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Assembly of water-soluble chlorophyll-binding proteins with native hydrophobic chlorophylls in water-in-oil emulsions

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ABSTRACT

The challenges involved in studying cofactor binding and assembly, as well as energy- and electron transfer mechanisms in the large and elaborate transmembrane protein complexes of photosynthesis and respiration have prompted considerable interest in constructing simplified model systems based on their water-soluble protein analogs. Such analogs are also promising templates and building blocks for artificial bioinspired energy conversion systems. Yet, development is limited by the challenge of introducing the essential cofactors of natural proteins that are highly water-insoluble into the water-soluble protein analogs. Here we introduce a new efficient method based on water-in-oil emulsions for overcoming this challenge. We demonstrate the effectiveness of the method in the assembly of native chlorophylls with four recombinant variants of the water-soluble chlorophyll-binding protein of Brassicaceae plants. We use the method to gain new insights into the protein–chlorophyll assembly process, and demonstrate its potential as a fast screening system for developing novel chlorophyll–protein complexes.

1. Introduction

Almost all the pigments that are directly involved in photosynthetic electron transport and light harvesting are bound to hydrophobic proteins within photosynthetic membranes. However, a few natural water-soluble proteins bind and assemble highly hydrophobic photosynthetic pigments such as chlorophyll (Chl), bacteriochlorophyll (BChl), and carotenoids. These include the Fenna–Mathews–Olson (FMO) protein of non-oxygenic photosynthetic green-sulfur bacteria [1,2], the peridinin-Chl protein (PCP) of dinoflagellates [3], and the water-soluble chlorophyll binding protein (WSCP) [4]. The latter, are unique among this group of Chl-binding proteins because they are not directly involved in primary photosynthetic reactions. WSCPs were identified in Brassicaceae, Polygonaceae, and Amaranthaceae plants [5,6]. The WSCP family is divided into two classes according to distinctive photophysical properties [4,5]. Class I WSCP from *Chenopodium album* undergoes photoconversion upon illumination, while class II WSCPs from *Brassica* plants are not photoconvertible. Class II is further sub-divided according to sequence similarity and biophysical properties into class IIa that includes cauliflower (*Brassica oleracea* var. *botrys*), Brussels sprout (*B. oleracea* var. *gemmifera*), and Japanese wild radish (*Raphanus sativus* var. *Raphanistroides*), and class IIb that includes Virginia pepperweed (*Lepidium virginicum*) [5]. The most prominent difference between the sub-classes is the position of Chl *a* lowest energy Qy absorption band at 672 nm in class IIa, vs. 663 nm in class IIb.

The precise physiological function and mode of action of WSCPs still remain unclear. Plenty of evidence suggests a stress response activity, and a photoprotective function of WSCPs [5–8]. Another role as transient carriers or scavengers of Chl metabolites was also speculated [9] but was not substantiated by physiological evidence [6,7]. Regardless of their specific native function, WSCPs may be very useful as simple systems for studying binding and assembly of Chl–protein complexes, Chl–Chl and Chl–protein interactions [4], and as templates for constructing artificial light-harvesting protein complexes. A crystal structure of Virginia pepperweed WSCP is available at a resolution of 2.0 Å. It reveals a Chl–protein complex comprised of four identical protein subunits, each of which binds a single Chl, bringing the four pigments into a closely packed arrangement within the hydrophobic core of the protein homotetramer [8]. Intriguingly, although WSCP is not a light-harvesting protein, this densely packed assembly of four Chls within the protein core does not lead to significant quenching of their fluorescence yield. Such a reduction of quenching is a hallmark of natural light-harvesting complex (LHC) functionality [10,11]. This unique property of WSCP is attracting the attention of optical spectroscopists attempting to understand its underlying physical principles. Most of the spectroscopic research so far focused on class IIa WSCPs from cauliflower [12–15]

Abbreviations: CaWSCP, water-soluble chlorophyll binding protein from cauliflower; BoWSCP, Brussels sprout; LvWSCP, Virginia pepperweed; RshWSCP, Japanese wild radish; W/O, water-in-oil

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instead of the class IIb WSCP from Virginia pepperweed for which high resolution data is available. Only recently, Alster et al. presented a 2D spectroscopy study of WSCP from Virginia pepperweed [16].

Spectroscopic studies require assembling the protein–Chl complexes in vitro. This is because the native proteins are assembled with Chl *a* and Chl *b*, hence the purified native complexes always contain a heterogeneous mixture of tetramers with different combinations of Chl *a* and Chl *b* [17]. Unfortunately, introducing the highly water-insoluble native Chls into the water-soluble proteins in-vitro is very challenging. One way to overcome this issue is by mixing the apoproteins with thylakoid membranes [15,17], but this method is limited to the native Chls present in the thylakoids. Another method for assembling several Chl and BChl derivatives with WSCP from cauliflower (CaWSCP) recombinantly expressed in *Escherichia coli* was previously demonstrated by Schmidt et al. [18]. It was based on immobilizing a histidine-tagged protein onto a Ni-affinity column and introducing Chl derivatives solubilized in detergents. More recently, this method was successfully used for reconstitution of recombinant WSCP from *Arabidopsis thaliana* [7], and modified and applied to recombinant versions of WSCP from Brussels sprout (BoWSCP), Japanese wild radish (RshWSCP) and Virginia pepperweed (LvWSCP) [19–21].

In this work we present a new method for assembling Chls with WSCP that is general, straightforward, and does not require tagging or immobilizing the proteins. It relies on encapsulating the water-soluble apoproteins in water-in-oil (W/O) microdroplets by preparing emulsions from their aqueous solutions in mineral oil. The high surface to volume ratio of the aqueous W/O microdroplets enables the introduction of hydrophobic cofactors that are dissolved in the oil phase. We demonstrate complete assembly of Chl *a* with the apoproteins of CaWSCP, BoWSCP, RshWSCP, and LvWSCP that were recombinantly expressed in *E. coli*. This method has enabled us, for the first time, to carry out a rigorous comparison of the spectral properties of the four different WSCP variants, and to resolve the issue of Chl:protein binding stoichiometry in the different variants. Finally, we demonstrate the potential of the method as a screening system for developing novel Chl–protein complexes.

2. Materials and methods

2.1. Expression and purification of recombinant WSCPs in E. coli

Recombinant LvWSCP, CaWSCPC, BoWSCP, and RshWSCP were expressed in *E. coli* as fusion proteins linked to Profinity eXact™ tag (Bio-Rad). In order to overexpress LvWSCP, the cDNA sequence of LvWSCP from *L. virginicum* (PubMed Accession Number: 2DRE_AGI:122919965), without a signal peptide and C-terminal cleavage extension was synthesized and optimized for efficient expression in *E. coli* by Genwiz, Inc. The coding sequence for CaWSCP from *B. oleracea* var. *botrys* was kindly provided by Prof. H. Paulsen (University of Mainz, Germany).

The modified cDNA of LvWSCP was cloned into the expression vector pETTRX-Pro-WSCPL by means of Ligation-Independent-Cloning (LIC) [22]. The same method was used for cloning the genes coding for CaWSCP, BoWSCP and RshWSCP into expression vectors pETTRX-CaWSCP, pETTRX-BoWSCP and pETTRX-RshWSCP, respectively. The primer sequences used for cloning are provided in Supplementary Table 1.

In order to overexpress CaWSCP, BoWSCP, RshWSCP, and LvWSCP, *E. Coli* BL21 bacteria were transformed with individual plasmids. These were grown in 1 l of LB medium at 37 °C until an OD of 0.3–0.6 was reached. Protein expression was induced by addition of 1 mM IPTG. After induction, bacteria were grown at 30 °C for 12–16 h and then were harvested by centrifugation at 7000 rpm for 10 min at 4 °C. Expressed WSCPs were purified using Bio-Scale Mini Profinity eXact Cartridges (Bio-Rad), according to manufacturer's instructions. Afterwards, the proteins were further purified on an anion exchange HiTrap™ Q HP column (GE Healthcare) equilibrated with 10 column volumes of 20 mM Tris–HCl buffer, pH 7.2. The proteins were eluted from the column with 20 mM Tris–HCl, pH 7.2, and 100 mM NaCl buffer. Finally, the elution buffer was exchanged to 50 mM sodium phosphate buffer, pH 7.8 using HiTrap™ desalting columns (GE Healthcare). All the chromatographic steps were carried out using an AKTA-Purifier system (GE Healthcare) at 4 °C, and the elution was monitored by absorbance at 280 nm. The purified proteins were used immediately for reconstitution with Chl *a*, or stored in the same buffer at 4 °C.

Protein purity was verified by SDS-PAGE using 10% polyacrylamide gel. Protein bands were visualized by ready-to-use Coomassie® stain, Instant Blue™ (Expedeon). Protein concentrations were determined spectrophotometrically using extinction coefficient and molecular weights of 25,565 cm^{-1} M^{-1} and 19,155 g/mol, 25,565 cm^{-1} M^{-1} and 19,665 g/mol, 27,055 cm^{-1} M^{-1} and 19,874 g/mol, and 25,565 cm^{-1} M^{-1} and 19,574 g/mol for CaWSCP, BoWSCP, RshWSCP, and LvWSCP, respectively. Both the extinction coefficient and molecular weights were determined from the amino acid sequence by using the algorithm available at http://www.biomol.net/en/tools/proteinextinction.htm.

2.2. Chl a extraction

Purified Chl *a* was extracted from lyophilized *Spirulina platensis* (kindly provided by Prof. Sammy Boussiba, Blaustein Institute for Desert Research, Ben-Gurion University at Sde-Boker, Israel) using standard protocols [23]. Briefly, methanol extracts from the dry cyanobacterium were loaded on DEAE Sepharose Fast Flow (GE Healthcare) column, and Chl *a* was eluted from the column using a step gradient of acetone followed by a 10:3 v/v acetone/methanol mixture. Pigment purity was analyzed on each step of extraction by thin layer chromatography using a 68:25:5:2 dichloromethane/isopropanol/n-hexane/methanol (v/v) mixture as eluent. The quantitative determination of Chl *a* was done spectrophotometrically using an extinction coefficient of 74,400 cm^{-1} M^{-1} in ethanol at 663 nm [24]. Aliquots of Chl *a* were dried by evaporation and stored at −20 °C under inert (Argon) atmosphere.

2.3. Water-in-oil emulsion preparation

W/O emulsions were prepared as described by Miller et al. [25] with some modifications. Typically, 5 ml of oil–surfactant mixture (4.5% (v/v) Span80, and 0.4% (v/v) Tween80 in mineral oil) were cooled on ice in a glass vial. Then, 1 ml of ice-cold aqueous phase was added to the organic mixture and an emulsion was generated by homogenization at 9500 rpm for 2 min using a homogenizer (IKA Ultra Turrax T25 basic). In order to breakdown the W/O emulsion and separate the water droplets from the organic phase, the emulsion was transferred to 1.5 ml tubes and centrifuged at 14,000 rpm for 5 min at room temperature. The upper oil phase was disposed, and the surfactants were removed by five rounds of extraction with 1 ml of mineral oil and centrifugation at 14,000 rpm for 5 min at room temperature. Finally, traces of mineral oil were removed from the aqueous phase by double extraction with 1 ml of water-saturated diethyl-ether and centrifugation as above.

2.4. Reconstitution of recombinant WSCPs with Chl a in water-in-oil emulsion

In order to reconstitute recombinant WSCPs with Chl *a,* the W/O emulsions were prepared as described above using a solution of the protein in 50 mM sodium phosphate buffer, pH 7.8 as the aqueous phase. Typically, 1 mg of protein was reconstituted with a 10 fold molar excess of Chl *a* (456 μg). A small volume (typically 20 μl) of concentrated Chl *a* dissolved in 100% ethanol was added to the emulsion and the pigment was thoroughly dispersed in the emulsion by flicking and inverting the glass vial. Then, the emulsion was incubated for 1–2 h on ice in the dark. After incubation, the water phase containing reconstituted WSCP/Chl *a* complexes was recovered from the emulsion by centrifugation

and washing with mineral oil and diethyl ether, as described above. Finally, the aqueous solution of reconstituted protein–pigment complexes was loaded on a PD-10 desalting columns (GE Healthcare) and eluted with 50 mM sodium phosphate buffer, pH 7.8 and concentrated in Amicon Ultra Centrifugal Filter Units (10 kDa MW cutoff, Millipore), to an OD at 663 nm for LvWSCP, and 672 nm for BoWSSCP, CaWSCP and RshWSCP of about 0.5–1.0 at a path length of 1 cm.

2.5. Reconstitution of crude extracts of recombinant CaWSCP with Chl a in water-in-oil emulsion

Recombinant CaWSCP containing His-tag at the N-terminus (CaWSCP-His) was overexpressed as described above using 250 ml of transformed BL21 bacteria. After harvesting, the pellet was dissolved in 1–2 ml of 50 mM sodium phosphate buffer pH 7.8, sonicated and centrifuged at 12,000 rpm for 30 min at 4 °C. Then, 125 μl of the supernatant was diluted to 1 ml with 50 mM sodium phosphate buffer, pH 7.8. This was used as the aqueous phase in the W/O emulsion preparation.

2.6. Size-exclusion chromatography

Size-exclusion chromatography (SEC) of WSCP samples was carried out using Superdex 200 10/300GL prepacked column, (GE Healthcare) on an AKTA-Purifier system (GE Healthcare) at 4 °C. The column was equilibrated with 50 mM Tris–HCl, 150 mM KCl, pH 7.7. Then, 250 μl of concentrated WSCP sample was loaded on the column and the elution was monitored online for aromatic amino acid absorbance at 280 nm, and at 420 and 660 nm for Chl absorbance. The column was calibrated with Molecular Weight Markers for Gel Filtration Chromatography (Sigma).

2.7. Spectroscopic measurements

UV–visible-NIR absorption was recorded at room temperature with a Jasco V-7200 spectrophotometer using a quartz cuvette with a path length of 10 mm. Circular dichroism (CD) spectra were measured at the same conditions with a Jasco J-815 CD spectropolarimeter using a scan speed of 100 nm/min, bandwidth of 4 nm, data pitch of 0.5 nm and response time of 2 s. Data from five consecutive measurements of each sample were averaged.

Measurements of Chl *a* room-temperature fluorescence were carried out in a 10 × 10 mm quartz cuvette using a Fluorlog-3 spectrofluorometer (Horiba). Emission spectra were obtained by excitation at 420 nm, and excitation spectra were obtained by monitoring the emission at 682 nm (BoWSCP, CAWSCP, RshWSCP) and 672 nm (LvWSCP). Fluorescence yield was calculated by dividing the area under the emission band of each WSCP sample by that of Chl *a* in acetone, and assuming the yield of the latter to be 30% [26]. More specifically, the WSCP fluorescence yield, Φ_{WSCP}, is given by [27]

$$\Phi_{WSCP} = \frac{F_{wscp}/f_{wscp}}{F_{Chl}/f_{Chl}} \left(\frac{n_{water}}{n_{acetone}}\right)^2 \Phi_{Chl}$$

where F_{WSCP}, and F_{Chl} are the areas under the fluorescence emission bands of WSCP in aqueous solution, and Chl *a* in acetone, respectively, f_{WSCP} and f_{Chl} are the respective absorption factors at 420 nm, n_{water} and $n_{acetone}$ are the refractive indices of water and acetone, respectively, and Φ_{Chl} is the fluorescence yield of Chl *a* in acetone. The absorption factor, f is related to the absorbance, A, by $f = 1 - 10^{-A}$. For each sample, the value F/f was obtained by measuring the emission spectra of four samples with absorbance at 420 nm ranging from 0.03 to 0.1. At this range F is to a good approximation linearly correlated with f; thus F/f can be obtained from the slope of a line fitted to a plot of F vs. f. Values of 1.33, and 1.36 were used for the refractive indices of water, and acetone, respectively [19].

2.8. Scanning fluorescence microscopy

Emulsions were inserted into an elongated rectangular capillary (VitroTubes W5010) and sealed with 5-minute epoxy. The droplets were visualized on a Leica SP5-X confocal microscope by exciting the sample at 660 nm and monitoring the fluorescence at 682 nm.

3. Results

3.1. Reconstitution of recombinant WSCP variants with Chl a

All the recombinant WSCPs were readily expressed in *E. coli* and purified from the soluble fraction of the bacterial lysate with good yields. The purified proteins were stable in aqueous-solutions, had the expected molecular weight according to gel-electrophoresis analysis, and featured a typical UV absorption spectrum with a peak at 280 nm and a tryptophan shoulder (data not shown). Aqueous solutions of the proteins were used for reconstitution with Chl *a* in W/O emulsions as described in Section 2 "Materials and methods". The absorption and CD spectra of the reconstituted complexes are shown in Fig. 1. As expected, the spectra of class IIa complexes CaWSCP, BoWSCP and RshWSCP were almost identical and distinct from the spectrum of class IIb LvWSCP. The absorption spectra were similar in band shape and position to the respective, previously reported spectra of native CaWSCP [17], BoWSCP [28], and LvWSCP [29]. An exception is the band at 468 nm that was observed only in the native complexes, and is attributed to the absorption of Chl *b*. The CD spectra were characteristic of excitonically coupled Chls, particularly the conservatively split CD band of the Qy electronic transition.

3.2. Size exclusion chromatography of WSCP/Chl a complexes

WSCP samples that were reconstituted with ten-fold molar excess of Chl *a* were eluted on a size exclusion column, and monitored for protein and Chl *a* absorption at 280 nm and 660 nm, respectively. The elution profiles of all four WSCP variants similarly revealed a predominant band corresponding to molecular weights around 80 kDa (Fig. 2, Table 1) in both the 280 nm and 660 nm profiles. This is equivalent to the expected molecular weight of a tetrameric WSCP/Chl *a* complex. Another small band corresponding to a high molecular weight species was observed in both the 280 nm and 660 nm profiles. Another minor population of monomeric WSCP was detected exclusively in the 280 nm elution profile (Fig. 2). The elution profiles of the different variants were similar, yet small but significant differences were observed between class IIa, and IIb protein complexes (Fig. 2). The monomer, and high molecular weight minor bands of class IIb LvWSCP were more pronounced and an additional minor band showed up in the 280 nm elution profiles corresponding to the molecular weight of apoprotein dimers. Interestingly, low Chl *a* concentrations increased the population of monomers and dimers in class IIb LvWSCP but not in class IIa WSCPs. By contrast, increasing the molar ratio of Chl *a* to apoprotein during reconstitution of class IIa and IIb WSCPs beyond ten-fold had no effect on the elution profiles. Altogether, our results suggest that the apoproteins of class IIa WSCPs are more stable to misfolding and aggregation in the W/O droplets than those of class IIb LvWSCP.

3.3. WSCP/Chl a stoichiometry

Previous studies reported different Chl binding stoichiometries for different WSCP variants. The numbers of Chls per WSCP tetramer ranged from one in BoWSCP [19], two in CaWSCP [18], and four in LvWSCP [8]. In this work, we were able to explore this issue more rigorously by comparing Chl complexes of all four WSCP variants prepared under the same conditions. We found that the absorption spectra of reconstituted BoWSCP, CaWSCP, RshWSCP and LvWSCP have similar ratios of the 280 nm protein peak, and the Chl *a* Qy absorption peaks

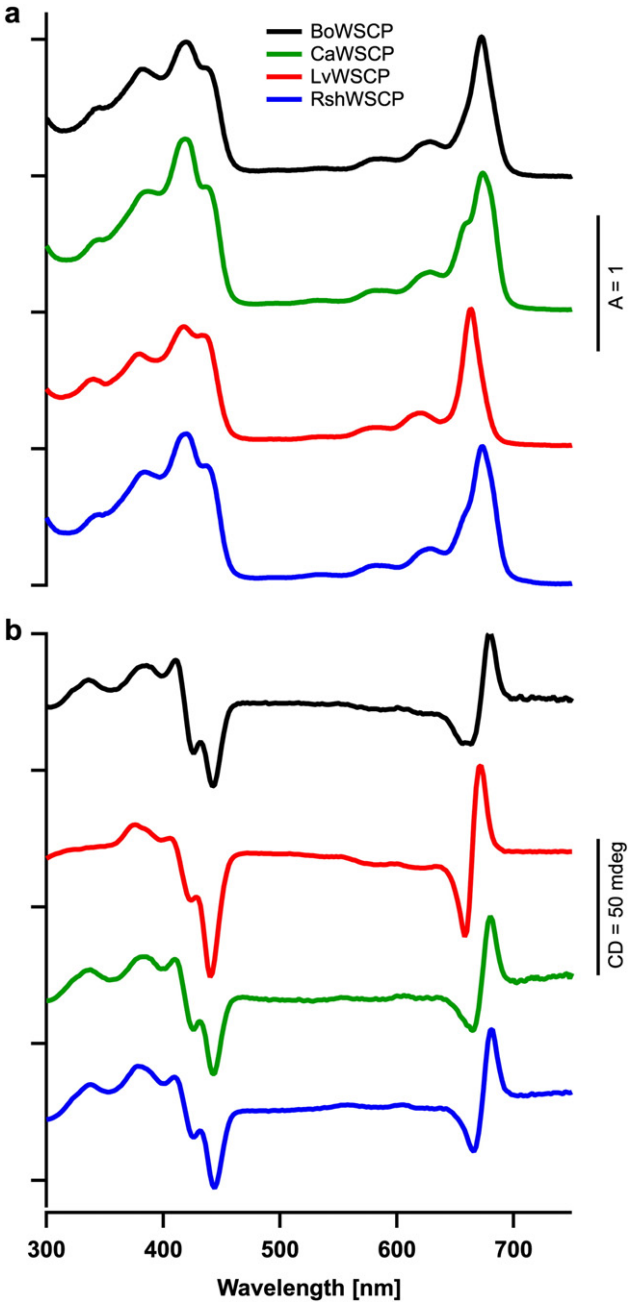

Fig. 1. Absorbance and CD spectra of four WSCP variants reconstituted with ten-fold molar excess of Chl *a* using the water-in-oil emulsion system. The absorption spectra were normalized to 1 at 673 nm for BoWSCP, CaWSCP, and RshWSCP, and 663 nm for LvWSCP, and the same normalization factors were then applied to the CD spectra.

(672 nm for BoWSCP, CaWSCP, RshWSCP, and 663 nm for LvWSCP) (Fig. 3). Furthermore, our size exclusion chromatography results clearly show that Chls do not bind to WSCP monomers or oligomers other than tetramers. This implies that, in contrast to previous reports, all WSCP variants have the same Chl:protein stoichiometry. Based on the LvWSCP structure, it is most reasonable to assume that the binding ratio is four Chl *a* molecules per WSCP tetramer.

3.4. Chl a fluorescence of recombinant WSCP/Chl a complexes

The implications of excitonic interactions between Chl *a* molecules within WSCP were evaluated by recording the fluorescence excitation and emission spectra of WSCP/Chl *a* complexes at room temperature (Fig. 4). Excitation at 420 nm resulted in Chl *a* fluorescence emission

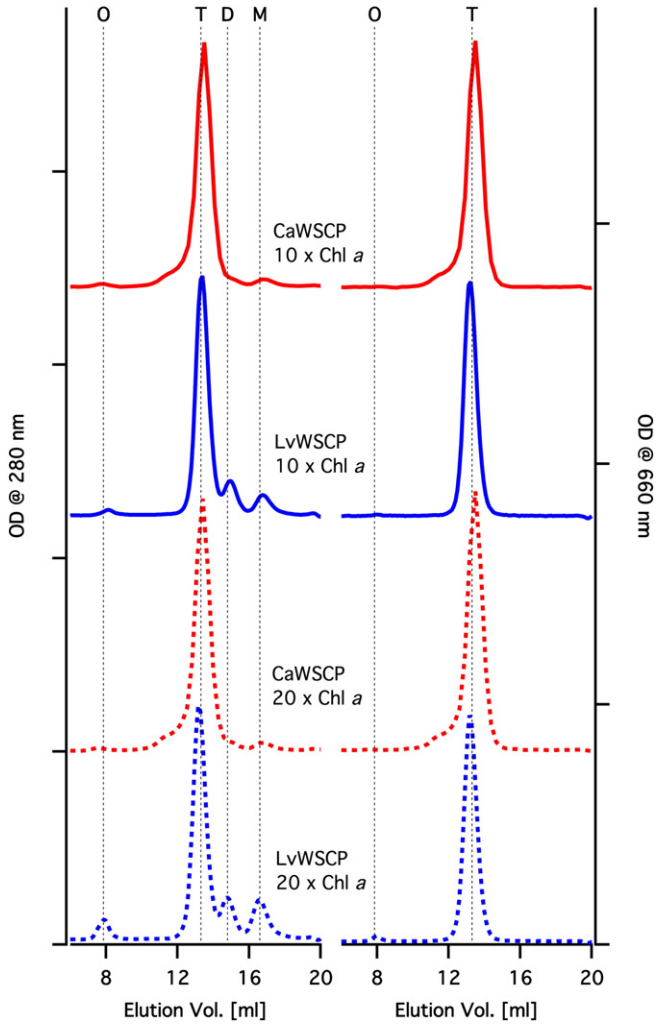

Fig. 2. Size exclusion chromatography traces of CaWSCP and LvWSCP. Solid, and dashed lines represent reconstitution with ten- and twenty-fold molar excess of Chl *a*, respectively, monitored by absorption at 280 nm and 660 nm. The markers M, D, T, and O indicate the expected elution volumes of WSCP monomers, dimers, tetramers and higher oligomer, respectively.

bands with a peak at 682 nm in BoWSCP, CaWSCP and RshWSCP, and 672 nm in LvWSCP (Fig. 4b). The excitation spectra reflected the absorption spectra of each sample (Fig. 4a). The fluorescence quantum yield of Chl *a* in WSCP was between 58 and 62% of the fluorescence quantum yield of Chl *a* in acetone (Fig. 4, Table 2).

3.5. Water-in-oil reconstitution for fast screening of WSCP/Chl a interactions

In order to demonstrate the potential of W/O emulsion as a fast screening system for positive reconstitution of WSCP proteins, we overexpressed CaWSCP in *E. coli* cells, broke down the bacterial cells

Table 1
Sizes and oligomerization states of WSCP/Chl *a* complexes.

Sample	Apparent complex MW [kDa]	Predicted apoprotein MW [g/mol]	Calculated oligomerization state*
BoWSCP	80	19,665	4.07
CaWSCP	82	19,155	4.28
RshWSCP	75	19,874	3.80
LvWSCP	88	19,574	4.50

* Calculated by the molecular weight ratio of the apparent WSCP/Chl *a* complex as measured by size exclusion chromatography to the monomeric apoprotein as predicted from the amino-acid sequence.

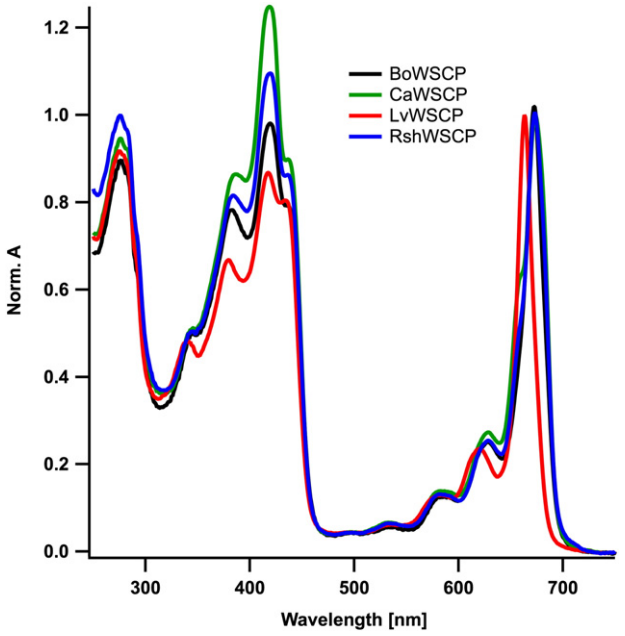

Fig. 3. Absorbance of BoWSCP, CaWSCP, RshWSCP and LvWSCP reconstituted with 10 fold molar excess of Chl *a*. The spectra were normalized to 1 at 672 nm for BoWSCP, CaWSCP and RshWSCP, and at 663 nm for LvWSCP.

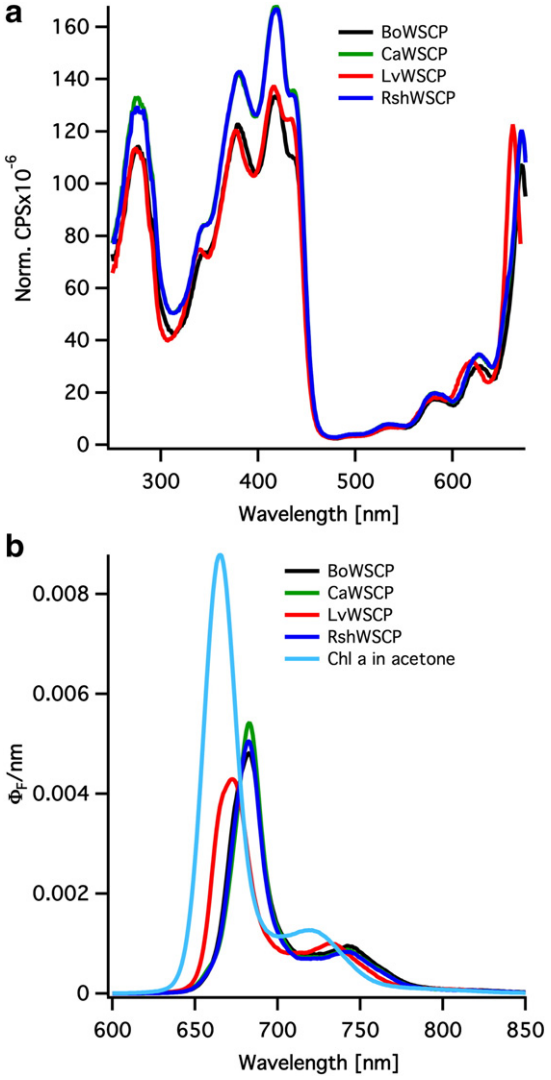

Fig. 4. Fluorescence spectra of reconstituted WSCP–Chl *a* complexes and Chl *a* in acetone. (a) Excitation spectra obtained by monitoring the emission at 682 nm for BoWSCP (black), CaWSCP (blue), RshWSCP (green) and at 675 nm for LvWSCP (red). The absorbance of each sample was 0.074, 0.074, and 0.075 at 673 nm, and 0.065 at 663 nm, respectively. These values were used for normalizing each spectrum by dividing it by the respective absorbance. (b) Fluorescence emission spectra of WSCP–Chl *a* complexes (color-coded as in a), and Chl *a* in acetone (cyan) excited at 420 nm. Each spectrum was normalized such that the area under the curve is the quantum yield of fluorescence (Φ_F).

by sonication and centrifugation, and used the supernatant for reconstitution with Chl *a* in the W/O emulsions. Extracts from bacteria that did not overexpress CaWSCP were used as negative controls. After breaking the W/O emulsion, the assembly of Chl *a* with CaWSCP was clearly observed by the green color of the aqueous phase, but only in extracts containing overexpressed CaWSCP (Fig. 5 a). Scanning confocal microscope fluorescence images of W/O emulsions in which the water phase was composed of either crude extracts of CaWSCP proteins, or a negative control containing only 50 mM sodium phosphate buffer in the aqueous phase (Fig. 5b) demonstrate the possibility of detecting assembled WSCP/Chl complexes directly in the W/O droplets. Chl *a* fluorescence from droplets containing the reconstituted WSCP/Chl *a* could be observed over the fluorescence background of Chl *a* from the oil phase. By contrast, the droplets of the negative control were not fluorescent and appeared as dark spots over the Chl fluorescence background from the organic phase. Thus, screening for positive reconstitution can be done without further steps of separating the droplets from the oil phase.

4. Discussion

In this work we have established W/O emulsions as a new general system for assembling water-soluble proteins with hydrophobic cofactors, and applied it to the assembly of recombinant WSCP apoproteins with their highly hydrophobic native Chl *a* cofactors. The main advantage of our WSCP/Chl reconstitution method over the method of Schmidt et al. [18] is that it does not rely on immobilizing the proteins on a solid support. Thereby, it does not require adding specific tags to the proteins. Binding to a solid surface as well as the tag itself may affect the protein structure and the assembly process with cofactors. For example, the six-histidine tag that was added to WSCP in previous studies may lead to non-specific binding of Chls by ligation to the histidines of the tag. In this work, the sequences of the recombinant WSCPs were identical to the native sequences without any tag. Another important advantage of our method is that the high surface to volume ratio of the aqueous microdroplets within the oil facilitates introduction of hydrophobic cofactors from the oil phase. This prevents cofactor aggregation that may influence reconstitution efficiency, oligomerization, and changes in protein/cofactor stoichiometry. In previous WSCP/Chl reconstitution methods Chls were dissolved either in buffers containing detergents, or in 40% aqueous methanol [18,19]. This may lead to Chl aggregation and/or non-specific Chl binding to WSCP. The latter case is not an issue in the new method since the hydrophobic pigments cannot enter the aqueous phase unless they are actively assembled by WSCP.

The method based on mixing apo-WSCPs with native thylakoids that was previously used for assembly with Chls [15,17] is similar to the W/O emulsion method in that it does not require tagging the proteins, or solubilizing Chls in aqueous mixtures of detergents or organic solvents.

Table 2
Absorption and emission properties of Chl *a* in WSCP.

Chl *a* in	Absorption [nm]	Emission [nm]	Stokes shift [cm^{-1}]	Fluorescence yield
Acetone	660	665	114	0.30 [26]
BoWSCP	672	682	218	0.185 ± 0.007
CaWSCP	672	682	218	0.178 ± 0.005
RshWSCP	672	682	218	0.173 ± 0.004
LvWSCP	663	672	202	0.177 ± 0.006

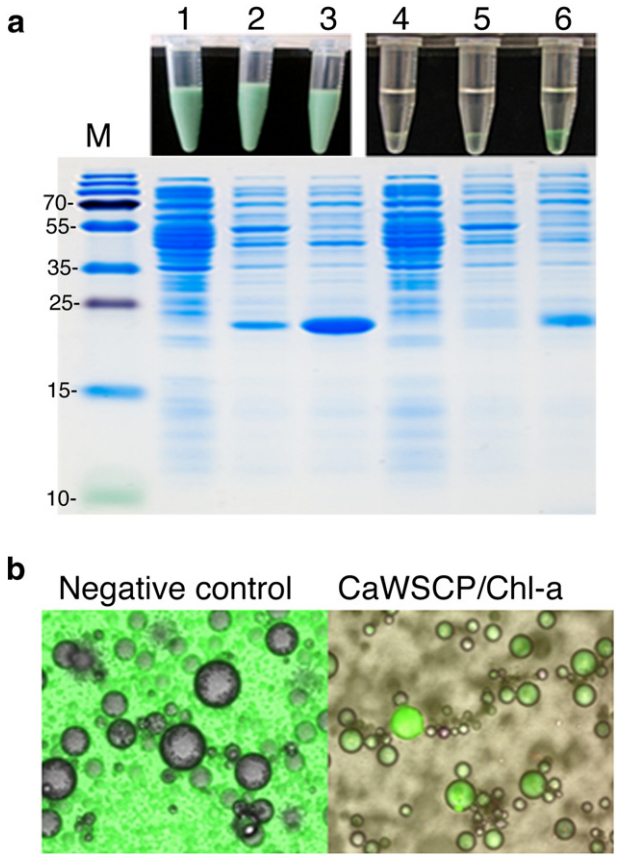

Fig. 5. Scanning fluorescence microscopy and visual screening of WSCP/Chl complex assembly. (a) SDS-PAGE of *E. coli* BL21 cell lysates before and after reconstitution with Chl *a* in the W/O emulsions. Lane 1: BL21 cells without the CaWSCPHis plasmid, lane 2: BL21 cells with the CaWSCPHis plasmid without induction by IPTG, lane 3: BL21 cells with the CaWSCPHis plasmid induced with IPTG. Lane 4, 5 and 6 are the same samples as lanes 1, 2 and 3, respectively, but after separation of the water-phase from the organic-phase of the emulsion. Small aliquots of each sample were run on a SDS-protein gel. Lane M: protein size marker. Pictures of the samples before and after phase separation are shown on top of each lane. (b) Confocal microscope images of W/O droplets prepared according to the reconstitution protocol with Chl *a* in the oil-phase. The droplets on the left image did not contain any protein whereas those on the right image contained CaWSCPHis protein. Fluorescence was monitored at 682 nm.

However, the previous method is limited to the Chls that are present in the thylakoid membranes. Thus, while it was possible to assemble WSCP with Chl *a*, or Chl *d* from thylakoids of *Synechocystis* PCC 6803 or *Acaryochloris marina*, respectively, assembly of Chl *b*-only WSCP complex was not possible because Chl *b* is always accompanied by Chl *a* in thylakoids. The new method is limited only by the solubility of pigments in the oil phase and therefore suitable for WSCP assembly with any natural or artificial Chl, BChl or porphyrin derivatives. Furthermore, it does not require the preparation of photosynthetic membranes and is free from interference of membrane or extra-membrane proteins such as phycobilisome components that may be present in such preparations.

The successful assembly of BoWSCP, CaWSCP, RshWSCP, and LvWSCP with Chl *a* in the W/O emulsion system has provided new insights into the different WSCP/Chl complexes and their assembly process. Most importantly, we found that all four WSCP variants assemble as homotetrameric complexes containing four Chls per complex. These findings contradict previous reports of different Chl–protein stoichiometries for different WSCP variants ranging from one to four Chls per tetrameric complex [17,18,28,30]. However, in contrast to the previous quantifications of Chls per protein that relied on extracting Chls from the WSCP complexes with organic solvents [17], we relied on a straightforward direct comparison of Chl–protein stoichiometry based on absorption spectra (Fig. 3). We suspect that in some of the previous cases Chl extraction might not have been complete because of tight binding of Chls to the WSCP complexes [31,32]. In the case of immobilized WSCP/Chl complexes, extraction was preceded by thoroughly washing with detergents [18], which may lead to dissociation of less stably bound Chl molecules. Considering the crystal structure of LvWSCP [8] that revealed four Chls in a homotetrameric complex, our experimental data strongly suggest that all the WSCP species bind four Chls per tetramer. This conclusion is easier to reconcile with the high sequence homology of the different WSCPs, and the symmetry of the WSCP tetrameric complex, than the previous assumptions of different Chl:protein ratios in different species.

SEC results confirm that WSCPs assemble with Chl *a* predominantly into tetrameric complexes (Fig. 2). In addition, we found minor fractions of Chl-containing higher oligomers, and Chl-free monomers. Similar species were previously observed in recombinant WSCP from cauliflower [18] and *A. thaliana* [7]. Interestingly, the distributions of oligomerization products in class IIa WSCPs differ significantly from class IIb LvWSCP. The fraction of Chl-free monomers was significantly larger in the latter. In addition, we found significant fractions of Chl-free dimers and Chl-containing higher oligomers that were not observed in class IIa WSCPs. Increasing the total Chl *a* content from ten to twenty-fold excess over the protein increased the sizes of these minor fractions in LvWSCP but had almost no effect on class IIa WSCPs. The higher quantities of Chl-free dimers and monomers in LvWSCP suggest that apo-LvWSCP is more prone to aggregation and/or misfolding at the oil–water interface of the water droplets than class IIa apo-WSCPs. The higher tendency of LvWSCP/Chl *a* to form higher oligomers may be a consequence of misfolding or instability, but may also reflect the lower affinity of LvWSCP to Chl *a* with respect to class IIa WSCPs [5].

The CD spectra of the WSCP/Chl *a* complexes closely resemble native WSCPs with the exception of the contribution from Chl *b* [17,19,28,33]. The CD spectra of class IIa BoWSCP, CaWSCP, and RshWSCP and class IIb LvWSCP (Fig. 1) reveal very similar shapes of the Soret and the Qy CD bands, indicating very similar excitonic interactions among the bound Chls. This is in agreement with previous analyses of absorption and CD spectra [30] demonstrating that the spatial arrangement of Chls in class IIa CaWSCP is approximately the same as in class IIb LvWSCP. The latter was obtained from the crystal structure of native LvWSCP. The arrangement revealed two pairs of Chls, each forming an “open sandwich” conformation with an opening angle of 27° [8]. Intriguingly, the excitonic interactions within the closely packed Chl dimers that are potentially powerful quenchers of fluorescence [34] lead to only moderate reduction of fluorescence quantum yield in WSCP. In all the variants that were measured in this work, the fluorescence quantum yield of Chl *a* ranged from 0.173 to 0.185, which is about 58 to 62% of the quantum yield of Chl *a* in acetone (Fig. 4). This feature was already observed in CaWSCP [14] and is apparently a general trait of WSCP/Chl complexes. Its possible function remains an open question. Maintaining large enough fluorescence quantum yield is critical for the functionality of light harvesting complexes, but WSCP is not involved in light harvesting processes [4]. Yet, regardless of the relevance to the physiological function of WSCP, its ability to maintain a set of excitonically interacting Chls without significantly affecting their singlet excited state lifetime makes it an ideal system for studying the underlying principles of tuning Chl excited state dynamics by their protein surroundings. The new WSCP/Chl reconstitution method presented in this work, particularly its potential use for fast screening of assembly (Fig. 5), opens up a wide range of possibilities for rigorously studying the mechanisms of tuning Chl excited state lifetimes by Chl–Chl and Chl–protein interactions.

5. Conclusion

The W/O emulsion method presented here is a general approach, suitable for reconstitution of water-soluble proteins with hydrophobic pigments. Its advantages were demonstrated by the successful assembly of Chls with four recombinant WSCPs from *Brassica* plants.

Particularly, we were able to show that all four variants have the same Chl/protein binding stoichiometry of four Chls per homotetrameric complex. Its applicability as a screening method was demonstrated by assembling WSCP/Chl complexes using *E. coli* cell lysate overexpressing recombinant WSCPs and a crude Chl extract. This does not require purifying the recombinant WSCPs, and the assembly can be visualized directly in water droplets without the need of separating the water phase from the organic phase of the emulsion. Using the method for assembling other water-soluble chlorophyll-binding proteins like FMO and PCP is currently being tested in our laboratory. In addition, the new method is a very promising tool for designing and constructing simplified artificial water-soluble protein analogs of large and elaborate transmembrane protein–cofactor complexes of primary metabolism. These can serve as model systems for studying cofactor binding and assembly, and energy- and electron transfer mechanisms in natural systems, and as templates for constructing artificial bio-inspired energy conversion devices.

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