Role of specific subunits for stability and stress resistance of cyanobacterial photosystem II

Die Rolle spezifischer Untereinheiten für die Stabilität und Stressresistenz von cyanobakteriellem Photosystem II

Dissertation zur Erlangung des Grades eines Doktors der Naturwissenschaften der Fakultät für Biologie an der Internationalen Graduiertenschule Biowissenschaften der Ruhr-Universität Bochum

Angefertigt am Lehrstuhl für Biochemie der Pflanzen

Vorgelegt von
Julia Eva-Maria Lax
aus Minden

Bochum
November 2005
Für Luise

Wenn Gott will, ist eine Nachtmütze bombenfest
(Aus Schlesien)
Teile dieser Arbeit wurden bereits veröffentlicht:


Referent: Prof. Dr. Matthias Rögner, Lehrstuhl für Biochemie der Pflanzen
Koreferent: Prof. Dr. Egbert J. Boekema, Department of Electron microscopy
Tag der Abgabe: 30. November 2005
## Contents

1 Introduction.......................................................................................................... 1

1.1 Cyanobacteria.................................................................................................. 1

1.2 Morphology of the cyanobacterial cell .......................................................... 1

1.2.1 The cyanobacterium *Thermosynechococcus elongatus*-BP1................ 5

1.3 Photosynthesis............................................................................................... 6

1.4 Photosynthetic and respiratory electron transport in cyanobacteria .......... 7

1.4.1 Photosynthetic electron transport.......................................................... 8

1.4.2 Respiratory electron transport reactions ............................................... 9

1.5 Photosystem II.............................................................................................. 10

1.6 Interrelationship between iron homeostasis and oxidative stress .......... 19

1.7 Adaptation of cyanobacteria to iron limitations ........................................... 20

1.8 Iron limitation and photosynthesis .............................................................. 22

1.9 Iron stress-induced protein A (IsiA)........................................................... 23

1.10 Iron deficiency-induced protein A (IdiA).................................................... 25

1.11 Aims of this work........................................................................................ 29

2 Material and Methods...................................................................................... 31

2.1 Biochemical methods.................................................................................... 31

2.1.1 Sterilisation.......................................................................................... 31

2.1.2 Bacterial strains................................................................................... 31

2.1.3 Cultivation of *T. elongatus* cells........................................................ 31

2.1.4 Estimation of the cell density............................................................... 33

2.1.5 *T. elongatus* growth curves ................................................................. 33

2.1.6 Harvesting of *T. elongatus* cells and preparation of thylakoid membranes ................................................................. 33

2.1.7 PS II isolation from *T. elongatus* ........................................................ 34

2.1.8 Other chromatographic methods.......................................................... 36

2.1.9 Sucrose density gradient centrifugation ............................................. 37

2.1.10 Concentration of proteins.................................................................. 37

2.1.11 TCA precipitation of proteins according to Peterson (1979).............. 37

2.1.12 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)....................... 38

2.1.13 Green native gradient Gel................................................................... 40

2.1.14 Western blot analysis.......................................................................... 42

2.1.15 Cross-linking of isolated PS II complexes ......................................... 44

2.2 Biophysical methods.................................................................................... 45

2.2.1 UV/VIS Spectroscopy.......................................................................... 45

2.2.2 Oxygen evolving activity measurements .......................................... 46

2.2.3 Low temperature fluorescence spectroscopy....................................... 47

2.2.4 Peptide Mass Fingerprint (PMF)........................................................ 47

2.2.5 MALDI-ToF of intact protein complexes............................................. 49

2.2.6 Electron microscopy............................................................................. 49

3 Results............................................................................................................. 51

3.1 Isolation of PS II complexes from *T. elongatus* wild type ................. 52

3.2 Characterisation of isolated PS II complexes .......................................... 54

3.2.1 Determination of the absorbance characteristics .................................. 54

3.2.2 Determination of the oxygen evolving activity ................................... 55

3.2.3 Analysis of the oligomerisation state of isolated PS II complexes....... 55

3.2.4 Subunit composition of isolated PS II complexes.................................. 57

3.2.5 Pigment analysis of isolated PS II complexes...................................... 62
3.3 Partial characterisation of a ΔPsbK mutant ................................................ 64
  3.3.1 Growth curves of *T. elongatus* wild type and ΔPsbK ....................... 65
  3.3.2 77 K pigment fluorescence measurements of whole cells ............... 65
  3.3.3 Pigment analysis of whole cells .................................................... 67
  3.3.4 Sucrose density gradient centrifugation of solubilised thylakoid membranes ................................................................. 68
  3.3.5 Comparative characterisation of isolated PS II complexes from *T. elongatus* wild type and the ΔPsbK mutant ................................. 69
3.4 Partial characterisation of a ΔPsbTc mutant .............................................. 73
  3.4.1 Growth of the ΔPsbTc mutant .......................................................... 74
  3.4.2 77 K pigment fluorescence of whole cells ....................................... 74
  3.4.3 Pigment analysis of whole cells .................................................... 75
  3.4.4 Sucrose density gradient centrifugation of solubilised thylakoid membranes ................................................................................. 76
  3.4.5 Comparative characterisation of isolated PS II complexes from *T. elongatus* wild type and the ΔPsbTc mutant ................................. 80
3.5 Characterisation of PS II from *T. elongatus* cells grown under iron deficiency 84
4 Discussion ......................................................................................................... 93
  4.1 Part 1: Role of specific subunits in PS II of *T. elongatus* ...................... 93
    4.1.1 Characterisation of isolated PS II complexes from *T. elongatus* wild type 93
    4.1.2 Pigment analysis via reversed phase chromatography .................... 97
    4.1.3 Electron microscopy for structural studies of PS II ....................... 98
    4.1.4 Role of the PsbK subunit .............................................................. 99
    4.1.5 Role of the PsbTc subunit ............................................................. 104
  4.2 Part 2: The effect of iron deficiency on PS II from *T. elongatus* ...... 109
    4.2.1 How does PS II cope with stress? .......................................... 113
  4.3 Future prospects ...................................................................................... 115
5 Summary ........................................................................................................ 117
6 Zusammenfassung ........................................................................................... 118
7 References ...................................................................................................... 120
8 Abbreviations ............................................................................................... 133
9 Curriculum vitae ............................................................................................ 135
1 Introduction

1.1 Cyanobacteria

Cyanobacteria are a remarkable group of organisms. In evolutionary terms they form the link between heterotrophically growing eubacteria and higher plants. Up to now, approximately 2000 species of cyanobacteria are known. Because of their high capability of adaptation, their occurrence is nearly ubiquitous. They can be found in fresh water (e.g. Oscillatoria-, Synechocystis-, and Synechococcus species), as well as in marine environments (e.g. Trichodesmium and Synechococcus). Even at arid places, for example, barks or limestones, they are present. In addition, thermophilic species exist (Thermosynechococcus), which are able to grow in hot springs, and psychrophilic species (Chroococcidiopsis) have been described, which can be found in the Arctica or Antarctica.

According to the endosymbiotic theory (Margulis, 1970), cyanobacteria are the ancestors of chloroplasts. During evolution they were incorporated by heterotrophically growing eukaryotes and in the course of time the cyanobacterial genome was partially integrated into the host genome. Therefore, chloroplasts contain still parts of the original DNA and their ribosomes correspond to cyanobacterial ribosomes (Stackebrandt, 1985).

Because of their biological variety, it is difficult to carry out an universally applicable classification. Therefore, previous classifications are based on morphologically characteristics.

Cyanobacteria are an excellent research model system especially for oxygenic photosynthesis and for adaptational processes. They grow relatively fast, are easy to handle, and can be manipulated genetically. Moreover, the genomes of many cyanobacteria such as Synechocystis sp. PCC 6803 (Kaneko et al., 1996) and Thermosynechococcus elongatus BP-1 (Nakamura et al., 2002) are completely sequenced. Isolation of stable protein complexes from thermophilic species facilitates various structural investigations.

1.2 Morphology of the cyanobacterial cell

Figure 1.1 shows a schematic model of a typical vegetative cyanobacterial cell. The cyanobacterial cell is surrounded by a cell wall consisting of an outer membrane, a peptidoglycan layer, and the cytoplasmic membrane. Many cyanobacteria have an
additional layer, the glycocalyx, which surrounds the cell wall. The function of this glycocalyx is on the one hand the protection against desiccation and on the other hand it has the function of a diffusion barrier of dissolved substances in aqueous environments. Thus, it plays an important role in the assimilation of nutrients. The glycocalyx is tightly associated with the outer membrane, which mainly consists of proteins and lipopolysaccharides. Additionally, it contains carotenoids and lipids which are not connected to carbohydrates (Omata and Murata, 1984, Jürgens and Weckesser, 1985, Murata and Omata, 1988). Furthermore, there are specific transport proteins, which are permeable to specific nutrients.

![Figure 1.1: Cross section of a typical cyanobacterial cell (such as *Synechocystis*)](Van den Hoek et al., 1995, modified).

The peptidoglycan layer subdivides the periplasmatic space into an outer and an inner compartment. This layer mainly provides mechanical stability. Moreover, it is likely that the inner and outer compartment have specific functions. The peptidoglycan layer of cyanobacteria has structural similarity to that of Gram-negative prokaryotes, but it has a larger diameter. Jürgens and Weckesser (1985) classified the cyanobacterial peptidoglycan layer as an independent layer in comparison with the peptidoglycan layer of typical Gram-negatives. The outer membrane is connected with the cytoplasm membrane by protein complexes. Outer membrane, peptidoglycan layer, and cytoplasm membrane interact in the transport of essential compounds into the cell. Apart from the various transport systems the cytoplasm membrane also contains a fully functional respiratory
Introduction

electron transport chain with components such as the cytochrome oxidase and ATP synthase (Sherman et al., 1994).

Within the cell, another membrane system, the thylakoid membrane, is located, which is partially connected to the cytoplasmic membrane. It contains the components of the photosynthetic as well as a second respiratory electron transport chain, including ATP synthase complexes. Both share in part identical components.

The cytoplasm is the place of major metabolic reactions such as nitrogen and glucose metabolism, and it contains small gas vacuoles, which allow the regulation of buoyancy in water (van den Hoek et al., 1995).

Because cyanobacteria have no eukaryotic type of compartmentation, it is important that they are able to convert a surplus of metabolic substances into osmotically neutral forms. This is achieved by the formation of different granules, such as carboxysomes, phosphate granules or glycogen granules. The nucleoplasm in the centre of the cells contains the cyanobacterial chromosomes, which are unbound and free of histones. In addition, most cyanobacteria contain plasmids of different size.

A further characteristic feature of some cyanobacteria is their pigmentation which in part depends on the light conditions (chromatic adaptation). Besides chlorophyll a (chl a) and different carotenoids which mainly play a role in the protection against light stress, they contain phycobilins (Sidler, 1994). Phycobilins are linear tetrapyrroles which are connected to phycobiliproteins via a thioester bond. They function as the primary light harvesting complexes of photosystem I and II in cyanobacteria and Rhodophyceae. Phycobiliproteins absorb light in a spectral range of 450 nm (green) to 665 nm (red). Therefore, cyanobacteria have, for instance, an advantage in deeper water, where only green and blue light, which cannot be efficiently absorbed by green algae, is available.

In the cyanobacterial cell phycobiliproteins form multimeric complexes, the phycobilisomes, which are attached to the thylakoid membrane (see Figure 1.2). In most cyanobacteria the phycobilisomes consist of a bi- or tricylindrical core with six peripheral rods in a hemidiscoidal arrangement.
Figure 1.2: Structure of a phycobilisome. (Heldt, 1996, modified) Each component consists of three α- and three β-subunits. The phycobilisome is connected onto the thylakoid membrane via linker polypeptides. Three aggregates consisting of four to five (αβ)₃-subunits form the core, to which cylindrical rods are bound.

The main components are allophycocyanin ($A_{\text{max}}$ 650 nm) in the centre of the core complex followed by phycocyanin ($A_{\text{max}}$ 620 nm) and phycoerythrin ($A_{\text{max}}$ 565 nm). While allophycocyanin and phycocyanin are present in all known species, phycoerythrin is absent in some species, such as *Synechococcus elongatus* PCC 7942 or *Synechocystis* sp. PCC 6803 (Bald et al., 1996). Because of the close packaging of the pigments, the transfer of excitons directly to chl a can occur with a high efficiency (Sidler, 1994).

Phycobilisomes also contain linker polypeptides which play a role in the assembly of the phycobilisomes.

The phycobilisomes are attached preferentially to PS II, but similar to LHC II of higher plants, they can transfer excitation energy also to PS I under changing light conditions. This process is called state-transition (Mullineaux and Allen, 1990). The mechanism of the movement of phycobilisomes is only poorly understood so far (see Figure 1.3).
Besides their function as accessory pigments, phycobilisomes also serve as nitrogen store (Allen and Smith, 1969, Wymann et al., 1985), especially in case of some **Synechococcus** species, which lack the genes for cyanophycin synthesis (Allen et al., 2005).

### 1.2.1 The cyanobacterium *Thermosynechococcus elongatus*-BP1

*Thermosynechococcus elongatus*-BP1 (*T. elongatus*) is a unicellular thermophilic fresh water cyanobacterium which shows optimally growth at a temperature around 55°C. It was first isolated from the Beppu hot spring in Japan (Yamaoka, 1978). This organism is an ideal model organism for the study of photosynthetic processes and the preparation of stable membrane protein complexes. Up to now the crystal structures of PS I (Jordan et al., 2001) and PS II (Zouni et al., 2001; Ferreira et al., 2004) could be gained from this organism. *T. elongatus* is easy to cultivate, has a relatively short generation time and the genome has been fully sequenced (Nakamura et al., 2002). This provides a basis for broad genome analyses and facilitates the strategies of site-directed mutagenesis.
1.3 Photosynthesis

The process of photosynthesis allows photoautotrophic organisms to use the energy of the sunlight to synthesise high-energy organic substances out of low-energy inorganic compounds. Two different groups of photosynthetic organisms can be distinguished. The first group contains anoxygenic photosynthetic organisms such as purple bacteria, green bacteria, and heliobacteria. They have only one photosystem and use anorganic electron donors with a low redox potential such as reduced sulphur compounds or organic compounds, such as succinate, coupled to a reverse electron flow to NAD, utilising the energy generated by the cyclic electron flow around the reaction centre (Drews and Imhoff, 1991).

The second group consists of oxygenic photosynthetic organisms like cyanobacteria, green algae, and higher plants. These organisms contain two photosystems and use water as electron donor, which is combined with the evolution of molecular oxygen. During the light reactions of oxygenic photosynthesis, sun energy is converted into chemical energy like ATP and reducing equivalents (NADPH+H⁺). These compounds are used in the Calvin cycle to synthesise glucose from ribulose-1,5-bisphosphate and CO₂.

Equation 1.1 summarises the overall photosynthetic reaction.

\[
6 \text{CO}_2 + 12 \text{H}_2\text{O} \rightleftharpoons \text{C}_6\text{H}_{12}\text{O}_6 + 6 \text{H}_2\text{O} + 6 \text{O}_2 \quad \text{Equation 1.1}
\]

\[\Delta G = 2868 \text{kJ/mol}\]
1.4 Photosynthetic and respiratory electron transport in cyanobacteria

Figure 1.4: Components of the photosynthetic and respiratory electron transport chain in cyanobacteria. CM = cytoplasmic membrane, TM = thylakoid membrane, PC = plastocyanin, PQ = plastoquinone, Fd = ferredoxin, FNR = ferredoxin NADP oxidoreductase, SDH = succinate dehydrogenase (Complex II), NDH-1 = NAD(P)H-dehydrogenase Typ 1 (Complex I), Cyt b6f = cytochrome b6f complex, Cyt bd: cytochrome bd oxidase, Cyt aa3 = cytochrome aa3 complex, Cyt c\textsubscript{553} = cytochrome c\textsubscript{553}, PS 1 = photosystem I, PS 2 = photosystem II (Teuber et al., 2001)

In contrast to higher plants, where the components of the photosynthetic and the respiratory electron transport chain are located in different compartments (chloroplasts and mitochondria), cyanobacterial thylakoid membranes contain both components. However, in the cytoplasmic membrane presumably only the components of the respiratory electron transport chain are functional (Figure 1.4) while components of the photosynthetic chain like the photosynthetic reaction centre complexes are proposed to represent early steps of biogenesis which are then transferred to the thylakoid membrane (Zak et al., 2001). Additionally, essential components of the electron transport chain in the thylakoid membranes (plastoquinone, the cytochrome b6f complex, and cytochrome c\textsubscript{553} /plastocyanin) are shared between respiratory and photosynthetic electron transport (Scherer, 1990, Vermaas et al., 2001).
1.4.1 Photosynthetic electron transport

The two photosystems of the oxygenic-type in the cyanobacterial photosynthetic apparatus are linked by a linear electron transport system (Figure 1.5), consisting of the cytochrome $b_{6}f$ complex and the mobile electron carriers plastoquinone, cytochrome $c$ (and/or plastocyanin) as well as ferredoxin, and the ferredoxin:NADP oxidoreductase.

While PS II complexes can exist as monomers and dimers in the thylakoid membrane, PS I can exist in the monomeric form (such as in higher plants, algae, and cyanobacteria, Golbeck, 1994) as well as in a trimeric form (in cyanobacteria, Karapetyan et al., 1999).

In the light reactions water is oxidised to molecular oxygen and the electrons are used to reduce NADP$^{+}$. These reactions also lead to the formation of an electrochemical proton gradient (Proton-motif force) via the thylakoid membrane which is the driving force for ATP synthesis (Lengeler et al., 1999). NADPH+H$^{+}$ and ATP are subsequently used in the Calvin cycle reactions (Heinecke, 2001).

**Linear photosynthetic electron flow:**

$H_{2}O$ as electron donor

$H_{2}O \rightarrow$ PS II $\rightarrow$ PQ pool $\rightarrow$ Cyt $b_{6}f$ $\rightarrow$ PC/Cyt $c_{553}$ $\rightarrow$ PS I $\rightarrow$ Fd $\rightarrow$ FNR $\rightarrow$ NADP$^{+}$

Figure 1.5: Linear electron transport in cyanobacteria with water as electron donor (Michel, 2003).

In addition to this linear photosynthetic electron transport, a cyclic electron transport around PS I exists. However, up to now it is not clear, how many different pathways exist and which components, besides PS I, are involved. Figure 1.6 shows the postulated pathways.

**Cyclic photosynthetic electron flow around photosystem I:**

NDH-1 $\rightarrow$ PQ pool $\rightarrow$ Cyt $b_{6}f$ $\rightarrow$ PC/Cyt $c_{553}$ $\rightarrow$ PS I

PS I $\rightarrow$ Fd $\rightarrow$ FNR $\rightarrow$ NADPH

PQ pool and/or Cyt $b_{6}f$ $\rightarrow$ PC/Cyt $c_{553}$ $\rightarrow$ PS I

Figure 1.6: Cyclic electron transport routes around PS I in cyanobacteria (Michel, 2003).
1.4.2 Respiratory electron transport reactions

During respiration organic carbon compounds become oxidised. Glycolysis, tricarboxylic acid cycle and the respiratory electron transport chain lead to ATP synthesis, formation of CO$_2$, and replenishment of C-metabolites. In cyanobacteria, the respiratory electron transport chain is located in the thylakoid membrane as well as in the cytoplasmic membrane. In the thylakoid membrane, the NDH-1 complex, the succinate dehydrogenase (SDH, Vermaas, 2001), other substrate dehydrogenases, the Cyt $b_6/f$ complex as well as the soluble electron carriers PC/Cyt $c_{553}$/Cyt$_M$, and the terminal oxidases Cyt c $aa_3$ and Cyt $bd$ (Vermaas, 2001) are involved (Figure 1.7).

**Respiratory electron transport in thylakoid membranes:**

\[
\text{SH} \rightarrow \text{NDH-1} \rightarrow \text{PQ pool} \rightarrow \text{Cyt } b_6/f \rightarrow \text{PC/Cyt } c_{553}/\text{Cyt}_M \rightarrow \text{Cyt c oxidase } aa_3\text{-type} \rightarrow \text{O}_2
\]

\[
\text{SH} \rightarrow \text{Substrate-DH} \rightarrow \text{PQ pool} \rightarrow \text{Cyt } b_6/f \rightarrow \text{PC/Cyt } c_{553}/\text{Cyt}_M \rightarrow \text{Cyt c oxidase } aa_3\text{-type} \rightarrow \text{O}_2
\]

**Figure 1.7: Respiratory electron transport chain in thylakoid membranes.** In addition to Cyt $c_{553}$, Cyt $c_M$ may participate in electron transport to terminal oxidases (Michel, 2003).

The cyanobacterial NDH-1 complex is an analogue of the eubacterial and eukaryotic mitochondrial respiratory complex I. In cyanobacterial cells, different types of NDH-1 complexes exist, each with different NhD and NhF subunits, performing different functions (Ohkawa et al., 2000; Herranen et al., 2004) A common characteristic of cyanobacteria is that the $ndhD$ and the $ndhF$ ($ndhF1,3-4$) genes exist as gene families with six ($ndhD1-6$) and three members, respectively, although other $ndh$ genes exist as single copy genes (Kaneko et al., 1996). Additionally, NDH-2, a second type of NADH dehydrogenase occurs in cyanobacteria, consisting of a single polypeptide chain. NDH-2 does not participate in the respiratory transport chain and was suggested to play a major role as redox sensor, for instance for the redox state of the PQ pool (Howitt et al., 1999).

**Respiratory electron transport in cytoplasmic membranes:**

\[
\text{SH} \rightarrow \text{Substrate-DH} \rightarrow \text{PQ pool} \rightarrow \text{Cyt } b_6/f \rightarrow \text{PC/Cyt } c_{553} \rightarrow \text{Cyt } c \text{ aa}_3/\text{Cyt } bd \text{ oxidase} \rightarrow \text{O}_2
\]

**Figure 1.8: Respiratory electron transport chain in cytoplasmic membranes.** In addition to Cyt $c_{553}$, Cyt $c_M$ may participate in respiratory electron transport to terminal oxidases (Michel, 2003).

Since the respiratory electron transport chain in the cytoplasmic membrane most likely lacks NDH-1 complexes (Ohkawa et al., 2001), the major electron donors for
Introduction

the cytoplasmic respiratory electron transport chain are still unknown. Possible candidates are succinate oxidised \textit{via} the SDH (Vermaas, 2001), pyruvate oxidised \textit{via} the pyruvate dehydrogenase (Engels et al., 1997), and L-amino acids \textit{via} L-amino acid dehydrogenases (Bockholt et al., 1996) or NAD(P)H \textit{via} NDH-2 (Figure 1.8).

1.5 Photosystem II

Since PS II is the central part of my work, the state of the art will be presented in more detail.

![Figure 1.9: Cyanobacterial photosystem II complex with attached phycobilisome (Michel and Pistorius, 2003. D1 = PsbA, D2 = PsbD, PCY = phycocyanin, AP = allophycocyanin, CP43 = PsbC, CP47 = PsbB, Cyt b\textsubscript{559} = cytochrome b\textsubscript{559} subunit, MSP = PsbO, P\textsubscript{680} = reaction center chlorophyll of PS II Pheo = pheophytin, Q\textsubscript{A} = tightly bound quinone, Q\textsubscript{B} = loosely bound quinone, Z = Tyr161 residue on PsbA.]

PS II is a membrane protein complex, which carries out the light-driven oxidation of water and the reduction of bound quinones (Figure 1.9).

According to its function, it can be classified as a water:plastoquinone oxidoreductase. The primary electron donor or reaction centre chlorophyll of PS II is called P\textsubscript{680}. After illumination an electron is transferred from the excited state of P\textsubscript{680} to a pheophytin (Pheo). The radical pair P\textsubscript{680}+/Pheo\textsuperscript{-} is formed. P\textsubscript{680}+ is the strongest oxidising redox component of PS II. It is reduced by Z, a tyrosine residue in position 161 on the D1 protein (Ke et al., 2001). Pheo\textsuperscript{-} donates the electron to a tightly bound quinone (Q\textsubscript{A}), located on the D2 protein, which then reduces a loosely bound exchangeable quinone (Q\textsubscript{B}), located in a pocket on the D1 protein. The function of the non-heme iron located between D1 and D2 is still unknown. Each Q\textsubscript{B} molecule is
able to accept two electrons from water and two protons from the stroma site. Thus, it represents a switch from a one electron to a two electron reaction. The reduced plastoquinone (PQH$_2$) is released into the membrane matrix. At the catalytic site of water oxidation, a cluster of four manganese atoms (Mn) cycles through a series of oxidation states designated as S$_0$–S$_4$ cycle (Joliot et al., 1969; Kok et al., 1970). After the extraction of four electrons from two water molecules, using calcium (Ca$^{2+}$) and chloride (Cl$^-$) as inorganic cofactors, one oxygen molecule is released. Thus, the Mn cluster accumulates four positive charges before the water oxidation process takes place.

Although the minimal oxygen evolving PS II complex contains only seven major proteins (D1, D2, CP43, CP47, Cyt b$_{559}$ α and β subunit, and PsbO) and several other small peptides below 5 kDa (Oelmüller et al., 1999), the native and functional PS II complex contains more than 25 subunits which are listed in Table 1.1 for PS II from _T. elongatus_. The function of many of the low molecular weight proteins is still unknown.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Subunit</th>
<th>Molecular mass [kDa]</th>
<th>TMH</th>
<th>Location</th>
<th>Possible function</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>psbA</em></td>
<td>D1</td>
<td>38</td>
<td>5</td>
<td>Integral</td>
<td>RC core; coordination of P$_{680}$, Pheo, Q$_B$, Y$_Z$, non-heme iron</td>
</tr>
<tr>
<td><em>psbB</em></td>
<td>CP47</td>
<td>56.3</td>
<td>6</td>
<td>Integral</td>
<td>Internal antenna; binding of Chl$_a$</td>
</tr>
<tr>
<td><em>psbC</em></td>
<td>CP43</td>
<td>50.1</td>
<td>6</td>
<td>Integral</td>
<td>Internal antenna; binding of Chl$_a$</td>
</tr>
<tr>
<td><em>psbD</em></td>
<td>D2</td>
<td>39.2</td>
<td>5</td>
<td>Integral</td>
<td>RC core; binding of Q$_A$</td>
</tr>
<tr>
<td><em>psbE</em></td>
<td>Cyt b$_{559}$$\alpha$</td>
<td>9.2</td>
<td>1</td>
<td>Integral</td>
<td>Coordination of b-type heme; involved in photoprotection and assembly</td>
</tr>
<tr>
<td><em>psbF</em></td>
<td>Cyt b$_{559}$$\beta$</td>
<td>4.4</td>
<td>1</td>
<td>Integral</td>
<td>Coordination of b-type heme; involved in photoprotection and assembly</td>
</tr>
<tr>
<td><em>psbH</em></td>
<td>PsbH</td>
<td>7.7</td>
<td>1</td>
<td>Integral</td>
<td>Involved in Q$_A$ to Q$_B$ transfer; PS II repair cycle</td>
</tr>
<tr>
<td><em>psbl</em></td>
<td>Psbl</td>
<td>4.2</td>
<td>1</td>
<td>Integral</td>
<td>Involved in dimerisation; photoprotection</td>
</tr>
<tr>
<td><em>psbj</em></td>
<td>Psbj</td>
<td>4.1</td>
<td>1</td>
<td>Integral</td>
<td>Putatively involved in assembly</td>
</tr>
<tr>
<td><em>psbk</em></td>
<td>Psbk</td>
<td>4.3</td>
<td>1</td>
<td>Integral</td>
<td>Putatively involved in stabilisation and Pigment binding</td>
</tr>
<tr>
<td><em>psbl</em></td>
<td>Psbl</td>
<td>4.4</td>
<td>1</td>
<td>Integral</td>
<td>Involved in Q$_A$ function and electron transfer at donor side</td>
</tr>
<tr>
<td><em>psbm</em></td>
<td>Psbm</td>
<td>3.7</td>
<td>1</td>
<td>Integral</td>
<td>Unknown</td>
</tr>
<tr>
<td><em>psbn</em></td>
<td>Psbn</td>
<td>4.7</td>
<td>1</td>
<td>Integral</td>
<td>Unclear if present in cyanobacteria</td>
</tr>
<tr>
<td>Gene</td>
<td>Protein</td>
<td>Mw (kDa)</td>
<td>Membrane Position</td>
<td>Function</td>
<td></td>
</tr>
<tr>
<td>------</td>
<td>---------</td>
<td>----------</td>
<td>-------------------</td>
<td>----------</td>
<td></td>
</tr>
<tr>
<td><em>psbO</em></td>
<td>PsbO</td>
<td>33</td>
<td>Peripher</td>
<td>Involved in oxygen evolution, stabilises Mn-cluster</td>
<td></td>
</tr>
<tr>
<td><em>psbP</em></td>
<td>PsbP</td>
<td>23</td>
<td>Peripher</td>
<td>Involved in assembly and stabilisation of Cl⁻ and Ca⁺</td>
<td></td>
</tr>
<tr>
<td><em>psbQ</em></td>
<td>PsbQ</td>
<td>16.5</td>
<td>Peripher</td>
<td>Involved in assembly and stabilisation of Cl⁻ and Ca⁺</td>
<td></td>
</tr>
<tr>
<td><em>psbT</em></td>
<td>PsbT</td>
<td>4</td>
<td>Integral</td>
<td>Involved in dimerisation, recovery of PS II activity after photoinhibition</td>
<td></td>
</tr>
<tr>
<td><em>psbU</em></td>
<td>12 kDa protein</td>
<td>12</td>
<td>Peripher</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td><em>psbV</em></td>
<td>Cyt c₅₅₀</td>
<td>17.8</td>
<td>Peripher</td>
<td>Coordinates c-type heme, Involved in water oxidation</td>
<td></td>
</tr>
<tr>
<td><em>psbX</em></td>
<td>PsbX</td>
<td>4.1</td>
<td>Integral</td>
<td>Putatively involved in Qₐ function</td>
<td></td>
</tr>
<tr>
<td><em>psbY</em></td>
<td>PsbY</td>
<td>4</td>
<td>Integral</td>
<td>Involved in Ca²⁺-binding and integrity of water oxidising complex</td>
<td></td>
</tr>
<tr>
<td><em>psbZ</em></td>
<td>PsbZ</td>
<td>12</td>
<td>Integral</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td><em>psb27</em></td>
<td>Psb27</td>
<td>11</td>
<td>Peripher</td>
<td>Putatively involved in assembly of PS II</td>
<td></td>
</tr>
<tr>
<td><em>psb28</em></td>
<td>Psb28</td>
<td>0</td>
<td>Peripher</td>
<td>Unknown</td>
<td></td>
</tr>
</tbody>
</table>

Table 1.1: Genes and proteins of cyanobacterial PS II.

Recently, Ferreira et al. (2004) published the crystal structure of PS II from the cyanobacterium *T. elongatus* at a resolution of 3.5 Å which is shown in Figure 1.10.
For the first time it was possible to assign 90% of the amino acid residues, enabling a detailed structural model of PS II.

According to the structure, the reaction centre proteins D1 (PsbA) and D2 (PsbD) consist of five transmembrane helices, each arranged very similar to the L- and M-subunit of the reaction centre of photosynthetic purple bacteria. They bind the cofactors for the light driven electron transport process (see Figure 1.11). Six chlorophyll molecules, two pheophytins, two plastoquinones (Q\textsubscript{A} and Q\textsubscript{B}), and one non-heme iron which mediates electron transfer from Q\textsubscript{A} to Q\textsubscript{B} are involved. With the exception of Q\textsubscript{A}, all redox-active cofactors are located on the D1 protein.
The subunits CP43 and CP47 are closely associated to the reaction centre proteins. Each subunit consists of six transmembrane helices which are circular arranged similar to the N-terminal domains of the PsaA and PsaB proteins of PS I. CP47 and CP43 form the inner antenna of the complex and bind 30 chl a molecules (see Figure 1.12).
The overall organisation of chlorophylls in PS II differs significantly from that in PS I. This difference probably explains the well known slow trapping of excitation energy in PS II in comparison with PS I (de Weerd et al., 2002).

Seven all-trans carotenoids, which are assumed to be $\beta$-carotenes, could be assigned (see Figure 1.12). However, biochemical analyses indicate the possible presence of more carotenoid molecules. Four of them are located between Cyt $b_{559}$ and CP43, possibly stabilised by the cluster of small transmembrane subunits in that region, and two are located on the CP47 side. The electron density map also indicates that CP47 may contain two to three $\beta$-carotenoids close to the monomer/monomer interface in the dimeric complex. Six of the seven $\beta$-carotene molecules are in close contact with chlorophyll head groups as required for facilitating energy transfer from $\beta$-carotene to chlorophyll and for quenching chlorophyll triplets to prevent photoinhibition.

Additionally, 13 transmembrane helices were assigned to specific low molecular weight subunits based on their amino acid sequences (see Figure 1.13).

**Figure 1.13: Overall structure of PS II from *T. elongatus* (Ferreira et al., 2004).** View of the PS II dimer along the membrane normal from the luminal side. The cofactors were removed for a better overview. Helices are shown in different colours with capitals corresponding to the respective genes. PDB-code: 1S5L. Figure adapted from Nowaczyk (2005).

PsbE and PsbF are the $\alpha$- and $\beta$-subunits of cytochrome $b_{559}$, which is closely associated with the reaction centre. Both subunits contain a single transmembrane
helix and a stromally-exposed N-terminus (Tae et al., 1988). And both coordinate a $b$-type heme via a histidine residue (Babcock et al., 1985). The slow photoreduction kinetics indicates that cytochrome $b_{559}$ is of minor importance for the primary electron transport process (Stewart and Brudvig, 1998). Probably, it is part of an alternative secondary electron transport pathway, which protects the PS II complex against photoinhibition (Poulson et al., 1995). Additionally, there is evidence that it plays a role in the assembly of functional complexes (Tae and Cramer, 1992).

PsbH is localised between CP47 and PsbX. In higher plants PsbH is phosphorylated at the N-terminus (Michel and Bennet, 1987), which is reduced and probably not phosphorylated in cyanobacteria (Pursiheimo et al., 1998). The function of the phosphorylation is not yet clear. In $Synechocystis$ sp. PCC 6803, deletion of the $psbH$ gene leads to a modified $Q_A$ to $Q_B$ transfer and a high sensitivity to photoinhibition (Mayes et al., 1993). The function of PsbH has also been discussed in connection with the PS II repair cycle (Bergantino et al., 2003).

PsbI is localised in the immediate vicinity to PsbA and CP43. Its function is unknown and the $psbl$ gene can be deleted in $Chlamydomonas$ without impairing PS II assembly and photoautotrophic growth. However, the mutant is very susceptible to photoinhibition (Kuenstner et al., 1995). In comparison to the wild type, PsbI deletion mutants of $T. elongatus$ showed a lower oxygen evolving activity (Katoh and Ikeuchi, 2001). Additionally, it was not possible to isolate dimeric PS II complexes, indicating that PsbI plays a role in the stabilisation of the dimeric PS II complexes (Katoh and Ikeuchi, 2001).

In the periphery of the PS II complex a cluster of four small subunits is localised: PsbJ, PsbK, PsbZ, and one unknown subunit. Five of the seven $\beta$-carotenes are localised in this region. Deletion of the $psbJ$ gene in $Synechocystis$ sp. PCC 6803 diminished, but did not prevent the activity of PS II or the growth of the organism under photoautotrophic conditions (Lind et al., 1993). The mutant was only able to accumulate 50 % of PS II complexes as compared to the wild type. The function of these complexes seems to be normal, suggesting that PsbJ is involved in the assembly of PS II rather than in primary photochemical reactions. PsbK is synthesised as a precursor with an N-terminal extension and is processed to its mature form. Deletion of the $psbK$ gene in $Synechocystis$ sp. PCC 6803 had little
or no effect (Ikeuchi et al., 1991, Zhang et al., 1993). This leads to the suggestion that PsbK plays a role outside of the photochemical reactions of PS II. A corresponding deletion in *Chlamydomonas reinhardtii* resulted in poor assembly of PS II and loss of the ability to grow photoautotrophically (Takahashi et al., 1994). These results suggest that in *Chlamydomonas reinhardtii* PsbK plays an important role in the assembly or stability of PS II.

The function of PsbZ is unknown. Deletion of the *psbZ* gene in *Chlamydomonas reinhardtii* showed no phenotype in comparison to wild type (Swiatek et al., 2001).

At the monomer/monomer interface, three of the small subunits are located: PsbL, PsbM, and PsbT. PsbL seems to be required for the normal function of the QA site, since the QA redox-activity decreases extremely compared to wild type, when isolated PS II core complexes are depleted of this polypeptide (Kitamura et al., 1994; Nagatsuka et al., 1991). In *Synechocystis* sp. PCC 6803, deletion of PsbL leads to a total loss of water splitting activity (Anbudurai and Pakrasi, 1993).

Ikeuchi et al. (1989) identified PsbM in *Synechococcus vulcanus* but the function and location within PS II is unknown. Although found in all types of oxygenic organisms, PsbM has not been examined so far.

A chloroplast encoded gene, *ycf8*, was identified as PsbT in the green algae *Chlamydomonas reinhardtii* by gene disruption and immunodetection (Monod et al., 1994). Unfortunately, the same name was also given to a different protein encoded by a nuclear gene, which encodes a hydrophilic protein (Kapazoglou et al., 1995). In order to avoid confusion, the chloroplast-encoded gene is now designated *psbTc*, while the nuclear-encoded one is denoted *psbTn*. PsbTc is not dispensable for photoautotrophic growth (Monod et al., 1994, Iwai et al., 2004). Further characterisation of PsbTc in *Chlamydomonas reinhardtii* showed that it is involved in the rapid recovery of PS II activity after photoinactivation (Ohnishi and Takahashi 2001). A disruption of the *psbTc* gene in *Synechocystis* sp. PCC 6803 showed no effects at the cellular level (Iwai et al., 2004), and a deletion mutant of *T. elongatus* showed no significant difference in photoautotrophic growth and oxygen evolution in comparison to wild type. However, the amount of monomeric isolated PS II complexes increased considerably (Iwai et al., 2004). The *T. elongatus* protein contains a cystein residue at position 12, which is lacking in all other species with known sequence (Iwai et al., 2001).
The affiliation of PsbN to the PS II complex is unclear (Kashino et al., 2002). As the assignment to the structure was done without knowing the amino acid sequence, it can not be excluded that this helix is part of another subunit, for instance PsbY. Deletion of psbN in *Synechocystis* sp. PCC 6803 neither prevented PS II assembly nor photoautotrophic growth (Mayes et al., 1993). The product of the *psbN* gene has not yet been detected in higher plants.

PsbX is located between the PsbH and PsbE subunits nearby the D2 protein. Similar to PsbK, PsbX is processed to its mature form. Deletion of the *psbX* gene in *Synechocystis* sp. PCC 6803 leads to a slightly reduced PS II content in the mutant compared to wild type (Funk, 2000). Analogous deletion of PsbX in *T. elongatus* showed a reduced growth under CO₂-limiting conditions (Katoh and Ikeuchi, 2001). Possibly, PsbX plays a role for the function of Qₐ similar to PsbL (Nagatsuka et al., 1991).

Although the subunit PsbY could not be assigned in the crystal structure, it might be that the helix assigned to PsbN in reality is PsbY. This is suggested by the fact that PsbY could be detected in many PS II preparations in contrast to PsbN (Kashino et al., 2002; Kashino et al., 2002). Deletion of the *psbY* gene in *Synechocystis* sp. PCC 6803 showed no significant changes with respect to growth, electron transfer rate, and PS II content when cells were cultivated with light of 50 µE m⁻² s⁻¹ (Meetam et al., 1999). However, when the light intensity was raised to 200 µE m⁻² s⁻¹ then the mutant showed a reduced growth, when the Ca²⁺ concentration was reduced suggesting that the protein might help to stabilise Ca²⁺ (Neufeld et al., 2004). Initially, PsbY was isolated from spinach utilising its low L-arginine metabolising activity in the presence of added MnCl₂ (Gau et al., 1998).

In addition to the already discussed intrinsic peptides of PS II, PS II from *T. elongatus* contains three extrinsic proteins, which bind to the luminal side of the complex. PsbV, PsbU, and PsbO. PsbV is also known as cytochrome c₅₅₀. It plays a role in water oxidation and its absence does not prevent photoautotrophic growth (Shen et al., 1995), although, *psbV* deletion mutants contain only about 60 % of wild type PS II and show a 60 % decreased oxygen evolution rate. PsbV/PsbO double mutants of *Synechocystis* sp.
PCC 6803 are unable to grow photoautotrophically, contain less than 20 % of wild type PS II, and show less than 10 % oxygen evolving activity. They are also more sensitive towards photoinhibition (Shen et al., 1995).

Like PsbV, PsbU (12 kDa protein) is only present in cyanobacteria. Its function is unknown. Deletion of the *psbU* gene in a mutant showed no significant differences regarding activity and stability of PS II compared to wild type (Shen et al., 1998).

In higher plants and cyanobacteria the PsbO protein is highly conserved. Although mature PsbO is often referred to as the 33-kDa protein, its calculated molecular mass is about 26.5 kDa in cyanobacteria (Nixon et al., 1992). It is an extrinsic protein with a high number of β-sheets (Xu et al., 1994) and it plays an important role in maintaining an optimal environment for water oxidation. Various studies indicate that it does so by stabilising the Mn cluster, but there is no evidence that it binds Mn directly. Indeed, deletion of the *psbO* gene in *Synechocystis* sp. PCC 6803 does not inhibit oxygen evolution or photoautotrophic growth in the mutant (Burnap and Sherman, 1991; Mayes et al., 1991; Philbrick et al., 1991). Under these conditions the function of the 33 kDa protein may be carried out by the PsbV protein (Shen et al., 1995).

Additionally to these extrinsic proteins, further proteins have been identified which are at least transiently associated with the complex. In a proteomic study with PS II complexes from *Synechocystis* sp. PCC 6803, PsbQ, Psb27, and Psb28 were detected (Kashino et al., 2002). The genomes of *Synechocystis* sp. PCC 6803 and *T. elongatus* further showed an open reading frame with homology to PsbP. Up to then it was assumed, that these proteins are only present in eukaryotes. Deletion of the *psbP* and *psbQ* genes in a *Synechocystis* sp. PCC 6803 mutant showed a reduced photoautotrophic growth and a reduced oxygen evolving activity under CaCl₂-limited conditions (Thornton et al., 2004). While Psb27 seems to be involved in PS II assembly (Nowaczyk, 2005), the function of Psb28 is still unknown.

### 1.6 Interrelationship between iron homeostasis and oxidative stress

A system immanent reaction during photosynthetic and respiratory electron transport is the transfer of electrons to molecular oxygen resulting in the production of reactive oxygen species (ROS) such as superoxide anion, hydroxyl radicals or hydrogen peroxide. Thus, oxygenic photosynthetic organisms have developed different
mechanisms to detoxify ROS. Superoxide anion $\text{S(O}_2^-$, the primary product of oxidative stress, are immediately dismutated to $\text{H}_2\text{O}_2$ and $\text{O}_2$ by the superoxide dismutase, and $\text{H}_2\text{O}_2$ is reduced to $\text{H}_2\text{O}$ mainly by ascorbate peroxidase (in plants) or by peroxidase or catalase (in cyanobacteria). Moreover, peroxiredoxin contributes to the detoxification of ROS (Dietz, 2005). It is well documented that ROS are extremely reactive and cause severe damage to cell components by, for example, inactivation of proteins, cleavage of DNA, and peroxidation of unsaturated fatty acids in cell membranes. The damage caused by ROS is increased in the presence of iron. Iron reacts with $\text{H}_2\text{O}_2$ in the Haber-Weiss-Fenton reaction by forming highly reactive hydroxyl radicals (Elstner, 1990). Thus, oxygenic photosynthetic organisms have developed mechanisms to utilise iron effectively for metabolic processes, but in parallel they also have developed strategies to reduce the damaging effects of iron. This implies a complex interrelationship between oxidative stress and iron homeostasis. However, not only a surplus of iron is a problem: Iron limitations also lead to increased oxidative stress which is explained in more detail in the following section.

1.7 Adaptation of cyanobacteria to iron limitations

For optimal growth photosynthetic organisms have to adapt to their environment. Cyanobacteria have a large pool of genetic information encoding biochemical pathways to achieve optimal utilisation of absorbed light energy, to evade oxidative damage caused by excessive light (photoinhibition), and to coordinate photosynthesis effectively with the overall cellular metabolism.

With the exception of some lactobacilli, iron is an essential element for all organisms (Archibald, 1983). Although it is the fourth most abundant element on earth, its biological availability is limited due to its physicochemical properties (Frausto da Silva and Williams, 1993; Lippard and Berg, 1994). Iron deficiency e.g. occurs during cell division which requires about the double amount of iron, often resulting in limitation of cell growth (Straus, 1994). In addition to the involvement in the photosynthetic electron transport chain, iron is an essential component of the respiratory electron transport chain, in nitrogen assimilation, dinitrogen fixation, chromophore biosynthesis, and deoxyribonucleotide synthesis. It also plays an important role as metal ion cofactor of ROS-detoxifying enzymes like catalase, peroxidase, and superoxide dismutase (Briat et al., 1995). Thus, iron limitation often leads to a
decreased decomposition of ROS (Lundrigen et al., 1997), and this results in an even stronger oxidative stress. This stress causes damage of the photosystems, especially PS II (Aro et al., 1993; Bhaya et al., 2000).

High iron concentrations in turn can also be deleterious (section 1.6; Elstner, 1990). Due to this problem organisms have developed strategies and effective control systems by which uptake, transport and storage of iron is maintained, while toxic effects are minimised.

The adaptation strategies of cyanobacteria under nutrient limitation are manifold. Common and unspecific adaptations are, for instance, a reduction of growth or structural changes of the cell. Specific adaptations are alterations in metabolism in a way that the requirement of nutrients is limited or that the limited nutrient is substituted by adequate substances.

Studies on the effects of iron deprivation reveal a variety of responses that range from the reduction or loss of structures or molecules which are important for the cell under non-stress conditions to the synthesis of new compounds. According to Straus (1994), these responses can be divided at least into three different categories: acquisition, compensation, and retrenchment.

Acquisition is an adaptational process by which the cell enhances its ability to assimilate iron from the environment by producing special iron-chelating molecules called siderophores. Siderophores have been reported in higher plants, fungi, bacteria, and cyanobacteria (Braun and Winkelmann, 1987).

Compensation is characterised by the synthesis of new proteins or increased synthesis of proteins that are capable to compensate the function of proteins which are reduced during iron stress. A prominent example is the replacement of ferredoxin at PS I by flavodoxin. The function of flavodoxin does not depend on iron availability.

Retrenchment describes the changes and the reduction of cellular structures and physiological processes. This response is often characterised by a large decrease in iron-containing molecules and molecules whose synthesis is directly or indirectly dependent on iron. Iron deficiency directly causes a degradation of cellular pigments, especially of chlorophyll-containing proteins and phycobilisomes as shown for Synechococcus elongatus PCC 7942 and Synechocystis sp. PCC 6803 (Hardie et al., 1983; Sherman and Sherman, 1983; Riethman and Sherman, 1988). The velocity of pigment degradation is variable. For instance, the ratio of phycocyanin to chlorophyll decreases (Guikema and Sherman, 1984), while the ratio of chlorophyll to
P$_{700}$ increases. Although iron is not a component of the light-harvesting phycobilisome complex itself, it plays an important role in the synthesis of phycobilin chromophores, since it is a cofactor of the heme oxidase.

In addition to these three adaptation mechanisms, the expression of iron-regulated proteins which have a protective function has recently attracted major attention. This includes, for instance, the increased expression of IdiA (Iron deficiency-induced protein A) and IsiA (Iron stress-induced protein A; reviewed in Michel and Pistorius, 2004).

### 1.8 Iron limitation and photosynthesis

Iron plays a major role in the photosynthetic electron transport chain. As shown in Figure 1.14 a minimum of 23 to 24 iron atoms are involved in the photosynthetic electron transport (Ferreira and Straus, 1994). As previously mentioned (see section 1.5) PS II contains a non-heme iron between QA, the primary electron accepting quinone that is bound to D2, and QB, the plastoquinone bound to D1.

![Figure 1.14: Iron atoms in the photosynthetic electron transport chain of cyanobacteria. Fe atoms total = 23-34. Figure adapted from Michel and Pistorius (2004).](image)

The exact function of this non-heme iron is not known and no redox turnover has been observed under physiological conditions. However, the presence of the non-heme iron is essential, since its extraction or replacement with other ions results in a loss of PS II activity (Kurreck et al., 1996). In addition, PS II contains two cytochromes b$_{559}$ located on the subunits PsbE and PsbF. The cytochrome b$_{5f}$ complex comprises five iron atoms which are bound in two hemes of cytochrome b$_{5}$, one heme in cytochrome f and of two iron atoms within the 2Fe-2S centre of the Rieske protein.
PS I contains three 4Fe-4S centres (F_x, F_A and F_B) and Ferredoxin two iron atoms in the 2Fe-2S centre. Cyanobacteria using cytochrome c_553 instead of plastocyanin contain one additional heme.

Iron deficiency leads to a situation in which the photosynthetic electron transport chain produces high amounts of ROS while the level of detoxifying enzymes (catalase, peroxidase and superoxide dismutases) is low, since their synthesis and function partly also depend on iron as cofactor (Lundrigen et al., 1997). As a result oxidative stress is increasing. Iron deficiency has been shown to induce a number of adaptive responses in the photosynthetic metabolism of cyanobacteria. In addition, all iron-containing components of the photosynthetic electron transport chain, which have been examined, show a decreased content in several cyanobacteria (Straus, 1994; Ivanov et al., 2000).

Two such modifications of the photosynthetic electron transport chain are the strongly induced expression of the proteins IsiA and IdiA. IdiA is assumed to protect the acceptor side of PS II against oxidative stress under conditions of mild iron limitation, whereas prolonged iron deficiency leads to the synthesis of a chlorophyll a antenna around PS I-trimers consisting of IsiA protein molecules (Boekema et al., 2001).

1.9 Iron stress-induced protein A (IsiA)

Up to now, the proteins IsiA and IsiB are the best characterised proteins shown to be highly upregulated under iron-deficient conditions (Laudenbach and Straus, 1988). IsiB is a flavodoxin which functionally replaces the iron-containing protein ferredoxin (PetF) in many cyanobacteria such as *Synechococcus*, *Synechocystis*, and *Anabaena*. Investigations of an IsiB-free *Synechocystis* sp. PCC 6803 mutant indicated that IsiB is not absolutely required for growing under iron-deficient conditions (Kutzki et al., 1998). The *isiA* gene encodes a 37 kDa protein, with homology to PsbC (CP43) of PS II, and therefore it was named CP43'. Like PsbC, IsiA is predicted to have six transmembrane helices and since the chlorophyll-binding motives are conserved it is assumed to bind 12 chlorophyll molecules (Bricker and Frankel, 2002). In contrast to PsbC, IsiA lacks 100 amino acid residues of the large
hydrophilic luminal loop between helices V and VI. The upregulation of IsiA leads to a blue-shift in the red absorbance maximum (680 to 673 nm) of chl $a$ at room temperature, and the chl $a$ fluorescence at 77K at 685 nm becomes dominant (Öquist, 1974; Burnap et al., 1993).

Despite its similarity to PsbC, expression of IsiA cannot compensate PsbC in a PsbC-free mutant of *Synechocystis* sp. PCC 6803, even when grown under iron-deficient conditions (Rögner et al., 1991).

The chlorophyll protein complex CPVI-4 (Riethman and Sherman, 1988) is expressed in the cyanobacterium *Synechoccus elongatus* PCC 7942 under iron deficiency. As Burnap et al. (1993) showed, this complex contains IsiA. It has been postulated, that IsiA has a function as a chlorophyll depot under iron-deficient conditions and helps to recover the photosystems upon availability of iron (Riethman and Sherman, 1988). Investigations of an IsiA free *Synechococcus* PCC 7942 mutant and another mutant which overexpresses IsiA, suggested a protective role for PS II with IsiA having a function in dissipation of excitation energy (Park et al., 1999; Sandström et al., 2001). Pakrasi et al. (1985) also suggested for IsiA a light-harvesting function under iron stress, which works mainly for PS II, but probably also for PS I.

At the same time, Bibby et al. (2001) and Boekema et al. (2001) were able to show that in *Synechocystis* sp. PCC 6803 and *Synechococcus elongatus* PCC 7942, respectively, trimeric PS I complexes are modified by IsiA under iron deficiency resulting in a PS I-IsiA supercomplex (see Figure 1.15).

![Figure 1.15: Modification of PS I by IsiA under iron deficient conditions.](image)

---

**Figure 1.15: Modification of PS I by IsiA under iron deficient conditions.** 18 IsiA molecules form a functional accessory light-harvesting antenna around a trimeric PS I complex. The expression of IsiA is transcriptionally repressed by Fur (Ghassmenian and Strauss, 1996).
The authors showed by single particle analysis, that the trimeric PS I complex was surrounded by a ring of 18 IsiA molecules. Results of Andrizhiyevskaya et al. (2002) suggest that IsiA is a functional antenna for PS I, for instance, under iron stress the size of the light-harvesting antenna of PS I is extremely enlarged. This function of IsiA, assembled under continuous iron stress, does not exclude an additional PS II-related function under weak iron stress (Burnap et al., 1993; Sandström et al., 2001).

1.10 Iron deficiency-induced protein A (IdiA)

Under iron stress, IdiA is expressed in the two closely related cyanobacteria *Synechococcus elongatus* sp. PCC 6301 and PCC 7942 at high amounts (Michel and Pistorius, 1992, Michel et al., 1996, 1998, 1999, Exss-Sonne et al., 2000). A similar protein, Tll0513, is also present in *T. elongatus* (Michel et al., 1998; Exss-Sonne et al., 2000) and *Synechocystis* sp. (Tölle et al., 2002). Although an IdiA-homologe protein is present in all cyanobacteria, whose genomes are fully sequenced, no IdiA immunologically related proteins have been found in *Chlamydomonas reinhardtii*, *Euglena gracilis* or *Chlorella vulgaris* by data bank analysis (Michel and Pistorius, 2004). IdiA is a 35 kDa protein which is mainly located intracellularly and is preferentially associated with the cytoplasmatic side of the thylakoid membrane as has been shown by cell fractionation methods and immunocytochemical techniques (Michel et al., 1998).

The encoding gene *idiA* was identified and partially characterised in *Synechococcus elongatus* PCC 6301 (Michel et al., 1996), and *Synechococcus elongatus* PCC 7942 (Michel, 1999). Southern blot analysis showed, that *idiA* exists as a single copy gene on the chromosome. The 1101 bp gene encodes a protein with a molecular mass of 39.2 kDa which is synthesized as a precursor and processed by cleaving between two alanine residues (position 36 and 37) into the mature form of 35.2 kDa. It is a basic protein (pl 10.49), contains no transmembrane helices, and most important- is homologous to periplasm-located iron-binding proteins of the SfuA/FbpA families.
The complete HindIII DNA fragment (Figure 1.16), containing the idiA gene (ORF3), was sequenced and analysed resulting in the identification of six further ORFs (Michel et al., 1999).

![Figure 1.16: Physical map of the 5763 bp HindIII DNA fragment of Synechococcus PCC 6301. Size and orientation of the genes are shown by the shaded arrows. The position of restriction sites are marked (Michel et al., 1999).](image)

Mutational analysis of ORF4 and ORF7 of Synechococcus elongatus PCC 7942 mutants showed that these gene products are involved in the activation of IdiA expression under iron deficiency. ORF4 is called idiB and encodes a putative helix-turn-helix transcriptional regulator (Michel et al., 1999; 2001) of the Crp/Fnr family (Holm et al., 1994). The IdiB-free Synechococcus elongatus PCC 7942 mutant exhibited no IdiA expression, suggesting that IdiB is a positive acting transcription factor (Michel et al., 1999). This has been proven by a corresponding promoter analysis (Michel et al., 2001).

A DpsA (ORF7)-free mutant showed a significant reduction of idiA, idiB and irpA expression. The isiAB expression was reduced by up to 50% in the mutant under iron-deficient conditions (Michel et al., 2003). DpsA belongs to a group of bacterial stress-inducible proteins that bind DNA and confer resistance to peroxide damage. Under metal ion limitation and peroxide treatment, the expression of dpsA is increased (Almiron et al., 1992). Pena and Bullerjahn (1995) showed that the DpsA protein in Synechococcus elongatus PCC 7942 has a weak catalase activity. A DpsA-free mutant showed extremely reduced growth under high light conditions and was more sensitivity towards paraquat and peroxide treatment (Dwivedi et al., 1997).

Analysis of an IdiA-free Synechococcus elongatus PCC 7942 mutant showed that besides a general reduction of cell growth the PS II activity is much lower under iron-
deficient conditions than in wild type, while the PS I activity seems to be not affected which was not observed in wild type cells. The reduced PS II activity is associated with an accelerated degradation of the D1 protein (Michel et al., 1996; Exss-Sonne et al., 2000). Investigations of the IdiA-free mutant and a PsbO-free mutant in comparison with wild type, using different electron acceptors and room temperature fluorescence measurements, provided evidence that the reduction of the PS II activity was mainly caused by damage of the acceptor side of PS II in the IdiA-free mutant. This assumption was also verified by a treatment of wild type and IdiA-free mutant cells with hydrogen peroxide (H$_2$O$_2$). In wild type cells the treatment resulted in a transient decrease of PS II activity paralleled by a transient decrease of the D1 protein and a transient increase of the IdiA protein. In contrast to wild type, H$_2$O$_2$ caused a significantly higher and longer lasting damage of PS II activity, while the PS I activity was not affected in the IdiA-free mutant (Exss-Sonne 2000; Exss-Sonne et al., 2000). In agreement with a PS II acceptor side function of IdiA is the finding that the inhibitory effect of the herbicide bentazone, which is assumed to bind to the acceptor side of PS II, is extremely reduced, when IdiA is expressed (Bagchi et al., 2003).

Figure 1.17: Modification of PS II by IdiA under iron-deficient conditions in Synechococcus elongatus PCC 6301/PCC 7942. PS II is modified by the IdiA protein, which is predicted to protect the acceptor side of PS II against oxidative stress under mild iron limitation either by interacting with the non-heme iron or by providing PS II with iron during the high turn-over of the D1 protein. The degradation of phycobilisomes unter iron limitation is indicated by a light blue colour – suggesting either a reduction of number or size of the phycobilisomes. Whereas the wild type expresses IdiA, an IdiA-free mutant is more accessible for damage by ROS under iron limitation. Figure adapted from Michel and Pistorius (2003).
Because of these results it has been postulated, that IdiA plays a role in the stabilisation of PS II by protecting the acceptor side of PS II against oxidative damage, especially, when oxidative stress is caused by iron limitation (see Figure 1.17; Michel and Pistorius, 1992; Michel et al., 1996; 1998 and 1999; Exss-Sonne et al., 2000). The exact stabilising and protecting mechanism of IdiA is not yet clear. A possible function of IdiA could be that it interacts with the non-heme iron located between QA and QB or that it acts as an iron storage or iron transport protein during the high turn-over of PsbA when oxidative stress occurs (Michel and Pistorius, 2004).

In addition, the time course of transcription of idiA and isiA mRNA was investigated (Michel and Pistorius, 2004). idiA mRNA was detected after 6 h reaching a maximum after 24 h, while isiA mRNA was detected after 24 h reaching a maximum after 60 h of growth under iron-deficient conditions. This indicates, that shortly after the onset of iron-deficient conditions the more labile PS II is modified by IdiA which has a postulated protective function of the acceptor side of PS II either by interacting with the non-heme iron between QA and QB or by supplying iron to PS II during the high turn-over of the D1 protein under iron deficiency or oxidative stress. After prolonged iron starvation, when damage of PS II cannot any longer be effectively prevented, modification of PS I by a newly synthesized chl a antenna, consisting of IsiA molecules, takes place (Figure 1.18). As a physiological consequence, a reduction of PS II activity occurs which results in a reduction of linear electron flow and an increase in cyclic electron flow activity around PS I. This has been shown by acridine yellow fluorescence measurements (Michel et al., 2003).
Figure 1.18: Model of the modification of PS II and PS I by IdiA and IsiA in *Synechococcus elongatus* PCC 7942/ PCC 6301 under iron deficiency. PS II is modified by the expression of IdiA, which is suggested to protect the acceptor side of PS II against oxidative damage in a yet unknown way especially when oxidative stress is caused by iron limitation. PS I is modified under prolonged iron limitation by expression of IsiA. 18 IsiA molecules generate a functional accessory light-harvesting antenna around a PS I-trimer. Figure adapted from Michel (2003).

1.11 Aims of this work

To immobilise isolated Photosystem II complexes onto electrodes and to use them in a semiartificial device where they should provide the electrons for hydrogen production from water, basic investigations of structure and function of the membrane protein complex are essential. PS II complexes from *T. elongatus* wild type should be isolated and characterised with a broad spectrum of methods in order to get a deeper view into basics of the structure and function of this membrane protein complex which are still unknown. For a more detailed characterisation a pigment analysis method should be established to examine the pigment content of the isolated complexes. In addition also PS II complexes from a *T. elongatus* wild type strain containing a *His*<sub>10</sub>-tag were obtained. Furthermore a *T. elongatus* ΔPsbK and a ΔPsbTc mutant should be functional characterised.
Moreover, it has been suggested that IdiA, a protein which is expressed under iron deficiency in *Synechococcus elongatus* PCC 7942 and PCC 6301, has a function in protecting the acceptor side of PS II under oxidative stress, especially when oxidative stress is caused by iron deficiency. An IdiA homologue protein is also present in *T. elongatus* (Michel et al., 1998; Exss-Sonne et al., 2000). Therefore, a partial characterisation of PS II complexes isolated from *T. elongatus* grown under iron-deficient conditions should also be done in order to get an electron microscopy structure of the better protected PS II complexes containing IdiA.
2 Material and Methods

2.1 Biochemical methods

2.1.1 Sterilisation

Growth media, solutions and materials were sterilised by heat autoclaving for 45 min at 121˚C (Varioklav H-P Labortechnik, Oberschleissheim, Germany).

2.1.2 Bacterial strains

Strains used in this work are listed and described in Table 2.1. For isolation of thylakoid membranes and for further isolation of PS II complexes, a *T. elongatus* wild type strain was used, which was a kind gift of Prof. Dr. E. K. Pistorius (Bielefeld). Additionally a *T. elongatus* strain was used, which was genetically modified for isolation of *His*-tagged PS II complexes (Prodöhl, 2002; Prodöhl et al., 2004). PsbTc and PsbK deletion mutants were a kind gift of Prof. Dr. M. Ikeuchi (Tokyo).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Thermosynechococcus elongatus</em> BP-1</td>
<td>Wild type</td>
</tr>
<tr>
<td><em>Thermosynechococcus elongatus</em> BP-1 CP43-His</td>
<td>Replacement of the endogenous <em>psbC</em>-Gene by a Variante, which encodes a Fusionprotein with a <em>His</em>-tag at the C-terminus of CP43. Contains a Chloramphenicol resistance cassette (Prodöhl, 2002; Prodöhl et al., 2004)</td>
</tr>
<tr>
<td><em>Thermosynechococcus elongatus</em> BP-1 ΔPsbK</td>
<td>Deletion of the <em>psbK</em> gene (Katoh and Ikeuchi, 2001)</td>
</tr>
<tr>
<td><em>Thermosynechococcus elongatus</em> BP-1 ΔPsbTc</td>
<td>Deletion of the <em>psbTc</em> gene (Iwai et al., 2004)</td>
</tr>
</tbody>
</table>

Table 2.1: Cyanobacterial strains used in this work

2.1.3 Cultivation of *T. elongatus* cells

*T. elongatus* cells were grown in BG-11 medium (Rippka et al., 1979) at 45˚C in a stream of 5 % (v/v) CO₂ in air. Cells cultivated under iron deficient conditions were kept in BG-11 medium without ferric ammonium citrate. The ΔPsbK mutant was maintained with 7 µg chloramphenicol and the ΔPsbTc mutant was maintained with 5 µg chloramphenicol. Wild type and mutant pre-cultures were illuminated with light of 50 µE m⁻²s⁻¹. Small scale (1-4l) cultures for iron-deficiency experiments were illuminated with light of 80 - 100 µE m⁻²s⁻¹.
Material and Methods

BG-11 Medium

5 mM HEPES NaOH pH 8.2
1 % (v/v) BG-FPC
6 x10^-4 % (w/v) Ferric ammonium citrate, iron content 16.5-18.5 %
0.190 mM Sodium carbonate, Na2CO3
0.175 mM Potassium phosphate, K2HPO4

BG-FPC

1.76 mM Sodium nitrate, NaNO3
30.39 mM Magnesium sulphate, MgSO4
24.49 mM Calcium chloride, CaCl2
3.14 mM Citric acid
0.28 mM Na-EDTA
10 % (v/v) Trace elements

Trace elements

46.26 mM Boric acid, H3BO4
9.15 mM Manganese chloride, MnCl2
0.77mM Zinc sulfate, ZnSO4
1.61 mM Sodium molybdate, Na2MoO4
0.32 mM Copper sulfate, CuSO4
0.17 mM Cobaltous nitrate, Co(NO3)2

2.1.3.1 Large scale cultivation of T. elongatus cells in a photobioreactor

20 l cultures of T. elongatus were grown in photobioreactors (Airlift Visual Safety Fermenter, Bioengineering AG, Wald, Switzerland). Polyamide foil was used as light-permeable reactor material. In general, 20 l of BG-11 medium were sterilised by autoclaving. The bioreactor was inoculated with 350 ml of pre-cultured cells, including all needed additives. During the first 12 hours, light was provided at 100 µE m^-2 s^-1. After that, light was provided at 150 - 200 µE m^-2 s^-1. The cell cultures were grown three to four days and harvested with an optical density (OD) at 750 nm of approximately 3.
2.1.4 Estimation of the cell density

For estimation of the cell density, 5 ml of cell culture were spun down (10 min, 2900xg, 4°C) in a hematocrit tube. The packed cell volume in µl per 5 ml culture could be estimated from the tube's scale. This value was used to calculate the cell density in form of µl packed cell volume per ml culture (µl PZV/ml).

Another possibility for estimation of the cell density is the measurement of the optical density at 750 nm. Up to a total absorbance of <1 an OD\text{750nm} value of 0.1 corresponds to about 0.1 µl packed cell volume per ml culture (Flores et al., 1982).

2.1.5 *T. elongatus* growth curves

To estimate the growth rates of *T. elongatus* wild type or mutant, a cell culture was grown for seven days in an Erlenmeyer flask. Then it was diluted into fresh medium to an OD\text{750nm} of 0.3. After seven days this culture was diluted again to an OD\text{750nm} of 0.3 in a 50 ml growth tube. After three days this culture was used to inoculate three parallel cultures with an OD\text{750nm} of 0.3 in a 150 ml growth tube. Growth was monitored two to three times daily by measuring the OD at 750 nm.

2.1.6 Harvesting of *T. elongatus* cells and preparation of thylakoid membranes

In general 20 l cultures were harvested after three to four days, when their OD at 750 nm reached approximately 3. First the cells were concentrated through an filtration cell (Amicon DC10 LA) to about 2 l. Cells were pelleted by centrifugation (GSA-Rotor, 18 min, 11000 rpm, 4°C, Sorvall) and washed once with buffer A (20 mM MES pH 6.5, 10 mM CaCl\textsubscript{2}, 10 mM MgCl\textsubscript{2}). Fresh weight of the cells was noted and the pellet was resuspended in 200 ml buffer B (20 mM MES pH 6.5, 10 mM CaCl\textsubscript{2}, 10 mM MgCl\textsubscript{2}, 0.5 M mannitol) containing 0.2 % (w/v) lysozyme (Sigma). After incubation for 90 min at 37°C, cells were broken in a Parr bomb at 2000 psi. Thylakoid membranes were pelleted by centrifugation (GSA-Rotor, 18 min, 11000 rpm, 4°C, Sorvall). To remove associated phycobilisomes, the thylakoid membranes were washed with buffer A. This step was repeated three times. After one more washing step with buffer B, the membranes were resuspended in a small volume of buffer D (20 mM MES pH 6.5, 10 mM CaCl\textsubscript{2}, 10 mM MgCl\textsubscript{2}, 0.5 M mannitol, 20 % (v/v) glycerol). The thylakoid membranes were frozen in liquid nitrogen and stored at -70°C until further use.
2.1.7 PS II isolation from *T. elongatus*

The method to isolate photosystem II from *T. elongatus* was carried out by the combination of two high performance liquid chromatography steps according to Kuhl et al. (2000) with some modifications. Thawed thylakoid membranes isolated from *T. elongatus* as described in section 2.1.6 were filled up with buffer B to a final volume of 80 ml, homogenised by a glass homogeniser and pre-extracted with 0.05 % n-Dodecyl β-D Maltoside (β-DM, Glycon) to remove the main part of phycobilisomes. After the addition of β-DM, the membranes were centrifuged (SS34-Rotor, 30 min, 18000 rpm, 4°C, Sorvall). The deep-blue supernatant was discarded and the pellet was resuspended in extraction buffer (20 mM HEPES pH 7.5, 10 mM CaCl$_2$, 10 mM MgCl$_2$, 2.65 % (w/v) ammonium sulphate) with a final chlorophyll concentration of about 1 mg/ml. The solubilisation followed by addition of the non-ionic detergents β-DM (1.2 % (w/v)) and sodium cholate (0.5 % (w/v), Sigma). After 30 min of stirring, unsolubilised material was sedimented by ultracentrifugation (Ti70-Rotor, 60 min, 42000 rpm, 4°C, ultracentrifuge, Beckman). To remove lipids and carotenoids, this solution was furthermore purified via overnight sucrose density gradient centrifugation. This was done with 14 % (w/v) sucrose in buffer B containing 0.03 % (w/v) β-DM. The bottom of the centrifuge tube was filled with a pillow of 80 % (w/v) sucrose in buffer B containing 0.03 % (w/v) β-DM to protect the protein against damage via high pressure. Solubilised proteins were loaded onto the gradient and centrifuged (SW28-Rotor, 25000 rpm, 18 h, 4°C, ultracentrifuge, Beckman). After removing the yellow-orange part from the sucrose gradient, the blue-green fraction was used for the further purification.

2.1.7.1 Chromatographic methods for purification of Photosystem II

The chromatographic steps during PS II-isolation were performed with a PerSeptive Biocad 700 E chromatography system (Applied Biosystems).

2.1.7.1.1 *Hydrophobic interaction chromatography (HIC)*

For hydrophobic interaction chromatography (HIC) a 36 ml Waters AP-2 column, self-packed with Poros 50 OH material (Applied Biosystems) was used. The sample was adjusted to 1.65 M ammonium sulphate. The high ionic strength starting buffer was 20 mM HEPES pH 7.5, 10 mM CaCl$_2$, 10 mM MgCl$_2$, 0.03 % (w/v) β-DM, 1.65 M ammonium sulphate. The sample (up to 5 mg chl a) was loaded onto the column and
PS II was eluted by a continuous gradient from 1.65 M to 0.6 M ammonium sulphate during 4 column volumes (Flow rate 10 ml/min). By a second gradient segment from 0.6 M to 0 M ammonium sulphate in one column volume the column was regenerated.

The PS II containing fractions were collected and concentrated through an ultrafiltration cell (Amicon) to about 10 ml. It followed dialysis over night against buffer B containing 0.03 % β-DM to prepare the sample for the ion exchange chromatography.

**2.1.7.1.2 Immobilised metal affinity chromatography (IMAC)**

In order to speed up and simplify the preparation a His\textsubscript{10}-tag was introduced at the C-terminus of the CP43 subunit of PS II which enabled the purification via an IMAC column. After solubilisation of the thylakoid membranes as explained in section 2.1.7, the extract was directly (without sucrose density gradient) loaded onto a nickel containing matrix (17 ml Chelating Sepharose Fast Flow, Amersham Biosciences). The starting buffer was 50 mM MES pH 6.5, 10 mM CaCl\textsubscript{2}, 10 mM MgCl\textsubscript{2}, 300 mM NaCl, 0.5 M mannitol, 1 mM histidine, 0.03 % (w/v) β-DM. After a washing step of about 4 column volumes, the PS II complexes were eluted with a continuous gradient from 1 to 100 mM histidine in one column volume (Flow rate 1 ml/min). PS II containing fractions were collected and prepared for ion exchange chromatography as described in section 2.1.7.1.1

**2.1.7.1.3 Ion exchange chromatography (IEC)**

For anion exchange chromatography (IEC) an UNO Q-6 column (BioRad) was used. After dialysis, the sample was directly loaded onto the column. Starting buffer was 20 mM MES pH 6.5, 10 mM CaCl\textsubscript{2}, 10 mM MgCl\textsubscript{2}, 0.5 M mannitol, 0.03 % (w/v) β-DM. After washing with 3 column volumes, the PS II complexes were eluted with a linear MgSO\textsubscript{4}-gradient. The concentration of MgSO\textsubscript{4} was increased to 200 mM within 2 column volumes. To regenerate the column, a final washing step with 500 mM MgSO\textsubscript{4} was performed. This chromatographic step yielded in 3 main peaks: monomeric PS II, dimeric PS II with high activity and dimeric PS II with low activity. These three peaks were collected separately and concentrated (Centricon 100, Millipore). It followed a desalting step via a size exclusion chromatography column.
(10 DG, BioRad) with buffer B containing 0.03 % β-DM. The samples were frozen in liquid nitrogen and stored at -70˚C.

2.1.8 Other chromatographic methods

Size exclusion chromatography (SEC) and reversed phase chromatography (RPC) were performed on a Waters system (two pumps, model 510 fitted with preparative pump heads and Rheodyne injector, Model 9125i for reversed phase and one analytically pump model 510 for size exclusion chromatography) connected to a diode array detector (PDA 996, Waters, Milford, MA, USA).

2.1.8.1 Size exclusion chromatography

To determine the monomer/dimer ratio, analytically size exclusion chromatography was performed. For this, a sample (5 µg chl a) was filtered through a 0.45 µm filter (Schleicher & Schuell). The column (TSK 4000 SWXL or TSK 3000 SWXL, Tosoh Haas) was equilibrated with running buffer (20 mM MES pH 6.5, 30 mM Ca$_2$Cl, 10 mM MgCl$_2$, 0.5 M mannitol, 0.02 % (w/v) β-DM) for at least 2 column volumes. The elution of PS II was done isocratically with a flow rate of 0.3-0.5 ml/min depending on system pressure.

2.1.8.2 Reversed phase chromatography

For quantification of pigments and to determine whether the Q$_B$-binding site in the PS II preparations is occupied, a reversed phase method was established. Pigment analysis was done according Patzlaff and Barry (1996) with some modifications.

The sample (40 µg chl a) or the pigment standard, respectively, was added to 80 % (v/v) acetone and 20 % (v/v) methanol. After mixing for one minute, the sample was sonicated in ice water for 5 min. Protein compounds were pelleted by centrifugation in an Eppendorf centrifuge (13000 rpm, 1 min, 4˚C). Supernatant was filtrated as described in section 2.1.8.1.

After equilibration of the column (Spherisorb ODS-2, Macherey & Nagel) with methanol for at least 2 column volumes, the elution was done isocratically with a flow rate of 1 ml/min.

For calculation respective pigment standards were used. Using their respective peak areas and extinction coefficients, the amount could be calculated exactly.
2.1.9 Sucrose density gradient centrifugation

20 \% (w/v) sucrose in buffer A (20 mM MES NaOH, pH 6.5, 10 mM CaCl$_2$, 10 mM MgCl$_2$) was filled into 36 ml ultracentrifuge tubes (polyallomer tubes, 14 x 89 mm, No. 331372, Beckman). At -20˚C the filled tubes were frozen over night. The defreezing was done at room temperature. This resulted in a linear gradient from 10-30 \% (w/w) sucrose. Samples were added onto the gradient and the gradient was centrifuged for at least 18 hours (Beckman ultracentrifuge, SW28 rotor, 25000 rpm, 4˚C). The gradients were harvested by inserting a syringe tip at the bottom of the tube and collecting of 0.5 ml fractions with the help of a peristaltic pump.

2.1.10 Concentration of proteins

For concentrating proteins without precipitation, several filtration devices were used. Volumes of less than 1 ml were concentrated using Microcon Spin Filtration devices (Millipore). Volumes up to 10 ml were concentrated using Amicon ultra centrifugal filter devices (Millipore).

All devices were used with membranes of 10 kDa, 30 kDa or 100 kDa cutoff, depending on the size of the protein of interest.

2.1.11 TCA precipitation of proteins according to Peterson (1979)

To concentrate proteins, a protein solution was mixed with ¼ volume of cold 100 \%(v/v) trichloroacetic acid (TCA). After precipitation for one hour on ice, proteins were pelleted by centrifugation in an Eppendorf centrifuge (11600 x g, 5 min, 4˚C). After removal of the supernatant, the centrifugation step was repeated once and the resulting pellet was resuspended in the desired buffer.
2.1.12 **SDS-polyacrylamide gel electrophoresis (SDS-PAGE)**

SDS-PAGE was performed according to Laemmli (1970) or Schägger & von Jagow (1987) respectively.

### 2.1.12.1 SDS-PAGE according to Laemmli (1970)

For SDS-PAGE according to Laemmli, a Mini-Protean III electrophoresis chamber (BioRad) was used.

<table>
<thead>
<tr>
<th>Substance</th>
<th>Separation gel (pH 8.8)</th>
<th>Stacking gel (pH 6.8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide</td>
<td>12.5%</td>
<td>5 %</td>
</tr>
<tr>
<td>Tris-HCl</td>
<td>0.75 M</td>
<td>0.125 M</td>
</tr>
<tr>
<td>SDS</td>
<td>0.1 %</td>
<td>0.2 %</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.050 %</td>
<td>0.067 %</td>
</tr>
<tr>
<td>Ammoniumperoxodisulfate</td>
<td>0.050 %</td>
<td>0.063 %</td>
</tr>
</tbody>
</table>

Table 2.2: Composition of SDS-PAGE according to Laemmli (1970).

After preparation of the separation gel, it was coated with isopropanol to guarantee an even surface structure. After polymerisation, the isopropanol was removed and the surface was washed with water to add the stacking gel onto the separation gel.

For denaturation, samples were mixed with 5 x loading buffer (5 % (w/v) SDS, 12.5 % (w/v) β-Mercaptoethanol or 0.5 M DTT respectively, 25 % (v/v) glycerine, 0.5 mg/ml bromphenol blue, 160 mM Tris-HCL pH 6.8) and incubated for 3 min at 95°C. After incubation, the samples were centrifuged (13000 rpm, 2 min, 4°C) and the supernatant was loaded onto the gel. To avoid aggregation, samples with isolated PS II complexes were loaded directly onto the gel without incubation.

Electrode buffer was 50 mM Tris-HCL pH 8.3, 0.192 M glycine and 0.1 % (w/v) SDS. Small gels (100 x 70 x 0.75 mm) were electrophoresed for approximately one hour at 30 mA per gel.
2.1.12.2 SDS-PAGE according to Schägger & von Jagow (1987)

For SDS-PAGE according to Schägger & von Jagow, a Protean II xi System (BioRad) was used.
The Schägger & von Jagow system allows especially the separation of small proteins (5-20 kDa).

<table>
<thead>
<tr>
<th>Substance</th>
<th>Separation gel (pH 8.45)</th>
<th>Stacking gel (pH 8.45)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide</td>
<td>12 %</td>
<td>6.6 %</td>
</tr>
<tr>
<td>Tris-HCL</td>
<td>1 M</td>
<td>0.75 M</td>
</tr>
<tr>
<td>Urea</td>
<td>6 M</td>
<td>-</td>
</tr>
<tr>
<td>Glycerine</td>
<td>9.8 %</td>
<td>-</td>
</tr>
<tr>
<td>SDS</td>
<td>0.1 %</td>
<td>0.1 %</td>
</tr>
<tr>
<td>Ammoniumperoxodisulfate</td>
<td>0.05 %</td>
<td>0.063 %</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.05 %</td>
<td>0.067 %</td>
</tr>
</tbody>
</table>

Table 2.3: Composition of SDS-PAGE according to Schägger & von Jagow (1987).

Preparation of the gels and sample preparation was done according to section 2.1.12.1.
Electrophoresis was carried out by applying an electrical field of 60 mA until the samples reached the separation gel and then 15 mA overnight. Electrode buffer was different for anode (0.1 M Tris pH 8.9) and cathode (0.1 M Tris pH 8.25, 0.1 M Tricine, 0.1 % (w/v) SDS).

2.1.12.3 Staining of polyacrylamide gels

After electrophoresis, polyacrylamide gels were stained with Coomassie Brilliant Blue.
The gel was incubated for one hour in Coomassie solution (0.2 % (w/v) Coomassie Brilliant Blue R, 40 % (v/v) methanol, 7 % (v/v) acetic acid). Background staining was removed with incubation in destaining solution (40 % (v/v) methanol, 7 % (v/v) acetic acid) over night.
Schägger & von Jagow gels were incubated 30 min in fixation solution (50 % (v/v) methanol, 10 % (v/v) acetic acid) before staining.

### 2.1.12.4 Marker for SDS-Gels

As Marker for SDS-Gels peqGOLD Protein-Marker (peqlab) was used.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Organism</th>
<th>Molecular weight [kDa]</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Galactosidase</td>
<td><em>E. coli</em></td>
<td>116.0</td>
</tr>
<tr>
<td>Bovine Serum-Albumine</td>
<td>Cow</td>
<td>66.2</td>
</tr>
<tr>
<td>Ovalbumine</td>
<td>Chicken</td>
<td>45.0</td>
</tr>
<tr>
<td>Lactat-Dehydrogenase</td>
<td>Pig</td>
<td>35.0</td>
</tr>
<tr>
<td>RE Bsp981</td>
<td><em>E. coli</em></td>
<td>25.0</td>
</tr>
<tr>
<td>β-Lactoglobuline</td>
<td>Cow</td>
<td>18.4</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>Chicken</td>
<td>14.4</td>
</tr>
</tbody>
</table>

Table 2.4: Composition of peqGOLD Protein-Marker.

### 2.1.13 Green native gradient Gel

Stock solution: 57.66 g glycine
12.02 g Tris-HCL, pH 8.6

Acrylamide solution: Serva 37.5:1

APS: 10 % (w/v) in aqua bidest

TEMED: conc.

Stacking gel buffer: 50 % (v/v) stock solution-HCL, pH 6.6

Anode buffer: 62 ml stock solution
428 ml aqua bidest
Cathode buffer: 62 ml stock solution
1 ml deriphat
427 ml aqua bidest

Solubilisation buffer: 25 ml stock solution
30 ml glycerine
45 ml aqua bidest

The gradient gel was prepared with a gradient mixer and a peristalsis pump to have a steady flow of solution into the gel plates. The gel composition for a midi-gel is listed in Table 2.5.

<table>
<thead>
<tr>
<th>Substance</th>
<th>4% Separating gel</th>
<th>6% Separating gel</th>
<th>3% Stacking gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide</td>
<td>0.66 ml</td>
<td>0.99 ml</td>
<td>0.57 ml</td>
</tr>
<tr>
<td>Deriphat</td>
<td>22.2 µl</td>
<td>22.2 µl</td>
<td>-</td>
</tr>
<tr>
<td>Buffer</td>
<td>1.67 ml</td>
<td>1.67 ml</td>
<td>1.9 ml</td>
</tr>
<tr>
<td>Glycerine</td>
<td>-</td>
<td>0.75 ml</td>
<td>-</td>
</tr>
<tr>
<td>Aqua bidest</td>
<td>4.23 ml</td>
<td>3.15 ml</td>
<td>4.2 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>7.5 µl</td>
<td>7.5 µl</td>
<td>25 µl</td>
</tr>
<tr>
<td>APS</td>
<td>80 µl</td>
<td>80 µl</td>
<td>75 µl</td>
</tr>
</tbody>
</table>

Table 2.5: Composition of green native gradient gel.

The samples of about 200 µg chl a were mixed with 90 µl solubilisation buffer and 10 µl 10 % (w/v) β-DM. After 30 min incubation at room temperature, non-solubilised parts were spinned down (13000 rpm, 15 min, 4°C). After chl a determination of the supernatant, samples containing 15-40 µg chl a were loaded onto the gel unless noted otherwise.

Isolated PS II complexes were loaded onto the gel without solubilisation. They were mixed with 20 µl of solubilisation buffer.

The electrophoresis was performed at 25-30 mA for approximately 2 h.
### 2.1.14 Western blot analysis

#### 2.1.14.1 Antibodies

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Name of Antibody</th>
<th>Concentration used</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PsbA (D1)</td>
<td>58 A</td>
<td>1:1000</td>
<td>Engels et al., 1992; Specht et al., 1990</td>
</tr>
<tr>
<td>PsbD (D2)</td>
<td>57 A</td>
<td>1:1000</td>
<td>Engels et al., 1992; Specht et al., 1990</td>
</tr>
<tr>
<td>IdiA</td>
<td>38 D</td>
<td>1:500</td>
<td>Michel &amp; Pistorius, 1992</td>
</tr>
<tr>
<td>TII 0513</td>
<td></td>
<td>1:500</td>
<td>Michel &amp; Pistorius, 1992</td>
</tr>
<tr>
<td>PsbB (CP47)</td>
<td>24 A</td>
<td>1:500</td>
<td>Engels et al., 1992</td>
</tr>
<tr>
<td>PsbC (CP43)</td>
<td></td>
<td>1:500</td>
<td>Pistorius</td>
</tr>
<tr>
<td>PsbO (MSP)</td>
<td>25 A</td>
<td>1:1000</td>
<td>Engels et al., 1992</td>
</tr>
<tr>
<td>Phycocyanin</td>
<td></td>
<td>1:2000</td>
<td>From <em>Aphanothera halophytica</em>; Sigma</td>
</tr>
<tr>
<td>Allophycocyanin</td>
<td></td>
<td>1:2000</td>
<td>From <em>Anabaena variabilis</em>; Boehringer</td>
</tr>
</tbody>
</table>

Table 2.6: Antibodies used in this work. All antibodies were a kind gift of Prof. Dr. E.K. Pistorius, Bielefeld

#### 2.1.14.2 Western blot analysis with a secondary antibody coupled to peroxidase

The proteins were blotted from a SDS-Gel onto a nitrocellulose membrane (Protran BA 85S Cellulosenitrate, Schleicher & Schüll) using the capillary blot method. After finishing the electrophoresis, the gel was washed 3 x 10 min in transfer-buffer (10 mM Tris-HCL pH 8.8, 2 mM EDTA, 50 mM NaCl, 0.1 mM DTE). After incubation in transfer-buffer, the membrane and the blotting paper (GB002, Schleicher & Schüll) were used to prepare a blot sandwich consisting of three layers blotting paper, membrane, gel, membrane, and again three layers of blotting paper. The sandwich was packed between two glass plates inside a plastic bag. It was incubated with weight down of about 1 kg over night.

After washing for 3 x 10 min with CMF-PBS-buffer (137 mM NaCl, 2.7 mM KCL, 6.5 mM Na₂HPO₄, 1.5 mM KH₂PO₄), the membrane was blocked with CMF-PBS-buffer containing 5 % (w/v) skimmed milk powder for 1 h. After washing for 3 x 10 min with washing solution CMF-PBS-buffer containing 0.5 % (w/v) skimmed milk powder and 0.02 % (v/v) TWEEN 20, the first antibody was applied for two hours. Concentrations
used for the different antibodies are listed in section 2.1.6. The dilution was made with CMF-PBS-buffer containing 0.5 % (w/v) BSA. The membrane was washed again 3 x 10 min with washing solution. The incubation with the secondary antibody (Anti-rabbit horseradish peroxidase conjugate, Sigma) was done in the dark. It was used in 1:15000 dilution in CMF-PBS buffer containing 0.5 % (w/v) BSA.

After thorough washing with washing solution, the membrane was equilibrated with 50 mM Tris-HCL, pH 7.35.

To visualise the signals, developer (3 mg 4-chloro-1-naphtol, 1 ml methanol, 5 ml Tris-HCL pH 7.35, 1 µl 30 % H₂O₂) was added. The reaction was stopped by washing with aqua bidest.

**2.1.14.3 Western blot analysis with a secondary antibody coupled to alkaline phosphatase**

The proteins were blotted onto a PVDF membrane (Immobilon-P, Millipore, Bedford, MA, USA) in the same way as described in section 2.1.14.2. As transfer-buffer 20 mM CAPSO pH 11.2, 20 % (v/v) methanol was used.

After blotting, the membrane was blocked with TBST-buffer (10 mM Tris-HCL pH 8, 150 mM NaCl, 0.05 % (v/v) TWEEN 20) containing 5 % (w/v) skimmed milk powder for one hour. After washing for 2 x 5 min with TBST-buffer, the first antibody was applied for 2 h. It was diluted in TBST-buffer. The membrane was washed again 2 x 5 min with TBST-buffer and the secondary antibody (Anti-rabbit alkaline Phosphatase conjugate, Sigma) was applied for one hour. It was used in 1:30000 dilution in TBST-buffer. After washing 2 x 5 min with TBST-buffer, the membrane was equilibrated with alkaline phosphatase-buffer (100 mM Tris-HCL pH 9.5, 100 mM NaCl, 5 mM MgCl₂).

For staining, 10 µl NBT (50 mg/ml NBT in 70 % (v/v) N,N-dimethylformamide) and 10 µl BCIP (50 mg/ml BCIP in N,N-dimethylformamide) in 15 ml of alkaline phosphatase-buffer were added.

**2.1.14.4 Marker for Western blot analysis**

To visualise the protein size on the blots, a prestained standard molecular weight marker was applied onto the gel.

For this, thepeqGOLD prestained protein-marker (peqlab) was used (Table 2.7).
### Material and Methods

#### Table 2.7: Composition of peqGOLD Prestained Protein-Marker.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Organism</th>
<th>Molecular weight [kDa]</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Galaktosidase</td>
<td>E. coli</td>
<td>116.0</td>
</tr>
<tr>
<td>Bovine Serum-Albumine</td>
<td>Cow</td>
<td>66.2</td>
</tr>
<tr>
<td>Ovalbumine</td>
<td>Chicken</td>
<td>45.0</td>
</tr>
<tr>
<td>Lactat-Dehydrogenase</td>
<td>Pig</td>
<td>35.0</td>
</tr>
<tr>
<td>RE Bsp981</td>
<td>E. coli</td>
<td>25.0</td>
</tr>
<tr>
<td>β-Lactoglobulin</td>
<td>Cow</td>
<td>18.4</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>Chicken</td>
<td>14.4</td>
</tr>
</tbody>
</table>

#### 2.1.15 Cross-linking of isolated PS II complexes

Chemical cross linkers contain reactive groups which are primary N-hydroxysuccinimidyl ester, imido ester and carbodiimides. These groups are specific to functional groups which are primary amines on the respective proteins. The cross linked PS II complexes should be used for electron microscopy. For cross linking of PS II complexes four different cross linker reagents were used which are summarised in Table 2.8.

#### Table 2.8: Cross linker reagents used for PS II complexes.

<table>
<thead>
<tr>
<th>Cross linker</th>
<th>Reactive group</th>
<th>Spacer length</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDC</td>
<td>Carbodiimide</td>
<td>-</td>
<td>Carboxyl-specific: aspartate and glutamate</td>
</tr>
<tr>
<td>DSG</td>
<td>Succinimidyl ester</td>
<td>7.7Å</td>
<td>Aminospecific: lysine and arginine</td>
</tr>
<tr>
<td>AMAS</td>
<td>Maleimid Succinimidyl ester</td>
<td>4.4Å</td>
<td>Aminospecific: lysine, arginine Sulfhydryl-specific: cystein</td>
</tr>
<tr>
<td>SMCC</td>
<td>Maleimid Succinimidyl ester</td>
<td>11.6Å</td>
<td>Aminospecific: Lysine, arginine Sulfhydryl-specific: cystein</td>
</tr>
</tbody>
</table>

Table 2.8: Cross linker reagents used for PS II complexes.
For cross linking experiments, 20 µg of Protein were mixed with a 500-fold excess of the respective cross linker. After incubation for 2 h at 26°C, the reaction was stopped by addition of SDS-sample buffer or 1 M Tris, pH 7.5. SDS-PAGE analysis according to Laemmli (1970) was performed followed by Western blot analysis.

2.2 Biophysical methods

2.2.1 UV/VIS Spectroscopy

Measurements of the OD and absorbance spectra were performed with a Beckman DU 7400 Diodearray.

2.2.1.1 Determination of chlorophyll content

10 µl of sample (whole cells, membranes or extracts) where added to 990 µl 100 % (v/v) methanol. After mixing, the sample was centrifuged at 13000 rpm for 2 min in an Eppendorf centrifuge. The OD of the supernatant was measured at 652 nm, 665.2 nm and 750 nm. The chl a content was calculated with the following Equation 2.1.

\[
\text{Chl}_{a} = \frac{[(\text{OD}_{665.2 \text{nm}} - \text{OD}_{750 \text{nm}}) \times 16.29] - [(\text{OD}_{652 \text{nm}} - \text{OD}_{750 \text{nm}}) \times 8.54]}{100}
\]  

Equation 2.1

2.2.1.2 Determination of phycobiliproteins

The determination of phyocyanin (PC) and allophycocyanin (AP) was done according Tandeau de Marsac and Houmard (1988) with cell-free extracts. Crude cell-free extract was precipitated with 1 % (w/v) streptomycin sulphate for 30 min at 4°C and centrifuged at 10000 x g for 10 min at 4°C in order to eliminate membrane fragments containing chlorophyll. The amounts of PC and AP in the supernatant fraction were calculated from measurements of the OD at 620 and 650 nm, respectively, using Equation 2.2.
Material and Methods

\[
PC \left[ \frac{mg}{ml} \right] = \frac{OD620nm - 0.7 \times OD650nm}{7.38} \\
AP \left[ \frac{mg}{ml} \right] = \frac{OD650nm - 0.19 \times OD620nm}{5.65} 
\]

Equation 2.2

2.2.1.3 Chlorophyll a and β-carotene determination of whole cells

Chl a and β-carotene ratios were calculated according to Kern (2005). A pigment extract in 80 % (v/v) acetone was prepared and absorbance spectra from 340 nm to 740 nm were recorded. To obtain a pure chl a spectrum a respective pigment standard was used. The spectra were normalised and absorbance differences between spectra of pigment-extracts and chl a standard were calculated using Origin. For chl a the molar extinction coefficient at 664 nm (76800 M⁻¹ cm⁻¹) and for carotenoids the extinction coefficient for β-carotene at 454 nm (144000 M⁻¹ cm⁻¹) was used.

2.2.1.4 Determination of protein content

The determination of protein content was done with the BCA Protein Assay (Pierce) according to manufacturer’s instructions.

The BCA-assay (Smith et al., 1985) is based on the reduction of Cu²⁺ to Cu⁺ by proteins under alkaline conditions (Biuret reaction), giving rise to a light blue complex. Cu⁺ is then chelated by bincinchoninic acid, resulting in a purple coloured solution.

2.2.2 Oxygen evolving activity measurements

Activity measurements of isolated PS II complexes were done with a FIBOX 2 (PreSens) system via dynamic luminescence quenching by oxygen. The collision between the indicator luminescence dye in its excited state and the quencher (oxygen) results in radiationless deactivation and is called dynamic quenching. After collision energy transfer takes place from the excited indicator molecule to oxygen which consequently is transferred from its ground state to its excited singlet state. As a result, the indicator molecule does not emit luminescence and the measurable luminescence signal decreases. Oxygen evolving measurement was done at 30°C.

For calibration of the system, oxygen saturated water (100 % oxygen) and water containing a pinch of Na₂S₂O₃ (0 % oxygen) were used.
Material and Methods

The measurement was done with a total volume of 1 ml activity buffer (20 mM MES NaOH, pH 6.5, 1 M Betaine, 30 mM CaCl₂, 10 mM MgCl₂, 0.03 % β-DM) containing 1 mM DCBQ, 5 mM Ferricyanid and 3-5 µg chl a. After 2 min equilibration in dark, the sample was illuminated with a light intensity of 3300 µE m⁻²s⁻¹. Evaluation of data was done via a personal computer which was connected to the FIBOX system.

2.2.3 Low temperature fluorescence spectroscopy

For low temperature fluorescence spectroscopy measurements a LM-2 fluorimeter was used. Cultures in the exponential growth phase were harvested and adjusted to either an optical density (750 nm) of about 1 or to a chl a content of 5 µg/ml. The cells were incubated 15 minutes in dark (state 2). For state transition experiments after dark incubation cells were illuminated either with white light or with red light (665 nm) to reach state 1.

After incubation the cells were frozen in liquid nitrogen and excited either with 435 nm light (for chlorophyll excitation) or 580 nm light (for phycobilisome excitation). Fluorescence emission was measured from 630 to 750 nm with a step size of 1 nm and a bandpass filter of 4 nm. The fluorescence spectra were corrected for the detector characteristics and normalised at 630 nm.

2.2.4 Peptide Mass Fingerprint (PMF)

To identify proteins separated on SDS-PAGE, the gel-bands can be digested with a sequence specific protease. The resulting fragment masses were determined with matrix-assisted-laser-desorption-ionisation time-of-flight (MALDI-ToF) mass-spectrometry (MS). Computer algorithms allow searching for proteins whose theoretical fragmentation patterns fit this detected masses.

Protein bands of interest were cutted out of the SDS-gel and destained in 2-3 washing steps with 50 % (v/v) acetonitrile in 25 mM NH₄HCO₃. After the gel pieces had been completely dried in a SpeedVac, they were incubated in trypsin-solution (12.5 ng/µl trypsin, 25 mM NH₄HCO₃) over night at 37°C. The peptide fragments were eluted from the gel matrix by addition of 10 µl 50 % (v/v) acetonitrile, 0.5 % (v/v) TFA and sonication for 20 min. The supernatant could be used directly for MALDI-ToF analysis. 0.6 µl of the sample were mixed with 0.6 µl matrix (10 mg/ml α-hydroxycinammmic acid in 60 % (v/v) acetonitrile, 1 % (v/v) TFA) on the target plate.
MALDI-ToF analysis of the samples was performed on a Voyager DE Pro (Applied Biosystems), operated with the Voyager 5.1 software using the following instrument settings:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acceleration voltage</td>
<td>20000 V</td>
</tr>
<tr>
<td>Grid</td>
<td>76</td>
</tr>
<tr>
<td>Guide wire</td>
<td>0.03</td>
</tr>
<tr>
<td>Delay time</td>
<td>180 nsec</td>
</tr>
<tr>
<td>Acquisition range</td>
<td>830-4000 m/z</td>
</tr>
<tr>
<td>Low mass gate</td>
<td>830</td>
</tr>
</tbody>
</table>

To reach a higher precision, the system was calibrated with a standard (Mix 1 and 2, Sequazyme Kit, Applied Biosystems) which was applied on the target plate directly beneath the sample.

The resulting spectra were analysed with the Data Explorer software (Applied Biosystems, version 4.0). After baseline correction and de-isotoping of the peaks, calibration was performed on the characteristic autolytic peaks of trypsin 842.51 and 2211.1046. The identification of proteins on the basis of the resolved masses was done with the MS-Fit program ([http://prospector.ucsf.edu/ucsfhtml4.0/msfit.htm](http://prospector.ucsf.edu/ucsfhtml4.0/msfit.htm)) using the following parameters:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Database</td>
<td>NCBI (most recent release)</td>
</tr>
<tr>
<td>Species</td>
<td><em>Thermosynechococcus elongatus</em></td>
</tr>
<tr>
<td>Minimum number of peptides required to match</td>
<td>2-4</td>
</tr>
<tr>
<td>Instrument</td>
<td>MALDI-TOF</td>
</tr>
<tr>
<td>Peptide masses</td>
<td>monoisotopic</td>
</tr>
<tr>
<td>Mass tolerance</td>
<td>+/- 50-200 ppm</td>
</tr>
<tr>
<td>Digest</td>
<td>Trypsin</td>
</tr>
<tr>
<td>Maximal number of missed cleavages</td>
<td>1-2</td>
</tr>
<tr>
<td>Modifications</td>
<td>oxidised methionine, N-terminal acetylation, Acrylamide modified cysteine</td>
</tr>
</tbody>
</table>
2.2.5 MALDI-ToF of intact protein complexes

To analyse the subunit composition of isolated PS II-complexes, the protein complexes were spotted in dried droplet application to the sample plate. As matrices, sinapic acid (Fluka, 10 mg/ml in 50 % (v/v) acetonitrile and 1 % (v/v) TFA) and ferulic acid (Fluka, 10 mg/ml in 60 % (v/v) acetonitrile and 1 % (v/v) TFA) were used. To reach a higher precision, the system was calibrated with a standard (Mix 3, Sequazyme Kit, Applied Biosystems) which was applied on the target plate directly beneath the sample.

For sinapic acid the following instrument settings were used:

- Accelerating voltage: 20000
- Grid: 95
- Guide wire: 0.1
- Delay time: 600
- Acquisition range: 1000-20000
- Low mass gate: 500

For Ferulic acid the following instrument settings were used:

- Accelerating voltage: 25000
- Grid: 93
- Guide wire: 0.1
- Delay time: 750
- Acquisition range: 1000-30000
- Low mass gate: 600

2.2.6 Electron microscopy

Transmission electron microscopy was performed with a Philips CM20FEG electron microscope using 200 kV at 66847 magnification. Negatively stained specimens were prepared on carbon-coated copper grids (staining solution was either 2% uranyl acetate, pH 4.5, phospho tungstic acid, pH 7 or 1% uranyl oxalate, pH 6.5).
A part of the grids was glow-discharged before preparing electron microscopy specimens to enhance the absorbance of PSII particles with their hydrophilic parts, which results in predomination of top-view projections. Other grids were used without this treatment and yielded electron microscopy specimens with mainly side-view projections. Images of 2000 x 2000 pixels were recorded with Gatan 4K slow-scan CCD camera with a step size of 15 µm, and a binning factor of 2, corresponding to a pixel size of 0.449 nm at the specimen level. Single particles projections were extracted from the digital images and analysed with Groningen Image Processing (GRIP) software on a PC cluster.
3 Results

In order to immobilise isolated Photosystem II complexes onto electrodes and to use them in a semiartificial device where they should provide the electrons for hydrogen production from water, basic investigations on the structure and function of the membrane protein complexes are essential. To contribute to this knowledge, PS II complexes of *T. elongatus* wild type and *T. elongatus* wild type containing a 10 x His-tag at the C-terminus of subunit CP 43 (PsbD) were investigated. In addition, PS II complexes isolated from two *T. elongatus* mutants, being a ΔPsbK and a ΔPsbTc mutant, were investigated. These mutants were chosen, because PsbK has been suggested to have a function in the stability of PS II complexes (Takahashi et al., 1994) and PsbTc is suggested to stabilise PS II dimers (Iwai et al., 2005).

Moreover, it has been suggested that the protein IdiA, which is expressed under iron deficiency in *Synechococcus elongatus* PCC 7942 and PCC 6301, has a function in protecting the acceptor side of PS II under oxidative stress, especially when oxidative stress is caused by iron deficiency. An IdiA homologue protein has been shown to also be present in *T. elongatus* (Michel et al., 1998; Exss-Sonne et al., 2000). Therefore, a partial characterisation of PS II complexes, isolated from *T. elongatus* grown under iron deficiency, were also included in my work. One major goal of my work was to identify PS II complexes which are best suited for electron donation for hydrogen production in artificial systems.
3.1 Isolation of PS II complexes from *T. elongatus* wild type

PS II of thylakoid membranes from *T. elongatus* was extracted using a combination of the non-ionic detergents β-dodecyl maltoside (β-DM) and sodium cholate as described in section 2.1.7. Although the usage of sodium cholate leads to reduced solubilisation efficiency compared to solubilisation with β-DM alone, monomerisation of PS II could be greatly avoided by using sodium cholate (Kuhl et al., 2000). This was an essential prerequisite for a homogenous preparation of PS II dimers with high oxygen evolution rates.

Figure 3.1: Sucrose density gradient of thylakoid membrane extract. The orange section (carotenoids and lipids) was removed and the blue green section was used for further purification.

To remove the main part of carotenoids and lipids the detergent extract was purified by sucrose density gradient centrifugation (Figure 3.1). After centrifugation for at least 18 hours the orange section containing carotenoids and lipids was removed and the blue-green section containing phycobilisomes, PS II, and PS I, was used for further purification.

The isolation of highly active dimeric PS II complexes was achieved by two high performance liquid chromatography (HPLC) steps:

1) Via hydrophobic interaction chromatography (HIC) PS II was separated from PS I, phycobilisomes and nucleic acids. The elution was done with an ammonium sulphate gradient from 1.65 M to 0.9 M and with a second gradient from 0.9 M to 0 M. The elution profile is shown in Figure 3.2. PS II-containing fractions were pooled, concentrated, and dialysed over night to remove the high salt content. The dialysed sample was directly applied onto an

2) Ion exchange chromatography (IEC) column. Using a magnesium sulphate gradient from 0.01 M to 0.4 M, highly active PS II complexes (Peak 2; Figure 3.3) could be separated from monomeric PS II complexes (Peak 1; Figure 3.3) and from dimeric PS II complexes with low activity (Peak 3; Figure 3.3).
Results

Figure 3.2: Elution profile of hydrophobic interaction chromatography. The HIC was done with an ammonium sulphate gradient from 1.65 M to 0.9 M and a second gradient from 0.9 M to 0 M. The corresponding conductivity in mS is shown by the blue line. Absorbance was recorded at 260 nm (red line) and at 280 nm (green line).

Figure 3.3: Elution profile of the ion exchange chromatography. The IEC was done with a magnesium sulphate gradient from 0.01 M to 0.4 M. The corresponding conductivity in mS is shown by the blue line. Absorbance was recorded at 260 nm (red line) and at 280 nm (green line).
The fractions containing Peak 1, Peak 2 and Peak 3 were pooled separately, concentrated and characterised with the following methods.

### 3.2 Characterisation of isolated PS II complexes

#### 3.2.1 Determination of the absorbance characteristics

To evaluate the degree of purification of the PS II complexes, room temperature absorbance spectra in the range of 250 to 780 nm were recorded. Figure 3.4 shows absorbance spectra of isolated PS II complexes in comparison to crude extracts. The spectrum of isolated complexes showed absorbance maxima at 674 nm and at 437 nm with a shoulder at 420 nm. These maxima are typical for PS II complexes (Dekker et al., 1988; Tang and Diner, 1994) while the absence of a peak in the longer wavelength (679 nm) range indicates the absence of PS I. In contrast to the spectrum of crude extract, where carotenoids (492 nm) and phycobilisomes (610 nm) were still detectable in high amounts, the spectrum of the isolated PS II complexes indicated a reduced carotenoid content, documented by the lower absorbance in the 492 nm region. The remaining absorbance is caused by carotenoids which are bound to the PS II complexes. Moreover, phycobilisomes were not present any more. As previous results showed, the 624 nm peak is not an artefact of phycobilisomes, but rather part of the chlorophyll $a$ ($chl a$) spectrum and also present in higher plant PS II preparations (Eijkelhoff et al., 1996).

![Figure 3.4: Absorbance spectra of crude extract (blue line) and isolated PS II complexes (red line).](image)

While phycobilisomes (610 nm) and carotenoids (492 nm) were still present in the crude extract, they were almost absent in isolated PS II complexes. The isolated PS II complexes showed the typical maxima for PS II which are 420 nm, 437 nm, and 674 nm according to Dekker et al. (1988) and Tang and Diner (1994).
3.2.2 Determination of the oxygen evolving activity

For investigation of the activity of the isolated PS II complexes, oxygen evolving measurements were performed. Oxygen evolution measurements were carried out at 30°C under continuous light with a FIBOX system via dynamic luminescence quenching as described in section 2.2.2. As artificial electron acceptors 2,6 dichloro-p-benzoquinone (DCBQ) and ferricyanide were used. The electrode was calibrated using air-saturated and nitrogen-saturated water. In Table 3.1 representative activities of isolated PS II complexes are given.

<table>
<thead>
<tr>
<th>Oxygen evolving activity</th>
<th>Peak 1 (Monomer)</th>
<th>Peak 2 (Dimer with high activity)</th>
<th>Peak 3 (Dimer with low activity)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[µmol O₂/mg Chl x h]</td>
<td>0</td>
<td>3800</td>
<td>1800</td>
</tr>
</tbody>
</table>

Table 3.1: Oxygen evolving activities of isolated PS II complexes, measured with a FIBOX system via dynamic luminescence quenching.

3.2.3 Analysis of the oligomerisation state of isolated PS II complexes

The oligomerisation state of isolated PS II complexes was investigated by analytical size exclusion chromatography (SEC). Using protein standards, the size of monomeric and dimeric PS II complexes could be estimated. Using this method, Kuhl et al. (2000) calculated a molecular mass of 300 kDa for monomeric PS II and of 500 kDa for dimeric PS II complexes including the detergent micelle. In my preparation PS II monomers eluted after 21 minutes and PS II dimers after 19 minutes (Figure 3.5). This corresponds to the molecular masses estimated by Kuhl et al. (2000). This analysis showed that PS II monomers and dimers were obtained in a nearly pure form.
Figure 3.5: Elution profile of size exclusion chromatography. The retention time for PS II monomers was 20.9 minutes (blue line) and the retention time for PS II dimers was 19.5 minutes (red line). Elution was done isocratically with a flow rate of 0.5 ml/min (TSK 4000 column).

Other possibilities to investigate the oligomerisation state and to separate monomeric and dimeric fractions are sucrose density gradient centrifugation and green native gels. For sucrose density gradients, a 20 % (w/v) sucrose solution was filled into centrifuge tubes and was frozen at -20°C (see section 2.1.9). Thawing was done at room temperature. The result was a linear gradient from 10-30 % (w/w) sucrose, as determined by refractometer measurements (Figure 3.6).

Figure 3.6: Sucrose density gradient centrifugation and estimation of sucrose density with a refractometer. A sucrose gradient was partitioned into 0.5 ml fractions. Each fraction was measured in a refractometer. PS II monomers and PS II dimers could clearly be separated into an upper and a lower band via the gradient.
Results

After centrifugation for at least 18 hours PS II monomers and dimers were separated in an upper and a lower band, respectively. Harvest of the gradient fractions was done with a peristaltic pump which was connected to a fraction collector.

Green native PAGE was prepared with a gradient of 4 to 6 % polyacrylamide. In the native PAGE, protein complexes maintain their native structure and are separated on the basis of their size. After mixing of purified PS II complexes with solubilisation buffer (see section 2.1.13), PS II complexes corresponding to 3 µg of chl a were loaded onto the gel. The separation of monomeric and dimeric PS II fractions is shown in Figure 3.7.

Figure 3.7: Green native PAGE of the three ion exchange chromatography fractions. The first peak shows monomeric PS II complexes and the second and third peak show dimeric PS II complexes. 4 to 6 % polyacrylamide gradient gel. Isolated PS II complexes corresponding to 3 µg chl a were loaded onto the gel.

3.2.4 Subunit composition of isolated PS II complexes

The separation of PS II subunits was done with 12 % SDS-PAGE containing 6 M urea according to Schägger and von Jagow (1987, see section 2.1.12.2). This system especially allows the separation of the small PS II subunits. The PS II subunits above 10 kDa were assigned according to their apparent molecular mass and peptide mass fingerprinting (PMF) after trypsin digestion of the respective gel bands. A typical pattern of PS II subunits is shown in Figure 3.8. In the dimeric fractions, all major PS II subunits were detected: D1 and D2 as central components of the PS II reaction centre, the inner antenna proteins CP47 and CP43, the α-subunit of cytochrome b$_{559}$ and also the extrinsic proteins PsbO, PsbV, and PsbU. In the monomeric PS II fraction the three extrinsic proteins PsbO, PsbV, and PsbU were not present but a further protein, Psb27, was found. Psb27 is a lipoprotein and is perhaps involved in PS II assembly (Nowaczyk, 2005). Furthermore, in comparison to dimeric PS II complexes, a higher contamination of monomeric PS II with additional protein is obvious. Below CP47 another band appeared which might be a CP47
degradation product. Additionally, several bands in the range of 14 to 21 kDa could be seen, which may belong to phycobilisome subunits.

Up to now it is not known why the third fraction exhibits a lower activity than fraction two. All major subunits for oxygen evolving activity were present in the PS II dimers with low activity. Additionally, a faint band of Psb27 could be observed in this fraction.

The composition of PS II with respect to the small subunits (below 10 kDa) in monomeric and dimeric PS II complexes showed no obvious differences in the SDS-PAGE.

The SDS-gel bands corresponding to subunits PsbE, PsbH, PsbF, PsbX, PsbJ, Psbl, PsbL, and PsbY could be identified via Liquid Chromatography/Electro Spray Ionisation (LC/ESI) MS/MS analysis (Nowaczyk, 2005). With this method proteolytic fragments were separated via LC and directly transferred into the ionic source. After ionisation with ES they were analysed in a quadrupole time-of-flight mass spectrometer. The identification of subunits occurred on basis of the peptide sequence which could be determined from the fragmentation data.

Up to now, 12 small intrinsic subunits are known. Because of their high grade of hydrophobicity and their small size, they could not be clearly separated in the SDS-PAGE.

Some subunits could not be stained with Coomassie Brilliant Blue. This is e.g. the case for PsbZ (Swiatek et al., 2001). PMF of the small subunits is also a problem, because digestion with a sequence specific protease results only in a small number

![Figure 3.8: SDS-PAGE of purified PS II complexes according to Schägger and von Jagow (1987). 12 % polyacrylamide gel containing 6 M urea. Each lane contains 40 µg of protein. Stained with Coomassie Brilliant Blue.](image)
of peptides. Using MALDI-ToF MS analysis of whole protein complexes, small subunits might be detected, which often are not clearly separated in SDS-PAGE analyses. Additionally, this method can provide information about post-translational modifications. A representative MALDI-ToF MS trace of PS II dimers from *T. elongatus* wild type can be seen in Figure 3.9. A mass range of 2500 to 10000 Da was analysed, showing multiple peaks, which could be matched to the PS II subunits.

![MALDI-ToF MS trace of dimeric PS II complexes from *T. elongatus* wild type.](image)

For the results summarised in Table 3.2, several concentrations in sinapinic acid matrix as well as a ferrulic acid based matrix have been tested. PsbE, PsbH, PsbZ, PsbF, PsbY, Psbl, PsbL, PsbX, PsbK, PsbJ, PsbM, and PsbT were identified. Subunits PsbE and PsbH were determined without N-terminal methionine. PsbX and PsbK were processed at the N-terminus to a mature form. PsbZ, PsbY, Psbl, PsbM, and PsbT contained the N-terminal methionine as formyl methionine. In PsbL the formyl residue is cleaved off. PsbF was found in the unmodified form, and PsbY contained a formylated N-terminus. PsbJ was identified in the acetylated form. PsbJ is hardly to detect, because the peaks of PsbJ and PsbM are very close to each other (4016.8 and 4009.9 for the processed mature forms). Mass deviations of the measurements are in a range of 0.8 to 9.8 Da. These variations are within range of error of the used Voyager DE Pro spectrometer. The assignment of the peaks was based on a previously study, were the mass peaks could be assigned with a higher degree of accuracy using a Q-Star mass spectrometer (<1.2 Da; Nowaczyk, 2005; cooperation with Jun.- Prof. B. Warscheid). MALDI-ToF analysis of monomeric PS II complexes and dimeric PS II complexes
with low activity showed, that all small subunits, being PsbK, PsbT, PsbJ, PsbM, PsbL, PsbX, PsbI, PsbY, PsbF, PsbZ and PsbH could be identified in both fractions.

Table 3.2: MALDI-ToF MS analysis of intact dimeric PS II complexes. Purified PS II complexes were applied to the target plate in either sinapinic acid matrix or ferrulic acid matrix. The theoretical masses of modified PS II subunits were calculated by Nowaczyk (2005). This method allows a reliable identification of the subunits including posttranslational modifications. The index numbers show the position of the N-terminal amino acid after processing of the polypeptide (Kashino et al., 2002; Gomez et al., 2002).

<table>
<thead>
<tr>
<th>Subunit (ORF-ID)</th>
<th>Mass&lt;sub&gt;c&lt;/sub&gt; unprocessed (mature form) [Da]</th>
<th>Modification</th>
<th>[m/z]</th>
<th>∆ mass&lt;sup&gt;mat&lt;/sup&gt;/mass&lt;sub&gt;c&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>PsbE (tsr1541)</td>
<td>9573.9 (9441.7)</td>
<td>NH&lt;sub&gt;2&lt;/sub&gt;-A&lt;sup&gt;2&lt;/sup&gt;</td>
<td>9438.7</td>
<td>-4.2</td>
</tr>
<tr>
<td>PsbH (tsr1386)</td>
<td>7355 (7222.6)</td>
<td>NH&lt;sub&gt;2&lt;/sub&gt;-A&lt;sup&gt;2&lt;/sup&gt;</td>
<td>7216.3</td>
<td>-7.5</td>
</tr>
<tr>
<td>PsbZ (tsr1967)</td>
<td>6765.3 (6792.3)</td>
<td>N-formyl methionine</td>
<td>6797.1</td>
<td>+3.8</td>
</tr>
<tr>
<td>PsbF (tsr1542)</td>
<td>5065.95</td>
<td>No modification</td>
<td>5069.7</td>
<td>+3.7</td>
</tr>
<tr>
<td>PsbY (tsr0836)</td>
<td>4773.9 (4613.7)</td>
<td>NH&lt;sub&gt;2&lt;/sub&gt;-(f)M&lt;sup&gt;3&lt;/sup&gt;</td>
<td>4603.9</td>
<td>-9.8</td>
</tr>
<tr>
<td>PsbI (tsr1074)</td>
<td>4406.3 (4434.4)</td>
<td>N-formyl methionine</td>
<td>4436.2</td>
<td>+1.9</td>
</tr>
<tr>
<td>PsbL (tsr1543)</td>
<td>4297.3</td>
<td>No modification</td>
<td>4300.7</td>
<td>+2.6</td>
</tr>
<tr>
<td>PsbX (tsr2013)</td>
<td>5233.4 (4188.5)</td>
<td>NH&lt;sub&gt;2&lt;/sub&gt;-T&lt;sup&gt;11&lt;/sup&gt;</td>
<td>4184.4</td>
<td>-4.7</td>
</tr>
<tr>
<td>PsbK (tsr0176)</td>
<td>5027.1 (4100.4)</td>
<td>NH&lt;sub&gt;2&lt;/sub&gt;-K&lt;sup&gt;19&lt;/sup&gt;</td>
<td>4094.0</td>
<td>-6</td>
</tr>
<tr>
<td>PsbJ (tsr1544)</td>
<td>4105.9 (4016.8)</td>
<td>Acetyl-M&lt;sup&gt;8&lt;/sup&gt;</td>
<td>4012.9</td>
<td>-2.9</td>
</tr>
<tr>
<td>PsbM (tsr2052)</td>
<td>3980.7 (4009.9)</td>
<td>N-formyl methionine</td>
<td>4007.9</td>
<td>-0.8</td>
</tr>
<tr>
<td>PsbT (tsr1531)</td>
<td>3875.8 (3903.1)</td>
<td>N-formyl methionine</td>
<td>3903.9</td>
<td>+0.1</td>
</tr>
</tbody>
</table>

Especially, the detection of PsbZ is interesting, because it could not be identified with alternative mass spectrometric methods like LC/ESI-MS/MS or Multidimensional Protein Identification Technology (MudPIT; Nowaczyk, 2005). The comparison of wild
type PS II and PS II complexes isolated from a wild type strain containing a 10xHis-tag at the C-terminus of subunit CP43 with MALDI-Intact-Mass-Tag-MS (MALDI-IMT-MS) analysis showed that PsbZ is not present in individual wild type preparations but is present in His-tagged PS II complexes. Because of these data, single particle analysis of His-tagged and wild type PS II complexes was done (Arteni et al., 2005).

Figure 3.10 Single particle analysis of wild type PS II complexes and His-tagged PS II complexes. A) Wild type PS II complex; B) His-tagged PS II complex; C) Overlay of wild type and His-tagged complexes; D) Overlay with the PS II crystal structure model (Ferreira et al, 2004). The overlay of wild type and His tagged PS II particles shows a difference in the peripheral region of the dimer. An overlay with the structure model shows that the respective density belongs to subunit PsbZ. (Arteni et al., 2005 in cooperation with Prof. E. J. Boekema and M. Nowaczyk).

Transmission electron microscopy was performed with a Philips CM20FEG electron microscope using 200 kV at 66847fold magnification. Negatively stained specimens were prepared on carbon-coated copper grids. The grids were glow-discharged before preparing electron microscopy specimens to enhance the absorbance of PSII particles with their hydrophilic parts, which results in predomination of top-view projections. Images were recorded and single particle projections were extracted and analysed with the Groningen Image Processing (GRIP) software. A total of 14611 particles was extracted from 350 images for wild type and 16828 particles were extracted from 400 images for the His-tag mutant.
Comparison of wild type (Figure 3.10, A) and *His-tagged* (Figure 3.10, B) PS II particles showed, that the *His-tagged* particles contained an additional density in the periphery of the dimer which was not present in wild type particles. An overlay (Figure 3.10, D) with the PS II structure model (Ferreira et al., 2004) confirmed the assignment of the PsbZ subunit. This example shows that the combination of mass spectrometry and electron microscopy is a powerful new approach in the field of characterisation of membrane protein complexes.

### 3.2.5 Pigment analysis of isolated PS II complexes

In order to quantify the amount of pigments and to determine whether the Q_B-binding site is occupied in the PS II preparations, a pigment analysis method has been established.

The total content of pigments in PS II complexes has been determined frequently but always with differing results depending on organism, preparation methods, and analytical methods used (Barbato et al., 1991; Yamaguchi et al., 1988; Kobayashi et al., 1990; Chang et al., 1994; Tang et al., 1994). Two pheophytine *a* molecules per reaction centre can be used as a reference for the determination of the stoichiometry of chlorophylls, carotenes and quinones.

In the crystal structure of PS II (Ferreira et al., 2004), 36 chl *a* molecules could be identified and seven β-carotenes were assigned; however, as is indicated by biochemical analyses (Tracewell et al., 2001) more carotenes may be present. Furthermore, each PS II reaction centre contains the plastoquinone molecules Q_A and Q_B. While Q_A is tightly associated with the D2 protein, Q_B functions as a mobile electron carrier which is released from PS II in the reduced form (see also section 1.5). In isolated PS II complexes an occupied Q_B binding site could play an important role for stability and activity.
Figure 3.11: Elution profile of reversed phase chromatography. Elution was done isocratically with methanol. Absorbance was measured at 255 nm. Peaks were assigned according to the elution time of the respective pigment standards and according to the absorbance spectra. Chl a elutes after 14.5 minutes, β-carotene after 49.8 minutes, pheophytine a after 76 minutes and plastoquinone after 85.1 minutes.

Pigment analysis was done by reversed phase chromatography according to Patzlaff and Barry (1996) with some modifications as outlined in section 2.1.8.2. After calibration of the column with several pigment standards (chl a, carotenoids, pheophytine a), the sample was eluted isocratically with methanol. This resulted in the elution profile shown in Figure 3.11.

The peaks were assigned according to the elution time of the respective pigment standards and the absorbance spectra. The calculation of different pigment ratios was done using the respective peak areas and the molar absorbance coefficients of the pigments in methanol based on two pheophytine a molecules per PS II reaction centre. For isolated dimeric PS II complexes from *T. elongatus* wild type, reversed phase chromatography of various preparations resulted in 35 to 41 chl a per two pheophytine a molecules. This is in good agreement with the 36 chl a molecules found per PS II reaction centre in the crystal structure of PS II of *T. elongatus* (Ferreira et al., 2004). Furthermore, a ratio of three to four β-carotene molecules per PS II reaction centre could be found. This differs from the expected seven β-carotene molecules determined in the structure. The peak assigned to carotenoids according to the absorbance characteristics show a significant shoulder towards longer retention times. This indicates that there are carotenoids present which are different
from β-carotene. It is not yet clear whether this form is already present in the fresh sample or whether it is formed during the pigment analysis procedure. Quantification of plastoquinones could not be done yet, because there was no adequate pigment standard available.

3.3 Partial characterisation of a ΔPsbK mutant

PsbK is one of the small subunits of PS II with a molecular mass of 4.3 kDa containing one transmembrane helix. PsbK is synthesised as a precursor with an N-terminal extension and is processed to its mature form. Deletion of the *psbK* gene in *Synechocystis* sp. PCC 6803 had little or no effect in comparison to wild type (Ikeuchi et al., 1991; Zhang et al., 1993). This leads to the suggestion that PsbK plays a role outside the photochemical processes of PS II. An analogous deletion of the gene in *Chlamydomonas reinhardtii* resulted in poor assembly of PS II and the loss of the ability to grow photoautotrophically (Takahashi et al. 1994). These results suggest that in *Chlamydomonas reinhardtii* PsbK plays an important role in the assembly and/or the stability of PS II. The function of PsbK in *T. elongatus* is still unknown. For the functional analysis of the PsbK subunit, a ΔPsbK mutant was constructed in the group of Prof. M. Ikeuchi in Tokyo (Katoh and Ikeuchi, 2001). In order to disrupt *psbK*, a chloramphenicol resistance cassette was inserted into the *psbK* gene. Cells were maintained in the presence of 7 µg ml\(^{-1}\) chloramphenicol. Segregation of the *psbK* disruption was confirmed by PCR in our laboratory (data not shown).
3.3.1 Growth curves of *T. elongatus* wild type and ΔPsbK

In order to investigate the effect of the psbK deletion on the fitness of the cells, growth curves in comparison to wild type have been recorded under identical conditions. Growth was determined by measuring the OD at 750 nm. Growth curves of the wild type and the ΔPsbK mutant under regular light conditions (80 µE m⁻² s⁻¹) are shown in Figure 3.12.

![Growth curves](image)

Figure 3.12: Growth of *T. elongatus* ΔPsbK compared to wild type under regular light conditions (80 µE m⁻² s⁻¹). Cells were grown at 45°C in BG 11 medium and bubbled with air enriched with 5% CO₂. Cells were inoculated with an OD at 750 nm of 0.3.

The ΔPsbK mutant was able to grow photoautotrophically at a rate comparable to that of wild type under light conditions in the range between 40 to 150 µE m⁻² s⁻¹ (data not shown). These results suggest that the PsbK subunit is dispensable for photoautotrophic growth under a wide range of light conditions.

3.3.2 77 K pigment fluorescence measurements of whole cells

In order to examine whether the stoichiometry of PS I and PS II is affected by psbK disruption, steady state 77 K pigment fluorescence spectra were recorded. Cells were harvested during the exponential growth phase at an OD = 1 at 750 nm. They were adapted for 10 min in the dark at 45°C (to be State 2; Fork and Satoh, 1986) before they were frozen in liquid nitrogen. Illumination of the cells with light of 440 nm leads to an excitation of chlorophyll molecules resulting in fluorescence of PS I and PS II. The fluorescence emission peak at 685 nm may originate from chlorophyll associated with either CP47 or CP43 (Shen and Vermaas, 1994). The peak at 695 nm represents the fluorescence of a single chl a molecule which is bound via His 114 to CP47. This low-energy chlorophyll is the main contributor to the 695 nm emission maximum (Shen and Vermaas, 1994; Funk, 2000), and the peak at 733 nm is due to PS I fluorescence (Mullineaux and Allen, 1990; van Thor et al., 1998).
Figure 3.13 shows PS I/PS II ratios from *T. elongatus* wild type in comparison to the ΔPsbK mutant. For comparison the spectra were corrected for instrument variation and normalised at 733 nm.

![77 K pigment fluorescence spectra of *T. elongatus* wild type cells and ΔPsbK cells.](image)

For comparison, spectra were corrected for instrument variation and normalised at 733 nm. *T. elongatus* wild type as well as the ΔPsbK mutant showed the typical emission spectrum without a variation of the PS II or PS I content. This was also true for preilluminated cells with red light or white light before freezing in liquid nitrogen (to convert the reaction centre to state 1; data not shown).

Illumination of the cells with light of 580 nm, excites the phycobilisomes and results in a fluorescence emission maximum at 690 nm if phycobilisomes are connected to PS II and at 730 nm if connected to PS I. The fluorescence at 690 nm and 730 nm is a result of resonance transfer of excitons from excited phycobilisomes onto chlorophyll molecules. An additional peak around 660 nm is due to free phycobilisomes (mainly allophycocyanin; Figure 3.14). The phycobilisome fluorescence also showed no difference between wild type and ΔPsbK mutant.
Figure 3.14: 77 K pigment fluorescence spectra of *T. elongatus* wild type cells and ∆PsbK cells. Cells were harvested with an OD at 750 nm of 1 and were incubated for 10 minutes in dark before freezing in liquid nitrogen. Excitation was done at 580 nm. The fluorescence emission peak at 690 nm represents the PS II-associated phycobilisomes, the peak around 730 nm represents the PS I-associated phycobilisomes, and the peak around 660 nm represents free phycobilisomes (allophycocyanin). For comparison, spectra were corrected for instrument variation and normalised at 733 nm.

### 3.3.3 Pigment analysis of whole cells

To determine the carotenoid/chlorophyll ratio of whole cells, absorbance spectra from 340 to 740 nm of freshly prepared pigment extracts in 80 % acetone were recorded. From the absorbance at 664 nm for chl *a* and 454 nm for β-carotene the concentrations of chl *a* and β-carotene were calculated using the respective extinction coefficients of the pigments. For chl *a* the molar extinction coefficient at 664 nm (76800 M⁻¹ cm⁻¹) and for carotenoids the extinction coefficient for β-carotene at 454 nm (144000 M⁻¹ cm⁻¹) was used. For the pigment extract, cells of *T. elongatus* wild type and the ∆PsbK mutant were grown for two or four days under regular light conditions (80 µE m⁻² s⁻¹) and for two days under high-light conditions (1500 µE m⁻² s⁻¹). In Table 3.3 the concentration of β-carotene and chl *a* are shown.

<table>
<thead>
<tr>
<th>Strain</th>
<th>T. elongatus</th>
<th>2 days 80 µE m⁻² s⁻¹</th>
<th>4 days 80 µE m⁻² s⁻¹</th>
<th>2 days 1500 µE m⁻² s⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>2.6x10⁻⁶</td>
<td>7.6x10⁻⁶</td>
<td>1.8x10⁻⁶</td>
<td>5.6x10⁻⁶</td>
</tr>
<tr>
<td>∆PsbK</td>
<td>3.0x10⁻⁶</td>
<td>8.9x10⁻⁶</td>
<td>2.2x10⁻⁶</td>
<td>6.6x10⁻⁶</td>
</tr>
</tbody>
</table>

Table 3.3: Concentrations of β-carotene and chl *a* of whole cells of *T. elongatus* wild type and the ∆PsbK mutant under different growth conditions.
Table 3.4 summarises the β-carotene/chl a ratios under various conditions.

<table>
<thead>
<tr>
<th>Strain</th>
<th>T. elongatus</th>
<th>β-car/chl a 2 days 80 µE m⁻² s⁻¹</th>
<th>β-car/chl a 4 days 80 µE m⁻² s⁻¹</th>
<th>β-car/chl a 2 days 1500 µE m⁻² s⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>0.34</td>
<td>0.32</td>
<td>0.94</td>
<td></td>
</tr>
<tr>
<td>ΔPsbK</td>
<td>0.34</td>
<td>0.32</td>
<td>1.26</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.4: β-carotene/chl a ratios of whole cells of T. elongatus wild type and the ΔPsbK mutant under different growth conditions.

Under regular light conditions with 80 µE m⁻² s⁻¹ the ΔPsbK mutant contained more β-carotene and more chl a than wild type, while the β-carotene/chl a ratio of wild type and the ΔPsbK strain were exactly the same. Under high-light conditions (1500 µE m⁻² s⁻¹) the mutant showed a higher β-carotene/chl a ratio of about 1.26 in comparison to 0.94 of wild type resulting from a higher β-carotene and a lower chl a content as compared to wild type under these conditions.

3.3.4 Sucrose density gradient centrifugation of solubilised thylakoid membranes.

In order to analyse the thylakoid membrane composition of T. elongatus wild type in comparison to the ΔPsbK strain, isolated thylakoid membranes were treated with 1.2 % β-DM and 0.5 % sodium cholate. For separation of the components, the thylakoid membrane extracts were added onto a 10-30 % (w/w) sucrose density gradient containing 0.03 % β-DM and centrifuged for at least 18 hours (for details see section 2.1.9).

![Figure 3.15: Sucrose density gradient centrifugation of solubilised thylakoid membranes from T. elongatus wild type and ΔPsbK. Thylakoid membranes were solubilised with 1.2 % β-DM and 0.5% sodium cholate. Thylakoid membrane extracts corresponding to 200 µg chl a were added onto each 10-30 % (w/w) sucrose density gradient containing 0.03% β-DM. Bands were assigned according to sucrose density gradients run with isolated monomeric and dimeric PS II complexes and according to their absorbance spectra.](image-url)
The thylakoid membrane composition analysed by sucrose density gradient centrifugation (Figure 3.15) showed no significant differences. From higher to lower densities, PS I trimers, PS II dimers, PS II monomers, phycobilisomes, free chlorophyll, and carotenoids could be identified in both, *T. elongatus* wild type and the ΔPsbK mutant.

### 3.3.5 Comparative characterisation of isolated PS II complexes from *T. elongatus* wild type and the ΔPsbK mutant

At the beginning of this work, two crystal structures of PS II complexes have been published from *T. elongatus* (Zouni et al., 2001) and *Thermosynechococcus vulcanus* (Kamiya and Shen, 2003), respectively. Interestingly, the subunit PsbK was assigned to different α-helices within the structures. In the PS II structure published by Kamiya and Shen, (2003) it was assigned to an α-helix in the periphery of the PS II dimer and in the structure published by Zouni et al., (2001) the PsbK subunit is located in the centre of the dimer close to the monomer/monomer interface (Figure 3.16). Based on the latter assumption it is possible that this subunit plays a role in the dimerisation of PS II complexes or stabilisation of dimeric complexes. To analyse this assumption, PS II complexes from the ΔPsbK mutant were isolated and the state of oligomerisation was probed by size exclusion chromatography.

![Figure 3.16: Assignment of PsbK in the crystal structures of PS II complexes from *T. elongatus* (Zouni et al., 2001) and *Thermosynechococcus vulcanus* (Kamiya and Shen, 2003). The PsbK α-helix (orange) has different locations in the two structures.](image)
PS II from the ΔPsbK mutant was isolated according to wild type (see section 2.1.7).

![Ion exchange chromatography elution profiles from three different PS II preparations from the ΔPsbK mutant.](image)

The elution profile of PS II complexes from the HIC was identical to the wild type elution profile shown in section 3.1, and the IEC also yielded all three PS II peaks. However, both ratio and elution time of the peaks differed from preparation to preparation in comparison to wild type. Figure 3.17 shows such elution profiles of three different preparations. It was not possible to reproduce the elution profile of ion exchange runs comparing six different preparations.

Analysis of the oligomerisation state of the three PS II peaks via size exclusion chromatography showed the same results as the corresponding fractions from wild type preparations. The first peak represents monomeric PS II and the second and third peak dimeric PS II which could be clearly separated (data not shown).

Oxygen evolution activity measurements of the second peak showed that the activity varies from 500 to 1300 $\mu$mol O$_2$/mg Chl a x h and was, thus, significantly lower than the activity of wild type PS II which had an average activity of 3800 $\mu$mol O$_2$/mg Chl a x h. Also pigment analysis of the isolated dimeric PS II complexes yielded highly different pigment compositions in comparison with wild type, varying from 43 to 71 chl a molecules and from 1 to 13 $\beta$-carotene per 2 pheophytine a molecules.
Results

Figure 3.18: SDS-PAGE of purified PS II complexes from *T. elongatus* wild type and the PsbK-minus mutant according to Schägger and van Jagow (1987). 12% polyacrylamide gel containing 6 M urea. Each lane contains 40 µg of protein. Stained with Coomassie Brilliant Blue.

Analysis of the subunit composition of the isolated dimeric PS II complexes via SDS-PAGE containing 6M urea according to Schägger and van Jagow (1987) showed that there is no obvious difference between wild type and ∆PsbK mutant with all major subunits being present in the mutant preparation. CP47, CP43, D1, D2, and the extrinsic subunits PsbO, PsbV and PsbU could be assigned according to their apparent molecular masses (Figure 3.18). A thick band of aggregates could be found around 115 kDa in the mutant PS II preparation, while PsbK as a small 4 kDa subunit is not detectable in the gel.

To analyse the successful deletion of *psbK* in the mutant and to investigate the composition of small subunits within the dimeric PS II complexes, MALDI-ToF MS analysis of whole PS II complexes was performed. Samples were measured on sinapic and ferulic acid matrices, respectively (Figure 3.19).

![Figure 3.18 SDS-PAGE](image)

![Figure 3.19 MALDI-ToF MS traces](image)

Figure 3.19 MALDI-ToF MS traces of intact isolated dimeric PS II complexes from *T. elongatus* wild type and the ∆PsbK mutant, respectively. Purified PS II complexes were applied to target plate and crystallised either in sinapic acid or ferulic acid matrix. The spectrum shows masses of the intact PS II subunits in an area from 3500 to 5000 Da.
The spectrum of dimeric PS II complexes from the ΔPsbK mutant showed a loss of the respective peak at 4100 m/z in comparison to wild type PS II complexes, indicating that the PsbK subunit is not present on protein level. Another difference is the presence of two additional peaks in the ΔPsbK mutant at 3853.3 and 3978.7 which could not yet be assigned. They may be impurities of the PS II complexes, since there could also be obtained impurities on the SDS-PAGE.

<table>
<thead>
<tr>
<th>Subunit (ORF-ID)</th>
<th>Mass C. unprocessed (mature form) [Da]</th>
<th>Modification</th>
<th>[m/z]</th>
<th>Δ mass M_/mass C.</th>
</tr>
</thead>
<tbody>
<tr>
<td>PsbE (tsr1541)</td>
<td>9573.9 (9441.7)</td>
<td>NH₂-A²</td>
<td>9450.6</td>
<td>+8.9</td>
</tr>
<tr>
<td>PsbH (tsr1386)</td>
<td>7355 (7222.6)</td>
<td>NH₂-A²</td>
<td>7216.3</td>
<td>-6.3</td>
</tr>
<tr>
<td>PsbZ (tsr1967)</td>
<td>6765.3 (6792.3)</td>
<td>N-formyl methionine</td>
<td>6768.8</td>
<td>+3.5</td>
</tr>
<tr>
<td>PsbF (tsr1542)</td>
<td>5065.95</td>
<td>No modification</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PsbY (tsr0836)</td>
<td>4773.9 (4613.7)</td>
<td>NH₂-(f)M³</td>
<td>4628.3</td>
<td>+14.6</td>
</tr>
<tr>
<td>PsbI (tsr1074)</td>
<td>4406.3 (4434.4)</td>
<td>N-formyl methionine</td>
<td>4436.8</td>
<td>+2.4</td>
</tr>
<tr>
<td>PsbL (tsr1543)</td>
<td>4297.3</td>
<td>No modification</td>
<td>4299.7</td>
<td>+2.4</td>
</tr>
<tr>
<td>PsbX (tsr2013)</td>
<td>5233.4 (4188.5)</td>
<td>NH₂-T¹</td>
<td>4190.8</td>
<td>+2.3</td>
</tr>
<tr>
<td>PsbJ (tsr1544)</td>
<td>4105.9 (4016.8)</td>
<td>Acetyl-M²</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PsbM (tsr2052)</td>
<td>3980.7 (4009.9)</td>
<td>N-formyl methionine</td>
<td>?</td>
<td>-</td>
</tr>
<tr>
<td>PsbT (tsr1531)</td>
<td>3875.8 (3903.1)</td>
<td>N-formyl methionine</td>
<td>3906.2</td>
<td>+3.1</td>
</tr>
</tbody>
</table>

Table 3.5: MALDI-ToF MS analysis of intact dimeric PS II complexes from the ΔPsbK mutant. Purified PS II complexes were applied to the target plate in either sinapinic acid matrix or ferrulic acid matrix. The theoretical masses of modified PS II subunits were calculated by Nowaczyk (MassC: PhD thesis, 2005). This method allows a reliable identification of the subunits including posttranslational modifications. The index numbers show the position of the N-terminal amino acid after processing of the polypeptide (Kashino et al., 2002, Gomez et al., 2002).
The MALDI-ToF analysis of dimeric PS II complexes isolated from the ΔPsbK mutant also enabled the identification of the other small (data summarised in Table 3.5). With the exception of PsbK, the mutant contained all small subunits of PS II. In contrast to wild type PS II complexes, where PsbE could only be found in the mature form, both unprocessed and mature form could be identified in the ΔPsbK mutant. Also, PsbZ could be identified in the unprocessed form while the wild type showed only the mature form. PsbJ and PsbM are hard to distinguish as their peaks are very close to each other (4016.8 and 4009.9 for the processed mature forms). Difficult to explain are the relative high mass deviations up to 16.8 Da of calculated and measured masses.

3.4 Partial characterisation of a ΔPsbTc mutant

PsbTc is a small one-helix subunit of the PS II complex with a molecular mass of approximately 4 kDa. In *T. elongatus* PsbTc contains a cystein residue at position 12 which so far has not been reported for any other species (Iwai et al., 2001). In the crystal structure of Ferreira et al. (2004) PsbTc is located in the centre of the dimer close to the monomer/monomer interface. PsbTc is not necessary for photoautotrophic growth (Monod et al., 1994), but in *Chlamydomonas reinhardtii* it is involved in the rapid recovery of PS II activity following photoinactivation (Ohnishi and Takahashi, 2001). For the functional analysis of the subunit, a PsbTc deletion mutant was constructed in the group of Prof. M. Ikeuchi in Tokyo (Iwai et al., 2004). The location of *psbTc* downstream of *psbB* with a short spacer of about 20 bp suggested a co-transcription. The genes, *psbB* and *psbTc* from *T. elongatus* were cloned using degenerate primers within *psbB*. In order to disrupt *psbTc*, a chloramphenicol resistance cassette was inserted into the *psbTc* gene. Cells were maintained with 5 μg ml⁻¹ chloramphenicol. Segregation of the *psbTc* disruption was confirmed by PCR in our laboratory (data not shown).

According to a recent publication (Iwai et al., 2004) the ΔPsbTc mutant is supposed to show no significant difference in photoautotrophic growth and oxygen evolution activity. However, the amount of monomeric isolated PS II complexes was highly increased.
3.4.1 Growth of the ΔPsbTc mutant

Growth of *T. elongatus* wild type and the ΔPsbTc mutant was determined under various conditions by monitoring the OD at 750 nm. Growth curves of the wild type and ΔPsbTc under regular light conditions (80 \( \mu \text{E m}^{-2} \text{s}^{-1} \)) are shown in Figure 3.20. No differences were obvious under these conditions.

![Growth Curve](image)

**Figure 3.20:** Growth of *T. elongatus* wild type and the ΔPsbTc mutant under normal light conditions (80 \( \mu \text{E m}^{-2} \text{s}^{-1} \)). Cells were grown at 45˚C in BG 11 medium, bubbled with air enriched with 2% CO\(_2\). Cells were inoculated with an o.D. at 750 nm of 0.3.

First results of experiments under high-light conditions (1500 \( \mu \text{E m}^{-2}\text{s}^{-1} \)) indicated that the growth of the ΔPsbTc mutant is slower than growth of wild type under these conditions (data not shown).

3.4.2 77 K pigment fluorescence of whole cells

In order to analyse the PS I to PS II ratio 77 K chlorophyll a emission spectra were recorded (Figure 3.21).

![Fluorescence Spectrum](image)

**Figure 3.21:** 77 K pigment fluorescence spectra of *T. elongatus* wild type cells and the ΔPsbTc mutant. Cells were harvested at an OD at 750 nm of 1 and were incubated for 10 minutes in dark before freezing in liquid nitrogen. Excitation was done at 440 nm. The fluorescence emission peak at 685 nm represents the PS II internal antenna fluorescence, the peak at 695 nm the fluorescence of a single chlorophyll a molecule bound to CP47, and the peak at 733 nm the PS I fluorescence. For comparison, spectra were corrected for instrument variation and normalised at 733 nm.
No significant changes in the shape of the pigment emission spectra with excitation of chlorophylls were observed in the ∆PsbTc mutant cells in comparison to wild type.

Figure 3.22: 77 K pigment fluorescence spectra of *T. elongatus* wild type and the ∆PsbTc mutant. Cells were harvested with an OD at 750 nm of 1 and were incubated for 10 minutes in dark before freezing in liquid nitrogen. Excitation was done at 580 nm. The fluorescence emission peak at 690 nm represents the PS II associated phycobilisomes, the peak around 730 nm represents the PS I associated phycobilisomes and the peak around 660 nm is due to free phycobilisomes. For comparison, spectra were corrected for instrument variation and normalised at 733 nm.

Phycobilisome fluorescence (Figure 3.22) showed a significant increase in the emission of PS II associated phycobilisomes (685 nm and 695 nm) and free phycobilisomes (660 nm) in the ∆PsbTc mutant compared to wild type.

### 3.4.3 Pigment analysis of whole cells

In order to investigate the carotenoid to chlorophyll ratio of whole wild type and ∆PsbTc mutant cells, absorbance spectra from 340 to 740 nm of a freshly prepared pigment extract in 80 % acetone were recorded. From the absorbance at 664 nm for chl *a* and 454 nm for β-carotene the concentrations of chl *a* and β-carotene were calculated using the respective extinction coefficients of the pigments. For the pigment extract, cells of *T. elongatus* wild type and the ∆PsbTc mutant were grown for two and four days, under regular light conditions (80 µE m⁻² s⁻¹) and for two days under high-light conditions (1500 µE m⁻² s⁻¹). In Table 3.6 the concentrations of β-carotene and chl *a* under various conditions are shown, and Table 3.7 summarises the corresponding β-carotene to chl *a* ratios.
Results

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>2 days</td>
<td>80 µE m^{-2}s^{-1}</td>
<td>2.6x10^{-6}</td>
<td>7.6x10^{-6}</td>
<td>1.8x10^{-6}</td>
<td>5.6x10^{-6}</td>
<td>2.9x10^{-6}</td>
</tr>
<tr>
<td>ΔPsbTc</td>
<td>2 days</td>
<td>80 µE m^{-2}s^{-1}</td>
<td>2.2x10^{-6}</td>
<td>4.3x10^{-6}</td>
<td>2.6x10^{-6}</td>
<td>4.2x10^{-6}</td>
<td>5.3x10^{-6}</td>
</tr>
</tbody>
</table>

Table 3.6: Concentrations of β-carotene and chlorophyll a of whole cells of T. elongatus wild type and the ΔPsbTc mutant under different growth conditions

<table>
<thead>
<tr>
<th>Strain</th>
<th>T. elongatus</th>
<th>β-car/chl a</th>
<th>β-car/chl a</th>
<th>β-car/chl a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>2 days</td>
<td>80 µE m^{-2}s^{-1}</td>
<td>0.34</td>
<td>0.32</td>
</tr>
<tr>
<td>ΔPsbTc</td>
<td>2 days</td>
<td>80 µE m^{-2}s^{-1}</td>
<td>0.50</td>
<td>0.63</td>
</tr>
</tbody>
</table>

Table 3.7: β-carotene to chlorophyll a ratios of whole cells of T. elongatus wild type and the ΔPsbTc mutant under different growth conditions.

Pigment analysis of whole cells showed an increased β-carotene to chl a ratio in the ΔPsbTc mutant in comparison to wild type under all three growth conditions. This is a result of a higher β-carotene content and lower chl a content in the mutant compared to wild type.

3.4.4 Sucrose density gradient centrifugation of solubilised thylakoid membranes

Figure 3.23: Sucrose density gradient centrifugation of solubilised thylakoid membranes of T. elongatus wild type and the ΔPsbTc mutant. Thylakoid membranes were solubilised with 1.2% β-DM and 0.5% Sodium cholate. The thylakoid membrane extract corresponding to 200 µg chl a were added onto each 10-30 % (w/w) sucrose density gradient containing 0.03 % β-DM. Bands were assigned according to sucrose density gradients run with isolated monomeric and dimeric PS II complexes and according to their absorbance spectra.

To analyse the thylakoid membrane composition sucrose density gradient centrifugation was performed. Isolated thylakoid membranes of T. elongatus wild type and the ΔPsbTc mutant were solubilised with 1.2 % β-DM and 0.5 % sodium cholate.
For separation of the components membranes corresponding to 200 µg Chl a were added onto a 10-30 % (w/w) sucrose gradient containing 0.03 % β-DM and centrifuged for at least 18 hours (Figure 3.23).

The wild type and the ΔPsbTc mutant showed significant differences in thylakoid membrane composition. The colour of the phycobilisome fraction of the ΔPsbTc mutant was more intensive and merged with the monomeric PS II fraction in contrast to wild type.

The major classes of phycobiliproteins present in the phycobilisomes are phycoerythrin (PE), phycocyanin (PC), and allophycocyanin (AP). Phycobiliproteins absorb visible light in the range of 450 to 660 nm (Figure 3.24). Using fluorescence emission spectroscopy, it could be shown that the energy absorbed by phycoerythrin could be transferred to allophycocyanin via phycocyanin (Sidler, 1994). The size, structure and number of phycobilisomes in cyanobacteria depend on the organism, and on the environmental milieu in which the organism is grown. Up to now it is not clear, whether T. elongatus contains phycoerythrin. It is not annotated in the gene data bank and similarity searches showed no results because of the high homology of the genes encoding the phycobilin chromophores. In the following analysis only allophycocyanin and phycocyanin were determined.

In order to investigate the phycobilisome composition, thylakoid membranes were treated with 0.05 % (w/v) β-DM. By this procedure loosely associated phycobilisomes were washed off and after centrifugation they were located in the supernatant. 200 µl of the supernatant of T.elongatus wild type and ΔPsbTc mutant thylakoid membranes were loaded onto a 30 % (w/v) sucrose density gradient (Figure 3.25).
The sucrose density gradients of the phycobilisomes from *T. elongatus* wild type and the ΔPsbTc mutant showed the differences in more detail which were already seen in the sucrose density gradients of solubilised membranes (Figure 3.23). The phycobilisome composition of the mutant differs significantly from that of wild type. In order to identify the different types of phycobiliproteins, the gradients were harvested in 0.5 ml fractions, and the absorbance spectrum in a range from 280 nm to 750 nm of each fraction was measured. The maxima were assigned according to the absorbance spectra of phycobiliproteins shown in Figure 3.24. This led to division into three fractions indicated by the three arrows in Figure 3.25.

The upper sucrose density gradient fraction of wild type phycobilisomes as well as of mutant phycobilisomes contained phycocyanin and allophycocyanin. The middle fraction contained for wild type only phycocyanin and for the mutant fraction also allophycocyanin. In contrast, the lower band contained only phycocyanin in the mutant fraction, while the wild type also contained allophycocyanin.

To verify these results, Western blot analysis with specific antibodies against allophycocyanin and phycocyanin was performed. Wild type cells as well as cells from the ΔPsbTc mutant were grown for two days under regular conditions and under iron-deficient conditions, which are known to degrade phycobilisomes quickly. After preparation, thylakoid membranes were treated with 0.05 % β-DM. After centrifugation, the phycobilisomes in the supernatant were used for Western blot analysis.
Results

Figure 3.26: Western blot analysis of phycobilisomes from *T. elongatus* wild type and the ΔPsbTc mutant. 20 µg of protein were separated on a 12 % SDS-PAGE according to Laemmli (1970). Western blot was performed with specific antibodies against phycocyanin (dilution 1:2000) and allophycocyanin (dilution 1:2000). The second antibody was coupled with alkaline phosphatase.

Since the antibodies showed specific reactions without additional bands or cross reactions, the bands could be clearly assigned to allophycocyanin and phycocyanin, respectively. The ΔPsbTc mutant showed a clear additional band in the phycocyanin blot when phycobiliproteins of the mutant were compared to wild type phycobilisomes. This additional band in the mutant disappeared under iron-deficient growth, indicating that this band contained a type of phycocyanin which was not present under initial iron-deficient conditions. In wild type a difference in phycobilin content was not obvious between iron-sufficient and -deficient conditions.

For estimation of the phycobiliprotein content cell cultures of *T. elongatus* wild type and the ΔPsbTc mutant were grown for two days and harvested by centrifugation. After resuspending the cells in sodium acetate buffer, pH 5.5, the cells were ruptured by French press treatment. After addition of 1% (w/v) streptomycin, they were incubated for 30 minutes. After centrifugation the absorbance at 620 nm for phycocyanin and at 650 nm for allophycocyanin was measured. The calculation of the phycobilin content was done on the basis of chl *a* and protein content, respectively, according to Tandeau de Marsac and Houmard (1988). The equations are given in section 2.2.1.2.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Allophycocyanin [mg/ml]</th>
<th>Phycocyanin [mg/ml]</th>
<th>PC/AP</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>T. elongatus</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>1.00</td>
<td>1.31</td>
<td>1.30</td>
</tr>
<tr>
<td>Δ PsbTc</td>
<td>1.26</td>
<td>1.52</td>
<td>1.21</td>
</tr>
</tbody>
</table>

Table 3.8 Content of phycobilins of *T. elongatus* wild type and the ΔPsbTc mutant.
These results, summarised in Table 3.8, indicated that the ΔPsbTc mutant has more phycobilisomes, or has phycobilisomes with a bigger size containing a higher phycocyanin and allophycocyanin content.

### 3.4.5 Comparative characterisation of isolated PS II complexes from *T. elongatus* wild type and the ΔPsbTc mutant

To analyse the effect of the deletion of the PsbTc subunit on isolated PS II complexes, preparation of PS II complexes from ΔPsbTc was done according to wild type preparation (see section 2.1.7). The results showed that the elution profiles from HIC as well as IEC were similar to the elution profiles obtained for wild type PS II preparation as shown in section 3.1. The oxygen evolving activity of the isolated dimeric PS II complexes was about 20% lower than the wild type activity being 3000 µmol O$_2$/mg Chl x h.

Analysis of the oligomerisation state via size exclusion chromatography showed that the first peak of the ion exchange chromatography contained monomeric PS II complexes, and the second and the third peak contained dimeric PS II complexes (data not shown).

Because Iwai et al., (2004) were not able to isolate dimeric PS II complexes, it was not expected that dimeric complexes could be purified in such a high amount similar to wild type PS II complexes as was possible in my preparation. To proof that the isolated, dimeric PS II complexes were present in the normal dimeric form or whether they were artificial aggregates which have accidentally the same size as dimeric PS II complexes, single particle analysis of these ΔPsbTc mutant complexes was performed. Therefore, negatively stained (2% uranyl acetate, pH 4.5) specimens were prepared on carbon-coated copper grids which were glow-discharged to enhance the amount of PS II topviews.

For a small image analysis, around 5000 particles were extracted and analysed with the GRIP software. As shown in Figure 3.27, image analysis showed normally arranged dimeric PS II particles for the ΔPsbTc mutant PS II complexes (Figure 3.27) being comparable to wild type particles. However, the centre of the dimer has a different pattern in wild type particles in comparison with the mutant dimer. Since PsbTc is located in the centre of the dimer, this could be the result of the deletion of PsbTc in the mutant, which is present in the wild type strain.
3.4.5.1 Subunit composition of isolated PS II complexes from the ΔPsbTc mutant

SDS-PAGE analysis of isolated PS II complexes from the ΔPsbTc mutant in comparison to wild type showed no significant differences with respect to the protein pattern. All major subunits were present as can be seen in Figure 3.28. CP47, CP43, PsbO, D1, D2, PsbV, PsbU, and Psb27 could be assigned according their apparent molecular mass.

In the low molecular weight range one band is missing in the ΔPsbTc mutant PS II complexes indicated by the red arrow. This band might be due to the absence of the subunit PsbTc.
For analysis of subunits having a size <10 kDa, MALDI-ToF MS measurements of whole PS II complexes in ferulic acid matrix were performed. The respective MALDI-ToF MS traces are shown in Figure 3.29. Data were obtained in the range between 3500 and 4800 Da. The spectrum of the ΔPsbTc mutant showed a loss of the respective peak at 3903 m/z, indicating that the PsbTc subunit is not present.

Figure 3.29: MALDI-ToF MS traces of whole PS II complexes from *T. elongatus* wild type and the ΔPsbTc mutant. Purified PS II complexes were applied to target plate and crystallised in ferrulic acid matrix. The spectrum shows masses of the intact PS II subunits in an area from 3500 to 5000 Da.

Table 3.9 summarises these results. With the exception of PsbTc, in the mutant all small subunits of PS II could be identified. In comparison to wild type PS II complexes, where PsbE could be only found in the mature form, in the ΔPsbK mutant complexes the unprocessed as well as the mature form could be identified. PsbZ could be identified in the unprocessed form while in the wild type PS II complexes only the mature form was identified. PsbJ and PsbM were hardly to distinguish because the peaks of PsbJ and PsbM are very to close to each other (4016.8 and 4009.9 for the processed mature forms).
<table>
<thead>
<tr>
<th>Subunit (ORF-ID)</th>
<th>Mass&lt;sub&gt;c&lt;/sub&gt; unprocessed (matureform)[ Da]</th>
<th>Modification</th>
<th>Measured masses [m/z]</th>
<th>Difference mass&lt;sub&gt;meas.&lt;/sub&gt; and mass&lt;sub&gt;calc.&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>PsbE&lt;sup&gt;(tsr1541)&lt;/sup&gt;</td>
<td>9573.9 (9441.7)</td>
<td>NH&lt;sub&gt;2&lt;/sub&gt;-A&lt;sup&gt;2&lt;/sup&gt;</td>
<td>9433.0</td>
<td>-8.7</td>
</tr>
<tr>
<td>PsbH&lt;sup&gt;(tsr1386)&lt;/sup&gt;</td>
<td>7355 (7222.6)</td>
<td>NH&lt;sub&gt;2&lt;/sub&gt;-A&lt;sup&gt;2&lt;/sup&gt;</td>
<td>7209.6</td>
<td>-13</td>
</tr>
<tr>
<td>PsbZ&lt;sup&gt;(tsr1967)&lt;/sup&gt;</td>
<td>6765.3 (6792.3)</td>
<td>N-formyl methionine</td>
<td>6780.9</td>
<td>-11.4</td>
</tr>
<tr>
<td>PsbF&lt;sup&gt;(tsr1542)&lt;/sup&gt;</td>
<td>5065.95 (4934.6) (4976.6)</td>
<td>No modification</td>
<td>5059.2</td>
<td>-6.75</td>
</tr>
<tr>
<td>PsbY&lt;sup&gt;(ts0836)&lt;/sup&gt;</td>
<td>4773.9 (4613.7)</td>
<td>NH&lt;sub&gt;2&lt;/sub&gt;-(f)M&lt;sup&gt;3&lt;/sup&gt;</td>
<td>4606.2</td>
<td>-7.5</td>
</tr>
<tr>
<td>PsbI&lt;sup&gt;(tsr1074)&lt;/sup&gt;</td>
<td>4406.3 (4434.4)</td>
<td>N-formyl methionine</td>
<td>4427.2</td>
<td>-7.2</td>
</tr>
<tr>
<td>PsbL&lt;sup&gt;(tsr1543)&lt;/sup&gt;</td>
<td>4297.3 ( -)</td>
<td>No modification</td>
<td>4291.3</td>
<td>-6</td>
</tr>
<tr>
<td>PsbX&lt;sup&gt;(tsr2013)&lt;/sup&gt;</td>
<td>5233.4 (4188.5)</td>
<td>NH&lt;sub&gt;2&lt;/sub&gt;-T&lt;sup&gt;11&lt;/sup&gt;</td>
<td>4182.2</td>
<td>-6.3</td>
</tr>
<tr>
<td>PsbK&lt;sup&gt;(ts0176)&lt;/sup&gt;</td>
<td>5027.1 (4100.4)</td>
<td>NH&lt;sub&gt;2&lt;/sub&gt;-K&lt;sup&gt;10&lt;/sup&gt;</td>
<td>4094.3</td>
<td>-6.1</td>
</tr>
<tr>
<td>PsbJ&lt;sup&gt;(tsr1544)&lt;/sup&gt;</td>
<td>4105.9 (4016.8)</td>
<td>Acetyl-M&lt;sup&gt;2&lt;/sup&gt;</td>
<td>4010.3</td>
<td>-6.5</td>
</tr>
<tr>
<td>PsbM&lt;sup&gt;(ts2052)&lt;/sup&gt;</td>
<td>3980.7 (4009.9)</td>
<td>N-formyl methionine</td>
<td>4004.6</td>
<td>-5.9</td>
</tr>
</tbody>
</table>

Table 3.9: MALDI-ToF MS analysis of whole dimeric PS II complexes from the ΔPsbTc mutant. Purified PS II complexes were applied to the target plate in either sinapinic acid matrix or ferrulic acid matrix. This method allows a reliable identification of the subunits including posttranslational modifications. The index numbers represent the position of the N-terminal amino acid after processing of the polypeptide (Kashino et al., 2002; Gomez et al., 2002).

### 3.4.5.2 Long term stability of dimeric PS II complexes from the PsbTc-minus mutant

In order to investigate the long term stability of isolated PS II complexes from the ΔPsbTc mutant, isolated dimeric complexes of *T. elongatus* wild type and ΔPsbTc mutant were incubated in dark on ice and at 45°C in dark, and in low-light. In between, the oligomerisation state was detected via size exclusion chromatography.
While long term stability of the PS II complexes from the $\Delta$PsbTc mutant was similar to wild type stability in dark conditions, on ice as well as at 45°C, the stability of the dimers illuminated with light of about 20 $\mu$E m$^{-2}$s$^{-1}$ was much lower. After 24 hours still 64% from the wild type PS II complexes were in the dimeric form, but only 31% from the $\Delta$PsbTc mutant complexes. After 48 hours, no more dimers in the $\Delta$PsbTc mutant complexes could be obtained but still 30% from the wild type PS II complexes were in the dimeric form.

### 3.5 Characterisation of PS II from *T. elongatus* cells grown under iron deficiency

Under iron deficiency, IdiA is expressed in the two closely related cyanobacteria *Synechococcus elongatus* sp. PCC 6301 and PCC 7942 at highly elevated amounts (Michel and Pistorius, 1992; Michel et al., 1996; 1998; 1999; Exss-Sonne et al., 2000). A similar protein, called Tll0513, is also present in *T. elongatus* (Michel et al., 1998; Exss-Sonne et al., 2000) IdiA is a 35 kDa protein in *Synechococcus elongatus* PCC 7942, which is mainly located intracellularly and is assumed to be preferentially associated with the cytoplasmatic side of the thylakoid membrane. IdiA is a basic protein (pI 10.49) and is predicted to contain no transmembrane helices (for details see section 1.10).

![Figure 3.30: Model of the function of IdiA in *Synechococcus elongatus* PCC 6301/PCC 7942.](image)

It has been postulated, that IdiA plays a role in stabilisation of PS II by protecting the acceptor side of PS II against oxidative damage, especially, when oxidative stress is caused by iron limitation (see Figure 3.30), Michel and Pistorius, 1992; Michel et al., 1996; 1998 and 1999; Exss-Sonne et al., 2000).
The exact mechanism of the protective action is not yet clear. Since IdiA is one of the two most prominent proteins expressed under iron starvation the association of IdiA with PS II complexes from *T. elongatus* was investigated. *T. elongatus* cells were grown under iron deficient conditions and PS II complexes were isolated and characterised from these cells. By these investigations it should be also clarified whether the PS II complexes are suitable for structural investigations via single particle analysis.

Cells were grown for four days under iron-deficient conditions in a four litre penicillin flask. To examine whether iron stress occurs, 77K steady state fluorescence spectra of whole cells were recorded. Iron-deficiency leads to an increase of the 685 nm peak due to the expression of IsiA under these conditions. IsiA is a 37 kDa protein, which has homology to PsbC (CP43) of PS II (for details see section 1.9). Since the fluorescence at 685 nm is caused by chl a molecules which are bound to CP43 and CP47 as well as to IsiA, this peak becomes dominant when the expression of IsiA is elevated (Figure 3.31).

![Figure 3.31: 77 K pigment fluorescence spectra of *T. elongatus* wild type cells, grown under iron-sufficient and iron-deficient conditions.](image)

When phenotypically measurable iron-stress occurred, cells were harvested by centrifugation and broken by French pressure treatment at 20000 PSI. PS II isolation was done according to wild type preparation (see section 2.1.7). HIC and IEC
Results

resulted in elution profiles similar to wild type under iron-sufficient conditions (see section 3.1). Characterisation of the oligomerisation state via size exclusion chromatography showed monomeric PS II complexes being present in the first ion exchange chromatography peak and dimeric PS II complexes being present in the second and the third peak (data not shown).

Figure 3.32: SDS-PAGE of isolated PS II complexes from *T. elongatus* wild type grown under iron-deficient conditions and according to Schägger and van Jagow (1976). 12 % polyacrylamide containing 6 M urea. Each lane contains 40 μg protein. Stained with Coomassie Brilliant Blue.

SDS-PAGE analysis of the iron-deficient PS II complexes showed the same pattern as the pattern from iron-sufficient PS II complexes (Figure 3.32). All major subunits were present. An additional band around 35 kDa which belongs to IdiA could not be observed.

Therefore, the three IEC peaks were characterised in more detail by Western blot analysis with specific antibodies against IdiA and PsbO. Starting with a 12% polyacrylamide SDS-PAGE according to Laemmli (1970). 20 μg of protein per sample were loaded onto an SDS-PAGE. The Western blot procedure was done according to 2.1.14.3 with a second antibody coupled to alkaline phosphatase. Since the antibodies showed specific reactions without additional bands or cross reactions, the bands could be clearly assigned either to IdiA or PsbO, respectively. The data presented in Figure 3.33 indicated, that IdiA was present in the dimeric PS II complexes but not in the monomeric fraction.
Results

Figure 3.33: Western blot analysis of PS II complexes isolated from *T. elongatus* cells grown under iron deficiency. The three ion exchange chromatography peaks (Peak 1 = monomeric PS II; Peak 2 = dimeric PS II; Peak 3 = dimeric PS II) were separated on a 12% polyacrylamide gel according to Laemmli (1970). Each lane contains 20 µg protein. Immunological analysis was carried out with antibodies against IdiA (dilution 1:500) and PsbO (dilution 1:1000). The second antibody was coupled to alkaline phosphatase.

To confirm these results, PMF of the respective gel-bands after trypsin digestion was done. The sample was crystallised onto α-hydroxycinnamic acid. After measuring in the mass spectrometer, the resulting mass values were evaluated with the MS-Fit software. The original data of evaluation are shown in Figure 3.34. Because IdiA and PsbO run very similar on SDS-polyacrylamide gels, as used in these experiments, it was difficult to separate the two bands. Therefore, both protein subunits could be detected in one band.

---

Figure 3.34 MS-Fit evaluation of PMF after trypsin digestion of the respective IdiA/PsbO gel band.

Since it could be clearly shown by Western blotting as well as by PMF that IdiA copurifies with dimeric PS II complexes, it was assumed that these particles are a good starting material for single particle analysis. Thus, electron microscopy was
Results of the single particle analysis are shown in Figure 3.35. It was very hard to obtain side views even when not glow-discharged grids were used. In all samples tested, it was not possible to detect any additional mass in the dimeric particles. Thus, although biochemical analyses clearly gave evidence of the presence of IdiA in dimeric PS II complexes, single particle analyses did not allow the detection of an additional peptide in the PS II complexes isolated from iron-deficient cells.

In order to find a reason for this discrepancy, SDS-PAGE and immuno-blot analysis of PS II complexes was done with freshly prepared PS II complexes, and also with PS II preparations which had been frozen after preparation. In each case, however, the whole sample containing all isolated proteins including IdiA had been analysed, irrespective of whether IdiA was still associated to the PS II complexes or already
dissociated. Single particle analysis was done with PS II preparations which were frozen and defrozen and dialysed against buffer without mannitol to remove the sugar because electron microscopy analysis is disturbed by sugar crystals in the background. Furthermore the PS II complexes were stained with uranyl acetate having a low pH (4.5). Thus, at least one of the procedures for preparing samples for single particle analysis could be responsible for the loss of IdiA, especially as it may be only loosely attached to PS II since IdiA has no transmembrane helix. In order to find this out, freshly prepared PS II complexes were treated in the same way as for electron microscopy. Treatment was observed by size exclusion chromatography, followed by SDS-PAGE and Western blot analysis of all eluted fractions from the size exclusion. This should tell whether IdiA was still bound to the PS II complexes.

3.5.1.1 Is the loss of IdiA caused by freezing or dialysing the sample?

The freshly prepared dimeric PS II complexes isolated from T. elongatus cells grown under iron-deficient conditions, containing IdiA, on the basis of Western blot analysis, were frozen in liquid nitrogen and thawed slowly on ice. The thawed sample was used to perform SEC. After pooling and concentrating of the fractions containing dimeric PS II complexes, protein estimation was done in order to use always the same protein amounts for SDS-PAGE and Western blot analysis. The same was done with the defrozen sample which was additionally dialysed against buffer without mannitol over night. Western blot was performed using the IdiA antibody and the PsbO antibody. The results shown in Figure 3.36 indicated that IdiA is still present after freezing and dialysing of the sample, respectively, i.e. IdiA seems to have a stronger interaction with PS II than expected and is not detached from PS II when the mannitol is removed.

![Figure 3.36 Western blot analysis of defrozen and dialysed dimeric PS II complexes containing IdiA after size exclusion chromatography.](image)

The samples were separated on a 12 % polyacrylamide SDS-PAGE according to Laemmli (1970). Each lane contains 20 µg protein. Immunological analysis was carried out with antibodies against IdiA (dilution 1:500) and PsbO (dilution 1:1000). The second antibody was coupled to alkaline phosphatase.
3.5.1.2 Is the lost of IdiA caused by the low pH of the staining solution?

Since both freezing and dialysis step could be excluded as reasons for the absence of IdiA, the low pH of the uranyl acetate staining solution of about 4.5 could be the reason for the detachment of IdiA from PS II dimers, especially since it is known that PS II is sensitive towards low pH. In order to mimic the effect of the staining solution on the PS II-IdiA association, the defrozen sample was washed with low pH buffer (20 mM Glycine buffer, pH 4.5) for five minutes. SEC followed again by SDS-PAGE and Western blot analysis could show the loss of IdiA from PS II due to this treatment. In parallel, also PsbO was washed off in high amounts (Figure 3.37).

Since the low pH of the staining solution seemed to be the reason for the loss of IdiA in the single particle analysis, an alternative staining solution with neutral pH, phospho tungstic acid, was tried. However, this procedure resulted in huge aggregates of PS II, which prevented single particle analysis (Figure 3.38).

![Figure 3.37: Western blot analysis for the detection of IdiA in PS II complexes washed with glycine buffer pH 4.5.](image)

![Figure 3.38 Single particle images of differently stained PS II complexes. A) PS II complexes negatively stained with uranyl acetate pH 4.5 B) PS II complexes negatively stained with phospho tungstic acid, pH 7.](image)
Another alternative for staining is a mix of 1% uranyl oxalate with oxalic acid. Uranyl oxalate is stable between pH 4.5 and 7. Although with this method, too, it was also hard to detect side views of PS II complexes, the images showed clear single particles similar to the uranyl acetate stained images. After picking of particles and alignment, it was possible to obtain a PS II structure with associated IdiA (Figure 3.39). The averaged PS II top view clearly shows that IdiA is located on CP43. Difference mapping (data not shown) showed that the difference is locally about 35% of the total density which is just the level of significance. Although a difference of 20-30% is still within statistical fluctuation, in this case the spot is quite special: It has never been observed before in data sets of PS II complexes from *T. elongatus* grown under iron-sufficient conditions. For a higher significance more particles have to be picked and analysed.

![Figure 3.39: Single particle analysis of PS II complexes from *T. elongatus* grown under iron-deficient and iron-sufficient conditions.](image)

A) Two-fold symmetrised image of about 6000 averaged PS II/IdiA projections from *T. elongatus* grown under iron-deficient conditions B) Two-fold symmetrised image of PS II from *T. elongatus* grown under iron-sufficient conditions. In red overlay: the density of the position of IdiA superimposed on the PS II map. C) Overlay with the PS II crystal structure (Ferreira et al., 2004) D) PS II crystal structure from Ferreira et al. (2004) and location of subunit CP43. In cooperation with A. Arteni in the group of Prof. E. J. Boekema in Groningen.
3.5.1.3 Cross linking of IdiA/PS II complexes

Another approach to obtain PS II complexes from *T. elongatus* with associated IdiA and to get informations about the nature of the association were cross linking methods which connect two molecules chemically by a covalent bond. This enables to determine immediate vicinity relationships, to analyse protein structures and to provide information on the distances between interacting molecules. Chemical cross linkers contain reactive groups which are primary N-hydroxysuccinimidy l ester, imido ester and corbodiimides. These groups are specific to functional groups which are primary amines on the respective proteins. The cross linked PS II/IdiA complexes should be probed by Western blotting with antibodies against the major subunits of PS II (D1, D2, CP43, CP47, cytochrome b$_{559}$) in order to investigate the interaction of IdiA with the PS II complex. Four different cross linker reagents were used for cross linking of PS II complexes: EDC, DSG, AMAS, SMCC (for details see section 2.1.15).

![Figure 3.40: Western blot of cross-linked PS II complexes.](image)

For cross linking experiments, 20 µg of Protein were mixed with a 500-fold excess of the respective cross linker (AMAS, SMCC). After incubation for 2 h at 26°C, the reaction was stopped by addition of SDS-sample buffer. SDS-PAGE analysis according to Laemmli (1970) was performed followed by Western blot analysis with an specific antibody against IdiA. The second antibody was coupled to alkaline phosphatase. Each lane contains 20 µg protein.

First results indicated that the cross linking of IdiA/PS II complexes was successful, with the cross linkers AMAS and SMCC which is obvious from additional band around 45 kDa indicated by the red arrow in Figure 3.40. While EDC and DSG showed no results. Since IdiA is probably associated with subunit CP43 as indicated by the single particle analysis, further cross linking experiments with AMAS and SMCC cross linkers should be done. Also, these results should be confirmed by mass spectrometry, which was most recently combined with cross linking methods in our laboratory (Gertz, 2005) and which is an excellent basis for further analysis of IdiA-PS II interaction.
4 Discussion

PS II is a membrane protein complex which is essential for the photosynthetic light reactions because of its capability of splitting water. Therefore, its structure and function relationships are an interesting and popular field of research. Recently, Ferreira et al. (2004) published the crystal structure of PS II complexes from the cyanobacterium *T. elongatus* at a resolution of 3.5 Å. For the first time it was possible to assign 90% of the amino acid residues, enabling a detailed structural model of PS II. However, many fundamental questions are still open. For example up to now the mechanism of water splitting and charge separation or the function of some of the small subunits having a molecular mass <10 kDa could not be clarified in detail.

The major goal of my thesis was to contribute to a better overall understanding of PS II in the thermophilic cyanobacterium *T. elongatus*. My investigations can be devided into two major parts.

4.1 Part 1: Role of specific subunits in PS II of *T. elongatus*

In the first part of my work, PS II complexes of different mutants of *T. elongatus* were investigated. These mutants were a ∆PsbK and a ∆PsbTc mutant and they were lacking the corresponding PSII peptides PsbK and PsbTc, respectively. These mutants were chosen, because PsbK has been suggested to have a function in the stabilisation of PS II complexes (Takahashi et al., 1994) and PsbTc was suggested to stabilise dimeric PS II complexes (Iwai et al., 2004). These mutants and isolated PS II complexes from these mutants were thoroughly characterised in comparison to wild type with respect to the function and stability of the PS II complexes.

The results of this first part of my work are discussed in the following sections in more detail.

4.1.1 Characterisation of isolated PS II complexes from *T. elongatus* wild type

The PS II preparation procedure established by Kuhl et al. (2000) yielded three subpopulations of PS II complexes which could be obtained in a nearly pure form as indicated by size exclusion chromatography. As alternative methods, to investigate the oligomerisation state of the fractions, sucrose density gradient centrifugation and green native polyacrylamide gels were used. In comparison to these techniques, the
advantage of SEC is that it can be performed in a relative short time (about half an hour per run) and also requires only a small amount of sample (5 µg chl a). Therefore, for further analytical experiments, for example long-term experiments, size exclusion chromatography was used.

The first subpopulation, obtained during PS II preparation after IEC, contained monomeric PS II complexes, which showed no oxygen evolving activity. Via SDS-PAGE analysis, it was shown that this fraction lacks the three extrinsic proteins PsbO, PsbU, and PsbV, which are involved in the water splitting mechanism of PS II. PsbV, also known as cytochrome c<sub>550</sub>, plays a role in water oxidation and its absence does not prevent photoautotrophic growth (Shen et al. 1995), although, psbV deletion mutants contain only about 60 % of wild type PS II and show a 60 % decreased oxygen evolution rate. PsbV/PsbO double mutants of *Synechocystis* sp. PCC 6803 are unable to grow photoautotrophically, contain less than 20 % of wild type PS II, and show less than 10 % oxygen evolving activity. They are also more sensitive towards photoinhibition (Shen et al., 1995). The function of PsbU is unknown. Deletion of the *psbU* gene in a mutant showed no significant differences regarding activity and stability of PS II compared to wild type (Shen et al., 1998). PsbO plays an important role in maintaining an optimal environment for water oxidation. Various studies indicate that it does so by stabilising the Mn cluster, but there is no evidence that it binds Mn directly. Indeed, deletion of the *psbO* gene in *Synechocystis* sp. PCC 6803 does not inhibit oxygen evolution or photoautotrophic growth in the mutant (Burnap and Sherman, 1991; Mayes et al., 1991; Philbrick et al., 1991). Under these conditions the function of the 33 kDa protein may be carried out by the PsbV protein (Shen et al., 1995). The absence of these three extrinsic subunits led to a loss of the water splitting activity in this subpopulation.

SDS-PAGE analysis additionally showed the presence of Psb27, which is a lipoprotein, containing two palmitine acid residues (C16:0) and one stearic acid residue (C18:0). It binds to the lumenal side of PS II and is probably involved in assembly of PS II complexes (Nowaczyk, 2005). This indicates that these monomeric PS II complexes are not a result of dimers, which were dissociated during the preparation, but are already present as monomers in the thylakoid membrane.
The second fraction after PS II purification, being the main fraction, contained dimeric PS II complexes with a high oxygen evolving activity of about 3800 µmol O₂/mg Chl x h.

Via SDS-PAGE analysis it was shown that this fraction contained all major subunits i.e. the reaction centre proteins PsbA and PsbD, the internal antenna proteins PsbB and PsbC and the three extrinsic proteins PsbV, PsbU and PsbO. SDS-Gel analysis also showed that no typical PS II contaminations like ATPase or phycobiliproteins (Dekker et al., 1988; Ichimura et al., 1992) were present in this fraction. Additionally, the small subunits with a molecular mass <10 kDa were identified with MALDI-ToF analysis of the whole PS II complexes. Altogether 19 subunits were identified, which is exactly the same number of subunits which could be assigned in the latest PS II high resolution crystal structure from Ferreira et al. (2004).

A discrepancy was the detection of PsbY, because it was not assigned in the crystal structure. Instead, PsbN was assigned to an α-helix which is located more peripheral in the PS II complex. However, the affiliation of PsbN is unclear and most probably was wrongly assigned (Kashino et al., 2002). As the assignment to the structure was done without knowing the amino acid sequence, it can not be excluded that this helix is part of another subunit, for example PsbY which was not assigned in the structure. This is suggested by the fact that PsbY could be detected in many PS II preparations in contrast to PsbN (Kashino et al., 2002 a; Kashino et al., 2002 b).

The third subpopulation of the IEC column contained dimeric PS II complexes with a 50 % lower activity than the second fraction. The reason for the lower activity is not clear yet. SDS-PAGE and MALDI-ToF analyses showed that in this fraction like in the second fraction all 19 subunits could be identified including the extrinsic proteins PsbO, PsbV and PsbU. But additionally Psb27 could be seen in the polyacrylamide gel. It is known, that PS II-Psb27 complexes can not be reconstituted with the extrinsic proteins, indicating that the lumen-exposed PS II domain which is required for binding of extrinsic PS II proteins is effectively blocked by Psb27 (Nowaczyk, 2005), and in this chromatography fraction also PsbO, PsbU and PsbV were present. However, the question, which could not be answered yet, is wether Psb27 and the oxygen evolving complex were present in stoichiometric amounts or wether this fraction is a mixture of intact PS II dimers containing oxygen evolving complexes and PS II-Psb27 dimers without these components. The lower content of intact dimeric
PS II complexes on chlorophyll basis could also explain the lower oxygen evolving activity.

Since the Psb27-PS II complex probably is an intermediate in PS II biogenesis, the question arised, at what stage of assembly of active PS II-complexes the Psb27-PS II complex has a function. The complex could play a role in assembly, deassembly and reassembly. The biogenesis and assembly steps of PS II in the thylakoid membrane are poorly understood. Zak et al., (2001) could show that the initial steps of biogenesis of cyanobacterial photosystems occur in the plasma membranes. Additionally, damaged PS II centres undergo a rapid and efficient repair mechanism. The repair of PS II via turnover of the damaged protein subunits is a complex process involving highly regulated reversible phosphorylation of several PS II core subunits, monomerisation and migration of the PS II core from the grana to the stroma thylakoids (in higher plants), partial disassembly of the PS II core monomer, highly specific proteolysis of the damaged proteins, and finally a multi-step replacement of the damaged proteins with de novo synthesised copies followed by the reassembly, dimerisation and photoactivation of the PS II complexes (Kanervo and Aro, 2004). During this procedure the extrinsic proteins (PsbO, PsbU and PsbV) and the oxygen evolving complex also have to be deassembled and reassembled. This could be perhaps the function of the PS II-Psb27 complex. To analyse the time dependent distribution of PS II subcomplexes, pulse label experiments using $^{15}$N enriched media combined with MALDI-ToF/ToF-MS analyses were performed (Nowaczyk et al., submitted). These experiments provided strong evidence that Psb27 plays a central role in the biogenesis of the water-splitting site which is most critical for the function of PS II. However, this was shown for monomeric PS II complexes isolated from a 10xHis-Tag mutant of T. elongatus. Not yet clarified is the fact, why Psb27 is also present in dimeric complexes isolated in my preparation.

Since the second ion exchange chromatography fraction was the main fraction and contained highly active, dimeric PS II complexes containing all subunits assigned in the crystal structure, further experiments and comparative studies with the PS II mutants were focussed on this fraction.
4.1.2 Pigment analysis \textit{via} reversed phase chromatography

Since a precise knowledge of pigment composition is a prerequisite for many types of biochemical and biophysical studies of PS II complexes, a pigment analysis method has been established in order to quantify the amount of pigments in the dimeric PS II complexes. The pigment content of PS II complexes is still under debate, because the total pigment content depends on the culture conditions of the organism, the preparation methods, and also on the analytical methods used (Barbato et al., 1991; Yamaguchi et al., 1988; Kobayashi et al., 1990; Chang et al., 1994; Tang et al., 1994). In general, existence of two pheophytin \( a \) molecules per reaction centre is assumed which represent the reference for the determination of the stoichiometry of chlorophylls and carotenoids.

In the crystal structure of PS II (Ferreira et al., 2004), 36 chl \( a \) molecules could be identified and seven \( \beta \)-carotenes were assigned. However, it is possible that there are more pigments as it was indicated by biochemical analyses (Tracewell et al., 2001). For pigment analysis a reversed phase method according to Patzlaff and Barry (1996) with some modifications as described in section 2.1.8.2 was used. The extracted pigments were loaded onto the reversed phase column and were eluted isocratically with methanol. The peaks were assigned according to the elution time of the respective pigment standards and the absorbance spectra. The calculation of different pigment ratios was done using the respective peak areas and the molar absorbance coefficients of the pigments in methanol based on two pheophytine \( a \) molecules per PS II reaction centre.

For the isolated dimeric PS II complexes from \( T. \) elongatus wild type analysis of various preparations resulted in \( 38 \pm 3 \) chl \( a \) per two pheophytin \( a \) molecules. This is in good agreement with the 36 chl \( a \) molecules found per PS II reaction centre in the crystal structure of PS II from Ferreira et al., (2004). Furthermore three to four \( \beta \)-carotene molecules could be identified per PS II monomer. This number differed from the expected seven \( \beta \)-carotene molecules determined in the crystal structure. The peak assigned to carotenoids according to the absorbance characteristics showed a significant shoulder towards longer retention times. This indicates that there were carotenoids present which are different from \( \beta \)-carotene. It is not yet clear whether this form is already present in the fresh sample or emerges during the pigment analysis procedure.
This method also enables the detection of plastoquinone, which could not be quantified yet, because of a missing standard since equilibration with decylplastoquinone was not successful due to the not comparable retention times of decylplastoquinone and the natural plastoquinone-9.

4.1.3 Electron microscopy for structural studies of PS II

In order to speed up and to simplify the preparation a \textit{His}_{10}\textsuperscript{-}tag was introduced at the C-terminus of the CP43 subunit of PS II which enabled the purification \textit{via} an IMAC column in our lab (Prodöhl, 2002; Prodöhl et al., 2003).

Since comparable analysis of wild type PS II complexes and PS II complexes isolated from the \textit{His-tag} mutant with MALDI-Intact-Mass-Tag-MS (MALDI-IMT-MS) analysis showed that subunit PsbZ is not present in individual wild type preparations but in \textit{His-tagged} PS II complexes (Nowaczyk, 2005). Electron microscopy was performed to test the possibilities of combining single particle electron microscopy with proteomics. Single particle analysis of \textit{His-tagged} and wild type PS II complexes was done in cooperation with Ana Arteni in the group of Egbert Boekema (Arteni et al., 2005). Comparison of \textit{T. elongatus} wild type and His-tagged particles showed an additional mass in the periphery of the PS II dimer in the \textit{His-tagged} particle which was not present in the wild type particle. An overlay with the PS II crystal structure from Ferreira et al., (2004) allowed the assignment of this density to subunit PsbZ, which was already indicated by mass spectrometry. PsbZ is a two transmembrane helix subunit of PS II located at the periphery of the complex. The function of this subunit is still unknown, since insertional inactivation of the \textit{psbZ} gene in \textit{Chlamydomonas reinhardtii} showed no phenotype in comparison to wild type (Swiatek et al., 2001).

This example showed that mass differences between membrane proteins in the order of 5\% are still detectable as could be shown for the PsbZ subunit of PS II.

Thus, the combination of mass spectrometry and single particle analysis is a useful new approach in the characterisation of membrane protein complexes.

The reason why PsbZ is lost during individual \textit{T. elongatus} wild type preparations could be explained by the peripheral location of this subunit. Therefore, it is more susceptible for being washed off from the PS II complex during the isolation procedure.
4.1.4 Role of the PsbK subunit

PsbK is one of the small subunits of PS II containing one transmembrane helix. It is synthesised as a precursor with an N-terminal extension and is processed to its mature form of 4.1 kDa. While PsbK deletion in *Synechocystis* PCC 6803 showed no significant differences in comparison to wild type (Ikeuchi et al., 1991) it was suggested, to have a function in the stabilisation of the PS II complex in *Chlamydomonas reinhardtii* (Takahashi et al., 1994). As the function of PsbK in *T. elongatus* was unknown a ΔPsbK mutant was constructed in the group of Prof. Ikeuchi in Tokyo. By comparing this mutant with *T. elongatus* wild type a number of differences were detected.

Characterisation of the ΔPsbK mutant was done on different levels. Cell cultures were analysed for growth under different light intensities and cells were characterised using fluorescence microscopy and pigment analysis. The thylakoid membrane composition was investigated via sucrose density gradient centrifugation. Furthermore, an extensive functional characterisation of isolated PS II complexes from the ΔPsbK mutant was done. The results obtained from the ΔPsbK mutant in comparison to *T. elongatus* wild type are summarised in Figure 4.1.
### Whole cells

- Growth was not affected in a light range from 80 to 150 µE m\(^{-2}\)s\(^{-1}\)
- PS I/PS II stoichiometry showed no differences
- 77K phycobilisome fluorescence was not affected
- β-carotene content was increased under high-light conditions

### Thylakoid membranes

- No obvious differences in thylakoid membrane composition

### Dimeric PS II complexes

- Isolation was not reproducible due to variations during ion exchange chromatography
- Oxygen-evolving activity was not reproducible but was significantly lower
- Pigment analysis was not reproducible
- PsbK was not present in PS II complexes
- D1, D2, CP43, CP47, Cyt b₅₅₉, PsbO, PsbU, PsbV were identified via SDS-PAGE analysis
- PsbTc, PsbJ, PsbM, PsbX, PsbL, Psbl, PsbY, PsbF, PsbZ, PsbH and PsbE were identified via MALDI-ToF of whole dimeric PS II complexes

**Figure 4.1: Comparison of the *T. elongatus* ΔPsbK mutant in comparison to wild type: summarised results.**

Since no significant differences became obvious during determination of growth curves under different light conditions, it can be concluded that the PsbK subunit is dispensable for photoautotrophic growth under a wide range of light conditions. Furthermore 77K pigment fluorescence measurements showed no effect on the PS I and PS II stoichiometry indicating that Psbk is not critically involved in the assembly process of PS II complexes. The phycobilisome content of association with PS II was not affected, too.

First differences of the ΔPsbK mutant in comparison to wild type were obvious when the carotenoid to chlorophyll ratio of whole cells was estimated. Under regular light conditions (80 µE m\(^{-2}\)s\(^{-1}\)) the ΔPsbK mutant contained a higher β-carotene and a higher chl a content compared to wild type, while the β-carotene to chl a ratio of wild
type and the ΔPsbK strain was exactly the same. Under high-light conditions (1500 µE m⁻²s⁻¹) the mutant showed a higher ratio of β-carotene to chl a in comparison to wild type. This was a result of a higher β-carotene and a lower chl a content compared to wild type under these conditions. β-carotene can partially serve as a light-absorbing pigment. However, its main function seems to be the protection of chl a near the reaction centre from photooxidation. Due to their ability to quench oxygen radicals, carotenoids are efficient anti-oxidants and thereby protect cells from oxidative damage. The change of the number of carotenoids and their composition occurs in response to light quantity and light quality (Hartmut and Lichtenthaler, 1987). Especially at high-light conditions, the protective role of carotenoids in cyanobacteria is important (Kellar and Paerl, 1980; Codd, 1981). Cells of a Synechococcus elongatus PCC 7942-strain containing less carotenoids showed a higher sensitivity to photoinhibition (Sandmann et al., 1993). Thus, the general increase of the β-carotene content of T. elongatus wild type as well as from the ΔPsbK mutant is a result of adaptation to high-light conditions in order to protect the photosynthetic apparatus against photooxidation. The higher β-carotene content in the mutant under high-light conditions in comparison to wild type might indicate, that the mutant cells are more sensitive to photooxidation under these conditions.

Using sucrose density centrifugation the composition of the thylakoid membrane from the ΔPsbk mutant was investigated in comparison to wild type. All components, PS II dimers, PS II monomers, PS I trimers, phycobilisomes, carotenoids and free chlorophyll were observed at about similar amounts in both, T. elongatus wild type and the ΔPsbK mutant, suggesting that the absence of PsbK has no obvious effect on the composition of the thylakoid membranes.

PS II isolation from the ΔPsbK mutant was done according to the PS II preparation of T. elongatus wild type. While the thylakoid membrane preparation and the first PS II isolation steps including extraction and HIC showed no differences between wild type and the ΔPsbK mutant, the IEC showed substantial deviations. The elution profile showed three peaks similar to the wild type PS II complexes, but their relative amount and elution time of the peaks differed from preparation to preparation in comparison to wild type.
Thus, monomeric and dimeric PS II complexes were isolated from the ΔPsbK mutant but not in a reproducible way and in about 50 % lower amounts than for wild type. The separation via IEC is due to different characteristics of the protein surface. Alterations during the separation procedure were probably caused by changed surface properties of PS II in the ΔPsbK mutant. The loss of subunit PsbK in PS II complexes might also result in PS II complexes which are more unstable during the isolation procedure in comparison to wild type PS II complexes. To verify this assumption long term stability experiments of isolated ΔPsbK mutant PS II complexes should be done.

Analysis of the oligomerisation state of the three IEC peaks via SEC showed the same results as the corresponding fractions from the wild type preparation. Therefore, the initial assumption suggesting that the subunit PsbK may be involved in dimerisation of PS II complexes indicated by the PS II high resolution structure from *T. elongatus* from Zouni et al. (2001) where PsbK was located close to the monomer/monomer interface, was not confirmed. This was supported by the recent PS II crystal structure from Ferreira et al. (2004) in which the PsbK subunit is located in the periphery of the PS II complex as in the structure from Shen et al. (2003).

Oxygen evolution activity of the dimeric PS II complexes from the ΔPsbK mutant was measured in presence of the artificial electron donor DCBQ. The activity changed from preparation to preparation, but was always significantly lower than in wild type. These data are corresponding to results from Katoh and Ikeuchi (2001) who showed that the optimum concentration of DCBQ for PS II complexes from the ΔPsbK mutant was two to three times higher than in wild type, indicating that the affinity of PS II to quinone is affected by the absence of the PsbK subunit.

![Figure 4.2: Structure of the PsbK subunit and association of two β-carotene molecules.](image)

Pigment analysis via RPC of the isolated dimeric PS II complexes yielded large variations in the pigment contents as compared to wild type, varying from 43 to 71 chl a molecules and 1 to 13 β-carotene molecules per 2 pheophytin a.
molecules. Since in the PS II high resolution crystal structure from Ferreira et al. (2004) PsbK is located in immediate vicinity to two carotenoid molecules (Figure 4.2), it is possible that the loss of the PsbK subunit has an influence on carotenoid binding and stability within the PS II complex. Additionally, it has been reported, that PsbK in PS II complexes from *Chlamydomonas reinhardtii* is specifically bound to CP43 (Sugimoto and Takahashi, 2001). Interestingly, in the PS II crystal structure (Ferreira et al., 2004) the PsbK helix is closely associated with CP43. Provided that the interaction between PsbK and CP43 is rather strong, a loss of PsbK could affect the stability of CP43 and as a result it might have an influence on chl *a*-binding of CP43.

Analysis of the subunit composition of the isolated dimeric PS II complexes via SDS-PAGE showed that there were no obvious differences between the *T. elongatus* wild type and the ∆PsbK mutant. The lack of subunit PsbK could be confirmed via MALDI-ToF analysis as well as via PCR. Furthermore, two additional peaks in the ∆PsbK mutant at 3853.3 and 3978.7 were detected via MALDI-ToF analysis of whole PS II complexes, which may be impurities although they could not yet be assigned.

Finally, it can be concluded, that the PsbK subunit might play a role in the pigment binding, especially of carotenoids, in PS II complexes. As shown by a deletion mutant, the lack of this subunit leads to a higher sensitivity against photoinhibition under high-light conditions.
### 4.1.5 Role of the PsbTc subunit

<table>
<thead>
<tr>
<th>Whole cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>➤ Growth was not affected under regular light conditions but slightly decreased under high light conditions</td>
</tr>
<tr>
<td>➤ PS I/PS II stoichiometry showed no differences</td>
</tr>
<tr>
<td>➤ Fluorescence of free phycobilisomes and phycobilisomes connected to PS II was highly increased</td>
</tr>
<tr>
<td>➤ β-carotene content was increased and chlorophyll α content is decreased under regular as well as under high light conditions</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Thylakoid membranes</th>
</tr>
</thead>
<tbody>
<tr>
<td>➤ Membrane composition was changed due to an additional phycobilisome fraction</td>
</tr>
<tr>
<td>➤ Higher content of phycocyanin and allophycocyanin</td>
</tr>
<tr>
<td>➤ Contained additional phycocyanines</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Dimeric PS II complexes</th>
</tr>
</thead>
<tbody>
<tr>
<td>➤ PS II preparation was reproducible</td>
</tr>
<tr>
<td>➤ Dimeric PS II complexes could be isolated</td>
</tr>
<tr>
<td>➤ The dimeric PS II complexes were no artificial aggregates</td>
</tr>
<tr>
<td>➤ Oxygen evolving activity was about 20 % lower</td>
</tr>
<tr>
<td>➤ Long term stability of the dimers was lower during light exposition at 45°C</td>
</tr>
<tr>
<td>➤ PsbTc was not present in the PS II complex</td>
</tr>
<tr>
<td>➤ D1, D2, CP43, CP47, Cyt b559, PsbO, PsbU, PsbV were identified via SDS-PAGE</td>
</tr>
<tr>
<td>➤ PsbK, PsbJ, PsbM, PsbX, PsbL, PsbI, PsbY, PsbF, PsbZ, PsbH and PsbE were identified via MALDI-ToF of whole dimeric PS II complexes</td>
</tr>
</tbody>
</table>

Figure 4.3: Comparison of the *T. elongatus* ΔPsbTc mutant and wild type: summarised results.

PsbTc contains one transmembrane helix and is located close to the monomer/monomer interface in the high resolution crystal structure of PS II (Ferreira et al., 2004). PsbTc of *T. elongatus* contains a cystein residue at position 12 which
has so far not been reported in any other species (Iwai et al., 2001). For a functional analysis of the subunit, a PsbTc deletion mutant was constructed in the group of Prof. M. Ikeuchi in Tokyo (Iwai et al., 2005). In a recent publication from the same group (Iwai et al., 2005), PsbTc is supposed to play an essential role in dimerisation of PS II complexes.

The ΔPsbTc mutant was characterised on different levels. Cell cultures were analysed by fluorescence spectroscopy, pigment analysis, and by measuring growth curves under different light intensities. The thylakoid membrane composition was analysed via sucrose density gradient centrifugation. Furthermore, an extended functional characterisation of isolated PS II complexes from the ΔPsbTc mutant was done. Figure 4.3 shows the summarised results obtained from the *T. elongatus* ΔPsbTc mutant in comparison to the wild type are shown – differences are discussed in the following section.

The ΔPsbTc mutant was able to grow photoautotrophically at a rate comparable to that of the wild type under regular light conditions. In contrast, the growth rates of the mutant in comparison to wild type were reduced when cells were cultivated under high-light conditions indicating that the loss of PsbTc results in a higher sensitivity against photoinhibition. These results are confirmed by Ohnishi and Takahashi (2001) who showed that deletion of the PsbTc gene in *Chlamydomonas reinhardtii* leads to photoinhibition under high-light conditions. Already Monod et al. (1994) identified PsbTc as a subunit of PS II in *Chlamydomonas reinhardtii* which maintains photosynthetic activity under adverse growth conditions.

The carotenoid to chlorophyll ratio of whole cells grown under different light conditions showed an increased β-carotene to chl a ratio in the ΔPsbTc mutant in comparison to wild type under regular- as well as under high-light conditions. This was a result of a higher β-carotene content and a lower chl a content in the mutant compared to wild type. In section 4.1.4 it was mentioned that the carotenoid content and composition changed as an adaptational process under different light conditions, but other growth conditions may also affect the carotenoid content. In *Pseudanabaena* M2, grown under nitrogen deficiency, an increase in carotenoid content, accompanied with a degradation of phycocyanin has been observed (Canto de Loura et al., 1987). Since the *T. elongatus* ΔPsbTc mutant showed also
differences in phycobilisome content and composition (see following sections) in comparison to wild type, the higher \( \beta \)-carotene content of the \( \Delta \text{PsbTc} \) mutant under the different conditions might be due to the modification of the phycobilisomes. Additionally, the PS II crystal structure from Ferreira et al. (2004) showed potential \( \beta \)-carotene densities at the monomer/monomer interface. Therefore, loss of PsbTc, located close by in the structure could result in a malfunctioning of \( \beta \)-carotene-binding to PS II.

As it was shown via 77K pigment fluorescence measurements the PS I to PS II ratio was not affected in the mutant, while upon excitation of cells with light of 580 nm, the fluorescence emission between 650 and 710 nm, representing the fluorescence of free phycobilisomes and PS II associated phycobilisomes, was significantly higher in the \( \Delta \text{PsbTc} \) mutant than in wild type. The higher phycobilisome fluorescence might be due to the disconnection of phycobilisomes from PS II or slightly disassembled PS II centres. Additionally, it is known that cyanobacteria alter the composition of their phycobilisome structure in response to different light conditions (Bogorad, 1975; Tandeau de Marsac, 1983; Tandeau de Marsac et al., 1988). This process of chromatic adaptation depends on light quality as well as light quantity. In general, low-light intensities stimulate the synthesis of phycobilisomes and the rods may increase in length (Öquist, 1974; Grossman et al., 1993). An increase in the size or number of phycobilisomes under such conditions may help to balance the electron flow between PS I and PS II (Melis et al., 1989). In contrast, high-light conditions lead to a decrease of the size or number of phycobilisomes (Miskiewicz et al., 2002). Therefore, a perturbation of the electron transfer between phycobilisomes and PS II may result in a higher number or bigger size of phycobilisomes.

Significant differences also became obvious, when the thylakoid membrane composition of the \( \Delta \text{PsbTc} \) mutant was compared to wild type via sucrose density centrifugation. The phycobilisome composition of the mutant differed significantly from that of wild type. These results confirmed the results of 77K pigment fluorescence spectroscopy, since the \( \Delta \text{PsbTc} \) mutant seems to have an increased number or size of phycobilisomes. Verification via Western blot analysis and quantification of phycobilins indicated that the mutant contained a higher amount of phycocyanin as well as allophycocyanin in comparison to wild type indicating that the efficiency of light collecting is influenced by a lack of the PsbTc subunit. The mutant
seems to produce phycobilisomes in a higher amount or bigger size, because either the connection of phycobilisomes to PS II is disturbed or the energy transfer from the phycobilisomes into the reaction centre is interrupted.

Isolated PS II complexes from the ΔPsbTc mutant showed no differences concerning the oligomerisation state of the complexes. The activity of the main fraction was about 20% lower than the wild type activity. Since Ohnishi and Takahashi (2001) showed, that the decrease in PS II activity of a ΔPsbTc Chlamydomonas reinhardtii mutant was a result from accelerated photoinactivation of PS II or decreased repair activity of the photodamaged PS II and since oxygen evolving activity measurements in my work were performed under saturating light intensities, this could be the reason for the lower activity of the PS II complexes from the T. elongatus ΔPsbTc mutant.

SDS-PAGE and MALDI-ToF analysis showed no differences in subunit composition of the PS II complexes, suggesting, that the deletion of PsbTc had no effect on PS II subunit composition as it was indicated by Iwai et al. (2004). They suggest an additional loss of PsbM in the ΔPsbTc mutant. But there were relatively high mass deviations, up to 16 Da difference between masses measured and masses calculated, in my MALDI-ToF results, which might be due to the spectrometer used. And since it is very difficult to distinguish between subunit PsbM and PsbJ, because of the similar calculated masses of about 4016.8 for PsbJ and 4009.9 for PsbM, these results have to be confirmed using a spectrometer with a higher precision. Via MALDI-ToF analysis and PCR it could also be shown, that PsbTc is not present in the mutant.

Iwai et al., (2004) suggested that subunit PsbTc plays an important role in dimerisation of the PS II complex, because they could not prepare dimeric PS II complexes from the ΔPsbTc mutant. Therefore, it was not expected to isolate dimeric PS II complexes in a ratio similar to wild type. Eijckelhoff et al. (1996) showed that artificial dimeric aggregations of PS II core complexes have been induced by size exclusion chromatography. To exclude an artificial dimeric aggregation, single particle analysis was performed, which showed that the isolated PS II complexes from the ΔPsbTc mutant were arranged in the normal dimeric form. The centre of the PS II dimer from the ΔPsbTc mutant looked slightly different from the wild type PS II dimer which may be due to the fact that the PsbTc subunit is located in the centre of the dimer in immediate vicinity of the monomer/monomer interface.
SEC and single particle analysis of the isolated PS II complexes from the ΔPsbTc mutant showed that it was possible to isolate normally arranged dimeric PS II complexes from the mutant in similar amounts to wild type PS II complexes. Supposing that the dimeric PS II complexes from the ΔPsbTc mutant are more unstable in comparison to wild type PS II complexes, which was indicated by long term experiments in this work, the monomerisation of the complexes might be influenced by the PS II preparation procedure. Iwai et al. (2004) broke the cells using a bead-beater. This is a very harsh method in comparison to Parr bomb or French press treatment which I used for cell rupture. Furthermore, Iwai et al. (2004) used the detergent heptyl-β-D-thioglcoside (HTG) to solubilise the PS II complexes from the thylakoid membrane and crude PS II particles were isolated by differential ultracentrifugation. HTG is a non-ionic detergent which was suggested to be very usefull for solubilisation of membrane proteins (Shimamoto et al., 1985) and was also used for solubilisation of monomeric spinach PS II complexes for two-dimensional crystallisation and cryo-electron microscopy (Nakazato et al., 1996). Iwai et al. (2004) could also isolate dimeric PS II complexes from T. elongatus wild type, using HTG for solubilisation but the ratio of monomeric to dimeric PS II complexes was about one to one. Kuhl et al. (2000) showed that solubilisation with β-DM alone led to a higher content of monomeric PS II complexes. As addition of sodium cholate stabilises the dimeric PS II complexes, the reason for obtaining only monomeric PS II complexes from the ΔPsbTc mutant in the publication from Iwai et al. (2004) might be just a consequence of the isolation procedure used.

Finally, it can be concluded, that subunit PsbTc in PS II of T. elongatus plays a role in phycobilisome binding which led in the mutant to a higher sensibility against damage by photoinhibition due to a disturbed light energy transfer. PsbTc is not essential for dimerisation of the PS II complex, but plays a role in stabilisation of dimeric complexes.
4.2 Part 2: The effect of iron deficiency on PS II from *T. elongatus*

In the second part of my work, I investigated PS II complexes which were isolated from *T. elongatus* cells cultivated under mild iron limitation. In previous investigations by Michel et al. (Michel and Pistorius, 1992; Michel et al., 1996, 1998, 1999; Exss-Sonne et al., 2000) it had been shown that under oxidative stress, especially when oxidative stress was caused by iron deficiency, the expression of a protein called IdiA (iron deficiency induced protein A - 39.2 kDa calculated molecular mass and 35 kDa mass of processed form) was strongly up-regulated in the mesophilic cyanobacteria *Synechococcus elongatus* PCC 7942 and PCC 6301. The so far obtained results gave evidence that this protein protects PS II from oxidative damage, and it has been suggested that the protein mainly protects the acceptor side of PS II. Moreover, it has been shown that IdiA is clearly detected in partially purified PS II complexes from the mesophilic cyanobacteria *Synechococcus elongatus* PCC 6301 and PCC 7942 (Michel, 1996; Lax, 2002) and that crude PS II preparations from this cyanobacterium had a higher oxygen evolving activity on chlorophyll basis when this protein was present (Michel, 1996). However, so far it had not yet been shown that this protein indeed tightly interacts with PS II and that it is still a peptide in highly purified PS II. The thermophilic cyanobacterium *Thermosynechococcus elongatus* BP-1 contains a similar protein to IdiA being Tll0513 which has a calculated molecular mass of 39.8 and which is also called IdiA. The protein IdiA of *T. elongatus* has 50 % identity and 75 % similarity to IdiA of *Synechococcus elongatus* PCC 7942 (Michel, 2003) and gives a good cross-reaction with the antibody raised against IdiA of *Synechococcus elongatus* PCC 7942 (Exss-Sonne et al., 2000). Moreover, it is well documented in the literature that PS II complexes from this cyanobacterium are substantially more stable than PS II complexes isolated from mesophilic cyanobacteria. Therefore, the major goal of this part of my thesis was to investigate whether this protein is an associated part of isolated and highly purified PS II complexes when cells are cultivated under iron-deficient conditions. The obtained results are discussed in the following section.

PS II complexes from cells which have been grown for four days under iron deficient conditions, were isolated and in part characterised to mainly answer the question whether the protein IdiA is a component of highly purified PS II complexes. The occurrence of iron deficiency in these cells was documented by measuring the
increase in chlorophyll fluorescence at 685 nm at 77K. This fluorescence is due to the expression of the protein IsiA (Öquist, 1974; Burnap et al., 1993) which forms a new antenna around PS I under iron limitation (Boekema et al., 2001; Bibby et al., 2001).

After harvesting of the *T. elongatus* cells from iron-deficient cultures, the PS II preparation was done according to the procedure established for *T. elongatus* wild type grown under iron-sufficient conditions. The isolation procedure of PS II complexes from the iron-deficient cells gave similar results as those obtained with iron-sufficient cells leading to separation in three fractions. The subsequently performed SDS-PAGE analysis of the three PS II peaks showed the same protein pattern as the pattern from iron-sufficient PS II complexes. However, an additional band in the molar range of about 35 kDa, which should indicate the presence of IdiA, was not seen. Therefore, the three fractions were characterised in more detail with the help of Western blot analysis and with specific antibodies against IdiA and PsbO. Since the antibodies are highly specific for IdiA and PsbO and since no additional bands or cross reactions with other proteins were obtained with these antisera, the detected bands could be clearly assigned to either IdiA or PsbO. The results gave clear evidence that PsbO is present in all three PS II fraction (also slightly in the monomeric fraction), but that IdiA was only present in dimeric PS II complexes. The Western blot analysis also showed that the IdiA band and the PsbO band were located at the same height on the gel indicating that the running behaviour of these two proteins in a SDS-gel is very similar and that this is the reason why no additional band was identified in a Coomassie Brilliant Blue stained gel.

To further confirm the result of the presence of IdiA in purified PS II dimers, peptide mass fingerprint of the respective gel-bands after trypsin digestion was done. The results clearly provided evidence that also via MALDI-ToF analysis it was possible to identify IdiA in dimeric PS II complexes. Thus, on the basis of these results obtained by Western blot analysis and by PMF, it can be concluded that IdiA is present in highly purified PS II complexes isolated from the thermophilic cyanobacterium *T. elongatus* when cultivated under iron limitation. Moreover, the results also provide evidence that IdiA is only present in dimeric but not in monomeric PS II complexes.

In the next step of my investigation single particle analyses were performed to see whether on the basis of this method a conclusion about the localisation of IdiA within
the dimeric PS II complex could be made. In cooperation with Ana Arteni in the group of Prof. Dr. E.J. Boekema electron microscopy was performed. The initial results of these investigation were extremely disappointing, since no additional electron density could be detected in dimeric PS II complexes. Moreover it was not possible to obtain side views. Thus, although biochemical analyses had given clear evidence of the presence of IdiA in dimeric PS II complexes, single particle analyses did not allow the detection of an additional peptide in the PS II complexes isolated from iron-deficient cells. Therefore, the question was asked what the reason could be for these deviating results. One of the treatments used in the procedure for preparing samples for single particle analysis could be the responsible for the detachment of IdiA from PS II complexes, since IdiA has no transmembrane helices. Therefore, IdiA can only be a peripheric protein. To answer the question of whether one of the treatments, used to prepare PS II complexes for single particle analysis, might be the reason for the detachment of IdiA from PS II, the PS II complexes were submitted to these procedures and then investigated for the presence of IdiA by the biochemical analyses. The investigations gave evidence that after freezing, thawing, and dialysis, IdiA was still bound to the dimeric PS II complex, indicating, that IdiA is associated with PS II in a fairly tight way.

Since the freezing step and the dialysis step were excluded as a reason for the absence of IdiA, the low pH of the uranyl acetate staining solution of about 4.5 could be the problem, as it is known that PS II is sensitive towards low pH values. Investigations of a low pH onto the PS II/IdiA complexes resulted in a loss of IdiA from PS II complexes. Since the reason for the absence of IdiA in the single particle analysis was identified to be due to the low pH of the staining solution, an alternative staining solution was tried. However, this procedure resulted in a further problem. PS II complexes could only be obtained as huge aggregates which made a single particle extraction impossible. Finally, another staining method, using uranyl oxalate pH 6-7, led to the desired result. It was possible to get an electron microscopic structure of PS II complexes with an attachment of IdiA (Figure 4.4).
The single particle analysis of the dimeric PS II complexes stained with uranyl oxalate of pH 6-7 gave the following results:

- An additional density would clearly be identified in dimeric PS II complexes. Since IdiA was identified to be present in these complexes by Western blot and PMF analysis, this additional density can be attributed to IdiA.
- Two such densities could be detected per PS II dimer, and it is suggested that IdiA is associated with CP43.
- At the present time it is uncertain whether IdiA is located on the lumenal or on the cytoplasmic side of PS II.

Biochemical data gave evidence, that IdiA is located on the acceptor side of PS II. It was shown in investigations of an IdiA-free *Synechococcus elongatus* PCC 7942 mutant that besides a general reduction of cell growth the PS II activity is much lower under iron-deficient conditions than in wild type, while the PS I activity seems to be not affected which was not observed in wild type cells. The reduced PS II activity is associated with an accelerated degradation of the D1 protein (Michel et al., 1996; Exss-Sonne et al., 2000). Moreover, investigations of the IdiA-free mutant and a PsbO-free mutant in comparison with wild type, using different electron acceptors and room temperature fluorescence measurements, provided evidence that the reduction of the PS II activity was mainly caused by damage of the acceptor side of PS II in the IdiA-free mutant. This assumption was also verified by a treatment of wild type and IdiA-free mutant cells with hydrogen peroxide (H$_2$O$_2$). In wild type cells the treatment resulted in a transient decrease of PS II activity paralleled by a transient
decrease of the D1 protein and a transient increase of the IdiA protein. In contrast to wild type, \( \text{H}_2\text{O}_2 \) caused a significantly higher and longer lasting damage of PS II activity, while the PS I activity was not affected in the IdiA-free mutant (Exss-Sonne 2000; Exss-Sonne et al., 2000). The finding that the inhibitory effect of the herbicide bentazone, which is assumed to bind to the acceptor side of PS II, is extremely reduced, when IdiA is expressed is in agreement with a PS II acceptor side function of IdiA (Bagchi et al., 2003).

The side of the attachment of IdiA could yet not be confirmed by electron microscopy. For this purpose a larger data set of single particles is necessary to reach a higher significance of densities.

4.2.1 How does PS II cope with stress?

In conclusion, the second part of my work provided evidence that the protein IdiA is associated with isolated dimeric PS II complexes but not with isolated monomeric PS II complexes of \( \text{T. elongatus} \). The results also suggest that IdiA is fairly tightly associated with these dimeric PS II complexes, Thus, these results give evidence that under certain stress conditions, such as cultivation of cells under iron-deficient conditions, IdiA is tightly associated with PS II and obviously helps PS II to cope with this stress situation by making PS II more resistant towards oxidative stress.

In oxygenic photosynthesis various reactive oxygen species (ROS), which can damage cellular proteins, are generated as a result of the incomplete reduction of molecular oxygen in the photosynthetic electron transport (Asada, 1996 and 1999; Mongkolsuk and Helmann, 2002). When the absorbance of light energy by light harvesting complexes exceeds the capacity for utilisation of the energy in photosynthesis, the generation of ROS is greatly increased. The main target of photodamage is PS II and is mainly due to damage of the D1 protein and the subsequent rapid degradation of this subunit (Barber and Andersson, 1992; Aro et al., 1993; Golden, 1994; Andersson and Barber, 1996, Asada, 2000). It was shown that an increased intracellular concentration of ROS primarily inhibited the synthesis of the D1 protein \textit{de novo} at the transcriptional level (Nishiyama et al., 2001). In contrast to higher plants, where the D1 protein is encoded by a single \textit{psbA} gene, in cyanobacteria it is encoded by a \textit{psbA} gene family, which differs in size and products depending on the organism (Mate et al, 1998; Sane et al., 2002; Salih et al, 1997). Which gene is transcribed depends on the growth conditions of the organism i. e.
light intensity, light quality, temperature, and oxygen availability (Kulkarni et al., 1994; Campbell et al., 1995, and 1998; Sane et al., 2002). Recently it could be shown for the thermophilic cyanobacterium *T. elongatus* that three psbA genes are present and also three forms of the D1 protein *in vivo* are present (Nowaczyk, 2005) indicating, that all genes are functional and produce three different gene products. However, the function of the three copies is still unknown.

Iron deficiency leads to a situation in which the photosynthetic electron transport chain severely forms ROS but in which the detoxifying enzymes i.e. catalase and peroxidase, are only present in reduced amounts, since partly they also depend on iron as cofactor (Lundrigen et al., 1997). Consequently oxidative stress is getting more intensive. It was shown that the adaptation of the multi protein complexes PS II and PS I to iron deficiency is a sequential process, which includes the enhanced expression of two major iron-regulated proteins, IdiA and IsiA (Michel and Pistorius, 2004). These results suggest that IdiA protects the acceptor side of PS II against oxidative stress under conditions of mild iron limitation in a yet unclear way, whereas prolonged iron deficiency leads to the synthesis of a chlorophyll *a* antenna around PS I-trimers consisting of IsiA protein molecules (Michel and Pistorius 1992; Michel 1996; Michel et al., 1996; Michel et al., 1998; Michel et al., 1999; Exss-Sonne, 2000; Exss-Sonne et al., 2000; Boekema et al, 2001; Michel et al, 2003; Michel and Pistorius, 2004). The physiological consequences of these alterations under prolonged iron starvation are a reduction of linear electron transport activity through PS II and an increase of cyclic electron flow activity around PS I as well as an increase in respiratory activity as shown by acridine yellow fluorescence measurements. Moreover, a strong interrelationship between iron homeostasis and oxidative stress in *Synechococcus elongatus* PCC 7942 is suggested by the fact, that transcription of major iron-regulated genes such as *isiA, isiAB, idiA* and *idiB* is induced by oxidative stress within a few minutes following a treatment of cells with hydrogen peroxide or methylviologen (Yousef et al., 2003). However the exact way of protective function of IdiA on PS II is not yet clear. It is possible that it either interacts with the non-heme iron located between Q$_A$ and Q$_B$ or that it functions as an iron storage/iron transport protein during the high turn-over of PsbA during oxidative stress.
To my knowledge there is only one report in the literature providing evidence that an additional protein, not being present under regular growth conditions, becomes associated with PS II under certain stress conditions. Downs et al. (1999a and 1999b) have shown that a small heat-shock protein, called 22-Ku, which is induced under heat stress, is found in the lumen of subchloroplast fractions (thylakoid grana vs. stroma) of *Chenopodium album* and *Lycopersicum esculentum*. The results of this group further showed that this protein protects PS II against oxidative stress and against photoinhibition. Their investigations indicate that this protein interacts with proteins on the luminal side of PS II and that it seems to protect mainly the water oxidising process. However, so far it has not been shown yet whether this protein is tightly associated with PS II and whether it remains attached to highly purified PS II complexes.

The results by Downs et al., (1999) and my results provide evidence that under certain stress conditions an additional protein, which is not a regular component of PS II, can interact with PS II and then protect PS II from stress – at least under mild stress conditions. Most likely such PS II complexes are better suited than wild type PS II complexes in an artificial system in which PS II is supposed to provide the electrons for hydrogen reduction. This is also indicated by results which showed that thylakoid membranes, treated with sodium cholate exhibit a higher oxygen evolution activity, when the cells were grown under iron-deficient conditions (Michel, 1996).

My own results give evidence for the first time that such a protein can indeed be a tightly associated part of PS II. This result opens many promising possibilities for further research with the goal to transform oxygenic photosynthetic organisms to obtain a more stable PS II complex which is the most labile part of the over-all photosynthetic process.

**4.3 Future prospects**

Since by now several methods and techniques are available, which can be used routinely, a good basis for the investigation of PS II complexes from various cyanobacterial mutants has been achieved. This should help to obtain extensive information on PS II in general and on the differences which exist in PS II complexes isolated from various mutants of *T. elongatus* and other cyanobacteria. The methods used in this work should be extended, especially with respect to analyses of the
electron transport within PS II and of the excitation energy transfer, e.g. by utilising fluorescence induction techniques. To analyse the subunit composition of different PS II mutants with respect to posttranslational modifications, MALDI-ToF measurements of intact PS II complexes can be done. This method has already been well established by Nowazcyk (2005) in cooperation with the Proteincentre of the Ruhr-Universität Bochum.

My results have shown that in *T. elongatus* IdiA copurifies with dimeric PS II complexes and that IdiA is a fairly tightly associated protein with these complexes. From the literature (review Michel and Pistorius 2003) it is well documented that this protein protects PS II from oxidative damage, especially when oxidative damage is caused by iron deficiency. Since by now highly purified PS II complexes of *T. elongatus* with and without this protein are available, the exact localisation of this protein within PS II should be investigated by doing more extensive single particle analyses and by cross-linking experiments. Moreover, these PS II complexes should be characterised with respect to their oxygen evolving capacity and their long-term stability, and also their stability towards elevated temperatures and other stress conditions. In addition, biophysical measurements should be applied for a thorough characterisation of the IdiA-containing PS II complexes in comparison to those PS II complexes not containing IdiA. The overall goal is to find out in which way IdiA protects PS II from oxidative damage. Further goals could be to expose *T. elongatus* cells to other nutrient-limiting growth conditions besides iron limitation and to investigate their effect on PS II. Such stress conditions could be calcium or magnesium limitation or growth with ammonium as N-source, since these growth conditions have previously already shown that they will lead to an expression of IdiA in the mesophilic cyanobacteria *Synechococcus elongatus* PCC 7942 and PCC 6301 (Michel and Pistorius, 1992; Michel et al., 1996; 1998; 1999; Exss-Sonne et al., 2000). Possibly such more severe growth conditions could lead to even more stable PS II complexes than those obtained from cells grown under mild iron limitation.
5 Summary

For immobilising of isolated Photosystem II complexes onto electrodes and to use them in a semiartificial device where they should provide the electrons for hydrogen production from water, basic investigations of structure and function of the membrane protein complex are essential. To contribute to this knowledge, PS II complexes of _T. elongatus_ wild type and different PS II mutants were investigated.

A new established combination of single particle analysis technique of isolated PS II complexes and MALDI-ToF analysis was used as a powerful approach in characterisation of membrane protein complexes.

Investigations of a _T. elongatus_ ΔPsbK mutant indicated, that the loss of the PsbK subunits led to a higher sensitivity against photoinhibition under high-light conditions which might be due to a loss of pigment-, especially β-carotene-binding. The loss of PsbK also resulted in a lower stability and activity of isolated PS II complexes from the mutant.

Investigations of a _T. elongatus_ ΔPsbTc mutant indicated that this subunit PsbTc might play a role in phycobilisome binding which led in the mutant to a higher sensibility against damage by photoinhibition because of a disturbed light energy transfer. Apparently, PsbTc is not essential for dimerisation of the PS II complex, but rather plays a role in the stabilisation of dimeric complexes.

Moreover, a partial characterisation of PS II complexes isolated from _T. elongatus_ grown under iron deficient conditions was also included in my investigations, to obtain PS II complexes with an attachment of IdiA.

It was possible to isolate iron-deficient PS II complexes. Western blot analysis with specific antibodies against IdiA showed clearly, that IdiA copurifies with the dimeric PS II complexes while it is not present in the monomeric fraction. With MALDI-ToF analysis after Trypsin digestion the presence of IdiA at the dimeric PS II complexes could be verified. Single particle analysis led to an electron microscopic structure, where IdiA is shown to become an associated part of the dimeric PS II complexes under iron-deficient conditions. These data also indicated, that IdiA binds to subunit CP43 of PS II. In conclusion, could be shown for the first time that a protein, which is expressed under certain stress conditions, associates tightly with PS II and forms a modified PS II complex.
6 Zusammenfassung

Für die Immobilisierung von isolierten PS II Komplexen auf Elektroden für die Anwendung in einem semiartifiziellen System, indem PS II Elektronen für die Wasserstoffproduktion aus Wasser liefern soll, sind grundlegende Untersuchungen der Struktur und Funktion des Membran Protein Komplexes essentiell. Um zu diesem Wissen beizutragen, wurden PS II Komplexe aus T. elongatus Wildtyp und verschiedenen PS II Mutanten untersucht. Hierbei wurde auch eine neu etablierte Kombination der Einzelpartikel und MALDI-ToF –analyse von isolierten PS II Komplexen als leistungsfähige Methode angewandt.

Untersuchungen einer T. elongatus ΔPsbK Mutante zeigten, dass der Verlust der Untereinheit PsbK unter hochlicht Bedingungen zu einer höheren Sensitivität gegenüber Photoinhibition führt, was in einem Verlust der Pigmentbindung, insbesondere der β-carotin Bindung begründet sein könnte. Der Verlust von PsbK resultierte weiterhin in einer geringeren Stabilität und Aktivität der isolierten PS II Komplexe aus der Mutante.

Untersuchungen einer T. elongatus ΔPsbTc Mutante zeigten, dass die Untereinheit PsbTc eine Rolle bei der Bindung von Phycobilisomen an PS II spielt, was aufgrund eines gestörten Lichtenergietransfers zu einer höheren Sensibilität gegen Photoinhibition führt. Weiterhin spielt diese Untereinheit offenbar keine Rolle in der Dimerisierung der PS II Komplexe, sondern eher in der Stabilisierung dimerer Komplexe.

welches unter verschiedenen Stressbedingungen exprimiert wird, fest an PS II assoziiert ist und einen modifizierten PS II Komplex bilden.
7 References


Asada, K. (1999), Responses to active oxygens, strong and weak lights, an overview. Tanpakushitsu Kakusan Koso 44 (15): 2230-2231


Bagchi, S. N., Pistorius, E. K. and Michel, K. P. (2003), A Synechococcus sp. PCC 7942 mutant with a higher tolerance towards bentazone. Photosynth Res. 75 171-182

Bald, D., Kruip, J. and Rögner, M. (1996), Supramolecular architecture of cyanobacterial thylakoid membranes: How is the phycobilisome connected with the photosystems? Photosynth Res. 49 103-118


Grimme, L. H. and Boardman, N. K. (1972), Photochemical activities of a particle fraction P1 obtained from the green alga Chlorella fusca. Biochem Biophys Acta. 49 1617-1620


References


Mullineaux, C. W. and Allen, F. (1990). State 1-State 2 transitions in the cyanobacterium Synechococcus 6301 are controlled by the redox state of electron carriers between photosystem I and II. Photosynth Res. 23 297-311


Nixon, P. J. and Diner, B. A. (1992), Aspartate 170 of the photosystem II reaction center polypeptide D1 is involved in the assembly of the oxygen-evolving manganese cluster. Biochemistry. 31 (3):942-8


Oelmüller, r., Meurer, J. and Pakrasi, H. B. (1999), Struktur, Funktion, Biogenerese und Evolution des Photosystem II. BIUZ. 1 36-43


References


Yousef, N., Pistorius, E. E., Michel, K.T., (2003), Comparative analysis of idiA and isiA transcription under iron starvation and oxidative stress in *Synechococcus elongatus* PCC 7942 wild type and selected mutants, Arch Microbiol 180: 471-483


## 8 Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>(m)A</td>
<td>(milli) Ampere</td>
</tr>
<tr>
<td>A</td>
<td>Absorbance</td>
</tr>
<tr>
<td>Å</td>
<td>Ångström</td>
</tr>
<tr>
<td>AMAS</td>
<td>N-(α-Maleimidoacetoxy)-Succinimidylester</td>
</tr>
<tr>
<td>AP</td>
<td>Allophycocyanin</td>
</tr>
<tr>
<td>APS</td>
<td>Ammoniumperoxodisulfate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosintriphosphate</td>
</tr>
<tr>
<td>β-DM</td>
<td>β-dodecyl maltoside</td>
</tr>
<tr>
<td>β-car</td>
<td>β-carotene</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>C</td>
<td>Celsius</td>
</tr>
<tr>
<td>Chl</td>
<td>Chlorophyll</td>
</tr>
<tr>
<td>CM</td>
<td>Cytoplasmic membrane</td>
</tr>
<tr>
<td>CP</td>
<td>Chlorophyll binding Protein</td>
</tr>
<tr>
<td>Cu</td>
<td>Copper</td>
</tr>
<tr>
<td>Cyt</td>
<td>Cytochrome</td>
</tr>
<tr>
<td>(k)Da</td>
<td>(kilo) Dalton</td>
</tr>
<tr>
<td>DNA</td>
<td>Desoxyribonucleic acid</td>
</tr>
<tr>
<td>DCBQ</td>
<td>2,6 dichloro-p-benzoquinone</td>
</tr>
<tr>
<td>DTT</td>
<td>1,4-Dithiothreitol</td>
</tr>
<tr>
<td>(µ)E</td>
<td>(micro) Einstein</td>
</tr>
<tr>
<td>EDC</td>
<td>N-(3-Dimethylaminopropyl)-N’-ethylcarbodiimide-hydrochlorid</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetate</td>
</tr>
<tr>
<td>e.g.</td>
<td>For example</td>
</tr>
<tr>
<td>ESI</td>
<td>Electro spray ionisation</td>
</tr>
<tr>
<td>Fd</td>
<td>Ferredoxin</td>
</tr>
<tr>
<td>Fe</td>
<td>Iron</td>
</tr>
<tr>
<td>FNR</td>
<td>Ferredoxin NADP Oxidoreductase</td>
</tr>
<tr>
<td>GRIP</td>
<td>Groningen Image Processing</td>
</tr>
<tr>
<td>h</td>
<td>Hour</td>
</tr>
<tr>
<td>HEPES</td>
<td>2-[4-(2-Hydroxyethyl)1-1 piperaziny1] ethansulfonic acid</td>
</tr>
<tr>
<td>HIC</td>
<td>Hydrophobic interaction chromatography</td>
</tr>
<tr>
<td>HPLC</td>
<td>High pressure liquid chromatography</td>
</tr>
<tr>
<td>HTG</td>
<td>Heptyl β-D thioglucoside</td>
</tr>
<tr>
<td>IEC</td>
<td>Ion exchange chromatography</td>
</tr>
<tr>
<td>IdiA</td>
<td>Iron deficiency Protein A</td>
</tr>
<tr>
<td>IMAC</td>
<td>Immobilised metall affinity chromatography</td>
</tr>
<tr>
<td>IMT</td>
<td>Intact mass tag</td>
</tr>
<tr>
<td>IsiA</td>
<td>Iron stress induced Protein A</td>
</tr>
<tr>
<td>(kJ)</td>
<td>(kilo) Joule</td>
</tr>
<tr>
<td>K</td>
<td>Kelvin</td>
</tr>
<tr>
<td>L</td>
<td>Litre</td>
</tr>
<tr>
<td>LC</td>
<td>Liquid chromatography</td>
</tr>
<tr>
<td>M</td>
<td>Mol</td>
</tr>
<tr>
<td>MALDI</td>
<td>Matrix-assisted-laserdesorption-ionisation</td>
</tr>
<tr>
<td>MES</td>
<td>2-(N-Morpholino) ethane-sulfonic acid</td>
</tr>
<tr>
<td>Min</td>
<td>Minute</td>
</tr>
<tr>
<td>Mn</td>
<td>Manganese</td>
</tr>
</tbody>
</table>
Abbreviations

MS   Mass spectroscopy
MSP  Manganese stabilising protein
MudPIT Multidimensional Protein identification technology
NAD  Nicotinamidadenindinucleotid
NADP Nicotinamidadenindinucleotidphosphate
NDH  NAD(P)H dehydrogenase
nm   Nanometer
OD   Optical density
ORF  Open reading frame
PAGE Polyacrylamide gel
P₆₈₀ Reaction centre of Photosystem II
PC   Plastocyanin
PE   Phycoerythrin
PDA  Photo diode array
PDB  Protein data bank
pH   Pondus hydrogenii
Pheo Pheophytine
PMF  Peptide mass fingerprint
PQ   Plastoquinone
PS I Photosystem I
PS II Photosystem II
PVDF Polivinylidenfluoride
PZV  Packed cell volume
Q    Quinone
ROS  Reactive oxygen species
RPC  Reversed phase chromatography
RPM  Rounds per minute
SEC  Size exclusion chromatography
SMCC 4-(N-Maleimidomethyl)cyclohexancarboxylsäure-N-
      hydroxysuccinimidylester
(m)S  (milli) Siemens
s    Second
SDH  Succinate dehydrogenase
SDS  Sodium dodecyl sulfate
SW   Swing out
T. elongatus Thermosynechococcus elongatus BP-1
TCA  Trichloro acetic acid
TEMED (N,N,N',N'-Tetramethylenelethendiamin)
ToF  Time of flight
Tris  Tris-(hydroxymethyl)-aminomethan
Tween Polyoxyethylene sorbitan monolaureate
v    Volume
(k)V  (kilo) Volt
w    Weight
Z    Tyrosine 161 residue
9 Curriculum vitae

Personal data:
Name: Julia Eva-Maria Lax
Date of birth: 08.10.1976
Place of birth: Minden (Westfalen)
Citizenship: German
Marital status: Single

School attendance:
1982-1986: Basic primary school; Königschule Minden
1986-1995: Comprehensive secondary school; Kurt-Tucholsky-Gesamtschule Minden
June 1995: Acquisition of the general qualification for university (Abitur)

Professional training:
1995-1996: Study of biology at Gesamthochschule Kassel
1996-1999: Study of biology at Universität Osnabrück
1999-2002: Study of biology at Universität Bielefeld
August 2002: Acquisition of the diploma-grade of biology in the department of cell physiology at the university of Bielefeld. Title of the diploma thesis (translated): “Characterisation of photosystem II complexes from the cyanobacteria Synechococcus elongatus PCC 7942 and Synechocystis PCC 6803”
Tutor: Prof. Dr. E. K. Pistorius
Oktober 2002: Affiliation into the European Graduate College 795 (Regulatory Circuits in Cellular Systems: Fundamentals and Biotechnological Applications)
Begin of the dissertation in the department of plant biochemistry at the Ruhr-University of Bochum. Tutor: Prof. Dr. M. Rögner.
Title of the thesis: “Role of specific subunits for stability and stress resistance of cyanobacterial photosystem II”.
**Posters:**


**Publications:**


Danksagungen


- Herrn Prof. Dr. Matthias Rögner, dafür das er mir im Rahmen des Europäischen Graduiertenkollegs 795 die Möglichkeit zur Promotion an seinem Lehrstuhl gegeben hat.
- Herrn Prof. Dr. E.J. Boekema für die freundliche Übernahme des Zweitgutachtens und für die Möglichkeiten an seinem Lehrstuhl im Labor zu arbeiten.
- Frau Prof. Dr. E. K. Pistorius und PD. Dr. K.-P. Michel für die großartige Unterstützung vor allen Dingen in den letzten Zügen der Arbeit.
- Ana Arteni für die unermüdliche Hilfe und die viele Arbeitszeit für die Elektronenmikroskopie.
- auch Thomas Schott und Wolfgang Schiefer für das Korrekturlesen meiner Arbeit.
- Claudia König für die unzähligen Membranpräparationen
- Gertrud Lideka und Christiane Wüllner für die Hilfsbereitschaft und Unterstützung bei formellen Dingen
- Berndt Esper und Wolfgang Schiefer für die prompte Hilfe immer wenn der PC oder der Drucker mal wieder gestreikt hat und bei Daniela Schlüsener, wenn es die Waters-Anlage war und bei Thomas Schröder im Falle der BioCad-Anlage…
- allen Kollegen und Kolleginnen des Lehrstuhls für Biochemie der Pflanzen, dafür, dass sie mich so nett aufgenommen haben und für das angenehme Arbeitsklima
- ganz besonders meiner Familie, insbesondere meinem Vater, für die finanzielle und stets verständnisvolle Unterstützung, durch die mir das Studium ermöglicht wurde.
- und natürlich Gerrit Lühn, für seine unermüdliche Geduld und aufbauende Unterstützung besonders während der vereinzelten trüben Tage meines Studiums und der doch gehäufte trüben Tage in den letzten Wochen.
Erklärung

Weiterhin erkläre ich, dass digitale Abbildungen nur die originalen Daten enthalten und in keinem Fall inhaltserändernde Bildbearbeitung vorgenommen wurde.

Bochum, den 30.11.05

___________________

Julia Lax