Recombinant Water-Soluble Chlorophyll Protein from *Brassica oleracea* Var. Botrys Binds Various Chlorophyll Derivatives†

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ABSTRACT: A gene coding for water-soluble chlorophyll-binding protein (WSCP) from *Brassica oleracea* var. Botrys has been used to express the protein, extended by a hexahistidyl tag, in *Escherichia coli*. The protein has been refolded in vitro to study its pigment binding behavior. Recombinant WSCP was found to bind two chlorophylls (Chls) per tetrameric protein complex but no carotenoids in accordance with previous observations with the native protein [Satoh, H., Nakayama, K., Okada, M. (1998) *J. Biol. Chem. 273*, 30568−30575]. WSCP binds Chl *a*, Chl *b*, bacteriochlorophyll *a*, and the Zn derivative of Chl *a* but not pheophytin *a*, indicating that the central metal ion in Chl is essential for binding. WSCP also binds chlorophyllides *a* and *b* and even the more distant Chl precursor Mg-protoporphyrin IX; however, these pigments fail to induce oligomerization of the protein. We conclude that the phytol group in bound Chl plays a role in the formation of tetrameric WSCP complexes. If WSCP in fact binds Chl or its derivative(s) in vivo, the lack of carotenoids in pigmented WSCP raises the question of how photooxidation, mediated by triplet-excited Chl and singlet oxygen, is prohibited. We show by spin-trap electron-paramagnetic resonance that the light-induced singlet-oxygen formation of WSCP-bound Chl is lower by a factor of about 4 than that of unbound Chl. This as-yet-unknown mechanism of WSCP to protect its bound Chl against photooxidation supports the notion that WSCP may function as a transient carrier of Chl or its derivatives.

Water-soluble chlorophyll-binding proteins (WSCPs)[1] have been isolated from *Amaranthaceae*, *Chenopodiaceae*, *Polygonaceae* (class I), and *Brassicaceae* (class II) (*1*). The two classes of WSCP differ in that the former change their absorption spectra upon illumination, whereas the latter show no photoconversion of their absorption behavior. The physiological function of WSCPs is not known yet. The pigment contents reported for various WSCPs vary between 1 chlorophyll (Chl) per protein of about 22 kDa (*2*) and 1 Chl per protein tetramer (*3*). This low pigment content per protein together with the fact that WSCPs clearly are not located in the thylakoid membrane makes it unlikely that WSCPs are involved in the light reaction of photosynthesis, although they share a short sequence motif with Chl-*a*/*b* light-harvesting proteins (*1*).

The expression of various *Brassicaceae* WSCPs has been reported to be induced by drought (*2*) and heat stress (*3*) or leaf detachment (*4*), which has led to the suggestion that WSCP is involved in Chl scavenging during stress conditions (*5*). Specifically, WSCP has been hypothesized to be a Chl carrier transporting Chls from the thylakoid to the inner envelope membrane where chlorophyllase is thought to reside (*6*), the enzyme that starts the biodegradation of Chls. As another clue to their potential function, WSCPs contain a sequence segment that bears similarity to Künitz-type protease inhibitors (*1*, *4*); however, no significant protease inhibitor activity of WSCPs has been found yet.

A WSCP cDNA from cauliflower (*Brassica oleracea* var. Botrys) has been expressed in *Escherichia coli*; the recombinant WSCP, fused to maltose-binding protein, is functional in vitro since it binds Chls and organizes into tetrameric pigment−protein complexes very similar to the ones isolated from cauliflower (*5*). In the present paper, we extend this work by studying the pigment binding behavior of recombinant WSCP fused only to a His tag. We have analyzed this by reconstituting recombinant WSCP with a number of Chl derivatives.

If WSCP in fact functions as a carrier for Chl or Chl derivatives, one would expect it to bind carotenoids also to

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[1] Abbreviations: BChl, bacteriochlorophyll; BPhe, bacteriopheophytin; Chl, chlorophyll; Chlide, chlorophyllide; EPR, electron-paramagnetic resonance; Gnd, guanidinium hydrochloride; His, hexahistidyl; LM, dodecylmaltoside; Mg-Proto IX, Mg-protoporphyrin IX; OG, octyl-β-D-glucopyranoside; Phe, pheophytin; Pheide, pheophorbide; RT room temperature; TEMP, 2,2,6,6-tetramethylpiperidine; TEMPO, 2,2,6,6-tetramethylpiperidine-1-oxyl; recWSCP, recombinant water-soluble chlorophyll protein.

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prevent Chl-induced photooxidation. Chl molecules whose excitation energy is not rapidly quenched, either by charge separation, energy transfer to other chromophores, or heat dissipation, can change to the triplet-excited state which upon contact with oxygen gives rise to the potentially very harmful singlet-excited oxygen. Carotenoids ease this risk, as they quench both the Chl triplet- and the oxygen singlet-excited states. Consistently, carotenoids are found in virtually all Chl- or bacteriochlorophyll (BChl)-containing complexes. Surprisingly, no carotenoids have been found in native WSCPs. To establish whether some alternative mechanism may prohibit WSCP-bound Chl from photooxidation, we measured the formation of singlet oxygen in WSCP compared to unbound Chl.

MATERIALS AND METHODS

Proteins. Native WSCP was isolated from adult cauliflower leaves (*4*) obtained from local farmers. For expressing recombinant WSCP (recWSCP), the expression construct for WSCP fused to maltose-binding protein (*5*) was modified such that the maltose-binding protein was replaced with a hexahistidyl (His) tag. The sequence coding for WSCP was cloned into a pDS12/RBSII vector (*7*) using SphI, EcoRI, and HindIII restriction enzymes. The His tag was introduced by using a synthetic oligonucleotide (cgtcaccatcaccatcaccatgct). recWSCP was overexpressed in *E. coli* strain JM101 as described (*8*) except that bacteria were lysed by using a French press. After centrifugation (25 min, 10000*g*, 4 °C) of the cell lysate, the supernatant contained soluble recWSCP, but the major part of the protein was present in the pellet as insoluble inclusion bodies. The pellet was resuspended in 1−2 mL of 10 mM sodium phosphate pH 9.0 and treated with 10 μL of DNase I (1 mg/mL in 20 mM Tris-HCl pH 8.0, 50 mM NaCl, 1 mM DTT, 0.1 mg/mL BSA, 50% [v/v] glycerol) per 100 mL bacterial culture. After 15 min incubation at room temperature (RT) and another 15 min at 37 °C, recWSCP inclusion bodies were again pelleted and resuspended in 1−2 mL of 10 mM sodium phosphate pH 9.0.

Pigments. Total pigment extract, as well as purified Chls and carotenoids, were isolated from pea plants as described before (*8*). Pheophytin *a* (Phe *a*) was prepared by dissolving 0.5 mg/mL Chl *a* in diethyl ether and adding the same volume 10% HCl. After change of color into brown-red, Phe *a* was extracted with diethyl ether. Zn insertion into Phe *a* based on the standard protocol (*9*) was done by dissolving the dried pigment (0.5 mg/mL) in chloroform, heating to 35 °C, and slowly adding 1.5 mL of saturated Zn-acetate in chloroform per milligram of pigment. After incubation of the sample at 35 °C for 1 h, Zn-Phe *a* was extracted with diethyl ether. To synthesize pheophorbide *a* (Pheide *a*), Chl *a* was dissolved to 1 mg/mL in trifluoroacetate and incubated under nitrogen for 15 min at RT. Pheide *a* was extracted with diethyl ether. Zn-Pheide *a* was synthesized in the same way from Zn-Phe *a*. BChl *a* was isolated from *Rhodovulum sulfidophilum* following the procedure described in ref *10*. Synthesis of Cu-BPhe *a* as well as Cu-Phe *a* was done as in ref *11*. Isolation of chlorophyllase and preparation of chlorophyllide *a* (Chlide *a*) were performed as in ref *12* except that the reaction was done with purified Chl *a* in 30% acetone for 3 h at RT.

Reconstitution of recWSCP. To refold recWSCP, 300 μg of protein was resuspended in 10 μL of 50 mM sodium phosphate pH 7.4 and denatured by adding solid guanidinium hydrochloride (Gnd) until the protein inclusion bodies were dissolved. Total pigment extract from pea thylakoids (100 μg of Chl, 10-fold molar excess over protein) was dissolved in 10 μL of ethanol and then mixed with 50 μL of 2.5% [w/v] octyl-β-D-glucopyranoside (OG) in reconstitution buffer (100 mM lithium borate pH 9.0, 12.5% [w/v] sucrose) and vortexed at RT for 30 s. Protein and pigment solutions were mixed, vortexed for 30 s, and incubated at RT for 5 min. The reconstitution mixture was diluted with reconstitution buffer to a final concentration of 1% OG and centrifuged for 2 min (4 °C, 10000*g*). For analysis of reconstitution products on sucrose density gradients, 1 mg of recWSCP was refolded according to the procedure described above except that dilution to 1% OG was done with 100 mM lithium borate pH 9.0. Alternatively, surface-immobilized recWSCP was reconstituted. A 10-mL column was packed with 1 mL of Chelating Sepharose fast flow (Amersham) and loaded with Ni^{2+} ions according to the manufacturer's instructions. One milligram of recWSCP was added and unbound protein was washed away using one column volume 100 mM sodium phosphate pH 7.8. Pigments (200 μg of Chl, 5-fold molar excess over protein) were dissolved in 10 μL of ethanol and transferred under mixing into 500 μL of OG buffer (100 mM sodium phosphate pH 7.8, 1% OG [w/v], 12.5% [w/v] sucrose). The pigment solution was then added to the column, suspended with the column material, and incubated at RT for 45 min. Unbound pigment was removed by washing the column with several volumes of OG buffer until the flow-through became colorless. To obtain refolded recWSCP in detergent-free solution, the column was then washed with at least 10 volumes of 100 mM sodium phosphate pH 7.8 while occasionally resuspending the column material. Reconstituted recWSCP was eluted with 1−1.5 volumes 10 mM sodium phosphate pH 7.8, 300 mM imidazole. Reconstitution products were analyzed on a 10% PAGE (*13*) using either detergent-free running buffer (50 mM Tris, 384 mM glycine, 1 mM EDTA) or buffer containing Deriphat as a detergent (0.15% [w/v] Deriphat, 48 mM glycine, 12 mM Tris). Sucrose density gradients with 0.1% [w/v] dodecylmaltoside (LM) and without any detergent were used for purification of pigmented recWSCP complexes. For the gradients, 0.4 M sucrose, 5 mM sodium phosphate pH 7.5 were subjected to three freeze−thaw cycles which establishes a gradient between 0 and 0.8 M sucrose. Centrifugation was done at 4 °C for 16 h at 175000*g*.

Spectroscopic Analyses. Absorption of WSCP complexes was measured at RT using a Shimadzu UV−Vis-scanning spectrophotometer (UV-2101PC). The spectra were taken between 350 and 750 nm with medium scan speed and bandwidths between 0.5 and 1 nm. Circular dichroism was measured in a CD spectropolarimeter (J-810-S, Jasco) at 4 °C in a quartz cuvette between 350 and 750 nm at 2-mm path length, scan speed 50 nm/min, data pitch 0.5 nm, response time 4 s.

Molar Protein and Pigment Stoichiometries. For Chl−protein stoichiometries in recWSCP, refolding was done on a Ni^{2+}-chelating column. The complexes were purified and isolated on a detergent-free PAGE as described for native WSCP (*4*). Pigments were extracted with 2-butanol as

described (*14*), except that prior to extraction at RT, recWSCP complexes were denatured by adding 1% [w/v] SDS, and a final concentration of 120 mM NaCl was used. Pigments were quantified by HPLC-analysis. Pigment species were separated by using a reversed phase column (Cromolith SpeedROD RP-18e 50−4,6; Merck) and a linear gradient from 30 to 70% acetone over a time period of 6.5 min. Eluting material was detected by measuring the absorption at 440 nm. Pigments were quantified according to calibration curves based on the algorithm of Porra et al. (*15*) for Chls and the extinction coefficient for carotenoids of Britton (*16*). Protein quantification was done photometrically in the protein solution remaining after pigment extraction using a recWSCP extinction coefficient of 28910 $(M\ cm)^{-1}$ determined from the amino acid sequence by using an algorithm available at http://paris.chem.yale.edu/extinct.html.

Spin-Trapping of $^1O_2$ by TEMP. Spin-trapping assays were performed in 10 mM sodium phosphate pH 7.8 with a concentration of 1 μg of Chl/mL, 100 mM TEMP (2,2,6,6-tetramethylpiperidine), and methanol (ultrapure), final concentration 3.5% (v/v). To improve the solubility of Chl, 0.1% LM was added to all samples. Samples were illuminated for a given time with white light (intensities of 150, 1000, 2500 μmol of photons $m^{-2}\ s^{-1}$) in a water bath at 20 °C. The electron-paramagnetic resonance (EPR) signals of the samples were measured before and after exposure to light with a Bruker ESP 300 spectrometer. X-band spectra were recorded at RT with 9.7 GHz microwave frequency, modulation frequency 100 kHz, modulation amplitude 2 G. To remove impurities in the spin-trap TEMP purchased from different manufacturers, purification was performed by vacuum distillation at 15 mbar in the presence of analytic traces of zinc powder to provide a reducing atmosphere. A fraction distilled at 42 °C showed the best properties with respect to stability and purity.

RESULTS

A recombinant WSCP from cauliflower with an N-terminal His tag (recWSCP) has been expressed in *Escherichia coli*. Substantial amounts (aproximately 46 mg per liter of culture medium) of the protein were accumulated mostly in the form of insoluble inclusion bodies. These were used for reconstitution in vitro with chlorophyll by complete protein denaturation in Gnd and dilution into OG and pigments extracted from pea thylakoids, containing Chl *a* and Chl *b* at a ratio of approximately 3. The pigmented recWSCP was then separated from excess pigments by partially denaturing gel electrophoresis in the presence of Deriphat as a detergent. Figure 1 shows that this procedure results in green chlorophyll−protein complexes (lanes 1 and 2) that comigrate with native WSCP isolated from cauliflower leaves (lane 5). The green band of native WSCP represents protein tetramers (*10*); therefore, pigmented recWSCP is also organized in tetramers. Oligomer formation clearly is dependent on the presence of pigments, as a mock reconstitution of recWSCP, following the reconstitution protocol except that no pigments are added, results in monomeric WSCP apoprotein exclusively (lane 3).

Pigment complexes of recWSCP could also be isolated by ultracentrifugation through a sucrose density gradient, either containing 0.1% LM or no detergent at all (not shown).

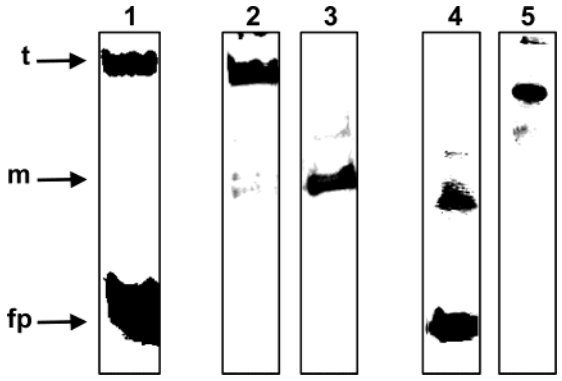

FIGURE 1: Refolding of recWSCP with Chls leads to tetramerization. Lane 1 shows recWSCP refolded with total pea pigments on a green gel. Lanes 2−4 show Coomassie stains after reconstitution from Gnd with total pea pigment (2), without pigments (3), and Chlide *a* (4). Line 5 shows a Coomassie stain of native WSCP from cauliflower. t, tetramers; m, monomers; fp, free pigment.

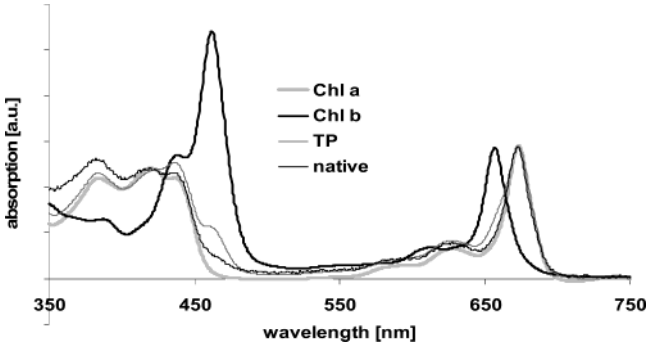

FIGURE 2: Absorption spectra of refolded recombinant and native WSCP. recWSCP reconstituted with Chl *a*, Chl *b* and total pea pigments (TP, Chl *a*/*b* = ∼3/1). Native WSCP was isolated from cauliflower leaves (Chl *a*/*b* = ∼17/1).

In the presence of 0.1% LM, the complexes sedimented more slowly than unbound pigment while in the absence of detergents in the gradient solution, the unbound pigments formed a pellet. Alternatively, the soluble fraction of the recWSCP overexpression product was refolded on a Ni^{2+}-chelating column where unbound pigments could efficiently be removed. By using detergent-free PAGE, unfolded protein could be completely separated from pigmented WSCP complexes.

Absorption spectra were measured of recombinant compared to native WSCP (Figure 2). RecWSCP was reconstituted on a Ni^{2+}-chelating column either with a pigment extract from thylakoids or with purified Chl *a* or Chl *b* (Figure 2). Column-bound recWSCP was carefully washed with detergent to remove unbound pigments and then eluted in detergent-free buffer. Spectra of recWSCP refolded with Chl *a* resemble those of native complexes (Figure 2) with major bands at 380, 415, 435, and 671 nm. With increasing Chl *b* content in the reconstitution mixture, additional absorption peaks (460, 656 nm) become visible, indicating Chl *b* binding. Reconstitution of recWSCP with Chl *b* exclusively results in the main absorption bands at 460 and 656 nm.

The pigment content of recWSCP, reconstituted with pigments on the Ni^{2+}-chelating column, was compared to that of the native protein by extracting the pigments and quantifying them by HPLC. Native WSCP isolated from cauliflower leaves contains 2 Chl molecules per tetrameric

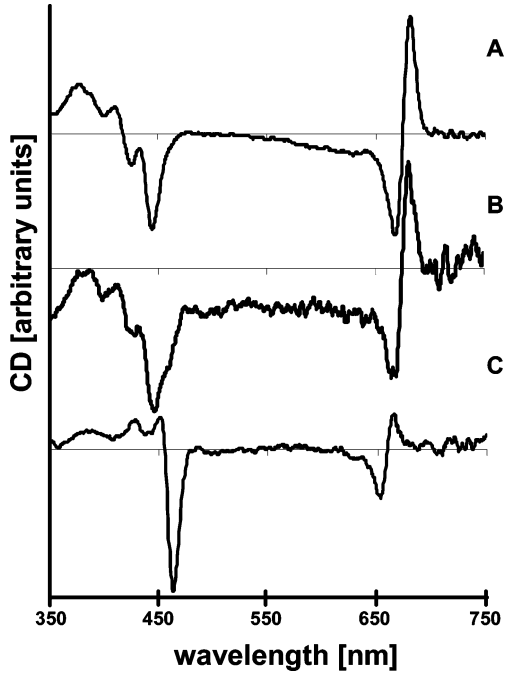

FIGURE 3: CD spectra of reconstituted recWSCP in comparison to native WSCP. (A) native WSCP, (B) recWSCP refolded with Chl *a* on a Ni^{2+}-chelating column, (C) recWSCP refolded from Gnd with Chl *b*.

complex (*5*). RecWSCP reconstituted with Chl *a* was found to contain 0.5 ± 0.2 Chl molecules per apoprotein, corresponding to about 2 Chl molecules per WSCP tetramer. Native WSCP did not contain any carotenoids, confirming analyses by Murata et al. (*17*). Neither did recWSCP even when a pigment extract from pea leaves, containing 9 carotenoid molecules per WSCP apoprotein (lutein/neoxanthin/violaxanthin/β-carotene = 51:21:12:1), was used in the reconstitution. Consistently, attempts to reconstitute recWSCP in the presence of thylakoid carotenoids as the only pigment component resulted neither in carotenoid binding nor in the appearance of WSCP tetramers (not shown).

A stoichiometry of about 2 Chls per WSCP tetramer raises the question whether intracomplex Chl−Chl interaction exists within a WSCP complex. As an indicator for excitonic interactions between Chl molecules, we measured the circular dichroism (CD) of WSCP in the spectral range of Chl absorption. The CD spectrum of recWSCP reconstituted with Chl *a* closely resembles that of native WSCP from cauliflower (Figure 3) with negative maxima around 443 and 665 nm. It is also similar to the CD spectrum of *Lepidium* WSCP (*18*), except that the latter has additional signals at Chl *b* absorption bands due to its higher Chl *b* content. When Chl *a* in recWSCP was replaced by Chl *b*, the signals in the 450 nm spectral region shifted to the red, whereas the signals in the 670 nm region shifted to the blue, demonstrating that these signals in fact were due to Chl *a* and Chl *b*.

The results so far demonstrate that both Chl *a* and Chl *b* can be reconstituted into recWSCP. To see which other structural variations in the Chl molecule are compatible with its binding into the WSCP complex, we reconstituted recWSCP with a number of Chl derivatives. In each case, the Gnd-solubilized apoprotein was reconstituted with the Chl derivative as the only pigment offered. Gel electrophoresis was used to determine whether pigmented monomeric or tetrameric WSCP was formed in these reconstitutions (Table 1). BChl *a* resembled Chl *a* and Chl *b* in that it was bound into tetrameric recWSCP. The Chl derivative with its central Mg^{2+}-ion removed, Phe *a*, was not detectably bound to recWSCP. However when Mg^{2+} was replaced by Zn^{2+} (Zn−Phe *a*), both pigment binding and tetramer formation were restored. The copper derivatives of Chl *a* (Cu−Phe *a*) and of BChl *a* (Cu−BPhe *a*), on the other hand, were not bound to recWSCP. When the phytol chain was removed in Chl *a* or Chl *b*, leading to Chlide *a* or *b*, green monomeric WSCP appeared on the gel, indicating that Chlide *a* was bound to WSCP but failed to promote oligomerization of the protein (Table 1, see also Figure 1). The same was true for the Zn-derivative of Chlide *a* and as well for Mg-Proto IX. However, binding of the latter was less stable than that of Chlide (not shown). Consistently, when both the Mg^{2+} ion and the phytol chain were missing in Pheide *a*, no pigment binding to WSCP was detectable.

Table 1: Reconstitution of recWSCP with Chl Derivatives[a]

pigment	reconstitution	multimers
Chl *a*	+	+
Chl *b*	+	+
BChl *a*	+	+
Zn- Phe *a*	+	+
Cu- Phe *a*	−	−
Cu- BPhe *a*	−	−
Chlide *a*	+	−
Chlide *b*	+	−
Mg- Proto IX	+	−
Zn- Pheide *a*	+	−
Phe *a*	−	−
Pheide *a*	−	−

[a] Reconstitution was started from Gnd-dissolved WSCP apoprotein and reconstitution products were analyzed on a 10% PAGE using Deriphat as detergent. When a pigmented band was detected, successful reconstitution is indicated by (+). When a band was detected that comigrated with native WSCP tetramers, this revealed multimerization of WSCP, also indicated by (+).

Compared to Chl-containing proteins of the thylakoid membrane, WSCP is unusual in that it does not contain any carotenoid to prevent photodynamic damage due to triplet-excited Chl. Therefore, we tested the photostability of Chl *a* in native and recombinant WSCP by exposing the complex to moderately strong illumination (500 μmol of photons m^{-2} s^{-1}) and measuring the loss of Chls as indicated by the decrease in Chl fluorescence. After 60 min of illumination, roughly half of the Chl fluorescence had disappeared in recWSCP (Figure 4); a similar behavior was observed of native WSCP (not shown). When detergent-dissolved Chl *a* at the same concentration was illuminated for 60 min under the same conditions, roughly 90% of the Chl was no longer fluorescent. This indicates that Chl bound to WSCP is protected to some extent against photodegradation.

Considering this result, the question arises whether the protein itself plays a role in the protection of the bound pigment. To investigate this, spin-trap EPR measurements were done on native and recombinant complexes and the development of singlet oxygen ($^1O_2$) was measured. Figure 5 shows the characteristic EPR signal of the nitroxyl radical 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO), which is formed by the reaction of TEMP with $^1O_2$. $^1O_2$ was produced by free Chl exposed for 10 min to illumination with white light (intensity: 150 μmol of photons m^{-2} s^{-1}) (Figure 5b).

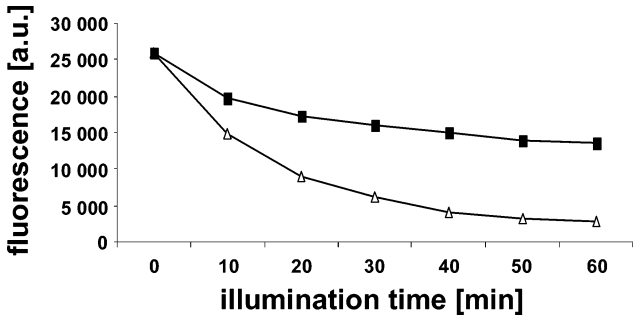

FIGURE 4: Photostability of reconstituted recWSCP. recWSCP was refolded from its Gnd solution with Chl *a* and purified on a sucrose density gradient containing 0.1% LM (squares). A mock reconstitution was performed lacking the protein and diluted in sucrose gradient buffer at the same Chl concentration (open triangles). Both solutions were illuminated with 500 μmol of photons m^2 s^{-1} for 0−60 min. After excitation at 410 nm, emission detection at 676 nm was plotted against time.

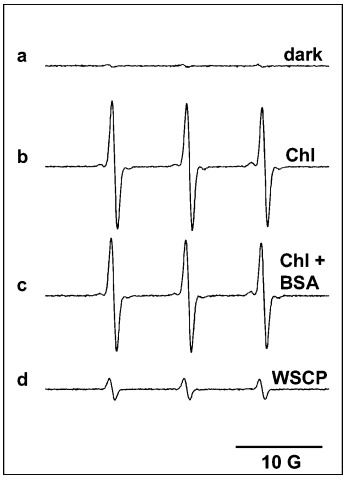

FIGURE 5: Spin-trap EPR measurements on native WSCP complexes. Native WSCP as well as the controls Chl *a* and Chl *a* with BSA as protein component were adjusted to the same Chl concentration. Evolved $^1O_2$ was detected via TEMPO-formation after illumination at 150 μmol of photons m^{-2} s^{-1} for 10 min. Background signals were detected prior to illumination (a, dark control with solubilized Chl *a* (1 μg of Chl/mL) prior to illumination b, solubilized Chl *a* (1 μg of Chl/mL) upon illumination; c, 1 μg of Chl/mL + 125 μg of BSA/mL upon illumination; d, native WSCP (1 μg of Chl/mL + approximately 86 μg of WSCP/mL) upon illumination.

Addition of BSA to the sample prior to the illumination decreased the size of the EPR signal only slightly (Figure 5c). When, however, instead of unbound Chl plus or minus BSA, native WSCP was used in the same buffer at the same Chl concentration, the signal was significantly lower at the given light intensity and illumination time (Figure 5d). Obviously, the binding of Chl to WSCP reduces the yield of $^1O_2$ formation. When recombinant His-tagged WSCP was used instead of the native protein, almost the same reduction of the TEMPO signal was seen (not shown). This indicates that the additional histidine residues in the His-tagged recombinant protein do not act as an efficient scavenger in recWSCP although histidine is a known $^1O_2$ quencher (*19*). A very small signal observable prior to illumination of the samples is due to the impurity of the spin-trap even after the purification protocol used (Figure 5a).

To further investigate the protecting effect of WSCP against singlet oxygen formation by Chl, the time dependence

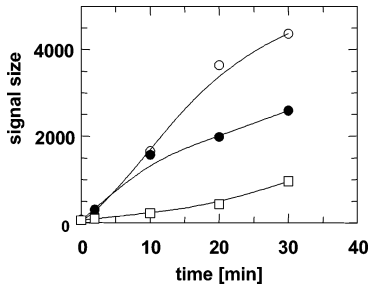

FIGURE 6: Time dependency of $^1O_2$ formation. Recombinant WSCP (squares) as well as the controls Chl *a* (open circles) and Chl *a* + BSA (filled circles) were illuminated at 150 μmol of photons m^{-2} s^{-1} in the presence of TEMP. The size of the EPR signal indicative for the amount of TEMPO-formation was plotted against time.

of TEMPO formation was measured. As shown in Figure 6, singlet oxygen production as indicated by the EPR signal of WSCP-bound Chl was only about 25% compared to that of free Chl, even at longer illumination time (30 min). To test whether this effect was merely due to the presence of protein rather than specific for WSCP, we added BSA to the chlorophyll solution. Since the aqueous solution contained LM as a detergent, Chl was not likely to become attached to BSA. Only when the samples were illuminated for more than 10 min, the addition of BSA to the Chl solution also reduced the yield of $^1O_2$ formation to some extent. Similar results were obtained at higher light intensities (1000 and 2000 μmol of photons m^{-2} s^{-1}, not shown).

WSCPs from *Chenopodium* and Brussels sprouts have been reported to show a remarkable stability at high temperatures (*20*, *21*). We found that the same is true for native and recombinant WSCP from cauliflower. The complexes withstood 20 min at 80 °C or 5 min at 100 °C in buffered solution without any detectable damage in pigmented complexes as visualized by CD (vis) spectroscopy. On the other hand, treatment in 1% SDS at 100 °C for 5 min fully dissociated the complexes (not shown).

DISCUSSION

In this work, recombinant His-tagged WSCP apoprotein was complexed with pigments in vitro and studied with regard to its pigment-binding properties. The recombinant complex closely resembles native WSCP according to its biochemical and spectroscopical properties tested. It also behaves quite similarly to the fusion protein of WSCP and maltose-binding protein (*5*) with the exception that with His-tagged WSCP we only observed monomeric and (pigmented) tetrameric complexes, whereas the pigmented fusion protein also formed dimers, trimers, and higher oligomers. One possible explanation for this difference is that the fusion to maltose-binding protein gave rise to all these different oligomers. Another explanation is that only the tetrameric complexes of recWSCP but not the other oligomers were stable enough to survive partially denaturing electrophoresis used in this work whereas in the previous paper, the complexes were separated by detergent-free electrophoresis. Partially denaturing electrophoresis separates unbound or unspecifically bound Chls even from the highly hydrophobic Chl-binding proteins of the thylakoid membrane; therefore, we can rigorously exclude any weakly or nonspecifically bound Chls from our analyses of recombinant WSCP complexes. The same is true for the quantification of

pigments per protein. To do this, the recombinant complexes have been immobilized on a Ni^{2+}-chelating sepharose column and extensively washed with detergent, again a procedure that is suitable to remove unspecifically bound pigments from membrane proteins of the photosynthetic apparatus (*22*). To purify pigmented WSCP complexes from free protein that could be retained on the column after the different washing steps, a preparative detergent-free-PAGE was added. Using this stringent method, the stoichiometry of 2 Chls per recWSCP tetramer was obtained; the value is consistent with the ones for native and MBP-WSCP (*5*). We cannot exclude that during the rigorous washing of the complexes some less stably bound Chl molecules dissociated, so the stoichiometry of 2 Chls per tetrameric complex should be regarded as a lower limit.

The two Chl molecules in tetrameric WSCP undergo excitonic interaction as judged from their CD signals in both the red and Soret absorption region. As the CD spectra of native WSCP and recWSCP refolded in the presence of Chl *a* are very similar, we conclude that the pigments are bound in approximately the same spatial arrangement relative to one another. RecWSCP complexes reconstituted with Chl *b* show a similar CD spectrum except that signals around 650 nm are shifted to the blue and signals around 450 nm are shifted to the red, in accordance with the shifts in absorption bands of Chl *b* compared to Chl *a*. This indicates that Chl *b* is bound to WSCP in a very similar fashion as Chl *a*.

WSCP is an unusual Chl-binding protein in that it does not bind carotenoids. All Chl-binding light-harvesting or reaction-center proteins of the photosynthetic apparatus in plants contain carotenoids. These quench the potentially dangerous triplet-excited states of Chls that otherwise give rise to the production of singlet-excited oxygen molecules which in turn destroy Chls and other components of the photosynthetic apparatus. When Chl *a* in aqueous detergent solution is illuminated, it is rapidly degraded, presumably due to the photodynamic effect described above. However, the same concentration of WSCP-bound Chl *a*, dissolved in the same solution and illuminated under the same conditions, disintegrates at a significantly slower rate, indicating that in the WSCP complex Chl *a* is somewhat protected against photodegradation. Consistently, as shown in Figures 5 and 6, singlet-oxygen production is lower for WSCP-bound Chl *a* than for unbound Chl *a*. This can in principle be due to several reasons: (i) The lifetime of the singlet-excited state of Chl *a* is shortened in the WSCP complex by some structural effect accelerating internal conversion, i.e., heat dissipation of excitation energy (*23*), by energy transfer to some unknown, energy dissipating component in the WSCP complex, or by a fast, reversible photoreaction possibly related to the photoconversion observed in class-I WSCPs. (ii) The rate of intersystem crossing from the excited singlet to the triplet state is altered thereby leading to a lesser population of the triplet state. (iii) The intrinsic lifetime of the Chl triplet state (without quencher) is shortened by the protein environment so that the probablitity of a reaction with O_2 is lowered. It is known that, in the absence of O_2, the P700 triplet state has a lifetime of about 6 μs compared to 1 ms for free Chl (*24*). (iv) Some component in the WSCP complex takes on the role of carotenoids in other Chl−protein complexes in that it quenches either triplet-excited Chl or singlet-excited oxygen or both. (v) The Chl is tightly enclosed by the tetrameric protein structure so that O_2 is excluded from the Chl binding site and, thus, cannot be excited into its singlet state. Assessment of the lifetimes of Chl excited states in WSCP by time-resolved spectroscopic measurements should enable us to distinguish between these possibilities.

The case of a plant protein containing chlorophyll without the protection by additionally bound carotenoids is unprecedented. Cytochrome b_6/f has recently been found to contain a Chl molecule whose function is not yet known, and this Chl is accompanied by only a substoichiometric amount of carotenoids (*25*). However, cytochrome b_6/f forms a dimer, so it is possible that one carotenoid exerts is protective effect on two Chls. No trace of carotenoids has been found in native or recombinant WSCP, so we can exclude carotenoids as photodynamically protective components.

We tested various Chl derivatives in the reconstitution of WSCP complex in vitro to obtain information about which structural components of the Chl molecule are essential for its interaction with WSCP. In accordance with previous results (*5*), we found that Chl *b* also binds efficiently into the complex. This is similar to some binding sites in LHCIIb that during complex reconstitution in vitro can accommodate either Chl *a* or Chl *b* (*26*). By contrast, BChl *a*, which is not at all bound into LHCIIb (*27*) is accepted as a ligand to WSCP. BChl *a* differs from Chl *a* by various side groups in the periphery of the tetrapyrrole ring and by a saturated C−C bond in ring B where Chl *a* possesses a double bond. This latter distinction leads to a different distribution of pi electrons over the mesomeric systems in Chl *a* and BChl *a*. Even so, both pigments are bound, indicating that recognition of the pigment by WSCP is not highly selective with regard to structural details of the tetrapyrrole ring.

Whereas structural alterations in the periphery of the Chl *a* molecule seem to be tolerated by WSCP, the central metal atom turns out to be essential. Phe (or BPhe) is not a ligand for WSCP. However, binding can be restored by replacing Mg^{2+} in Chl *a* with Zn^{2+}. Both Mg^{2+} and Zn^{2+} prefer pentacoordinate structures and thus form a square-pyramidal structure with porphyrins with an external axial ligand, whereas Cu^{2+} prefers tetracoordinate complexes and thus is thought to exhibit a planar geometry with the tetrapyrrol ring without an axial ligand (*28*). This may prevent Cu-Phe *a* from binding to WSCP. A similar observation has been made with different metallo-Phe *a* in the reconstitution of LHCIIb: Zn-Phe *a* can replace Chl *a* molecules, whereas Cu-Phe *a* cannot (C. Eisen and H. Paulsen, unpublished observations).

A special role in WSCP binding seems to be performed by the phytol chain in Chl *a*. Phytol is not required for pigment binding to WSCP as shown by pigmented recWSCP complexes containing Chlide *a* and Zn-Pheide *a*. However, the phytol appears to be essential for the oligomerization of the protein. Whereas WSCP with Chl *a* bound is predominantly found in its tetrameric state, the complexes with Chlide *a* and Zn-Pheide *a* comigrate with the nonpigmented monomeric protein in partially denaturing gel electrophoresis and apparently do not form any oligomers. One explanation is that the phytol chains of bound Chl molecules mediate or stabilize protein−protein interactions between WSCP monomers in the tetrameric complex. Consistently, Chlide *b* and

an even earlier Chl-precursor, Mg-Proto IX, were found to bind to the protein but failed to cause multimerization.

The function of WSCP still is a matter of speculation, partially because its intracellular location is unclear. WSCP has been detected in the soluble fraction of chloroplasts (*29*); however, the presence of a signal peptide in WSCP targeting it to the secretory pathway indicates to its (possibly transient) location in the endoplasmatic reticulum (*5*). It has been hypothesized that WSCP is a Chl carrier during senescence (*1*) transporting Chl molecules from the thylakoid to the inner envelope membrane where chlorophyllase initiates the Chl degradation process (*30*). Another possibility is that WSCP serves as a carrier not primarily of Chl but of precursors, serving during Chl biogenesis, or of a degradation product, functioning during the breakdown of Chl. Many Chl precursors or breakdown products are efficient photosensitizers, able to catalyze the light-induced production of potentially dangerous singlet oxygen. Therefore, it is conceivable that the localization of these Chl derivatives is strictly controlled during their metabolism, possibly necessitating carrier molecules chaperoning Chl derivatives from one enzymatic conversion to the next. Our data put some limitations on the Chl derivatives that are potentially transported by WSCP. Components of the degradation pathway that may bind to WSCP include only Chl and Chlide. The next degradation step is the demetalation resulting in Phe, and our data clearly show that Phe *a* is no ligand of WSCP. Chlide *a* is also an intermediate of Chl *a* synthesis via Chl synthase (*31*) and of Chl *b* synthesis via Chlide *a* oxygenase (*32*). Thus, WSCP as a potential carrier of Chlide and Mg-Proto IX (and possibly other Chl precursors) may be involved in the Chl biosynthesis pathway. Of course, it still needs to be determined whether the monomeric WSCP complexes provide protection against photooxidation as shown for the WSCP tetramer. The spectroscopic and biochemical characterization of monomeric WSCP−pigment complexes is presently underway along with studies of whether WSCP-bound Chlide is a substrate for either Chl synthetase or Chlide oxygenase.

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