CHAPTER 1

Introduction
1.1. The basic principles of photosynthesis

Photosynthesis is the vital biological process by which, in a series of events, the sun’s energy is captured and converted into chemical energy. This process also provides the primary source of molecular oxygen for life on Earth. Photosynthesis is carried out by a wide variety of organisms including cyanobacteria, algae and plants. All these photosynthetic organisms utilize the light energy to synthesize organic compounds and more generally provide the means for maintenance and growth.

The photosynthetic process in plants and algae occurs in organelles known as chloroplasts, whereas more primitive photosynthetic organisms such as oxygenic cyanobacteria, prochlorophytes and anoxygenic photosynthetic bacteria lack such organelles. Photosynthetic reactions are traditionally divided into two groups: the "light reactions", which consist of electron and proton transfer reactions leading to the synthesis of ATP and NADPH and the "dark reactions" which consist of the biosynthesis of carbohydrates from CO₂.

Light reactions occur in a complex membrane system called the thylakoid membrane which houses five major membrane protein complexes in the case of oxygenic photosynthesis. The complexes are known as Photosystem II (PSII), Photosystem I (PSI), the cytochrome b₆f (Cyt b₆f) complex, the light-harvesting complex (LHC) and ATP synthase (Figure 1).
**Figure 1.** Schematic representation of the major components of oxygenic photosynthesis. Chlorophyll molecules are excited by light, which are located in the two LHCI and LHCII, represented by square symbols. Solid lines indicate non-cyclic electron transfer movements. The dashed line represents the alternative cyclic electron transfer pathway in PSI. Abbreviations: LHC-light harvesting complex; Pheo-pheophytin; PQ-plastoquinone; PQH$_2$-plastoquinol; PC-plastocyanin; A$_1$-phyloquinone; A$_0$-chlorophyll a; F$_B$F$_{s}$-iron sulphur clusters; Fd-ferredoxin.

The primary photosynthetic process in the PSII complex starts with the absorption of light by the light-harvesting antenna (LHCII) of PSII. After absorption of light the excitation energy is transferred to the reaction centre, where a chl molecule known as P680 (Primary electron donor absorbing light at 680 nm) is located. Excitation of P680 causes charge separation and a (P680$^+$Pheo$^-$) primary radical pair is formed. P680$^+$ withdraws one electron from a tyrosine residue in the D1 protein which in turn is reduced by electrons from the manganese cluster. This cluster oxidises water, and releases O$_2$ and protons (H$^+$) into the lumen. From the Pheo$^-$ the electron is then transferred to the primary electron acceptor, a quinone molecule (Q$_A$), after which the electron passes to a secondary electron acceptor (Q$_B$). Each plastoquinone (PQ) can be reduced by two electrons from Q$_B$ and accepts two protons from the stroma. The reduced plastoquinol (PQH$_2$) is released into the lipid phase of the membrane and carries the electrons to the Cyt b$_{6f}$ complex.

The overall reaction carried out by PSII is given in Equation 1.1:

$$h\nu \quad 2H_2O + 2PQ \rightarrow O_2 + 2PQH_2$$  \hspace{1cm} 1.1

The PQH$_2$ is oxidised by the transfer of two electrons through the Cyt b$_{6f}$ complex to the plastocyanine (PC) and subsequently two protons are released into the inner thylakoid space (equation 1.2):

$$PQH_2 + 2PC_{\text{oxidized}} \rightarrow PQ + 2PC_{\text{reduced}} + 2H^+$$  \hspace{1cm} 1.2

In PSI light is absorbed by antenna pigments in a light harvesting system (LHCl) and excitation energy is transferred to the primary electron donor of PSI P700 (Primary electron donor absorbing light at 700 nm) that leads to charge separation between P700 and the primary electron acceptor A$_0$ (a monomeric Chla
molecule). The P700$^+$ cation thus formed in turn is reduced by plastocyanin. The electron on A$_0$ passes towards the stroma side via phylloquinone molecule (A$_1$) and a number of iron-sulfur (Fe-S) clusters (F$_x$, F$_A$, F$_B$). The terminal Fe-S cluster subsequently reduces the water-soluble protein named ferredoxin (Fd). The reduction occurs on the stromal side of the thylakoid membrane. The overall reaction that is driven by PSI is given in Equation 1.3:

$$ h\nu \quad PC_{\text{reduced}} + Fd_{\text{oxidized}} \rightarrow PC_{\text{oxidized}} + Fd_{\text{reduced}} $$

1.3

The reduced equivalents from reduced ferredoxin are used to reduce NADP$^+$ to NADPH, which reaction is catalysed by the enzyme Fd-NADPH reductase.

The overall flow of electrons in non-cyclic (linear) electron transport is described as follows:

$$ H_2O \rightarrow \text{PSII} \rightarrow \text{PQ} \rightarrow \text{Cyt b$_{6f}$} \rightarrow \text{PC} \rightarrow \text{PSI} \rightarrow \text{Fd} \rightarrow \text{NADP}^+ $$

An alternative pathway is cyclic electron transport. It occurs when the stromal NADP$^+$ concentration is too low to accept electrons from reduced ferredoxin. If this is the case the electrons are transferred back to plastocyanin via the Cyt b$_{6f}$ complex. It does not require the input energy by PSII and thus it does not involve in production of O$_2$. In cyclic electron transport the energy is used only for the generation of a proton motive force and NADPH is not formed. This is termed cyclic photophosphorylation. The overall flow of electrons in cyclic electron transport is described as follows:

$$ \text{PSI} \rightarrow \text{Fd} \rightarrow \text{Cyt b$_{6f}$} \rightarrow \text{PC} \rightarrow \text{PSI} $$

The electrochemical proton gradient generated by the light reactions is used by ATP synthase to synthesize ATP from ADP and Pi. The next step of photosynthesis is the consumption of NADPH and ATP for the assimilation of CO$_2$ in the Calvin cycle, resulting in the formation of carbohydrates.

**Organization of the thylakoid membrane in chloroplasts**

A striking feature of the thylakoid membrane is its structural differentiation in granal and stromal regions. Folded thylakoid membrane parts that are closely associated into stacks are called granal thylakoid membranes and the non-stacking parts are known as stromal thylakoid membranes. This fine-structure of the
thylakoid membrane has been studied by electron microscopy for over 50 years, mostly with the aid of thin-sectioning, negative staining and freeze-etching EM techniques (Weier, T. E. 1963, Staehelin, L. A. 1975, Miller, K. R. and Staehelin, L. A. 1976, Staehelin, L. A. 1976, Miller, K. R. 1976, Stoylova, S. et al. 2000, Ford, R. C. et al. 2002). Thin sections have presented the first visualization of the organization of the thylakoid membrane within chloroplasts. It was subsequently proposed that all grana are interconnected by tubes forming a net (Weier, T. E. 1963). Considering all the artifacts of this technique the model is now abandoned. Instead, an alternative model was proposed by Andersson et.al.(1980) and called the folded-membrane model. Here, stroma thylakoids do not directly continue from the granum thylakoids, but form a fork, where the continuous membrane folds in such a way that the “upper” and “lower” membranes originate from a layer “above” and a layer “below” the stacked pair of membrane (Andersson, B. and Anderson, J. M. 1980, Mustardy, L. and Garab, G. 2003). This organization provides a rapid, reversible folding. Environmental conditions can lead to the partial unfolding of the granum stacks, and this triggers the redistribution of absorbed excitation energy within the thylakoid membrane. (This mechanism is called “state transition” and will be discussed in more detail below) (Allen, J.F. 1992)).

Andersson et. al. (1980) also demonstrated the lateral heterogeneity in the distribution of chlorophyll-protein complexes in the thylakoid membrane. In this work it was illustrated that in higher plants PSI and PSII are spatially separated, with PSII almost exclusively located in the granum partition of the thylakoid membrane and PSI in both the stroma lamellae and on the ends of the grana stacks. The ATP synthase enzyme is entirely localized in the stroma lamellae and the cytochrome $b_{6}f$ complex is nearly equally distributed between the two types of membrane. A schematic representation for the organization of the thylakoid membrane is depicted in Figure 2.

The possible functions of such lateral distribution for the membrane-bound complexes are still not fully understood. For example, in green algae and cyanobacteria the thylakoid membrane does not exhibit the profound grana stacking and lateral distribution of the photosynthetic complexes observed in higher plants (Goodenough, U. W. and Staehelin, L. A. 1971). However, several suggestions have been made to explain the functional advantages for grana formation and lateral heterogeneity. The formation of grana prevents competition between linear electron transport and cyclic electron transport in which only PSI and the Cyt $b_{6}f$ are involved. The grana PSI complex reduces ferredoxin, which in its turn reduces NADPH, used for driving the Calvin cycle. The stroma-bound PSI
Figure 2. Organisation of the thylakoid membrane in higher plant chloroplasts. Vertical distance between membranes are to scale, in horizontal direction an average grana stack would be twice as large in diameter.

participates in the cyclic pathway, in which it reduces ferredoxin, which itself reduces the components of a cyclic photophosphorylation pathway (Bendall, D. S. and Manasse, R. S. 1995). Grana stacking is also necessary to prevent “spillover” of excitation energy from PSII to PSI, which is possible due to the difference in absorption properties of chlorophylls within the photosystems (Bendall, D. S. and Manasse, R. S. 1995). In addition, PSI and PSII are distinguished by their excitation trapping kinetics: PSII is three times slower than PSI. In order to avoid quenching of PSII, Trissl and Wilhelm (1993) proposed that granum formation is a strategy developed by nature to separate physically “slow” PSII from “fast” PSI (Trissl, H. W. and Wilhelm, C. 1993). Moreover, there is much evidence that the function of the latter segregation is related to the regulation of antenna function and consequently directly related to the quality and quantity of light harvesting. It has been observed that in conditions of low light the plants have more grana than in high-light grown plants where the unstacked membranes dominate (Andersson, J. M and Andersson, B. 1988). This is due to the fact that in stacked membranes
the pigment proteins are more closely packed leading to an increased antenna size. This is critically important because at low light intensity light harvesting is the limiting factor in photosynthetic efficiency.

**Regulatory mechanisms**

Oxygenic photosynthesis relies on the function of both PSII and PSI which operate in series. The absorption properties of the light harvesting systems differ between PSI and PSII. In case of PSI the LHCI antenna pigments absorb in the far red region of the spectrum, whereas in PSII LHCII absorbs at shorter wavelengths in the red region. Natural environmental conditions, such as quality and quantity of light are fluctuating which may alter the balance in absorption of light energy between the two photosystems. Under these circumstances, regulation of light harvesting is necessary to balance the absorption of light energy. Therefore the spectral imbalance of the exciting light results in an imbalance of excitation between the two photosystems.

Under conditions when light excites preferentially either PSII or PSI the redistribution of excitation energy is regulated by a special mechanism, which is called “state transition” (Bonaventura, C. and Myers, J. 1969, Murata, N. 1970, Bennett, J. et al. 1980, Bennett, J. et al. 1988, Allen, J.F. 1992). This mechanism is based on the reversible phosphorylation of LHCII by a thylakoid kinase (Bennett, J. 1979, Rintamaki, E. et al. 1997, Pursiheimo, S. et al. 1998, Carlberg, I. et al. 1999). Zito et al. showed that the kinase activation signal is transduced through the cytochrome b$_{6}$f complex, where it requires quinol binding at the (Q$_{0}$) site of Cyt b$_{6}$f complex (Zito, F. et al. 1999). When the light is absorbed preferentially by PSII (state 2) the PQ pool is reduced and binds to the quinol oxidation (Q$_{0}$) site of the cytochrome b$_{6}$f, and this activates the kinase. As a result, LHCII becomes phosphorylated, dissociates from PSII and migrates to PSI. As a consequence, the antenna size of PSII is reduced and the antenna in PSI is enlarged which balances the absorption of light energy. Phosphorylation of the LHCII leads to the unstacking of the grana which facilitates the migration of the LHCII complexes within the thylakoid membrane. When excitation of PSI is favored (state 1) the PQ pool is oxidized and the kinase deactivated, which allows the mobile LHCII antenna proteins to become dephosphorylated by a phosphatase (Bennett, J. 1980) and subsequently re-associated with the PSII complex.

Under conditions where the photosynthetic organism receives more sunlight than it can use for photosynthesis, regulation of the utilisation of light energy is necessary in order to minimise potential photooxidation damage. Non-photochemical quenching (NPQ) is the mechanism that quenches singlet-excited
chlorophyll molecules and harmlessly dissipates excess excitation energy as heat (Horton, P. et al. 1996). It has been shown that the antenna protein PsbS, which is a member of the Cab (Chlorophyll a/b binding) family, plays an important role in protective energy dissipation (Li, X. P. et al. 2000). They suggested that the binding of protons to the PsbS protein triggers a conformational change of this protein leading to a quenching state. To extend this idea it has been shown that the protonation of PsbS induced the dissociation of dimers into monomers, where the monomeric form is believed to be the active form of the protein involved in NPQ (Bergantino, E. et al. 2003).

1.2. Photosystem II

Photosystem II is a multisubunit complex embedded in the thylakoid membranes of higher plants, algae and cyanobacteria that uses solar energy to drive the photosynthetic water-splitting reaction. The PSII core consists of a membrane-embedded reaction centre, a core antenna complex and a complex of water-soluble extrinsic proteins in the lumen, called the Oxygen-Evolving Complex (OEC) that carries out water oxidation. At the periphery, membrane associated chlorophyll-proteins form a peripheral light harvesting complex, which funnels the energy to the reaction centre. PSII is the only biological system known that is capable of oxidizing water to molecular oxygen, so it has been extensively studied, trying to understand how the natural system works, but also in the context of artificial systems for energy conversion. In attempts to elucidate the structure and function two specific preparations have been very useful. The first, a preparation of the active PSII complex which is capable of oxygen evolution, was called BBY, named after Berthold, Babcock and Yocum, who first reported it (Berthold, D. A. et al. 1981). It contains all the components of the reaction centre described below, plus two core antenna complexes known as CP43 and CP47 (chlorophyll proteins with apparent molecular mass of 43 and 47 kDa, respectively) as well as LHClI antenna complexes and additional proteins of the oxygen-evolving complex. Three extrinsic proteins, PsbO (33-kDa subunit), PsbP (23-kDa subunit), and PsbQ (17-kDa subunits), together form the OEC. The OEC also includes the Mn cluster which is involved in the optimization of its function in water splitting. The second preparation that has been widely studied is the PSII reaction centre, consisting of two chlorophyll-binding proteins, known as D1 and D2, along with the α and β subunits of a membrane-bound cytochrome b559 and a small peptide, the PsbI gene product, reported by Nanba, O. & Satoh, K. (1987). This reaction centre complex binds six chlorophyll molecules, two pheophytins, and two β-carotenoids.
and is capable of primary photochemistry, but not O₂ evolution or secondary electron transfer involving the quinones (Nanba O and Satoh K 1987).

There are over 29 subunits associated with the PSII complex of higher plants and green algae. Almost all of these subunits have been studied and the function of each of them is summarised in Table 1 (Hankamer, B. et al. 1997a). Two of these subunits, D1 and D2, are homologous to the L and M proteins in purple bacteria and some of the functions could be similar (Zouni, A. et al. 2001). However, all other subunits in higher plants have no homology with proteins from other types of prokaryotic photosynthetic organisms. It therefore remains a particularly interesting challenge to find and elucidate the function of especially the low molecular weight proteins, which do not function in harvesting of light due to the lack of bound chlorophyll. At present, there are 12 proteins, which have been structurally characterised and known to have a mass of less than 10 kDa, which are associated with the PSII complex (Table 1). However, the exact number and functions of the small subunits associated with PSII in vivo remains to be established.

Table 1. Photosystem II subunits of cyanobacteria and green plants

<table>
<thead>
<tr>
<th>Gene</th>
<th>Subunit</th>
<th>Molecular mass (kDa)</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>psbA</td>
<td>D1</td>
<td>38</td>
<td>Binding P680, Pheo, Qₜ, Qₐ</td>
</tr>
<tr>
<td>psbD</td>
<td>D2</td>
<td>39</td>
<td></td>
</tr>
<tr>
<td>psbE</td>
<td>α cyt b559</td>
<td>1</td>
<td>Binding heme, photoprotection</td>
</tr>
<tr>
<td>psbF</td>
<td>β cyt b559</td>
<td>1</td>
<td>Binding heme, photoprotection</td>
</tr>
<tr>
<td>psbB</td>
<td>CP47</td>
<td>56</td>
<td>Excitation energy transfer</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Binding 33 kDa protein</td>
</tr>
<tr>
<td>psbC</td>
<td>CP43</td>
<td>50</td>
<td>Excitation energy transfer</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Binding 33 kDa protein</td>
</tr>
<tr>
<td>psbI</td>
<td>I protein</td>
<td>4</td>
<td>Stabilisation of PSII</td>
</tr>
<tr>
<td>psaH</td>
<td>H protein</td>
<td>7.7</td>
<td>Photoprotection, PSII assembly</td>
</tr>
<tr>
<td>psbK</td>
<td>K protein</td>
<td>4.3</td>
<td>PSII assembly</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Binding PQ</td>
</tr>
<tr>
<td>psbL</td>
<td>L protein</td>
<td>4.4</td>
<td>Involvement in Qₐ function</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>PSII assembly</td>
</tr>
<tr>
<td>psbM</td>
<td>M protein</td>
<td>3.75</td>
<td>?</td>
</tr>
<tr>
<td>psbN</td>
<td>N protein</td>
<td>4.7</td>
<td>?</td>
</tr>
<tr>
<td>psbO</td>
<td>33 kDa protein</td>
<td>26.5</td>
<td>Stabilises Mn cluster</td>
</tr>
</tbody>
</table>
## Structure of Photosystem II complexes

An essential precondition for understanding the reactions that are involved in water oxidation is a detailed model of the three-dimensional structure of the complex. Both electron microscopy and X-ray crystallography have contributed to the elucidation of the structure of the PSII complex. Most crystallographic investigations have been performed with core complexes or subcomplexes. In total the PSII core complex consists of over 20 subunits, where all cofactors involved in the charge separation in PSII are bound to the RC proteins D1 and D2, and closely associated with these two proteins are the inner antenna proteins CP43 and CP47; all cofactors which are required for water-splitting activity, are bound to three extrinsic proteins of the oxygen-evolving complex, PsbO, PsbP, PsbQ proteins.

Medium-resolution structures of the PSII CP47-reaction centre, isolated from higher plants, have been obtained by electron microscopy of two-dimensional
Intracellular crystals (Rhee, K. H. et al. 1997, Rhee, K. H. et al. 1998). A 3D structure at 8 Å resolution showed the position of 23 transmembrane α-helices, including the D1, D2 and the largest proximal antenna subunit CP47. However, the crystal structure lacked information about both the three luminal extrinsic subunits of the oxygen-evolving complex and the CP43 subunit. In subsequent investigations the structure of a more complete core complex was solved (Hankamer, B. et al. 1999), where in addition to the CP47, D1 and D2 proteins, the crystals contained CP43, one of the proteins of the OEC PsbO protein and several low molecular weight subunits including PsbH. This study revealed that the CP43 and CP47 subunits are located on opposite sides of the D1/D2 heterodimer (Figure 3). Together with other EM studies (Zheleva, D. et al. 1998, Boekema, E. J. et al. 1998a, Barber, J. et al. 2000) these initial investigations also revealed that the CP47-CP43-RC complexes have a dimeric organisation. The precise organisation of the PSII core complex from higher plants has not yet been determined, but X-ray crystallographic studies have been carried out on complexes from two different species of the cyanobacterium *Thermosynechococcus*. Cofactors bound to the RC and CP43 and CP47 and the α-carbon backbones of all helices at 3.8 Å and 3.7 Å resolution, respectively have been revealed (Zouni, A. et al. 2001, Kamiya, N. and Shen, J. R. 2003). These structures are a good match for the major protein elements of the higher plant CP47-CP43-RC complex structure (Rhee, K. H. et al. 1997, Hankamer, B. et al. 1999), which can be explained by the fact that the sequence of the main subunits CP43, CP47, D1 and D2 in higher plants and cyanobacteria have a high degree of identity. Differences between the cyanobacterial and plant structures mostly concern helices attributed to the small subunits.

The main discrepancy between the organisation of the PSII complex of cyanobacteria and higher plants is in the light harvesting system. The higher plants possess a unique transmembrane light-harvesting complex whereas in cyanobacteria this is substituted by a large water-soluble phycobilisome antenna complex, which is attached to the cytoplasmic side of the cyanobacterial PSII complex.

The peripheral antenna system in the higher plants PSII complex is quite diverse in terms of structure and relative position in the energy transfer sequence. The PSII complex has two types of antenna proteins. Of these, there are three monomeric proteins, that are located closest to the core complex, which are known as the minor antenna proteins CP29, CP26 and CP24 (or Lhcb4, Lhcb5 and Lhcb6, respectively). The minor antenna proteins mediate the binding of more peripheral heterotrimeric LHCII complexes, which are composed of Lhcb1, Lhcb2 and Lhcb3 proteins. Electron microscopy has been employed to determine the structure of the trimeric LHCII complex (Kuhlbrandt, W. and Wang, D. N. 1991). The structure
was determined at 3.4 Å resolution, and this revealed that each monomer consists of three transmembrane helices that coordinate 12 chlorophyll \( a \) and \( b \) molecules. Due to the high sequence identity between the Lhcb proteins, especially in the membrane spanning regions, the structure of CP26 and CP29 proteins could also be reasonably well modelled (Sandona, D. et al. 1998).

The first investigation into the detailed structure of a complete PSII-LHCII complex was done by electron microscopy and image analysis (Rogner, M. et al. 1987, Boekema, E. J. et al. 1995, Boekema, E. J. et al. 1998b, Boekema, E. J. et al. 1999a, Boekema, E. J. et al. 1999b). These studies revealed the presence of supercomplexes consisting of a dimeric PSII core complex, designated as “C” and trimeric LHCII complexes in three different types of binding positions. These positions were named S, M and L in the case of higher plants, in which “S” refers to a strongly, “M” to a moderately and “L” to a loosely bound trimeric LHCII complex (Boekema, E. J. et al. 1999b). Several combinations of attached LHCII complexes have been found and named in a way that the number of LHCII complexes attached is indicated. Among them there are \( C_2S_2, C_2S_2M, C_2S_2M_2, C_2S_2M_2L \) supercomplexes. The example of \( C_2S_2M_2 \) supercomplexes is depicted in Figure 3, where besides the trimeric LHCII complexes the three peripheral monomeric LHCII proteins CP29, CP26 and CP24 are shown on each side of the complex.

The locations of CP29, CP26 and CP24 have tentatively been proposed from cross-linking experiments (Bassi, R. and Dainese, P. 1992, Harrer, R. et al. 1998). In chapter 3 direct evidence is provided, based on analysis of antisense plants lacking CP26 or CP29, showing that the model presented in Figure 3 is correct.

**Figure 3.** A schematic representation of the \( C_2S_2M_2 \) PSII-LHCII complex. The position of core subunits: CP43, CP47, D1, D2 together with the minor antenna proteins: CP24, CP26, CP29 and LHCII S, M trimers is indicated. The dimeric core complex is depicted in white. The minor antenna proteins are light grey. LHCII trimers are dark grey.
Interestingly, the CP24 subunit is only present in large supercomplexes which are containing “M” and “L” LHCII trimers. Therefore, the tentative position could be in close vicinity of these LHCII trimers. In addition to the supercomplexes, it was found that two C\textsubscript{2}S\textsubscript{2}M or C\textsubscript{2}S\textsubscript{2}M\textsubscript{2} PSII-LHCII supercomplexes are able to interact to form “megacomplexes” (Boekema, E. J. et al. 1999a) where the interface of the two supercomplexes was formed by “M” trimers and CP26 and CP24 subunits. In total three different types of megacomplexes the so-called type I, type II and type III association were found, in which the M trimers, CP26 and CP24 of the two supercomplexes interact in different ways. This flexibility in organisation could be the first indication for the structural dynamics in the PSII organisation \textit{in vivo}.

Biochemical data have revealed that eight LHCII trimeric complexes should be present for every dimeric PSII core complex (Dainese, P. and Bassi, R. 1991, Peter, G. F. and Thornber, J. P. 1991, Jansson, S. 1994). However, even megacomplexes of PSII can not accommodate such a large number of LHCII complexes (Boekema, E. J. et al. 1999a).

Consequently, this would lead to a large proportion of LHCII being unaccounted for. Recently, in the non-bound population of LHCII, a remarkable complex was found and analysed using electron microscopy and image processing (Dekker, J. P. et al. 1999) (Figure 4). These so-called icosienamers (21-mers) consist of 7 LHCII trimers. However, no specific association with PSII particles has yet been detected. Further investigations are required to elucidate whether and how icosienamers and PSII particles interact.

**Figure 4.** The icosienamer. The supermolecular complex of seven LHCII trimers. The tripods indicate trimer (Dekker, J. P. et al. 1999).

The next intriguing question is how the various PSII-LHCII supercomplexes make up the complete grana membrane. Investigation of the intact thylakoid membrane started by freeze-fracture, freeze-etch techniques (Miller, K. R. and Staehelin, L. A. 1976, Miller, K. R. 1976). The low resolution data monitored the
outer and internal surface of the thylakoid membrane and showed the location of
the components within the stacked and unstacked parts of the membrane. These
low resolution techniques were also able to recognize rows of protein complexes at
some places of the thylakoid outer surface. In the 1976, Miller reported “I have no
explanation for the occurrence of such arrays, but they clearly provide a
convenience aid in measuring particles and observing their substructure” (Miller,
K. R. 1976). Further to this and based on the surface size it was suggested that in
the stacking part of the thylakoid membrane the PSII particles are responsible for
these arrays, which was later confirmed by several authors (Tsvetkova, N. M. et al.
1995, Boekema, E. J. et al. 2000a, Ford, R. C. et al. 2002). At present, the
appearance of the regular lattices still remains an enigma.

The step forward towards elucidation of the organisation of the thylakoid
membrane was made by EM and image analysis (Boekema, E. J. et al. 2000a).
Useful objects of investigation were fragments of grana membranes, in which two
membranes were attached inside-out (“paired inside-out membranes”). Analysis
showed that some fragments appeared to contain semi-crystalline domains with
rows of PSII complexes spaced by 23 or 26.3 nm (Boekema, E. J. et al. 2000a).
The packing was compatible with a repeating unit consisting of PSII-LHCII
supercomplexes. Although the resolution in the light harvesting area of the
complexes appeared to be low, fitting of the supercomplexes within the lattice
could be done due to the accurately determined position of core PSII part. A
comprehensive analysis revealed two types of supercomplex lattices which were
shown to be composed of $C_2S_2$ and $C_2S_2M$ particles. In chapter 2 of this Thesis,
similar studies are described and provide information about the organization of the
thylakoid membrane in Arabidopsis thaliana plants, where the resolution in the
light harvesting area was sufficient to visualize the LHCII trimers directly. In this
way, the $C_2S_2M_2$ supercomplexes as a building block were accurately determined.

It should be noted that only a part of the isolated thylakoid grana membranes is
organized in ordered lattices, consistent with observations made on complete
chloroplasts with freeze-fracture EM. This could be explained by the high degree
of heterogeneity in supercomplex antenna size within one membrane, leading to a
considerable degree of distortions of crystalline lattices.

**Perspective**

To summarise the ongoing research it should be remarked that there has been
considerable progress towards elucidating the structure of PSII in the last few
years. Several preliminary 3D structures have been published, though none at
atomic resolution (Zouni, A. et al. 2001, Kamiya, N. and Shen, J. R. 2003). In
addition to this, structures of supermolecular complexes containing LHCII proteins have been investigated using EM and feasible models have been obtained (Boekema, E. J. et al. 1998b, Boekema, E. J. et al. 1999b, Nield, J. et al. 2000b, Nield, J. et al. 2000c). Furthermore, the structural organisation of PSII in paired grana membranes has been shown (Boekema, E. J. et al. 2000a). However there are still many questions need to be answered:

1. The position of PsbS subunit remains elusive. Comparison of the wild type thylakoid membrane containing a PsbS subunit with thylakoid membrane purified from the npq4-1 mutant (PsbS lacking mutant) would be useful.

2. The position of some of the small subunits needs to be determined.

3. It would be useful to look from a biochemical perspective to the precise function of the crystalline organisation of PSII-LHCII within the thylakoid membrane. It is also of interest to study the appearance of the crystallinity with wild type or Lhcb mutants grown under different light conditions.

1.3. Photosystem I

Photosystem I (PSI) is a large multi-subunit protein complex, embedded in the photosynthetic thylakoid membrane of higher plants, algae and cyanobacteria, which catalyses the light-induced electron transfer from plastocyanin (cytochrome c) on the luminal side of the membrane to ferredoxin (flavodoxin) on the stromal side of the membrane. The PSI complex may be considered as having two functional domains: a core complex and a peripheral light-harvesting complex (LHC). The peripheral LHCI complex subunits are typical for higher plants.

The core complex contains at least 12-13 different proteins, which are named according to their genes PsaA to PsaX (Table 2) (Chitnis, P. R. 2001).

<table>
<thead>
<tr>
<th>Subunit</th>
<th>Molecular mass (kDa)</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>PsaA</td>
<td>83</td>
<td>Light harvesting</td>
</tr>
<tr>
<td>PsaB</td>
<td>82</td>
<td>Charge separation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Electron transport</td>
</tr>
<tr>
<td>PsaC</td>
<td>8.8</td>
<td>Electron transport</td>
</tr>
</tbody>
</table>
The protein subunits of PSI play different roles in the function and organisation of the PSI complex (Scheller, H. V. et al. 2001, Chitnis, P. R. 2001, Fromme, P. et al. 2001). The functional role of PSI subunits can be correlated with the binding of inorganic and organic co-factors and with binding of other proteins that associate with the PSI complex.

The two major core subunits of PSI, the transmembrane proteins PsaA and PsaB, form the central part of the complex and bind almost all electron transport co-factors including the primary electron donor P700 and primary electron acceptors. Moreover, it was suggested that the loop regions of the PsaA and PsaB subunits at the luminal surface are involved in the interaction with plastocyanin (Krauss, N. et al. 1993, Krauss, N. et al. 1996, Jordan, P. et al. 2001). The outer surface of PsaA/B on the stromal side forms the binding interface for the PsaC,

The C-terminus of the PsaC interacts with the PsaD subunit, which plays an important role in assembly and stability of the PSI complex (Naver, H. et al. 1996). In antisense plants reduction of the amount of PsaD leads to a decrease in all other subunits of PSI including the light harvesting complex (LHCI), suggesting that PSI cannot be properly assembled in the absence of PsaD (Haldrup, A. et al. 2003). In plants with reduced amounts of PsaE the growth of the plants is limited to 50% of normal values and this is explained by the importance of PsaE for the stability of PSI complex (Varotto, C. et al. 2000). There are three membrane proteins, PsaF, PsaG and PsaK, which have been shown to be in direct contact with LHCI (Kjaerulff, S. et al. 1993, Jansson, S. et al. 1996, Jensen, P. E. et al. 2000, Haldrup, A. et al. 2000).

The PsbH subunit is proposed to have an important role under certain physiological conditions (Lunde, C. et al. 2000). Under limiting light conditions the additional LHCII antenna is associated with PSI complex and PsaH forms the binding site for the interaction. In PsaH-less Arabidopsis plants LHCII cannot transfer energy to PSI and hence state transitions are impaired (Lunde, C. et al. 2000).

**Structure of Photosystem I complex**

Over the least 20 years considerable efforts have been made in obtaining structural data on the PSI complex. Three structural models of PSI were subsequently published by the same groups of investigators (Krauss, N. et al. 1993, Krauss, N. et al. 1996, Schubert, W. D. et al. 1997, Klukas, O. et al. 1999a, Klukas, O. et al. 1999b). Initially the structure of a trimeric complex from cyanobacteria was resolved by X-ray at 6 Å resolution which could later be improved to 4 Å resolution. At present, the structure of the cyanobacterial PSI core complex is known at 2.5 Å resolution (Jordan, P. et al. 2001), which for the first time provides detailed insight into the molecular architecture of the core complex and for the first time reveals the organization of 12 different protein subunits within a monomer and 127 cofactors, including 4 lipids and a putative Ca$^{2+}$ ion.
Although most of the subunits in the eukaryotic core complexes have homologues in the cyanobacterial PSI core complex, there are exceptions, such as the PsaG, PsaH, PsaN and PsaO subunits, which are only found in eukaryotes. In contrast, PsaM and PsaX are only present in cyanobacteria (Table 2). Another difference between the cyanobacterial PSI complex and PSI core complex from eukaryotes is the supermolecular organization. Cyanobacterial PSI exists as trimers and monomers (Boekema, E. J. et al. 1987, Shubin, V. V. et al. 1993, Tsiotis, G. et al. 1995, Kruip, J. et al. 1999), whereas there is no evidence that plant PSI assembles into larger structures than monomers. It is not fully understood why the cyanobacterial PSI complex occurs in two different aggregation forms, but it was shown that during the isolation the ratio between trimers and monomers can be regulated through the ionic strength of the medium (Karapetyan, N. V. et al. 1997). It has also been shown that the PsaL subunit is a prime candidate for the trimerization site (Chitnis, V. P. and Chitnis, P. R. 1993). Indeed, based on the 3D structure (Jordan, P. et al. 2001), PsaL is located close to the 3-fold symmetry axis in a trimerization domain, forming the contact between the monomers. In the case of higher plants and algae only monomeric PSI has been found, although the PsaL is present. The trimerization of PSI is conceivably prevented by the presence of the PsaH subunit which is a unique feature of green plants PSI. The location of PsaH subunit has been determined from the cross-linking experiments which revealed that PsaH is indeed located in close proximity to PsaL (Jansson, S. et al. 1996) and thus may prevent trimerization by steric hindrance.

The main discrepancy between the PSI complexes from eukaryotes and cyanobacteria is in the light harvesting antenna which is completely different in these two groups of organisms. In cyanobacteria, the peripheral light harvesting complex is a giant water-soluble complex, the phycobilisome, whereas in plants and algae the antenna is composed of membrane-bound Chl $a$ and $b$ containing proteins. In higher plants, this LHCI antenna contains four chlorophyll $a/b$ binding proteins, Lhca1, Lhca2, Lhca3 and Lhca4, with molecular masses of 21-24 kDa, but in some species the number of subunits is higher. For example, in Arabidopsis, two additional genes, Lhca5 and Lhca6, were identified (Jansson, S. 1999). Green algae like *Chlamydomonas reinhardtii* (*C. reinhardtii*) do not possess the same set of six Lhca proteins as found in higher plants (Durnford, D. G. et al. 1999). Instead, there are approximately ten Lhca proteins of which the sequence is not homologous to the higher plant Lhca 1-4 types (Hippler, M. et al. 2001, Stauber, E. J. et al. 2003). In general, this means that a divergence exist within the LHCI superfamily.

In order to obtain information about the structural organisation of the whole PSI-LHCI complex, electron microscopy with single particle analysis has therefore
been applied. Initially, a model was suggested in which the core complex is completely surrounded by a monolayer of eight LHCI monomers (Boekema, E. J. et al. 1990). However, more recently better resolved EM data became available, which indicated that the LHCI subunits are only present on one side (Boekema, E. J. et al. 2001b) (Figure 5). The actual number of copies could not be determined but suggested to be about 4-5 if a packing density similar to the antenna of PSII is assumed (Boekema, E. J. et al. 2001b). However, additional chemical cross-linking and mutagenesis studies suggested that the core complex could be surrounded by eight copies of the LHCI proteins, located on the one side of the PSI core complex. This model of the PSI-LHCI complex suggested that the LHCI proteins are connected to the core complex via PsaF, PsaJ, PsaG and PsaK subunits, which are known to be involved in interactions with the light harvesting proteins (Kjaerulff, S. et al. 1993, Jansson, S. et al. 1996, Jensen, P. E. et al. 2000, Haldrup, A. et al. 2000).

Figure 5. A schematic representation of plant PSI-LHCI complex. A model according to Scheller et al. (Scheller, H. V. et al. 2001)

A more refined model of the cyanobacterial PSI (Fromme, P. et al. 2001) suggested that both PsaG and PsaK subunits should be located away from a pseudo two-fold axis, pointing towards an external location of the two subunits. In Figure 5, the PsaK and PsaG subunits are located on opposite sides of the core complex connecting the Lhca2 and Lhca3 homodimers to the core complex (Kjaerulff, S. et al. 1993, Jansson, S. et al. 1996). Very recently this model was verified at much higher resolution with data obtained from an X-ray crystallographic study of PSI from pea. The new 3D structure of the plant PSI–LHCI complex has been solved at 4.4 Å resolution (Ben-Shem, A. et al. 2003) and for the first time reveals the detailed organisation of the LHCI proteins around the PSI core complex (Figure 6).
In short, the structure shows that there are only four copies of LHCl proteins located on one side of the core complex. They are postulated to interact as two dimers: one as Lhca1-Lhca4 and the other as Lhca2-Lhca3. It should be noted that this conclusion has been made mostly according to the published biochemical and mutagenesis studies. The role of PsaG and PsaK subunits in the binding of LHCl proteins has been confirmed, with the Lhca1 having a strong attachment to one of the helices of PsaG and most likely the Lhca3 interacts with PsaK. The other two subunits, Lhca4 and Lhca2, have been shown to have a weak association with the PsaF and PsaJ subunits, respectively. Finally, the location of the PsbH close to the PsaL subunit was unambiguously assigned from additional cross-linking data (Jansson, S. et al. 1996).

**Figure 6.** A near-atomic model of plant PSI at 4.4 Å represented as C\textsubscript{\textalpha} backbone. PSI is viewed from the stromal side of the thylakoid membrane. The positions of subunits F, G, H and K of the reaction centre, together with the four light-harvesting proteins (Lhca 1–4), have been indicated. The relative wide space between Lhca2 and 3 and the core complex is mostly filled with additional chl molecules (not shown), which explains the discrepancy between the estimated number of about 4-5 LHC subunits from EM and the actual number of 4 (Ben-Shem, A. et al. 2003).


**Perspectives**

Despite the fact that the 3D structure of plant PSI-LHCI complex has been solved to near-atomic resolution some intriguing questions remain to be answered about the organisation of PSI-LHCI complex in higher plants:

1. The function of the PsaH subunit has been described as a binding site for the LHCII proteins in the state 2, yet there is no structural data available about the actual binding of LHCII proteins.

Although *C. reinhardtii* has been used as a model system for elucidating the assembly and regulatory processes of the photosynthetic machinery its light harvesting system is still not very well understood. Recently, proteomics data predicted the presence of at least nine different *lhca*-related gene products (Stauber, E. J. et al. 2003). Therefore the next logic steps would be:

2. To identify and localise these proteins within the PSI complex.

3. To identify the stoichiometry of individual Lhca proteins.

4. To monitor how the relative abundance of individual Lhca (Lhcb) proteins changes under different physiological conditions.

**1.4. Protein structure determination**

In the current genomics era there is still a strong need for understanding the processes underlying the passage from the sequence to the structure and from the structure to its function and dynamics. There are many disciplines in the structural biology field that can contribute to answering these fundamental questions. Among these, X-ray crystallography, nuclear magnetic resonance (NMR) and electron microscopy (EM) are the most powerful approaches for macromolecular structure determination. So far, X-ray crystallography has been extremely successful as an approach to solve the structure from small molecules to very large assemblies such as viruses and ribosomes with molecular masses of Megadaltons. Solution NMR is the other well-established method for determining atomic structures of relatively small proteins and allows the dynamics of a protein to be measured. Recently, progress towards assessing the structure of large complexes by NMR has been reported (Fiaux, J. et al. 2002). However, many biological proteins do not arrange
themselves in perfectly ordered 3D crystals necessary to perform X-ray diffraction; many other proteins are simply too large to be approached by NMR spectroscopy. Electron microscopy has become a progressively more important technique for structural determination of large macromolecules. It does not require a large amount of material and it is also a powerful tool for studying conformational changes in large assemblies, with the so-called “spray method” developed by Berriman & Unwin (Berriman, J. and Unwin, N. 1994). EM structure analysis can be performed on samples from ordered assemblies such as 2D crystals to asymmetric randomly oriented individual particles. Electron crystallography of two-dimensional crystals yields the highest resolution data, since crystals present the largest numbers of proteins in well defined positions and orientations. Moreover it has the potential to provide structural information at near-atomic level. The most successful examples of atomic structures determined by electron crystallography are the light-harvesting complex II (Kuhlbrandt, W. et al. 1994), aquaporin (Murata, K. et al. 2000, Ren, G. et al. 2001), bacteriorhodopsin (Henderson, R. et al. 1990) and tubulin (Nogales, E. et al. 1998). These studies have yielded high-resolution maps (3-4 Å) of sufficient quality to define an atomic model of the proteins. The reconstitution of membrane proteins into lipid bilayer also offers an opportunity to visualize the structure of the proteins in a rather native environment as well as in biologically active conformations. However, the homogeneity of the purified proteins and difficulties in obtaining crystals are the main limitations of this approach. Alternatively, large proteins and complexes, which are difficult to purify in homogeneous form or to reconstitute into periodic 2D arrays, can be better reconstructed using single particle methods. So far one of the highest resolutions (7.4 Å) has been obtained with the hepatitis B core particle (Bottcher, B. et al. 1997). Although lagging behind in resolution with respect to electron crystallography, this technique can reveal structural features and conformational changes that underlie the function of the protein. Attainment of near-atomic resolution is feasible in principle, provided a sufficient number of particles to increase the signal-to-noise ratio. (These averaging techniques will be discussed in more detail below).

A common scheme for structure determination by single particle EM is depicted in the Figure 7. Briefly, a specimen must be prepared in a relatively homogeneous form as a suspension of single particles (2D crystals), rapidly frozen (vitrified) as a thin film or negatively stained, transferred into the electron microscope and photographed. The resulting images if recorded on film must be then digitized and subsequently processed using the single particle image analysis.
**Figure 7.** The main steps involved in electron microscopy from sample preparation to map interpretation. Three main steps: Sample preparation, Electron microscopy and Single particle image analysis are described in the text.

**EM specimen preparation**

EM sample preparation techniques aim to overcome fundamental problems in the radiation sensitivity of the biological material and protection of the specimen from the structural damage caused by dehydration. For individual particles two main specimen preparation methods have been developed to achieve this.

Negative staining is the conventional method, that was introduced by Brenner and Horne at the end of the fifties (Brenner, A. and Horne, R.W. 1959). The idea of negative staining is to enhance the visibility of a weakly scattering biological object by surrounding it with strongly scattering heavy-metal salts. To achieve this, the sample is mixed with a solution of a heavy metal salt. A support film with a thin layer of an amorphous heavy-metal salt with embedded proteins is obtained by air-drying. Brenner and Horne presented data showing that the biological material was surrounded by a heavy-metal ("negative stain") layer, which considerably increased the scattering contrast lacking in the absence of stain. This method is not only straightforward and very fast, but also highly reproducible. Moreover, samples prepared in this manner have demonstrated good sustaining and protection from radiation damage.
Amongst the most effective heavy metal salts that have been found to be suitable for this method are uranyl acetate, uranyl formate, sodium/potassium phosphotungstates and ammonium molybdate. In addition, a glow-discharge device is often employed to make the surface of the support film more hydrophilic, to facilitate the absorption of the protein to the film and a better (more equal) stain distribution. Using this method, the signal-to-noise ratio is generally extremely good. However, the image signal can be disturbed by a grainy noise originating from the carbon film beneath the sample and/or from the dried stain. Negative staining is effective at revealing the outer surface and therefore the quaternary structure of a protein molecule. Under standard conditions it is possible to achieve a routine resolution anywhere between 10-20 Å. This resolution will not allow to reveal high-resolution details, although catalase crystals show diffraction to 4 Å resolution after negative stain embedding (Massover, W. H. et al. 2001). This illustrates that the negative staining method does not itself destroy the high-resolution structure information, but only “covers it”.

Fast preparation of a thin layer of hydrated specimen at liquid nitrogen temperature is the second and advanced preparation technique. In this method biomolecules are embedded in a thin layer of amorphous ice and imaged in the EM in the frozen state, the so-called Cryo-EM technique. Preparation methods to obtain ice-embedded protein specimens were developed in the 80-ties by Dubochet and colleagues (Adrian M. et al. 1984). A solution of molecules is rapidly frozen by plunging the EM grid into liquid ethane and then transferring the grid to the electron microscope in special nitrogen-cooled holders. The Cryo-EM technique overcomes two problems: the native hydrated macromolecular structure is preserved in the microscope vacuum by the amorphous ice layer, and the low temperature of the specimen strongly reduces radiation damage (Stark, H. et al. 1996). Consequently, it enables the use of a higher electron dose. In the frozen hydrated specimen, the native structure is maintained although with very low contrast. An alternative method to prevent denaturation and dehydration of protein and supermolecular assemblies is air-drying in the presence of sustaining media such as glucose (Unwin, P. N. and Henderson, R. 1975) or trehalose (Hebert, H. et al. 1997, Walz, T. et al. 1997). Embedding in sugar in this way generates a non-volatile water-like environment for protein molecules, thus sustaining the specimen, and takes advantage of the decrease of radiation damage at low temperature. Unfortunately the contrast using this method is even lower than in ice, since sugar almost matches the density of the protein. This is usually not a problem with membrane crystals, where the contrast depends on the differential electron scattering density of protein, but makes sugar-embedding impractical for single particles.
Another revolutionary method was invented in 1998 by Dubochet and colleagues, who combined the two techniques of vitreous-ice embedding and negative staining (Adrian, M. et al. 1998). In this case the specimen preparation is very similar to the thin-film vitrification technique used for preparing Cryo-EM specimens, except for the addition of a heavy metal salt at a concentration of several percent. Correspondingly, the vitrified sample also appears to be negatively stained. The advantages of this method are two-fold. Firstly, the high contrast of a Cryo-negatively stained sample can be imaged close to focus and thus better preserves the high-resolution information. Secondly, the signal-to-noise ratio is higher, so improving the visualisation of the sample with a high amount of detail.

In summary, all described techniques have in common that a small amount of purified sample is deposited on a support film, which is usually either a carbon or a plastic film, directly mounted on a copper or molybdenum electron microscopy grid.

1.5. Electron Microscopy

The first electron microscope was developed at the beginning of the 1930’s, and had a modest resolution of 200 Å at best (Knoll, M. and Ruska, E. 1932). Towards the end of the 1940s, after the Second World War, the equipment was greatly improved resulting in the production of electron microscopes capable of attaining a resolution of better than 10 Å. These days, state of the art electron microscopy is capable of reaching a resolution of about 1 Å. This capability of performing imaging at very high resolution carries implications for the whole design of the instrument. In particular, the performance of electron microscopes has benefited from enhancements to the optical system and the electron source.

Image formation

A schematic representation of the image formation in the electron microscope is depicted in Figure 8. The electron gun (filament) provides the illumination source. A coherent beam of electrons is then projected onto the specimen via the condenser lens. In normal imaging mode, a single or double condenser lens ensures that the incident electron beam is parallel (for simplicity this is not depicted on the picture). The incident electron beam passes through the specimen with the electrons being scattered or non-scattered by the atoms of the specimen. Scattered electrons further interact with the magnetic field of the objective lens as depicted in the Figure 8 as a dashed line. The objective lens magnifies the image approximately 30 times, but in practice this is not enough and further magnification
is achieved by three or four projection lenses that produce the final image in the **image plane**.

**Figure 8.** Schematic diagram showing the principle of image formation in an EM. The dash line indicates the scattered electrons, which are removed by the objective aperture. An arrow shows the part of the image which is magnified by the sets of the objective and projection lenses and reprojected on the image plane.

If the electrons are considered to interact with the specimen atoms as elementary particles the contrast in an electron micrograph arises from elastic and inelastic scattering. When the beam of electrons is deflected by the positively charged nucleus without loss of energy, the deviation angle is mostly large and scattered electrons can be removed by the objective aperture. This is called *scattering contrast*. It is the major contrast mechanism for thick specimens or for heavy-metal stained specimens. When the electrons undergo inelastic scattering with loss of energy, the interaction with the lenses is different due to chromatic aberration effects, which will not be discussed here in detail. The result is a
blurring of the image and the consequence of the energy loss is an increase in background noise and radiation damage as a side-effect.

If the electron beam is considered to be a wave, the contrast arises from a phase shift between the scattered and non-scattered waves in combination with an additional phase shift to the scattered electrons created by defocusing of the objective lens and spherical aberration. This is called phase contrast. Thin specimens or unstained specimens are weakly scattering and such specimens are considered as phase objects. In practice the final image always results from a combination of phase contrast and scattering contrast at a ratio depending on the type of specimen.

During imaging the high-resolution structural information is easily lost due to radiation damage. Most biological specimens are able to withstand an electron dose that does not exceed 1000-5000 e/\text{nm}^2. Nevertheless, the highest resolution features of the specimen are already affected at electron exposures as low as 100 e/\text{nm}^2 or less. Specimen damage is primarily caused by ionizations resulting from the inelastic interaction of electrons with the orbital electrons of organic material. This, in turn, leads to rearrangements of chemical bonds, formation of free-radicals and diffusion of the fragments. Whereas elastic interactions produce image contrast but no damage, inelastic interactions can produce permanent changes. By reducing the electron exposure, the situation may be such that the specimen details are well preserved, but cannot be observed because of lower signal-to-noise ratio (SNR) of the image. This generally leads to hazy images. At high resolution there does not seem to be any way to escape this dilemma except through the use of image averaging techniques. Over the last 40 years, special image processing software has been developed to overcome this inherent noise problem.

1.6. Image analysis as a method to increase signal-to-noise ratio

In general an electron microscopy image of biological material suffers from a very low signal-to-noise ratio. To overcome this problem and to increase the SNR, image enhancement programs have been developed. Thus, averaging of a large number of copies of molecules leads to a tremendous improvement in the SNR of the data. The idea of increasing the SNR in electron images was pioneered by Nobel prize winner A. Klug and colleagues in the sixties. Application to unstained specimens was first discussed by Glaeser in 1971 (Glaeser, R. M. 1971) and applied to bacteriorhodopsin crystals in a classical paper (the most cited one in protein EM) by Unwin & Henderson (Unwin, P. N. and Henderson, R. 1975).
Single particle analysis started in the seventies as well. Because this technique is widely applied in this thesis, the basic steps of this technique are described below.

**Fundamentals of single particle processing**

Once images have been obtained, scanned and the quality of each micrograph determined, the real image processing can begin. The first step in this procedure is the particle picking. *Particle selection* can be performed manually or by using automatic particle picking software. Automation of particle selection may facilitate this process but the result needs to be often verified visually and thus it may not necessarily always save time compared to manual selection. In the second step, a set of particle projections is *aligned* with respect to each other or with respect to a reference image. There are several approaches used for repositioning each individual particle projection within its frame such that after alignment all are positioned similarly. In the straightforward concept, alignment is achieved by using cross-correlation functions (CCFs) and auto-correlation functions (ACFs) and by comparing the particles one by one against a reference image (van Heel, M. et al. 1992). A CCF will show a peak (after a certain translational shift) at the place where a motif present in both images matches maximally, therefore the best alignment is achieved when the CCF is maximal. The search for ACF peaks after rotational shifts is performed in a similar way. By imposing the translational and rotational shifts on each of the images, alignment of the complete data set can be achieved. However, alignment of a set of images with respect to a reference image tends to bias the data set towards the appearance of the specific reference image. The reason of this is that the alignment is complicated by the low contrast and random noise present in electron microscopy images, and in particular in the reference. This problem can only partially be solved by using averaged projections as next improved references. To overcome this reference bias a multi-reference alignment procedure and a reference-free alignment scheme have been proposed. The first is especially useful for heterogeneous data sets or for data sets of randomly oriented single particles (van Heel, M. and Stoffler-Meilicke, M. 1985). Aligned images are usually submitted to *multivariate statistical analysis* (MSA), which is applied to discriminate between different types of molecular projections prior the averaging. In the MSA approach aligned images are submitted to multivariate statistical analysis usually in the form of correspondence analysis (CA) where the program determines the direction of the interimage variance and calculates the image coordinates in a system spanned by the newly determined axes (van Heel, M. and Frank, J. 1981, Frank, J. and van Heel, M. 1982). Each image of x×x pixels can be considered as a point in an (x×x)-dimensional space, where each axis represents the density value of a single pixel. Aligned images of similar
molecular views are represented as a cloud of points in a multidimensional coordinate system. The size and “local density” of the cloud represents the variations among the images. In order to interpret this cloud the MSA compresses the large quantities of image data present in images into eigen vectors which correspond to the axes of a new system of coordinates and eigenvalues are used to evaluate the distance of the vectors. The main vector runs parallel to the prominent directions into which the cloud extends and this vector is the major component of variations in a new coordinate system. The second vector represents the largest remaining interimage variance, and so on. The unit vectors in a new system correspond to a decrease amount of image variance. Usually, the use of the first 4 to 8 vectors is sufficient to describe the most important differences between projections in a data set. The final step in single particle image analysis is classification, during which similar projections are grouped into classes.

The final result of the single particle image analysis is averaged classes or class-sums, which represent all the views of the molecules present in a data set. The averaging procedure boosts the common signal by a factor of $n^{1/2}$, where $n$ is the number of images; therefore the class-sums have a substantial higher SNR than the initial images thus making the interpretation much more straightforward than with initial images.
Outline of this Thesis.

This Thesis focuses on an investigation of the structure of the photosynthetic proteins Photosystem II (PSII) and Photosystem I (PSI) by electron microscopy in combination with single particle image analysis. In chapter 2, the results of an analysis of Arabidopsis plants PSII-LHCII supercomplexes, is presented. This section also describes a comparison of spinach PSII-LHCII with the Arabidopsis PSII-LHCII supercomplexes. It also shows new insight into the organisation of inside-out membranes, in which PSII is packed in specific ways. Analysis of PSII obtained from three different types of mutants of Arabidopsis is described in chapters 3, 4 and 5. The effect of a deletion of the PSII antenna proteins CP26+CP29, Lhcb2/Lhcb1, PsbS has been analysed and described, respectively. Chapter 3 describes the results of a comparison of PSII membranes isolated from a CP26 mutant with wild type PSII membranes. The main result of this work is that the location of the minor antenna protein CP26 in PSII complex could be revealed. Chapter 4 describes the effect of the lack of Lhcb1/Lhcb2 proteins, which are the major components of the major LHCII trimer. It reports how the absence of the Lhcb1/Lhcb2 proteins has been compensated by the CP26 protein. In chapter 5 the PSII-LHCII megacomplexes and native membranes isolated from a PsbS mutant are analysed. Chapter 6 describes the analysis of PSI-LHCI complexes purified from the green alga Chlamydomonas reinhardtii. These experiments demonstrate that the PSI-LHCI complex in green algae have a larger antenna than the PSI complex of spinach.