In vitro investigation of the photoprotection mechanism of Light Harvesting Complex II

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CHAPTER 1

Introduction

PHOTOSYNTHESIS

"Photosynthesis is a process in which light energy is captured and stored by an organism, the stored energy is used to drive energy-requiring cellular processes [1]"

This definition of photosynthesis includes all kind of systems that use solar energy to survive, from algae to plants to bacteria.
The oxygenic photosynthetic process can be described by this minimal equation:

$$6CO_2 + 6H_2O \rightarrow C_6H_{12}O_6 + 6O_2$$

Julius Robert Mayer, in 1864, discovered that carbohydrates, in the form of starch, were accumulated in the portion of the leaf directly exposed to the light. The understanding of this observation was that, under illumination, the absorbed carbon dioxide in the presence of water was converted into organic carbon, in the form of carbohydrates, with O_2 as a side product.
As displayed in Figure 1.1, photosynthesis takes place in the chloroplast, within the thylakoid membrane compartments and in the soluble phase named stroma. Two components of the thylakoid membrane have been identified: a cylindrical stacked system called granum and the un-stacked interconnecting region named stroma lamellae [2]. The thylakoid membrane is a lipid bilayer, mainly composed of galactolipids and phospholipids, which hosts protein complexes and cofactors [1].
The photosynthetic process consists of two phases which are the light and dark reaction respectively described below:

- Light reaction: involves harvesting of the light energy, which is converted to chemical energy and temporarily stored in the form of ATP and NADPH.
- Dark reaction: involves the conversion of CO_2 using the energy of ATP and NADPH into other compounds used for long-term energy storage, via reactions involved in the Calvin-Benson cycle.

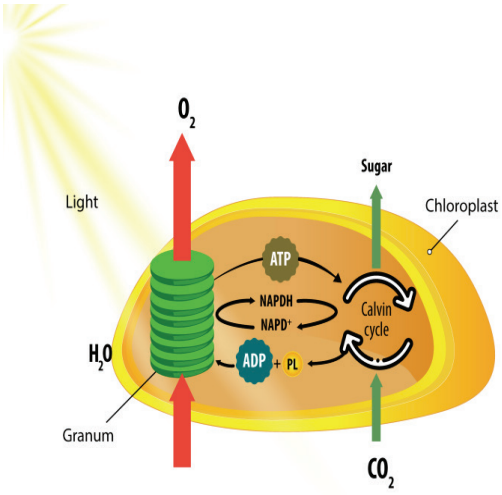

Figure 1.1 Representation of the two main stages of photosynthesis. On the left side, the light reactions are presented and, on the right side the dark reactions carried out in the chloroplast are shown.

Solar energy is primarily absorbed by two groups of pigments, chlorophylls (Chls) and carotenoids (Cars). These pigments are bound to Light-Harvesting Complexes (LHCs) and to both reaction centre complexes, Photosystem I (PSI) and Photosystem II (PSII). The representation in Figure 1.2 shows the linear electron flow upon light absorption. Excitation energy is transferred to the PSII reaction centre Chl special pair (P680), creating a charge-separated state. An electron is donated from P680 to pheophytin and consequently to the plastoquinone (PQ) bound to the complex, Q_A. $P680^+$ is reduced back to P680 via the active Tyr (Y_z) of PSII. Y_z is reduced back using electrons extracted from water at the oxygen-evolving complex (OEC) while protons are released into the lumen. The electron, from the Q_A site, is transferred to a PQ in the Q_B site. After two turnovers, the reduced Q_B-PQ accepts two protons from the stroma and is released from the PSII complex in the form of PQH_2 [3,4]. This molecule, after diffusion through the membrane, reaches the Cyt b_6f in which the Q-cycle takes place. The two electrons are donated to the blue copper plastocyanin (PC) which is a redox protein able to diffuse to PSI that similarly to the PSII has a reaction centre Chl special pair P700, and the four protons are released into the lumen. Excitation of PSI leads to charge separation from the P700 special pair producing an electron that is used to reduce ferredoxin (Fd). $P700^+$ is then reduced back accepting electrons, from the PC while the Fd electrons are donated to $NADP^+$-oxide reductase to produce NADPH. The net reaction can be written as:

$$2H_2O + 2NADP^+ + 2H^+ \rightarrow O_2 + 2NADPH + 4H^+$$

The electron flow involves the generation of reducing power used to produce ATP. The protons, which accumulated at the lumen, generate a ΔpH used by the ATP synthase complex to drive the conversion of ADP with inorganic phosphorus into ATP. The ATP and NADPH produced are used to fixate CO_2 into carbohydrates.

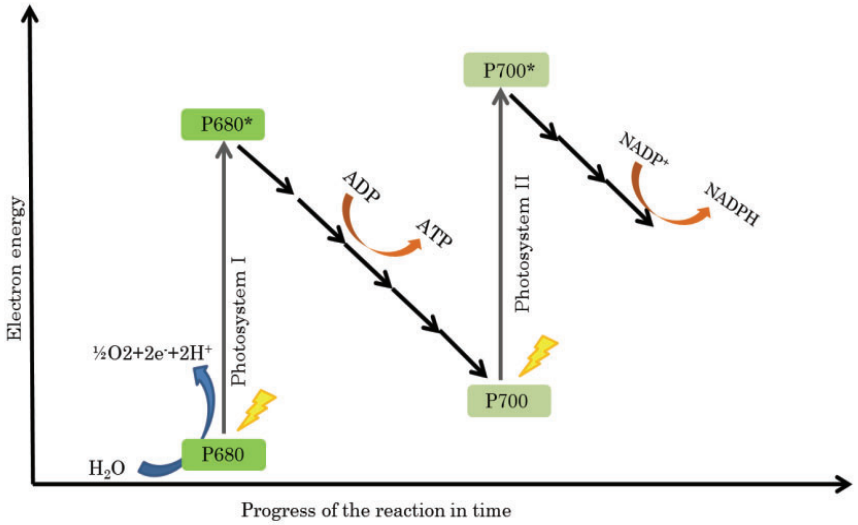

Figure 1.2 Representation of the linear electron flow in the light reactions process according to Hohmann-Marriot et al. [1].

PHOTOPROTECTION

Photosynthetic systems are exposed to intermittent sunlight. Sudden changes in the sunlight exposure can saturate quickly the electron transport chain preventing further use of the absorbed energy. The consequence of the unused energy is the formation of triplet $^3Chl^*$, after inter-system crossing (ISC) of $^1Chl^*$, which can react with oxygen to produce singlet oxygen $^1O_2$ causing oxidative damage to proteins, lipids, and pigments [5,6]. To reduce the negative effect of light stress, plants and algae have developed a variety of photoprotective mechanisms that can be activated in the long and in the short term. The first group is characterized by an acclimation of the system to stressful conditions. The second group is, instead, characterized by a short-term response to sudden changes in the light exposure known as non-photochemical quenching (NPQ), which are the mechanisms of interest in this dissertation.

With the increase of irradiation, the electron flow increases and with it, the ΔpH across the membrane. The system responds to ΔpH with various mechanisms aimed to avoid overload of the electron flow. The ΔpH activates the regulatory protein PsbS and the xanthophyll-cycle in which de-epoxidase enzymes convert violaxanthin to zeaxanthin [7,8].

Figure 1.3 shows the differences between the utilized energy and absorbed. In the case of high light, photosynthetic systems keep absorbing energy photons but only a fraction will be actually involved in the photosynthetic cycle while the rest is dissipated as heat to prevent overexcitation. At the molecular level, the absorbed energy, when in excess compared to what is the capacity of the carbon fixation, leads to lumen acidification, which initiates processes that down-regulate photosynthesis [9].

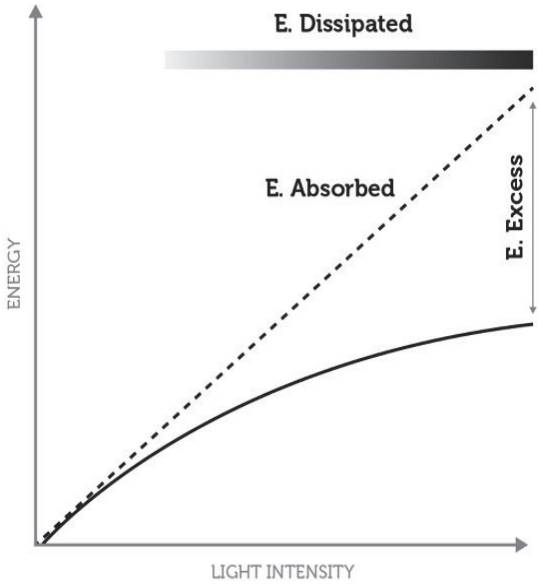

Figure 1.3 The graphic illustration, according to Ruban et al., shows how in high light condition is necessary the photoprotection of the photosystem II. The 'excess energy' is defined as the difference between the light absorbed and the utilized, which can cause photo-oxidative damage to the photosynthetic system. This excess will be dissipated via NPQ processes as heat [10].

Examples of such down-regulating processes are excitation quenching and the xanthophyll cycle [9].
Several mechanisms of photoprotection have been identified involving different components, among which the major Light-Harvesting antenna Complex (LHCII) plays a central role. NPQ can be differentiated into three processes as described below:

- qI slow relaxing process related to photoinhibition, which is defined as a light-induced decrease of the quantum yield of photosynthetic carbon fixation [11].
- qZ is related to the presence of zeaxanthin (Zea). When NPQ is activated, a significant amount of epoxide carotenoids, predominantly violaxanthin, (Vio) is converted into non-epoxide zeaxanthin (Zea) via the de-epoxidase enzyme, in a process known as the xanthophyll cycle. This VDE cycle is reversible in the dark [12-15]. The full understanding of the role of this pigment in the photoprotection is still under investigation.
- qE is the fastest component of NPQ that is activated and relaxes very quickly [16]. As for qZ, this mechanism is activated by the acidification of the thylakoid lumen. The qE process is called energy-dependent quenching and is a nonphotochemical quenching, through which the excess absorbed light energy is safely dissipated as heat [16,17].

This dissertation will focus on the molecular mechanisms involved in the fast component of NPQ, qE. Researchers have proposed different mechanisms involving either Chl–Cars [18-22], or Chl–Chl interaction [23,24] to explain the photophysical cause of excitation energy dissipation. Molecular models for non-photochemical quenching involving Chls-lutein interactions, and consequently quenching of lutein excited states, have been supported recently by *in silico* models predicting that small changes in orientation of the lutein might be sufficient to tune excitation quenching [25].

LIGHT-HARVESTING ANTENNAS

The primary function of the antenna is to absorb light and transfer the energy to the RC, which is a trap. In 1936 Gaffron and Wohl imagined that the energy was transferred from one pigment to the other, but pigments are not capable to carry out the photosynthesis by themselves [26]. Therefore, it was conjectured that a photosynthetic unit must consist of a collection of pigments. This was later confirmed by Franck and Teller in 1938 [27].
A variety of antennas have been identified in organisms able to perform photosynthesis. Antenna complexes can be divided into two main classes: integral membrane antenna complexes and extrinsic antenna complexes. The first class consists of proteins with buried pigments and are embedded in the lipid bilayer of photosynthetic membranes. The second class, instead, consists of extrinsic antenna complexes that are associated with transmembrane elements. The energy absorbed by the extrinsic complexes is first transferred to the integral membrane from which it is redirected to the reaction centre [1].

The existence of an antenna is essential for sufficient light collection and photoprotection in photosynthesis. Not only the antennas are necessary to harvest photons and efficiently transfer the energy to the reaction center, but for oxygenic photosynthetic organisms that are exposed to full sunlight, the energy excess has to be dissipated to avoid photodamage. Examples of proteins that have both light-harvesting and photoprotective functions are the LHC pigment-protein complexes [1]. This class of proteins is still under investigation to understand how the molecular mechanism of photoprotection is activated and which kind of structural rearrangements are needed to promote this function.

LIGHT-HARVESTING COMPLEX II

Light-Harvesting Complex II (LHCII) associates with PSII to form a multisubunit pigment-protein complex located in the thylakoid membrane of algae and higher plants. LHCII is the first chlorophyll-binding protein to be identified and the most abundant membrane protein on Earth [28,29]. The protein belongs to the multi-gene family of LHC proteins. All LHC proteins are encoded by Lhc genes and form complexes containing three membrane-spanning helices, with the exception of Psbs that has four helices, with three invariant amino acids Glu, Arg, several well conserved Gly residues and a conserved sequence for the generic LHC motif in the stretch ELINGRLAMLGFLGFLVPELIT, which is the transmembrane core of the complex [30]. Two of the transmembrane helices are kept together by Arg-Glu salt bridges and contain the majority of the binding sites for chlorophylls and carotenoids [31]. The LHCII trimeric complexes of higher plants are assembled from polypeptides encoded from three genes, Lhcb1, Lhcb2, and Lhcb3, while Lhcb4, Lhcb5, and Lhcb6 encode for the monomeric core antenna complexes.

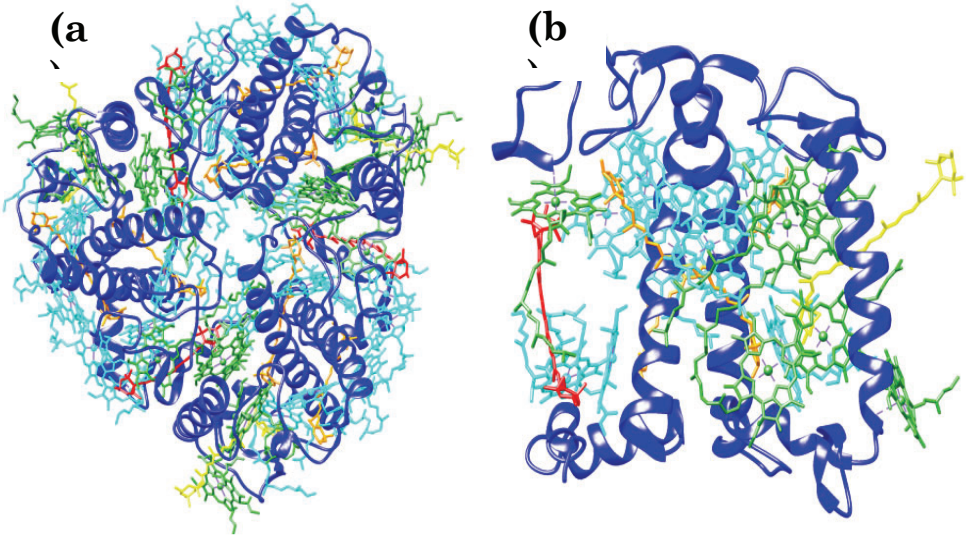

Figure 1.4 LHCII trimeric crystal structure (a) and monomeric (b) from spinach (PDB- 1RWT). In green Chl b, in cyan Chl a, in orange Lut1 and Lut2, in red Vio and in yellow Neo.

As shown in the paper of Caffarri *et al.*, Lhcb1 and Lhcb2 have similar but non-identical pigment binding and spectral properties, and the presence of Lhcb3 together with the other holoproteins lead to the formation of a heterotrimer [32].
The primary function of the LHCII protein is to bind pigments in an arrangement that ensures efficient absorption and excitation transfer of solar energy. In the case of excess of light, in order to prevent photodamage, the LHCII protein switches from a light-harvester function into a photoprotector function [33].

PHOTOSYNTHETIC PIGMENTS AND THEIR CHARACTERISTICS

In the following paragraph Chlorophylls and carotenoids, which are the active components involved in light harvesting, are described.

CHLOROPHYLLS

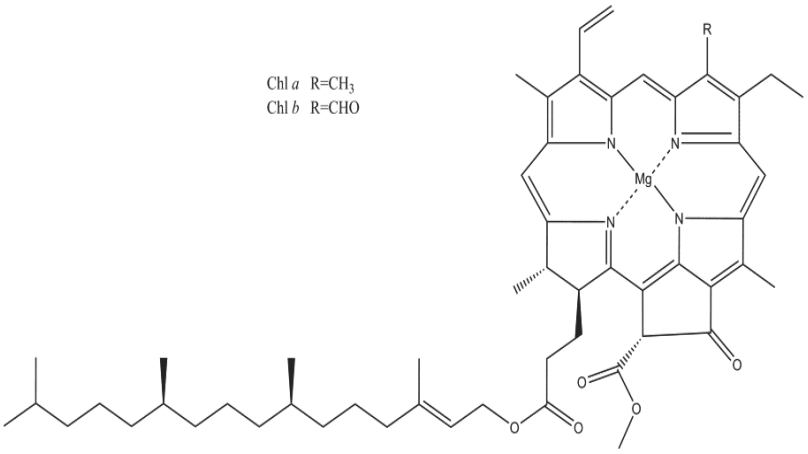

Figure 1.5 Chl chemical structure. The difference between Chl a and Chl b lies in the group R in C-7 which is respectively a methyl or a formyl group.

The first researchers investigating Chl pigments were Pelletier and Caventou in 1818 [1]. Several kinds of Chl pigments have been discovered in higher plants and algae, which are named in order of discovery from *a-f*. The Mg^{2+} coordinates the four nitrogen atoms, which are the part of a pyrrole ring, as represented in Figure 1.5 [34]. The hydrocarbon tail is attached to the fifth ring, isocyclic ring, which derived from protoporphyrin.
Chl *a* and Chl *b* are abundant in the eukaryotic photosynthetic organisms. As illustrated in Figure 1.5 the two pigments differ only for the group in the C-7 position. Both pigments absorb in the blue and red region of the visible spectrum. These properties are correlated to the conjugation system, given by the π-electrons, which extends over the planar chlorine macrocycle. The electronic transitions can be explained with the theory of the four orbitals model [35].

The presence of the formyl group gives more symmetry to the macrocycle, which means that the absorption spectrum is more similar to the porphyrin one, and is responsible for the spectral shift to shorter wavelengths compared to Chl *a*. The two types of Chls are not uniformly distributed and in fact, while Chl *a* is present in the reaction centre, Chl *b* not. In the absorption spectra of Chl *a*, as shown in Figure 1.6, the excited state is populated by blue light absorption, the Soret band in the region 400-480 nm, and relaxes, via heat loss, to the energy level accessed by red light absorption for the Q bands in the region 600-700 nm [36].

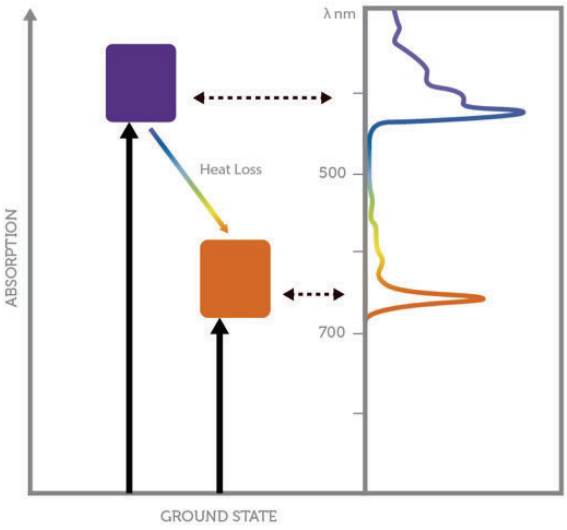

Figure 1.6 Simplified absorption energy level diagram for Chl a according to what proposed from Blankenship et al. [36].

CAROTENOIDS

Cars have in common the delocalization of the π-electrons. The Cars family is divided into two subgroups, unoxygenated and oxygenated. ß-Carotene and α-Carotene are part of the first group, in which no oxygen atoms are present in the structure [37]. In the other subgroup, at the end of each chain there are usually ring structures containing one or more oxygen atoms as part of a hydroxyl group or epoxide group. Figure 1.7 shows the chemical structures of the most common carotenoids which are bound to the LHCs.

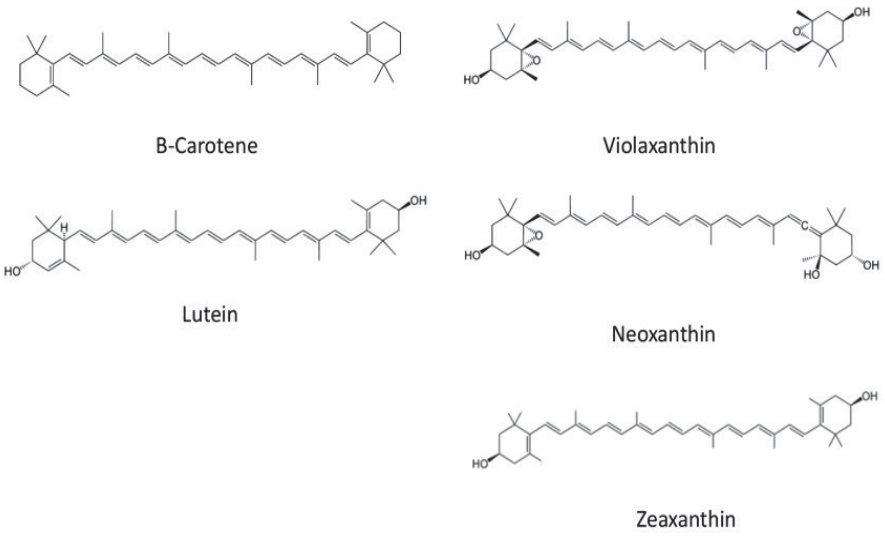

Figure 1.7 Chemical structures of carotenoids with different conjugation lengths. Neo has N=8. Vio has N=9, Lut has N=10 and Zea has N=11 double bonds

Carotenoids have the primary function of increasing the absorption of LHCs in the blue-green region (400-500 nm) with their conjugated polyene chain. The transition from S0 to S2 is promoted by light absorption, while the direct transition to S1 is forbidden due to the molecular symmetry of the S0 and S1 state. The S2 excited-state lifetime is in the order of femtoseconds and the S2 state decays by internal conversion to S1 from which the molecule relaxes to the ground state via non-radiative internal conversion.

The excitation energy levels of carotenoids are inversely dependent on their conjugation length (N). Longer conjugation lengths lower the energy of the S1 states [38]. Carotenoids increase the energy absorption cross section of the protein but also play an important role in the promotion of the protein folding, as will be further discussed in Chapter 5 [39]. Indisputably, another important role of these molecules resides in the participation to the photoprotection mechanism because they are strong antioxidants. Excess of energy promotes the formation of harmful long-lived $^3Chl^*$ which reacts with O_2 forming $^1O^*$ [5]. This mechanism is prevented owing to the proximity of Cars to Chls, which allows the transfer of the Chl triplet energy to the carotenoid and consequently the energy is dissipated via a non-radiative process [40].

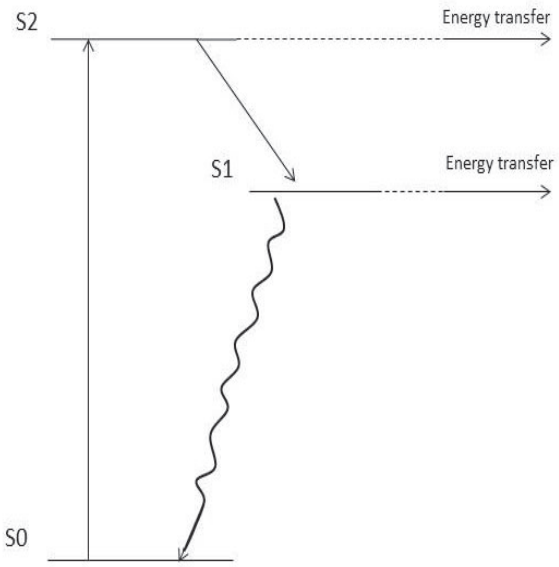

Figure 1.8 Schematic energy level diagram of carotenoids according to Blankenship [1].

PsbS

The photosystem II subunit S protein (PsbS) is an active participant in qE-type non-photochemical quenching, which protects plants from photodamage under excess light conditions. The activation of PsbS, triggered from low pH that protonates the two glutamates in the lumenal loop, initiates qE processes [41]. PsbS, as mentioned before, is part of the LHCs family. The protein structure is characterized by four transmembrane helices, as shown in Figure 1.9, which determine a compact structure that is unable to selectively bind any pigments. Fan *et al.* have proposed that *in vivo* the active form of PsbS during qE, at pH 5.0, most probably is a stable dimer [41]. No clear explanation of how PsbS is exactly involved in the NPQ has been commonly agreed upon so far and the molecular mechanism of PsbS is still unresolved [8,10,42].

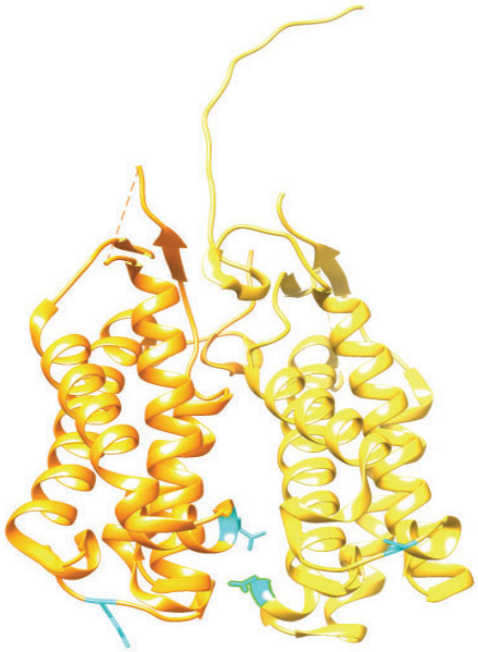

Figure 1.9 PsbS crystal structure (PDB-4RI2) from spinach. In cyan are underlined the 2 glutamate sites active in the protection mechanism. Glu 173 is in the loop while Glu 69 is in the helix.

One hypothesis is that PsbS is originally associated with the PSII core, and under high-light conditions migrates toward LHCs [42]. Considering that the protein does not bind any pigment molecules, the activated dimeric PsbS protein, located among the antennae, might play the role of an attenuator slowing down the energy flow amongst the LHCs. It has been conjectured that PsbS is able to promote thylakoid membrane reorganization, responsible for the promotions of the quenching states [41,43]. Evidence for direct interaction between PsbS and LHCs during qE has been validated with pull-down assays [44] and by *in vitro* reconstitution of a proteoliposome system containing PsbS, LHCII, and Zea [45]. This interaction is analysed in Chapter 3. It is also possible that in qE processes PsbS acts as pH-sensing trigger or as a catalyst [45].

MIMICKING A MEMBRANE ENVIRONMENT

For structural and functional studies, the membrane environment plays an essential role in membrane proteins [46-48]. It would be ideal to be able to study a protein of interest in its native environment but due to the membrane complexity, alternatives have to be found [49]. For membrane protein analysis, it is, therefore, a standard procedure to isolate the one of interest to avoid any interferences with other proteins or membrane constituents [50].

This dissertation focuses on the membrane protein LHCII, which in the native systems is located in the thylakoid membrane. LHCII is a hydrophobic protein and for this reason, the isolated protein can be studied either in detergent micelles or can be inserted in model membranes, such as liposomes and nanodiscs.

As mentioned earlier in this chapter, LHCII can switch between two functions. *In vitro*, it is possible to reproduce these two activities by inserting the protein in nanodiscs or in liposomes as is presented and discussed in Chapters 2 and 3. In nanodiscs, due to the relatively large size of the LHCII pigment-protein complex compared to the nanodiscs sizes, only one protein per disc is inserted [51], preventing LHCII-LHCII interactions, while in liposomes, LHCII aggregates can be formed.
Membrane proteins tend to be hydrophobic and can be solubilized only by agents, detergents, which are defined as amphipathic molecules forming micelles in water.
In the mixture of biological membrane and detergent, the latter brings the membrane proteins into solution as detergent-protein complexes, although some lipid molecules may remain attached to the protein [50].

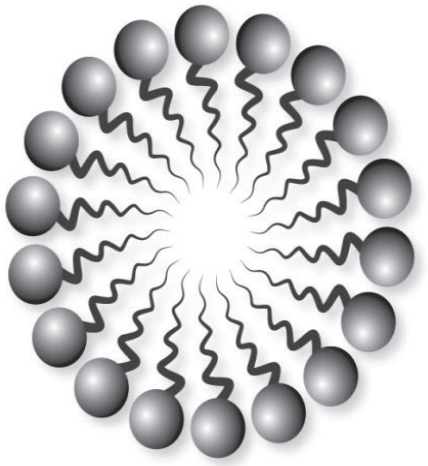

Figure 1.10 Cross section of a detergent micelle. The circle of hydrophilic heads point toward the water solution, while the hydrophobic tails are buried inside the micelle.

The structural biology of membrane proteins occupies a central place in current biophysics, biochemistry and cell biology investigations. Membrane proteins often display altered or loss of activity and function outside the phospholipid environment. For many systems, it is possible to reproduce the native behaviour in a membrane model [52].
In the 1970s the pioneering work of Racker and colleagues, started a new era for membrane protein characterization started [49,53]. Similarly to detergent molecules, lipids interact with the hydrophobic portions of the membrane protein, usually are the α helices, so that the protein is embedded in the phospholipid bilayer, as it is *in vivo* [50].

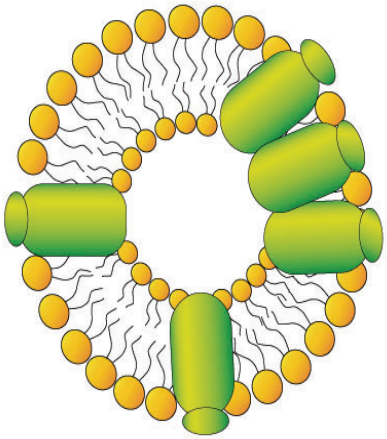

Figure 1.11 A liposome is a system formed by lipids, organized in a double layer similar to a natural biological membrane, creating a cavity filled by an aqueous solution [54]. This is a schematic cross-section representation of a proteoliposome. In yellow, the lipids are shown that are assembled via hydrophobic interactions between their acyl chains, which allows forming the characteristic bilayer. In green, a schematic representation of reconstituted protein is displayed. The "empty" cavity inside the proteoliposome is filled with a water-based solution which is often the buffer applied for protein solubilisation.

Reconstitution of purified membrane proteins into liposomes makes possible to have accurate control of several factors such as lipid composition and proteins interaction which affect the function of the target protein [49].

Alternative to liposomes is possible to use nanodiscs. As displayed in Figure 1.12, a nanodisc consists of a membrane patch that has been solubilized by two amphipathic proteins, called membrane scaffold proteins (MSPs). MSPs wrap around the hydrophobic core of the lipids, effectively creating a soluble portion of the membrane. When prepared properly, nanodiscs are uniform in size and allow the study of isolated membrane proteins *in vitro*, while maintaining a native-like membrane environment [55].

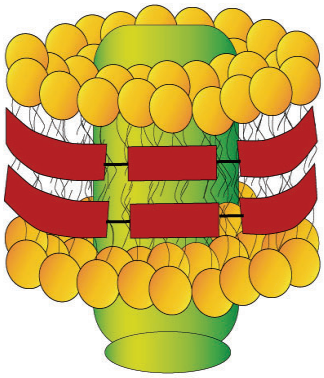

Figure 1.12 Representation of a nanodisc with inserted protein. In yellow the lipids forming the bilayer, in green, the inserted protein and in red the MSPs are shown which embrace the phospholipids bilayer. The size of the disc is determined by the length of the MSP and the lipids stoichiometry.

Nanodiscs have proven to be an invaluable tool for revealing the structure and function of isolated membrane proteins. There are several advantages of using nanodiscs over liposomes. In nanodiscs, both the C- and N-terminus of the protein are accessible and it is possible to have only one protein per disc, avoiding aggregation. Nanodiscs are increasingly being used as 'cassettes' that allowing investigation of membrane proteins without denaturation through a variety of analytical methods [52].

SCOPE OF THE THESIS

The scope of this thesis is to investigate *in vitro* the photoprotection mechanism of Light Harvesting Complex II. In Chapter 2 we aimed to study and differentiate the effect of the protein environment in the fluorescence quenching. Further investigation, whether the LHCII fluorescence quenching is the results of solo LHCII-PsbS interaction were performed in Chapter 3 by studying the two proteins co-inserted in a model membrane. In Chapter 4 we described the NMR quantity overproduction of recombinant Lhcb1, in *E. coli*, refolded in presence of pigments of which only lutein is ^{13}C labelled. With this selective labelling, the aim was to obtain a simplified protein NMR spectra from which only peaks relative to the labelled lutein are visible. Next up in Chapter 5 we focused in solid state NMR analysis of ^{13}C lutein-rLhcb1 in the unquenched and quenched state, respectively in detergent and in the aggregate state. The aim was to follow how lutein chemical shift is influenced upon conformational changes that undergo in the transition from unquenched to quenched state. Conclusion and future outlook of the thesis are discussed in Chapter 6.

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