The PSII-S Protein of Higher Plants: A New Type of Pigment-Binding Protein†

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ABSTRACT: An intrinsic 22 kDa protein of photosystem II has been shown to possess high sequence homology with the CAB gene products, but differs from these proteins by an additional putative fourth transmembrane helix. This protein, designated PSII-S in accordance with the assignment of the name *psb*S to its gene, has been isolated by nonionic detergents and preparative isoelectric focusing in this study. The isolated PSII-S protein was shown to bind 5 chlorophyll molecules (*a* and *b*) per protein unit and also several different kinds of carotenoids. The room temperature absorption spectrum of the Q_y transition of the chlorophylls bound to the isolated protein is characterized by a broad band with a maximum at 671 nm. The 77 K fluorescence spectrum exhibits a peak at 672 nm. A single photon counting technique was applied to resolve the room temperature decay kinetics of the first excited singlet states in the chlorophyll ensemble of the PSII-S protein. The data can be satisfactorily described by triexponential kinetics with lifetimes of $\tau_1 = 1.8$ ns, $\tau_2 = 4.4$ ns, and $\tau_3 = 6.1$ ns and normalized amplitudes of 0.09, 0.60, and 0.31, respectively. Circular dichroism spectra suggest that, in contrast to LHCII, virtually no pigment coupling exists in the PSII-S protein. Two copies of the PSII-S protein were found per PSII in spinach thylakoids. It displays an unusually extreme lateral heterogeneity, since the PSIIβ centers located in the stroma exposed thylakoid regions contained only residual amounts of the PSII-S protein. Although the protein is unambiguously shown to bind pigments, it has several features distinct from previously described chlorophyll binding proteins. Therefore, it seems very likely that the PSII-S protein is involved in additional and/or alternative functions to normal light-harvesting.

The process of photosynthetic cleavage of water into dioxygen and hydrogen takes place in a multimeric thylakoid protein complex, photosystem II (PSII)[1] (for recent reviews, see Debus (1992), Rutherford et al. (1992), and Renger (1993)). The PSII of green algae and higher plants contains more than 25 different polypeptides with molecular masses ranging from 3 to about 50 kDa (see Andersson and Franzén (1992), Renger (1992), and Vermaas et al. (1993)). Several of these are known to contain pigments, preferentially chlorophylls, but also carotenoids. At least two types of chlorophyll-binding proteins can be distinguished. The first group is represented by the pigment proteins forming the PSII core complex, i.e., the gene products of *psb*B (CP47) and *psb*C (CP43). CP47 and CP43, each containing 15−22 chlorophyll *a* molecules (Chang et al., 1994; Alfonso et al., 1994), are assumed to act as the inner or core antenna. The redox active groups that catalyze a stable charge separation (the photoactive chlorophyll *a* designated as P680 and the acceptors pheophytin and plastoquinone) are located in a heterodimer consisting of polypeptides D1 (*psb*A) and D2 (*psb*D) that are closely associated with three smaller polypeptides, the two subunits of Cyt b_{559} (*psb*E and -F) and the *psb*I gene product. The D1/D2 heterodimer binds 4−6 chlorophyll *a* molecules, 2 pheophytins, and 1−2 carotenes (Barber et al., 1987; Kobayashi et al., 1990; Gounaris et al., 1990).

The second major type of chlorophyll-binding proteins is the family of CAB gene products forming the peripheral antenna system which plays a key role in light-harvesting and is also involved in acclimation of the photosynthetic apparatus to different light environments. The proteins belonging to the CAB gene family contain chlorophyll *b* in addition to chlorophyll *a*. The dominant complex is the trimeric LHCII that forms the major part of the antenna system and accounts for about 50% of all chlorophylls in the thylakoid membrane (see Green et al. (1991) and Jansson (1994)). The minor chlorophyll *a*/*b* antenna proteins are designated CP29, CP26, CP24, and CP14 according to size (Green et al., 1988; Dunahay & Staehelin, 1986; Bassi et al., 1987; Henrysson et al., 1989; Irrgang et al., 1993; Zucchelli et al., 1994).

In addition to these pigment binding proteins, the PSII complex contains several nonpigmented proteins. Some of them are of functional relevance in electron transport while others appear to be regulatory subunits. In the latter category the best studied is the PSII-O (33 kDa) protein that binds extrinsically at the lumenal side of the PSII complex and which is essential for stabilizing the manganese cluster of

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[1] Abbreviations: CAB, chlorophyll *a*, *b* binding protein; car, carotenoids; CD, circular dichroism; Chl, chlorophyll; ELIP, early light induced protein; FCP, fucoxanthin *a*, *c* proteins; LHC, light harvesting complex; OTG, octyl β-D-glucothiopyranoside; OGP, *n*-octyl β-D-glucopyranoside; p*I*, isolelectric point; PSII, photosystem II.

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the water oxidizing complex. Likewise, the PSII-P (23 kDa) and PSII-Q (16 kDa) proteins are also relevant as regulatory subunits in modulating the Ca^{2+} and Cl^- demand of the water oxidizing process in plants (for review see Debus (1992) and references therein).

A subunit of 22 kDa, encoded by the nuclear *psb*S gene, exhibits unique properties but has so far escaped unambiguous assignment of its functional role. This hydrophobic protein was originally identified and later isolated by Ljungberg et al. (1984, 1986). It has been purified by different methods and was reported to contain neither pigments nor metallic cofactors (Ljungberg et al., 1986; Bowlby & Yocum, 1993; Mishra & Ghanotakis, 1993). Based on coprecipitation studies using antibodies against the extrinsic 33 and 23 kDa proteins, it was suggested that the 22 kDa protein is part of, or at least closely associated with, the oxygen evolving complex. However, this hypothesis was recently questioned by the findings that PSII membrane fragments depleted of the 22 kDa protein retained all three extrinsic regulatory PSII-O, PSII-P, and PSII-Q proteins (Mishra & Ghanotakis, 1993) and that rebinding of the latter two proteins to PSII core complexes could be achieved without the 22 kDa protein (Merrit et al., 1987). Also a stabilizing function for the primary electron acceptor Q_A has been suggested for the PSII-S protein (Henrysson et al., 1987; Dekker et al., 1988), but this has not been further substantiated (Bowlby & Yocum, 1993).

A different alternative became apparent when the sequence of the *psb*S gene was determined and indicated homology between the PSII-S and the CAB family (Wedel et al., 1992; Kim et al., 1992), ELIPs (early light induced proteins) (Adamska et al., 1993), and FCPs (fucoxanthin–chlorophyll *a*, *c* antenna proteins) (Green & Pichersky, 1994). However, one important difference is found in the derived protein sequence and hence in the predicted folding pattern: the PSII-S protein is inferred to have four transmembrane helices (Wedel et al., 1992; Kim et al., 1992; Wallbraun et al., 1994), while only three are predicted in the CAB proteins and ELIPs (Grimm et al., 1989). The latter structural feature has been experimentally demonstrated for LHCII by electron crystallography (Kühlbrandt et al., 1994). Recent biochemical studies support the predicted pattern of four transmembrane helices for the PSII-S protein (Kim et al., 1994). Within the structural pattern derived from hydropathy analysis, the first helix and third helix of this protein are homologous in the same manner as in CAB proteins, while the second helix is related to the fourth additional helix (Wedel et al., 1992; Kim et al., 1992).

Regardless of the details of the folding pattern, the sequence similarities between *psb*S and the CAB genes suggest that the PSII-S protein could be pigment binding, and experimental indications for this possibility were recently obtained by mild SDS–PAGE analysis (Funk et al., 1994). In this present study we conclusively demonstrate that the PSII-S protein can bind chlorophyll and carotenoids by using an isolation procedure based upon nonionic detergents and isoelectric focusing. The isolated protein was characterized by biochemical and biophysical analyses and shown to possess both common and unique properties with respect to the known CAB proteins.

MATERIALS AND METHODS

Isolation of the 22 kDa Protein. Spinach was grown in a growth chamber in hydroponic culture for 8 weeks with 12 h alternating light (350 $\mu E/m^2s$) and dark periods, at a temperature of approximately 20 °C. PSII membrane fragments isolated from spinach leaves as described by Berthold et al. (1981) were solubilized with octyl thioglucopyranoside (OTG) with some minor modifications as described in Funk et al. (1994). After centrifugation, a so-called OTG pellet is obtained that is highly enriched in the 22 kDa protein (Mishra & Ghanotakis, 1993).

For preparative isoelectrofocusing a bed (100 mL) containing 5% Ultradex (Pharmacia), 2% Ampholine carrier ampholites (pH 4–6.5), 1% glycine, and 1% octyl glucopyranoside (OGP) was prepared according to Dainese et al. (1990a), poured on a tray (21 × 13 cm), and placed in a fumehood to evaporate excess water (10%). After solubilization with OGP (detergent/chlorophyll ratio 30:1) for 30 min on ice at 4 °C, the sample was applied near the cathode to the precooled bed (1 °C). The electrophoresis was run at 2 W for 30 min and at 8 W for 14 h. Finally, the green bands were collected with a spatula.

To remove the bed material, the different fractions were filtered through disposable polypropylene columns (Pierce) with small volumes of a buffer containing 25 mM Mes–NaOH (pH 6.5) and 1% OGP. The samples were concentrated using Centricon-10 tubes (Amicon) and dialyzed against 0.1% OGP in 25 mM Mes–NaOH buffer (pH 6.5).

Gel Electrophoresis and Immunological Techniques. Polypeptide analysis was performed by SDS–PAGE according to Laemmli (1970) using 17.5% acrylamide and 4 M urea. The gels were either stained with silver nitrate (Oakley et al., 1980) or electroblotted onto PVDF membranes and incubated with the antibody against the PSII-S protein (Ljungberg et al., 1986) followed by immuno decoration with ^{125}I iodinated protein A, alkaline phosphatase (Bio-Rad), or enhanced chemoluminescence (ECL, Amersham). To check for the purity of the obtained protein, the relevant fraction was immunoblotted with antibodies raised against LHCII, CP29 (Henrysson et al., 1989), CP26 (Di Paolo et al., 1990), CP24 (Spangfort et al., 1990), and CP14 (Irrgang et al., 1993). The yield of the PSII-S protein during the various fractionation steps was determined by quantitative Western blotting.

Pigment and Protein Determination. The chlorophyll content was determined in 80% acetone, calculated as in Porra et al. (1989). Protein concentration was determined according to Lowry et al. (1951) with modifications as described in Markwell et al. (1981). For chlorophyll and protein determination the same sample was used, chlorophyll being measured after extraction in acetone and the amount of protein after acetone precipitation. Pigment analysis was performed according to Thayer and Björkman (1990) using a reversed phase HPLC column at room temperature with 80% acetone.

Spectroscopic Analysis. Absorption spectra were recorded using a Shimadzu UV-3000 double-beam spectrophotometer. Steady state fluorescence was measured at 77 K with a Perkin Elmer luminescence spectrometer. For monitoring emission spectra, the samples were excited at 435 nm; for the excitation spectra the emission was measured at 671 nm. Circular dichroism spectra were measured with a JASCO

J-720 spectropolarimeter at 4 °C, scanning from 400 to 700 nm with a speed of 50 nm/min and a sensitivity of 20 mdeg. To obtain a good signal to noise ratio, 3 scans were averaged.

Fluorescence decay curves were monitored by using a time-correlated single photon counting technique as outlined in Liu et al. (1993), with some modifications as described in Bernarding et al. (1994). The sample was excited at 650 nm by laser pulses with a FWHM ≤15 ps at a repetition rate of 800 kHz. Fluorescence was selected by an interference filter, $\lambda_{max} = 684$ nm. During excitation, the sample was stirred in a 10 mm × 10 mm cuvette at 4 °C. Resolution of the time-to-amplitude converter was 50 ps/channel. The fluorescence decay data were analyzed by a fit program based on the Levenberg−Marquardt algorithm, assuming multiexponential decay kinetics. Fit quality was judged by reduced χ^2 values and weighted residual plots.

Stoichiometry and Lateral Distribution of the 22 kDa Protein. The stoichiometry of the PSII-S protein per PSII was estimated by quantitative Western blotting. Known amounts of the protein purified according to Ljungberg et al. (1986) and thylakoid membranes of specified chlorophyll concentrations were subjected to SDS−PAGE. The samples were electrotransferred to PVDF membranes and incubated with the antibody raised against the PSII-S protein and ^{125}I-protein A, and the obtained autoradiograms were scanned by laser densitometer (Molecular Dynamics). The ratio between the PSII-S protein and the PSII reaction center (P680) was determined using a typical chlorophyll/P680 ratio of 360 (Melis, 1984). Thylakoid membranes were subfractionated by differential centrifugation and aqueous polymer two-phase partition into inside-out and stroma lamellae thylakoid vesicles representing appressed thylakoids and nonappressed thylakoids, respectively (Andersson & Åkerlund, 1978). The subfractions were subjected to quantitative Western blotting, performed as described above using antibodies against the PSII-S protein. For a comparison, antibodies against the extrinsic PSII-O protein (33 kDa protein) were also used.

RESULTS

In order to investigate the pigment-binding properties of the PSII-S protein and its possible function, it was isolated in a native state by using a procedure based upon nonionic detergents and preparative isoelectric focusing (Dainese et al., 1990a). An extract, obtained after short solubilization with octyl thioglucopyranoside (OTG) of PSII enriched membrane fragments (Mishra & Ghanotakis, 1993), was used as starting material. As shown in lane 3 of the denaturing SDS−polyacrylamide gel depicted in Figure 1, this OTG extract is highly enriched in the PSII-S protein, but also contains residual amounts of the PSII-R protein and ferredoxin−NADP reductase. In spite of the drastic reduction of all other chlorophyll-binding proteins, in particular, LHCII, this OTG pellet has a green color. This observation provides initial support for the possibility of the PSII-S protein being a chlorophyll-binding protein.

For further analysis, the hydrophobic OTG extract was solubilized with octyl glucopyranoside (OGP) and then separated by flat bed isoelectric focusing (IEF) in the pH range of 4−6.5. The results obtained are illustrated in Figure 2. One major green band focusing at pH 6.1, near the sample application site, and two minor green bands at pH 5.7 and

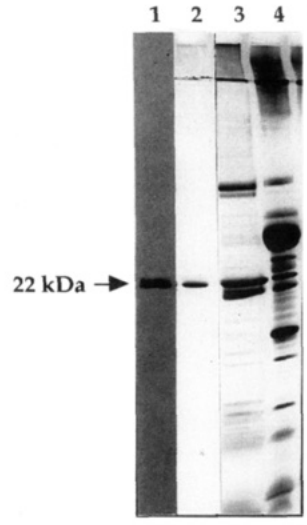


FIGURE 1: Denaturing silver-stained SDS−PAGE of PSII membrane fragments (4), the OTG pellet (3), and the green IEF fraction at pH 6.1 (2) showing the purity of the isolated 22 kDa (PSII-S) protein. Lane 1 shows this IEF fraction blotted against the anti-PSII-S antibody.

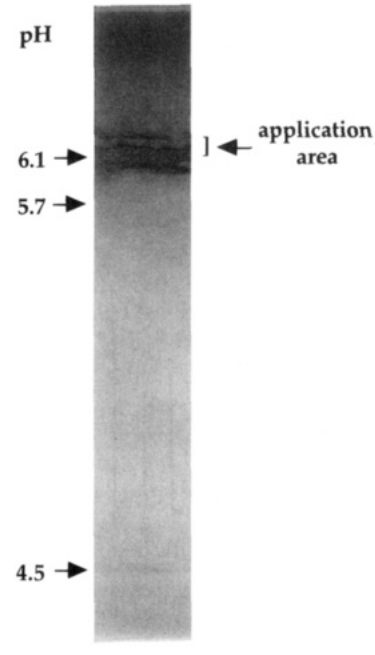


FIGURE 2: Fractionation of OTG extract from BBY by preparative isoelectric focusing in 1% octyl glucopyranoside. On the left side, the p*I* of the different green bands is indicated.

4.5 were resolved. After protein elution from the gel bed, the main band and also the two minor bands gave clear green solutions. Denaturing SDS−PAGE as shown in Figure 1, lane 2, reveals that the main green band at pH 6.1 is the highly purified 22 kDa protein, while the two minor bands contained a complex of the 22 kDa protein together with the ferredoxin−NADP reductase (FNR) (band 2, pH 5.7) and a minor fraction of LHCII (pH 4.5). Antibodies raised against the *psb*S gene product immunodetected the isolated PSII-S protein (Figure 1, lane 1), whereas antibodies against LHCII, CP29, CP26, CP24, and CP14 did not react with the PSII-S protein fraction (data not shown).

The yield of the PSII-S protein starting from the PSII membrane fragments was typically 10%, as judged by quantitative Western blotting. Predominantly, the loss of the protein occurred during the OTG extraction step while the

Table 1: Comparison of the Pigment Composition of the PSII-S Protein with the Other Chlorophyll *a/b* Binding Proteins in Photosystem II[g]

	PSII-S	LHCII[a]	CP29[a]	CP26[a]	CP24[a]
chl/car	4.9	5.6	3.1	3.9	3.0
chl *a/b*	5.9−6.1	1−1.4[b]	2−4[c]	2−3[d,e]	1.2−1.6[f]
chl/prot	5	12−13[b]	4−12[c,e]	5−9[d,e]	5[f]
car/prot	1	2	1−3	1−2	1−2
mol/100 mol of chl *a*:					
neoxanthin	6	6.2	7.7	5.7	tr
violaxanthin	5	0.9	14.2	8.7	18
antheraxanthin					
lutein	12	23.6	21.3	22.5	38.2
zeaxanthin					
β-carotene	1		1.7	1.6	3.5
chl *b*	17	73.7	38.4	51.6	81.2

[a] Bassi et al., 1993. [b] Kühlbrandt et al., 1994. [c] Henrysson et al., 1989. [d] Barbato et al., 1989. [e] Bassi & Dainese, 1990. [f] Bassi et al., 1990. [g] chl, chlorophyll; car, carotenoids.

yield in the subsequent purification step was over 90%. Starting from PSII membrane fragments corresponding to 40 mg of chlorophyll, approximately 0.5 mg of PSII-S protein could be obtained.

The PSII-S protein isolated by isoelectric focusing (IEF) was further characterized in terms of its chlorophyll content and the chlorophyll/protein ratio. Acetone extraction of the 22 kDa protein revealed a chlorophyll *a/b* ratio of 6:1 as judged by spectroscopical analysis. Determination of the protein content in the precipitate after the acetone extraction showed a chlorophyll/protein molar ratio of 5 ± 1.

For a more detailed analysis of the pigment composition, the purified PSII-S protein was analyzed by reversed phase HPLC. The results obtained are summarized in Table 1 and show that the PSII-S protein binds a rather small amount of chlorophyll *b*. The chlorophyll *a/b* ratio of 5.9 determined by HPLC is in close correspondence with the value of 6.1 obtained from the spectral determination of the acetone extract. On the other hand, it markedly deviates from the value of 2.2 recently reported for the PSII-S protein resolved by SDS−PAGE (Funk et al., 1994). The relative amount of bound carotenoids is also low compared to that of the CAB proteins. The most abundant carotenoid in the PSII-S protein and all CAB proteins is lutein. On the basis of 100 chlorophylls there are 12 luteins in the PSII-S protein in comparison to more than 20 in the CAB proteins. Significant amounts of neoxanthin and violaxanthin are also present. With respect to the content of β-carotene (1 β-car/100 chlorophyll *a*) the *psb*S gene product is more related to the isolated minor antenna proteins (CP29, CP26, CP24) than to LHCII (Bassi et al., 1993). The elution profile also revealed the presence of very small amounts of antheraxanthin and zeaxanthin. In addition, an unknown pigment eluted between antheraxanthin and lutein (data not shown). In comparison with the CAB proteins, the stoichiometry of the pigments of the isolated PSII-S protein is different. Among all CAB proteins so far identified, the PSII-S protein has the highest chlorophyll *a/b* ratio and together with LHCII the highest chlorophyll/carotenoid ratio.

The spectral properties were analyzed to allow further characterization of pigment organization in the isolated PSII-S protein. Figure 3 shows the absorption spectrum measured at room temperature in the range of 400−750 nm. The red region exhibits a pronounced peak at 671 nm which

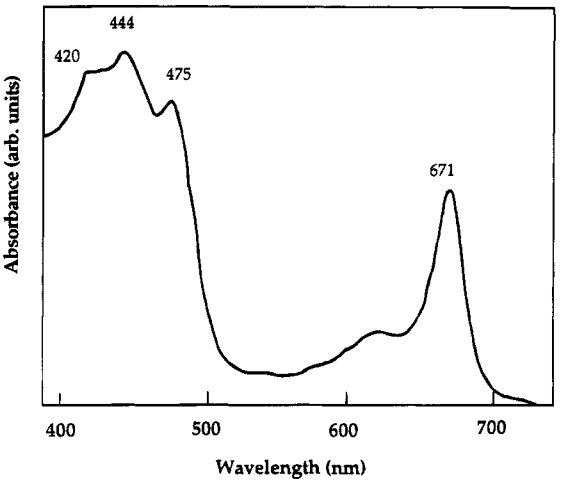


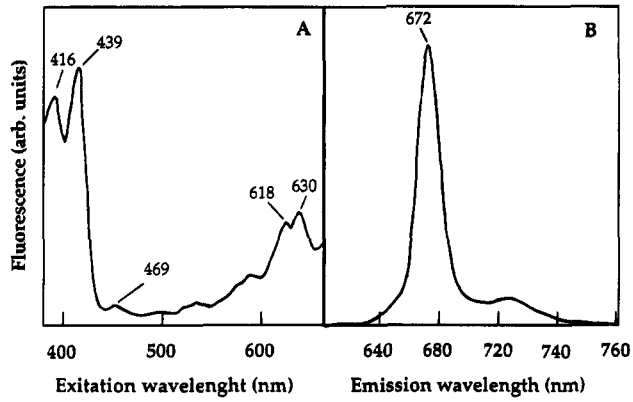
FIGURE 3: Absorption spectrum of the purified PSII-S protein at room temperature.

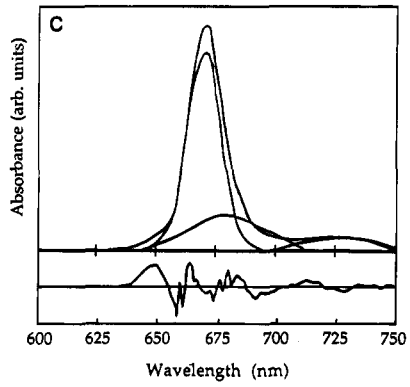
is typical for chlorophyll *a* species. Moreover, a second peak or a distinct shoulder in the region between 640 and 650 nm is lacking, consistent with the low amount of chlorophyll *b*. The peak near 620 nm represents the vibronic 0 → 1 satellite of the Q_y transition of chlorophyll *a*. In the blue region three bands can be distinguished with peaks at 420, 444, and 475 nm.

Steady state fluorescence measurements were performed at 77 K. The emission spectrum measured at an excitation wavelength of 435 nm is depicted in Figure 4B. It is characterized by a main band peaking at 672 nm and a small vibronic 1 → 0 satellite near 730 nm. These features are typical for chlorophyll *a*, but the overall band maximum is blue-shifted compared with other isolated CAB proteins (see Discussion).

A deconvolution into Gaussians as described previously by Maciy et al. (1992) revealed that a satisfactory fit of the 77 K emission spectrum (Figure 4C) can be achieved by a dominating band peaking at 671 nm (FWHM of 16−18 nm), together with the broad vibronic 0 → 1 satellite at 730 nm. Both bands together account for at least 75% of all the emission. A minor band with a peak at 680 nm and a normalized extent of about 20% could also be determined from this analysis. However, it has to be emphasized that this band is unusually broad (FWHM about 30 nm) and is not indispensable in order to achieve a satisfying description of the experimental data by Gaussian deconvolution (data not shown). Therefore, the existence of this longer wavelength band cannot be considered to be experimentally proven.

The action spectrum of the fluorescence emission measured at 672 nm is shown in Figure 4A. It exhibits a structured feature with two dominating bands at 416 and 439 nm in the blue region and two smaller bands in the red with peaks at 618 and 630 nm (for technical reasons the measurements were limited to wavelengths below 650 nm). In addition, small peaks were resolved at around 469, 540, and 589 nm. Except for the 469 nm band, which is probably due to singlet−singlet excitation energy transfer from carotenoids to chlorophyll, all other peaks are assumed to originate mainly from chlorophyll *a*. A surprising and somewhat puzzling feature is the appearance of two bands in the wavelength region between 618 and 630 nm where the 0 → 1 vibronic satellite of the Q_y transition in chlorophyll





position (nm)	FWHM-values (nm)	amplitude (arb. units)	(%)
670.4	15.8	13.2	69.8
680.0	31.0	2.2	22.7
725.9	31.6	0.7	7.5

FIGURE 4: Fluorescence spectrum of the purified PSII-S protein, recorded at 77 K. (A) Excitation spectrum with emission measured at 672 nm. (B) Emission spectrum obtained upon excitation at 435 nm. (C) Deconvolution of the emission peak at 672 nm into Gaussian bands.

a is located. The most likely explanation of this phenomenon is the existence of at least two types of chlorophyll *a* molecules with different spectral properties. This possibility is supported by the observation of two peaks in the region of the Q_x transition, i.e., at 580 and 590 nm, and by the deconvolution of the room temperature absorption spectrum into Gaussian bands (Schrötter, Funk, and Renger, unpublished results).

Time-resolved fluorescence measurements provide further information on the properties of chromophores bound within a protein matrix. This has been recently demonstrated for LHCII (Liu et al., 1993). The fluorescence decay profiles of trimeric LHCII isolated by detergent solubilization exhibit multiexponential kinetics that depend on the solubilization procedure as well as the type and concentrations of detergent used (Ide et al., 1987; Bassi et al., 1991; Mullineaux et al., 1993). The average lifetime of the overall decay was found to become significantly shortened upon aggregation of LHCII. This phenomenon reflects the formation of a quenching state due to interaction between trimeric units (Mullineaux et al., 1993). Figure 5 shows fluorescence decay curves obtained by single photon counting measurements in samples of the PSII-S protein. A numerical analysis of the data obtained reveals that a satisfactory fit can be achieved by triexponential kinetics as is shown by the residuals presented in the lower part of the figure. The fit procedure leads to the following lifetimes and normalized amplitudes

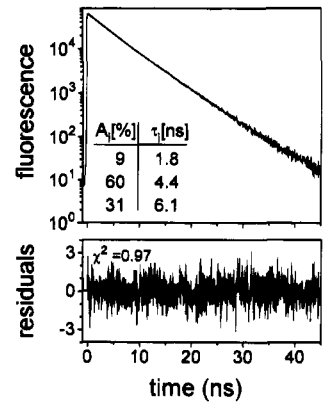


FIGURE 5: Fluorescence lifetime measurement of the purified PSII-S protein at 4 °C (top). Fit quality given by χ^2_{Red} values and weighted residual plots (bottom). Experimental conditions as described in Materials and Methods.

(given in parentheses): $\tau_1 = 1.8$ ns ($a_1 = 0.09$), $\tau_2 = 4.4$ ns ($a_2 = 0.60$), and $\tau_3 = 6.1$ ns ($a_3 = 0.31$). The first two kinetics closely resemble those observed in solubilized nonaggregated LHCII with $\tau_1 = 2.2$ ns ($a_1 = 0.12$) and $\tau_2 = 4.3$ ns ($a_2 = 0.88$) (Liu et al., 1993). In marked contrast to LHCII isolated by sucrose gradient centrifugation (Irrgang et al., 1988), however, a third kinetic with a longer lifetime of 6.1 ns is also observed in the isolated PSII-S protein (Figure 5). A value of about 6 ns and $a = 0.31$ is reminiscent of the lifetime of chlorophyll *a* in ethanoic solution (6.3 ns; see Butler & Norris, 1963). Similar values were observed for free chlorophyll in liposomes (Liu et al., 1993). It is therefore tempting to assign the 6 ns component to chlorophyll molecules which are comparatively loosely associated with the protein. This interpretation is further supported by recent time-resolved fluorescence decay measurements of isolated CP29. It was found that the normalized extent of the 6.1 nm kinetics markedly increased in preparations that were obtained by a harsher isolation procedure (Funk, Irrgang, Napiwotzki, and Renger, unpublished results). Likewise, Sukenik et al. (1989) observed that the presence of Triton X-100 can interrupt singlet excitation energy transfer from chlorophyll *b* to chlorophyll *a* in LHCII. This transfer of excitation energy normally takes place in the subpicosecond and picosecond time domain both at room temperatures (Bittner et al., 1994) and at 12 K (Bittner et al., 1995). Moreover, modifications of excitation energy transfer were also found in LHCII solubilized with various types of detergents (Ide et al., 1987; Bassi et al., 1991). Therefore, if in a rough approximation the 6.1 ns component is used as a measure of dislocated chlorophyll *a*, our present results suggest that about 70% of the chlorophyll of the isolated PSII-S protein is bound to the protein matrix in the same or similar way as in a native complex. The remaining 30% of the chlorophyll molecules are inferred to be still associated with the protein but in a highly nonordered manner. According to the isolation procedure of the PSII-S protein nonattached chlorophyll molecules can be excluded as a source of the 6.1 ns components, since it has a p*I* different from pH 6.1 and would therefore not comigrate with the PSII-S protein during the IEF separation.

For a further spectral characterization, circular dichroism spectra were measured at 4 °C in the range of 400−700 nm. The data obtained for the PSII-S protein are depicted in Figure 6A. A rather flat spectrum is observed within the

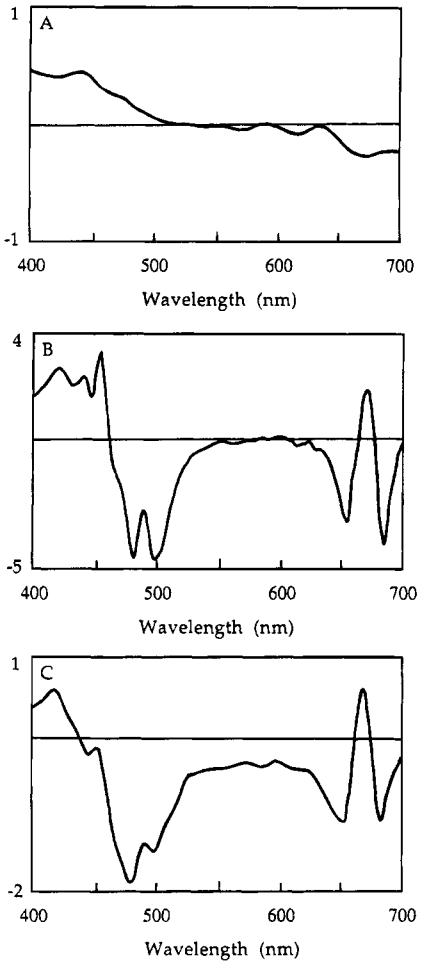


FIGURE 6: Circular dichroism measurement of the purified PSII-S protein at 4 °C in the range of 400−700 nm. (A) PSII-S protein; (B) LHCII; (C) CP29. Chlorophyll concentration in all samples: 5 μg/mL.

whole range. This feature is in marked contrast to that which is found for CAB proteins like LHCII (see Figure 6B) and CP29 (see Figure 6C). The latter spectrum slightly differs in its shape from recently published results (Dainese et al., 1990b) probably due to complex formation with CP26 and/or another CP29 form. Regardless of details in the spectral shape, it is clear that the PSII-S protein lacks typical features that are indicative for strong pigment−pigment interaction, e.g., the w-like structure in the red region (Q_y transition) of aggregated LHCII (Barzda et al., 1994). It has to be emphasized that all three spectra were measured at the same chlorophyll concentration of 5 μg/mL. The spectrum of PSII-S rather resembles a chlorophyll *a* Triton X-100 extract of CP47 (Kwa et al., 1994). It is therefore inferred that the chlorophyll molecules bound to the PSII-S protein are not excitonically coupled. This conclusion is in part understandable by the fact that the chlorophyll/protein ratio in the PSII-S protein is smaller by a factor of about 3 compared with LHCII and about 2 compared with CP29. In addition, the absence of excitonic coupling in the PSII-S protein might also suggest a different mode of binding to the protein matrix.

The experimental findings presented above provide unambiguous evidence for the PSII-S protein to be pigment binding. This property is understandable by its homology to the CAB proteins, but it has to be emphasized that the mode of binding exhibits some different features. It has previously been shown that PSII-S is closely associated with the PSII core complex (Ghanotakis et al., 1987). Another relevant parameter for consideration on its possible functional and/or structural role is the abundance of the PSII-S protein in relation to other PSII proteins and the lateral distribution between the appressed and nonappressed thylakoid regions. Therefore, the relative amount of the *psb*S gene product per PSII was determined in intact thylakoids as well as in subfractions. Antibodies to the PSII-S protein were used to obtain Western blots of the purified protein and of thylakoid membranes of known total chlorophyll amount. For quantification, the blots were scanned by laser densiometry. A ratio of 200 ± 50 chlorophylls per one PSII-S protein was determined for intact thylakoids. This value is consistent with an overall stoichiometry of about two PSII-S proteins per reaction center on the basis of a chlorophyll/P680 stoichiometry of 360 gathered from typical data reported in the literature.

The amount of the PSII-S protein in the different thylakoid membrane regions was compared with the relative content of the PSII-O protein in subfractions derived from appressed and nonappressed thylakoids. The amount of the PSII-O protein was found (on chlorophyll basis) to be 4 times higher in appressed compared to stroma exposed thylakoid regions, typical for the lateral distribution of PSII components between the two thylakoid regions (Anderson & Andersson, 1982). Notably, the PSII-S protein deviated from this distribution in showing an extremely low abundance in the stroma exposed thylakoid. Thus, the amount of the PSII-S protein was on average 54 times higher in the appressed thylakoid regions than in the stroma exposed regions. It is therefore concluded that the PSII-S protein is associated with PSIIα but not with PSIIβ.

DISCUSSION

The present biochemical and biophysical data give strong support to the implication from the sequence data (Wedel et al., 1992; Kim et al., 1992) that the PSII-S protein can bind pigment. The PSII-S could consequently serve as another light-harvesting protein to PSII, but it could potentially have other functions related to its pigment binding, yet to be identified. It shares several properties of the other CAB proteins, but there are also several distinct features including a unique polypeptide folding with a fourth transmembrane helix, different pigment content and organization as well as an unusually extreme lateral distribution to the appressed thylakoid regions.

The pigment analysis of the PSII-S preparation suggests that there are on average 4−6 chlorophyll molecules per isolated protein. Considering that the protein was isolated by a procedure involving detergents, some of the chlorophyll may have been detached during the isolation. This may in turn imply that in the isolated sample there is a heterogeneity with respect to bound pigment molecules. The content of chlorophyll *b* is quite low, giving a chlorophyll *a*/*b* ratio of 6, which in turn indicates approximately one molecule of chlorophyll *b* per protein. In a previous study based upon SDS−PAGE (Funk et al., 1994) a chlorophyll *a*/*b* ratio of 1.6−2.2 was estimated. However this value was combined with a chlorophyll/protein ratio as low as 3, indicating preferential loss of chlorophyll *a* induced by the presence of the anionic detergent. Among the chlorophylls present in the PSII-S protein, the biophysical measurements suggest

that 30% of chlorophyll *a* molecules are uncoupled, indicating that at least two chlorophyll *a* molecules are only loosely attached to the protein.

Thus, according to our analysis, the isolated PSII-S protein is suggested to contain 6 chlorophyll *a* (*vide supra*), 1 chlorophyll *b*, and 1 carotenoid. For comparison, LHCII is reported to bind 7 chlorophyll *a* molecules, 6 chlorophyll *b* molecules, 2 luteins, and 1 neoxanthin (Bassi et al., 1993; Kühlbrandt et al., 1994), CP29 contains 6 chlorophyll *a*, 2 chlorophyll *b*, and 2−3 carotenoids, CP26 contains 6 chlorophyll *a*, 3 chlorophyll *b*, and 3 carotenoids, and CP24 contains 3 chlorophyll *a*, 2 chlorophyll *b*, and 1−2 carotenoids (Bassi et al., 1993). Thus, the overall pigment content, and in particular the amount of chlorophyll *b*, is lower for the PSII-S protein as compared to CAB proteins in general. As is also the case for these proteins, the PSII-S preparation contains substoichiometric amounts of several carotenoid molecules. This could be explained by the possibility that some of these pigments may be bound in between subunits of an oligomeric complex in analogy to what was found for other CAB proteins (Bassi et al., 1991, 1993; Dainese & Bassi, 1991). Alternatively, the carotenoids could be bound on the surface of the complexes. Recent work on the fucoxanthin−chlorophyll *a*, *c* protein assembly showed that xanthophyll is mainly bound at the interface between lipid molecules and polypeptide chain (Mimuro & Katoh, 1991).

It is important to note that the isolated PSII-S protein contains violaxanthin (Table 1) known to play an essential role in mediating nonphotochemical quenching via the violaxanthin ↔ antheraxanthin ↔ zeaxanthin cycle (Demmig & Björkman, 1987; Noctor et al., 1993; Ruban et al., 1994; Crofts & Yerkes, 1994). It could therefore be considered that the PSII-S protein could be essential for this process. Nonphotochemical quenching has been associated in several studies with the light-harvesting antenna of PSII and in particular LHCII (Horton et al., 1991). However, other experimental data (Dainese et al., 1992) and theoretical considerations (Crofts & Yerkes, 1994) suggest that the minor CAB proteins such as CP29, CP26, and CP24 are the site of the xanthophyll interconversion. However, the barley mutant *Chlorina* f2, which lacks all CAB proteins, even the minor ones, when grown under intermittent light conditions, still possesses a functional violaxanthin cycle (Krol et al., 1995). It was suggested that ELIPs could account for this observation, but the presence of the PSII-S protein in this mutant provides an alternative explanation in line with the proposal made above.

The PSII-S protein exhibits the same overall abundance as the PSII-O protein, i.e., two subunits per reaction center (Andersson et al., 1984). The PSII-S protein is characterized by a more pronounced heterogeneity of the lateral distribution than other subunits of PSII and its light-harvesting antenna. It is almost exclusively confined to the grana partition regions and exists only in trace amounts in the stroma exposed thylakoid regions. It even remains in the grana partitions after *in vitro* induced photoinhibitory damage of the PSII reaction center, when other PSII subunits migrate to the stroma exposed thylakoid regions (Hundal et al., 1990). Consequently, the PSII-S protein appears to be exclusively present in PSIIα and not, or only to a low extent, in PSIIβ. The differences between these two types of photosystem II centers are the antenna size (PSIIβ consists mainly of the inner LHCII) and poor Q_A to Q_B electron transfer in PSIIβ centers (Anderson & Melis, 1983). Thus, a function of the PSII-S protein as a linker between the antenna and the PSII−core complex was postulated also by Kim et al. (1994).

For a further discussion concerning the function of the pigment binding PSII-S protein, one has to raise the question as to whether this protein is an antenna protein with a relatively normal light-harvesting function or if it has another possibly unknown function. Some of our data like the pigment analysis, absorption spectrum, or 77 K fluorescence emission spectrum support the possibility that the PSII-S protein has a light-harvesting function in funneling light energy to the PSII reaction center as the conventional antenna proteins. However, other data raise doubts to this possibility; despite the overall sequence homology, only two amino acids of the predicted chlorophyll binding sites in the PSII-S are identical (B. R. Green, personal communication) to the ones of LHCII (Kühlbrandt et al., 1994). Like LHCII, the PSII-S protein could bind one chlorophyll *a* molecule on amino acid Glu-180 (chlorophyll *a*1), one chlorophyll *a* molecule on Glu-65 (chlorophyll *a*4), and possibly another chlorophyll *a* molecule on Gly-78 (chlorophyll *a*6). Other chlorophyll binding sites deduced from the *psb*S sequence are clearly possible, but still speculative. The excitation spectrum of the fluorescence emission at 77 K suggests that there is a double peak in the vibrational sub-band. This could indicate that two populations of chlorophyll *a* (or two chlorophyll *a* molecules) exist bound to the PSII-S protein that do not interact with each other. The latter idea is supported by circular dichroism measurements which are indicative of an extremely weak coupling of the chlorophylls. Further investigations are in progress concerning the mode of chlorophyll binding in the PSII-S protein. Moreover, the PSII-S protein differs from the other CAB proteins in that it is stable in the absence of chlorophyll. As judged by immunodetection, it is present in etiolated material as well as in mutants lacking or depleted of chlorophyll (Funk, Adamska, Green, Andersson, and Renger, to be published elsewhere). If it is not involved in light-harvesting, what could be the functional significance of the PSII-S protein? We propose, based upon a combination of the biochemical, biophysical, and sequence information, that it could bind chlorophyll transiently during conditions of biosynthesis, degradation, and turnover of chlorophyll binding proteins. Such a function would be in line with the atypical chlorophyll binding properties of the protein as well as its stability in the absence of bound pigments. More specifically, such a function could involve donation of newly synthesized chlorophylls to the antenna proteins during their assembly. Notably, the synthesized proteins are inserted in the stroma exposed thylakoid regions, but the ligation of pigments occurs in the grana regions (Yalovsky et al., 1990), the site of the PSII-S protein.

Yet another function could be to scavenge potentially dangerous chlorophyll, liberated during degradation of chlorophyll binding proteins such as the D1/D2 reaction center dimer (Barber & Andersson, 1992) and LHCII (Lindahl et al., 1995). The PSII-S protein could even serve as a chlorophyll exchange protein during the turnover of the PSII reaction center at high-light stress conditions, a function previously proposed for the ELIPs (Adamska et al., 1993).

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