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Regulation of photosynthesis

- Cytochrome b6f in redox regulation

- Two novel proteases acting on an N-terminal peptide of LHCII

Jörgen Ström

LUND UNIVERSITY

Plant Biochemistry

2005

Doctoral dissertation

By due permission of the Faculty of Science at Lund University, to be defended in public at the Center for Chemistry and Chemical Engineering (Kemicentrum), Hall A, Sölvegatan 39, Lund, on the 7th of June 2005 at 13.15 for the degree of Doctor of Philosophy. Faculty opponent is Dr Thomas Pfannschmidt, Institut für Botanik und Pflanzenphysiologie, Jena University, Germany.

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Abbreviations

b6f- Cytochrome b6f-complex

cGEP- chloroplast glutamyl endopeptidase

DBMIB- 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone

DCMU- 3-(3,4-dichlorophenyl)-1,1-dimethylurea

D1- reaction center protein of PSII, PsbA gene product.

D2- reaction center protein of PSII, PsbD gene product.

EPR- Electron paramagnetic resonance

HGT- horizontal gene transfer

Lhcb1- the most common LHCII protein

LHCII- light harvesting complex II

MCD- Magnetic circular dicroism

PQ- Plastoquinone

PSI- Photosystem I

PSII- Photosystem II

ROS- reactive oxygen species

List of publications

This thesis is based on the following publications, which will be referred to in the text by their Roman numerals:

I **Cytochrome b6 in redox regulation of photosynthesis and chloroplast gene expression**
Ström, J, Puthiyaveetil, S. Forsberg, J. and Allen J.F.
(2005), Photosynthesis: Fundamental aspects to Global Perspectives, International Society of Photosynthesis, in press.

II **Low-Temperature Absorption and Magnetic Circular Dichroism of the Four Haems of the Cytochrome b6f Complex**
Årsköld-Petersson, S., Ström, J., Allen, J.F. and Krausz, E.
(2005), Photosynthesis: Fundamental aspects to Global Perspectives, International Society of Photosynthesis, in press.

III **Protease activities in the chloroplast capable of cleaving an LHCII N-terminal peptide**
Forsberg, J., Ström, J., Kieselbach, T., Larsson, H., Alexciev, K., Engström, Å. and Åkerlund, H.-E. (2005), Physiol. Plant. **123**, 21-29

IV **A thylakoid membrane associated protease that acts upon an LHCII N-terminal motif under reducing conditions**
Ström, J., Forsberg, J., Danielsson, R. and Åkerlund H.-E. (2005), manuscript

Additional publications, not included in this thesis:

V **Energy transduction anchors genes in organelles**
Allen, J.F, Puthiyaveetil, S., Ström, J. and Allen, C.A.
(2005) BioEssays, **27**, 426-435.

1. From basic chemistry to real life.

Life, as we know it, is all about redox chemistry. A definition of what life is could include the following conditions: one or several cells that can grow and develop, reproduce, react to stimuli, and most important have a metabolism. By this definition, viruses are generally not considered to be a true form of life. To do all these things energy is needed, and that energy has to come from either simple chemicals or complex molecules, chemical or electrical gradients, or from radiation. In the case of plants the radiation comes from sunlight. Often all these different sources of energy are utilized in the cell.

A starting point for understanding how the chloroplast works is to look at the very beginning of what we call "life". This is a difficult task since the evidence in the form of the fossil record is limited. When it comes to early life forms, the absolute earliest forms of life have probably disappeared due to competition with later more vigorous species, the prokaryotes. However, looking at the fossil record, prokaryotes appeared 3.5 billion years ago and eukaryotes appeared only 1.5 billion years ago (Awramik, 1992; Blankenship, 1992).

What predated the free living prokaryote? What can fill the gap between chemistry and free living entities? According to an updated model (Martin and Russell, 2003) of how life originated; *".. in structured iron monosulphide precipitates in a seepage site in a hydrothermal mound at a redox, pH and temperature gradient between sulphide-rich hydrothermal fluid and iron(II)-containing waters of the Hadean ocean floor."* In other words, the beginning of life emerges when there is a redox gradient that can be utilized to do chemistry. Older, still persisting theories about how life once started, concerning the prebiotic soup, seem less likely considering that a solution at equilibrium will never do chemistry, or as W. Martin puts it; " ..*once autoclaved, a bowl of chicken soup left at any temperature will never bring forth life."* To get life, as we know it started, there are several requirements that have to be fulfilled.

Compartmentalization in the form of iron monosulphide precipitate formations suggests something that could predate the cell. The cell is a dominant feature of what is called "life". Energy to drive synthetic reactions

is also needed. Reactions that need energy will not randomly happen to the extent that it becomes a steady supply of building blocks for more complex molecules. As an example, the well known photosynthetic reaction

$$CO_2 + H_20 \text{ --> } (CH_20) + O_2$$

that involves many enzymatic steps in the chloroplast, has an equilibrium constant of about 10^{-500} (Taiz and Zeiger, 1998). In other words, carbon dioxide and water will never spontaneously form simple sugar molecules. Chemical and temperature gradients generated from the submarine hydrothermal vents flowing through iron monosulphide cavities may have generated the energy needed to do redox chemistry (Martin and Russel 2003). The surface of the monosulphide formations would not only act as a compartment but also catalyze some of the anticipated redox chemistry in those early "cells". From this, it is believed that the "RNA world" evolved. The RNA world was composed mostly of RNA, fueled with substrates from FeS-catalyzed reactions using substrates from the hydrothermal vent and the surrounding ocean (Russell and Hall, 1997). Once the ribosome had evolved, the DNA world and protein synthesis commenced: the early origin of a common ancestor was not far away (Woese, 2002). The next step in early life was the synthesis of membrane lipids. Here a great divide was seen between eubacteria and archaebacteria, which have very different sets of membrane lipids. From now on, the early cells were free living and not confined to the ironsulphate structure. The early cells probably had basic chemotrophic enzymes to do redox chemistry. A theory for evolution is that the early cells experienced a phase of dynamic horizontal gene transfer (HGT) that drastically affected the genomic content. This had a great effect on the cell complexity and is called the "Darwinian Threshold" (Woese, 2002). The first eukaryotic cell was suggested to have arisen from endosymbiotic origin of mitochondria in an archaebacterial host (Martin and Müller, 1998; Martin and Russell, 2003)

2. Photosynthesis

Photosynthesis appeared very early in the history of life. Geological evidence indicates that, about 2 billion years ago, oxygen started to accumulate in the atmosphere. However, photosynthetic oxygen evolution probably started much earlier. The reason why oxygen is not seen in the geological record is because it was masked by vast amounts of Fe^{2+} ions in the oceans that were oxidized to Fe^{3+}. The appearance of oxygen in the atmosphere had an enormous impact for the early life on earth. For flora and fauna that had been developed in a reducing atmosphere, the increased levels of oxygen were lethal. The organisms that survived did it either by hiding in anoxygenic places or by adopting an oxygenic metabolism. The diverse flora we have today is a result of the evolution that followed this crucial adaptation. It is estimated that, 300,000-500,000 species of plants, algae and bacteria perform oxygenic photosynthesis. Another major change connected to an oxygen-rich atmosphere, was the formation of an ozone (O_3) layer that protects earth from harmful UV radiation. This made it possible for life to spread from the oceans 400 million years ago, and plants began to grow on land.

The oxygen-sensitive or anoxygenic photosynthetic organisms from the domain Bacteria have representatives in four phyla – Purple Bacteria (e.g. *Rhodopseudomonas viridis* and *Rhodobacter sphaerodies*), Green Sulphur Bacteria (*Chlorobium*), Green Sliding Bacteria (*Chloroflexus*) and Gram Positive Bacteria. These are of ancient origin and use light to extract electrons from molecules other than water. Their reaction centres resemble those of PSI and PSII which were merged into the electron transport chain that we find in cyanobacteria and eukaryotic plant cells. Another group that uses light to harvest energy is Archaea, which includes organisms known as halobacteria. *Halobacterium salinarium* uses bacteriorhodopsin to convert light into chemical free energy. However, the mechanism by which halobacteria convert light energy is fundamentally different from that of higher organisms because there is no redox chemistry and halobacteria can

not use CO_2 as their carbon source. Consequently some biologists do not consider halobacteria as photosynthetic (Gest, 1993).

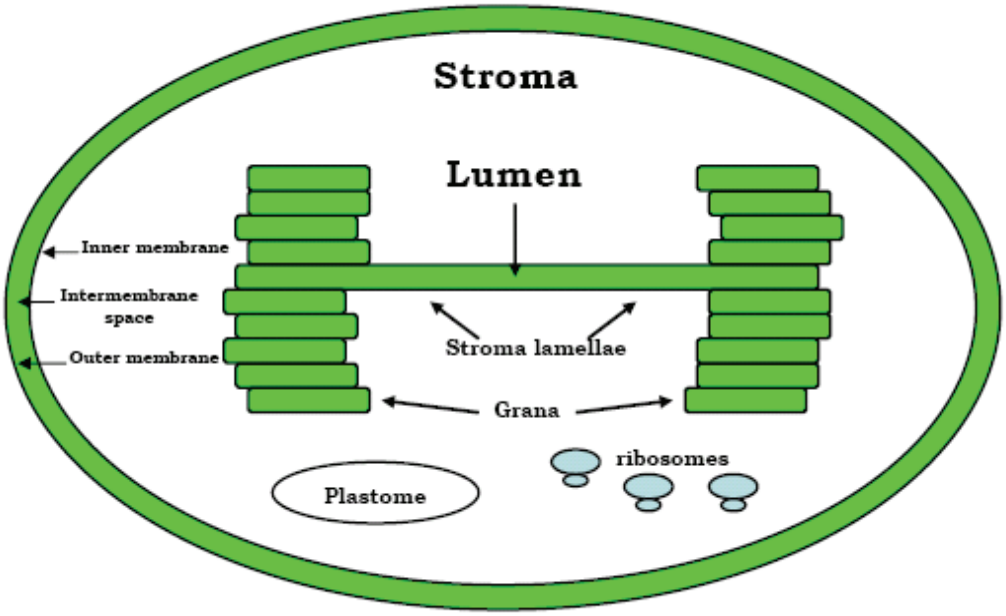

Figure 1. Chloroplast with the thylakoid membrane that can be divided into stroma lamellae and grana. In the stroma are the plastome, ribosomes and the enzymes involved in CO_2 fixation found.

2.1 The chloroplast

In plant cells, photosynthesis takes place in the chloroplast. The number of chloroplasts that each cell contains varies from one up to a few hundred. The chloroplast is surrounded by an outer membrane followed by an inner membrane creating an intermembrane space (figure 1). Inside the inner membrane are the stroma and the thylakoids. The thylakoids form a membrane system that contains all the protein complexes needed to do photosynthesis. The thylakoid membrane separates the lumen, the inside of the thylakoids, from the stroma. In the thylakoid membrane is where the light reactions take place. Light is harvested so that electrons can be extracted from water and protons pumped across the membrane, resulting in formation of ATP and NADPH. The stroma is where the dark reactions take place, also called the carbon assimilation process. In the stroma, the chloroplast harbors its own DNA, called the plastome. The plastome contains

about 100-120 different genes (Race et al., 1999). The stroma is also where the organellar ribosomes are found, part of a complete machinery to express genetic information (Stern et al., 1997). Most proteins in the chloroplast are nuclear-encoded (Abdallah et al., 2000) and imported post-translationally into the chloroplast (Jarvis and Soll, 2001). The thylakoid membrane has a very distinct, folded structure that can be divided into grana lamellae (grana) and stroma lamellae. Grana are the stacked regions of the thylakoids, and contain a higher proportion of PSII whereas the stroma lamellae have a higher proportion of PSI (Albertsson, 2001). The structural difference between grana and stroma lamellae is followed by a functional difference: most of the linear electron transport is believed to take place in the grana region, while cyclic electron transport occurs in the stroma lamellae. An overview of the heterogeneity of the thylakoid membrane was provided by Dekker (Dekker and Boekema, 2005) and the dynamics in the thylakoid membrane will be addressed later.

2.2 A subset of genes remain in the chloroplast.

Chloroplasts originated from bacterial symbionts that provided their host cells with oxygenic photosynthesis. The host cell acquired genetic information from the symbiont, and over time incorporated a large part into the nucleus of the host cell. But, why has not all the DNA moved over to the cell nucleus?

Several reasons have been proposed why the chloroplast still has DNA in the stroma. “The lock-in hypothesis” (Bogograd, 1975) proposes that the core protein must be synthesised in the compartment where it will be active. This is dismissed since the mechanism of protein import and targeting exists (Schatz, 1998). The “Hydrophobicity barrier” hypothesis and the “Frozen accident” hypothesis (von Heijne, 1986) are not likely either (Allen, 2003b). The hypothesis that “The transfer of genes from organelle to the nucleus is under way, but incomplete” (Adams et al., 2000), is strongly disfavoured due to the fact that the genes transferred are not randomly selected (Martin et al., 1998).

The reason for the remaining genes in the chloroplast may simply be that they are needed to be close to the electron transport chain in the thylakoid membrane so that efficient regulation of photosynthesis is maintained during changing light conditions. The is hypothesis is called CORR, *co*-location for *r*edox *r*egulation of gene expression (Allen, 1993; Allen and Raven, 1996; Allen, 2003b; Allen et al., 2005). This hypothesis predicts which genes that remain in the chloroplast and which genes that are transferred into the nucleus. If the gene is closely involved in the redox chemistry in the organelle or important for the redox signalling they are maintained in the chloroplast. If the chloroplast loses that ability to perform photosynthesis, then it quickly starts to lose the genes coding for the proteins involved in photosynthesis (Wilson et al., 1996).

2.3 Electron transport and proton pumping.

The thylakoid membrane contains the major complexes of photosynthesis. These are photosystem I (PSI) (Ben-Shem et al., 2003), phostosystem II (PSII) (Zouni et al., 2001), Cytochrome b6f (b6f) (Kurisu et al., 2003; Stroebel et al., 2003), ATP-syntase (Junge, 1999) and the movable antenna Light harvesting complex II (LHCII) (Lui et al., 2004). These complexes are all involved in the light reactions: extracting electrons from water; transporting them through the thylakoid membrane generating NADPH; pumping protons across the thylakoid membrane, thus creating an electro chemical gradient which is used for ATP production (figure 2). PSII contains several chlorophyll binding proteins; CP24, CP26, CP29 ,CP43 and CP47 that compose the stationary antenna of PSII (Green and Durnford, 1996). The moveable antenna complex LHCII is composed mainly of the chlorophyll binding proteins Lhcb1 and Lhcb2.

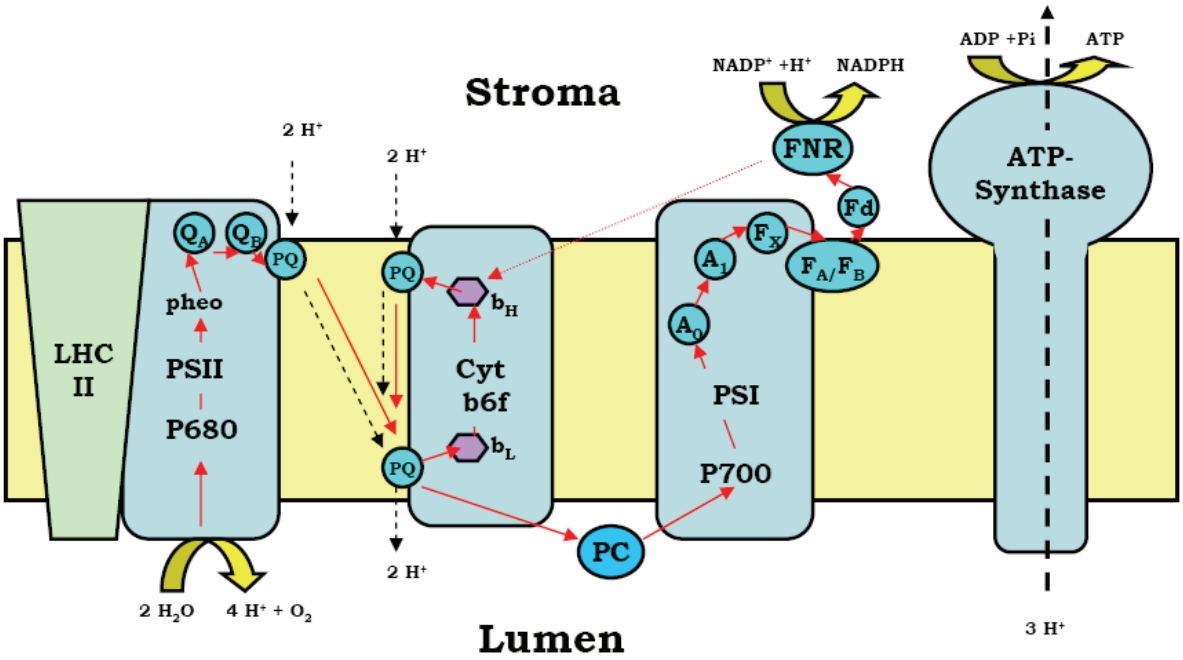

Figure 2. Electron transport chain in the thylakoid membranes. Here we see four major complexes (PSI, PSII, cytochrome b6f and ATP-synthase) and the moveable antenna LHCII.

The antenna proteins together with bound chlorophyll funnel light towards the reaction centre. The core of the reaction centre is a chlorophyll pair called P680 where the first step in photosynthesis takes place. An excited electron from P680 is moved away to Q_B via pheophytin and Q_A. The electron hole in $P680^+$ is filled from the manganese cluster, which extracts electrons from water. At the Q_B, site plastoquinone is reduced in two steps to plastoquinol and at the same time two protons are picked up from the stroma. Plastoquinol then moves to the cytochrome b6f-complex where at the Qo site, it gives away its two electrons, one electron goes to the Rieske protein and the other electron goes to the cytochrome b_L and then to cytochrome b_H in the cytochrome b6 part of the b6f-complex. The protons are then released into the lumen. The electron from Rieske centre is transferred to cytochrome f and then further to plastocyanin which becomes reduced. A second plastoquinol is reduced at the Qo site as mentioned above. The electron at cyt b_H together with the second electron reduces a quinone at the Qi site where it also abstracts two protons from the stroma and thereby produce a quinone that leaves the complex. In PSI absorbed light will finally excite a special pair of chlorophylls (P700) in the reaction

centre. Electrons will leave P700 and after passing a few redox carriers end up in Ferredoxin (Fd) located on the stromal side of the membrane. Ferredoxin-NADP oxidoreductase (FNR) transfers two electrons from Fd to $NADP^+$ together with one H^+ generating NADPH. An electron from plastocyanin restores P700 from its oxidized state. The production of protons at PSII in the water splitting machinery, and the pumping of protons across the membrane, facilitated by the plastoquinone pool, drives the generation of ATP, through the ATP syntase complex. The above reactions comprise the Z-scheme (Hill and Bendall, 1960), forming the linear electron flow through the thylakoid membrane (figure 2). The synthesis of ATP requires a H^+ gradient over the thylakoid membrane (Mitchell, 1961), and is also called photosynthetic phosphorylation (Arnon et al., 1954). When the need for ATP is greater than the need for reducing power in form of NADPH, the chloroplast turns to cyclic phosphorylation. A suggested explanation is that the main part of cyclic phosphorylation is taking place in the stroma lamellae (Albertsson, 2001). How this shift to cyclic photophosphorylation is regulated is not known. It is possible that the redox state of the plastoquinone pool and the low concentration of ATP play an important part. Clearly there seems to exist a correlation between state transition and the regulation between linear and cyclic electron flow (Finazzi et al., 2002). According to Zhang (Zhang et al., 2001), the cyclic electron transport involves the cytochrome b6f-complex where electrons from PSI via FNR (ferredoxin:$NADP^+$ oxidoreductase) are cycled back into the b6f-complex. A second way to generate a proton gradient without generating more reduced NADPH is called pseudocyclic photophosphorylation. Pseudocyclic phosphorylation involves the linear electron transport except that the oxygen that is generated in the manganese cluster at PSII acts as an electron sink at PSI instead of $NADP^+$. The net result is a proton gradient across the membrane utilized for ATP-synthesis. Pseudocyclic phosphorylation is believed to occur when overreduction causes the Calvin cycle to be unable to utilize NADPH due to insufficient levels of ATP (Heber and Walker, 1992). Whether pseudocyclic phosphorylation is a real alternative to cyclic phosphorylation is still under debate (Allen, 2003a).

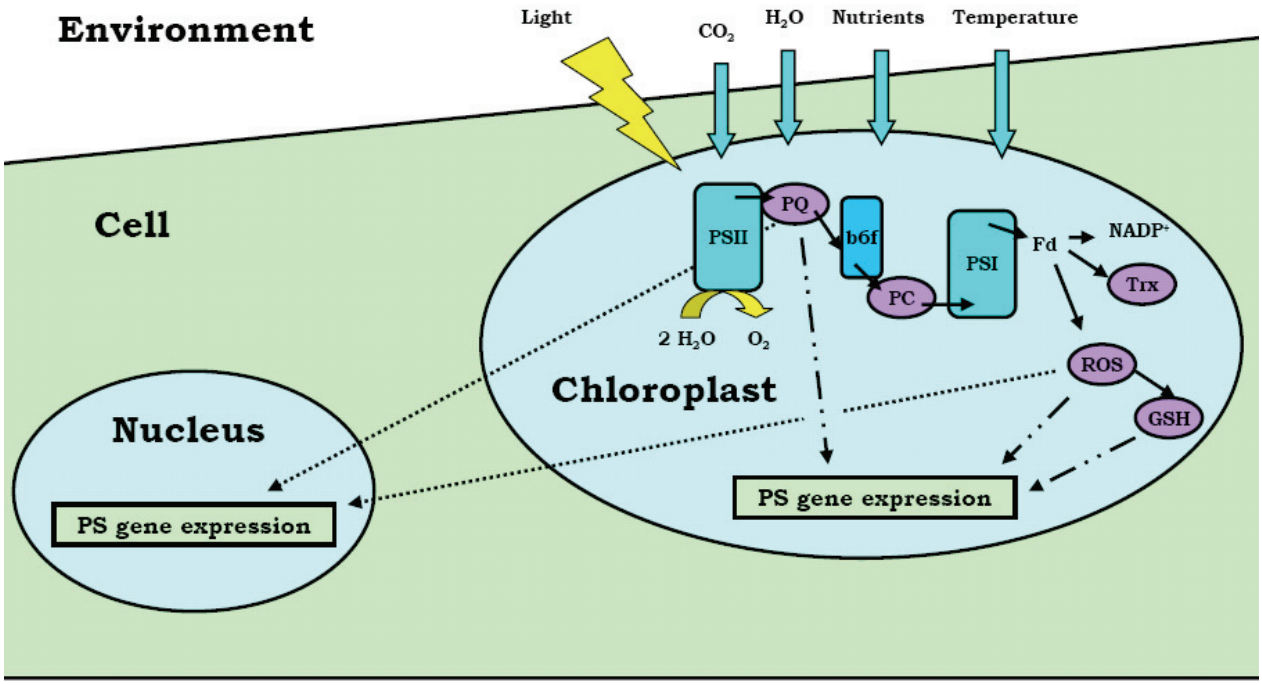

Figure 3. Redox control in the chloroplast (adapted from Pfannschmidt 2001).

3. Redox control of gene expression.

Redox chemistry in the form of photosynthesis is the most important reaction that takes place in the chloroplasts. The small amount of DNA in the chloroplast, derived from the DNA of the ancestral endosymbiotic cyanobacterium, corresponds to 0.1% of the genes that the cyanobacteria once had.

There seem to be at least two independent systems signalling to the nucleus from the chloroplast (Surpin et al., 2002). One involves regulation of the nuclear cab-genes coding for LHCII-proteins. This is done by elevated substrate levels of tetrapyrrol intermediates. These are precursors to chlorophyll which turn down the Lhcb-expression in the nucleus (Oster et al., 1996). The other system mediates redox signaling, both to nucleus and to chloroplast DNA. Only the redox-mediated signaling is addressed in this thesis (figure 3).

The redox-mediated signalling involves different parts of the electron transport system in the thylakoid membrane. This means that there is a

range of signals coming from different parts of the electron transport chain between PSII and PSI and also immediately after PSI. The signalling goes both to genes in the chloroplast and to genes in the nuclear DNA (Pfannschmidt, 2003). The main reason for the regulation is to maintain photosynthetic efficiency at as high a level as possible, especially under unfavorable conditions. The redox regulation reacts mainly to optimize photosynthesis or, in extreme conditions, to minimize the induced damage. A sensitizer of the redox poise in the electron transport chain is the redox state of the plastoquinone-pool (PQ). This poise of the plastoquinone pool is connected to state transition, cytochrome b6f and LHCII-kinase activation (Allen, 1992a).

There is a range of levels where redox regulation interacts with gene expression. Not only transcription of genes, but also splicing of mRNA seems to be regulated. Stabilization versus degradation of mRNA is another point of regulation, in addition to both ribosome loading and translation of mRNA (Pfannschmidt, 2003).

The rate of *psaAB* transcription (*psaA* and *psaB* are central components of PSI) is affected by the quality of the light. The transcription of these genes decreases with light directed towards PSI and increases with light directed towards PSII (Pfannschmidt et al., 1999). Addition of DCMU gives the same effect as light towards PSI, and addition of DBMIB gives the effect of light towards PSII. This means that the PQ-pool regulates transcription of a subset of chloroplast genes.
PsbA codes for the D1 protein, a central component of PSII. The expression of *psbA* is dependent on the redox state of thioredoxin in the chloroplast. The levels of RNA do not seem to be affected, but the protein synthesis is increased 50-to 100-fold after exposure to light (Danon and Mayfield, 1994). A nuclear-encoded activator protein binds to the *psbA* mRNA forming a RNA-protein complex in the presence of reduced thioredoxin. In the higher plants there exists mediated redox control during RNA maturation or transcription (Nickelsen and Link, 1993). The endoribonuclease activity is enhanced by

phosphorylation and by oxidized glutathione, and on the other hand dephosphorylation and treatment with reduced glutathione reduces the activity (Liere and Link, 1997). A kinase (plastid transcription kinase) was found that is involved with the plastid-encoded RNA polymerase that phosphorylates σ-factors. Its activity is regulated by phosphorylation and the redox state via GSH (Baginsky et al., 1999). In *Synechocystis* PCC 6803, a two-component system consisting of a sensor histidine kinase (RppB) and a response regulator (RppA) is involved in the transduction of the PQ redox signal. A corresponding system has not yet been found in higher plants (Pfannschmidt, 2003). Nevertheless, sequence analysis suggests that some two component receptors have potential organellar target sequences (Forsberg et al., 2001). In paper I (in part 6) phosphorylation of the b6f-complex was investigated as a potential candidate for redox signalling.

When it comes to nuclear encoded genes, the redox state of the PQ-pool seems to be involved in the expression of *Lhcb* genes (Escoubas et al., 1995). At higher irradiation levels, the redox signaling is mediated via the glutathione cycle and reactive oxygen species (ROS) (Pfannschmidt et al., 2001). ROS can also appear due to stress (high temperature, lack of nutrients, or low levels of CO_2) in the plant cell. The presence of ROS (hydrogen peroxide, superoxide, singlet oxygen and hydroxyl radicals) is a strong signal that originates from both PSI and PSII. Under extreme situations when everything is reduced or where there is an ATP deficiency which brings the Calvin cycle to a halt, electrons from PSI are transferred to oxygen (Pfannschmidt, 2003). The ROS formation in PSII comes mainly from in increased triplet chlorophyll causes singlet oxygen formation, but most electron carriers in the chloroplast has a potential for ROS generation (Edreva, 2005). In *Arabidopsis* the appearance of ROS activates the APX (ascorbate peroxidase) gene family, which is important in scavenging hydrogen peroxide using ascorbate as electron donor, turning the peroxideinto water (Mullineaux et al., 2000).

3.1 Protein phosphorylation in the chloroplast

Phosphorylation of proteins is, in nature, a common way to modify their structure and function. The addition of a negatively charged phosphate group to the peptide often results in a local conformational change, but can also affect the structure of the whole protein. The effect of the phosphorylation in terms of regulation is that the protein is given different properties and this affects its function. For example, the protein may lose affinity to one complex and gain affinity to another. The conformational change can also lead to activation of an enzyme as well as deactivation. An added phosphate group can e.g. sterically hinder the substrate from accessing the catalytic site. Enzymes catalyzing are called kinases. They are classified according to which amino acid residue they modify. Serine, threonine and tyrosine are the residues that are most commonly phosphorylated, but also histidine, asparagine, aspartate and glutamine can become phosphorylated (Cozzone, 1998). The removal of phosphate groups on proteins is catalyzed by phosphoprotein phosphatases.

Reversible phosphorylation in the chloroplast of LHCII has been described by Bennett (Bennett, 1977) and Allen (Allen, 1992b; Allen and Forsberg, 2001), but also by several others over the years (Vener et al., 1995; Gal et al., 1997; Rintamäki et al., 1997; Vener et al., 1997; Rintamäki et al., 2000; Vener et al., 2001). In the chloroplast several phosphoproteins have been found. The major ones are the PSII proteins D1, D2, CP43 and the *PsbH* gene product; the two major chlorophyll *a/b* binding LHCII-proteins, Lhcb1 and Lhcb2 are phosphorylated under specific conditions. The minor chlorophyll *a/b* binding protein CP29 is phosphorylated under both light and cold stress conditions (Pursiheimo et al., 2001). A 12 kDa protein is shown to be partially released from the membrane when it becomes phosphorylated (Carlberg et al., 2003). Subunit V of the cytochrome b6f-complex seems to undergo reversible phosphorylation (Hamel et al., 2000). Another subunit of Cytochrome b6f, b6 becomes phosphorylated under reducing conditions (Gal et al., 1992). The reversible phosphorylation of PSII proteins D1, D2 and CP43 is believed to involve the repair cycle of photoinhibited PSII (Rintamäki et al., 1996). The

D1 and D2 proteins are phosphorylated under reducing conditions and remain phosphorylated at elevated light levels. Phosphorylation of CP43 is also seen in darkness. This is believed to be connected to the fact that CP43 gives stability to the PSII complex, preventing premature dissociation of the complex (Baena-Gonzalez et al., 1999). The LHCII protein phosphorylation appears to be under more delicate control of the chloroplast redox environment. At moderate light and redox conditions in the chloroplast, the LHCII-kinase is activated via the cytochrome b6f-complex and down-regulated via reduced thioredoxin (Rintamäki et al., 1997). The phosphorylation pattern of phosphoproteins in the thylakoid membrane is affected by the redox conditions, which implies the presence of different regulatory pathways and possibly different pathways for the dephosphorylation as well (Pursiheimo et al., 2003) .

3.2 The Light-harvesting complex II

Light-harvesting complex II (LHCII), is the most abundant chlorophyll binding protein complex in plants. It alone binds more than 50% of all the pigments that are involved in photosynthesis (Nilsson et al., 1997). The structure is composed of a polypeptide of about 230 amino acids, 12 chlorophyll molecules and at least 2 luteins. LHCII exists as trimers caused to a large extent by interaction between monomers at their amino- and carboxyl-terminal loops. LHCII contains four α-helices of which three span the thylakoid membrane (Kühlbrant et al., 1994; Lui et al., 2004). LHCII preparations mainly contain three gene products encoded by *Lhcb1*, *Lhcb2* and *Lhcd3*. The composition of the different gene products in LHCII varies from 10:3:1 to 20:3:1 in content depending on plant growth conditions and the preparative method (Green and Durnford, 1996). As part of the light-regulating mechanism called state transition, LHCII becomes phosphorylated, (Thr-6 in the pea *Lhcb1* gene product) (Michel and Bennett, 1989). This triggers a conformational change in the structure of LHCII (Nilsson et al., 1997). This structural change has been proposed as the basis for light state transitions (Allen, 1992b; Nilsson et al., 1997; Allen and Forsberg, 2001). It has been shown that there are several phosphorylation

sites in LHCII besides Thr-6, both threonine (Dilly-Hartwig et al., 1998) and tyrosine residues (Forsberg and Allen, 2001).

3.3 State transitions

The thylakoid membrane is exposed to changing light conditions and is in need of regulation. State transitions are a way for eukaryotic plants to optimize photosynthetic performance and minimize radical-induced damage. If PSI and PSII are excited at different rates, the plastoquinone pool will become either reduced or oxidized. This will lead to efficient electron transport being either slowed down or stopped. State transitions are a way of adjusting the incoming light to the different needs of the two photosystems. The mechanism for this is reversible phosphorylation of LHCII. When PSII works at a higher rate (getting more incoming light) than PSI, the plastoquinone pool will quickly become reduced. LHCII-kinase will start to phosphorylate the LHCII-trimer. This in turn will trigger movement of the phosphorylated LHCII-trimer (LHCII-P) towards PSI. This will lead to a more balanced excitation of PSI and PSII. If too much light is absorbed by PSI the plastoquinone pool gets oxidized. The oxidized plastoquinone pool will result in the LHCII-kinase being turned off, LHCII-P becomes dephosphorylated, and returns back to PSII (Allen, 1992b). In species where cytochrome b6f is impaired, no activation of LHCII-kinase is seen (Gal et al., 1987). This means that cytochrome b6f is involved in the activation of the kinase. To activate the kinase, a plastoquinol is needed to be bound to the Qo site of the b6f-complex (Vener et al., 1995; Vener et al., 1997). Activation of LHCII-kinase occurs at low light levels when only a fraction of the cytochrome b6f pool is reduced. Maximal phosphorylation of LHCII is reached before the electron flow reaches saturation level. Close to and above the saturation level of electron flow LHCII phosphorylation is drastically lowered (Zer et al., 2003). It was reported recently that light seems to be involved in the accessibility for phosphorylation of the N-terminal part of LHCII. Light was shown to induce a conformational change that prevents the LHCII-kinase from phosphorylating the residue: this dependence is also correlated with a susceptibility to added trypsin (Zer et al., 2003). Since most of the PSII is

found in the grana and PSI is mostly found in the stroma lamellae, the question is what the driving force for the movement of the antenna is. When subunit H of PSI is not present, no state transition takes place. LHCII-kinase is activated via the plastoquinone pool. LHCII is phosphorylated, but LHCII remains at PSII. In other words, subunit H is essential for LHCII to interact with PSI (Lunde et al., 2000). It has been proposed that conformational changes within the lipid membrane layer of the LHCII protein is the driving force for the movement (Allen, 1992b), and not the large negative charge coming from the phosphate group. The discovery of subunit H in docking of LHCII confirms this. Two LHCII-kinases have been identified, Stt7 from *Chlamydomonas reinhardtii* (Depège et al., 2003) and STN7 from *Arabidopsis thaliana* (Bellafiore et al., 2005). These are structurally and functionally similar and needed for state transition. Another candidate for phosphorylation of LHCII is TAK-1 (Thylakoid associated kinase). TAK-1 that co-purifies with the cytochrome b6f-complex was shown to phosphorylate LHCII proteins *in vitro* (Snyders and Kohorn, 2001), but TAK-1 antisens plants exhibited reduced levels of phosphorylation of all the PSII phosphoproteins, questioning its specificity for phosphorylating LHCII.

For land plants about 15-20% of LHCII is mobile during state transition, whereas for *Chlamydomonas reinhardtii* as much as 80% of LHCII is mobile during state transition (Bellafiore et al., 2005). Despite the big difference in the fraction of LHCII that is being phosphorylated, state transition is important for adaptation to changing light conditions, and without this adaptation growth is considerly impaired in conditions where quantity and quality of light changes continuously.

4. Structure and function of Cytochrome b6f

The first purifications of the cytochrome b6f-complex, free from cytochrome b559, were a fortunate by-product of purification of ATP-synthase (Hauska, 2004). Much of the earlier structural knowledge of b6f was based on the structure from mitochondrial cytochrome bc-complex. Recent structures of cytochrome b6f from the thermophilic cyanobacterium *Mastigocladus laminosus*, at 3.0 Å resolution and the eukaryotic green alga *Chlamydomonas reinhardtii* at 3.1 Å resolution (Kurisu et al., 2003; Stroebel et al., 2003) have given more insight into the complex. These new structures showed that the mitochondrial and chloroplast complexes, on several features are strikingly similar.

The b6f-complex in the thylakoid membrane is dimeric in structure. The monomer of the complex contains one of each subunit; cytochrome f, cytochrome b6, Rieske FeS protein and subunit IV and the four small hydrophobic subunits, PetG, PetL, PetM and PetN. For eukaryotes, the Rieske FeS protein and all the minor subunits are encoded in the nucleus. In the structure there are four different hemes and one FeS-complex (figure 4). Cytochrome b6 and subunit IV together show good homology to cytochrome b of mitochondria (Berry et al., 2000). Cytochrome b6 contains three hemes, b_H, b_L and heme c_i. Subunit IV does not contain redox-active components but is needed for proper assembly of functional b6f. Parts of subunit IV together with b6 and Rieske FeS protein form the Qo-pocket. It has been shown that the conserved sequence PEWY in subunit IV is important for docking of plastoquinol to the Qo-site. Mutational studies where PEWY was changed into YWEP abolished binding of plastoquinol to the Qo-site with the result that LHCII-kinase could not be activated (Zito et al., 1999). The Qo-pocket is proposed to oxidize plastoquinol to plastoquinone in a concerted manner, minimizing or eliminating reactive semiquinone formation (Osyczka et al., 2004). Possibly, this is also the case when plastoquinone is reduced to quinol in the Qi-pocket involving both b_H and heme c_i.

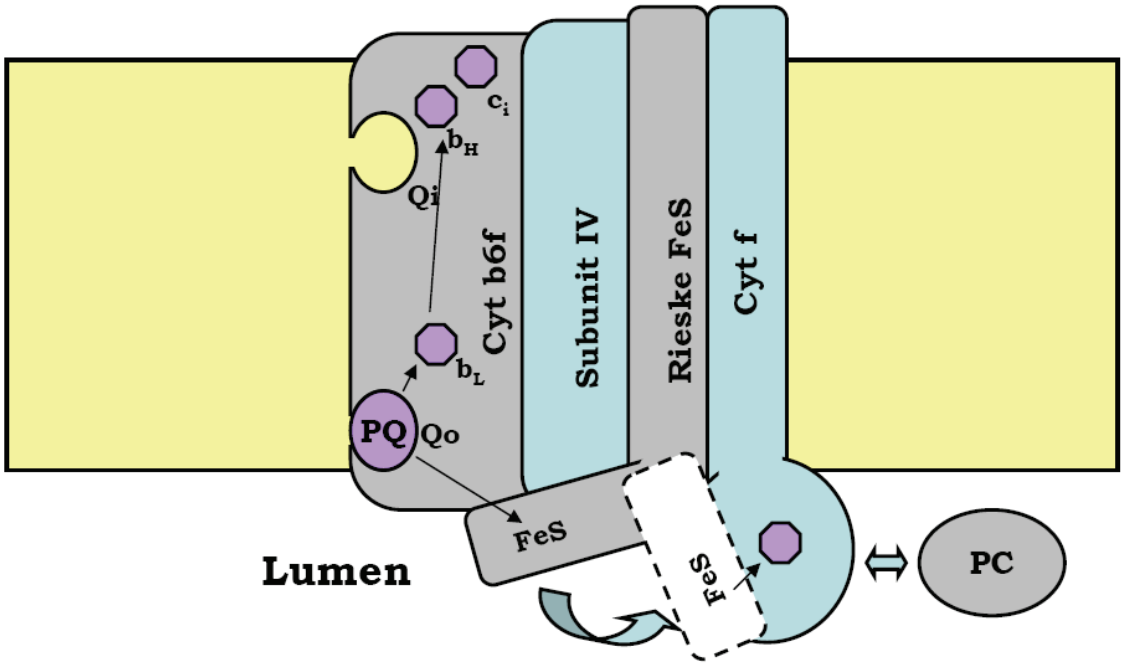

Figure 4. Schematic figure of the cytochrome b6f-complex, showing the major proteins cyt f, cyt b, Rieske FeS protein and subunit IV.

Movement of FeS is needed for reduction of cytochrome f; the electron for this is taken from plastoquinol which is oxidized in a bifurcated manner, giving one electron to b_H and the other to Rieske FeS. The Rieske FeS protein then moves towards cytochrome f where it reduces the heme c in the protein (Zhang et al., 1998). Rieske FeS protein has one transmembrane region with a large extrinsic part on the lumenal side of the membrane. The flexible part of Rieske contains the Fe_2-S_2 complex. A model that has been proposed for activation of the LHCII-kinase is movement of the Rieske FeS protein induced by reduction of the Rieske FeS protein. When the Rieske FeS protein is in its proximal position this inhibits LHCII-kinase activation. The activation is proposed to involve interaction with a putative transmembrane segment of the kinase on the lumenal side of the membrane (Vener et al., 1998). On the other hand, it has been shown consistently that acidic pH in the lumen favors proximal position of the Rieske FeS protein. Zito showed that binding of plastoquinol to Qo triggers LHCII-kinase activity on the stromal side of the thylakoid membrane (Zito et al., 1999). The plastoquinol binding favors the proximal position of Rieske FeS protein. Lowered pH in

the lumen also increases the affinity for proximal position of FeS. Thus it is also suggested that the transmembrane helix of the Rieske protein plays a critical role in the kinase activation (Zito et al., 1999).

Cytochrome f contains one heme. As in the case of the Rieske FeS protein, cytochrome f also has only one transmembrane region with a large extrinsic domain on the lumenal side and a small extrinsic domain on the stromal side of the membrane. The large extrinsic domain of cytochrome f contains a heme of type c. A cluster of basic residues at the domain interface is where the acidic plastocyanine is proposed to dock to cytochrome f (Berry et al., 2000).

The cytochrome b6f-complex also contains one chlorophyll-*a* and a carotenoid, but the function of these pigment molecules is still unknown**.** Low temperature redox difference spectroscopy measurements on the complex revealed a red shift of the chlorophyll and carotenoid when b6 is reduced. This indicates that the pigments are in close contact with the hemes and possibly involved in the electron transport (Wenk et al., 2005). The chlorophyll is located between two helices in the subunit IV protein and the phytyl chain ends within the Qo-pocket (Kurisu et al., 2003; Stroebel et al., 2003). The carotenoid is a β-carotene that has one of its cyclohexane rings 6 Å away from heme c_i. The chlorophyll, the carotene and the plastoquinone binding site are facing at the same direction, out of the complex. It is speculated that chlorophyll could act as a sensor in the Qo-site, and the carotene could act as a sensor in the Qi-site, interacting with PSI or possibly a kinase (Stroebel et al., 2003). In paper II (part 6) we are looking at the different hemes in the b6f-complex.

Two Cytochrome b6f-related proteins that are not seen in the structure are subunit V (nuclear gene PETO) (Hamel et al., 2000) and Ferredoxin:$NADP^+$ oxidoreductase (FNR) (Zhang et al., 2001). Subunit V, which only seems to be present in grana fractions (Romanowska and Albertsson, 1994) becomes phosphorylated under state II transition (Hamel et al., 2000). The presence

of heme c_i in the structure gives a clue as to how cyclic electron transport from PSI can take place (Stroebel et al., 2003). One other very interesting observation is that cytochrome b6 has been seen to become phosphorylated under reducing conditions (Gal et al., 1992). This observation can serve as an alternative way to activate LHCII-kinase and is possibly a redox signal to the chloroplast DNA.

5. Proteases in the chloroplast.

The chloroplast is a very dynamic organelle with the respect to light gathering, electron transport, proton pumping, state transitions, changing levels of mRNA synthesis and protein synthesis. To all this can be added another very important activity, namely proteolysis. Proteolysis in the chloroplast is often due to environmental stress responses: increased light at PSII causes photoinhibition (Haussuhl et al., 2001). A second reason is unavailability of cofactors, when no chlorophyll binds to the chlorophyll binding apoprotein (Hoober and Highes, 1992). Thirdly an imbalance in multisubunit complexes; adjustment of LHCII vs PSII at high light conditions, i.e. the total number of LHCII decreases (Lindahl et al., 1995). These last processes are addressed as acclimative proteolysis. Proteases in the chloroplast are either nuclear encoded or chloroplast-encoded, and most of them have prokaryotic homologs. They can also be divided according to where they operate, in the stroma, or the lumen, or in the thylakoid membranes (figure 5).

5.1 Stromal proteases

Import of protein into the stroma is completed only after removal of transient peptides. The removal is done by a chloroplast processing enzyme (CPE) which is a metalloprotease containing a Zn-binding motif. The enzyme functions as a general protease that removes stromal targeting peptides from all imported proteins (Richter and Lamppa, 1998). The family of Clp-proteases is also found in the stroma. These serine proteases are chloroplast-encoded and constitutively expressed. They are composed of two functionally distinct subunits, ClpP and ClpA or ClpX (Ostersetzer and Adam, 1996). Many of them are induced upon stress (Zheng et al., 2002) whereas others are housekeeping proteases (Halperin and Andersson, 1996). In paper III (part 6) a novel protease found in the stroma is presented.

5.2 Thylakoid membrane-associated proteases

In the thylakoid membrane there is a protease that cleaves leader sequences for proteins targeted to the lumen, called the thylakoid processing

peptidase, TPP (Chaal et al., 1998). An ATP-dependent metalloprotease called FtsH is an integral thylakoid-membrane protein with ATP- and Zn^{2+}-binding domains exposed to the stroma. FtsH degrades unassembled Rieske FeS protein (Ostersetzer and Adam, 1997) and is involved in the degradation of D1 protein that routinely is photoinhibited in PSII. SppA is found in the thylakoid membrane strongly bound to the stromal side of the membrane. Its content increases with increasing levels of light and it seems to have a role in the modulation of LHCII (Lensch et al., 2001). In paper IV (part 6) at novel membrane protease is described.

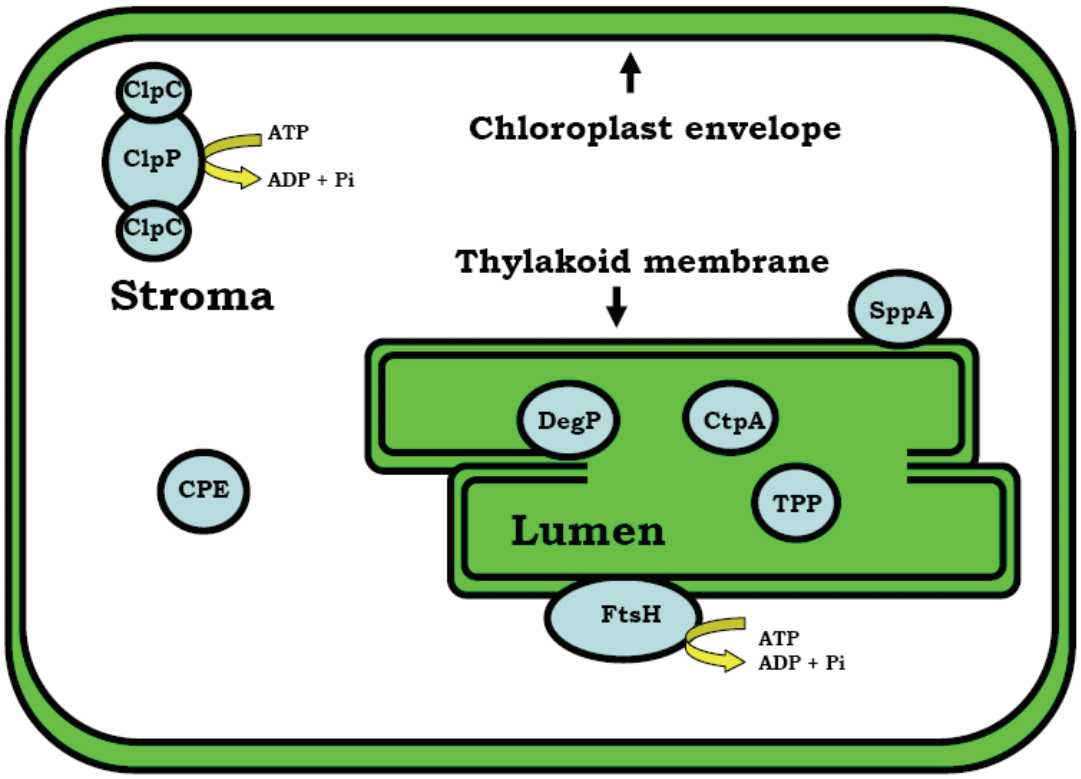

Figure 5. Proteases localized in the chloroplast (adapted from Adam 2000).

5.3 Lumenal proteases

In the lumen a protease belonging to another ATP-independent family of proteases is found, namely DegP. It is a heat-shock protease that is essential for survival at high temperatures. At low temperatures it acts as a chaperone, but at high temperatures it acts as a protease and is tightly

associated with the thylakoid membrane (Itzhaki et al., 1998). CtpA is found in the lumen and is involved in pre-D1 processing (Inagaki et al., 1996).

5.4 Acclimative proteolysis of PSII and LHCII.

Regulation of gene expression monitored through proteolysis has not yet been seen in the chloroplast. However, regulatory proteolysis is clearly seen in both prokaryotes and mitochondria (Adam, 2000).

One way to compensate for the imbalance between PSI and PSII in incoming light is to dismantle one of the photosystems and/or light-harvesting antenna to counteract the resulting imbalance in the plastoquinone pool. Most knowledge about degradation of PSII concerns photoinhibition. In PSII it is mainly the D1 protein that is damaged. To be able to insert a new D1-protein, the damaged D1 needs to be removed so that photosynthesis in that complex can commence again. A photoinhibited PSII is a source of even more damage caused to the membrane proteins. The ROS formation in PSII comes mainly from that increased triplet chlorophyll causes singlet oxygen formation. Additional PSII proteins that are degraded, at a lower rate, are D2, CP43, and a 6.1 kDa nuclear-encoded protein *PsbW* (Hagman et al., 1997). The degradation for D1 is believed to pass through several steps. D1 is first cleaved yielding 23 kDa and 10 kDa fragments via a process were GTP is required. The fragments are then further degraded in an ATP-dendent manner. FtsH is involved in the degradation of D1 (Adam, 2000). FtsH is a family of metallo-proteases that are ATP-dependent for their activity (Lindahl et al., 1996). FtsH is important in light-induced turnover of PSII D1 protein (Lindahl et al., 2000), but also degrades unassembled Rieske FeS protein.

A number of proteases are involved in the processing and degradation of LHCII. A Chlorophyll *a/b* binding apoprotein is degraded if no chlorophyll is bound to the protein; the protease acts only upon the apoprotein of LHCII and not the functional LHCII (Hoober and Highes, 1992). SppA which has a bacterial homolog in *Synechocystis* is an *Arabidopsis* protease which is encoded in the nucleus and predicted to be a serine protease. SppA is a thylakoid membrane-associated protease, found predominantly in stroma

lamellae. The substrate for SppA has not been clearly determined. The protease is shown to be light-induced when the mRNA levels reach a maximum 26-52 hours after light treatment. The acclimative degradation has been shown to have an induction phase of 48 h after transferring plants from low to higher light intensities (Lindahl et al., 1995; Yang et al., 1998). This correlates well with light-induced degradation of LHCII. Another unnamed protease with proteolytic activity against LHCII was found to be co-purified with the LHCII-trimer but not the LHCII-monomer (Anastassiou and Argyroudi-Akoyunoglou, 1995) (Georgakopoulos et al., 2002). This strange "self-digesting" protease was shown to also have proteolytic activity against D1 and D2 when purified PSII was added. The protease belonging to a cysteine family of proteases. The protease is activated by Mg^{2+}, and inhibited by Zn^{2+}, Cd^{2+} and EDTA. A strictly ATP-dependent unnamed protease was found to act against LHCII-proteins. The protease was found to be sensitive to salt washing and was to some extent purified. It also responded to increasing light levels by degrading LHCII. The apoprotein and the monomer of the holoprotein are targeted for degradation whereas the LHCII-trimer is not (Lindahl et al., 1995; Yang et al., 2000).

Evidence for acclimative proteolysis of b6f and PSI is rarer than for PSII and LHCII. However, a photoinhibition study carried out on PSI where ROS in combination with light triggered proteolytic degradation of the PsaB protein (Sonoike et al., 1997), whereas for cytochrome b6f only degradation of assembled Rieske protein was observed (Ostersetzer and Adam, 1997).

6. Present investigation

The aim of the present investigation was to understand some of the regulatory mechanisms that govern the chloroplast. Cytochrome b6f-complex is indirectly or directly involved in at least two regulatory processes in the chloroplast. One is the LHCII-kinase activation and the other is the regulation of expression of some of the genes for electron transport chain proteins. In our work we used wild type (wt) of *Pisum sativum* plants in the proteolytic and phosphorylation studies. In magnetic circular dichroism (MCD) measurements we used both pea and spinach.

Phosphorylated b6f-complex under reducing conditions (paper I).

In this study we wanted to investigate the role of possible phosphorylation of the cytochrome b6f-complex. Phosphorylation of proteins serves as regulatory mechanisms for many proteins, but is also important in cell signalling. It has been shown by several groups that cytochrome b6f is needed for LHCII-kinase activation (Gal et al., 1987; Vener et al., 1995; Zito et al., 1999) and has a possible role in regulation of gene expression. Our primary aim was to determine under which conditions b6f was phosphorylated. In order to do this, we used whole chloroplasts which were supplied with P^{32} and then subjected to in vivo phosphorylation protocols. Labelled membranes were collected and separated by Blue-native gel electrophoresis (Kügler et al., 1997). The first dimension gave a good separation between the different complexes in the chloroplast which where then identified using MS-MS, and we could show that the cytochrome b6f-complex is phosphorylated under reducing conditions. Our finding correlates with an earlier observation (Gal et al., 1992), however they used heme staining in SDS-page gels on b6f enriched thylakoid membranes to detect the phosphorylated b6. These gels had several unidentified membrane proteins present, whereas we could identify the different complexes in the Blue-native gel and show that phosphorylation was nearly absent in the cytochrome b6f-complex under oxidizing conditions. To determine which possible residue that could be phosphorylated, a sequence alignment of the 55 known sequences of b6 (*petB*, only 20 sequences shown in the paper I)

was done. In the sequences aligned we found 5 conserved histidines in all of the predicted gene products, whereas four of them where binding hemes (Stroebel et al., 2003). The remaining histidine was found on the N-terminal part of the b6 protein facing the stroma, in vicinity of cyt c_i. This gave us reason to believe that this histidine could be phosphorylated and vital for redox signaling in the chloroplast.

MCD-spectra of the b6f-complex. (paper II).

To get a picture of the redox events that are taking place in the cytochrome b6f-complex, we titrated the signal from the different cytochromes in the complex using redox agents, using MCD to detect the changes. In paper II we show the redox components of the cytochrome b6f-complex, were each component has a distinctive feature. A limiting factor is to have pure enough samples. The purity we had was about 50 Cytochrome b6f-complexes per one PSII-complex. The different hemes can be identified as low spin or high spin, but also the reduction state of the heme is determined. We see in the spectra all hemes known to be in the complex, low spin hemes cyt b_H, cyt b_L cyt f and the high spin heme cyt c_i. However the peak from c_i has a distinctive MCD signal, but is small in the presence of the other hemes which make redox quantification difficult. The different reduction states were achieved measuring on untreated sample, and sample reduced with dithionite and oxidized with periodate. In the untreated sample cyt f was fully reduced whereas cyt b_H, and b_L were fully oxidized. Periodate oxidized all the low spin hemes. In dithionite treated samples all low spin hemes were reduced. The recently discovered heme c_i is assigned to a peak at 430 nm which is typical for high spin hemes. We were only able to reduce 50% of the heme c_i, which could mean two things. Either is c_i in close vicinity to the Qi-pocket promoting heme c_i to frequently participate in redox chemistry or is the MCD-signal for c_i inherently weak.

cGEP, a protease possibly participating in degradation of LHCII (paper III).

We have identified a new protease, chloroplast glutamyl endopeptidase cGEP that is active in the stroma part of the chloroplast (paper III). cGEP is an endo-proteolytic protease of serine-type, cleaving on the carboxyl side of glutamic acid. As substrate we used a recombinant peptide corresponding to the N-terminal loop and the first turn in helix-B of light-harvesting complex II (Lhcb1 from pea). The protease was partially purified and could be correlated to a 95 kDa polypeptide band on SDS-polyacrylamide gels. MS-MS sequencing of peptides from *in gel* trypsination showed high similarity to sequences of a protein in *A. thaliana*. The *A. thaliana* protein showed similarity to several proteases in the NCBI public database that were annotated as glutamyl endopeptidases. The sequence of the nuclear-encoded holoprotein was, according to different programs, for subcellular location of proteins in most cases predicted to be localized to the chloroplast. The protease was not dependent on ATP, GTP or DTT for its activity. Whereas we have shown proteolytic activity against the LHCII N-terminal peptide we have so far not been able to show proteolytic activity against isolated LHCII. This suggests that the structure and availability of the substrate *in vivo* is a determining factor. However in a study (Harper et al., 2004) on *A. thaliana,* wild type (*wt*) and a mutant (*cch1*) were exposed to changing light conditions. The degradation of lhcb 1, 2, 3 were increased in the *cch1* mutant compared to *wt*. In an EST micro-array study on the *cch1* mutant, the expression levels of cGEP was found to be increased 2.5-fold in the *cch1* mutant compared to *wt*. This suggests that cGEP *in vivo* could be involved in LHCII degradation.

A novel membrane associated protease with potential to degrade LHCII (papers III and IV).

What we have done in paper III and IV is to show the presence of a membrane-associated protease of serine/cysteine-type in thylakoids. The protease cleaves specifically a peptide that resembles the N-terminal of Lhcb1, at the carboxyl side of the second alanine in the sequence, MRKSATTKKVASSGS. Activation of the protease was achieved by thiol

reducing agents (dithiothreitol (DDT), mercaptoethanol and thioredoxin). Mg^{2+}, GTP and ATP had no or very minor effects on the activity. Stroma lamellae showed 2-3 fold higher activity than grana fractions. The proteolytic activity is pH-dependent; at pH 7, resembling pH in the stroma in darkness, the activity was optimal. At pH 8, resembling pH in the stroma during photosynthesis, the proteolytic activity was significantly lower. In our activation measurements on light incubated whole chloroplasts the protease shows a negative dependence on light, and oxygen seems to be causing this effect. Purification attempts on the protease, submitting thylakoid membranes to alkaline treatment caused disappearance of proteolytic activity, which was retrieved in enrichment of supernatant. We have so far not been able to find conditions where the protease acts on the holoprotein of LHCII. However, Stauber (Stauber et al., 2003) saw in proteomic study of light-harvesting proteins that the N-terminal had been processed *in vivo* with a cleavage site correlating with the breakdown product attained when the reported protease cleaves the synthetic peptide. In the literature there are several membrane-associated proteases that act upon LHCII, however the membrane protease reported in paper III and IV appear to be distinct from them.

6.1. Conclusions and future perspective

We have seen that the environment around cytochrome b6f, the LHCII-kinase and LHCII is a complex one. To get a more complete picture, we have tried several approaches to target the signaling via the cytochrome b6f-complex to the LHCII-kinase. Part of our approach was to focus on signalling via protein phosphorylation. We saw that b6f-complex is phosphorylated under reducing conditions (paper I). This phosphorylation could be a signal for kinase activation. In sequence of cytochrome b6, we saw a conserved histidine on the N-terminal arm facing the stroma. In later experiments (not shown), the histidine is probably not the residue phosphorylated, since acid incubation of P^{32} labelled cyt b6 gave no loss of signal (Stock et al., 1989). The histidine is however still interesting due to its closeness to heme c_i, a novel cytochrome that sits close to the stromal side of the thylakoid membrane. The function of heme c_i is still unknown but it has been suggested that the heme c_i participates in cyclic electron transfer from PSI (Zhang et al., 2001).

An obvious continuation of this work is to identify the phosphorylation site. A possible site was found by sequence investigations using Prosite Scan View (http://us.expasy.org/cgi-bin/prosite/ScanView), a pair of amino acids, T21 and S22 in the N-terminal part of b6. By a combination of P^{32} labelling, Blue-native electrophoresis and MS-MS, we are now in progress to establish whether T21 and S22 are sites for phosphorylation or not.

An attempt to determine the redox potentials of the prosthetic groups in cytochrome b6f was done using MCD (paper II). The measurements showed that it was possible to see the different high and low spin cytochromes in the thylakoid membrane. What we see in the measurement is the well known cyt b_H, cyt b_L and cyt f, but most importantly the signal from heme recently discovered c_i. With the knowledge of how to detect heme c_i, it would be possible to determine the redox potential of this heme. It would be of interest to correlate the redox potential of heme c_i, with the common midpoint potential of E_m=+40 mV where several thylakoid proteins are known to

become phosphorylated (Silverstein et al., 1993). In the case of MCD measurements in paper II, we have showed that it was possible to distinguish the different components with good results, but so far we have not done any redox titration.

Related to this we have made preliminary EPR measurements on the Rieske FeS protein. It is suggested that a conformational change of the transmembrane part of the Rieske FeS protein is the triggering signal to activate LHCII-kinase (Vener et al., 1998). In this work we want to determine the redox potential needed to break a sulphur bridge in the Rieske FeS protein. Also here we are interested to correlate this with the phosphorylation of thylakoid proteins at the common midpoint of 40 mV (Silverstein et al., 1993). Preliminary data indicates a value around 0 mV, for the breakage of the sulphur bridge, but this needs to be verified. In the EPR spectra we see also signals from the possibly heme c_i, cyt b, and cyt f. The heme c_i peak appears between a redox potential of -90 to +100 mV, with a clearly visible peak at half its full height at a redox potential of 50 mV. These very early experiments suggest that Heme c_i could participate in the signalling to LHCII-kinase activation, also here, more measurements are needed. Redox measurements on heme c_i have been done by two different groups. Rappaport (Rappaport et al, conference abstract Montreal 2004), reported a redox potential of E_m= +75 mV measured on *Chlamydomonas reinhardtii*, whereas another group reports several possible values around 0 mV (Zhang et al., 2004) measured on *Mastigocladius laminosus*. Considering the remarkable similarity in the crystal structure of cytochrome b6f complexes from the eukaryote *C. reinhardtii* (Kurisu et al., 2003) and the cyanobacterium *M. laminosus* (Stroebel et al., 2003) the discrepancy in redox potential for c_i is not what one would expect. Since axial ligands not are present in heme c_i. Heme c_i could possibly act as an oxygen sensor apart of being involved in electron transport. The role of heme ci needs to be investigated futher.

In paper III and IV we present two novel proteases that are possibly involved in proteolytic activity against the light harvesting antenna, LHCII. We identified a stroma localised protease and suggested the name cGEP, chloroplast glutamyl endopeptidase. However mutant studies (Harper et al., 2004) and proteonomic data (se paper III) support that cGEP is likely to have a function in LHCII degradation. It is possible that cGEP is involved in proteolysis of other substrates as well. The membrane associated protease acts, as shown earlier, on the same substrate as cGEP but at different proteolytic sites. The membrane associated protease is more sensitive to changes in the environment, such as changes in redox state of the thiol pool. Changes in pH and O_2 concentration also have an affect on the protease. This leads us to believe that the membrane associated protease partakes in regulatory events in the chloroplast. A continuation of these protease studies would include attemps to find out under which conditions LHCII becomes accessible to the protease (e.g. phosphorylation, partial denaturation of substrate and ionic composition). Another option would be to determine if the proteases are active against other substrates in the thylakoid membrane. By making synthetic peptides corresponding to N-terminal arms and extrinsic loops originating from the protein complexes in the thylakoid membrane and then measure proteolytic activity on those peptides would give us a better understanding of the two proteases presented here.

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