and Bassi, R. (1990) *Photochem. Photobiol.* **51,** 693–703
17. Berthold, D. A., Babcock, G. T., and Yocum, C. F. (1981) *FEBS Lett.* **134,** 231–234
18. Bassi, R., Rigoni, F., Barbato, R., and Giacometti, G. M. (1988) *Biochim. Biophys. Acta* **936,** 29–38
19. Rogl, H., Kosemund, K., Kuhlbrandt, W., and Collinson, I. (1998) *FEBS Lett.* **432,** 21–26
20. Giuffra, E., Cugini, D., Croce, R., and Bassi, R. (1996) *Eur. J. Biochem.* **238,** 112–120
21. Remelli, R., Varotto, C., Sandona, D., Croce, R., and Bassi, R. (1999) *J. Biol. Chem.* **274,** 33510–33521
22. Schägger, H., and von Jagow, G. (1987) *Anal. Biochem.* **166,** 368–379
23. Bassi, R., Machold, O., and Simpson, D. (1985) *Carlsberg Res. Commun.* **50,** 145–162
24. Santini, C., Tidu, V., Tognon, G., Magaldi, A. G., and Bassi, R. (1994) *Eur. J. Biochem.* **221,** 307–315
25. Di Paolo, M. L., Peruffo dal Belin, A., and Bassi, R. (1990) *Planta* **181,** 275–286
26. Gabellini, N., Gao, Z., Eckerskorn, C., Lottspeich, F., and Oesterhelt, D. (1988) *Biochim. Biophys. Acta* **934,** 227–234
27. Plumley, F. G., and Schmidt, G. W. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 146–150
28. Paulsen, H., and Hobe, S. (1992) *Eur. J. Biochem.* **205,** 71–76
29. Wollman, F.-A., Minai, L., and Nechushtai, R. (1999) *Biochim. Biophys. Acta* **1411,** 21–85
30. Bossmann, B., Knoetzel, J., and Jansson, S. (1997) *Photosynth. Res.* **52,** 127–136
31. Simpson, D. J., Machold, O., Hoyer-Hansen, G., and von Wettstein, D. (1985) *Carlsberg Res. Commun.* **50,** 223–238
32. Hankamer, B., Nield, J., Zheleva, D., Boekema, E., Jansson, S., and Barber, J. (1997) *Eur. J. Biochem.* **243,** 422–429
33. Harrer, R., Bassi, R., Testi, M. G., and Schäfer, C. (1998) *Eur. J. Biochem.* **255,** 196–205
34. Bassi, R., and Dainese, P. (1992) *Eur. J. Biochem.* **204,** 317–326
35. Briantais, J.-M., Vernotte, C., Picaud, M., and Krause, G. H. (1979) *Biochim. Biophys. Acta* **548,** 128–138
36. Hoppe, J., Schairer, H. U., and Sebald, W. (1980) *Eur. J. Biochem.* **112,** 17–24
37. Ros, F., Bassi, R., and Paulsen, H. (1998) *Eur. J. Biochem.* **253,** 653–658
38. Pagano, A., Cinque, G., and Bassi, R. (1998) *J. Biol. Chem.* **273,** 17154–17165
39. Schmid, V. H. R., Cammarata, K. V., Bruns, B. U., and Schmidt, G. W. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 7667–7672
40. Dainese, P., and Bassi, R. (1991) *J. Biol. Chem.* **266,** 8136–8142
41. Kuttkat, A., Hartmann, A., Hobe, S., and Paulsen, H. (1996) *Eur. J. Biochem.* **242,** 288–292
42. Wedel, N., Klein, R., Ljungberg, U., Andersson, B., and Herrmann, R. G. (1992) *FEBS Lett.* **314,** 61–66
43. Bassi, R., Croce, R., Cugini, D., and Sandona, D. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 10056–10061
44. Pichersky, E., and Jansson, S. (1996) in *Oxygenic Photosynthesis: The Light Reactions* (Ort, D. R., and Yocum, C. F., eds) pp. 507–521, Kluwer Academic Publishers, Dordrecht, The Netherlands
45. Funk, C., Adamska, I., Green, B. R., Andersson, B., and Renger, G. (1995) *J. Biol. Chem.* **270,** 30141–30147
46. Jegerschöld, C., Rutherford, A. W., Mattioli, T. A., Crimi, M., and Bassi, R. (2000) *J. Biol. Chem.* **275,** 12781–12788
47. Jahns, P., and Junge, W. (1990) *Eur. J. Biochem.* **193,** 731–736
48. Walters, R. G., Ruban, A. V., and Horton, P. (1994) *Eur. J. Biochem.* **226,** 1063–1069
49. Briantais, J. M. (1996) in *Life Sciences, Light as an Energy Source and Information Carrier in Plant Physiology* (Jennings, R. C., Zucchelli, G., Ghetti, F., and Colombetti, G., eds) pp. 113–124, NATO ASI Series A, Plenum Publishing Corp., New York
50. Nield, J., Funk, C., and Barber, J. (2000) *Philos. Trans. R. Soc. Lond-Biol. Sci.* **355,** 1337–1344
51. Nield, J., Orlova, E. V., Morris, E. P., Gowen, B., van Heel, M., and Barber, J. (2000) *Nat. Struct. Biol.* **7,** 44–47
52. Frank, H. A., Das, S. K., Bautista, J. A., Bruce, D., Vasil'ev, S., Crimi, M., Croce, R., and Bassi, R. (2001) *Biochemistry* **40,** 1220–1225
53. Crimi, M., Dorra, D., Bosinger, C. S., Giuffra, E., Holzwarth, A. R., and Bassi, R. (2001) *Eur. J. Biochem.* **268,** 260–267
54. Moya, I., Silvestri, M., Vallon, O., Cinque, G., and Bassi, R. (2001) *Biochemistry* **40,** 12552–12561
55. Ruban, A. V., Pascal, A. A., Robert, B., and Horton, P. (2002) *J. Biol. Chem.* **277,** 7785–7789