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## **Involvement of Ycf3, Ycf4 and BtpA in photosystem I biogenesis of *Synechocystis* sp. PCC6803.**

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### **Introduction**

Membrane protein complexes are essential in many important biochemical processes. Unfortunately, their biogenesis is as yet hardly understood. We have chosen cyanobacterial photosystem I (PS I) as a model system to study the assembly of its -mainly hydrophobic - protein subunits and cofactors into a functional membrane protein complex. Cyanobacterial PS I comprises at least 11 protein subunits, 3 Fe<sub>4</sub>S<sub>4</sub> -clusters, 2 phylloquinones, 22 carotenoids, 4 lipids and 96 chlorophylls.

Several proteins, which seem to influence PS I biogenesis but are not part of the functional complex, have been described in the literature (for a review see (Schwabe and Kruip, 2000)). Our group focuses on three of these proteins, with the aim to understand their exact molecular function in the assembly process.

Ycf3 and Ycf4 are homologues of plastid-encoded proteins found in higher plants (ycf - hypothetical chloroplast open reading frame). Their importance for the assembly of functional PS I has been shown in tobacco (Ruf et al., 1997) and *Chlamydomonas* (Boudreau et al., 1997). Ycf3 (19.9 kDa) contains three TPR sequence motifs, corroborating the assumption that it is involved in protein-protein interaction (Lamb et al., 1995). Ycf4, a very hydrophobic protein with a molecular weight of 20.4 kDa shows no specific motifs.

Of BtpA (biogenesis of thylakoid proteins A, (Bartsevich and Pakrasi, 1997)), homologues have not been found in higher plants, but in other, non-photosynthetic bacteria as well as eukaryotes (e. g. the nematode *C. elegans*), implying a more general function for this protein than just the biogenesis of PS I or thylakoid proteins.

We present here our data on the characterization of the proteins themselves as well as on the studies of deletion strains in *Synechocystis* PCC6803.

### **Materials and methods**

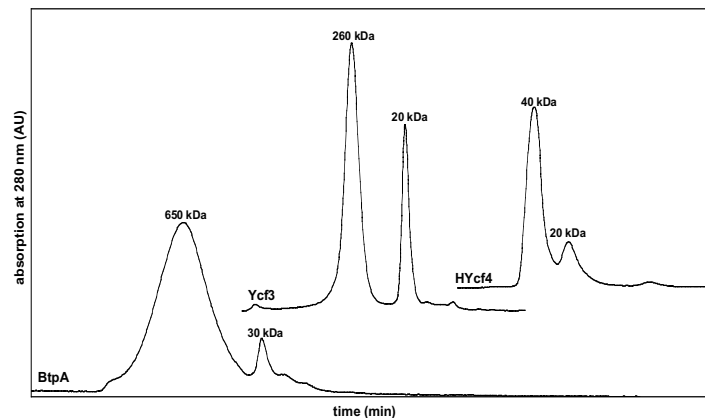
Recombinant protein was overexpressed in *E. coli* in a his-tagged (HYcf4) or native form (Ycf3, BtpA), purified (details to be published elsewhere) and characterized by size exclusion chromatography on a BioSilect 400 column. Deletion mutants were constructed from glucose-tolerant *Synechocystis* PCC6803 by complete replacement of the corresponding gene with a kanamycin resistance. Polyclonal antibodies raised in rabbit against his-tagged proteins were used to verify both purification and deletion mutants. Immunogold-localization studies were performed as described (Engels et al., 1997). *Synechocystis* was grown in BG-11 medium at 30 °C, 5 E (low light) or 50 E (normal light), supplemented with 10 mM glucose for photoheterotrophic growth if necessary. PS I was isolated from thylakoid membranes

according to (Wenk and Kruip, 2000). Low temperature emission spectra were obtained after excitation of whole cells at 435 nm using an Aminco Series 2 spectrometer.

## Results

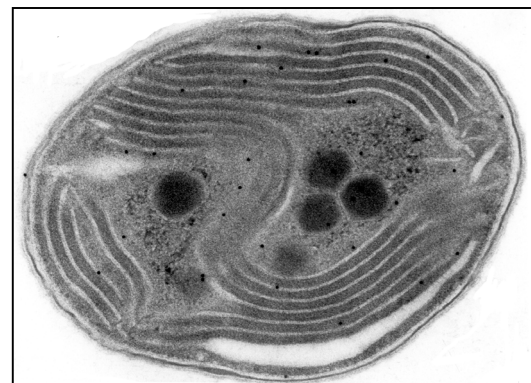
Of the three recombinantly expressed proteins, only Ycf3 could directly be recovered in soluble form. Both BtpA and HYcf4 had to be solubilized with chaotropic substances and refolded after purification. HYcf4 could only be kept soluble with the addition of detergents. Size exclusion chromatography (SEC) showed that the preparations were pure and that all three proteins exist in monomeric as well as in oligomeric forms.

**Fig. 1** Size exclusion chromatography of purified, recombinant proteins to determine purity of sample and oligomerization status. Samples were separated on a BioSilect 400 column, Gel Filtration Standard (BioRad) serving as molecular weight marker.



As can be seen in **Fig. 1**, HYcf4 forms dimers, whereas Ycf3 and BtpA show high molecular weight complexes corresponding to 260 kDa and 650 kDa respectively. The specific homo-oligomerization of Ycf3 and BtpA could also be verified in a yeast-2-hybrid system (data not shown).

**Fig. 2** Immunogold-localization of BtpA in *Synechocystis* sp. PCC6803. Dark dots indicating antibody-BtpA cross-reactions can clearly be seen to be localized on thylakoid membranes. Pre-Immune sera as negative controls showed no cross-reaction.



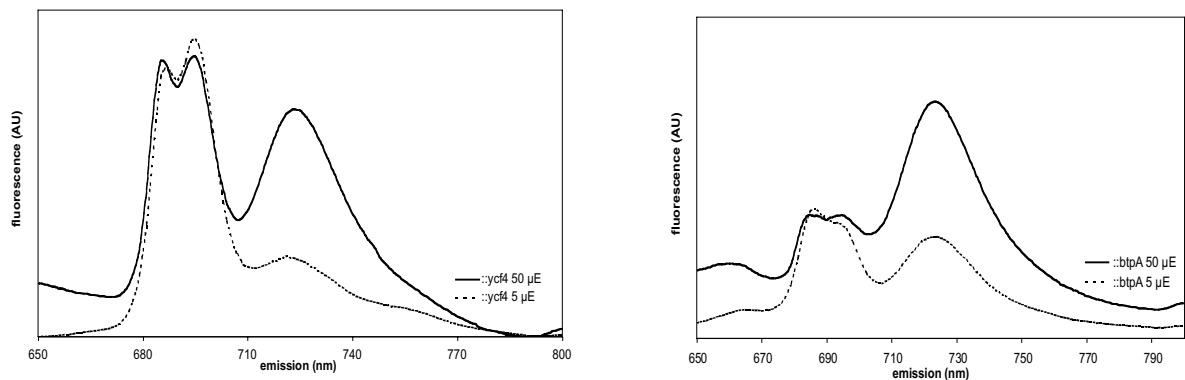
Immunogold-localization with polyclonal antibodies raised against the his-tagged proteins showed that all three proteins are located on the thylakoid membrane of *Synechocystis*. The result for BtpA-localization is shown here as a representative example (Fig. 2). Pre-Immune sera did not show any specific reactions against *Synechocystis* cells.

Before the deletion mutants were subjected to further analysis, complete segregation was established through PCR-analyses and southern blotting. As this was the case after a few rounds of streaking, one clone per transformation was selected for detailed phenotypic studies.

In order to ascertain whether these strains still contain assembled PS I, low temperature fluorescence spectra were recorded to assess the PS I to PS II ratio. A typical wildtype

spectrum would show a prominent PS I peak at 725 nm with two smaller PS II peaks at 685 nm and 695 nm, respectively.

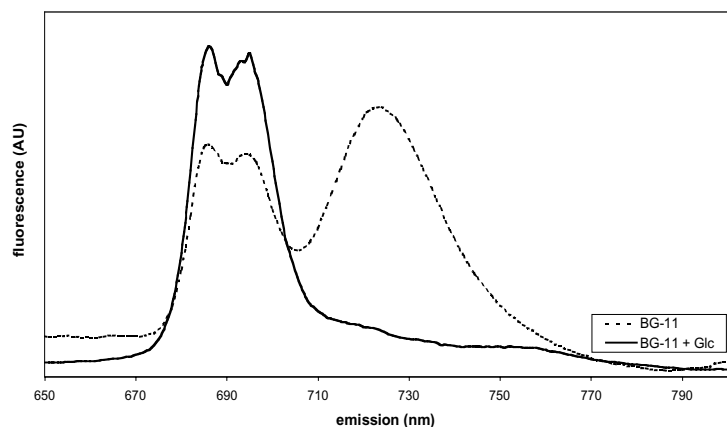
In deletion strains of *ycf3*, no PS I was detected with low temperature spectroscopy. These mutants are not able to grow photoautotrophically and have to be supplemented with glucose (data not shown). Deletion of *ycf4* as well as that of *btpA* causes a severe decrease in PS I content under normal growth conditions. However, both strains grow photoautotrophically. To further analyze these deletion mutants, we subjected them to either low (5 E) or normal (50 E) growth light (Fig. 3). Whereas the wildtype did not show significant changes in PS I content under these light conditions, both mutants did: under low light conditions, these strains reduce their PS I levels quite drastically to less than 30 % compared to the corresponding content under normal growth light conditions.



**Fig. 3** Low temperature fluorescence spectra after excitation at 435 nm, showing the PS I to PS II ratio. Wildtype cells show no change of PS I or PS II levels in response to low light (data not shown). Cells were grown at 30°C under either low light (5 E) or normal growth light (50 E). Spectra were normalized to the peak at 690 nm.

*Ycf4* deletion mutants grown on glucose as a carbon source refrain from assembling photosystem I. When the cells are suspended in a glucose-free medium, they restart PS I synthesis. The PS I complex can then be detected with fluorescence spectroscopy (**Fig. 4**) as well as isolated from thylakoid membranes.

**Fig. 4** 77K fluorescence spectra after excitation of whole cells at 435 nm, showing the PS I to PS II ratio in the *ycf4* deletion strain. WT cells show no change of PS I or PS II levels in photoautotrophic as compared to photoheterotrophic growth. (data not shown).



The PS I still assembled by the *ycf4* and *btpA* deletion mutants is functional, as can be inferred from their ability to grow without organic carbon source and from light-induced P700 reduction kinetics (data not shown). A detailed analysis of purified PSI-complexes from the

mutants did not show differences regarding subunit composition or oligomerization status in comparison to the wild type.

## Discussion

Here, we have presented an initial characterization of Ycf3, Ycf4 and BtpA. They all are thylakoid membrane-associated proteins, which do not or only transiently associate with functional PS I. Ycf3 and BtpA show specific homo-oligomerization both as isolated proteins and in a yeast-2-hybrid system. The latter analysis still has to be carried out for Ycf4, which is very hydrophobic and thus not very susceptible to experiments of this kind. Deletion of any of these proteins is not lethal for *Synechocystis*. Without Ycf3, however, only photoheterotrophic growth is possible and no PS I can be assembled. It can be concluded that to date, Ycf3 is the only essential protein for PS I biogenesis in cyanobacteria.

Studies by Rochaix et al. have shown that in *Chlamydomonas*, both Ycf3 and Ycf4 are essential for stable accumulation of the PS I complex (Boudreau et al., 1997). At least for Ycf4, the situation is different in *Synechocystis*. In this model organism, Ycf4 and BtpA have clearly a regulatory or stabilizing function, which is modulated by environmental factors such as light or nutrient availability.

Further studies will involve analysis of mRNA levels for PS I subunits and stability of the PS I complex in the deletion strains of *ycf4* and *btpA*. The hunt for interaction partners using antibody-affinity is well under way. In addition, a proteomics project to analyze changes in protein makeup of the deletion mutants has been initiated.

## Acknowledgments

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