S31-005

Functional studies on the small Cab-like proteins of *Synechocystis* sp. PCC 6803

L Hao[1], K Schmidt[2], H Paulsen[2] and C Funk[1]

[1]*Department of Biochemistry and Biophysics, Arrhenius Laboratories for Natural Sciences, Stockholm University, S-106 91 Stockholm, Sweden. Phone: +46-8-16 4392, Fax: +46-8-15 3679, E-mail:* Limin.Hao@dbb.su.se, *Christiane.Funk@dbb.su.se*

[2]*Institut für Allgemeine Botanik, Johannes Gutenberg Universität, Müllerweg 6, D-55099 Mainz, Germany, Phone: +49-6131-3924633, Fax: +49-6131-3923787 E-mail: krschmid@mail.uni-mainz.de,* paulsen@mail.uni-mainz.de

Keywords: cyanobacteria, HLIP, antenna, Cab-protein, chlorophyll

Introduction

The two photosystems in plants and cyanobacteria together catalyze oxygenic photosynthesis. Each of the photosystems consists of a reaction center, where light-induced charge separation takes place, and an antenna system that feeds excitation energy into the reaction center complex. For efficient and rapid energy transfer the light absorbing chlorophyll molecules in these organisms are bound to proteins. The biosynthesis of pigment-binding proteins is a sensitive process as on the one hand free chlorophyll can adopt potentially harmful triplet states and on the other hand pigment-binding proteins that are not stabilized by chlorophyll are degraded immediately.

Although the reaction centers are remarkably conserved throughout evolution, different types of organisms have adopted and retained different antenna pigment-protein complexes. In higher plants, the main peripheral antenna is the chlorophyll *a*/*b* binding light harvesting complex (LHC) (Jansson, 1994), which is encoded by a multi-gene family (*cab* gene family) with at least 10 different genes located in the nucleus. They are related to each other in terms of sequence similarity and assumed to share a common evolutionary origin. Most of the family members code for proteins with three transmembrane helices (TMH), the sequences of the first and third membrane-spanning regions being highly conserved. The crystal structure of LHCII, the main light harvesting protein of photosystem II, revealed that the first and the third TMH are crossing each other in the membrane and together bind most of the pigments (Kühlbrandt et al., 1994). The finding of early light induced proteins (Elips), which also have three TMHs and the PsbS protein, which has four TMHs expanded the *cab* gene family (Green and Pichersky, 1994). These proteins have been found to bind chlorophyll and play a different role from light harvesting (Funk et al., 1995a,b, Adamska et al., 1999). More distant relatives to the *cab* gene family are the

fucoxanthin-chlorophyll *a*/*c* binding proteins of the chromophytic algae (Green and Pichersky, 1994). It has been proposed for the evolution of the modern three-helix chlorophyll *a*/*b* and chlorophyll *a*/*c* light harvesting antenna proteins to originate from a four-helix protein, e.g PsbS, which itself evolved from one- and two-helix ancestors. Indeed, one-helix proteins (OHPs) and two-helix proteins (SEPs, stress enhanced proteins) have been found in the genome of *Arabidopsis thaliana* (Jansson et al., 2000, Heddad and Adamska, 2000).

In contrast to higher plants, the cyanobacterium *Synechocystis* sp. PCC 6803 lacks chlorophyll *b* as well as multi-helix CAB proteins, the major peripheral light-harvesting complex is the phycobilisome. Therefore it was very surprising when in the genome of *Synechocystis* 6803 five genes were identified that were homologous to the Cab-gene family (Funk and Vermaas, 1999). Four of these five genes are 174-213 bp in length and code for small proteins predicted to be similar to the first or the third TMH of the CAB proteins. However, the fifth sequence belongs to the C-terminal end of the ferrochelatase. The five gene products have been named small Cab-like proteins (SCPs) and their corresponding genes *scp*s. They were found to be expressed preferentially in the absence of photosystem I, but their transcription was not significantly enhanced at high light intensity (250 µE m^{-2} s^{-1}, Funk and Vermaas, 1999). However, at very high light (500 µE m^{-2} s^{-1}) the levels of their protein products were elevated (He et al., 2001).

The strongly conserved amino acid residues that are found in the SCPs as well as in the pigment binding CAB proteins suggest that the SCPs are capable of binding pigments. Localization studies using peptide directed antibodies recognized ScpD only in the thylakoid membrane. Here, we report our attempt of *in vitro* pigment reconstitution of these SCPs. To receive a large amount of the desired proteins, we overexpressed the proteins in *E.coli*, pigments were extracted from pea and *Synechocystis* 6803. With the help of *in vitro* reconstitution, we currently investigate whether the SCPs really are involved in pigment binding and are indeed a new family of antenna proteins in cyanobacteria.

Materials and Methods

Separation of outer membrane, plasma membrane and thylakoid membrane

Outer membranes, plasma membranes and thylakoid membranes were separated in a highly pure state by combining density centrifugation with aqueous polymer two-phase partitioning (Norling et al., 1998, Zak et al., 1999). The membranes were analysed on high-resolution or normal SDS-PAGE (Schägger and von Jagow, 1987; Laemmli, 1970) and analysed via immunoblotting (Towbin et al., 1979).

Gene cloning and overexpression in E.coli

The small Cab-like proteins were cloned into TOPO plasmid (In vitrogen Co.) and subcloned into expression vectors PT7-7 or pBADA/His. The overexpression was performed as in Paulsen et al. (1993).

Pigment isolation

Pigments were isolated from pea (*Pisum sativum*) leaves according to Paulsen et al. (1990). Chlorophyll content was estimated as described in Porra et al. (1989). Total cyanobacterial pigments were isolated from *Synechocystis* sp. PCC 6803 cells, that were pelleted and then disrupted by french press passages and further purified as mentioned above. Pigments were dried under nitrogen and stored in aliquots at –20°C.

In vitro pigment reconstitution

In vitro reconstitution experiments based on freeze-thaw cycles and detergent exchange were performed according to Paulsen et al. (1990 and 1993). Reconstitution experiments based on rapid mixing of equal volumes of pigment and protein solutions were as described by Booth and Paulsen, (1996, variatons to be published elsewhere).

Results and Discussion

Using a peptide-directed antibody against the N-termini of ScpC and ScpD (Funk and Vermaas, 1999) we wanted to identify the localization of these small Cab-like proteins in the cyanobacterial cell. As the small Cab-like proteins are highly expressed in PSI-less mutants (Funk and Vermaas, 1999) a PSI-less/*chlL*$^-$ mutant (Wu and Vermaas, 1995), which can synthesize chlorophyll only in light, was grown under light activated heterotrophic conditions (Anderson and McIntosh, 1991) for 2 weeks and then exposed to light (10 μmol m^{-2} s^{-1}). From the "etiolated" mutant as well as from samples taken during various "greening" time points plasma membranes and thylakoid membranes were isolated (Norling et al., 1998, Zak et al., 1999) and separated on SDS-PAGE. Fig. 1 shows an immunoblot performed with antibodies directed against NrtA and ScpC/ScpD. It can be seen that ScpC and ScpD are strictly localized in the thylakoid membrane, whereas NrtA, a protein involved in nitrate and nitrite transport over the plasma membrane (Kobayashi et al., 1997), indicates the purity of the preparation. However, inside the thylakoid membrane ScpC and D do not seem to be localized close to either photosystem I or II as was indicated after blue native PAGE run on wild type and mutant strains (not shown). Further localisation studies, involving also the other SCPs, are in progress.

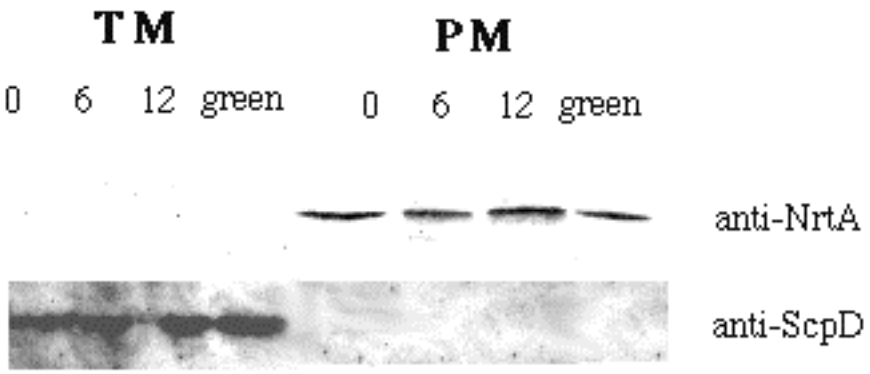

Fig. 1: Localisation of ScpC and ScpD. Separation of thylakoid (TM) and plasma membranes (PM) of the PSI-less/*chl*L$^-$ mutant was performed via aqueous polymer two phase partition. The mutant was grown at light activated heterotrophic conditions for 2 weeks (0 hours) and then illuminated. Immunoblots were performed on samples taken upon different illumination intervals (6 hours, 12 hours) and on cells grown in light (green).

Sequence comparison as well as primary results strongly suggest that the small Cab-like proteins are able to bind pigments (Funk and Vermaas, 1999). However, so far it is not clear whether a single one-helix protein can coordinate chlorophyll or if homo- and/or hetero-oligomers are necessary to form multi-helix pigment-protein complexes as seen in the Cab-antenna proteins of higher plants (Kühlbrandt et al., 1994). We therefore overexpressed the SCPs in *E.coli* and purified the proteins from inclusion bodies. Pigments were isolated from pea and *Synechocystis* 6803 as mentioned in materials and methods. In a first approach only single proteins and homo-oligomers were analysed. The SCPs one by one were reconstituted using the standard method

(Paulsen et al., 1990), but also the detergent exchange method (Paulsen et al., 1993) and the rapid mixing method (Booth and Paulsen, 1996). All these methods reconstituted LHCII successful (not shown), however, no pigment binding could be observed on the SCPs as judged from native gels and CD spectroscopy (not shown).
Currently we are trying to reconstitute heterooligomers of the SCPs with pigments. As a second approach we are also overexpressing these proteins directly in *Synechocystis* 6803 under the control of the strong *psb*A promotor. In this way we may be able to directly isolate the SCPs in a pigment-binding state by using an epitope tag.

Acknowledgements

The authors would like to thank Dr. B. Norling for the help with the membrane isolation and Dr. Omata for the NrtA antibody. Financial support from the Swedish Research Council and the Carl Tryggers Stiftelse is greatly acknowledged.

References

Adamska I, Roobol-Boza M, Lindahl M, Andersson B (1999) *European Journal of Biochemistry* **260**, 453-460.

Anderson SL, McIntosh L (1991) *Journal of Bacteriology* **173**, 2761-2767.

Booth PJ, Paulsen H (1996) *Biochemistry* **35**, 5103-5108.

Dolganov NA, Bhaya B, Grossman AR (1995) *Proceedings of the National Academy of Sciences USA* **92**, 636-640.

Funk C, Vermaas W (1999) *Biochemistry* **38**, 9397-9404.

Funk C, Adamska I, Green BR, Renger G, Andersson B (1995a) *The Journal of Biological Chemistry* **270**, 30141-30147.

Funk C, Schröder W.P, Napiwotzki A, Tjus SE, Renger G, Andersson B (1995b) *Biochemistry* **34**, 11133-11141.

Green BR, Pichersky E (1994) *Photosynthesis Research* **39**, 149-162.

He Q, Dolganov NA, Björkman O, Grossman AR (2001) *The Journal of Biological Chemistry* **276**, 306-314.

Heddad M, Adamska I (2000) *Proceedings of the National Academy of Sciences USA* **97**, 3741-3746.

Jansson S, Andersson J, Kim SJ, Jackowski G (2000) *Plant Molecular Biology* **42**, 345-351.

Jansson S (1994) *Biochimica et Biophysica Acta* **1184**, 1-19.

Kobayashi M, Rodriguez R, Lara C, Omata T (1997) *The Journal of Biological Chemistry* **272**, 27197-27201.

Kühlbrandt W, Wang D, Yoshinori F (1994) *Nature* **367**, 614-621.

Laemmli UK (1970) *Nature* **227**, 680-685.

Norling B, Zak E, Andersson B, Pakrasi H (1998) *FEBS Letters* **436**, 189-192.

Paulsen H, Rümler U, Rüdiger W (1990) *Planta* **181**, 204-211.

Paulsen H, Finkenzeller B, Kühlein, N (1993) *European Journal of Biochemistry* **215**, 809-816.

Porra RJ, Thompson WA, Kriedemann PE (1989) *Biochimica et Biophysica Acta* **975**, 384-394.

Schägger H, von Jagow G (1987) *Analytical Biochemistry* **166**, 368-379.

Towbin H, Staehelin T, Gordon J (1979) *Proceedings of the National Academy of Sciences USA* **76**, 4350-4354.

Wu Q, Vermaas W (1995) *Plant Molecular Biology* **29**, 933-945.

Zak E, Norling B, Andersson B, Pakrasi H (1999) *European Journal of Biochemistry* **261**, 311-316.