Video Article

Water in Oil Emulsions: A New System for Assembling Water-soluble Chlorophyll-binding Proteins with Hydrophobic Pigments

Dominika Bednarczyk[1], Dror Noy[2]

[1]Department of Biological Chemistry, Weizmann Institute of Science

[2]Migal-Galilee Research Institute

Correspondence to: Dror Noy at drorn@migal.org.il

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Abstract

Chlorophylls (Chls) and bacteriochlorophylls (BChls) are the primary cofactors that carry out photosynthetic light harvesting and electron transport. Their functionality critically depends on their specific organization within large and elaborate multisubunit transmembrane protein complexes. In order to understand at the molecular level how these complexes facilitate solar energy conversion, it is essential to understand protein-pigment, and pigment-pigment interactions, and their effect on excited dynamics. One way of gaining such understanding is by constructing and studying complexes of Chls with simple water-soluble recombinant proteins. However, incorporating the lipophilic Chls and BChls into water-soluble proteins is difficult. Moreover, there is no general method, which could be used for assembly of water-soluble proteins with hydrophobic pigments. Here, we demonstrate a simple and high throughput system based on water-in-oil emulsions, which enables assembly of water-soluble proteins with hydrophobic Chls. The new method was validated by assembling recombinant versions of the water-soluble chlorophyll binding protein of *Brassicaceae* plants (WSCP) with Chl *a*. We demonstrate the successful assembly of Chl *a* using crude lysates of WSCP expressing *E. coli* cell, which may be used for developing a genetic screen system for novel water-soluble Chl-binding proteins, and for studies of Chl-protein interactions and assembly processes.

Video Link

The video component of this article can be found at http://www.jove.com/video/53410/

Introduction

Hydrophobic pigments such as chlorophylls (Chls), bacteriochlorophylls (BChls) and carotenoids are the primary cofactors in photosynthetic reaction centers and light harvesting proteins that carry out electron transport, and light energy capture and transfer. The reaction centers and most of the Chl-binding light harvesting complexes are transmembrane proteins. The Fenna-Matthews-Olson (FMO) protein of non-oxygenic photosynthetic green-sulfur bacteria [1,2], and the peridinin-Chl protein (PCP) of dinoflagellates [3] are exceptional examples of water soluble light harvesting proteins. The water-soluble chlorophyll binding proteins (WSCPs) of *Brassicaceae*, *Polygonaceae*, *Chenopodiaceae* and *Amaranthaceae* plants [4,5] are another unique example, yet in contrast to FMO and PCP, these are neither involved in light harvesting nor in any of the primary photosynthetic reaction, and their precise physiological functions are yet unclear [5-8]. Their high Chl-binding affinity have led to a suggested function as transient carriers of Chls and Chl derivatives [9,10]. Alternatively, it was hypothesized that WSCP plays a role in scavenging Chls in damaged cells and protects against Chl-induced photooxidative damage [7,11-13]. More recently, it was suggested that WSCP functions as a protease inhibitor and plays a role during herbivore resistance as well regulates cell death during flower development [14]. WSCPs are divided into two main classes according to their photophysical properties. The first class (class I, *e.g.* from *Chenopodium album*) may undergo photoconversion upon illumination. Class II WSCPs from Brassica plants, that do not undergo photoconversion [5,10], are further subdivided into class IIa (*e.g.*, from *Brassica oleracea, Raphanus sativus*) and IIb (*e.g.*, from *Lepidium virginicum*). The structure of class IIb WSCP from *Lepidium virginicum* was solved by X-ray crystallography at 2.0 Å resolution [8]. It reveals a symmetric homotetramer in which the protein subunits form a hydrophobic core. Each subunit binds a single Chl which results in a tight arrangement of four closely packed Chls within the core.This simple all Chl arrangement makes WSCPs a potentially useful model system for studying binding and assembly of Chl-protein complexes, and the effects of neighboring Chls and protein environments on the spectral and electronic properties of individual Chls. Furthermore, it may provide templates for constructing artificial Chl-binding proteins that may be used for light-harvesting modules in artificial photosynthetic devices.

Rigorous studies of native WSCPs are not feasible because the complexes purified from plants always contain a heterogeneous mixture of tetramers with different combinations of Chl *a* and Chl *b* [9]. Thus, a method for assembling recombinantly expressed WSCPs with Chls *in vitro* is required. This is challenged by the negligible water-solubility of Chls which makes it impossible to assemble the complex *in vitro* by simply mixing the water-soluble apoproteins with pigments in aqueous solutions. *In vitro* assembly by mixing the apoproteins with thylakoid membranes [15] was demonstrated, but this method is limited to the native Chls present in the thylakoids. Schmidt *et al*. reported on assembling several Chl and BChl derivatives with WSCP from cauliflower (CaWSCP) by recombinantly expressing a histidine-tagged protein in *E. coli* immobilizing it onto a Ni-affinity column and introducing Chl derivatives solubilized in detergents [11]. Successfully reconstitution of recombinant WSCPs from *A.*

thaliana [6], and Brussels sprouts (BoWSCP), Japanese wild radish (RshWSCP) and Virginia pepperweed (LvWSCP) by a similar method were also reported.

Here, we present a novel, general, straightforward method for assembling Chls with WSCP that does not require tagging or immobilizing the proteins. It relies on preparing emulsions from their aqueous solutions of the water-soluble apoproteins in mineral oil. The proteins are thus encapsulated in water-in-oil (W/O) microdroplets with very high surface to volume ratio [16]. The hydrophobic cofactors are then dissolved in the oil and are readily introduced into the droplets from the oil phase. We report on using the method for assembling of several variants of WSCP apoproteins recombinantly expressed in *E. coli* with Chl *a*. We demonstrate the assembly from crude lysate of WSCP-overexpressing bacteria which may be used as a screening system for developing novel Chl binding proteins.

Protocol

1. Preparing Chl *a* Stock Solutions

1. CRITICAL STEP: Perform all the steps of chlorophyll preparation in a chemical hood, under green light (520 nm) or in the dark in order to minimize photodamage. Always add Nitrogen or Argon before freezing the pigments for storage. Ensure that all solvents are analytical grade.
2. Weigh about 5 mg of lyophilized Spirulina platensis cells or other cyanobacterium cells containing only Chl *a* in thylakoid membranes and crush it using a mortar and pestle.
3. Load crushed cells onto a glass column and wash with about 50-100 ml of 100% acetone in order to remove carotenoids. Discard the eluted orange/green fraction.
   Note: If the orange fraction is not eluted with 100 ml of acetone continue washing the cells with acetone until green fraction starts to elute.
4. Exchange acetone with 100% methanol and collect the green fraction containing Chl *a*. The volume of eluted fraction may vary between 50-100 ml. At the beginning, the eluted fraction has a dark green color, which changes into pale green at the end of elution. When the color of eluted fraction turns into pale green stop the elution.
5. Evaporate the methanol using a rotary evaporator until the extract is completely dry. Do not apply heat to the solution; ensure that the evaporator's water bath temperature does not exceed 30 °C.
   Note: The time of evaporation depends on the volume of methanol fraction being evaporated and may vary between 10-60 min. It is important to dry the extract completely.
6. Dissolve the pigments from the dried extract in a small volume of diethyl ether (about 5-10 ml), and filter through cotton wool. Ensure that pigments are completely dissolved in ether before filtering.
7. Evaporate the diethyl ether until the pigments are completely dry (10-30 min).
   Note: The dry pigments can be purged with and kept under Nitrogen or Argon at -20 °C, in the dark until further processing.
8. Dissolve the dry pigments in the smallest volume possible of 100% methanol (about 1 ml), even if not everything is completely suspended. Add 4 ml of acetone to the solution, and flick the glass gently in order to completely dissolve the pigments.
9. Using a Pasteur pipette, load the sample gently onto a column of DEAE sepharose equilibrated in 100% acetone.
10. Elute carotenoids (an orange-yellow band) with 100% acetone. Then, elute Chl *a* (green band) with 3:1 v/v acetone/methanol mixture.
    Note: The volume of acetone and acetone/methanol mixture is approximately equivalent to the volume of DEAE sepharose loaded on the column.
11. Verify Chl *a* purity by thin layer chromatography using a 68:25:5:2 dichloromethane/n-hexane /isopropanol /methanol (v/v) mixture as eluent [17].
12. Evaporate the solvent using a rotary evaporator until the Chl *a* is completely dry (10-60 min).
    Note: The dry Chl *a* can be purged with nitrogen or argon and stored under argon atmosphere at -20 °C in the dark.
13. Prepare Chl *a* stock solution by re-dissolving the dry Chl *a* in 2-4 ml of 100% ethanol.
    Note: The extinction coefficient of Chl *a* at 663 nm is 74,400 $cm^{-1}M^{-1}$ (83.3 cm^{-1} $(mg/ml)^{-1}$) in ethanol. A typical stock solution should have an OD of 1,860 corresponding to a concentration of 25 mM (23 mg/ml). Adding 20 µl of this stock to an emulsion containing 5 ml of organic phase, and 1 mg of WSCP in 1 ml of aqueous phase results in a mixture with 10 fold molar excess of Chl *a* vs. WSCP.

2. Preparing Organic Phase of the Emulsion

Note: The organic phase of the emulsion is composed of mineral oil containing 4.5% (v/v) Span 80, and 0.4% (v/v) Tween 80.

1. Weigh in a 50 ml tube 0.2 g of Tween 80, 1.8 g of Span 80, and 38 g of mineral oil. Mix well all components and cool down on ice.
   Note: The organic phase can be stored in 4 °C up to one week.

3. Preparing the Aqueous Phase of the Emulsion

Note: The aqueous phase of the emulsion may be composed of either purified WSCP, or crude extract of bacteria overexpressing WSCP.

1. Preparing an aqueous phase containing purified WSCP.
   1. Grow *E.coli* BL21 bacteria containing WSCP plasmid [12] in 1 L of LB medium at 37 °C until OD of 0.3-0.6.
   2. Induce protein expression by adding 1 mM IPTG. After induction grow bacteria at 30 °C for 12-16 hr.
   3. Harvest bacteria by centrifugation at 5,000 x g for 10 min at 4 °C.
   4. Dissolve the pellet in binding buffer and sonicate on ice (30 sec on, 15 sec off, five times). Use 10 ml of buffer for the pellet obtained from centrifugation of 250 ml of LB medium with cells overexpressing the protein.
      Note: Depending on purification method, the binding buffer may be composed of 100 mM phosphate buffer, pH 7.2 or 50 mM Tris, pH 7.5, 500 mM NaCl, 5 mM EDTA.
   5. Spin down the cell lysate at 12,000 x g for 30 min at 4 °C.

6. Purify WSCP by affinity chromatography. Depending on the tag fused to WSCP, purify recombinant proteins using the appropriate commercially available affinity chromatography system for protein purification following manufacturer instructions.
7. For emulsion preparation, use purified WSCP in 50 mM phosphate buffer, pH 7.8. Ensure that the final protein amount used for reconstitution is 0.5-1.0 mg per 1 ml of buffer.

2. Preparing an aqueous phase containing crude bacterial lysate with WSCP.
   1. Grow *E.coli* BL21 cells containing plasmid-expressing WSCP in 250 ml of LB medium at 37 °C until OD 0.3-0.6.
   2. Induce protein expression with 1 mM IPTG and grow bacteria overnight at 30 °C.
   3. Harvest bacterial cells by centrifugation at 5,000 x g for 10 min at 4 °C.
   4. Dissolve the pellet in 1-2 ml of 50 mM sodium phosphate buffer pH 7.8, sonicate (30 sec on, 15 sec off, three times) and centrifuge at 12,000 x g for 30 min at 4 °C.
   5. Prepare the aqueous phase of the emulsion by mixing 0.125 ml of supernatant with 0.875 ml of 50 mM sodium phosphate buffer pH 7.8.

4. Assembly of WSCP with Chl *a* in Emulsion

1. Transfer 5 ml of oil-surfactant mixture into a glass vial and cool it on ice. Before pipetting, verify that all the components of the organic phase are mixed thoroughly and there is no phase separation between surfactants and mineral oil.
2. Add 1 ml of ice-cold aqueous phase prepared as in section 3 to 5 ml of organic phase.
3. Mix both phases using a tissue homogenizer for 2 min at 9,500 rpm on ice.
4. CRITICAL STEP: From this stage on, perform all further steps under green light (520 nm) in order to minimize photodamage. Add 20 µl of 25 mM Chl *a* stock solution (see section 1.13) to the emulsion. Disperse by flicking and inverting the glass vial. Make sure that the Chl is evenly distributed in the emulsion.
5. Incubate the emulsion for 1-2 hr on ice in the dark.
6. In order to break down the emulsion and separate water droplets from the organic phase transfer the emulsion to 1.5 ml plastic tubes and centrifuge at 14,000 x g for 5 min at room temperature.
   Note: If the assembly is successful, the lower aqueous phase should have a green color.
7. Dispose the upper oil phase and add 1 ml of mineral oil. Mix well the mineral oil with the pelleted emulsion by vortex or by flipping the tube thoroughly. Spin down the sample at 14,000 x g for 5 min at room temperature. Repeat this step until a clear meniscus separating the aqueous and mineral oil phases, without any intermediate emulsion is obtained.
8. Perform this step in a chemical hood. After the aqueous and mineral oil phases are clearly separated, remove mineral oil and add 1 ml of water-saturated diethyl ether. Vortex and spin down the sample at 14,000 x g for 5 min at room temperature. Repeat this step twice.
9. After the second centrifugation, remove the diethyl ether and leave the tubes open for 5-20 min in the hood.
10. Finally, load the aqueous phase containing the WSCP/Chl *a* complex onto a desalting column and elute with buffer appropriate for further experiments.
    Note: The protein is stable in phosphate- and Tris buffers in a broad range of pH (6.0-7.5). The sample can be stored at 4 °C protected from light up to one month.

Representative Results

Recombinant WSCP apoproteins were assembled with Chl *a* in W/O emulsions according to the protocol described in the previous section. The protocol was implemented using aqueous phases containing either pure WSCPs, or lysates *E.coli* cells overexpressing WSCP (**Figure 1**). The protocol is simple, fast and does not require any special equipment except a tissue homogenizer.

The absorbance and CD spectra of Chl *a* complexes of four recombinant WSCP variants, namely RshWSCP, CaWSCP, BoWSCP and LvWSCP are shown in **Figure 2.** These are similar in band shape and position to previously reported spectra of the respective native complexes [10,18,19]. These results clearly show that WSCP/Chl complexes reconstituted in W/O emulsion system resembles the native complexes.

To demonstrate the potential of W/O emulsion as a fast screening system for positive assembly of WSCPs with Chls, crude lysates of *E.coli* cells expressing WSCPs were used as the aqueous phase of the emulsion. Lysates of bacteria that did not express WSCPs were used as negative control. Positive assembly of WSCP with Chl *a* is readily observed by the green color of the aqueous phase, but only in lysates of WSCP expressing cells (**Figure 3**). Droplets containing these lysates feature distinct Chl *a* fluorescence in confocal microscope images. This implies that successful assembly of WSCP/Chl complexes can be detected directly in the water droplets, which may be the basis for W/O emulsion-based screening systems.

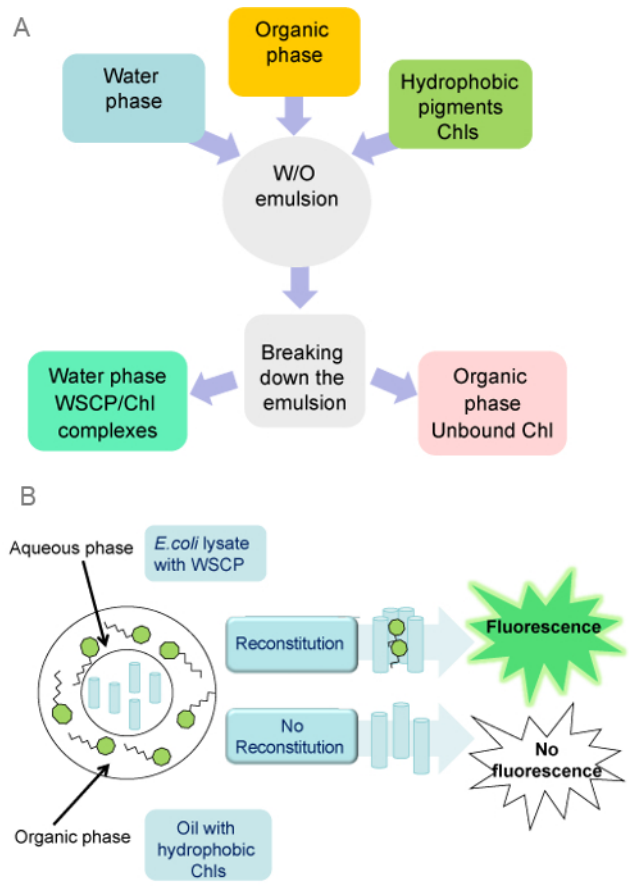

Figure 1: Assembly of WSCP with Chls in W/O Emulsions. (**A**) In a preparative protocol, the organic phase is mixed with an aqueous phase, which contains purified WSCP or *E.coli* cell lysates expressing WSCP. When the emulsion is ready, Chls are added to the emulsion. After reconstitution, water droplets are separated from organic phase by centrifugation. (**B**) W/O emulsion system can be used for fast and high throughput screening for positive reconstitution of WSCPs with Chls. The fluorescence of the WSCP/Chl complex can be detected directly from the water microdroplets by a confocal microscope. Please click here to view a larger version of this figure.

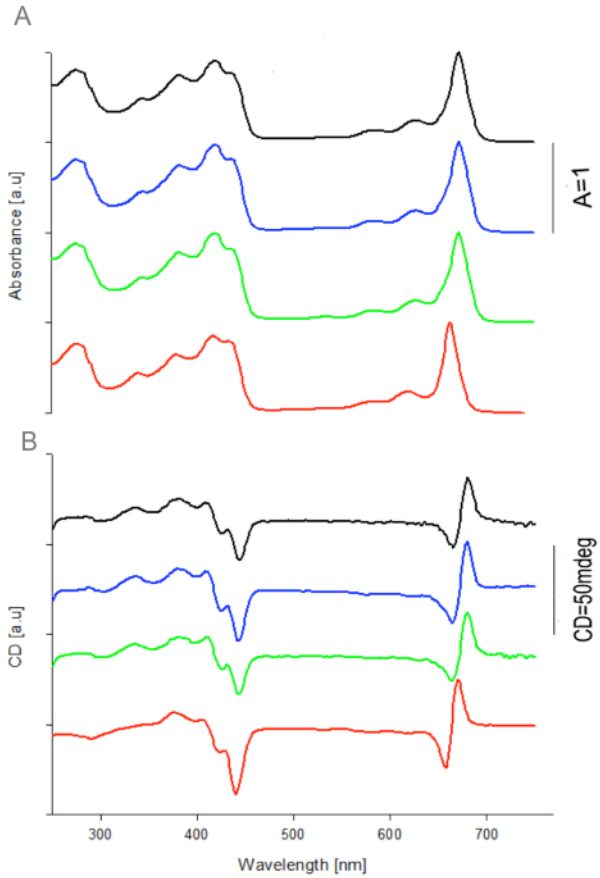

Figure 2: Absorbance and CD spectra of WSCP/Chl *a* Complexes. Apoproteins of four WSCP variants were reconstituted with 10-fold molar excess of Chl *a*. The absorbance spectra (**a**) were normalized to 1 at 673nm for BoWSCP (black), CaWSCP (green), and RshWSCP (blue) and 663nm for LvWSCP (red). The same normalization factors were applied to the CD (**b**). Reproduced with permission from [16]. Please click here to view a larger version of this figure.

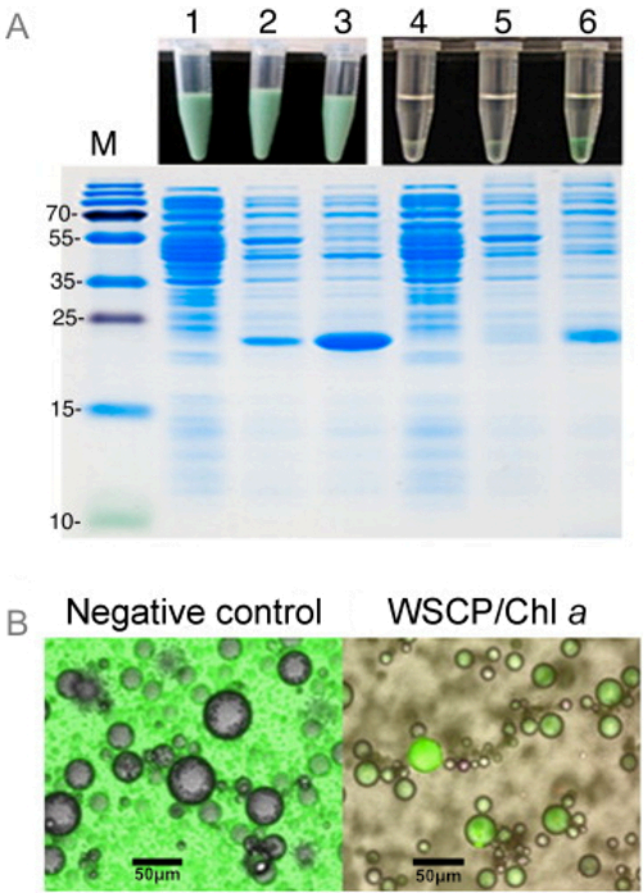

Figure 3: Scanning Fluorescence Microscopy and Visual Screening of WSCP/Chl Complex Assembly. (**A**) SDS-PAGE of *E. coli* BL21 cell lysates before and after reconstitution with Chl *a* in the W/O emulsions. Lane 1: BL21 cells without the WSCP plasmid, lane 2: BL21 cells with the WSCP plasmid without induction by IPTG, lane 3: BL21 cells with the WSCP 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 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 WSCP protein. Fluorescence was monitored at 682 nm. Reproduced with permission from [16]. Please click here to view a larger version of this figure.

Discussion

Our goal was to develop a new general system for assembly of water-soluble chlorophyll-binding proteins with hydrophobic pigments. Here it is shown that the new reconstitution system based on W/O emulsion is a general approach proven to work for assembly of WSCP apoproteins from Brussels sprouts, cauliflower, Japanese horseradish and Virginia pepperweed recombinantly expressed in *E. coli*. Here results are presented from reconstitution of 1 mg of WSCP with 10-fold molar excess of Chl *a*. However it is also possible to use lower concentrations of WSCP for reconstitutions and different Chl/protein molar ratios. The minimum amount of protein, which we were able to assemble in W/O emulsions was 100 µg, while the pigment to protein ratio could vary from 5:1 and up to 20:1. Also, the volume of aqueous phase could be lowered down to 50 µl. In this work the assembly of WSCP with Chl *a* is presented. Yet, it is also possible to assemble WSCP with other hydrophobic Chls, BChls and their derivatives. Currently, we are testing our system with other water-soluble pigment-binding proteins such as FMO and PCP.

Although, our method is fast and simple there are some critical steps, which need to be considered during W/O preparation and reconstitution of WSCP with Chl. The glass vials, which are used for emulsion preparation, need to be clean and free of detergent. Even small traces of detergents in vials will affect the emulsion preparation and result in low quality of emulsion. Also, the vials should be completely dry. Other critical step, which might influence the reconstitution efficiency, is thoroughly dispersion of Chl in organic phase of emulsion. Therefore, it is important to fully mix a small volume of Chl in 5 ml of emulsion. It is also important to do as many steps as necessary to break down emulsion and completely remove mineral oil from water phase. Any traces of mineral oil in water phase might influence further experiments.

W/O emulsion is an alternative approach for the method established by Schmidt *et al*. [11], which was based on immobilizing His-tagged WSCP on affinity chromatography column and incubating with Chls. Although, the method of Schmidt *et al.,* proved to work for different WSCPs, it requires His-tagged proteins and may result in non-specific binding of Chls by ligation to the histidines of the tag. Furthermore, immobilizing proteins on a solid surface may influence protein structure, thus influencing the assembly process with pigments. In addition, Chl, which was used for assembly of WSCPs in this system was dissolved either in buffer containing detergents or 40% methanol [11,12]. Such solvents might result in pigment aggregation and/or non-specific Chl binding to WSCP. Our new reconstitution system based on W/O emulsion does not rely on immobilizing the proteins to solid support. Therefore, no purification tag fused to WSCPs is required, and recombinant proteins with native

sequence can be assembled. Another important advantage of the W/O method is in minimizing pigments aggregation that may influence reconstitution efficiency, oligomerization and pigment/protein stoichiometry. This is because the hydrophobic pigments are dissolved in the oil phase and their introduction into the W/O droplets is enabled by the high surface to volume ratio of the latter. Furthermore, unspecific binding of Chls by WSCP is circumvented since hydrophobic pigments cannot diffuse between organic and aqueous phases of the emulsion. Only pigments that are actively assembled by WSCP during the reconstitution can enter into the water microdroplets of the emulsion.

The method of assembling WSCP with pigments by mixing WSCP with isolated thylakoids [10] is similar to the W/O emulsion system presented here. It does require detergents or organic solvents for solubilizing the Chls, nor tagging the proteins. However, the W/O method can be used with any pigments that is soluble in the oil phase whereas the previous method is limited to Chls that are natively present in thylakoid membranes. Thus, assembly with pure Chl *a* or Chl *d* is possible from because these are available from cyanobacterial thylakoids of *e.g. Synechocystis PCC 6803* or *Acaryochloris marina*, respectively. However, it is not feasible to reconstitute WSCP with Chl *b* since this pigment is always accompanied by Chl *a* in thylakoids. By contrast, WSCP assembly in W/O emulsion can be tested with any natural or artificial Chl, BChl or porphyrin derivatives. It does not require using photosynthetic membranes and thereby it is free from interference of extra-membrane components such as phycobilisome that may be attached to cyanobacterial membranes. The W/O 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.

In summary, we presented here a general method for assembling water-soluble proteins with hydrophobic pigments. Its advantages of were demonstrated by the successful assembly of Chl *a* with different recombinant WSCPs from *Brassica* plants as clearly indicated by spectroscopic and biochemical results. In addition, we demonstrated how the W/O assembly protocol can be used for fast screening of WSCP/Chl complex assembly by using *E.coli* cell lysates overexpressing recombinant WSCPs and crude Chl extracts. In this way we can skip time consuming protein purification steps and the need of breaking emulsions in order to separate the water droplets from the organic phase may also be avoided by using fluorescence microscopy for directly visualizing the assembly in water droplets. The general applicability of the new method makes it a useful tool for studying cofactor binding and assembly, and energy- and electron transfer mechanisms in transmembrane protein-cofactor complexes by designing and constructing their simplified artificial water-soluble protein analogues.

Disclosures

The authors declare that they have no competing financial interests.

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