Functional architecture of photosynthetic light harvesting complexes
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Photosynthesis is the process in which sunlight energy is used to convert CO₂ into organic compounds. To perform this process plants, algae and cyanobacteria are equipped with two multisubunit protein complexes, Photosystem I (PSI) and Photosystem II (PSII). Many of the subunits bind chromophores which absorb part of the visible light and efficiently transfer the excitation energy to a special chlorophyll molecule, where it is used to promote charge separation. The capture of the light energy is achieved by the light harvesting complexes (antenna complexes) of both photosystems, which coordinate chlorophyll and carotenoid (xanthophyll) molecules.

The photosynthetic efficiency (the ratio of the energy stored to the energy of light absorbed) varies under different environmental conditions. During their life, plants experience large fluctuations in light intensity, which occur on time scales ranging from seconds to seasons. In several conditions the amount of energy harvested by the plants exceeds the need of the cell and this excess energy can lead to the production of reactive oxygen species that can cause an inhibition of photosynthesis. To prevent this photoinhibition, plants have evolved several regulatory mechanisms. In excess light, part of the absorbed energy is thermally dissipated via a mechanism called Non-Photochemical Quenching (NPQ). The antenna are fundamental in the photoprotective responses, switching from a light harvesting form to an efficient thermal dissipation form. There is little understanding of the precise molecular mechanism through which the antenna could reversibly switch between fundamentally different states, in particular in how the pigment function can be altered within these complexes.

In this thesis I report results on the investigation of the functional architecture of light harvesting complexes integrating the available structural data of LHCII from Spinacea oleacea and of the two photosystems, with the spectroscopic properties of chlorophylls and carotenoids bound to these complexes.

It is known that pigments in different sites experience different interactions with their specific local protein environment, which modify their electronic structure. This can have a large effect on the spectroscopic properties of the protein-bound pigments and, thus, on their role in light-harvesting and photoprotection. Small differences in the environment of the pigments may lead to important changes in the spectroscopic features of the complex and thus, for instance, change the functional role of a specific pigment cluster. On the other end, the high sensitivity of the pigments to their local environment allows to use them as very sensitive structural probes.
Refolding by an *in vitro* procedure allows us to analyse individual antenna, identifying the spectroscopic and biochemical properties of each complex. Moreover, the creation of mutants by site direct mutagenesis, substituting the aminoacid coordinating a chlorophyll with a residue unable to coordinate the pigment, permits to elucidate the characteristics of individual cofactors and to investigate their role in the antenna complex. This technique, together with the purification of native complexes, provided the samples analysed in this thesis.

Part I focuses the attention on the determination of the spectroscopic properties of chlorophylls bound to monomeric antenna from PSI and PSII of higher plants, with particular attention to the low energy forms of both photosystems. These forms are particularly important because their represent a relative energy sink which makes them the best candidate for playing a primary role in the regulation of the energy flow through the system. By refolding *in vitro* several WT and mutant complexes were obtained.

In chapter 2 the Lhca4 antenna is analysed. A typical feature of the antenna complexes of Photosystem I is the presence of chlorophyll (Chl) molecules which absorb at energy levels lower than the reaction centre. These Chls act as a sink for the excitation energy, which is used for charge separation and should be transferred against a gradient. Among the four Lhca complexes the Lhca4 complex is known for its most red forms, which shows a fluorescence emission at 732 nm. In this chapter we have investigated the origin of the red forms in this complex, performing mutation analysis at the putative binding ligand of several Chls and comparing the spectroscopic properties of the mutants with that of the Wild Type. We could conclude that the red forms are the low energy band of an excitonic interaction involving two Chls $a$ molecules at sites 1015 and 1025. Mutations at neighbouring sites, which are not directly responsible for the red forms, lead to a change in the fluorescence properties of the complex, indicating that this feature is very sensitive to small changes in the structure of the protein. The spectroscopic properties of other Chls bound to the complex and the strength of their interaction were also analysed, furnishing a complete map of the spectroscopic properties of the individual chromophores bound to this antenna complex.

In chapter 3 we used mutation analysis and refolding *in vitro* to investigate the structure of Lhca3. From the first determined X-ray structure of PSI-LHCl it was suggested that Lhca3 has a different folding as compared with the other members of the Lhc family, with the two central helices being swapped. Spectroscopic features of individual chlorophylls are here used to probe the folding of this complex. Our results not only demonstrate that Lhca3 has the typical folding of Lhc complexes, but also reveal the presence of Chls 1013 (A3) and 1023 (B3), which were not observed in the crystal structure. The more recent
structure of PSI-LHCII confirms our findings. Moreover, we can assign the absorption characteristics to several Chls bound to the complex and we demonstrate that also in Lhca3 the low energy emission forms are originated by the excitonic interaction between Chls 1015 (A5) and 1025 (B5). We can conclude that the ‘red forms’ origin is conserved in all the Lhca and modulation of the emission energies is dependent on the pigments environment, distinctive for each complex.

In chapter 4, we studied by mutation analysis a particular domain of the antenna complexes of Photosystem II, composed of two Chls (612 and 611) and one xanthophyll (Lutein L1). It has been proposed that this domain is involved in the mechanism of energy dissipation which protects the system from high light damage. It was previously shown that in LHCII these two Chls represent the low energy state of the system and that in situations which simulate NPQ they can transfer energy to the neighbouring lutein. Here we demonstrate that in LHCII these Chls strongly interact with the lutein molecule, already in “normal” conditions and in the absence of quenching. A small change in the structure is thus sufficient to transform them in an efficient quenching site. The same experiments were repeated on the minor antenna complexes of Photosystem II. It is demonstrated that this domain is fully conserved in Lhcb4 and Lhcb5 and at least partially conserved in Lhcb6. Our results indicate that if this protein domain is responsible for the quenching in LHCII, all the antenna are prepared to be a quencher. This can explain the finding that for the NPQ all antenna complexes are dispensable, suggesting the presence of multiple quenching sites in the Photosystem II.

Part II focuses on the study of the carotenoids bound to the antenna complexes of both photosystems. These pigments play several roles in the photosynthetic process acting as accessory light-harvesting pigments, being fundamental for the structural stabilization of the complexes and, more important, they are involved in the photoprotection mechanisms. In high light conditions, when the amount of energy harvested by the system exceeds the capacity for electron transport to available sinks, the photochemistry is limiting and other de-excitation mechanisms become of importance. Part of the excitation energy decays via intersystem crossing, leading to the production of chlorophyll triplets which can react with molecular oxygen producing singlet oxygen, a very reactive and harmful species, leading to the oxidation of the photosynthesis machinery. The main role of the carotenoids is to protect the system from this damage by quenching the chlorophyll triplets and scavenging the singlet oxygen. The carotenoid composition of the antenna complexes is highly conserved through the plant kingdom and different carotenoids species have been observed to be bound to specific binding sites. In this section we have investigated the role of each of them in light harvesting and chlorophyll triplet quenching.
In chapter 5 the attention is focused on the structural and spectroscopic analysis of the carotenoids in the Lhca antenna. A complete map of the carotenoids distribution in all four Lhca complexes is given. Integrating spectroscopic measurements with mutation analysis it was possible to localize the individual binding sites, which were not resolved by X-ray crystallography and to determine the occupancy of each of them. The singlet and the triplet energy transitions of each xanthophyll in each binding site were also determined. This information, integrated with the analysis of mutants, permits to determine that the carotenoids occupying sites L1 and L2 are active in the triplet quenching. Both carotenoids decay with the same lifetime, but with distinguished spectroscopic features: the xanthophyll in L1 has a maximum at 507 nm and that in L2 at 522 nm. Moreover, the interaction Chl 1015 - Car L2 seems to influence the energy distribution of the carotenoid triplet decay, causing a shift to lower energies. Comparison of the LHCI native complex and the sum of individual Lhca Triplet minus Singlet spectra indicates that beta-carotene is active in the triplet quenching. Furthermore, our results suggest that energy equilibration is complete in the Lhca1-Lhca4 heterodimer, but not in Lhca2-Lhca3, at least in the fast scale.

In chapter 6 the carotenoids bound to the Lhcb antenna are investigated in order to elucidate their role and efficiency in the triplet quenching. Our findings demonstrate that in all the Lhc antenna only the carotenoids accommodated in the two internal sites L1 and L2 are responsible of the triplet quenching. In LHCII trimer the lutein in L1 site, localised in close proximity of the low energy state chlorophyll, is quenching the largest part of the triplets. The carotenoid in L2 has a longer lifetime and shows a red shifted maximum compared to the one in L1. The difference in lifetime depends on the occupancy of the N1 site, which acts as an oxygen barrier limiting the oxygen access to the inner Lhc domain and contributing to maintain the photostable conformation of the complex. The carotenoid triplet decay of monomeric Lhcb1, Lhcb4, Lhcb5 is monoexponential and shorter than in the LHCII trimer, indicating the absence of an oxygen barrier, protecting the internal sites. Furthermore it is shown that, different from previous reports, the chlorophyll to carotenoid triplet energy transfer has an efficiency of 95% allowing explanation of in vivo data.