Biochemical and Structural Studies of the Large Ycf4-Photosystem I Assembly Complex of the Green Alga *Chlamydomonas reinhardtii*  

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Ycf4 is a thylakoid protein essential for the accumulation of photosystem I (PSI) in *Chlamydomonas reinhardtii*. Here, a tandem affinity purification tagged Ycf4 was used to purify a stable Ycf4-containing complex of >1500 kDa. This complex also contained the opsin-related COP2 and the PSI subunits PsaA, PsaB, PsaC, PsaD, PsaE, and PsaF, as identified by mass spectrometry (liquid chromatography–tandem mass spectrometry) and immunoblotting. Almost all Ycf4 and COP2 in wild-type cells copurified by sucrose gradient ultracentrifugation and subsequent ion exchange column chromatography, indicating the intimate and exclusive association of Ycf4 and COP2. Electron microscopy revealed that the largest structures in the purified preparation measure 285 \times 185 Å; these particles may represent several large oligomeric states. Pulse-chase protein labeling revealed that the PSI polypeptides associated with the Ycf4-containing complex are newly synthesized and partially assembled as a pigment-containing subcomplex. These results indicate that the Ycf4 complex may act as a scaffold for PSI assembly. A decrease in COP2 to 10% of wild-type levels by RNA interference increased the salt sensitivity of the Ycf4 complex stability but did not affect the accumulation of PSI, suggesting that COP2 is not essential for PSI assembly.

**INTRODUCTION**

In oxygenic photosynthetic organisms, such as cyanobacteria, algae, and higher plants, the thylakoid membranes house two distinct types of photosystem, photosystem I (PSI) and photosystem II (PSII). Electron transfer from water to NADP⁺ is driven by the light-induced photochemical reactions of PSII and PSI, which act in series. The PSI reaction center (RC) complex consisting of two homologous subunits, PsaA and PsaB, contains the primary electron donor (P700) and acceptors of chlorophyll a (A₀), phyloquinone (A₁), and 4Fe-4S cluster (F₄). The reduced form of F₄ donates an electron to F₅ and subsequently to F₆, which are bound to PsaC, and finally reduces ferredoxin. The electrons transferred by PSI from plastocyanin on the luminal side to ferredoxin are used to generate NADPH that is subsequently used in the Calvin cycle reactions to fix carbon dioxide into organic compounds.

Recently, the structure of cyanobacterial PSI complex has been determined at 2.5-Å resolution (Jordan et al., 2001), while that of higher-plant PSI-light-harvesting complex I (LHCI) supercomplex has been obtained at 3.4-Å resolution (Amunts et al., 2007). For the green alga *Chlamydomonas reinhardtii*, a number of medium- to low-resolution structures of the PSI-LHCI supercomplex exist (Germano et al., 2002; Kargul et al., 2003). PSI is a multiprotein complex that consists of 12 subunits in cyanobacteria and 14 to 15 subunits in higher plants and algae and more than 120 cofactors, such as chlorophyll a, b-carotene, lipid, and iron-sulfur clusters (Nelson and Yocum, 2006). PSI redox components include six chlorophyll a, two phylloquinones, and one 4Fe-4S cluster, F₅. Together with the majority of antenna chlorophyll a and b-carotene, they are associated with the PSI RC, while several antenna chlorophyll a molecules are present on several peripheral subunits, PsaJ, PsaK, PsaL, PsaM, and PsaX in cyanobacteria (Jordan et al., 2001). On the stromal side (or on the cytoplasmic side in cyanobacteria) of the PSI complex, PsaC, PsaD, and PsaE form a cluster and provide a docking site for ferredoxin. By contrast, PsaF with a single transmembrane helix contains an N-terminal hydrophilic domain extending in the luminal space. In algae and higher plants, this luminal extension is larger than in cyanobacteria and provides a docking site for...
plastocyanin. In the vicinity of PsaF, two subunits are present: the peripheral PsaN on the lumenal side, which is absent from cyanobacteria, and the small hydrophobic PsaL with a single transmembrane helix. In algae and higher plants, PSI is tightly associated with the peripheral antenna complex, LHCl, containing chlorophyll a/b and xanthophylls to form the PSI-LHCl supercomplex (Amunts et al., 2007; Jensen et al., 2007). The three-dimensional structure of the plant PSI-LHCl supercomplex revealed that four LHCl complexes form a belt residing on one side of the PSI core complex (Amunts et al., 2007). On each edge of the LHCI belt, two subunits, PsaK and PsaG, containing two transmembrane helices, are located symmetrically. Three subunits, PsaH, PsaL, and PsaS, with two, one, and three transmembrane helices, respectively, are located on the opposite side of the LHCI belt. PsaH is absent from cyanobacteria but is involved in reversible binding of LHCl during state transitions in higher plants and algae (Lunde et al., 2000; Germano et al., 2002; Varotto et al., 2002). PsaL is required for the stable trimerization of the PSI complex in cyanobacteria (Chitnis and Chitnis, 1993), while the PSI complex is monomeric in higher plants and algae (Kargul et al., 2005; Amunts et al., 2007). Additional subunits, PsaO and PsaP, might be present in the vicinity of the PsaH-Psai-PsAI cluster in higher plants (Jensen et al., 2004; Khrouchtchova et al., 2005). In Chlamydomonas, Psao is present but not stably bound to the PSI complex, and the psap gene is undetectable even in the database (the Kazusa EST database, Joint Genome Institute [version 3.0], National Center for Biotechnology Information [NCBI; NC 005333 and NC 001638], and release 2 of the C. reinhardtii genome [chlre2]).

Questions arise as to how the photosystem gene products are targeted both spatially and temporally to the correct membrane location, how they are assembled into functionally active units, and what pathways underlie their regulation under different environmental conditions. Since a number of subunit components must be integrated properly into a functional structure, these components should be assembled in a stepwise manner. According to the three-dimensional structure of the PSI complex, it is likely that the assembly of two large RC subunits, Psaa and Psab, occurs as an initial assembly step followed by the subsequent integration of peripheral subunits. Mutants deficient in one of the RC subunits do not accumulate PSI complex (Girard-Bascou et al., 1987; Redding et al., 1999). Pulse-chase protein labeling experiments of Psaa- or Psab-deficient mutants of Chlamydomonas revealed that the synthesis of Psaa is dependent on the presence of Psab (Girard-Bascou et al., 1987). More recently, it was shown that assembly of PSI involves an assembly-dependent regulation of synthesis of the major chloroplast-encoded subunits (Wostrikoff et al., 2004). Mutants affected in the assembly of redox components, phyloquinone and Fx, ligated on PSI RC, also affect PSI complex assembly (Johnson et al., 2000; Shen et al., 2002; Shimada et al., 2005; Lefebvre-Legendre et al., 2007). Successful integration of peripheral subunits into the PSI complex was analyzed by pulse-chase labeling and subsequent fractionation of PSI polypeptides (Duhring et al., 2007). However, the detailed molecular mechanisms of the assembly still remain elusive.

It is expected that at least part of the stepwise integration of PSI subunits into the functional complex are assisted by protein factors, such as molecular chaperones. Several factors involved in assembly, stability, and regulation of PSI complexes have been reported so far (Schottler and Bock, 2008). The chloroplast-encoded Ycf3, which is also conserved in cyanobacteria, contains a tetratricopeptide repeat and is essential for the assembly of the PSI complex (Boudreau et al., 1997a; Ruf et al., 1997). This protein is located in the thylakoid membranes and is not involved in the transcription and translation of PSI polypeptides. It is thus most probably required for a posttranslational step of PSI assembly. A direct interaction of Ycf3 with Psaa and Psad was observed (Naver et al., 2001). Ycf37 also contains a tetratricopeptide repeat and is essential for the assembly of the PSI complex in Arabidopsis thaliana (also designated Pyg) (Stuckel et al., 2006), while this protein is not essential but plays a regulatory role in cyanobacteria (Wilde et al., 2001; Duhring et al., 2006, 2007). Ycf4 is a 22-kD protein with two putative transmembrane domains and is localized on the thylakoid membrane as part of a large complex (Boudreau et al., 1997a). This protein is encoded by the chloroplast genome in eukaryotes and is highly conserved among photosynthetic organisms from cyanobacteria to higher plants (Boudreau et al., 1997a). Ycf4 is an essential factor for PSI complex assembly in the green alga C. reinhardtii (Boudreau et al., 1997a), while a cyanobacterial mutant deficient in Ycf4 is still able to assemble the PSI complex although at a reduced level (Wilde et al., 1995). To investigate the biochemical properties of the large complex containing Ycf4, we successfully purified a Ycf4-containing complex and identified its protein components by N-terminal amino acid sequencing, immunoblot, and mass spectrometry analyses. Furthermore, the purified preparation was visualized by transmission electron microscopy and single particle analysis. This revealed that the Ycf4-containing complex is larger than 1500 kD and contains a retinal binding protein, COP2, and several PSI polypeptides, which appear to be assembled into an intermediate assembly subcomplex. It is thus anticipated that the purified Ycf4-containing complex plays a pivotal role in an initial assembly step of PSI by directly mediating the interactions between newly synthesized PSI polypeptides and in assisting the assembly of the PSI complex.

RESULTS

Generation of TAP-Tagged Ycf4

In C. reinhardtii, the ycf4 gene is present in the rps9-ycf4-ycf3-rps18 polycistronic transcriptional unit on the chloroplast genome and is necessary for PSI complex synthesis and/or stability (Boudreau et al., 1997a). This previous work also demonstrated that Ycf4 is part of a large complex, interacting with other biomolecules. In this study, we aimed to confirm the presence of this large complex and to identify its other components. To achieve a high purification, a two-step affinity column chromatography was performed in conjunction with tandem affinity purification (TAP)-tag technology (Rigaut et al., 1999). The TAP-tag consists of calmodulin binding peptide and Protein A domains separated by a tobacco etch virus protease cleavage site.

To generate chloroplast transformants in which the TAP-tag is genetically fused to the C-terminal end of Ycf4, a transformation
vector was constructed as described in Methods. Wild-type cells were cotransformed with a particle gun using a mixture of the pXK29-TAP plasmid and the pEX-50-AAD plasmid. The pEX-50-AAD plasmid contains the aadA cassette at the BamHI site downstream of the psbA gene (Takahashi et al., 1996). A control strain was generated by transformation with the pEX-50-AAD plasmid, which was resistant to spectinomycin but showed wild-type photosynthetic activity as described before (Takahashi et al., 1996). Transformants were first selected for resistance to spectinomycin and then screened for the presence of the TAP-tag by PCR between the EcoRI and ClaI restriction sites flanking the ycf4 gene (see Supplemental Figure 1 online). Wild-type and control strains produced a 676-bp fragment, whereas the transformant generated a 1235-bp product as expected from the insertion of the TAP-tag sequence (Figure 1). These results indicate that the generated TAP-tag strain was homoplasmic, with the wild-type chloroplast DNA copies totally replaced by the tagged copies by homologous recombination.

Characterization of the TAP-Tagged ycf4 Strain

We first checked the effect of the TAP-tag on Ycf4 stability. Whole-cell proteins were subjected to SDS-PAGE and polypeptides separated and analyzed by immunoblotting using anti-Ycf4 antibody. The wild type accumulated the 22-kD Ycf4, while the TAP-tagged strain produced a strong band of 44 kD but no signal of 22 kD (Figure 2). The increase in the size of Ycf4 corresponds to the fusion of TAP-tag. This observation indicates that no wild-type Ycf4 was expressed in the transformant. The strong signal of the TAP-tagged Ycf4 detected by immunoblotting is due to the presence of the Protein A sequence in the TAP-tag that reacts with IgG. To estimate the accurate level of Ycf4, the TAP-tagged Ycf4 in the thylakoids was digested with TEV protease to remove the protein A domain in the TAP-tag prior to the immunoblotting analysis. Treatment of wild-type thylakoids with TEV protease

Figure 1. Confirmation of the Segregated TAP-Tagged ycf4 Mutant (ycf4-TAP Mutant) by Restriction Fragment Length Polymorphism.

The EcoRI-ClaI fragment of ycf4 was amplified by PCR from total DNA of the wild type (1), control strain (2), and TAP-tag fused ycf4 mutant (3). The resulting DNA fragments were separated on a 1.5% agarose gel.

Figure 2. Effects of the TAP-Tag in Ycf4 on the Levels of Ycf4, PSI, and PSII Proteins.

(A) Accumulation of Ycf4 was estimated by immunoblot analysis. The signals were visualized by enhanced chemiluminescence. Total cell protein was loaded (equivalent to 1 μg chlorophyll), and signals were detected using an anti-Ycf4 antibody. A dilution series of the wild type was used to estimate Ycf4 accumulation in the ycf4-TAP mutant. +, Ycf4 not treated with TEV protease; +, Ycf4 treated with TEV protease. (B) Effects of the TAP-tag in Ycf4 on the structure of the Ycf4 complex and chlorophyll-protein complexes. The thylakoids from the wild type and ycf4-TAP were solubilized with DDM, and the resulting extracts were separated by sucrose density gradient centrifugation. The entire gradients were fractionated from the bottom (fraction 1) to the top (fraction 20) and were analyzed by immunoblotting using anti-Ycf4 antibody. (C) The level of PSI proteins (PsaA, PsaD, and PsaF) and PSII protein (PsbA) in the ycf4-TAP mutant was estimated by immunoblot analysis. Total cell proteins of the wild type and ycf4-TAP (equivalent to 1 μg chlorophyll) and a dilution series of the wild type were loaded.
did not affect the Ycf4 signal, indicating that nonspecific digestion did not occur. However, the digestion of TAP-tagged Ycf4 thylakoids produced a weaker signal at 28 kD, which corresponds to the size of Ycf4 containing calmodulin binding peptide as part of the TAP-tag but lacking the Protein A sequence (Figure 2A). It was estimated from the signal intensity that Ycf4 decreased to 25% of the wild-type level.

Because the Ycf4 protein is part of a large macromolecular complex (Boudreau et al., 1997a), we next analyzed whether the TAP-tagged Ycf4 is still part of a large complex by sucrose density gradient centrifugation after solubilization of the thylakoids with n-dodecyl-β-d-maltoside (DDM) (Figure 2B). TAP-tagged Ycf4 fractionated in the densest part of the gradient like wild-type Ycf4, indicating that such a large protein complex is maintained in the TAP-tagged strain. The broader distribution of TAP-tagged Ycf4 could be ascribed at least partly to the strong signal of TAP-tagged Ycf4.

We also checked the effect of the TAP tagging on photosystem subunit levels by immunoblotting using anti-PsaA, PsaD, PsaF, and PsbA antibodies. One of the PSI RC polypeptides, PsaA, as well as stromal and lumenal peripheral polypeptides, PsaD and PsaF, respectively, accumulated at wild-type levels (Figure 2C).

Fluorescence induction kinetics of dark-adapted cells confirmed that the TAP-tag strain displays PSI activity (see Supplemental Figure 2 online). Accordingly, the TAP-tag strain grew photoautotrophically in high salt minimum (HSM) medium under medium light (50 μE·m⁻²·s⁻¹) or high light (1000 μE·m⁻²·s⁻¹) like the wild type.

We confirmed that the function and structure of Ycf4 are not significantly affected by the fusion of the TAP-tag at the C terminus of Ycf4. Thus, the TAP-tagged Ycf4 should be useful for isolating the Ycf4-containing complex and for characterizing its structure. To purify the Ycf4 complex, a two-step affinity column chromatography was performed as described in Figure 3A. The extracts obtained by solubilization of thylakoid membranes with DDM from the TAP-tagged strain (fraction a) were applied to an IgG agarose column (first column). Since the adsorption of the TAP-tagged Ycf4 was not efficient, the thylakoid extracts were mixed with IgG agarose and incubated in a rotating column at 4°C overnight. After the incubation, we drained the unbound thylakoid extracts (fraction b). We found that 90% of Ycf4 in the extracts had been adsorbed to the IgG agarose (Figure 3B, top panel). After washing the column with excess buffer (fraction c), the IgG agarose was incubated with TEV protease at 18°C to cut the Protein A domain from the TAP-tag. After digestion, the

![Figure 3. Purification of TAP-Tagged Ycf4 by Affinity Chromatography.](image)

(A) Two-step affinity purification steps are schematically shown. Each fraction was, as designated, from a to g.

(B) Yield of TAP-tagged Ycf4 at every purification step was estimated by immunoblot analysis. Top panel: IgG beads column adsorbed 90% amount of Ycf4 from thylakoid extracts. Bottom panel: The calmodulin bead column adsorbed almost all of the extract digested by TEV protease. Asterisk indicates a band cross-reacting with the Ycf4 antibody. This is probably a light chain of IgG released from IgG beads.

digested TAP-tagged Ycf4 (fraction d) was subsequently incubated with a calmodulin resin in the presence of calcium ions in a rotating column for 2 h at 4°C. We drained the unbound fraction from the calmodulin column (fraction e) and washed the column with excess buffer (fraction f). Almost all the digested product was adsorbed to the calmodulin resin of the second column (Figure 3B, fractions d and e in the bottom panel). After washing, the bound material was released with EGTA (Figure 3A, fraction g). A broad signal below Ycf4 was detected in the TEV-cleaved eluate and calmodulin column flow-through fraction (Figure 3B, bottom panel, fractions d and e, indicated with an asterisk). According to the apparent size and cross-reactivity with the secondary antibody (anti-rabbit IgG antibody), it is likely that this broad signal corresponds to a light chain of IgG released from IgG agarose. This band was completely removed from the final preparation obtained after calmodulin column purification (fraction g). As a negative control, purification was performed...
using the same procedure on wild-type thylakoids. We note that the final sample from TAP-tagged Ycf4 thylakoid extracts was slightly green, while that from the wild-type extracts was colorless.

Characteristics of the Purified Ycf4

To determine which proteins interact with Ycf4, the purified samples were subjected to SDS-PAGE and polypeptides stained with Coomassie blue. The preparation obtained from wild-type thylakoid extracts contained almost no proteins (Figure 4A, lane 1). This indicates that the two-step affinity chromatography can be applied for the specific purification of TAP-tagged proteins. By contrast, the preparation from the TAP-tagged Ycf4 thylakoid extracts contained five main proteins of 120, 64, 32, 28, and 20 kD and a few minor bands (Figure 4A, lane 2).

The results of N-terminal amino acid sequencing of the proteins in the sample from TAP-tagged Ycf4 thylakoid extracts are summarized in Table 1. The 28-kD band corresponds to Ycf4. Although the N-terminal amino acid residue could not be determined, the amino acid sequences from the second to 15th residue except Leu-4 were the same as the sequence deduced from the ycf4 gene. It was also revealed that the first Met residue is processed in the TAP-tagged Ycf4. The 64- and 20-kD bands were identified as ASA1 and PsaF, respectively. The N-terminal ends of PsaF and ASA1 were as reported previously (Franzén et al., 1989; van Lis et al., 2003). It was not possible to determine the N-terminal amino acid sequence of the 32-kD protein and of the other minor proteins. ASA1 (for ATP Synthase-Associated1), which was first named MASAP (for Mitochondrial ATP Synthase-Associated Protein) (van Lis et al., 2003), is a mitochondrial protein; this protein has a presequence predicted to target to mitochondria and is actually present in pure mitochondrial preparations and furthermore copurifies with mitochondrial F0F1-ATP synthase.

Chlamydomonas F0F1-ATP synthase forms a dimer and contains nine ASA subunits (ASA1 to 9) (van Lis et al., 2003, 2007; Cardol et al., 2005). Thus, the presence of ASA1 in the purified Ycf4 preparation is rather unexpected.

We next sliced the gel and digested proteins with trypsin and analyzed the resulting polypeptides by liquid chromatography–tandem mass spectrometry (LC-MS/MS). The searching of MS/MS data was done within the Kazusa EST database, Joint Genome Initiative (version 3.0), and NCBI (NC_005353 and NC_001638), as well as within release 2 of the C. reinhardtii genome (chire2). We confirmed by MS the assignments of the 64-, 28-, and 20-kD proteins (Table 2). Interestingly, several peptide fragments derived from PsaA and PsaB were identified from the 64-kD band, indicating that PSI RC polypeptides copurified with the Ycf4-containing complex. In addition to those from PsaF, peptides derived from PsAD, which migrates close to PsaF on the polyacrylamide gel, were also detected in the 20-kD band.

Figure 4. Polypeptide Composition of the TAP-Tagged Purified Ycf4 Complex.

(A) Left two lanes: the samples purified from thylakoid extracts of the wild type (1) and ycf4-TAP (2). The polypeptides were separated by SDS-PAGE and stained with Coomassie blue. Right lanes: Immunoblot analysis of the polypeptides of the purified Ycf4 complex using anti-ASA1, -PsaA, -COP1, -COP2, -Ycf4, -PsaC, -PsAD, -PsaE, and -PsaF antibodies.

(B) Absorption spectrum of the purified Ycf4 complex.

(C) Coimmunoprecipitation with anti-Ycf4 or -COP2 antibody. The thylakoid membranes from the wild-type cells were solubilized with DDM, and the resulting extracts were incubated with Dynabeads Protein G cross-linked with preimmune (a), anti-Ycf4 (b), or -COP2 (c) serum. Coimmunoprecipitated proteins were analyzed by immunoblottings using anti-Ycf4, -COP2, -PsaF, -PsaA, -PsAD, -PsaE, -PsaC, -Lhca6, and -PsbA antibodies.
Although the determination of the N-terminal amino acid sequence of the 32-kD protein was unsuccessful, MS revealed that this protein is a chlamyopsin, either COP1 or COP2 (Fuhrmann et al., 2003). It was shown previously that the N-terminal residue of COP1 and COP2 is blocked (Deininger et al., 1995). COP1 and COP2 are opsins-related proteins originally isolated from Chlamydomonas eyespot preparations (Deininger et al., 1995). They are alternative splicing products of the **COP1** gene with exons 1 to 7 and 9 encoding COP1 and exons 1 to 8 encoding COP2. Later immunoblot analysis using specific antibodies raised against the amino acid sequences encoded by the exon 9 (COP1) and 8 (COP2), respectively, revealed that COP2 dominates over COP1 with a ratio of 50/1. Whereas most peptides occurred in both COP1 and COP2, a peptide fragment IVAEVKPGK (amino acids 205 to 213) specific to COP2 was assigned (Table 2). In our experiments, the sequence coverage of 35% was achieved for chlamyopsin, indicating that this is a major component of the protein band. Since no peptide fragment specific to COP1 was detected and no signal cross-reacted with anti-COP1 antibody (Figure 4A), the 32-kD protein is named COP2 in the following. COP1 and COP2 were reported not to be required for behavioral responses like phototaxis and photophobic responses (Fuhrmann et al., 2001). Thus, the functional role of COP1 and COP2 is currently elusive. The mitochondrial adenine nucleotide translocator (ANT) was identified in three peptides, suggesting that this protein is also present in this particular Coomassie-stained band (Sharpe and Day, 1993). However, ANT is more likely to be a contamination rather than a genuine component of the Ycf4-containing complex because it is a mitochondrial protein. In addition, the 120-kD band was identified as ASA1, which suggests that ASA1 formed an aggregate, most probably a dimer. In-solution digestion and LC-MS/MS were used to analyze the entire set of polypeptides in the Ycf4 complex purified from the thylakoid extracts of Ycf4-TAP and in a sample purified by the same procedure but from thylakoid extracts of the wild type. The identified proteins are summarized in Table 3. In addition to the proteins detected by the in-gel digestion analysis shown in Table 2, the chloroplast-encoded orf1995 (**ycf1** homolog) product was found in the Ycf4 complex. This protein is composed of an N-terminal hydrophobic domain, which is likely to be anchored in the chloroplast envelope membrane or in thylakoid membranes, and a C-terminal hydrophilic region enriched in positively charged residues able to bind DNA and RNA (Boudreau et al., 1997b). Disruption of this gene in Chlamydomonas and tobacco (**Nicotiana tabacum**) is impossible, suggesting that this gene is essential (Boudreau et al., 1997b; Drescher et al., 2000). It is noteworthy that this protein has an affinity for the Ycf4 complex, although the amount of this protein in the purified Ycf4 preparation appears to be very low. By contrast, no proteins found in the Ycf4 complex was detected in the sample from the wild type except for ASA1 (see Supplementary Table 1 online).

**Table 1. N-Terminal Amino Acid Sequences**

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<th>Sample Protein</th>
<th>Deduced Sequence</th>
<th>Determined Sequence</th>
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<td>66 kD ASA1</td>
<td>YVTALKVFSEGVAAPKNKESTA</td>
<td>YVTALKVFSEGVAAPKNKExTA</td>
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An “x” indicates a residue that could not be determined.

Immunoblotting analyses using anti-COP2 and -Ycf4 antibodies clearly showed cross-reactions with the 32- and 28-kD bands, respectively. By contrast, no signal was detected by immunoblot analysis using anti-COP1, suggesting that COP1, if any, is present at very low levels. The anti-ASA1 antibody cross-reacts with the 64- and 120-kD bands. Immunoblot analysis with anti-PsaD and -PsaF antibodies revealed a cross-reaction with the 20-kD band, although the amount of PsaD appeared to be much lower than that of PsaF (Figure 4A). The presence of PsaA was also confirmed by immunoblotting analysis. Furthermore, small amounts of PsaC and PsaE were detected by sensitive immunodetection as described in Methods. These proteins could not be detected with the LC-MS/MS probably because their amount was too small. PsaG, PsaH, and PsaL were not detected by sensitive immunodetection. It was previously proposed that a large Ycf4-containing protein complex acts in the assembly of the PSI core complex (Boudreau et al., 1997a). The copurification of the major PSI proteins strongly suggests that the Ycf4-containing complex plays a pivotal role in the biogenesis of PSI complex by mediating the interactions between PSI proteins. The presence of chlorophyll-containing polypeptides, such as PsaA and PsaB, in the purified TAP-tagged Ycf4 preparation is consistent with the observation that the final preparation is faintly green. The absorption spectrum of the purified sample revealed the presence of chlorophylls and carotenoids (Figure 4B). The red absorption maximum at 680 nm is specific for the PSI complex.

The interactions between Ycf4, Cop2, PsaF, and PsaA were confirmed by immunoprecipitation using anti-Ycf4 and -Cop2 (Figure 4C). However, PsaC, PsaD, and PsaE were not detected, suggesting that these proteins are only loosely associated with the Ycf4 complex and are dissociated during the solubilization and/or purification procedures. As expected, subunits of LHCI (Lhca6) and PSII (PsbA) were not detected, indicating that the immunoprecipitation was specific.

To confirm whether the purified Ycf4 complex maintained its large structure, we performed sucrose density gradient ultracentrifugation and gel filtration column chromatography. Ycf4, COP2, and PsaF were mostly present in the densest region of the gradient like Ycf4 from wild-type thylakoid extracts (Figure 5A) and were fractionated in a large size (>1000 kD) by the chromatography (Figure 5B). On the sucrose density gradient, the green color was also observed. These
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(Continued)
observations suggest that a small amount of chlorophyll in the purified Ycf4 complex is associated with PsaA copurified with TAP-tagged Ycf4. It is inferred that PsaA and most probably PsaB form a chlorophyll-containing subcomplex and are detached from the Ycf4 complex during sucrose density gradient centrifugation. It is clear that PsaF was not associated with but one of peripheral subunits, PsaD, cofractionated with the subcomplex. PsaC and PsaE could not be detected in these fractions, probably because the antibodies against these subunits are not strong enough. However, it is very likely that PsaC and PsaE remain associated with the subcomplex in the presence of PsaD (Mannan et al., 1994; Yu et al., 1995). Thus, it is likely that a PSI assembly intermediate subcomplex is weakly associated with the Ycf4 complex.

**EM and Single Particle Analysis on the Ycf4 Complex Preparation**

The high molecular weight fraction of Ycf4 from the sucrose density gradient (fraction 2 in Figure 5A) was imaged at room temperature, after being negatively stained, using an FEI-Tecnai T12 electron microscope. The images revealed a structurally heterogeneous preparation (Figure 6A). The micrographs chosen, judged to be high quality from their power spectra indicating negligible drift, astigmatism, and Thon ring extension to better than 10 Å, allowed for a data set of all possible single particles (>9200) to be extracted at 2.44 Å per pixel on the specimen scale. Reference-free alignment and classification procedures resolved these particles into several distinct populations based on overall size and shape, and further iterative multireference alignments improved their signal-to-noise ratio. The averages (Figure 6B) contained rectangular-shaped forms, some slightly dimeric in nature, consistently with dimensions of \(~285 \times 185 \text{ Å} \) (length \times width), which are similar to those observed for photosystems (Nield et al., 2000, 2003). No symmetry operators were applied. On occasion a more heavily stained central region, running the length of the averages, was apparent as indicated by white arrows (Figure 6B) lending a pseudodimeric appearance to these classes. In addition, smaller densities were often observed to extrude from the central region (Figure 6B, asterisks).

**Transient Association of PSI Polypeptides with Ycf4 Complex**

If the Ycf4 complex is involved in PSI complex assembly, one expects that the PSI polypeptides detected in the complex are newly synthesized and thus should be exclusively pulse labeled during a short period. To test this prediction, total cellular proteins of *Chlamydomonas* wild-type cells in logarithmic growth phase were pulse labeled with \([\text{35S}]\text{Na}_2\text{SO}_4\) for 5 min, and the thylakoid membranes were subsequently isolated. The thylakoid membranes were solubilized with DDM, and the resulting

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Table 2. (continued). The charge state (Z) of the measured ion, the calculated deviation (ΔM), and the cross correlation factor (Xcorr) calculated by the Sequest algorithm are given. The position of the peptide within the protein sequence is also listed.
extracts were separated by sucrose density gradient ultracentrifugation. Because we used a small swing rotor for sucrose density gradient ultracentrifugation, the separation was relatively poor; Ycf4-complex and mature PSI complex were mainly separated in fractions 1 to 5 and 4 to 10, respectively, while PSII and LHCII were separated in fractions 7 to 12 and 12 to 16, respectively (Figures 7A and 7B). Since PsaA and PsaB form diffuse bands on the gel, it was difficult to detect labeled PsaA and PsaB around 60 to 70 kD. Instead, we focused on a sharp labeled band around 20 kD as indicated in Figure 7B. Immunoblotting verified that the position of this band corresponds to PsaD and/or PsaF. Although thylakoid membranes contain a large number of proteins, PsaD/F are clearly identified on the gel after fractionation of chlorophyll-protein complexes on sucrose density gradient (Sugimoto and Takahashi, 2003). The 5-min pulse-labeling experiments indicated that the labeled PsaD and PsaF are present in multiple fractions, some of which also contain Ycf4 and the PSI-LHCl supercomplex. This observation reveals that a large portion of the newly synthesized PsaD/F was integrated into PSI-LHCl supercomplex, while a small portion of these proteins still cofractionated with the Ycf4-complex within the 5-min pulse. This rapid PSI complex assembly is consistent with the fact that it is difficult to detect PSI complex assembly intermediate subcomplexes in wild-type cells. After the chase, the labeled PsaD and PsaF in the Ycf4-complex (fractions 1 to 4) decreased, while those in the mature PSI complex (fractions 4 to 12) were stable. These observations suggest that the newly synthesized PSI polypeptide(s) are transiently associated with the Ycf4 complex and are subsequently shifted to the mature PSI complex.

To confirm the transient association between newly synthesized PSI subunits and Ycf4-complex, we performed immunoprecipitation of the newly synthesized PSI proteins with anti-Ycf4 antibody (Figure 7C). To assign the position of PSI polypeptides on the gel, we prepared PSI-LHCl supercomplex uniformly labeled with 14C. In Figure 7C, lane a, the positions of PsaA/B,
LHCI polypeptides, and PsaD/F are clearly shown. We pulse labeled total cell proteins, and the thylakoid membranes were isolated and solubilized as shown in Figure 7B. The thylakoid extracts were then immunoprecipitated using anti-Ycf4 antiserum, and the precipitated polypeptides were subjected to SDS-PAGE (Figure 7C, lane b). In addition to several labeled polypeptides in the 25 to 50 kD range, the band corresponding to PsaD and PsaF was clearly seen. By contrast, only a weak signal corresponding to PsaA/B could be detected, and the LHCI polypeptides were undetectable. The small amount of PsaA/B immunoprecipitated with Ycf4 antiserum is expected from the analysis of the Ycf4 complex, which contains only small amounts of PsaA/B (Figure 4A). It is likely that PsaA/B were dissociated from the complex, while PsaF remained stably associated with it. After the 20-min chase, the labeled PsaD/F was undetectable, demonstrating that the newly synthesized PsaD/F were transiently associated with the Ycf4-complex.

Copurification of Ycf4 and COP2 from Wild-Type Cells

As shown in Figure 4, COP2 copurified with Ycf4. To address quantitatively whether all Ycf4 and COP2 are present in the same large complex, Ycf4-containing fractions were isolated and purified from wild-type thylakoids. Ycf4 was found exclusively in the thylakoids, as previously reported (Figure 8A) (Boudreau et al., 1997a). Although COP2 was localized in the eye-spot apparatus (Deininger et al., 1995), it was specifically present in our thylakoid preparation and not in the soluble fractions. These data are consistent with a recent Chlamydomonas thylakoid proteomics study (Allmer et al., 2006), in which COP2 was also found in purified thylakoid membranes based on the identification of five distinct peptides. Since the eye-spot apparatus is located inside the chloroplast and is attached to a few thylakoid membranes (Schmidt et al., 2006), it may be associated with the thylakoid membranes.

To remove extrinsic proteins, the thylakoid membranes were washed with 2 M NaBr (Figure 8B). Approximately 90% of OEE2 (PsbP) and CF1 (AtpA and AtpB) was removed from the thylakoid membranes. Ycf4 and COP2 remained stably associated with the thylakoid membranes, whereas approximately half of ASA1 was removed by washing with 2 M NaBr or 2 M NaCl, indicating that the binding of ASA1 to the thylakoid membranes is weak. Thylakoid membranes were then solubilized with DDM and fractionated on a sucrose density gradient. Immunoblots using anti-Ycf4 and anti-COP2 antibodies revealed that almost all of COP2 was present in fractions 5 to 8, as was Ycf4 (Figure 8C). Further purification of these four fractions by DEAE ion exchange column chromatography also showed that COP2 and Ycf4 were present in the same fractions (fractions 12 to 14) (Figure 8D). These experiments confirmed that almost all COP2 and Ycf4 present in cells are both components of a high molecular weight complex.

PSI polypeptides, PsaA and PsaF, were detected in the highly purified Ycf4 preparation as shown in Figure 4. Very sensitive immunological detections using anti-PsaA and PsaF antibodies showed that a small amount of PsaA and PsaF still copurified with Ycf4 and COP2 from wild-type thylakoid extracts (Figure 8D). Thus, the results obtained from wild-type thylakoid membranes confirmed that the Ycf4 complex associates with PSI polypeptides. We also analyzed the distribution of ASA1 during the purification steps. Since the antibody against ASA1 was specific but not strong, the immunological detection of ASA1 was slightly hazy. However, only a portion of ASA1 copurified with Ycf4 and COP2 after sucrose density gradient
centrifugation (Figure 8C). Subsequent fractionation by DEAE chromatography also revealed that only a portion of ASA1 copurified with Ycf4 and COP2 (Figure 8D). These observations indicate that ASA1 has a weak affinity to the Ycf4 complex, which is consistent with the observation shown in Figure 5A.

The final preparation from the wild-type thylakoid extracts still contained numerous polypeptides when analyzed by SDS-PAGE (see Supplemental Figure 4 online). In contrast with the TAP-tagged purified Ycf4 complex in which Ycf4 was a prominent band, Ycf4 was present only as a minor component of the complex isolated from wild-type cells, indicating that the degree of purification was considerably lower in this case.

**COP2 Is Not Essential for PSI Complex Assembly**

To study the function of COP2 in PSI complex assembly, we used knockdown mutants of Cop2 (Cop2-RNAi [for RNA interference]) already reported by Fuhrmann et al. (2001). The Cop2-RNAi mutants accumulated Cop2 to 5 to 10% of wild-type level but Ycf4 and PSI to wild-type levels (Figure 9A) and thus grew photoautotrophically under low and strong light conditions (Figure 9B). These findings suggest that COP2 is not essential for PSI complex assembly. However, it cannot be completely excluded that COP2 is involved in the regulation of PSI complex assembly under certain growth conditions. It is also possible that minute amounts of COP2 are sufficient for assembly of PSI.

A small effect of the knockdown of COP2 on the stability of the Ycf4 complex was observed. The thylakoid membranes from Cop2-RNAi were solubilized and fractionated by sucrose density gradient ultracentrifugation, and Ycf4 was detected near the bottom of the sucrose gradient without NaCl as Ycf4 from wild-type cells (Figure 9C). However, when NaCl was added to the sucrose gradient, most Ycf4 from Cop2-RNAi cells fractionated near the top of the gradient, indicating that the Ycf4-complex is unstable. By contrast, the addition of NaCl to the sucrose gradient did not affect the stability of the Ycf4 complex from wild-type thylakoids. These observations are consistent with the fact that COP2 is a component of the Ycf4-complex and stabilizes the structure of the Ycf4 complex. The thylakoid membranes from Δycf4 were also solubilized and fractionated, and the separation of COP2 was analyzed (Figure 9C). COP2 was detected near the bottom of the sucrose gradient, and the separation pattern was not significantly affected even in the presence of NaCl. However, a slight shift of COP2 toward the lighter fractions of the gradient was observed, although the shift may be at the limit of detection in these sucrose density gradients. These observations suggest that the absence of Ycf4 might slightly, if any, affect the stability of the oligomeric structure of COP2.

**Ycf4 Complex Is Destabilized in the Absence of PsaF**

The Ycf4 complex purified by the affinity column chromatography contained a substantial amount of PsaF. We next fractionated

![Figure 8. Purification of Ycf4 and COP2 from Wild-Type Cells.](image-url)
thylakoid extracts from a PsaF-deficient strain (ΔPsaF) by sucrose density gradient ultracentrifugation to study the effect of the deletion of PsaF on the structure of Ycf4 complex. The ΔPsaF cells synthesize functional PSI complexes and thus are able to grow photoautotrophically (Farah et al., 1995). Figure 9C shows that Ycf4, present in fractions 2 to 6 near the bottom of the gradient from wild-type cells, was found in fractions 3 to 16 in ΔPsaF. This observation indicates that the Ycf4 complex was destabilized in the absence of PsaF. The distribution of COP2 was also shifted to the upper fractions, suggesting that part of COP2 was also dissociated but to a lesser extent.

We also fractionated thylakoid extracts from a PsaA/B-deficient strain (ΔpsaA/B) in which the other PSI polypeptides barely accumulate (Redding et al., 1998). Figure 9C shows that Ycf4 and COP2 were fractionated in fractions 2 to 7, indicating that the Ycf4 complex is stable in the absence of PSI RC. Sensitive immunodetection revealed that a small amount of PsaF also fractionated in fractions 2 to 7, indicating the exclusive copurification of PsaF with the Ycf4 complex. This finding is consistent with the model that PsaF, which is synthesized in the cytosol and transported into chloroplast, is specifically localized on the Ycf4 complex before it is integrated into a PSI assembly intermediate subcomplex.

DISCUSSION

Successful Ycf4 Purification by TAP-Tag Technology

In this study, we generated a chloroplast transformant in which Ycf4 was fused with an affinity tag (TAP-tag) at its C-terminal end, allowing the successful purification of a stable Ycf4-containing complex by two-step affinity chromatography. Since Ycf4 is known to be essential for PSI complex assembly and is part of a large complex in C. reinhardtii (Boudreau et al., 1997a), we verified that the fusion of the TAP-tag with Ycf4 does not affect PSI complex assembly as well as structure of the Ycf4 complex. The highly purified TAP-tagged Ycf4 formed a stable complex with a size estimated at ~1500 kD by sucrose density gradient centrifugation, gel filtration chromatography, and single particle analysis. The major protein constituents of the purified TAP-tagged Ycf4 were identified as being COP2, Ycf4, and PsaF via

(A) Accumulation of COP2, Ycf4, and PsaA proteins in COP2-RNAi strain was estimated by immunoblotting. Thylakoid membranes (equivalent to 1 μg chlorophyll) and a dilution series of the control strain (cw15) were loaded.

(B) Growth of control strain (cw15) and COP2-RNAi mutant. The cells were grown on TAP agar plate in the dark (TAP dark) or on HSM agar plates in medium light (50 μE m⁻² s⁻¹) or high light (300 μE m⁻² s⁻¹).

(C) Thylakoid membranes from the wild type, COP2-RNAi, ΔYcf4 ΔPsaF, and ΔPsaA/B were solubilized with DDM, and the resulting extracts were separated by sucrose density gradient centrifugation in the absence (−) or presence (+) of 100 mM NaCl. The entire gradients were fractionated from the top to the bottom, and the fractions were subjected to immunoblottings using anti-Ycf4, -Cop2, and -PsaF antibodies.
N-terminal amino acid sequencing, mass spectrometry, or immunoblotting and immunoprecipitation. A weak binding of ASA1 as well as PsaA, PsaC, PsaD, and PsaE to the TAP-tagged Ycf4 complex was also observed. In addition, MS revealed the presence of PsaB and chloroplast encoded Ycf1 (Orf1995). The presence of the PSI polypeptides, PsaA, PsaB, PsaC, PsaD, PsaE, and PsaF, suggests that Ycf4 is involved in PSI complex assembly by interacting directly with PSI polypeptides. The presence of COP2 in the Ycf4 complex represents a new finding.

Interaction of a PSI Subcomplex with Ycf4 Complex

The highly purified TAP-tagged Ycf4 contained a small amount of chlorophyll and carotenoids and showed an absorption peak at 680 nm, which is characteristic of PSI complexes (Figure 4B). Immunoblotting and MS revealed that the purified Ycf4 complex contains the two chlorophyll binding PSI RC subunits, PsaA and PsaB. When the purified Ycf4 complex was fractionated by sucrose density gradient centrifugation, the PsaA, PsaD, and pigments were detached from the Ycf4 complex and separated in the same fractions (Figure 5A). We could not determine the distribution of PsaB, PsaC, and PsaE in the sucrose density gradient because the corresponding antibodies were not strong enough. However, according to the position of PsaA on the sucrose density gradient, it is highly likely that this polypeptide was part of a subcomplex most probably consisting of PsaA and PsaB. Thus, these observations strongly suggest that a subcomplex consisting of the PsaA and PsaB heterodimer has an affinity for the purified Ycf4-containing complex. In addition to the heterodimer, the three subunits, PsaC, PsaD, and PsaE, which form a cluster on the stromal side of PSI, were detected. The copurification of PsaD with PsaA on sucrose density gradients suggests that PsaD was already assembled into the assembly subcomplex, whereas PsaF was not yet stably integrated into the subcomplex. Thus, the initial assembly steps are integration of the heterodimer of PsaA and PsaB, and subsequently PsaC, PsaD, and PsaE are assembled on the stromal side of the heterodimer. The integration of PsaF into the PSI subcomplex may occur at a later assembly step. This is consistent with the result that a PsaF-deficient mutant is able to assemble a structurally and functionally competent PSI complex (Farah et al., 1995). Moreover, it is noticeable that PsaF is an abundant protein in the Ycf4 complex in vast excess compared with the other PSI subunits detected in this complex. The observation that the Ycf4 complex is significantly destabilized in the absence of PsaF suggests that this protein plays an important structural role in this complex (Figure 9C). Because the PSI polypeptides appear to be weakly bound to the Ycf4 complex, it is possible that additional PSI polypeptides are present in the complex and that most of them are dissociated during the two-step affinity purification because this procedure involves a series of large volume washing steps. The pulse-chase results suggest a transient association of the newly synthesized PSI subunits with the Ycf4 complex, at least in the case of PsaD. It can therefore be concluded that PSI polypeptides were integrated into an assembly intermediate in the Ycf4 complex.

Ycf37/Pyg7 appears to play a similar role in PSI complex assembly. It is essential for PSI complex assembly and cofractionates with the PSI complex in Arabidopsis (Stöckel et al., 2006). In cyanobacteria, this protein mediates more efficient PSI accumulation and regulates the latest steps of PSI assembly (Dühring et al., 2006). It is of interest that the chloroplast-encoded Ycf3 also has interactions with PsaA and PsaD (Naver et al., 2001). Since Ycf3 is essential for PSI complex assembly in Chlamydomonas (Boudreau et al., 1997a) and tobacco (Ruf et al., 1997), it should be involved in initial assembly steps as an essential factor.

The electron microscopy results showed the purified Ycf4 complex with many heterogeneous views, but a number of stable populations were identified (Figure 6). The TAP-tagged Ycf4 preparation used for the electron microscopy lacked the PSI polypeptides because it was further purified by sucrose density gradient centrifugation. The observed Ycf4 complexes were all of similar size and shape, ~285 × 185 Å. Such dimensions in negative stain are similar to those observed for other electron microscopy-visualized photosynthetic complexes, including those of photosystems calculated in three dimensions (Nield et al., 2000, 2003). It is possible that the mass of these complexes exceeds 1000 kD, although no mass information can be gained from two-dimensional projections alone. The extruded density often observed, asterisks in various averages shown in Figure 6B, may indicate that multiple copies of Ycf4 and COP2 form an underlying molecular scaffold. However, no regular, symmetrical or otherwise, motifs have been observed in the preparations imaged. During extensive washing with low concentration of DDM in the two-step affinity column, some fine structure of the Ycf4-containing complex might be damaged. Because the Ycf4-containing complex has a large size and specific affinity with PSI polypeptides, it is hypothesized that the complex provides a scaffold where newly synthesized PSI polypeptides and cofactors are integrated and resulting assembly intermediates are stabilized.

Assembly of PSI Components

According to recent models for PSI complex assembly (Figure 10), the first step is the translation of PsaB, which is reported to enhance the translation of PsaA, and subsequently these two polypeptides form a heterodimer (Wostrzikoff et al., 2004). It was reported that the translation of chlorophyll binding polypeptides is stimulated in the presence of cofactors, such as chlorophylls (Kim et al., 1994) and carotenoids (Herrin et al., 1992). Thus, it is inferred that the integration of chlorophylls and carotenoids occurs cotranslationally. In addition to these pigments, phylloquinone and 4Fe–4S cluster (F_{X}) are integrated into the heterodimer. In phylloquinone-deficient Arabidopsis mutants, the PSI complex is destabilized (Shimada et al., 2005), while plastoquinone substitutes for the function and structure of phylloquinone in cyanobacteria and Chlamydomonas (Johnson et al., 2000; Lefebvre-Legendre et al., 2007). Integration of phylloquinone occurs before F_{X} is assembled (Shen et al., 2002). The assembly of F_{X} is required for the subsequent integration of PsaC, PsaD, and PsaE located on the stromal side (Shen et al., 2002). Since Ycf3 interacts with PsaA and PsaD, this factor may assist the assembly of the heterodimer and probably the three subunits on the stromal side, PsaC, PsaD, and PsaE (Figure 10). This study
revealed that the PSI subcomplex consisting of the heterodimer and the three subunits of the stromal side is associated with the Ycf4 complex. Ycf4 is not essential but is required for the efficient PSI complex assembly in cyanobacteria (Wilde et al., 1995). Thus, the Ycf4 complex plays a role as a scaffold for PSI complex assembly. When the heterodimer is formed and the three stromal subunits are integrated on the Ycf4 complex, assembly intermediate subcomplexes are stable and prevented from digestion by proteases. The protection of the subcomplex from damage by proteases should be more vital in Chlamydomonas than in cyanobacteria because Chlamydomonas has a more efficient system to remove damaged, misfolded, and mutated complexes than cyanobacteria. As an example, in the absence of PsaC, the PSI complex is rapidly degraded in Chlamydomonas (Takahashi et al., 1991) but is rather stable in cyanobacteria (Mannan et al., 1991). In subsequent assembly steps, PsaF would be transferred to the PSI subcomplex from the Ycf4 complex. PsaJ, which is located in close proximity to PsaF, may be assembled at the same time. However, it still remains elusive how and when the other subunits, PsaH, PsaL, PsaN, and PsaO, are integrated. Since PsaH, PsaL, and PsaN form a cluster at the periphery of the heterodimer, they will be integrated successively: PsaL/PsaN, which are located inside of the cluster, and then PsaH, which is present outside of the cluster. PsaO, which is most probably located on the exterior side of the PsaH/PsaL/PsaN cluster, is subsequently integrated. PsaK and PsaG are located on each edge of the LHCl belt, while PsaM is present on the luminal side of LHCl. Thus, it is likely that they are integrated into PSI complex when LHCl oligomer is integrated into PSI core complex.

**Retinal Binding Protein Is a Component of Ycf4 Complex**

One of the unexpected observations in the polypeptide composition of the Ycf4 complex purified by affinity chromatography is the presence of the retinal binding protein, COP2. COP2 was coimmunoprecipitated with the anti-Ycf4 antibody and vice versa, confirming the intimate interaction between Ycf4 and COP2 (Figure 4C). Since almost all COP2 copurified with Ycf4 (Figure 8), this polypeptide is specifically and stably bound to the Ycf4 complex. In the green alga C. reinhardtii, at least seven rhodopsin-related proteins with different functions exist. They are classified from COP1 to COP7. COP3 and 4 are now identified as Type I channel rhodopsin proteins (Hegemann et al., 2001; Nagel et al., 2002, 2003, 2005). COP1 and COP2 are unique proteins that are not conserved among plants and only found in invertebrates. COP1 and COP2 proteins result from alternative splicing of the COP gene transcripts with exons 1 to 7 and 9 and exons 1 to 8, respectively. The COP1 gene product is predicted to result from a splicing pattern that has so far not been observed. Initially, COP1 was identified as a novel Type II opsin protein in C. reinhardtii, standing for chlamyopsin. COP1 and COP2 retain a unique structure, and only four transmembrane helices could be identified by hydropathy plots, whereas other opsins have seven transmembrane domains. Retinal binding sites are conserved, and binding of 3H-retinal was confirmed by in vitro reconstitution (Deininger et al., 1995). Given these data, COP1/2 was expected to be involved in phototaxis. However, further studies involving COP gene silencing using RNAi methods did not reveal any effect on phototaxis in these strains (Fuhmann et al., 2001); hence, the function of COP1/2 remains unresolved. Because it is not conserved in plants, COP2 is not a universal component of the Ycf4 complex. One possibility is that in Chlamydomonas, COP2 functions as a sensory photoreceptor that regulates the biogenesis of PSI complex to allow for adaptation to varying light conditions. We are currently continuing to investigate the role of COP2 in PSI complex assembly.

COP2 was first isolated from the eye-spot apparatus, a visual system for measuring the light intensity and to detect the direction of the light source, which is located inside the chloroplast (Deininger et al., 1995; Schmidt et al., 2006). The eye-spot apparatus is composed of two highly ordered layers of carotenoid-containing lipid globules that are associated with thylakoid membranes (Schmidt et al., 2006). Thus, the question arises whether Ycf4 tightly associated with COP2 is also localized in the eye-spot apparatus. Recent proteome analyses of the highly...
purified but rather intact eye-spot apparatus from *Chlamydomonas* revealed the presence of a number of thylakoid proteins, such as subunits of PSI and PSII complexes, light-harvesting complexes, cytochrome *b6f* complex, and ATP synthase, as well as Ycf4 (Schmidt et al., 2006), although cross-contamination cannot be completely excluded. It is likely that Ycf4 is present in the thylakoid membrane fragments in the eye-spot apparatus. However, it is not known whether Ycf4 is enriched in these small thylakoid membrane fragments. If Ycf4 is exclusively located in the eye-spot apparatus, PSI complex and probably some other photosynthetic complexes might be synthesized and assembled in the thylakoid membrane regions included in the eye-spot. By contrast, it is reported that one of the PSII RC polypeptides, D1, is synthesized in discrete regions near the pyrenoid (Unicake and Zerges, 2007). With respect to the sites of synthesis of photosynthetic complexes, cyanobacterial Ycf4, which is not essential but required for efficient biogenesis of PSI complex (Wilde et al., 1995), and Ycf3, which is essential for PSI biogenesis, are exclusively detected in the plasma membrane rather than in the thylakoid membranes (Zak et al., 2001). It was proposed that the assembled PSI complexes are translocated to the thylakoid membranes. Thus, it is possible that the PSI complex might be assembled at specific sites or regions of the thylakoid membranes.

The functional role of COP2 in PSI complex assembly remains elusive. We analyzed the phenotype of COP2-RNAi cells in which COP2 accumulates to <10% of wild-type level. The COP2-RNAi cells were able to grow photototrophically under medium and high light conditions and accumulated PsA like wild-type cells, indicating that the reduction of COP2 by >90% does not affect PSI complex assembly under these growth conditions. However, the Ycf4 complex of the COP2-RNAi cells was more sensitive to salt treatment (Figure 9C), raising the possibility that COP2 might stabilize the Ycf4 complex. It is also possible that COP2 plays a regulatory role in PSI complex assembly to adjust the synthesis of the PSI complex under varying growth conditions. Alternatively, since excess amounts of COP2 are synthesized in wild-type cells, the small amount of COP2 in COP2-RNAi cells might be sufficient for PSI complex assembly. Further study with COP2-RNAi cells will be required to investigate the functional role of COP2 or knockout mutants of COP2 might be required for this analysis.

**Are ASA1 and Ycf1 Authentic Components of the Ycf4 Complex?**

ASA1 is a unique protein in *Chlamydomonas* with no known homolog in any other organism except for the closely related green alga, *Polytomella* sp Pringsheim 198.80 (van Lis et al., 2007). ASA1 is thought to be present on the mitochondrial inner membrane because it is identified as a mitochondrial ASA protein (van Lis et al., 2003, 2007). The purified TAP-tagged Ycf4 complex was not contaminated by the mitochondrial ATP synthase, and a small amount of ASA1 was detected in the negative control of the affinity purification (Table 3). In addition, ASA1 readily dissociated from the highly purified TAP-tagged Ycf4 complex (Figure 5) and fractionated nonspecifically on sucrose density gradient and DEAE chromatography from wild-type thylakoid extracts (Figure 8). Thus, it is concluded that ASA1 is not a genuine component of the Ycf4-containing complex. Given that ASA1 and Ycf4 are separately located in mitochondria and chloroplast, respectively, these two proteins should not form a complex in vivo unless ASA1 has a dual mitochondrial and chloroplast localization. It is known that most thylakoid preparations of *Chlamydomonas* are substantially contaminated by mitochondria. Thus, ASA1 and Ycf4 might form a nonspecific complex when these membranes were solubilized with DDM.

MS analysis additionally revealed the presence of the chloroplast-encoded Orf1995, a homolog of Ycf1, in the purified Ycf4 complex. Ycf1 is a large protein composed of an N-terminal hydrophobic region and a C-terminal hydrophilic domain enriched in positively charged residues (Boudreau et al., 1997b; Drescher et al., 2000). Because Orf1995/Ycf1 is essential (Boudreau et al., 1997b; Drescher et al., 2000), it is unlikely that this protein is uniquely involved in PSI complex assembly. However, the ability of the positively charged C-terminal domain to bind DNA and RNA may suggest its involvement in chloroplast gene expression. If this is the case, the site of translation of chloroplast-encoded PSI subunits, such as PsA, PsB, and PsAc, may be localized on the Ycf4 complex. The amount of Orf1995 in the Ycf4 complex appears to be very low based on the intensity of the stained band, raising questions on the specificity of the association of Orf1995/Ycf1 with the Ycf4 complex. This protein may transiently bind to the Ycf4 complex when the PSI subunits are actively synthesized. However, further studies are needed to elucidate the role of Orf1995/Ycf1 in PSI complex assembly.

**METHODS**

**Strains and Growth Conditions**

We used the green alga *Chlamydomonas reinhardtii* wild-type strain 137c, Ycf4-deficient mutant (ΔYcf4) (Boudreau et al., 1997a), PsA-deficient mutant (ΔPsA) (Farah et al., 1995), PsA/B-deficient mutant (ΔPsA/ΔPsB) (Redding et al., 1998), and Cop2-RNAi mutant (Fuhrmann et al., 2001). Cells were grown in Tris-acetate-phosphate or HSM media (Gorman and Levine, 1965) at 25°C. Chloroplast transformants were selected by three rounds of single colony purification on Tris-acetate-phosphate agar plates containing spectinomycin (150 μg mL⁻¹) and subsequently maintained on Tris-acetate-phosphate agar plates containing spectinomycin (25 μg mL⁻¹).

**Generation of TAP-Tagged ycf4**

A 2.9-kb Xbal-KpnI chloroplast DNA fragment containing ycf4 and ycf3 (accession number Y13655) was cloned into pBluescript digested with the same enzymes to generate pXK29 (see Supplemental Figure 1 online). To insert the TAP-tag at the C-terminal end of Ycf4, a new BamHI restriction site was created upstream of the ycf4 stop codon by performing a two-round PCR using pXK29 as template. A 220-bp fragment was created through the first-round PCR using two oligonucleotides, 5'-GGTTCTTTAGAAGCCATCTAAACTGATTTT-3' (underline indicates BamHI restriction site) and 5'-GAATTCCTAACT-3' (underline indicates Clal restriction site). The resulting fragment and oligonucleotide 5'-ATTTAGAATCTGAATGATACC-3' (underline indicates EcoRI restriction site) was used as primers during the second-round PCR to create a 0.7-kb EcoRI-ClaI DNA fragment with a new BamHI site. This fragment was digested with EcoRI and Clal and was inserted into pXK29.
digested with the same enzymes, thereby replacing the wild-type fragment by the corresponding DNA fragment with a new BamHI site to generate pXK29-BamHI. BamHI restriction sites at both 5’ and 3’ termini of the coding region of the TAP-tag (underlined sequences) were created by PCR using two oligonucleotides, 5’-GGATCCCTCAATGGGAA-AAGAGA-3’ and 5’-GGATCCGCGTCAACTCCCGGCGGCGG-3’, using pBS1479 plasmid containing TAP-tag (Rigaut et al., 1999). The resulting 560-bp fragment was digested with BamHI and was subsequently inserted at the BamHI site in pXK29-BamHI to generate pXK29-TAP. The orientation of the inserted TAP-tag sequence was determined by digesting the plasmid with HincII. Three HincII sites are present in pXK29-TAP: in the ycf4 gene and in the DNA fragment encoding TAP-tag as indicated in Supplemental Figure 1 online and in the multicloning site of the plasmid. Thus, pXK29-TAP with the TAP-tag sequence in right and opposite orientations provides three DNA fragments of 3067, 628, and 250 bp. The nucleotide sequence of PCR amplified fragments was determined using a 373 DNA Sequencer (Applied Biosystems).

Purification of Ycf4 Complexes by Affinity Column Chromatography

The TAP-tagging protocol used was essentially as described previously (Rigaut et al., 1999) with the following modifications. Thylakoids were isolated as described (Chua and Bennoun, 1975; Takahashi et al., 1991) and extrinsic proteins were removed by incubating the membranes (0.8 mg chlorophyll mL⁻¹) with 2 M NaBr on ice for 30 min. The extract was diluted sixfold with a solution of 5 mM HEPES-NaOH, pH 7.5, and 10 mM EDTA and centrifuged. The pelleted membranes were resuspended in this buffer to 0.8 mg chlorophyll mL⁻¹ and solubilized with 0.8% (w/v) DDM on ice for 30 min. After adding 150 mM NaCl to the extracts, insoluble materials were removed by pelleting the sample, and the resulting extracts (9 to 10 mL, fraction a in Figure 3A) were applied to 250 μL of rabbit IgG agarose (Sigma-Aldrich) equilibrated with the buffer (5 mM HEPES-NaOH, pH 8.0, and 150 mM NaCl, 0.02% DDM) in a polypropylene column (Bio-Rad). The column was rotated gently at 4°C overnight to keep the IgG agarose beads suspended, thus ensuring efficient adsorption of the sample. The column was subsequently washed with 40 mL IPP150 buffer (10 mM Tris-HCl, pH 8.0, and 150 mM NaCl, 0.02% DDM) and with 10 mL TEV cleavage buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5 mM EDTA, 1 mM DTT, and 0.02% DDM). After the TEV protease digestion (100 units mL⁻¹) at 18°C for 2 h, the eluate was collected (fraction d in Figure 3A) and was added to a final concentration of 3 mM. Subsequently, a tenth of the eluate volume (fraction d) was added and incubated on a Calmodulin affinity resin column (200 μL; Amersham Biosciences; Stratagene). Subsequently, the column was washed with 60 mL of calmodulin binding buffer. Finally, five separate fractions were eluted with EGTA-containing solution (g₁-g₅). The TAP-tag fused protein was eluted in the g₁ and g₅ fractions. The final preparation was concentrated via spin column (Microcon YM-100; Millipore) as needed. All buffers contained 0.02% DDM.

Purification of Ycf4 Complex from Wild-Type Cells

Ycf4-enriched fractions were separated from thylakoid extracts solubilized with DDM by sucrose density gradient centrifugation with an SW28 rotor (Beckman) at 141,000g for 24 h as described (Sugimoto and Takahashi, 2003) except that the gradient contained 100 mM NaCl. The Ycf4-enriched fractions were collected and diluted fourfold with 50 mM Tris-HCl, pH 8.0, and 0.05% DDM and applied onto an ion-exchange column (2.5 cm diameter × 5 cm height) containing DEAE Toyopearl 650S (Tosoh). Fractions were eluted using a linear NaCl gradient from 0 to 300 mM in buffer containing 50 mM Tris-HCl, pH 8.0, and 0.05% DDM.

Size Estimation of Ycf4 Complex

The purified Ycf4 complex and molecular marker proteins were separated by sucrose density gradient (0.1 to 1.3 M sucrose) centrifugation in an SW41Ti rotor (Beckman) at 200,000g for 14 h as described already (Takahashi et al., 2004) or by gel filtration column chromatography with Superose 6 HR 10/30 (Amersham Bioscience) using an FPLC system (Amersham Bioscience) as described by Sugimoto and Takahashi (2003). Molecular mass marker proteins were thyroglobulin (669 kD), apoferritin (443 kD), β-amylose (200 kD), alcoholdehydrogenase (150 kD), BSA (66 kD), and carbonic anhydrase (29 kD) (Sigma-Aldrich).

SDS-PAGE

SDS-PAGE was performed with 6 M urea in the resolving gel when detecting D1 (Takahashi et al., 1991) or without (Takahashi et al., 2004).

Antibodies

The anti-COP1 and -COP2 antibodies were gifts of Peter Hegemann (Humboldt-Universität zu Berlin). An anti-ASA1 antibody was generated against synthetic oligopeptide NKDXXLSYXAN conjugated with multiple antigenic peptides (Sigma-Aldrich) by immunization of rabbits.

Immunoblotting

Immunoblottings were performed as described previously (Hatano-Iwasaki et al., 2000), and signals were detected with a luminiscence image analyzer (LAS-4000 mini). For quantitative evaluation, signal intensity was analyzed with Multi Gauge ver.3.0 software (Fujifilm). When weak signals needed to be detected, CanGetSignal (TOYOBO) was used for the incubation of nitrocellulose filters with a primary antibody, and ECL Advance protein gel blotting detection kit (GE Healthcare) was used for the incubation of the filters with the secondary antibody.

Immunoprecipitation

Immunoprecipitation was performed with Dynabeads Protein G (Invitrogen) as described in the manufacturer’s manual. IgG was cross-linked with Dynabeads Protein G by dimethyl pimelimidate. Thylakoid membranes in 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 2 mM KH₂PO₄, pH 8.0, at 150 mM NaCl, 0.02% DDM and 0.02% DDM. After the TEV protease digestion (100 units mL⁻¹) at 18°C for 2 h, the eluate was collected (fraction d in Figure 3A) and was added to a final concentration of 3 mM. Subsequently, a tenth of the eluate volume (fraction d) was added and incubated on a Calmodulin affinity resin column (200 μL; Amersham Biosciences; Stratagene). Subsequently, the column was washed with 60 mL of calmodulin binding buffer. Finally, five separate fractions were eluted with EGTA-containing solution (g₁-g₅). The TAP-tag fused protein was eluted in the g₁ and g₅ fractions. The final preparation was concentrated via spin column (Microcon YM-100; Millipore) as needed. All buffers contained 0.02% DDM.

Identification of Polypeptides

N-terminal amino acid sequences were determined by a PSQ-1 protein sequencer (Shimadzu) as reported previously (Koike et al., 2007). Polypeptides were separated by SDS-PAGE and electroblotted onto a polyvinylidene difluoride filter. For MS of these polypeptides, gel bands were digested with trypsin, and analysis by MS was performed using an LCQ Deca XP or an LTQ ion trap mass spectrometer (ThermoFinnigan) as described by Stauber et al. (2003). In-solution digestion of the purified TAP-tagged Ycf4-complex was performed by incubating the complex with Lys-C (25 ng μL⁻¹) at 37°C for 16 h and subsequently with trypsin (30 ng μL⁻¹) at 37°C for 24 h. The resulting digests were purified by passing through a solid phase extraction tip (C-TIP; AMR) and were injected to an LTQ ion trap mass spectrometer.
Pulse-Chase Labeling of Total Cellular Proteins

Pulse-chase labeling experiments of total cellular proteins were performed as described by Ohnishi and Takahashi (2001) with some modifications. Cells grown to mid-log phase (4 × 10^6 cells mL^-1) in TAP medium containing reduced amount of sulfur were collected by centrifugation, resuspended at 25 μg chlorophyll mL^-1 in TAP medium containing no sulfur, and incubated for 2 h. Total cellular proteins were labeled for 5 min with [35S]Na$_2$SO$_4$ at 100 μCi mL^-1 (American Radiolabeled Chemicals) in light (100 μE m^-2 s^-1). Pulse-labeling was stopped by adding cycloheximide (10 μg mL^-1) and chloramphenicol (100 μg mL^-1). Chase of the labeled proteins was performed in the presence of 10 mM Na$_2$SO$_4$. The cells were broken by vigorous vortexing with glass beads, and the thylakoid membranes were purified by discontinuous sucrose gradient ultracentrifugation. The thylakoid membranes (0.8 mg chlorophyll mL^-1) were solubilized with 0.8% DDM, and the resulting extracts were fractionated by sucrose density gradient ultracentrifugation (0.1 to 1.3 M sucrose) in a Beckman TLS-55 rotor at 259,000g for 3 h. Labeled polypeptides were separated by SDS-PAGE and were radioluminographically detected using an imaging plate with a fluorescent image analyzer (FLA-7000; Fujiﬁlm). Labeled proteins were quantitatively estimated with MultiGauge (Fujiﬁlm).

Uniform labeling of total cellular proteins with $^{14}$C was performed using [14C]CH$_3$COONa (GE Healthcare) according to (Pierre et al., 1995) with some modiﬁcations. Cells were grown to mid-log phase (2 × 10^6 cells mL^-1) in HSM medium and then in HSM medium containing 2 μCi mL^-1 [14C]CH$_3$COONa in light (100 μE m^-2 s^-1) for 17 h.

Electron Microscopy and Single Particle Analysis

A dilution series of Ycf4 samples were applied to carbon-coated copper 300 mesh electron microscopy grids and negatively stained with 2% uranyl acetate. When the uniform distribution of protein complexes, observed as single particles, was achieved, imaging was performed at room temperature using a Philips-Fei T12 electron microscope, operating at 120 kV, as maintained by the Centre of Biomolecular Electron Microscopy, Imperial College London. Fourteen micrographs were recorded at 52,000 x and chosen to be scanned into the computing environment on the basis of minimum astigmatism and drift using a Nikon LS9000 Super Coolscan set to 6.35-μm step size. Subsequent calculation of the Fourier power spectra, on a per micrograph basis, showed that astigmatism and drift were indeed negligible and the first minimum consistently in the 17- to 18-A range. No correction was made for the contrast transfer function. Data sets were compiled using the automatic particle selection procedures of Boxer, a module of the EMAN software package (v1.7; Ludtke et al., 1999 and references therein). All subsequent image processing was performed using Imagic-5 (Image Science). Images were coarsened by a factor of two, resulting in 2.44 Å per pixel on the specimen scale. Reference-free alignment, followed by multivariate statistical analysis, allowed for initial two-dimensional class averages to be identified, and two main subpopulations were separated and treated de novo based on overall particle size. Iterative refinement on each subpopulation resulted in the final class averages shown (van Heel et al., 1996).

Accession Numbers

Sequence data from this article can be found in the Join Genome Institute or NCBI databases under the accession numbers listed in Tables 2 and 3.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Restriction Maps of the Chloroplast DNA Fragment Containing rps9-ycf4-ycf3 Genes in the Wild Type and of the TAP-tag ycf4 Genes.

Supplemental Figure 2. Fluorescence Induction Kinetics of Wild-Type and ycf4-TAP Cells.

Supplemental Figure 3. Growth of Wild-Type, Δycf4, and ycf4-TAP Cells.

Supplemental Figure 4. Polypeptides of the PSI-LHCI Supercomplex.

Supplemental Table 1. Peptides Detected by LC-MS/MS.

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REFERENCES


