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BRIEF COMMUNICATION

Isolation of the cyanobacterial YFP-tagged photosystem I using *GFP-Trap®*

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Abstract

A strain of *Synechocystis* sp. PCC 6803 expressing the yellow fluorescent protein (YFP) fused to the C-terminus of the PsaF subunit of PSI has been constructed and used to isolate native PSI complexes employing the *GFP-Trap®*, an efficient immunoprecipitation system which recognizes the green fluorescent protein (GFP) and its variants. The protein analysis and spectroscopic characterization of the preparation revealed an isolate of trimeric and monomeric PSI complexes, which showed minimal unspecific contamination as demonstrated by comparison with the wild type control. Interestingly, we detected CP43 subunits of PSII and small amounts of PSII core complexes specifically pulled-down with the YFP-PSI, supporting the association of PSII assembly modules and intermediate assembly complexes with PSI, as observed in our previous studies. The results demonstrate that the *GFP-Trap®* system represents an excellent tool for studies of PSI biogenesis and interconnection of PSI and PSII assembly processes.

Additional key words: assembly factor; pigment–protein complex; two-dimensional electrophoresis.

Photosystem I (PSI) is an integral membrane pigment–protein complex which utilizes light energy to mediate electron transport from plastocyanin to ferredoxin in the photosynthetic apparatus of algae, plants, and bacteria. Together with PSII and light-harvesting complexes it comprises a large proportion of the thylakoid membrane (TM). While in chloroplasts of algae and plants the PSI complex exists as a monomer associated with a membrane-bound light-harvesting complex, in most cyanobacterial species the dominant form of PSI is trimeric (Hladík and Šofrová 1991), although low levels of monomers, dimers, and in some species even tetramers can be detected (Watanabe *et al.* 2011). The first structural models of PSI with a resolution below 10 Å were obtained for trimeric PSI complexes isolated from thermophilic cyanobacteria thanks to their high stability in the isolated state (for review *see* Fromme *et al.* 2001). The study of Jordan *et al.* (2001) provides the most detailed molecular structure of trimeric PSI to date; with a resolution of 2.5 Å it revealed the presence of at least 12 different protein subunits and about 127 noncovalently bound cofactors per monomer including 96 chlorophylls (Chls), two phylloquinones, three iron-sulfur clusters, 22 carotenoids, four lipids, a putative Ca^{2+} as well as 201 water molecules. In comparison with the structure, the knowledge about PSI biogenesis is much less complete. The current view is mostly based on the study of PSI mutants in the green alga *Chlamydomonas reinhardtii* and involves the integration of the large Chl-binding subunit PsaB into the TM followed by integration of the second large subunit PsaA, which is dependent on the presence of PsaB. Together, the proteins are considered to form a bulky heterodimer which is assumed to serve as a platform for the assembly of the remaining subunits and cofactors (Wollman *et al.* 1999). This concept significantly differs from the concept of sequential PSII assembly, which assumes an initial formation of assembly modules including one large Chl-binding subunit, the small adjacent subunits, and the cofactors. These modules subsequently combine in a stepwise manner forming intermediate assembly complexes (Komenda *et al.* 2012b, Nickelsen and Rengstl 2013). This model was mostly obtained using mutants of the cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter, *Synechocystis* 6803) which allowed the isolation and

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Abbreviations: Chl – chlorophyll; CN – clear native; GFP – green fluorescent protein; PAGE – polyacrylamide gel electrophoresis; TM – thylakoid membrane; WT – wild type; YFP – yellow fluorescent protein.
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characterization of assembly modules and assembly intermediate complexes (Komenda *et al.* 2004). In this respect a comparably detailed study of PSI biogenesis in cyanobacteria is missing. In contrast to PSII, the PSI assembly process seems to be relatively fast as reports on detecting PSI intermediate assembly complexes are almost missing in the literature. One detailed study of a PSI assembly intermediate was performed by Ozawa *et al.* (2010) using *Chlamydomonas,* in which the complex was characterized as a monomer lacking several small subunits and a light-harvesting complex.

Recent identification of trimeric PSI as the main sink for newly synthesized Chl in *Synechocystis* 6803 led to a suggestion that the production of PSII assembly modules could occur in the absence of *de novo* Chl biosynthesis, using Chls released from trimeric PSI instead (Kopečná *et al.* 2012). Furthermore, PSII components like CP43 (Komenda *et al.* 2012a, Kopečná *et al.* 2015) and PSII assembly intermediates (Bečková *et al.* 2017) were identified in complexes with PSI. These results suggested a close relationship between the newly produced PSII complexes and PSI. To analyze this, a simple purification method for the isolation of pure PSI complexes is needed. Previously, cyanobacterial PSI complexes were isolated using sucrose-density gradient ultracentrifugation in combination with multiple ion exchange column chromatography steps (Witt *et al.* 1987, Fromme and Witt 1998). However, this approach is time-consuming and not effective enough concerning the purity and yield of the obtained PSI preparations. High yields can potentially be obtained using simple and fast methods such as Ni^{2+} affinity chromatography. This method is dependent on the construction of mutants that express an appropriate PSI subunit fused with a polyhistidine tag (His-tag). Several His-tagged subunits of PSI have been tested for their use in PSI purification in green algae as well as in cyanobacteria. Tagging of small transmembrane PsaK1 and PsaL subunits of *Synechocystis* 6803 PSI with the hexahistidine had adverse effects on PSI and the complex could not be purified in its active form (Tang and Chitnis 2000). On the other hand, active PSI complexes of *Chlamydomonas reinhardtii* have been isolated using His-tagged PsaA (Gulis *et al.* 2008) and the PsaF subunit has also been successfully used for PSI purification in *Thermosynechococcus elongatus* (Prodohl *et al.* 2004) as well as in *Synechocystis* 6803 (Kubota *et al.* 2010). Importantly, expression of the tag attached to the C-terminus of PsaF did not cause any adverse effects on the PSI complex (Kubota *et al.* 2010). Nevertheless, His-tag preparations of *Synechocystis* 6803 are characterized by significant unspecific protein binding (Boehm *et al.* 2009) and the study of the relationship between PSII biogenesis and PSI requires the introduction of a new, highly selective method for isolation of PSI.

In this study, we employed a *Synechocystis* 6803 strain expressing yellow fluorescent protein (YFP) fused to the C-terminus of the PsaF subunit of PSI which allowed us to isolate PSI complexes using the *GFP-Trap*® immunoprecipitation system which recognizes the green fluorescent protein (GFP) and its variants including YFP. Protein complexes attached to the fusion protein may be eluted either under acidic conditions to obtain native complexes or by hot SDS buffer for analysis of their subunit composition. The work presented here provides a simple and fast methodological approach for the isolation of pure PSI complexes under native conditions using low pH elution buffer.

To prepare the YFP-tagged PSI mutant (hereafter WT-YFP), the wild type (WT) cells were transformed with a pUC18:*psaF*-YFP-Cm^R plasmid. This vector was constructed by cloning the YFP-coding sequence to the 3´coding region of the *psaF* gene (*sll0819*) and the 5´coding region of the *psaJ* gene (*sml0008*). The chloramphenicol-resistance (Cm^R) located downstream of the *psaJ* gene was used as a selectable marker. Transformants were selected for chloramphenicol resistance, and PCR was used to show integration of YFP and elimination of the WT gene copies. The mutant and the WT did not differ in their cell absorption spectra (Fig. 1*A*). 77 K low temperature Chl fluorescence spectra revealed that the presence of the YFP-tag slightly affected the PSI/PSII ratio of the mutant cells (Fig. 1*B*). However, this had apparently no effect on the growth rate of the mutant as its duplication time was identical to that of WT (12.66 ± 0.63 h and 12.49 ± 0.76 h, respectively).

The WT-YFP and WT control cells were grown in conical flasks on a rotary shaker under moderate light conditions [40 μmol(photon) m^{-2} s^{-1}] at 30°C in liquid BG11 medium. Thylakoid membranes were isolated in buffer B (25 mM MES/NaOH, pH 6.5, 10 mM $CaCl_2$, 10 mM $MgCl_2$, 25% glycerol) by breaking the cells with zirkonia/silica beads using a *Mini-Beadbeater* (*BioSpec Products*, USA) as described in Komenda and Barber (1995). Chl concentration was measured in 100% methanol extracts according to Wellburn (1994). The immunoprecipitation procedure using the *GFP-Trap*® (*ChromoTek*, Germany) was basically done according to the producer´s protocol with some modifications as described here. TM suspension containing 75 μg of Chl *a* was solubilized with 1% (w/v) n-dodecyl-β-D-maltoside (DM) and then incubated with 25 μl of the equilibrated bead slurry (2 h, 10°C) on the spin column. Buffer B containing 0.04% DM was then used for the washing steps. The final elution was performed under native conditions, using 0.2 M glycine (pH 2.5) with 0.04% DM, and the eluate was immediately neutralized with 1 M Tris base.

Room temperature (RT) absorption and 77 K low temperature Chl fluorescence spectra were measured using a *Shimadzu UV-3000* spectrophotometer (*Shimadzu*, Japan) and an *Aminco Bowman Series 2* luminescence spectrometer (*Thermo Fisher Scientific*, USA), respectively.

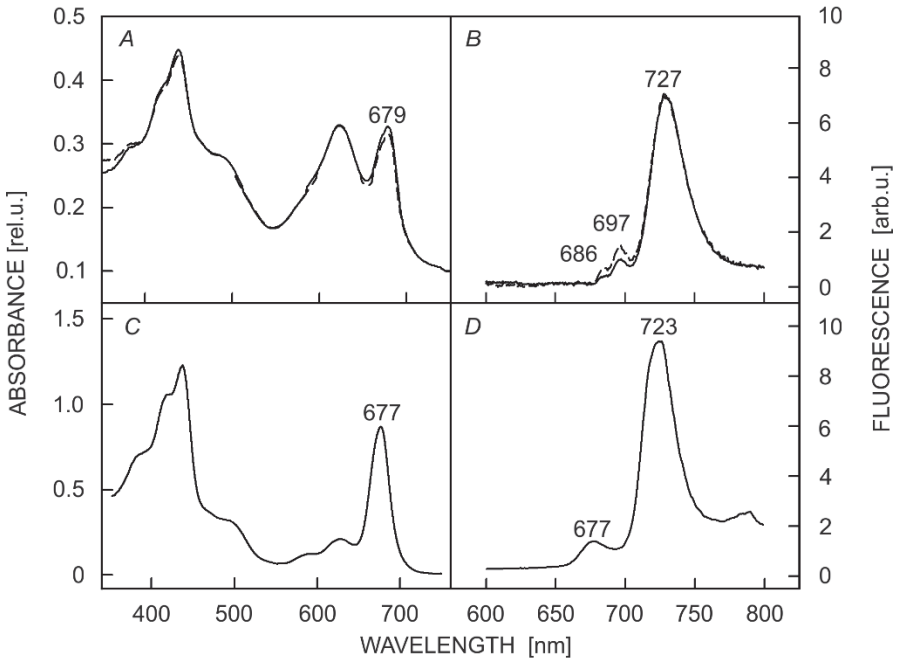

Fig. 1. Room temperature absorption (*A,C*) and 77 K low temperature chlorophyll fluorescence (*B,D*) spectra of the *Synechocystis* 6803 cells (*A,B*) of wild type (*solid line*) and mutant expressing PsaF-YFP (WT-YFP; *dashed line*), and of the preparation isolated using *GFP-Trap*® from the WT-YFP mutant (*C,D*). The numbers designate absorption and emission maxima in nm.

Protein complexes and their components were separated by two-dimensional polyacrylamide gel electrophoresis (PAGE) combining clear native (CN) electrophoresis in a 4–14% gradient gel with SDS-PAGE in a denaturing 16–20% gradient gel containing 7 M urea (Komenda *et al.* 2012a). Protein composition was also analyzed by one-dimensional SDS-PAGE using the same denaturing gels. Native gels were photographed and scanned for Chl fluorescence. SDS gels were stained by *SYPRO*® *Orange* (*Invitrogen*, USA) and scanned for fluorescence, then immunoblotted and probed with primary antibodies raised against the D1 and CP43 proteins (Knoppová *et al.* 2014). The presence of the YFP-tag was checked using an anti-GFP antibody (*Abcam*, UK). Alternatively, SDS gels were stained by *Coomassie Brilliant Blue G-250* (*Bio-Rad*, USA) and the identity of the stained PSI proteins designated in Fig. 2*B,C* was verified by mass spectrometry as described in Knoppová *et al.* (2014).

In order to verify the specificity of the WT-YFP preparation obtained using the *GFP-Trap*®, we applied the same isolation procedure to the solubilized membranes from the WT cells lacking the YFP-tagged proteins. While the WT isolate was colorless, the WT-YFP preparation was strongly pigmented. Measurement of its RT absorption spectra showed the presence of pigment–protein complexes containing carotenoids (broad absorption band with maxima at 450–490 nm) and Chl with red maximum at 677 nm (Fig. 1*C*), which is quite typical for the cyanobacterial PSI complexes (Knoppová *et al.* 2014). The 77 K Chl fluorescence spectrum of the WT-YFP preparation showed the main peak at 723 nm (Fig. 1*D*), again typical for cyanobacterial PSI complexes (Shen *et al.* 1993). Spectra did not show significant fluorescence emission at 685 and 695 nm typical for PSII complexes, nevertheless, there was a small peak at 677 nm, which most probably corresponded to a small number of Chl molecules released from PSI after the transient acidification during the elution.

The preparations were further analyzed by electrophoretic methods in order to evaluate the selectivity of the purification method and reveal possible contamination by other membrane complexes and proteins. The CN-PAGE showed the presence of large pigmented complexes with molecular sizes of about 700 and 250 kDa (Fig. 2*A*) corresponding well with the trimeric and monomeric forms of PSI complexes, respectively. Similar green complexes were resolved in the solubilized TM from the WT and WT-YFP strains (Fig. 2*A*), but strikingly, the membranes and especially the preparation from the WT-YFP showed a larger heterogeneity in the PSI complexes than the WT membranes. As expected, isolated PSI complexes showed only very weak RT Chl fluorescence despite their high Chl content (Fig. 2*B*, 1D fluor; compare to a free pigment zone, F.P.), similar to the RT fluorescence of much less abundant PSII Chl-binding antenna CP43 which was also detected in the preparation (Fig. 2*B*, arrow).

The analysis of the WT-YFP preparation using one-dimensional SDS-PAGE (Fig. 2*C*) as well as separation of protein subunits of complexes resolved by CN-PAGE in the second dimension (Fig. 2*B*) revealed the presence of

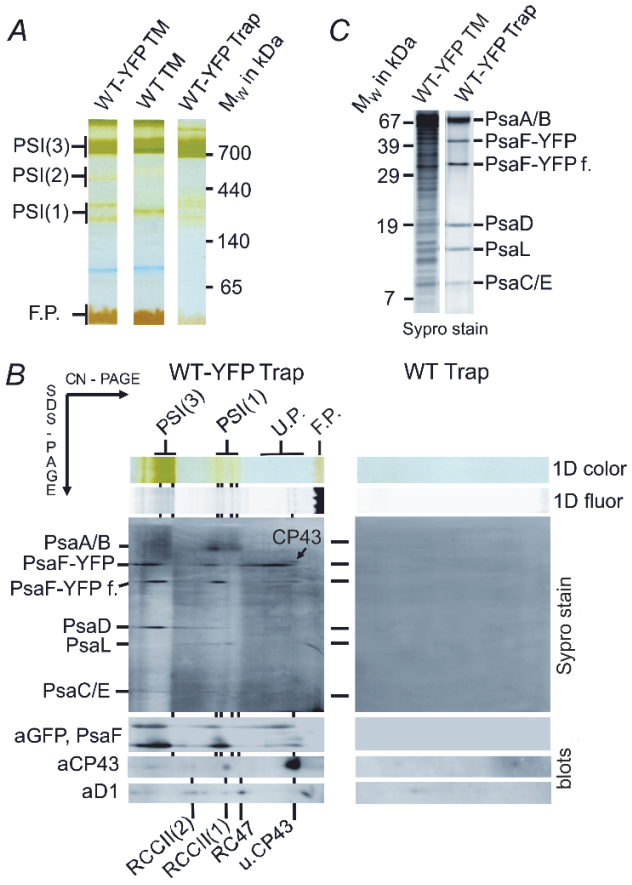

Fig. 2. Analysis of photosystem I complexes from the PsaF-YFP expressing mutant (WT-YFP) and the wild type control (WT) in thylakoid membranes (TM) and preparations isolated using *GFP-Trap*® (Trap). *A* – Separation of the complexes by clear native (CN) PAGE. *B* – Analysis of the preparations using two-dimensional PAGE. The 1st dimensional CN gels were photographed (1D color) and scanned for room temperature Chl fluorescence (1D fluor). The 2nd dimensional SDS gels were stained by *Sypro® Orange* (Sypro stain), blotted to a PVDF membrane, and D1, CP43, and YFP proteins were detected by the specific antibodies. PSI(3), PSI(2), and PSI(1) designate trimeric, dimeric, and monomeric PSI complexes, respectively; RCCII(2) and RCCII(1) designate dimeric and monomeric PSII core complexes; RC47 is the PSII core complex lacking CP43; u.CP43 is unassembled CP43; U.P. are unassembled proteins and F.P. free pigments; PsaF-YFP f. designates a folded form of the fusion protein; the arrow shows the stained band of unassembled CP43 in the 2D gel. 5 μg of chlorophyll were loaded for all samples with exception of WT Trap for which equivalent volume of isolate as for WT-YFP Trap was used. *C* – Analysis of the WT-YFP Trap preparation using one-dimensional SDS-PAGE. The separated proteins were visualized by *Sypro® Orange* (Sypro stain).

typical PSI subunits in all separated PSI forms. We confirmed the presence of the PsaA/PsaB pair, PsaC, PsaD, PsaL, and PsaE proteins in the observed PSI complexes by mass spectrometry. Interestingly, we also detected two proteins which were reactive with antibodies against GFP (Fig. 2*B,C*) and PsaF suggesting that they represent two distinct forms of PsaF-YFP differing in their electrophoretic mobility. Indeed, it was previously shown that GFP-tagged proteins move in the SDS gels in two forms: a smaller fluorescent one representing insufficiently denatured and still folded protein, and the larger fully denatured one (Aoki *et al.* 1996). Similar distinct forms of GFP-tagged proteins may originate from their partial denaturation by low pH (Enoki *et al.* 2006). Since we obtained the PSI-YFP preparation by elution using low pH buffer we assume that the observed two protein bands originated from the partial denaturation of a fraction of the PsaF-YFP protein by low pH. The protein denatured by low pH shows lower mobility, better corresponding to the theoretical molecular mass of the protein (Aoki *et al.* 1996) while the folded YFP (PsaF-YFP f.) shows a higher electrophoretic mobility than anticipated from its amino acid composition. Interestingly, only this folded form was detected in membranes from WT-YFP strain after their 2D gel analysis when the gel lane from the native gel was denatured by 2% SDS at room temperature. However, its level decreased reciprocally with appearance of the "larger" form, when the SDS treatment was done at increased temperature. As judged from the stained 2D gel (Fig. 2*B*), individual small subunits and folded or denatured PsaF-YFP were not present in all the individual PSI bands that were resolved in the CN gel. This variable content of these subunits is therefore most probably responsible for the size heterogeneity of PSI complexes observed in the membranes and especially in preparation from the WT-YFP strain (Fig. 2*A*). Importantly, the control, colorless preparation isolated from WT membranes lacked any visibly stained protein bands, confirming the high selectivity of the isolation method and the purity of the isolated PSI-YFP complexes (Fig. 2*B*, WT Trap).

To confirm the previously detected association of PSII components with PSI, we electroblotted the 2D SDS gels and immunodecorated the large PSII Chl-binding subunits CP43 and D1 in both preparations. The WT-YFP preparation contained immunodetectable CP43 (Fig. 2*B*, blots) which was detected mostly in the region of unassembled proteins and was also visible on the stained 2D gel (Fig. 2*B*, arrow). However, its amount was quite small in comparison with PSI subunits (its level was estimated to be below 5% of the level of stained PsaF-YFP in the preparation) and was not visible in the stained one-dimensional SDS gel (Fig. 2*C*). Its presence mostly in the region of unassembled proteins indicates that it detached from PSI complexes during CN-PAGE. A small fraction of CP43 was also found associated with complexes containing D1 and based on their sizes they represented monomeric and dimeric PSII core complexes (Fig. 2*B*). The D1 protein was additionally found in bands corresponding to the PSII core complexes lacking CP43. In contrast, the control WT preparation did not contain these PSII complexes and proteins. Thus, the results confirmed the specific binding of unassembled CP43 to PSI which was previously identified in the strain lacking the CP47

protein (Komenda *et al.* 2012a) or in the strain depleted in phosphatidyl glycerol with destabilized binding of CP43 within the PSII core complexes (Kopečná *et al.* 2015). The reason for the interaction of the newly synthesized CP43 with PSI is unknown but could be explained by the action of PSI as a scaffold protein providing Chl for the insertion into the nascent polypeptide chain of the synthesized CP43. Such a role for PSI could be further extended by the efficient energy transfer from CP43 to PSI which may protect the newly synthesized CP43 assembly module against photodamage (Komenda *et al.* 2012a). Similar scaffolding and protecting roles of PSI could also explain the formation of PSI–PSII supercomplexes (RCCS) recently identified in *Synechocystis* 6803 cells, especially under high light conditions (Bečková *et al.* 2017). Evidence for the formation of RCCS was also found in this study with the identification of PSII core complexes in the WT-YFP preparation. The previously identified RCCS consisted of a PSI trimer and two PSII monomers associated with Psb27 and Psb28 assembly factors (Komenda *et al.* 2012a, Bečková *et al.* 2017).

In conclusion, we have used the *GFP-Trap*® approach to isolate the pure YFP-tagged PSI complexes from *Synechocystis* 6803 cells. Compared to other isolation methods, this method is simple, fast and very selective, providing PSI preparation with a minimal level of unspecific protein contamination. This preparation is especially appropriate for study of PSI biogenesis, identification of PSI-associated assembly factors and interaction between PSI and PSII protein components. It could also be a tool for the study of other protein complexes in *Synechocystis* 6803 as long as successful fluorescent protein tagging is achieved.

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