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NOVEL CO_2 REGULATED PROTEINS IN *SYNECHOCYSTIS* PCC 6803

by

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LIST OF ORIGINAL PUBLICATIONS

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II. **Dalton Carmel, Natalia Battchikova, Maija Holmström, Paula Mulo and Eva-Mari Aro** (2011) Knock-out of low CO_2-induced *slr0006* gene in *Synechocystis* PCC 6803: Consequences on growth and proteome. Proceedings of the 15th International Congress on Photosynthesis, Beijing, China (in press).

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ABBREVIATIONS

Bis-Tris	Bis(2-hydroxyethyl)-amino-tris(hydroxymethyl)-methane
CA	Carbonic anhydrase
CCM	Carbon concentrating mechanism
CHAPS	3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate
C_i	Inorganic carbon
DBMIB	2, 5-dibromo-3-methyl-6-isopropyl-p-benzoquinone
DMBQ	2,6-dimethyl-*p*-benzoquinone
DCMU	3-(3',4'-dichlorophenyl)-1,1 dimethyl urea
DCBQ	2,6-dichloro-*p*-benzoquinone
DIGE	Differential in gel electrophoresis
DM	n-dodecyl-β-D-maltoside
DNAse	Deoxyribonuclease
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ESI	Electro spray ionization
ETC	Electron transfer chain
FMN	Flavin mononucleotide
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IEF	Isoelectric focusing
iTRAQ	Isobaric tag for relative and absolute quantification
kDa	Kilo Dalton
Km^r	Kanamycin resistant cassette
MALDI-TOF	Matrix assisted laser desorption/ionization-time of flight
MS	Mass spectrometry
NDH-1	Type-1 NADP(H) dehydrogenase
OG	n-octyl-β-D-glucopyranoside
PAGE	Polyacrylamide gel electrophoresis
PBS	Phycobilisome
PCC	Pasteur Culture Collection
PCR	Polymerase chain reaction
PDB	Protein data bank
PSI	Photosystem I
PSII	Photosystem II
RNAse	Ribonuclease
RuBisCO	Ribulose-1,5 bisphosphate carboxylase/oxygenase
SDS	Sodium dodecyl sulfate
Sp^r	Spectinomycin resistant cassette
TCA	Trichloro acetic acid
TES	2-[[1,3-dihydroxy-2-(hydroxymethyl)propan-2-yl]amino]ethanesulfonic acid
WT	Wild type

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ABSTRACT

The large biodiversity of cyanobacteria together with the increasing genomics and proteomics metadata provide novel information for finding new commercially valuable metabolites. With the advent of global warming, there is growing interest in the processes that results in efficient CO_2 capture through the use of photosynthetic microorganisms such as cyanobacteria. This requires a detailed knowledge of how cyanobacteria respond to the ambient CO_2. My study was aimed at understanding the changes in the protein profile of the model organism, *Synechocystis* PCC 6803 towards the varying CO_2 level. In order to achieve this goal I have employed modern proteomics tools such as iTRAQ and DIGE, recombinant DNA techniques to construct different mutants in cyanobacteria and biophysical methods to study the photosynthetic properties. The proteomics study revealed several novel proteins, apart from the well characterized proteins involved in carbon concentrating mechanisms (CCMs), that were upregulated upon shift of the cells from high CO_2 concentration (3%) to that in air level (0.039%). The unknown proteins, Slr0006 and flavodiiron proteins (FDPs) Sll0217-Flv4 and Sll0219-Flv2, were selected for further characterization. Although *slr0006* was substantially upregulated under C_i limiting conditions, inactivation of the gene did not result in any visual phenotype under various environmental conditions indicating that this protein is not essential for cell survival. However, quantitative proteomics showed the induction of novel plasmid and chromosome encoded proteins in Δ*slr0006* under air level CO_2 conditions. The expression of the *slr0006* gene was found to be strictly dependent on active photosynthetic electron transfer. Slr0006 contains conserved dsRNA binding domain that belongs to the Sua5/YrdC/YciO protein family. Structural modelling of Slr0006 showed an α/β twisted open-sheet structure and a positively charged cavity, indicating a possible binding site for RNA. The 3D model and the co-localization of Slr0006 with ribosomal subunits suggest that it might play a role in translation or ribosome biogenesis. On the other hand, deletions in the *sll0217-sll218-sll0219* operon resulted in enhanced photodamage of PSII and distorted energy transfer from phycobilisome (PBS) to PSII, suggesting a dynamic photoprotection role of the operon. Constructed homology models also suggest efficient electron transfer in heterodimeric Flv2/Flv4, apparently involved in PSII photoprotection. Both Slr0006 and FDPs exhibited several common features, including negative regulation by NdhR and ambiguous cellular localization when subjected to different concentrations of divalent ions. This strong association with the membranes remained undisturbed even in the presence of detergent or high salt. My finding brings ample information on three novel proteins and their functions towards carbon limitation. Nevertheless, many pathways and related proteins remain unexplored. The comprehensive understanding of the acclimation processes in cyanobacteria towards varying environmental CO_2 levels will help to uncover adaptive mechanisms in other organisms, including higher plants.

1. INTRODUCTION

1.1. Cyanobacteria – a general overview

The process of photosynthesis is one of the most important photochemical reactions on Earth that has led to the development of advanced life forms. Although the early evidence dates back to Archaean Eon (3.9-2.5 Gya), the evolution of photosynthesis has remained a great Precambrian puzzle. Based on the consistent inference, it is generally agreed that prehistoric photosynthetic activity was carried out by anoxygenic photosynthetic bacteria using reductants such as H_2, H_2S or ferrous iron (Olson 2006). Later due to evolutionary pressure, cyanobacteria - the architect of the early Earth's atmosphere carried out oxygenic photosynthesis by splitting water, generating ATP and reducing $NADP^+$, which in turn was used for reduction of CO_2 (Awramik 1992). The success of cyanobacteria has been suggested to be due not only to the advantage of oxygenic photosynthesis, but also to inhibit competitors by a potentially toxic agent O_2 (Xiong and Bauer 2002).

Cyanobacteria are considered to be resilient predecessors of all higher oxygenic phototrophs (McFadden 1999). They form diverse groups of self-sustaining communities, exhibiting versatile physiology and wide ecological tolerance that contribute to their competitive success over a wide range of environments, starting from various niches in Antarctic glaciers all the way to the Sahara desert (Rippka 1988). It is estimated that a significant fraction of global primary productivity is contributed by cyanobacteria (Price 2011). Morphologically, cyanobacteria include miscellaneous groups of solitary and colonial unicellular and filamentous species (Knoll 2008). Cyanobacterial cells divide either by binary fission or by multiple fission (Rippka et al. 1979). Besides, cells are able to differentiate either terminally or non-terminally: heterocyst-N_2 fixing cells (Kim et al. 2011; Cohen et al. 2006), hormogonia (motile multi cell filament) or akinates-resting cells (Singh and Montgomery 2011).

Cyanobacteria are prokaryotic organisms which perform oxygenic photosynthesis very similar to that of higher plants. Thus, they serve as a model system to resolve biological questions that are difficult to approach in higher plants. Many cyanobacterial strains are capable of DNA mediated natural transformation. Thus, not only questions associated with the photosynthesis, carbon fixation or light-regulated gene expression, but also those associated with, for example, the cell differentiation and resistance to environmental factors or stress may be easier to address with the power of molecular genetics in cyanobacteria (Cohen and Gurevitz 2006).

Synechocystis sp. PCC 6803 (hereafter referred as *Synechocystis*), a fresh-water unicellular and non-nitrogen fixing cyanobacterium was used in this present study. *Synechocystis* can grow either autotrophically or heterotrophically in the presence of glucose (Rippka et al. 1979). Each *Synechocystis* cells contain 7-10 copies of circular chromosomes and 7 plasmids. The genome of *Synechocystis* was the first photosynthetic microorganism to be sequenced (Kaneko et al. 1995).

1.2. Carbon Concentrating Mechanisms (CCMs)

The CO_2 level of the ancient atmosphere was probably over 100-fold higher than today (Kaufman and Xiao 2003). This, together with low O_2 conditions, indicates that the primitive cyanobacteria would not have needed a CCM to achieve effective photosynthesis (Badger and Price 2003). The geological records have shown the presence of low levels of CO_2 and high levels of O_2 in the Neoproterozoic era (600–950 Ma) compared to Paleoproterozoic era (2.1–2.5 Ga). These conditions which limited CO_2 resources for photosynthesis would have triggered a refined system for carbon concentration (Riding 2006; Janhunen et al. 2007). This subsequently would have provided a competitive survival advantage under C_i limitation, allowing both CO_2 and HCO_3^- species to be exploited. Today, CCMs occur in all the cyanobacteria examined, in most of the algae, embryophyta including anthocerotes, lycopsids, pteropsids and coniferopsids, and in a significant number of flowering plants (Raven et al. 2008).

CO_2 diffuses slowly in aqueous solutions, thereby limiting its availability (Lapointe et al. 2008) (Riebesell et al. 1993). Two important factors for photosynthesis in aquatic organisms are the availability of dissolved inorganic carbon and the kinetics of the primary carbon-fixing enzyme, Ribulose-1, 5 Bisphosphate Carboxylase/Oxygenase (RuBisCO). Although the CO_2 and O_2 compete for the active sites in RuBisCO, the CCM dramatically reduces photorespiratory costs by depressing oxygenase activity, thereby providing a competitive advantage with respect to efficiency of photosynthesis (Price et al. 1998). The main function of cyanobacterial CCMs is an active transport of CO_2 and HCO_3^- species, resulting in accumulation of HCO_3^- in the cell. This results in as high as a 1000-fold higher intracellular HCO_3^- concentration as compared to the external environment. Carbonic anhydrase generates elevated CO_2 levels from HCO_3^- within carboxysomes.

1.2.1. Major modes of C_i uptake

Generally, CCMs in cyanobacteria involve six functional elements: (1) Passive or energized entry of dissolved inorganic carbon (CO_2 and HCO_3^-) (2) Increase in HCO_3^- concentration in the cytosol (3) entry in carboxysome (4) saturation of CO_2 near RuBisCO (5) fixation of CO_2 (6) prevention of CO_2 leakage from the carboxysome and the cell (Kaplan and Reinhold 1999). Five different C_i uptake systems have been identified in cyanobacteria. Three of them are involved in the transport of bicarbonate and are located in the plasma membrane, while two others are localized in the thylakoid membrane and are involved in CO_2 uptake.

1.2.1.1. Bicarbonate Transporters

Growth of many species of cyanobacteria under conditions of low CO_2 concentration results in the induction of bicarbonate uptake systems. Concentration of C_i in the cells is an energy demanding process and consequently tightly regulated (Bloye et al. 1992). Previous studies have shown that HCO_3^- transport involves phosphorylation in several proteins by the serine-threonine protein kinase in *Synechococcus* PCC 7942 and

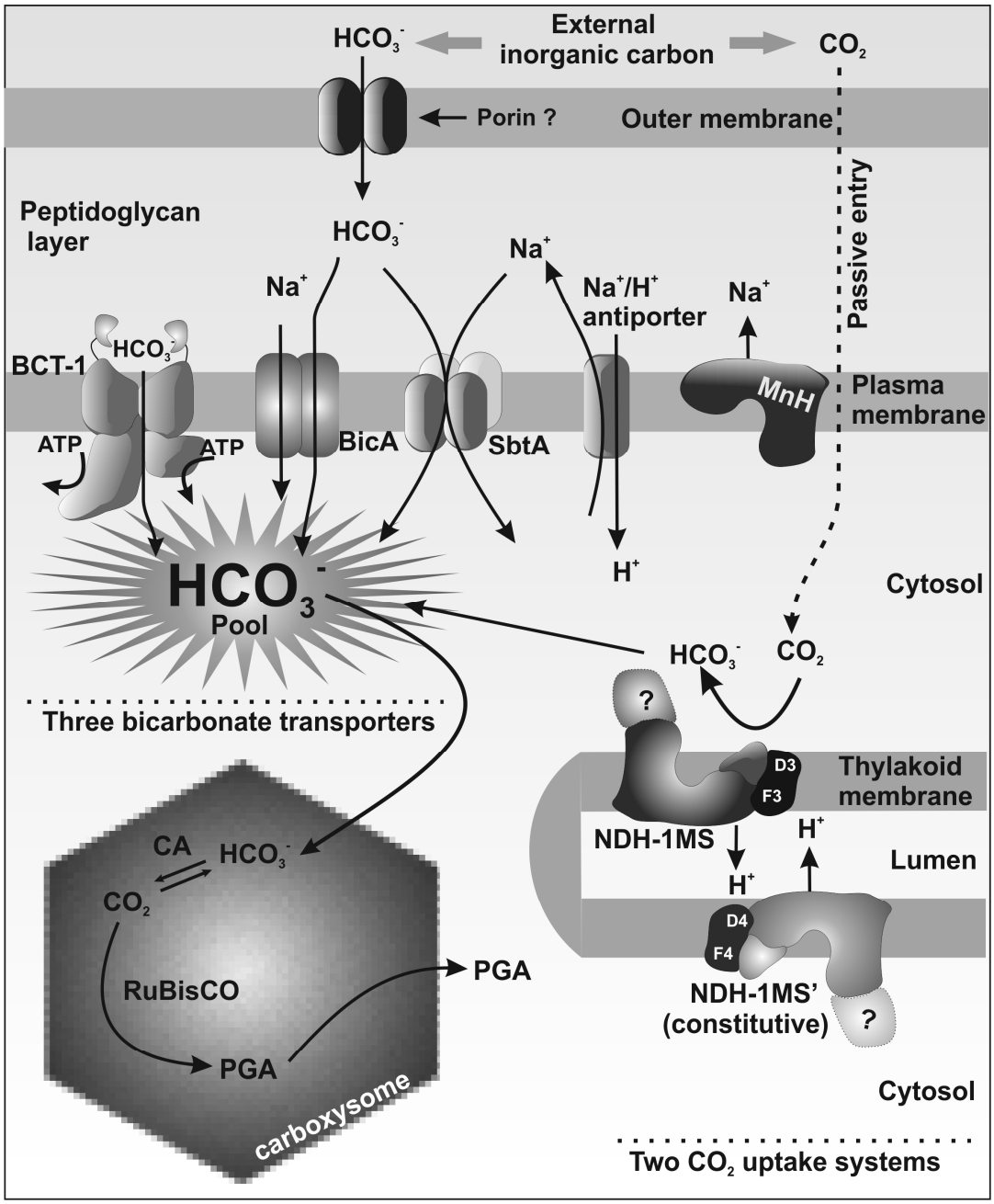

Figure 1: Major components of carbon concentrating mechanisms showing three distinct bicarbonate transporters (BCT1, BicA and SbtA) in the plasma membrane, inducible (NDH-1MS) and constitutively (NDH-1MS') expressed NDH-1 complexes in the thylakoid membrane and carboxysomes in the cytosol. The Mnh complex and the Na^+/H^+ antiporter located in the plasma membrane, were also induced by low C_i and presumed to maintain internal pH. (PGA- 3-phosphoglyceric acid; CA-carbonic anhydrase).

Synechococcus PCC 7002 under severe C_i limitation. Several transporters involved in the bicarbonate uptake in cyanobacteria have been well characterized (Sultemeyer et al. 1998).

The high affinity bicarbonate transporter (BCT1) (Omata et al. 1999) belongs to the ATP binding cassette (ABC) transporter family (Higgins 2001). BCT1 is encoded by the *cmpABCD* operon, which is highly expressed under C_i limitation (McGinn et al. 2003; Woodger et al. 2003; Wang et al. 2004). It is a complex of four different protein subunits, and is the only cyanobacterial example of uniporter for HCO_3^-. The CmpA is a lipoprotein bound to the plasma membrane, where it binds HCO_3^-. CmpB, a dimeric hydrophobic membrane protein probably forms a transport path through the membrane. The CmpC and CmpD subunits contain ATP binding sites and are located on the cytoplasmic face of CmpB (Price 2011; Omata et al. 2002). Comparative analysis of the known cyanobacterial genomes has indicated that BCT1 is only present in β-cyanobacteria (refer section 2.3.2 for classification) (Badger et al. 2006).

The sodium-dependent bicarbonate transporter (SbtA) is a single subunit type transporter that was originally identified in *Synechocystis* (Shibata et al. 2002). Recent studies have shown that the amount of *sbtA* is dramatically increased under C_i limiting conditions (Zhang et al. 2004). *sbtB*, a small gene downstream of *sbtA* in *Synechocystis*, is co-expressed with *sbtA* under C_i limitation-which suggests that the products have similar functions (Wang et al. 2004). Homologues of *sbtA* have been identified in many β-cyanobacteria (Badger et al. 2002; Badger et al. 2006; Badger and Price 2003) but they are absent from species such as *Thermosynechococcus, Gloeobacter violaceus*, and *Trichodesmium erythraeum*. To date, the SbtA proteins from *Synechocystis* (Shibata et al. 2002) and *Synechococcus* PCC 7002 (Price et al. 2004) are the only forms of β-cyanobacterial SbtA that have been confirmed by both genetic and physiological analysis.

SbtA from *Synechocystis* forms a tetrameric protein complex with an apparent molecular mass of around 160 kDa. SbtA possesses ten membrane spanning domains and two centrally located hydrophilic regions that probably face the cytoplasm (Price et al. 2011). It is a Na^+/HCO_3^- symporter driven by the electrochemical sodium gradient (Price et al. 2011). It is presumed that the dependence of HCO_3^- transport on the sodium ions might have significance in the maintenance of internal pH, by working together with Na^+/H^+ antiporter, during CO_2 fixation (Ogawa and Kaplan 2003). The rapid induction of HCO_3^- transport is also dependent on Na^+ (Amoroso et al. 2003), suggesting that SbtA could be a target of this activation response.

The BicA transporter is another Na^+-dependent HCO_3^- transporter, first identified in the marine cyanobacterium *Synechococcus* PCC 7002 (Price et al. 2004). BicA belongs to a large family of eukaryotic and prokaryotic transporters often described as sulphate transporters in many bacteria (the SulP family), and BicA homologues are widespread in both α- and β-cyanobacteria. Recently, these transporters were also identified and characterized from *Synechococcus* WH 8102 and *Synechocystis* (Xu et al. 2008). In *Synechococcus* PCC 7002, BicA expression is highly inducible under condition of C_i limitation, but it also appears to be expressed at low levels in cells grown at high CO_2 concentrations. However, in *Synechocystis* the BicA gene appears to be constitutively

expressed (Wang et al. 2004). The protein contains a domain designated STAS (sulphate transporter anti-sigma factor-like domain). The STAS domain of SulP transporters is known to be a regulatory domain in some mammalian homologues (Ko et al. 2004). BicA is potentially an important component of C_i uptake in many oceanic species of cyanobacteria.

1.2.1.2. CO_2 uptake systems

Cyanobacteria take up CO_2 as one of the forms of inorganic carbon. Although it is widely accepted that CO_2 enters the cyanobacterial cell by passive diffusion, some studies have shown that water channels such as aquaporin, that are present in the plasma membranes, assist in the entry of CO_2 (Tchernov et al. 2001). Previous studies provide clue that NDH-1 complex play role in conversion of CO_2 to bicarbonate and transport across thylakoid membrane but the exact mechanism remains unclear.

In cyanobacteria, the CO_2 uptake is dependent on two variants of NDH-1 complexes. The proton-pumping NDH-1 is a multisubunit complex in cyanobacteria that functionally resembles respiratory Complex I of the mitochondria and of many bacteria (Birungi et al. 2010). Several variants of NDH-1 may exist in a single cyanobacterial cell. The NDH-1M subcomplex, forms the core module which is present in all variants of the NDH-1 complexes. It is composed of fourteen subunits (Ndh-ABCEGHIJKLMNOS). In addition to the core module, various forms of NDH-1 contain different representatives of NdhD and NdhF families (Battchikova et al. 2011**;** Battchikova et al. 2007). The number of NdhD and NdhF family members varies in cyanobacteria (Battchikova et al. 2005). For example, *Synechocystis* encodes six NdhD (D1-D6) and three NdhF (F1, F3 and F4) proteins (Battchikova et al. 2010). In addition to NDH-1M subunits, the NDH-1L complex, contains NdhD1 and NdhF1 as well as two recently found subunits NdhP and NdhQ. The NDH-1L' complex is similar to NDH-1L except the fact that it has NdhD2 instead of NdhD1. NDH-1MS is composed of NDH-1M together with subunits NdhD3, NdhF3, CupA and CupS whereas NDH-1MS' is likely to include NDH-1M along with subunits NdhD4, NdhF4 and CupB.

NDH-1 variants perform different functions. Studies with different *ndhD* and *ndhF* mutants showed that NDH-1MS and NDH-1MS' are the two NDH-1 variants involved in CO_2 uptake (Shibata et al. 2001). Subunits CupA, CupS and CupB functionally associated with these two distinct NDH-1 complexes are presumed to have a role in the conversion of CO_2 to HCO_3^-, despite they don't show homology to any of the presently known carbonic anhydrases. NDH-1MS is inducible under low CO_2 condition whereas NDH-1MS' is constitutively expressed (Zhang et al. 2004). High-affinity CO_2 uptake involving NDH-1MS is dependent on linear electron transport (Maeda et al. 2002). However, the detailed mechanism how these complexes function is still unclear and the activity module for cyanobacterial NDH-1 remains unknown.

1.2.2. Carboxysomes

Carboxysomes, the proteinacious polyhedral compartments of cyanobacteria, are located in the cytoplasm. The carboxysome encapsulates two important enzymes, carbonic anhydrase (CA) and RuBisCO. Carboxysomes are found in all cyanobacteria and in some chemoautotrophic bacteria (Yeates et al. 2008). Bicarbonate that is accumulated in the cell is used in the generation of elevated CO_2 levels within carboxysomes (Yeates et al. 2011). Carboxysomes have been categorized into two types on the basis of the RuBisCO ortholog they contain and their shell protein composition (Tabita et al. 2008). α-carboxysomes are found in chemoautotrophs and in some marine cyanobacteria. The shell proteins of carboxysomes are encoded by *cso* operons of similar organization. By contrast, β-carboxysomes, which are found mainly in freshwater cyanobacteria, are encoded by *ccm* genes distributed throughout the genome (Price et al. 1998; Kerfeld et al. 2010; Shively et al. 1998). The composition of the protein shell has been most extensively studied in α-carboxysomes from chemoautotrophic proteobacteria such as *Thiobacillus*. It shows the presence of three proteins CsoS1, peptide A and peptide B. Homologues of these proteins have been identified in β-cyanobacteria which include CcmK, CcmL and CcmO. *Synechocystis* has two additional *ccmK* genes and a *ccmO* gene completely separate from *ccmKLMN* cluster (Cannon et al. 2001). Apart from these polypeptides, the carboxysome shell also appears to contain two polypeptides that have no homology to each other. In α-carboxysomes there is the CsoS2 (80-90 kDa) and the CsoS3 (55-65 kDa), while in β-carboxysomes these appear to be replaced by CcmM (55-70 kDa) and CcmN (26 kDa) (Price et al. 1998).

For CO_2 fixation to occur in β-carboxysomes, it is thought that HCO_3^- diffuses through the proteinaceous shell of the carboxysome where the carbonic anhydrase inside the structure acts to catalyze the formation of CO_2 from HCO_3^- at rates high enough to saturate the carboxylation reaction of RuBisCO (Reinhold et al. 1992). Carboxysome shells are also postulated to provide a shield against the CO_2 leakage. When cyanobacteria are subjected to C_i limitation (typically 20-50 ppm CO_2), they have the ability to induce a greatly enhanced level of CCM activity. This change is accompanied by increase in RuBisCO activity (Price et al. 1992) and carboxysome shell proteins (McKay et al. 1993), and results in the increased affinity for CO_2 and HCO_3^- uptake (Kaplan et al. 1994).

1.2.2.1. Carbonic anhydrase

The enzyme carbonic anhydrase catalyzes the inter-conversion of CO_2 to bicarbonate. In cyanobacteria, for the cytosolic HCO_3^- pool to be available for fixation by RuBisCO, it must be converted to CO_2. This reaction is catalyzed by the β-CA present inside the carboxysomes. Over recent years, carbonic anhydrase (CA) proteins and related genes have been identified in a wide range of Archaea, bacteria, eubacteria, algae, plants and animals (Kupriyanova and Pronina 2011). Four distinct classes of CA enzymes have been identified and termed as α-, β-, γ- and δ-type CAs (Tripp et al. 2001). Both α-CA and β-CA have been reported to exist in many cyanobacteria. All carbonic anhydrases are metalloenzymes which bind a single zinc ion at active sites despite the structural differences. The binding of zinc is very crucial for the enzyme activity (Cox et al. 2000). In

Synechocystis, the *icfA* gene (also known as *ccaA,* the gene for carboxysomal carbonic anhydrase protein A) encodes a functional β-CA that contains conserved residues (Cys39, Asp41, His98 and Cys101). These amino acids are involved in the coordination of Zn^{2+} at the active site (Mitsuhashi et al. 2000). Mutations in *icfA* resulted in impaired utilization of internal C_i and greatly reduced cell growth at air levels of CO_2 (even in the presence of glucose). These findings indicate the importance of carboxysomal CA for efficient operation of the CCM in cyanobacteria. Carboxysomal β-CA has been characterized at the gene and protein levels from *Synechococcus* PCC 7942 and *Synechocystis* and they have been shown to have common ancestry with prokaryotic β-CAs (Fukuzawa et al. 1992; Yu et al. 1992; So and Espie 1998).

The *Synechocystis* genome shows a putative homologue of α-CA, which is presumed to be present in the periplasm with no established functional role (So et al. 1998). The observation that CO_2 can be taken up and delivered to the cytosol as HCO_3^- has led to speculation about the potential involvement of a CA-like activity in this process (Badger 2003). In most of the cyanobacteria, the CO_2 uptake activity is associated with NDH-1 complex in most of the cyanobacteria. The CupA / CupB (Shibata et al. 2001) or ChpX / ChpY (Maeda et al. 2002) proteins associated with the NDH-1 complex are involved in light-dependent CO_2 hydration and may be responsible for the CA-like activity. The available evidence indicates that irrespective of the substrate, HCO_3^- is the species that accumulates within the cytoplasm. The advantage of accumulating HCO_3^- is that, being an ionic form of C_i, its ability to permeate to the plasma membrane is 1000-fold less than that of the uncharged CO_2 molecule (Price and Badger 1989). The cyanobacterial genome sequences do not appear to code for a CA that is expressed in the cytosol which explains how the wasteful dissipation of this HCO_3^- pool is prevented (Badger et al. 2002).

1.2.2.2. RuBisCO

In cyanobacteria, the enzyme RuBisCO (Ribulose1, 5-bisphosphate carboxylase oxygenase) located mainly in the carboxysome, catalyzes the first step of CO_2 fixation in the Calvin-Benson cycle. The active site of all RuBisCO proteins is formed by the interaction of two monomeric catalytic subunits, with the C-terminus of one monomer binding to the N-terminus of a second monomer in a specific manner to form two active sites per dimer (Bracher et al. 2011). Distinct residues from both monomeric units make up the active site, which is required for both carboxylation of CO_2 and the fixation of molecular oxygen, with the two gaseous substrates clearly competing for the same active site. The relative rate of carboxylation and oxygenation of RuBP define the protein's catalytic efficiency or ability to provide a cell with the carbon required. There are four known forms or types of RuBisCO found in nature: Form I, II, III, and IV (Tabita et al. 2008). The classification is based on the sequence, the arrangement of genes in the genome, and the subunit composition. Of the four forms, form I is the most abundant and is found in both eukaryotes and bacteria. This form of RuBisCO is composed of eight large subunits and eight small subunits (L8S8).

The phylogentic tree of RuBisCO derived from various photosynthetic bacteria has shown that cyanobacterial species either contain form 1A or form 1B type of RuBisCO

(Delwiche 1999). Based on the type of RuBisCO, the cyanobacteria are classified as either α-cyanobacteria with form 1A RuBisCO (eg. *Prochlorococcus marinus* MEO4) or β-cyanobacteria with form 1B RuBisCO (e.g. *Synechocystis*) (Badger and Price 2003). Form IB enzymes have been sub-classified into IB (eukaryotes and green algae) and IBc to differentiate the form IBc in cyanobacteria which is associated with carboxysomes (Badger et al. 2002; Badger and Bek 2008).

1.3. Signaling and Regulation of CCM

Relatively little is known about signaling mechanisms through which cyanobacterial sense the fluctuations in CO_2 / HCO_3^- levels. However, several possibilities for mechanism of sensing the C_i limitation have been suggested by recent studies, including (1) changes in the redox states in the photosynthetic electron transport chain (Kaplan et al. 2004) (2) photorespiratory metabolite based mechanism (Huege et al. 2011) and (3) direct sensing of the depleted internal C_i pool (Price et al. 2005). In the latter case, cells might sense the Ci pool decline by HCO_3^- binding sensory protein. Recent studies have shown that HCO_3^- stimulates signaling proteins, soluble adenylate cyclases (sACs), which are involved in cAMP-mediated signal transduction (Zippin et al. 2001; Raven 2006). *Synechocystis* encodes two sACs, Sll1161 and Slr1991. It was observed that HCO_3^- regulation by AC is conserved in cyanobacteria (Hammer et al. 2006).

In proteobacteria, clustered genes coding for enzymes of the Calvin-Benson cycle are transcribed under the control of a repressor gene called *cbbR* (Kusian and Bowien 1997; Vichivanives et al. 2000; Schell 1993). CbbR belongs to the LysR family of transcriptional regulators, in which a DNA-binding domain is connected to a sensor domain that is often involved in the perception of stress conditions (Schell 1993). *Synechocystis* has three homologues of the proteobacterial *cbbR*-like genes: *sll0998* (RbcR), *slr1594* (NdhR or CcmR) and *sll0030* (CmpR). Reverse genetics studies have shown that *sll0998* is essential for cell survival, and it mainly involves the regulation of *rbcLS* operon, whereas *sll0030* and *slr1594* are dispensable and not involved in regulating the Calvin-Benson cycle enzymes – but instead have an essential role in regulating C_i responsive genes (Figge et al. 2001).

Several other regulatory genes are also known to be involved in gene regulation during C_i limitation, including *slr1214* (Rre15), a two-component response regulator (Wang et al. 2004), sigma factors *sll0184* (SigC) (Tuominen et al. 2008), *sll0359* and *sll0822* (cyAbrB- like proteins) (Lieman-Hurwitz et al. 2009), IcfG (Beuf et al. 1994) and PII (Garcia-Dominguez and Florencio 1997). However, further studies are needed to reveal the detailed mechanisms involved in the signaling process during C_i limiting conditions.

2. AIMS OF THE STUDY

Efficient biofuel production (eg. biohydrogen) in bioreactors requires detailed knowledge about cyanobacterial physiology including responses to fluctuating CO_2 concentration. The effects of CO_2 concentration and the efficiency of CCM on the growth and development of cyanobacteria are crucial global questions since cyanobacteria are the most important primary producers in oceans and heavily contribute for the total global biomass production. The CO_2 concentrating mechanisms in cyanobacteria are well characterized nowadays due to the fact that cyanobacteria are the potential candidates for solving primary energy problems that we are facing today. However, some other mechanisms that are induced by low CO_2 induced and related proteins have received only minor attractions.

The aim of my work was

1. To reveal the changes in the protein profile of *Synechocystis* PCC 6803 during low CO_2 acclimation.
2. To identify novel CO_2 responsive proteins.
3. To characterize the low CO_2 induced protein Slr0006.
4. To characterize the low CO_2 induced proteins Flv4-Flv2.

3. MATERIALS AND METHODS

3.1. Synechocystis strains and construction of mutants

The *Synechocystis* PCC 6803 glucose tolerant strain (wild type-"WT") and several specific inactivation strains, especially mutants lacking subunits of NDH1-MS, NDH-1MS' and bicarbonate transporter, were used in this study. Moreover the deletion mutants of *slr0006*, *flv2* and *flv4* were constructed in order to understand the functional role of these proteins.

Strains	Function of deleted protein	References
Δ*ndhD3::Km*^{r}	NDH-1MS	(Ohkawa et al. 2000)
Δ*ndhD4::Sp*^{r}	NDH-1MS'	(Ohkawa et al. 2000)
Δ*ndhD3*::*Km*^{r} / Δ*ndhD4*::*Sp*^{r}	NDH-1MS and NDH-1MS'	(Ohkawa et al. 2000)
Δ*cupA::Km*^{r}	NDH-1MS	(Ohkawa et al. 2000)
Δ*cupA::Km*^{r} / Δ*cupB::Cm*^{r}	NDH-1MS'	(Shibata et al. 2001)
Δ*ndhD1*::Km^r / Δ*ndhD2*::*Cm*^{r}	NDH-1L and NDH-1L'	(Ohkawa et al. 2000)
Δ*sbtA::Km*^{r}	Bicarbonate transporter	(Shibata et al. 2002)
Δ*ndhR::Sp*^{r}	Transcriptional regulator	(Wang et al. 2004)
Δ*ndhB::Km*^{r} (M55)	NDH-1	(Ogawa 1991)
Δ*slr0006::Km*^{r}	unknown protein	Present study (Paper II)
Δ*sll0217::Km*^{r} (*flv4*)	unknown protein	Present study (Paper V)
Δ*sll0219:: Sp*^{r} (*flv2*)	unknown protein	Present study (Paper V)
Δ*sll0218-19::Hyg*^{r}	unknown protein	Present study (Paper V)

Glucose-tolerant *Synechocystis* was used to generate deletion mutation in the *slr0006*, s*ll0219*, and s*ll0217* genes by replacement of part of the target gene with an antibiotic resistant cassette. The upstream and downstream genomic regions of the target genes were amplified by PCR, followed by restriction enzyme digestion. The digested fragments were cloned into the pUC18 vector together with kanamycin from pUC4Kan vector (Taylor, Rose 1988) or the spectinomycin resistance cassette from pHP45 (Prentki and Krisch 1984) instead of deleted region. The plasmid with the direct orientation of the antibiotic cassette was used to transform *Synechocystis* cells as described by Zang et al. (2007). The complete segregation of the mutant strain was verified using PCR with gene specific primers.

The primers and the length of the amplicon are discussed extensively in the respective articles (Paper II & Paper IV)

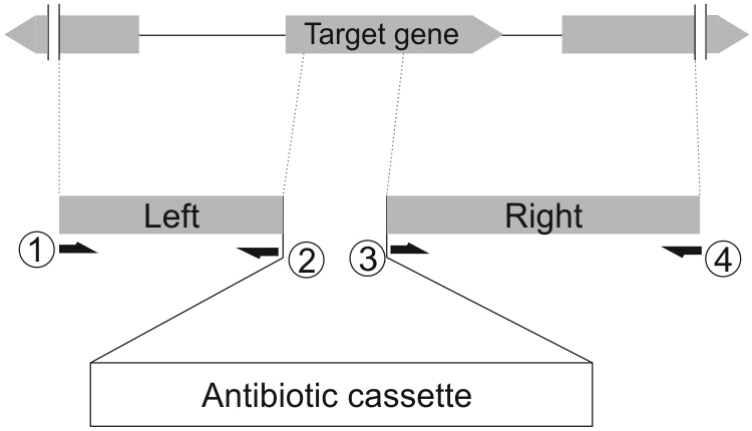

Figure 2: Deletion in the target genes was introduced by cloning the upstream sequence (amplified using primers 1 and 2) and downstream sequences (amplified using primers 3 and 4) in pUC18 vector. Antibiotic cassette digested with the same restriction enzyme as that of in primer 2 and 3, was introduced in between the cloned fragments in the vector.

3.2. Growth conditions

Synechocystis WT and mutant strains were grown in BG-11 medium (Rippka 1988) at 30°C under continuous illumination of 50 µmol photons m^{-2} s^{-1} and shaking at 110 rpm. The medium was buffered either with 20 mM HEPES pH 7.5 or 20 mM TES pH 8.3. For high CO_2 conditions, cells were grown in chambers filled with 3% CO_2. For low CO_2 cells were grown in air level CO_2 (0.039 %) and Na_2CO_3 was omitted in the BG-11. The standard growth conditions were modified in certain experiments as described in detail in the respective papers. For mutants, antibiotics were added in the following concentrations: kanamycin - 50 µg/µL, spectinomycin - 25 µg/µL, chloramphenicol - 5 µg/µL and hygromycin - 10 µg/µL. For high CO_2 to low CO_2 shift experiments, the cells were grown in 3% CO_2 until OD_{750} = 0.8-1.0, centrifuged and resuspended in fresh BG-11 medium lacking Na_2CO_3. After re-suspending the cells, they were grown under low CO_2 conditions for either 24 h or 72 h, depending on the experimental setup. For some experiments, the photosynthetic electron chain inhibitors, DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea) or DBMIB (2, 5-dibromo-3-methyl-6-isopropyl-p-benzoquinone) were added in fresh BG-11 medium during the shift from high CO_2 to low CO_2 and the cells were incubated for 24 h. 5 mM ascorbate was supplemented along with 25 µM DBMIB. For dark treatment, the flask was covered with aluminum foil.

3.3. RNA isolation and RT-qPCR analysis

Total RNA of *Synechocystis* cells was isolated from 10 ml culture by the Trizol method according to McGinn et al. (2003) followed by the DNAse treatment (Ambion Turbo DNase kit,USA). cDNA was synthesized using the Bio-Rad iScript cDNA Synthesis kit (Bio-Rad Laboratories) as described in Zhang et al. (2007). The primers (400-500 bases) used for RT-qPCR analysis are described in our previous study (Zhang et al. 2009) except for primers specific for the *sll0218* (5'- CTCTCAA CCAATGTGGATTCG-3' and 5'-CCAGACTGACGAATTTGATGG-3'). The primer pairs were tested *in silico* using the Amplify software (B. Engels, 2004, University of Wisconsin) and were compared using BLAST against the *Synechocystis* genome. The RT-qPCR was performed with an optimized annealing temperature in Bio-Rad IQ5 system using iQ SYBR Green Supermix as previously described (Sicora et al. 2006). Normalization was performed for each sample based on the expression level of *rnpB*. Melting curve analysis was always performed to ensure the specificity of the products.

3.4. Protein analysis

3.4.1. Isolation of proteins

For western blot analysis, *Synechocystis* cells were washed with 50 mM HEPES pH 7.5 and resuspended in lysis buffer containing 50 mM HEPES pH 7.5, 800 mM sorbitol, and 1 mM ε-amino-*n*-caproic acid (ACA). Routinely, the isolation procedure was carried out in the presence of 30 mM $CaCl_2$ (Gombos et al. 1994). When indicated, the isolation was performed in the presence of different concentrations (0 mM, 1 mM, 10 mM or 25 mM) of $CaCl_2$ or $MgCl_2$ in the lysis buffer. In addition, NaCl (5 mM and 50 mM) was added to the lysis buffer in the studies of FDPs. The cells were broken with glass beads (150-212 microns; Sigma) with six repeated cycles of 1 min vortexing and 1 min cooling in ice water bath. The unbroken cells and glass beads were removed (500 g, 5 min, 4ºC), and cell extracts were centrifuged (30000 *g*, 30 min, 4ºC) to separate the total membrane and soluble fractions. Pellets containing most of the membranes were suspended in the storage buffer (50 mM Tricine pH 7.5, 600 mM sucrose, 30 mM $CaCl_2$, and 1 M glycinebetaine). The crude membrane and crude soluble fractions were stored at -80^{o}C until use. The entire protein isolation procedure was carried out in dim light at 4^{o}C. Depending on the experiment, the total, soluble or membrane proteins were treated with DNAse, RNAse, detergents and/or high salt as described in Paper III.

For aqueous two-phase partitioning, the total membranes were isolated as described above, except for the lysis buffer, which contained 5 mM potassium phosphate pH 7.4, 800 mM sorbitol, 25 mM $MgCl_2$ and 1 mM ACA.

For DIGE analysis, 30 mL of cell cultures (OD = 0.8) were harvested, washed in 50 mM HEPES pH 7.5 and broken using glass beads (1 min vortex and 1 min interval on ice) in the buffer containing (30 mM Tris-Cl pH8.5, 8 M urea, 2 M thiourea and 4%

CHAPS). Extracts were centrifuged at 150 g to remove any cell debris. The supernatant was stored at -80°C until analysis.

For iTRAQ, the proteins were extracted from the cells in the buffer containing 50 mM HEPES pH 7.5, 0.1% SDS and 0.1% Triton X-100. Cells were broken with glass beads (150-212 μm) in a bead beater (Mini-Bead-Beater-8, Unigenetics Instruments Pvt. Ltd., India) six times, 1 min each with at least 3-min incubation on ice between runs. Crude cell extracts were centrifuged for 5 min at 1000 *g* to remove the unbroken cells and glass beads. The resulting supernatant was incubated for 30 min with 2% SDS (final concentration) to facilitate extraction of membrane proteins. Samples were centrifuged for 20 min at 12 000 *g*, and the supernatant was saved for the future use.

The protein content of the membranes and soluble fractions was determined using the Bio-Rad Dc protein assay kit.

3.4.2. Aqueous two-phase partitioning

Two-phase partitioning was performed according to Norling et al. (1998) by constituting total membranes with 10-g two-phase system. Similarly, a 30-g repartitioning system without the sample was prepared. The tubes containing two phase system were mixed and centrifuged to separate the phases. Thereafter, a fresh lower phase solution (5.8% Dextran) was added to the upper phase sample, and a fresh upper phase solution (5.8% polyethylene glycol) was added to the lower phase sample and the repartitioning was repeated three times to improve the purification. The final upper and lower phases were diluted and centrifuged at 125000 *g* for 45 min. The pellets were washed with the two phase buffer and the centrifugation was repeated. Finally the membranes were resuspended in a small volume of 20 mM Tricine-NaOH pH 7.5, 10 mM NaCl, 10 mM $MgCl_2$ and 500 mM sucrose.

3.4.3. BN/SDS-PAGE and western blotting

Protein complexes either in the membrane or in the soluble fractions were separated by blue native PAGE (Herranen et al. 2004). Samples were resuspended in the buffer containing 25 mM bis-tris pH 7.0, 20% glycerol (w/v), 1 mM pefabloc and 10 mM $MgCl_2$. Solubilization of the protein samples was carried out for 20 min on ice in the same buffer containing 2.5% n-dodecyl β –D-maltoside (DM). Solubilized samples were separated in 5-12% or 6-13% gradient PAGE. For analyses of the protein complex in the 2D BN/SDS-PAGE, the BN-PAGE strips were incubated in the Laemmli sample buffer containing 5% β-mercaptoethanol and 6 M urea at RT for 2 h. For denaturing electrophoresis, proteins were separated in 12.5% SDS-PAGE containing 6 M urea (Laemmli 1970) using a Mini-PROTEAN II electrophoresis cell (Bio-Rad). The proteins from the gels were electroblotted onto PVDF membrane Immobilon-P; Millipore) using standard procedures and immuno-labeled using different antibodies.

In addition to the antibodies described in Papers I, II, III and IV, antibodies against Slr0006 and FDP proteins were produced and used in this study. Two peptide from Slr0006 protein, CGDRAMDVAKIPPA and CGEPVTSFEAASRY, were synthesized and conjugated separately with KLH carrier. The peptides were then mixed and rabbits were immunized to generate polyclonal antibodies using standard 84-day protocol (Innovagen AB, Sweden). The antibody was verified by comparing different bleeds with pre-immune serum against total protein extracts from the control strain and corresponding mutant strains. The linearity of the antibody detection was verified against different concentration of total proteins from *Synechocystis* WT.

3.4.4. Isoelectric focusing and Differential In-Gel Electrophoresis

For Differential In Gel electrophoresis (DIGE) quantitation, total proteins from the control strain and Δ*slr0006* were labeled using CyDye DIGE Fluor minimal dyes (GE Healthcare) according to the Ettan™ 2D DIGE protocol. The pooled, labeled proteins were focused in pH 3–11NL IPG strips using the Ettan™ IPGphor™ IEF system (GE Healthcare) according to the manufacturer's instructions. SDS-PAGE in the second dimension was performed using a Protean II 2D cell (Bio-Rad) and low-fluorescence glass plates (Jule Inc.). Image acquisition was carried out using the Geliance 1000 imaging system (Perkin Elmer) and the images were analyzed using ProFINDER2D software (Perkin Elmer). The identification of proteins was performed by electrospray ionization tandem mass spectrometry (ESI MS/MS) as in Battchikova et al. (2005).

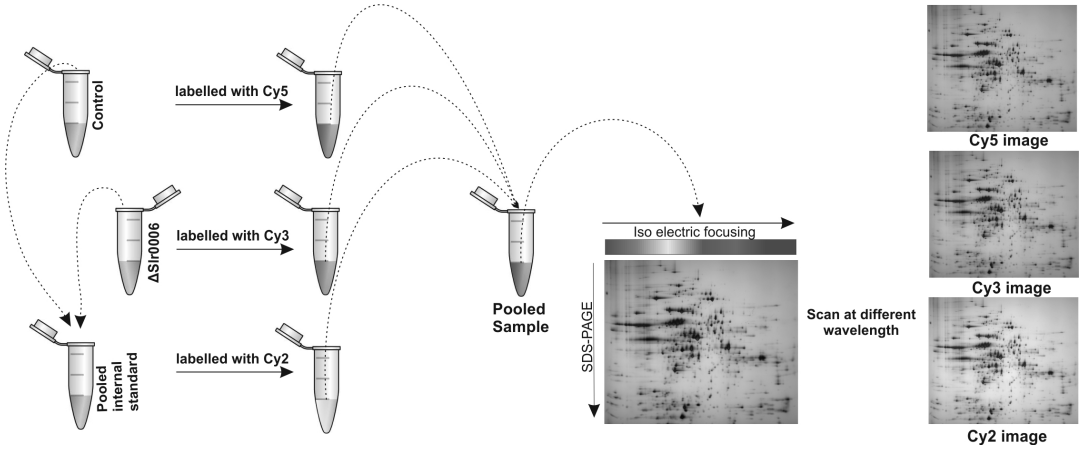

Figure 3: Differential in gel electrophoresis work flow. The Protein samples from the WT and mutant were labeled with respective dye and separated by IEF/SDS-PAGE. Imaging was carried out at different channels and analysed.

3.4.5. Isobaric Tag for Relative and Absolute Quantification

Proteins (100 µg/sample) were precipitated with cold acetone followed by reduction and alkylation according to the iTRAQ kit manufacturer's instruction (Applied Biosystems). Further, proteins were digested with trypsin and labeled with the iTRAQ reagents according to manufacturer's instructions. The labeled peptides were pooled within every experiment and vacuum-dried. Peptides were prefractionated using PolySULFOETHYL A column (PolyLC, Columbia, MD) on Agilent 1100 HPLC system (Agilent). Fractions containing labeled peptides were pooled on the basis of peak intensity at 214 nm (totally 16 fractions), vacuum-dried, and stored at -20 °C prior to mass spectrometric analysis.

Desalting was performed using a combination of POROS R2 and R3 microcolumns followed by drying in speed vac. The peptides were dissolved in 2% formic acid and separated by reverse phase chromatography using a C18 PepMap column. The nano-HPLC system (Famos, Switchos and Ultimate from Dionex/LC Packings, Amsterdam, The Netherlands) was connected in-line with API-QSTAR (Applied Biosystems) mass spectrometer for tandem mass spectrometry analysis

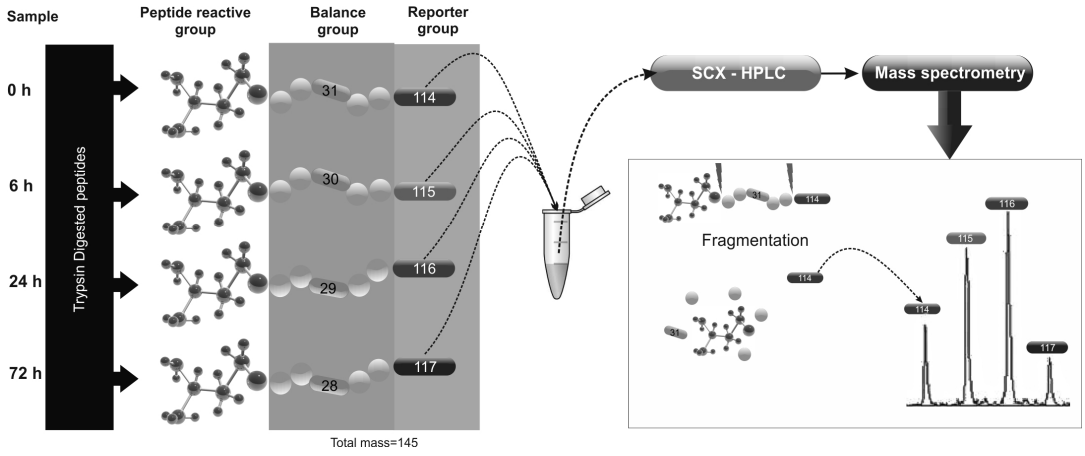

Figure 4: iTRAQ workflow. The samples collected at different time points were digested and labeled. The labeled peptides were separated and analyzed by mass spectrometry.

3.5. Structural modeling

Homology models of Slr0006 were constructed using three crystal structures as templates: Sua5 from *Sulfolobus tokodaii* [(PDB code 2EQA; (Agari et al. 2008)], YrdC from *Escherichia coli* [(PDB code 1HRU;(Teplova et al. 2000)] and YciO from *E. coli* [(PDB code 1KK9;(Jia et al. 2002)]. A structure-based sequence alignment was made by

superimposition of the templates using the program VERTAA in the BODIL modeling environment (Lehtonen et al. 2004). The Slr0006 sequence was then aligned to the fixed structure-based alignment using the program MALIGN in BODIL (Lehtonen et al. 2004). The models were analyzed and compared to the templates by superimposition using the program VERTAA in BODIL (Lehtonen et al. 2004).

Similar to Slr0006, the 3D structural models for Flv2 and Flv4 were produced using MODELLER (Sali and Blundell 1993) and evaluated with Procheck (Laskowski et al. 1993). The crystallographic structure of $F_{420}H_2$ oxidase from the methanogenic archaea *M. marburgensis* (closed conformation, PDB ID: 2OHI; open conformation PDB ID: 2OHJ) (Seedorf et al. 2007) were used as a template to model the open and closed conformation of the β-lactamase-like and flavodoxin domains of the *Synechocystis* Flv2/Flv4 heterodimer. Separately, based on *M. thermoacetica* FprA (PDB ID: 1YCF) closed conformation, a model was created for the Flv2/Flv4 heterodimer. To model the C-terminal flavin reductase domains of Flv2 and Flv4 the crystallographic structure of *A. fulgidus* ferric reductase was used (PDB ID: 1I0S) (Chiu et al., 2001).

3.6. Ribosome enrichment

Isolation of the polysome-enriched fraction was carried out by centrifugation of the sample in a sucrose gradient according to Tyystjarvi et al. (2001). The gradient supernatant was fractionated into 200-µL fractions followed by precipitation of proteins with acetone at -20^{o}C, overnight. The protein precipitates collected from gradient fractions as well as the pellet formed during the sucrose gradient centrifugation were dissolved in 1x Laemmli buffer (63 mM Tris-HCl pH 6.8, 10% glycerol, 2% SDS, 0.0025% bromophenol blue and 6 M urea) and separated by SDS-PAGE.

3.7. Fluorescence spectrometry

77K fluorescence emission spectra of the whole cells were measured with a USB4000-FL-450 spectrofluorometer. Cells suspended in fresh BG-11 medium, corresponding to 5 µg/mL of chlorophyll concentration were acclimated to growth conditions for 1 h and then quickly frozen. The samples were excited with 580-nm or 440-nm light obtained via 10-nm width filters. The fluorescence spectra were analyzed with Gaussian deconvolution, and the areas of all fluorescence sub-bands were calculated.

3.8. Oxygen evolution measurements

Steady-state oxygen evolution was measured with a Clark-type oxygen electrode (Hansatech, DW1, UK) at 30°C under saturating light conditions. Measurements of net photosynthesis rates from low CO_2 cells were carried out at 500 µmol photons m^{-2} s^{-1} without adding bicarbonate. The whole chain photosynthesis electron transfer rates (H_2O to CO_2) and the PSII electron transfer rates (H_2O to quinone), in the presence of an artificial electron acceptor, either 0.5 mM DCBQ (2, 6-dichloro-*p*-benzoquinone) or 2 mM DMBQ (2,6-dimethyl-*p*-benzoquinone), were measured both in the presence and absence of 10 mM $NaHCO_3$.

3.9. Bioinformatics tools used

Several bioinformatics tools were used to analyze protein and nucleotide sequences. They are listed below as follows:

Tool	**Web address**
BLASTP	http://blast.ncbi.nlm.nih.gov/Blast.cgi
CyanoBase	http://genome.kazusa.or.jp/cyanobase
Expasy	http://expasy.org
Pattern Finder	http://www.algosome.com/resources/pattern-finder.html
SIGNALP	http://www.cbs.dtu.dk/services/SignalP
SMART	http://smart.embl.de
STRING	http://string-db.org
UniProt	http://www.uniprot.org

4. RESULTS

4.1. Synechocystis proteome changes upon a shift from high to low CO_2

We have carried out the quantitative proteome analysis of *Synechocystis* cells using isobaric tags (iTRAQ) to revealed the changes in *Synechocystis* proteome during acclimation of cells to the CO_2 starvation and to find novel proteins involved in this mechanism (Paper I). *Synechocystis* cells grown in high CO_2 (3%) were shifted to air level CO_2 followed by quantitative analysis of proteins isolated from the cells harvested at 0, 6, 24, and 72 h after the shift.

In general, the observed differential regulation was consistent in all biological replicas. The changes in expression levels were significant (>1.5-fold up-regulation or <0.7-fold down regulation) for 76 proteins (Paper I – Figure 1).

4.1.1. C_i uptake systems

A remarkable response was observed for CCM components participating in both CO_2 and bicarbonate uptake systems. The subunits of NDH-1MS complex, CupA (Sll1734), CupS (Sll1735), and NdhD3 (Sll1733), were among the most highly up-regulated proteins (Paper I). Seven hydrophilic subunits, NdhH (Slr0261), NdhI (Sll0520), NdhJ (Slr1281), NdhK (Slr1280), NdhM (Slr1623), NdhN (Sll1262), and NdhO (Ssl1690), and two hydrophobic subunits, NdhA (Sll0519) and NdhG (Sll0521), which are common for all forms of *Synechocystis* NDH-1 complexes, were also up-regulated. However, their expression levels were lower than for CupA, CupS and NdhD3. Besides up-regulation of the CO_2 uptake system, bicarbonate transporters located in plasma membrane were also triggered in response to the CO_2 downshift. The Na^+-dependent bicarbonate transporter SbtA (Slr1512) and SbtB (Slr1513) were increased during the shift to low CO_2. Furthermore, the subunits of the ATP-dependent BCT-1 transporter and CmpA-D (Slr0040-0043) accumulated to the maximum level already at 6 h, and both subunits of RuBisCO (RbcL, Slr0009 and RbcS, Slr0012) were found to be among the most up-regulated proteins. In addition, the carboxysome shell components CcmK1 (Slr1029), CcmK2 (Slr1029), and CcmM (Sll1031) showed slight upregulation (Paper I).

4.1.2. Photosynthesis and photoprotection

The subunits of ATP synthase and cytochrome b_6f complex as well as soluble electron carriers, plastocyanin (PetE, Sll0199) and ferredoxin-$NADP^+$ oxidoreductase (PetH, Slr1643), remained fairly unchanged during the low CO_2 shift. Subunits of PSI, PSII, and phycobilisomes, however, showed slight downregulation in the course of the experiment. At 72 h of the low CO_2 treatment, the proteins involved in chlorophyll biosynthesis, porphobilinogen synthase (HemB, Sll1994), coproporphyrinogen III oxidase (HemF, Sll1185), geranylgeranyl hydrogenase (ChlP, Sll1091), magnesium-protoporphyrin IX monomethyl ester cyclase (Sll1214) and magnesium protoporphyrin IX chelatase subunit H (ChlH, Slr1055), demonstrated marked repression (Paper I).

Synechocystis, like all other photosynthetic organisms, use light and convert it to a form of energy suitable in supporting intracellular metabolic processes. Nevertheless, the excess of excitation energy results in the damage to components of the photosynthetic apparatus and generates harmful reactive oxygen species (ROS). The water-soluble orange carotenoid protein (OCP, Slr1963), which is essential in non-photochemical quenching (Boulay et al. 2008), showed a steady increase during acclimation to low CO_2. Two flavodiiron proteins, Flv2 (Sll0219) and Flv3 (Sll0550), were also significantly up-regulated. Flv2 showed a fast induction and reached a plateau level at 6 h. The Flv3 protein which functions in Mehler reaction (Allahverdiyeva et al. 2011), accumulated steadily during the entire course of the treatment. Among other enzymes protecting cells against ROS, glutathione peroxidase Gpx2 (Slr1992) demonstrated fast response to low CO_2 conditions whereas superoxide dismutase (SodB, Slr1516) and bifunctional catalase peroxidase (KatG, Sll1987) remained unchanged.

4.1.3. Nitrogen uptake

The balance between carbon and nitrogen assimilation is vital for the optimization of cell metabolism (Singh et al. 2008). Cyanobacteria use ammonium, nitrate, nitrite, and urea as their major sources of nitrogen. Our results in Paper I showed that the CO_2 downshift led to a fast and significant decrease in expression of all detected and quantified transporters participating in the uptake of fixed nitrogen. High-affinity ammonium/methyl ammonium permease (Amt1, Sll0108) was distinctly down-regulated in response to the CO_2 limitation. The urea transport system substrate-binding protein UrtA (Slr0447) was found to be repressed. Similarly, the ATP-dependent nitrate/nitrite transporter encoded by *nrtABCD* operon (*sll1450-sll1453*) showed a strong decline in the expression upon CO_2 limitation at 24 h for NrtA (Sll1450), NrtC (Sll1452), and NrtD (Sll1453), (NrtB, Sll1451 was not detected). Our results clearly showed strong down regulation of ferredoxin-dependent nitrite reductase (NirA, Slr0898). However, the enzymes participating in GS-GOGAT pathway, glutamine synthetase GlnA (Slr1756), ferredoxin-dependent glutamate synthetase GltS (Sll1499), and NADH-dependent glutamate synthetase GltD (Sll1027), which are involved in the conversion of ammonium to glutamate, did not show marked changes in response to CO_2 limitation. The expression of GlnB (Ssl0707), the important regulatory protein involved in nitrogen and inorganic carbon uptake, was not changed by low CO_2. In contrast, the glutamine synthetase, GlnN (Slr0288), decreased significantly during the treatment (Paper I).

4.1.4. Ribosomes and chaperones

CO_2 limitation resulted in a decline of a protein synthesis since quantified protein components of the 50S and 30S ribosomal subunits showed decrease during the course of the C_i downshift experiment (Paper I). The decline was minor and could be noticed only at 72 h, however, the response was quantified with a high confidence. Further, chaperonins GroEL1 (Slr2076), GroES (Slr2075), and HtpG (Sll0430) were steadily down regulated during the course of low CO_2 treatment. The Usp1 protein (Slr0244), a member of the universal stress protein-family, also declined.

4.1.5. Unknown proteins

A number of unknown proteins were quantified as being significantly up or downregulated in the iTRAQ experiment (Paper I). The functions of these proteins and their roles in acclimation of cell to the CO_2 downshift remain unknown. The Slr0006 were among 20 unknown proteins whose expression was significantly upregulated upon CO_2 limitation. This protein became the subject for further characterization.

Table 1: Bioinformatics sequence analysis of unknown proteins detected in iTRAQ experiment.

Protein	AA	SS	TM	Domain	Y2H Interaction*
Sll0023	447	N	N	Glutathionyl spermidine synthase	NarB, OdhB, Sll0022
Sll0588	153	N	N	-	Sll0588, PdhB
Slr0006	217	N	N	Sua5/YciO/YrdC	DHNA-CoA thioesterase, KatG, Slr0209
Slr0453	821	N	N	Phosophoketolase	-
Slr0476	136	N	N	-	Sll1647, Sll0645, AroB, GyrB, Slr2018
Slr0606	317	N	N	Glycosyl transferases	FtsY
Slr0729	101	N	N	DUF3181	NatA, CcmK1
Slr1624	458	Y	N	-	-
Slr1926	223	N	N	ARGINASE_1 : Hydrolase	Sll0499, Sll0585, RecJ, SepF, SpkD
Slr2025	153	N	N	DUF1821	PbsA2, Slr1444, HoxH, Slr2037
Slr2032	248	N	N	DUF561	-
Slr2101	143	Y	Y	Cupin_2	-
Slr2144	301	N	N	-	Sll0837, Slr0793, Sll0408
Ssl0294	70	N	N	-	Sll1647, Sll1696, ArgG, GyrB, Sll1942, Slr1117, Sll0236, Slr1103, Sll0103, Sll0178
Ssl0352	58	N	N	-	UvrC, Slr1942, Sll0149
Ssl2874	89	N	N	DUF370	-
Ssr0692	51	N	N	-	Sll0149, Slr0909, EpsB, PolA, Sll1528, NdhH, Ssr0692, MtfB, Slr1968
Ssr1528	94	N	N	-	Sll0488
Ssr3341	70	N	N	Sm-like ribonucleoproteins	-
Slr0151	320	N	Y	TPR	-

AA- length of the amino acid; SS- Signal sequence; TM- Transmembrane helix;* data obtained from Yeast two hybrid interaction database from Cyanobase

4.2. Characterisation of Slr0006

4.2.1. Regulation and structural model of Slr0006

The unknown Slr0006 protein, showed remarkable increase in our CO_2 limitation study in *Synechocystis* similar to different CCM related proteins (Paper I). The *slr0006* ORF is a single-copy gene encoding a 23-kDa uncharacterized protein. The genes in the vicinity of the *slr0006* gene, including that for fructose bisphosphate aldolase (*sll0016*), *rbcL* (*slr0009*) and *rbcS* (*slr0011*) have vital roles in carbon metabolism. The protein-protein blast using the BLASTP algorithm showed that the Slr0006 protein exhibits 60% identity with an unknown protein from *Cyanothece sp. ATCC 51142* whereas the sequence identity is relatively low for other proteins. Sequence analysis of the Slr0006 protein showed the presence of a domain that belongs to the Sua5/YciO/YrdC protein superfamily composed of Sua5, YciO, YrdC, and YwlC proteins which share structural similarities (Teplova et al. 2000, Kuratani et al. 2011). Such domains are widely distributed in eukaryotes and prokaryotes and occur as: (1) independent proteins, (2) with C-terminal extensions, and (3) as domains in larger proteins. Slr0006 is independent protein containing the domain extended from amino acid position 24-195.

In order to understand the functional role of this protein, the expression of Slr0006 was tested in a response to different environmental cues. Temperature is one of the factors that determine the availability of CO_2 in the medium. Less CO_2 is available in BG-11 medium at high temperature than at low temperature (Carroll et al. 1991). To test the expression of the Slr0006 protein at high and low temperature, the control strain was grown in chamber filled with 3% CO_2 until OD_{750} = 0.8, precipitated and re-suspended in fresh BG-11. The growth of cells was subsequently continued under low CO_2 conditions either at 30°C, 37°C or 22°C for 72 h. Equal amounts of cells were harvested, and total protein fractions were analyzed by western blotting. Although no distinct differences in the amount of Slr0006 could be detected between the control and high temperature-treated cells, exposure to low temperature apparently prevented the expression of the *slr0006* gene (Paper II).

Also, expression of the Slr0006 protein under acidic and alkaline conditions was tested in the control strains (Paper II). The cells from 3% CO_2 were suspended in fresh BG-11 (OD_{750} = 0.8) buffered with either 20 mM MES-KOH, pH 6.0, or 20 mM CHES-KOH, pH 9.0 and the growth was continued at air level CO_2 for 72 h. Western blot showed that the amount of Slr0006 was higher in cells grown under acidic conditions as than in those grown in an alkaline environment.

To verify whether the Slr0006 protein cooperates with some of the known CCMs, we analyzed the expression of the Slr0006 protein in several CCM mutants involved in carbon uptake and respiration (*ΔndhD3, ΔndhD4, ΔcupA, ΔcupA/ΔcupB, ΔndhD3/ΔndhD4*, *ΔndhD1/ΔndhD2,* M55 (*ΔndhB)*, and *ΔsbtA*). Western blot analysis of the *Synechocystis ΔndhD3, ΔndhD4, ΔcupA, ΔcupA/ΔcupB,* and *ΔsbtA* strains revealed that the level of Slr0006 was similar between the control and mutant strains indicating that upregulation of the *slr0006* gene was not required to compensate for the impaired function of the CCM

complexes studied (Paper II). On the contrary, in the Δ*ndhD3*/Δ*ndhD4* mutant that completely lacks the CO_2 uptake system, the accumulation of Slr0006 was slightly higher than in the control. Marked downregulation of Slr0006 expression was observed in Δ*ndhB* and Δ*ndhD1/*Δ*ndhD2*. The deletion of the *ΔndhB* gene in *Synechocystis* results in both impaired CO_2 uptake and cyclic electron transport around PSI, and the deletion of *ndhD1/ndhD2* lead to impaired cyclic electron transport. Recent studies have shown that, the deletion of *ndhB* or *ndhD1/ndhD2*, also results in decreased net photosynthetic activity (data not shown).

The response of Slr0006 to the changing redox state of the plastoquinone (PQ) pool was investigated using two electron transport inhibitors: DCMU and DBMIB. DCMU blocks the Q_B site of PSII, resulting in an oxidation of the PQ pool, while DBMIB prevents electron transfer to the cytochrome b_6f complex, which leads to a highly reduced state of the PQ pool. The high CO_2 grown cells were shifted to low CO_2 and incubated for 24 h in BG-11 medium containing either DCMU (10 μM) or DBMIB (25 μM). DBMIB, in turn, was reduced by 5 mM sodium ascorbate, in order to eliminate its effects as an electron acceptor or quencher of chlorophyll fluorescence (Trebst 2007). Our results showed that the accumulation of Slr0006 in low CO_2 condition was completely prevented in the presence of DCMU or DBMIB. Moreover, no Slr0006 protein could be detected in the low CO_2 grown cultures upon treatment of the cells in the dark for 24 h, not even in the presence of glucose. Taken together, our results suggest that linear electron transfer *per se* regulates the expression of Slr0006 (Paper II and Paper IV).

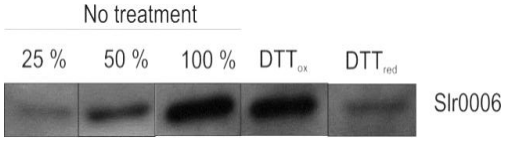

Figure 5: Effect of DTT (oxidized and reduced forms) on the accumulation of Slr0006 after the shift to low CO_2 conditions.

Although DCMU and DBMIB have opposite effects on the redox state of the PQ pool, their influence on the expression of Slr0006 was similar, which indicates that the signal is not sensed by the redox state of the PQ but somewhere else. We therefore used DTT, a redox reagent that mainly targets the disulphide bonds, rather than the redox components located between PSII and PSI (Sippola and Aro 2000). Our results clearly showed that the expression of Slr0006 is downregulated in the presence of reduced DTT, indicating that the thiol redox state might play a role in deciphering the signal during C_i limiting conditions (Fig.5). These results do not, however, enable us to make any final conclusions about the redox regulatory network of Slr0006 expression.

Investigation of the upstream sequence of *slr0006* revealed a unique signature sequence, T(N_{11})A, which is a putative binding site for the NdhR, a regulatory protein for various C_i responsive genes (Wang et al. 2004; Woodger et al. 2007). The expression of the *slr0006* gene in Δ*ndhR::Sp*^{r} was studied upon shift of the high CO_2 grown cells to low CO_2. Slr0006 protein started to accumulate already under high CO_2 in the Δ*ndhR::Sp*^{r}, whereas no protein could be detected in the control strain. Moreover, a pronounced

accumulation of Slr0006 protein in the Δ*ndhR::Sp*^{r} mutant as compared to the control strain was observed at 6 h indicating that the Slr0006 gene has the same regulator as other CCM genes (Paper IV).

To evaluate the structural characteristics of Slr0006, the 3D structure of the protein was modeled from three different crystallographic structural templates: Sua5 from *Sulfolobus tokodaii strain 7* (2EQA), YrdC from *Escherichia coli* (1HRU) and YciO from *E. coli* (1KK9). Despite the low sequence identity (20%) between Slr0006 and the crystallographic structures, all the models of Slr0006 had an α/β twisted open-sheet structure typical of the Sua5/YrdC/YciO protein family. To obtain a highly reliable and good-quality model of Slr0006, all modeled structures were validated using WHATCHECK, PROCHECK and ProSA-web. The 3D structural models of Slr0006 showed the presence of a positively charged cleft, but the structural details of the cleft varied depending on the template used (Paper IV).

4.2.2. Localisation of Slr0006

The programs (TMHMM http://www.cbs.dtu.dk/services/TMHMM/) used to predict the presence of transmembrane helices and signal peptide revealed that Slr0006 is likely a soluble protein. The first step in identifying the cellular location was to separate the total membrane and soluble fractions of *Synechocystis* after breaking the cells. We routinely use lysis buffer containing 30 mM $CaCl_2$ to separate the membrane fraction from the soluble pool in *Synechocystis* (Gombos et al. 1994). Analysis of the membrane and soluble fractions by western blot revealed that the Slr0006 protein was mainly located in the membrane fraction. However, lysis of cell with buffers that differed in the concentration of divalent cations (either $CaCl_2$ or $MgCl_2$) showed that reducing the concentrations of divalent cations in the lysis buffer drastically changed the location of the Slr0006 protein from the membrane fraction to the soluble fraction. To detremine whether the membrane attachment of the Slr0006 protein was dependent on the presence of divalent ions, the total *Synechocystis* cell extract, isolated in the absence of divalent ions, was reconstituted with 30 mM $CaCl_2$. Clearly, addition of the cations to the cell extract after breakage of the cells only had a minor effect on the membrane binding of Slr0006. Moreover, cell lysis in the presence or absence of DNAse or RNAse had no influence on the location of Slr0006 (Paper III).

Further, the total membranes containing Slr0006 were washed with 2 M NaBr to separate the peripheral and integral proteins. Furthermore, the isolated membranes were solubilized with different nonionic, anionic and zwitterionic detergents. Our results showed that Slr0006 was strongly bound to the membrane, since all the detergents except SDS proved to be ineffective in releasing it from the membranes.

The 3D model of Slr0006 clearly showed the presence of a positively charged cleft that is possibly involved in binding to dsRNA. These results prompted us to check whether Slr0006 is associated with ribosomes. In *Synechocystis,* ribosomes are mainly located in the cytoplasm, while some are distributed between the plasma membrane and the thylakoid membranes. Isolation of the soluble and membrane bound polysomes from the high-to-low

CO_2 shifted cells showed co-localization of Slr0006 protein with the S1 protein of the 30S ribosomal subunit, both in the soluble and in the membrane-bound ribosomes. Interestingly, the S1 protein of the 30S ribosomal subunit also behaved similarly to Slr0006 protein when isolated in the absence or in the presence of cations (e.g 0 or 30 mM $CaCl_2$). Most of the ribosomal RNA was present in the soluble pool when the proteins were isolated in the absence of cations, while a fraction of the ribosomal RNA could be found in the membrane pool when the cells were broken in the presence of 30 mM $CaCl_2$ (Paper IV).

4.2.3. Functional analysis of Slr0006

To understand the functional role of Slr0006 protein, a deletion mutant for *slr0006* gene was constructed by replacing part of the *slr0006* ORF with the kanamycin resistance cassette (Km^r). The complete segregation was verified by PCR analysis. The following western blot analysis revealed that the *Δslr0006* was completely devoid of the Slr0006 protein. The control and mutant strains were studied in various controlled environmental stress conditions in order to understand the function of Slr0006.

We tested how the *slr0006* inactivation effected the growth of the cells upon standard light conditions (50 µmol photons $m^{-2}s^{-1}$) or high light conditions (250 µmol photons $m^{-2}s^{-1}$) in various conditions including different pH (6, 7, 8 and 9), presence or absence of sodium carbonate in the medium, at low temperature (18°C), under phototrophic and mixotrophic conditions. The growth patterns of WT and inactivated strain remained similar under these different conditions, except at 18°C where *Δslr0006* showed severe chlorosis and slow growth (Paper IV).

In addition, a global proteome analysis of Δ*slr0006* was carried out using Differential In-Gel Electrophoresis. Solubilized proteomes of the control and Δ*slr0006* strains were labeled with Cy3 or Cy5. Cy2 was used as an internal standard to avoid gel-to-gel variation. The pooled samples were separated by 2D IEF/SDS-PAGE. The Cy3 and Cy5 images taken from each gel were virtually overlaid to study the difference between the control strain and Δ*slr0006*. Five individual proteins were consistently down-regulated in Δ*slr0006* compared to the control strain. As expected, the Slr0006 protein was absent in the Δ*slr0006* strain (175-fold down-regulated). The degree of down-regulation for other proteins of unknown function (Slr0105, Sll6012, Slr7087 and Slr7090) was approximately 2-fold. Slr0006 and Slr0150 are encoded by the *Synechocystis* chromosome, whereas Slr7087, Slr7090 and Sll6012 are encoded by the pSYSA and pSYSX plasmids. The kanamycin resistance cassette was the only protein found to be upregulated in *Δslr0006* as compared to the control strain (Paper II).

4.3. Characterisation of flavoproteins

Flavodiiron proteins (FDP), earlier known as A-type flavoproteins, are involved in detoxification of O_2 and NO in anaerobic bacteria and archaea. *Synechocystis* encodes four flavodiiron proteins - Flv1 (*sll1521*), Flv2 (*sll0219*), Flv3 (*sll0550*) and Flv4 (*sll0217*). Previous studies have shown that Flv1 and Flv3 are involved in Mehler's reaction and Flv2

and Flv4 are involved in photoprotection of the cell (Zhang et al. 2009; Allahverdiyeva et al. 2011). The flavodiiron proteins are a family of enzymes generally able to reduce dioxygen to water, and/or nitric oxide to nitrous oxide. Goncalves et al. (2011) have shown the strong induction of all the FDP under nitrosative stress after 12 h.

4.3.1. Localization of Flv2 and Flv4 proteins

The *flv4*, *sll0218* and *flv2* genes are highly conserved and clustered together to form a single operon in the *Synechocystis* chromosome. These genes were strongly induced when *Synechocystis* cells were shifted to low CO_2 conditions (Paper I). Transmembrane prediction using program TMHMM showed that the Flv2 and Flv4 are devoid of transmembrane helices whereas Sll0218 possesses four α-helices and is therefore likely to be present at the membrane. Surprisingly, both Flv2 and Flv4 behaved very similar to Slr0006 with respect to localization in the presence and in the absence of divalent cations (Paper III). On the other hand, Sll0218 was detected only in the membrane fraction irrespective of the buffer system. To precisely localize the Flv4, Sll0218 and Flv2 proteins, the thylakoid and plasma membranes from *Synechocystis* cells were purified by the two phase partitioning method (Norling et al. 1998) and were probed with protein specific antibodies. The Flv4 and Flv2 were associated with the plasma membrane and Sll0218 was associated with the thylakoid membrane. As for Flv2 and Flv4, in the presence of divalent ions Slr0006 was also identified in the plasma membrane (Paper III and Paper V).

We investigated the expression of Flv2 and Flv4 proteins in the WT and in the mutants containing interruption of the *flv2-flv4* operon at different positions. When either *flv2* alone, or both the *flv2* and the *sll0218* genes were inactivated, there was a subtantial decrease (up to 50%) in the expression of Flv4 compared to WT. Interestingly, an elevated expression of Sll0218 was observed in the *flv2* mutant and the transcript level of the *flv4* gene was up-regulated five-fold.

4.3.2. Dimer formation and structural model of FDPs

Generally, FDPs adopt a homodimer or a homotetramer structure and dimerization is often essential for the proper function (Vicente et al. 2008). As FDPs showed a strong association with the membrane in the presence of divalent cations, the solubilization process became extremely challenging. Thus, soluble fraction prepared using a buffer, that lacks divalent ions was used for further analysis. Our results (Paper V) clearly show that the Flv2 protein with a molecular weight of 65 kDa formed a complex of roughly 140 kDa, which corresponded to the dimer form of Flv proteins in BN-PAGE. Since the anti-Flv4 polyclonal antibody gave a high background signal, Flv4 was fused with a FLAG-tag and was expressed both in the WT and in the Δ*sll0218-19* mutant. The FLAG-tag antibody revealed two specific bands in the *flag-flv4* strain, corresponding to dimeric and monomeric forms. Moreover, the dimeric FLAG-Flv4 form disappeared from the Δ*sll0218-19*/*flag-flv4* strain indicating that Flv2 is necessary for the dimer formation. However, compared to the dimeric form, the monomeric FLAG-Flv4 remained abundant which may have resulted from introduction of the FLAG tag impairing dimer formation.

The Sll0218 protein, on the contrary, was found to be associated with a much larger complex, more than 500 kDa in size, between the PSII dimer and the NDH-1L complex in BN-PAGE (Paper V). The possibility of association between FDPs and the NDH-1 complex can be excluded since the level of NdhJ in Δ*sll0218-19* and in the WT remained similar. Likewise, the Sll0218 complex was formed even in the M55 strain.

To understand the possible formation of Flv2/Flv4 heterodimer and its unique membrane association property we have constructed Flv2/Flv4 homology models of the β-lactamase-like domain, containing the diiron site and the flavodoxin domain containing the FMN binding site (Paper V). The heterodimer is formed by head-to-tail arrangement, to facilitate the iron binding site of the Flv2 monomer to face the FMN-binding site of the Flv4 monomer and vice versa. The models showed several conserved metal-binding sites. The crystallographic structure of the reduced state (PDB ID: 2OHI) and oxidized state (PDB ID: 2OHJ) of the $F_{420}H_2$ oxidase of *Methanothermobacter marburgensis* was used as a template to investigate the structural changes of the enzyme during electron transfer in the Flv2/Flv4 dimer. The sequence identity between $F_{420}H_2$ oxidase and *Synechocystis* Flv2 and Flv4 is 29% and 26%, respectively. The FMN binding at the interface of the monomers is supported by the fact that there are six conserved FMN-binding residues (Ser285, Thr290, Thr337 from Flv2, and Glu99, His164, Trp165, Asp42 from Flv4) in the Flv2 monomer and by residue Trp175 in Flv4.

In almost all members of the FDP family, a conserved Trp residue stacks with the FMN isoalloxazine ring whereas it is replaced by Gly in methanogens and some cyanobacteria (Vicente et al. 2008). This Trp residue is conserved in Flv2 (Trp371) and in *M. thermoacetica* FprA (Trp347), but Flv4 and in *M. marburgensis* $F_{420}H_2$ oxidase have a Gly in the corresponding position. In the open conformation of the Flv2/Flv4 model, which is based on *M. marburgensis* $F_{420}H_2$ oxidase structure, Trp371 is turned away from the binding site resulting in the formation of a cavity which could accommodate another co-factor.

The oxidation rearranges the bound irons to form an open conformation. In $F_{420}H_2$ oxidase of *M. marburgensis* the first iron is coordinated similarly, but the second iron is moved and coordinated by His26, Glu85 and His267 from the other monomer. The corresponding residues in the Flv4 monomer are Asp42 and Glu99 from Flv4 and Tyr287 from Flv2, while in the Flv2 monomer they are Glu51 and Asn108 from Flv2 and Tyr277 from Flv4. Furthermore, a third iron is coordinated by His151, Asp320 and a water molecule at the surface of *M. marburgensis* $F_{420}H_2$ oxidase stabilizing the switch loop (148-PLLH-151) in the open conformation (Seedorf et al. 2007, Vicente et al. 2008). In the Flv4 monomer, His151 is conserved (His164) and Asp320 is substituted with Ser330. In Flv2, the corresponding residues are Arg174 and Ser340. The conserved switch loop is much more conserved in the Flv4 monomer (161-PNLH-164) than in the Flv2 monomer (171-PTPR-174). When the loop opens, Trp152 in *M. marburgensis* $F_{420}H_2$ oxidase and Trp165 in Flv4 (Trp175 in Flv2) move away, creating a cavity next to the FMN. The conformational changes at the FMN binding sites of Flv2 monomer and the Flv4 monomer iron binding site are conserved and functional concluding that the two reactive sites in the Flv2/Flv4 heterodimer are distinct from each other (Paper V).

4.3.3. Functional analysis of the flv mutants

Previous study has shown the possible participation of flavoproteins in the photoprotective pathway (Zhang et al. 2009). All the *flv4-flv2* mutants (described in paper V) showed strong light-dependent decline of functional PSII centers under conditions of low CO_2. The PSII content of the *flv* mutants decreased compared to that in WT. Not only the content, but also the proportion of the dimeric PSII form and the monomeric form greatly differed in *flv* mutants. Even so, the mechanism behind the photoprotective role of FDPs is still unclear. To find the role of FDPs, the 77K fluorescence emission spectra were recorded from the cells grown under conditions of low and high CO_2. The excitation of chlorophyll a using 440 nm light resulted in fluorescence emission spectra that showed two main peaks characteristic to PSII (685 nm and 695 nm) and PSI (723 nm). Essentially, no differences could be traced between WT and the *flv* mutants under both high and low CO_2. Instead when excitation light was shifted to 580 nm which specifically excites PBS, three fluorescence emission maxima were recorded which originate from PBS (650 and 665 nm), PSII (685 and 695 nm) and PSI (723 nm) emission, respectively. The PSII fluorescence emission at 685 and 695 nm was greatly enhanced in all FDP mutants when compared to WT under low CO_2, which completely disappeared under high CO_2. The peak at 685 nm corresponds to emission from the internal antenna chlorophylls in CP47 and the peak at 695 nm represent emission from both the core antenna chlorophylls in CP43 and the allophycocyanin emitters (Vernotte et al. 1992; Karapetyan 2008). The 695 nm emission peak did not increase proportionately with the 685 nm peak in any of the *flv* mutants indicating that the increase in 685 nm fluorescence peak is likely to be mainly due to terminal phycobilin emitters rather than to the core CP43 antenna chlorophylls (Vernotte et al. 1992). These results show that the FDP mutants show inefficiency in energy transfer from PBS to PSII under condition of low CO_2.

Photosynthetic electron transfer capacity was monitored with oxygen evolution measurements using different electron acceptors. High CO_2 conditions did not affect the oxygen evolution in WT and in FDP mutants. Nevertheless under low CO_2 a significant increase was noticed after addition of 10 mM $NaHCO_3$ as a terminal electron acceptor. DMBQ (2,6- dimethyl-*p*-benzoquinone) which accepts electrons after Q_B and DCBQ (2,6-dichloro-*p*-benzoquinone), which accept electrons directly from Q_A were used to study the oxygen evolution in the WT and FDP mutants (Graan and Ort 1986). In particular, the rates of oxygen evolution in the FDP mutants were in line with the reduced accumulation of PSII centres. Higher oxygen evolution was observed in WT in the presence of DMBQ than in the presence of DCBQ whereas all the *flv4-flv2* operon mutants showed higher rate of oxygen evolution in the presence of DCBQ than in the presence of DMBQ. These results provide evidence for the presence of an additional electron exit site in close vicinity to the Q_B site under low CO_2 which apparently becomes inhibited by binding of DCBQ to the Q_B site. The lack of this phenomenon in the WT cells grown under high CO_2 and from all the *flv* mutants grown either at low or high CO_2 suggests that Flv2/Flv4 is involved in a novel, previously unknown electron transfer route from PSII (Paper V).

5. DISCUSSION

5.1. C_i limitation induces changes in the proteome of Synechocystis

Cyanobacterial photosynthesis occurs in radically diverse habitats and uses various forms of carbon concentrating mechanisms featuring multiple C_i transporters. Cyanobacteria from dynamic environments can change the CCM activity depending on C_i availability, but the molecular basis of this regulation is unclear (Woodger et al. 2007). Acclimation to C_i starvation involves a complex network of proteins in *Synechocystis*. Our results showing dynamic changes (changes in protein abundances with respect to time) in the *Synechocystis* proteome (Paper I) are consistent with previous microarray studies (Wang et al. 2004; Eisenhut et al. 2007) and other studies related to acclimation of *Synechocystis* cells to low CO_2 (Zhang et al. 2004). The protein expression levels at 72 h and after one week of low-CO_2 treatment were comparable, indicating that the cells generally become acclimated to low CO_2 within 3 days. However, for some proteins, more than one week of acclimation is needed to achieve steady-state expression levels (Paper I). In general, the hydrophobic proteins are under-represented in this study, including many membrane-embedded subunits of the photosynthetic machinery, which are known to be strongly and constitutively expressed in *Synechocystis* cells. This might be resulted from the technical difficulties associated with the isolation methods.

The CCM components that participates in both CO_2 and bicarbonate uptake showed the most intense response when *Synechocystis* cells were subjected to CO_2 starvation (Paper I). The BCT-1 transporter was induced first, and followed by SbtA/SbtB. Based on these changes, one can presume that at the early stages of CO_2 limitation, *Synechocystis* cells mainly use bicarbonate. Although depletion of bicarbonate turns on the CO_2-uptake system, bicarbonate uptake mechanisms remain at higher levels even after prolonged growth under low CO_2 conditions, indicating that the cells are able to take up inorganic carbon of all possible forms. Amongst all the variants of NDH-1 complexes, only the subunits belonging to NDH-1MS and NDH-1MS' complexes were found to be strongly up regulated. Intracellular CO_2 is converted to bicarbonate which is then converted to CO_2 by the carbonic anhydrase inside carboxysomes and made readily available for CO_2 fixation by RuBisCO. Since carboxysomes contain most of the RuBisCO (Price 2011) the increase of shell components showed analogous up-regulation of the enzyme.

During this study, a number of unknown proteins were quantified as being significantly up or downregulated. The functions of these proteins and their roles in cell acclimation to the CO_2 downshift remain unknown. The unknown Slr0006 protein was significantly upregulated during the low-CO_2 treatment. Of 19 other unknown proteins whose expression was significantly upregulated under CO_2 limitation, the highest induction was detected for Slr2032, Ssl0352 (NdhS), Ssr0692, and Ssr1528 proteins. Their expression was increased more than doubled and the changes were statistically significant (Paper I). These proteins constitute an interesting group and their role in acclimation of *Synechocystis* to low CO_2 remains to be determined.

5.1.1. Slr0006, Flv2 and Flv4 are involved in C_i acclimation

It has been shown that transcription of *rbcL* (*slr0009*), *rbcX* (*slr0011*), and *rbcS* (*slr0012*) is induced by C_i limitation (Wang et al. 2004). These three genes are members of a low C_i activated gene cluster in which *slr0006* was the most strongly induced (Fig. 6). It is worth mentioning, however, that Slr0006 does not belong to the same operon as the RuBisCO genes. The induction of *slr0006* transcription already at 3 h suggests that the protein is needed as soon as the depletion of the internal C_i level has been sensed (Wang et al. 2004). Our proteomics results also confirm the dramatic induction of Slr0006 under low CO_2 at the protein level (Paper I). The immediate and strong induction of Slr0006 raised our interest in studying whether it might have a role in deciphering the signal of C_i depletion inside the cell. We also focused on the flavodiiron proteins since Sll0217 and Sll0219 proteins showed strong upregulation during C_i limitation and high light [(Zhang et al. 2009) , Paper I]. An unknown ORF, *sll0218*, physically clustered with *sll0217* and *sll0219*, was co-transcribed with *sll0217* (*flv4*) and *sll0219* (*flv2*) (Wang et al. 2004), and has also been characterized. Previous studies with other FDPs, Flv1 and Flv3 have shown that they are involved in the Mehler reaction (Allahverdiyeva et al. 2011). Because the genetic architecture of the FDPs is highly conserved in the cyanobacteria, one might assume that they possibly share structural and functional similarities (Dandekar et al. 1998).

Figure 6: Low CO_2 induced gene cluster which includes *slr0006* and RuBisCO genes (Wang et al. 2004).

5.2. Ambiguous localization of Slr0006, Flv2 and Flv4

Characterization of novel proteins involves identification of their respective location inside the cell. Since bioinformatics analysis predicted an absence on transmembrane helices in the Slr0006 protein, we expected to find it in the cytoplasmic fraction. Routinely, we break cyanobacterial cells in buffers containing high concentration of divalent ions especially $CaCl_2$ in order to keep photosynthetic complexes intact (Gombos et al. 1994). Surprisingly, Slr0006 was found to be strongly associated with the membranes.

The most interesting observation was that decrease in the concentration of $CaCl_2$ or $MgCl_2$ during the breaking of cells resulted in a shift of the Slr0006 protein from a (very tightly) membrane bound fraction to the soluble fraction (Paper III). As Flv2 and Flv4 also behaved similarly, it appears that the ambiguous location of Slr0006 is not unique for this

protein but may be shared with a group of other proteins yet to be characterized. Even so, the Sll0218 encoded by the *flv2-flv4* operon was unequivocally associated with a large thylakoid membrane complex. It is noteworthy that even the S1 protein of the 30S ribosomal subunit and most of the ribosomal RNA behaved similarly to Slr0006 both in the presence and in the absence of divalent cations (Paper IV). Although the mechanism behind this phenomenon is not currently known, description of the process will help cyanobacteriologists to avoid mis-localization of novel proteins due to variations in buffer composition.

The protein surface of the Flv2/Flv4 heterodimer model showed putative metal-binding sites that were also found in some homologous structures (Paper V). The metal ions on the previously studied structures originated from the crystallization solution, but some of the metal-binding residues are conserved or substituted with similar residues in our model. These residues may have a role in the cation-dependent association of Flv2 and Flv4 with the membrane fraction. However, it should be noted that the model of Slr0006 showed a positively charged cavity and no metal-binding surface, and yet it can be present in the membrane in the presence of divalent ions (Paper IV).

5.3. Slr0006 mimics YciO protein but is functionally related to protein synthesis

5.3.1. Slr0006 is not essential for cell survival

To understand the role of Slr0006 during C_i limitation we took the reverse genetics approach and constructed a deletion mutant lacking functional Slr0006 (Paper II). The growth of both WT and Δ*slr0006* strains was compared under different environmental conditions. The interruption of *slr0006* did not led to any visible phenotype in most of the conditions, indicating that Slr0006 is dispensable for cell survival and that its absence may be compensated for some unknown mechanism(s). The solubility of CO_2 varies greatly with both temperature and the partial pressure of the gas itself (Carroll et al. 1991). As the expression of Slr0006 was apparently downregulated at low temperature (Paper II), it appears that CO_2 serves as the primary C_i source required for the induction of the *slr0006* gene. Interestingly, when the cells were grown continuously at 18°C, the Δ*slr0006* strain showed severe defects in pigmentation and grew poorly. This phenotype is probably linked to the putative ribosome-related function of Slr0006 (see section 3.4, Paper IV)

Differential in-gel electrophoresis is a powerful gel-based quantitative proteomics approach that is routinely employed to reveal differences in the proteome profile (Marouga et al. 2005). To understand the functional role of Slr0006 under conditions of carbon limitation, proteomes from WT and from Δ*slr0006* were compared using DIGE analysis. The mutant strain in which the *slr0006* gene is inactivated consistently showed 2-fold downregulation of four unknown proteins (Slr0105, Sll6012, Slr7087 and Slr7090). Slr0105 is a protein encoded by gene present in the *Synechocystis* chromosome, whereas the other proteins Sll7087, Sll7090 and Slr6012 are encoded by the genes in the plasmids pSYSA and pSYSX (Table 1). The product of kanamycin resistance cassette was the only upregulated protein detected in Δ*slr0006* as compared to the control strain (Paper II). It

might be possible that upregulation of other proteins could have been detected under some other environmental conditions.

Table 2: List of genes encoding proteins with changes in the expression level in the *slr0006* mutant identified in DIGE

ORF	Gene Location	TM*	Length	Domain	Predicted function
slr6012	pSYSX:11315..13507	1	730	AAA_5	ATP binding
sll7087	pSYSA:82630..83436	ND#	268	RAMPs	CRISPR
sll7090	pSYSA:85199..88138	ND#	979	Cas	CRISPR
slr0105	Chr:2978508..2979896	ND#	462	CibA	Cobalamin synthesis

*Transmembrane helix; #ND- not detected; Length denotes number of amino acids

The predicted domains provide clues to the possible function of the identified proteins. Slr6012 protein contains a transmembrane helix and is predicted to be involved in the ATPase activity associated with some ABC transporters (Higgins 2001). It is worth mentioning that such kind of transporters are directly involved in C_i uptake in *Synechocystis* and were recognized in our proteomics study (Paper I). The proteins Sll7087 and Sll7090 possibly function as clustered regularly interspaced palindromic repeat (CRISPR)- associated (Cas) proteins. These CRISPR-Cas systems include a unique family of regulatory RNA that function as an adaptive immune system in bacteria and archeae to reject invading DNA molecules (Marraffini and Sontheimer 2010). Recently, it has been shown that cAMP signal transduction, a pathway induced under carbon-limiting conditions, is involved in the induction of the *cas* gene in *Thermos thermophiles* (Marraffini and Sontheimer 2010).

5.3.2. The slr0006 gene is negatively regulated by NdhR

LysR-type transcriptional regulators (LTTRs) activate the divergent transcription of linked target genes or unlinked regulons encoding proteins with extremely diverse functions (Schell 1993) and they comprise the largest family of prokaryotic regulatory proteins identified so far. The family has expanded to over 100 members that have been identified in diverse bacterial genera (Tropel and van der Meer 2004). LysR-family transcription factors, which resemble the Calvin Benson cycle regulator CbbR from proteobacteria have been implicated in the expression of C_i transporter genes in cyanobacteria (Woodger et al. 2007). In *Synechocystis*, NdhR is one of the LysR proteobacterial-type transcription factors, which has been shown to regulate the expression of many genes involved in inorganic carbon (C_i) uptake, including the *ndhF3/ndhD3/cupA*, *ndhD5/ndhD6*, and *sbtA/sbtB* operons (Wang et al. 2004). The possible DNA-binding region of NdhR has been proposed based on previous studies that showed a unique signature sequence $T(N_{11})A$ (Figge et al. 2001). Sequence analysis of the promoter region of Slr0006 showed the existence of the $T(N_{11})A$ motif. In addition, the deletion of *ndhR* resulted in the increased accumulation of the Slr0006 protein already under high CO_2

conditions. The binding of NdhR appears to regulate the expression of Slr0006 under carbon-limiting conditions, as in most other CCM-related genes (Paper IV).

5.3.3. Interruption of linear electron transfer chain suppresses Slr0006 induction

The observations that i) the *slr0006* gene exhibits strict regulation by NdhR, (Paper IV) and ii) it is strongly induced by low CO_2 (Paper I), prompted us to study the level of accumulation of Slr0006 in different mutants with apparent defects in carbon uptake and in the photosynthetic electron transport chain. In Δ*ndhD3*/Δ*ndhD4*, the carbon uptake system is not functional whereas the deletion of the *ndhD1/ndhD2* gene resulted in a low respiration rate and partially disrupted the PSI-dependent cyclic electron transport (Shibata et al. 2001). Δ*ndhB*, which lacks the assembled NDH-1 complex, shows defects in carbon uptake and cyclic electron transport (Ohkawa et al. 2000). In addition, the photosynthetic activity is hampered in M55 (Cournac et al. 2004) and in Δ*ndhD1/*Δ*ndhD2*. The induction of the Slr0006 protein was severely hindered in M55 and partially in Δ*ndhD1/*Δ*ndhD2,* while it was slightly up regulated in Δ*ndhD3*/Δ*ndhD4* cells (Paper IV). These results indicate that upregulation of the *slr0006* gene is not required to compensate for an impaired function of the CCM complexes. This conclusion is supported by the results showing that the expression of the *slr0006* gene did not change in Δ*ndhD3,* Δ*ndhD4,* Δ*cupA,* Δ*cupA/*Δ*cupB* and Δ*sbtA* strains (Paper II), which are deficient in carbon uptake (Ogawa 1992; Ohkawa et al. 2000; Shibata et al. 2001).

Changes in the intersystem redox state of the electron transport chain have been implicated in controlling the transcriptional activators of photosynthetic gene expression in cyanobacteria (Sippola and Aro 2000; Hihara et al. 2003). Slr0006 induction was completely prevented in the presence of inhibitors DCMU or DBMIB and in cells incubated in darkness. These results clearly indicate that photosynthetic electron transfer *per se* is a prerequisite for induction of the Slr0006 protein (Paper IV).

5.3.4. Slr006 model resembles YciO protein structure from E.coli

The Slr0006 sequence shows the presence of a conserved domain that belongs to the Sua5/YciO/YrdC protein superfamily. The domain extends throughout the protein sequences. Slr0006 sequence analysis showed neither the presence of transmembrane helix or signal sequences. The members of this family have high structural similarity, but they have relatively low sequence identity (Jia et al. 2002; Fu et al. 2010). However, it is widely accepted that the conservation in structure is far higher than the sequence itself (Illergard et al. 2009). The 3D models of Slr0006 show a typical α/β twisted open-sheet structure with both parallel and anti-parallel β-strands, which is a characteristic feature of the members of this protein family. Proteins that belong to this family are characterized by a positively charged cleft which binds dsRNA (Teplova et al. 2000; Jia et al. 2002; Yee et al. 2002; Agari et al. 2008) indicating that Slr0006 might also bind RNA as well. Co-localisation of Slr0006 with the S1 ribosomal subunit and rRNA indicates that indeed Slr0006 might indeed function together with ribosomes. The amino acids Lys50 and Arg52 in YrdC were used to distinguish between the YrdC and YciO families. In contrast to YrdC protein, YciO proteins have a positively charged amino acid at position 50 and a hydrophobic

amino acid in position 52. The Slr0006 contains Lys59 and Phe61 in the corresponding positions, which indicates that Slr0006 resembles more the YciO structure than YrdC structure. Based on complementation studies, El Yacoubi et al. (2009) suggested that only Sua5 and YrdC are involved in t^6A modification, which is clearly evident from the fact that *yciO* deletion mutant could not compensate for the loss of t^6A. Moreover it has been found that deletion of *yciO* leads to enormous accumulation of glycogen inside the *E. coli* cells (Montero et al. 2009). However, the deletion of the *slr0006* gene did not alter the internal level of glycogen (data not shown), suggesting that despite the structural similarities, Slr0006 may not function as YciO protein (Paper IV).

5.4. Flv2 and Flv4 form a heterodimer and optimize energy transfer from PBS to PSII

Cyanobacteria show the presence of multiple genes encoding FDPs: These are categorized as being either FlvA (including Flv2) or FlvB (including Flv4) based on the sequence analysis (Zhang et al. 2009). The FDPs studied so far function as homodimers or homotetramers *in vitro* (Vicente et al. 2008; Vicente et al. 2009). Also, Flv2 and Flv4 appear to form a heterodimer, as demonstrated by (1) structural modelling (2) stability of the proteins in mutants and by (3) by BN-PAGE analysis (Paper V).

Phycobilisomes (PBS) are the major antenna for light harvesting in cyanobacteria which is attached to the thylakoid membrane on the cytoplasmic surface (Grossman et al. 1993). PBSs are generally associated with the reaction centres under normal growth conditions and uncoupled PBSs are present in very low amounts (Mullineaux and Holzwarth 1991). Energy transfer from PBS to the reaction centres of the mutants were studied by the fluorescence spectra at 77K. All *flv* inactivation strains showed significant increase at 685 nm. The fluorescence emission at 685 nm in the *flv* mutants results from partial uncoupling of the terminal emitter of PBS from PSII. Interestingly, the *flv* mutants exhibit higher amounts of decoupled PBS under low C_i compared to WT. The PBS are connected to PSII and PSI through flexible interactions (Barber et al. 2003). Especially the ApcE, a linker protein connecting the phycobilisome and the thylakoid membrane has been shown to be crucial (MacColl 1998). These results allow us to presume that the Flv2/Flv4 heterodimer is involved in the interaction between PBS and PSII at low C_i (Paper V).

Apart from being induced under low C_i conditions, the *flv2* and *flv4* genes have been shown to respond to high light conditions (Hihara et al. 2001; Zhang et al. 2009). Previous studies have shown possible role of Flv2 and Flv4 in photoprotection of PSII under low CO_2 condition (Zhang et al. 2009). The mutant strains showing low efficiency of energy transfer from PBS to PSII, due to uncoupled PBS, suggests that the Flv2/Flv4 heterodimer is involved in dynamic interaction between PBS and PSII under low C_i condition. The changes in dimer to monomer ratio of PSII in *sll0218*- inactivated strains may suggest that Sll0218 cause subtle changes in the confirmation of the PSII dimer leading to the energy transfer between PBS and PSII. Furthermore, our results show a clear inhibition of PSII oxygen evolution in cells grown under high CO_2 - both WT and all *flv* mutants - when DCBQ is used as an external electron acceptor. This result suggests the presence of a novel electron transfer route from PSII to the Flv2/Flv4 heterodimer (Paper V, Fig .9).

CONCLUSIONS

Taken together, *Synechocystis* serves as an excellent model to study the changes in proteins during CO_2 starvation. This knowledge combined with other CCM related (proteomics) studies will help to understand adaptive mechanisms in other organisms, including higher plants.

C_i starved Synechocystis cells exhibit changes in protein profile

- Investigation of the proteome changes in *Synechocystis* under C_i limiting conditions showed major changes in the proteins involved in inorganic carbon uptake, CO_2 fixation, nitrogen transport and assimilation.
- The initial response started with induction of inorganic carbon transporters followed by the proteins involved in CO_2 uptake and fixation
- Carbon starvation downregulated nitrogen transport to maintain the carbon-nitrogen balance.
- Our study on the C_i limitation revealed several hypothetical proteins of unknown functions

Novel proteins involved in CO_2 limitation

- Slr0006 and FDP (Sll0217 and Sll0219) showed drastic upregulation during carbon limitation.
- These proteins are negatively regulated by NdhR and show dramatic changes of location in the presence or in the absence of divalent ions.
- Presence of a positively charged cavity and co-localization of Slr0006 with ribosomal proteins and rRNA would suggest that Slr0006 plays a role in ribosome associated processes.
- The photosynthetic electron transfer chain regulates the expressions of Slr0006.
- FDPs show possible formation of heterodimer and additional cofactor binding.
- FDPs participate in energy transfer from PBS to PSII and protect PSII against photodamage.

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