From Disorder to Order: The Functional Flexibility of Single Plant Light-Harvesting Complexes

Kruger, T.P.J.

2011

document version
Publisher's PDF, also known as Version of record

1 Introduction

It may be obscured in the vast Milky Way. But there is something remarkable about this place, something captivating, enchanting: LIFE! Revolving around a huge life-sustaining source of heat and light is the home of thriving populations of staggering variety and complexity. Countless conditions have been fine-tuned to sustain life on this planet. Life! How can it be fathomed?

1.0. Prelude

The beauty of physics is often encapsulated by its simplicity. Complex problems can be broken down into ever simpler parts, each described by familiar physical laws. However, trying to use such a reductionistic approach in the domain of biology quickly brings about the realisation that *life is not all that simple!*

What makes life so complex? One important factor is that a living system is much more than the sum of its constituents. Biological systems are organised and integrated into levels of increasing complexity, where each added level of complexity creates emergent phenomena that do not follow simply from those of the simpler system. Put differently: simple things interacting in simple ways can yield surprisingly complex outcomes.

The synergy, rich diversity, and striking organisation can be appreciated on all levels of complexity in living systems. Indeed, *life is beautiful*! However, unravelling how the fundamental functions in a living system are performed remains a formidable task. This quest brings us to the level of the macromolecule, where proteins are actively involved in virtually all functions exhibited in living organisms. The ability of these molecular machines to perform a specific function is closely related to their flexible structure, which can be altered significantly upon interaction with the local environment or other molecules (2). This molecular switching behaviour often needs precise regulation in order to prevent disarray in the generally dense and heterogeneous environments and to maintain a sustainable balance between the cellular demand and supply. Such regulation is crucial for the survival of an organism, because *life out there can be demanding*!

Life is also *full of decisions*. In most proteins, conformational switching between different functional states is controlled by the binding of effector ligands, which can range from small molecules to other proteins (2). However, conformational changes in some other proteins are induced more subtly, *e.g.*, thermally, by means of the pH, or indirectly via light when pigment molecules are bound to the protein. In such cases, a situation may be reached where the protein is faced with the necessity to “choose” among different functional states, all of which have a comparable probability to be accessed. This situation is further complicated by the protein’s intrinsic disorder at physiological conditions, a property which may strongly influence the switching capability of the protein. Indeed, *disorder is part of life.*

How do the proteins in these equilibrium conditions decide which structural state(s) to access? Does this behaviour form the basis for the general way in which these proteins exhibit multifunctionality? What role does disorder in the protein and its local environment play in these protein dynamics? How is a protein’s functionality connected to its flexibility?

These questions have been investigated primarily for a protein which likely serves as the most abundant membrane-bound protein on earth (3) and which functions as part of the main light-harvesting pigment-protein complex in the primary events of photosynthesis in plants. Before the questions are addressed, we first need to introduce the physiological context of our protein system, the relevant physical and chemical processes involved, and the experimental technique that was used.

1.1. Photosynthesis

Photosynthesis is the process by which plants, algae and many species of bacteria convert light energy by means of a complicated chain of events into biochemical energy. All processes up to the early stages of energy storage are carried out in lipid bilayers, generally referred to as thylakoid membranes. The whole photosynthetic process is initiated by the absorption of a quantum of light (a photon), the energy of which is used to transport an electron from the inner, luminal side of the thylakoid membrane to the opposite, stromal side, and simultaneously transferring a proton in the opposite direction. The resulting transmembrane pH gradient, or electrochemical potential, serves as a biobattery to drive the reactions that eventually lead to the production of carbohydrates. Most organisms extract the electrons from water, thus releasing oxygen as a by-product. All oxygen-consuming organisms on earth are dependent on this free oxygen for cellular respiration. In addition, the carbohydrates formed as the end product of photosynthesis are used directly or indirectly as food by nearly all living organisms on earth. As such, photosynthesis is indispensable for sustaining virtually all life on earth.

1.2. Structural Architecture of the Photosystems

Light absorption is performed by generally large arrays of pigment molecules that are non-covalently bound to protein scaffolds. These ingeniously designed pigment-protein systems are known as light-harvesting complexes (LHCs) and act as antenna systems to increase the absorption cross-section in a conceptually similar manner as a satellite dish. The “receiver” to which the excitation energy is guided is called the reaction centre (RC), the photochemical system in which the ultrafast electron transfer reactions as initial stabilisation of the energy take place. Two types of RCs exist in vascular plants, each associated to different LHCs. The two LHC-RC assemblies are known as photosystem (PS) I and II, respectively. Although PSI and PSII work concurrently, the PSI RC requires the electrons from the PSII RC. Electron transport between PSI and PSII is performed by a separate pigment-protein system, known as the cytochrome b_6f complex, which also transports protons across the thylakoid membrane. PSII is located primarily in dense, stacked regions of the thylakoid, known as grana, whereas the other major protein complexes are found in the unstacked regions, called stroma lamellae, which connect the grana stacks (4).

The structure of PSI from vascular plants has been resolved to 3.4 Å, revealing 17 protein complex subunits (5). The outer antenna comprises the four complexes Lhca1–4, which are organised into the two adjacent dimers Lhca1/4 and Lhca2/3 (6-8). These LHCs are organised in a half-moon shape on one side of the large core antenna (6). PSII is an even larger multicomplex, comprising at least 27-28 subunits (4). Although it has not yet been possible to obtain high-quality crystals of PSII for green plants, information about its architecture can be derived from a combination of cryoelectron microscopy structures (4, 9), the crystal structures of individual LHCs (10, 11), and the related crystal structure of the PSII oxygen-evolving centre from cyanobacteria (12). The latter structure, which has recently been elucidated to a remarkable 1.9 Å resolution (12), gives an estimate for the plant RC

and inner antenna complexes, CP43 and CP47, collectively known as the core complexes. The peripheral antenna system of plants is composed of six nearly identical types of pigment-protein complexes (13), Lhcb1–6. The first three, Lhcb1–3, form heterotrimers of different compositions (14) and are known as the major outer antenna LHCII, while the minor outer antenna consists of the three monomers Lhcb4–6, also known as CP29, CP26, and CP24, respectively.

While PSII generally contains two copies of each minor Lhcb complex, up to six LHCII trimers likely associate with it (4). The varying number of LHCII complexes in this so-called PSII-LHCII supercomplex (9) suggests that LHCII can bind to the core with varying strengths (4). Under normal growth conditions, about 50% of the LHCII trimers are disconnected from PSII (4), some of which may aggregate, while some others diffuse to the lamellae where they may bind to PSI. The movement of LHCII between PSI and PSII is referred to as a state transition and serves to redistribute the excitation energy between PSI and PSII (15-17).

1.3. Photosynthetic Pigments: Chlorophylls and Carotenoids

Chlorophylls (Chls) are the most abundant pigments in LHCs, characterised by a tetrapyrrole ring which coordinates a central Mg ion (Fig. 1.1a). The extensive conjugated π-electron system of this ring is responsible for very intense absorption bands in the visible region of the electromagnetic spectrum. The two main electronic transition dipole moments of Chl are polarised along the *y* and roughly *x* molecular axes of the ring and are associated to the electronic transitions Q_y and Q_x, respectively. A third, large absorption band typically occurs in the blue and is known as the Soret band, comprising several transitions with mixed polarisation states. Fig. 1.2 shows the three pronounced absorption bands of Chl *a* and *b*. Compared to Chl *a*, Chl *b* has one methyl group exchanged for a formyl group (see Fig. 1.1a), which is responsible for the significant absorption differences between the two molecules. The long phytol chain of Chls serves to anchor them into their protein scaffolds (18).

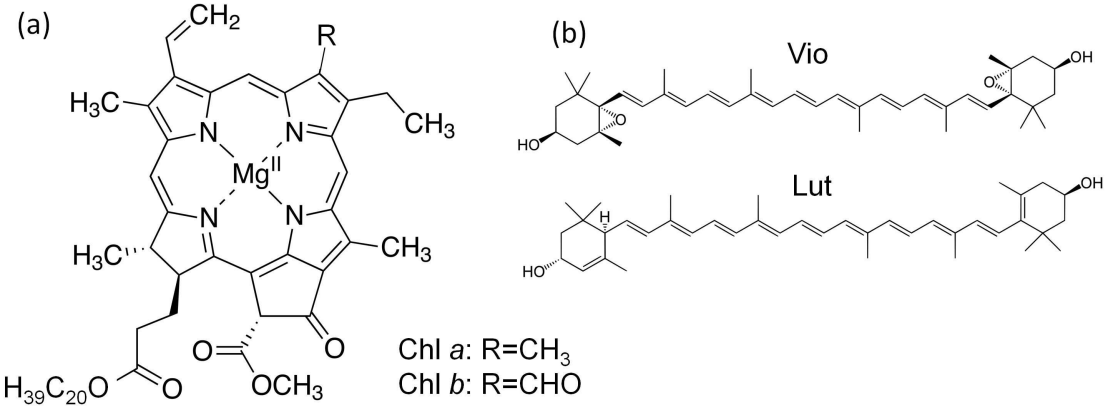

Figure 1.1. *Structure of some pigments present in plant LHCs: (a) Chl a and b, which differ only by the residue denoted by R; (b) The xanthophylls violaxanthin (Vio) and lutein (Lut).*

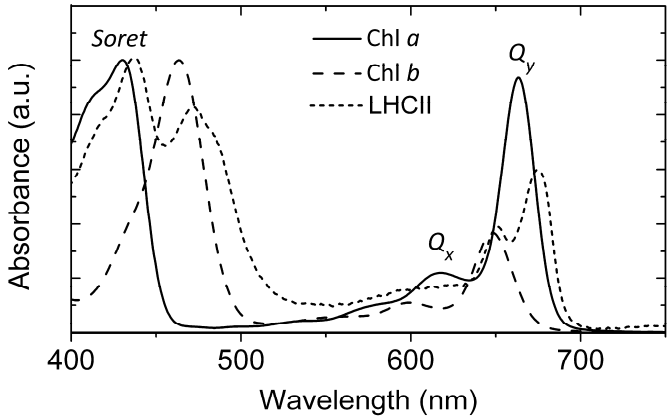

Figure 1.2. *Room-temperature absorption spectrum of Chl a (solid line) and b (dashed line) in ethanol, and LHCII trimers (dotted line), normalised to the maximum absorbance. The absorption bands of Chl a are indicated.*

Carotenoids (Cars) are another vital group of pigments in photosynthesis. Not only do they serve as accessory light-harvesters but they also stabilise their surrounding protein structures (19) and function as important photoprotectors (20). These pigments are characterised by their long, linear, π-conjugated polyene chains (see Fig. 1.1b). The resulting strong absorption bands are responsible for the bright colours of many vegetables, fruit and flowers as well as the ornamental colouring encountered in many birds and fish. The Cars present in plant LHCs contain oxygen in their terminal rings and are known as xanthophylls (Xans). They absorb light in the blue-green region of the electromagnetic spectrum where Chls absorb only weakly, thereby increasing the absorption cross-section of an LHC complex.

The most imperative function of Cars is their participation in photoprotection. An excited Chl recurrently enters a triplet state (^{3}Chl*), a state with high enough energy to react with a ground-state triplet oxygen ($^3O_2$) to generate highly reactive and thus deleterious singlet oxygen:

$$^3\mathrm{Chl}^* + {}^3\mathrm{O}_2 \rightarrow {}^1\mathrm{Chl} + {}^1\mathrm{O}_2^* .$$

To prevent this sensitisation of oxygen, Cars are capable of both quenching ^{3}Chl* and scavenging $^1O_2^*$ via their triplet state. This strong antioxidative property of Cars makes them essential for the survival of nearly all organisms on earth (21).

Cars are also essential components in the regulation of energy transfer in LHCs, a property which is discussed in more detail in Section 1.8.

1.4. The Peripheral LHCs of Plants

The crystal structure of the LHCII trimer was first resolved to 3.4 Å (22) and several years later to 2.72 Å (10)and 2.5 Å (3). The highly resolved structures revealed the location and orientation of 8 Chls *a*, 6 Chls *b*, and 4 Cars in each monomeric subunit. The Cars consist of 2 luteins (Luts), 1 neoxanthin (Neo), and, depending on the light conditions, either a violaxanthin (Vio) or zeaxanthin (Zea). The absorption spectrum of this complex is displayed in Fig. 1.2, indicating the broadening and redshift of the Chl transition bands due to excitonic interactions and the effect of the protein environment.

The very recently resolved structure of CP29 (11) indicates that the protein structure of this complex is almost identical to that of the monomeric subunits of LHCII. Even though the pigment composition is somewhat different, the pigments bind to the same sites as in LHCII,

giving rise to a very comparable room-temperature absorption spectrum. CP24 and CP26 exhibit similar absorption spectra, which can be explained on the same grounds.

In contrast, Lhca complexes exhibit a peculiar spectroscopic feature, despite the high structural homology of their proteins and similar pigment composition as compared to Lhcb (5, 10, 11, 13). These complexes are capable to absorb at considerably lower energies as the result of a charge-transfer (CT) state in a particular Chl dimer that mixes with the lower excitonic states of the complex (23-27). This particular arrangement of the Chl dimer enhances the light absorption of shaded leaves in dense canopies (28).

1.5. Excitonic Interactions

The exciton concept was mentioned in the Section 1.4. This is a particularly important property of LHCs to ensure fast and efficient transfer and trapping of excitation energy. In general, excitonic interactions are relevant for systems involving strong Coulombic interactions between pigment molecules. As a consequence, the electronic properties of such systems are determined by the coupling between the local excited states of the interacting pigments. Detailed descriptions of this process can be found in Refs. 29 and 30. A few concepts will be highlighted in this section.

As illustration, the excitonic coupling between a pair of identical molecules will be considered. For such an excitonically coupled dimer, Coulombic interactions occur between the electrons and nuclei of one molecule with those of the other one. For uncharged molecules this interaction can be approximated by a dipole-dipole interaction. As a result of this interaction, the excited state is split into two states with distinct energies (known as excitonic states), such that the splitting between the excitonic states is twice the coupling energy (29, 30). In addition, the average of these two levels shifts with respect to the ground state, and the excitation is delocalised over both molecules. This excitonic splitting is illustrated in Fig. 1.3a for a strongly coupled dimer. When the two molecules are in a “head-to-tail” configuration, the lowest energy transition is strongly allowed and the excitonic state is redshifted, while a blueshift occurs for a “sandwich” dimer. The absorption spectrum of a system is therefore very sensitive to the relative positions and orientations of the pigments.

The large number of interactions experienced by photosynthetic pigments clearly gives rise to complicated excitonic manifolds. In an LHC, the pigments do not only interact with one another but also with their local protein environment. As the protein is highly heterogeneous, every pigment experiences a unique local environment, which causes each pigment to have a different transition energy, also called site energy. These site energies are not constant but fluctuate on many different timescales due to the large structural flexibility of proteins (see Section 1.6). In other words, structural disorder of the protein gives rise to energetic disorder of the pigments. The time-averaged behaviour of this site heterogeneity is commonly modelled by a Gaussian distribution around the average value for each pigment.

Photosynthetic pigments experience two additional types of interactions that also contribute to significant disorder, *viz.*, exciton-phonon and exciton-vibrational (vibronic)

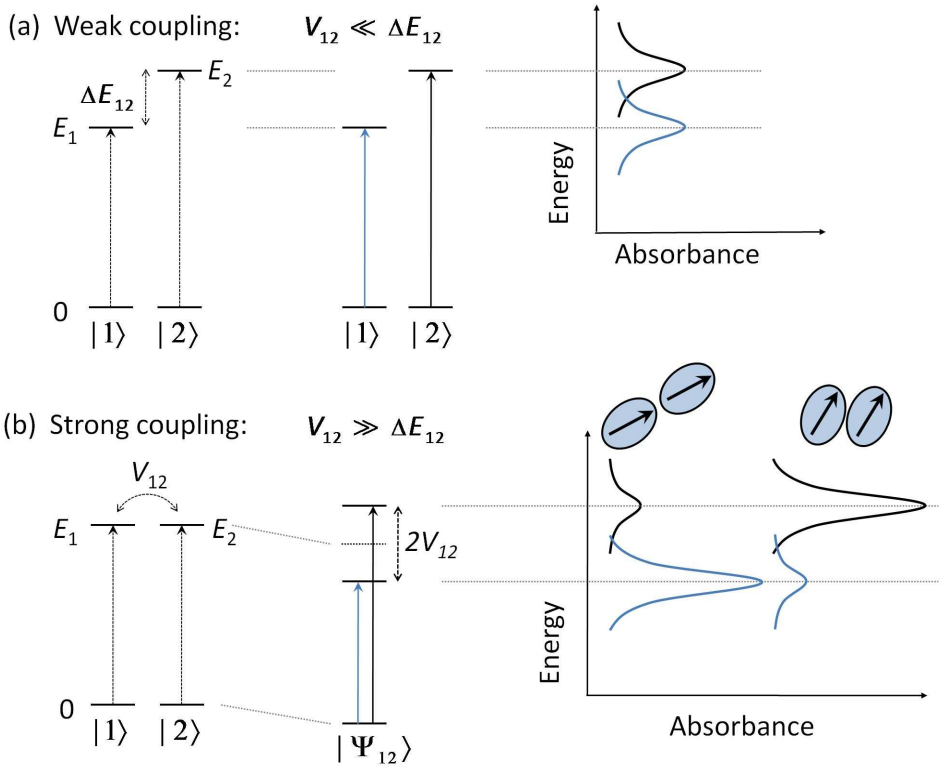

Figure 1.3: *Effects of the interaction (V_{12}) and site-energy difference $\Delta E_{12} = |E_1 - E_2|$ on the energy levels and spectra of two monomer pigments with states $|1\rangle$ and $|2\rangle$. (a) In the weak-coupling limit the interaction between the pigments is weak compared to their site-energy difference and the resulting absorption spectrum is independent of the mutual orientation of the pigments. (b) In the strong-coupling limit the difference in site energies is negligible and two new, delocalised exciton states are created. Spectra are shown for the two dipoles arranged in a "head-to-tail" and a "sandwich" configuration. Adapted from Ref. 1.*

coupling. The first denotes interactions between the pigment electronic transitions and the fast nuclear vibrational modes of the protein, while the second kind of interaction originates from the coupling between the electronic transitions of the pigments with their intramolecular vibrations. Since the phonons and molecular vibrations form a quasi-continuum of modes, these energies are often referred to as a bath. Coupling between the excitons and this bath produces an ultrafast energy cascade from higher to lower levels. This process constitutes the basic mechanism of efficient light harvesting in photosynthesis (31).

Dipole interactions are frequently described in one of two limits: that of the weak or the strong coupling, both of which are illustrated in Fig. 1.3. In the weak-coupling case (Fig. 1.3a) the interaction between the transition dipole moments is much smaller than the difference in site energies of the two pigments. As a result the coupling to the bath dominates and the excited-state wave functions are mainly localised on individual pigments. The spread in absorption bands consequently directly reflects the spread in site energies. In this regime, the energy transfer between pigments occurs via resonance and is typically described by using Förster theory (32). In the strong-coupling limit (Fig. 1.3b) the transition-dipole interaction dominates the site energy difference and bath interaction, and the excited-state wave functions are delocalised over the interacting pigments. This is generally the case for

interpigment distances smaller than 2 nm, a property of most LHCs. Redfield theory (33) provides a realistic description in this regime (31). If the dipolar coupling and site energy difference are comparable, the expected behaviour of energy transfer is intermediate between the weak and strong coupling limits.

Pure Redfield theory fails to provide an accurate estimation of the energy-transfer dynamics in LHCII (34, 35). By treating exciton-phonon coupling differently, a modified Redfield approach (36, 37) has proven a successful description (31, 35, 38). Combining this picture with the results from various time-resolved nonlinear spectroscopic techniques, the pathways and timescales of energy transfer in LHCII were elucidated, thus providing a comprehensive picture of the energy equilibration in this complex (39).

1.6. Protein Conformational Fluctuations

Crystal structures may give the apparent notion that proteins are static structures. On the contrary, these structures represent averages of a myriad of conformations. The polypeptide backbone and especially the side chains are in constant motion due to thermal energy and numerous intrinsic and extrinsic interactions. In addition, the close packing of a protein frequently leads to the conflict of different side-chains trying to occupy the same space, a property often referred to as frustration. A theoretical study of protein motions is restrained by the intrinsic complexity of even the simplest proteins. Their complex structures give rise to a bewildering variety of motions. Since the average displacement of protein atoms is generally larger than those found in crystals and glasses but smaller than liquids, protein motions are often called semi-liquid.

A highly acclaimed conceptual framework for protein motions has emerged from the pioneering experiments of Frauenfelder and co-workers on the well-studied myoglobin protein (40, 41) and complimentary molecular dynamics simulations (42, 43). In this context the concept of an energy-level scheme is replaced with the abstract idea of a conformational landscape, which describes all the possible conformations of the protein and the associated potential or free energy of each conformation. Such an energy hypersurface exists in the high-dimensional space where each degree of freedom of the protein adds another coordinate. To illustrate the landscape, a one-dimensional (1D) projection or cross-section is generally presented to demonstrate an arrangement of interchanging energy barriers and minima between them (Fig. 1.4). The local minima correspond to conformational substates (CSs), and the average height of the energy barriers gives rise to a

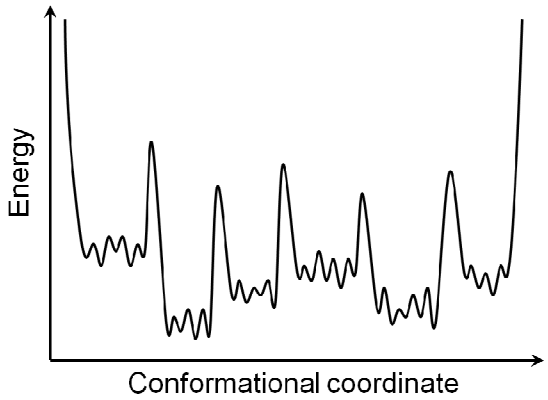

Figure 1.4: *Cartoon of the cross-section through a highly simplified energy landscape of a protein, illustrating the hierarchical arrangement of barrier heights which separate local energy minima. See text for details.*

hierarchical arrangement of these CSs. In such a 1D representation, the barrier heights directly reflect the activation energy required for a protein to escape from a particular CS and acquire another conformation. These heights consequently determine the extent of the structural arrangement required to alter the conformation and also the probability or typical timescale of such a change. For example, motions of electrons, H atoms, and specific atomic groups occur typically within ~1 ps, whereas domain movements take place on ms–s timescales (44). In this way the timeframe of different types of motions can be connected to the stability of the associated CS.

When energy is added to a protein system, *e.g.*, by excitation of an embedded pigment, the probability of crossing relatively large energy barriers increases and extended regions of the landscape can be traversed. Such a perturbation additionally leads to fluctuations of the barrier heights according to the energy equilibration in the system. An important difference between the behaviour of an excited electron in a molecule and protein movements according to the landscape model is that the former involves a moving particle that can tunnel through potential barriers, while the latter denotes searching of an energy minimum that can only be attained by crossing over energy barriers.

Unless the time-dependent structural dynamics of protein systems are synchronised, all information about these dynamics is averaged out in conventional ensemble experiments. These techniques inevitably consider all protein systems to be identical, whereas in reality, significant heterogeneity results from differing interaction strengths with the local environment. One powerful way to circumvent these problems is to investigate one protein system at a time. This method is briefly introduced in the next section.

1.7. Single-Molecule Spectroscopy (SMS)

Detection of single molecules represents the ultimate level of sensitivity. Great technological advances in the last few decades have realised this possibility. Information on single molecules and their interactions can be acquired most effectively by means of fluorescence spectroscopy due to the high selectivity and sensitivity of this technique. Even though this field, called single-molecule spectroscopy (SMS), can be considered a mere 20 years old, it has been expanding so much that a complete overview of the field is no longer possible. A few reviews in the earlier stages of the field are, *e.g.*, given by Refs. 45 and 46. In recent years, several books on this topic have appeared (see, *e.g.*, Refs. 1 and 47).

The new information provided by SMS as compared to ensemble-averaging techniques can be summarised as follows:

- The probability distribution of an observable is obtained instead of a single, mean value. This enables identification of subpopulations.
- Static and dynamic heterogeneity of observables can be separated on accessible timescales.
- Time-dependent processes can be elucidated without the difficulty, or often impossibility, to synchronise these processes in many molecules.

- Statistically rare events can be explored, enabling the possibility to detect new phenomena.

A large amount of information can be acquired from a single, isolated fluorescing system when it is illuminated continuously for extended periods of time. A common approach is to immobilise such a system onto a substrate. This approach often reveals three types of fluorescence fluctuations, namely that of the intensity, spectral shape, and polarisation. At cryogenic temperatures, large-scale motions of a protein are frozen out (48) and the width of emission spectra consequently narrows down considerably, often revealing vibrational substructure. Low-temperature spectral studies on bacterial PSI complexes made it possible to resolve some of their energy-transfer dynamics (49, 50), while low-temperature spectral and polarisation studies on bacterial LHCs have provided valuable insights on the structure and energy dynamics of these systems (see Ref. 51 for a recent review).

At room temperature, which is obviously much more representative of physiological conditions, a protein can traverse extended regions of its energy landscape. Large spectral fluctuations of up to 60-70 nm have been observed for bacterial LHCs (52-54). These spectral dynamics have been successfully simulated using Modified Redfield Theory (55, 56), strongly suggesting that the spectral changes are associated to changes in the pigment site energies that are modulated by relatively slow protein conformational changes.

Rapid fluorescence intensity fluctuations are often considered the fingerprint of a single quantum system. Virtually all single, fluorescent systems have shown the ability to switch off their emission entirely at random times and for random intervals (57-62). This phenomenon has become known as fluorescence intermittency or blinking. Despite extensive theoretical and experimental research, the nature of these fluctuations remains unresolved for most systems. For LHCs, this phenomenon has drawn only little attention as yet (57, 62-66).

Before introducing a typical experimental setup for room-temperature SMS (in Chapter 2), another important process in plants will be considered first.

1.8. Nonphotochemical Quenching (NPQ)

Stress is not confined to humans only. Plants also need to cope with demanding environmental conditions, such as large fluctuations in temperature and light intensity. The highly efficient LHCs of plants are designed to function optimally under low light conditions. As a result, the amount of light absorption quickly exceeds the plant's physiological needs during exposure to high irradiation levels. Without proper regulation the highly reactive intermediate products generate harmful reactive oxygen species (67).

Since sunlight can fluctuate frequently and dramatically in both intensity and spectral quality during the day, mechanisms of energy regulation are required on many different timescales. The capacity for light harvesting is adjustable not only on the macroscopic scale, for example by stomatal responses as well as leaf and chloroplast movements, but also on the molecular scale, where a large number of mechanisms are employed. On this scale, energy dissipation can be observed as the nonphotochemical quenching (NPQ) of Chl

fluorescence and is conventionally divided into the three components qE, qT, and qI according to their relaxation kinetics in the dark. The process of NPQ has drawn much attention and several reviews have been written (see, *e.g.*, Refs. 68-71). Most excess energy is quenched by thermal dissipation, a rapidly activated process that is reversible in the dark and often termed qE. The excess excitation energy can also be redistributed by means of state transitions (qT), thus altering the effective antenna sizes of PSI and PSII, a process which occurs in the time frame of tens of seconds. During long-term acclimation to a particular environment, the antenna sizes may be further modified through changes in gene expression and/or proteolysis (70). Despite the efficiency of qE and qT, photoinhibition still occurs frequently under sustained thermal dissipation. This process results in an additional reduction of the measured fluorescence and is thus referred to as photoinhibitory quenching (qI). Repair of inflicted systems, in particular the PSII RC, is mainly responsible for the slow relaxation kinetics of qI, often involving several hours. In this thesis only the component qE is considered.

qE is triggered by the build-up of the transthylakoid proton gradient (ΔpH), consequently activating the violaxanthin de-epoxidase enzyme, which converts Vio into Zea via the Xan cycle (68), and protonating the LHC-related PsbS protein (72) and the LHCs of PSII (73). Despite extensive investigations, the exact site of qE within the PSII antenna and the photophysical mechanism remain under debate. The early work of Horton and co-workers suggested that qE is promoted by aggregation of the PSII LHCs and controlled by the Xan cycle Cars (74). This allosteric regulatory behaviour of Vio and Zea was elaborated in further studies (75, 76), where LHCII was proposed to be the site of qE and that the conformational change into a dissipative state is promoted by Zea as well as the protonated PsbS protein. This conformational transition between the quenched and unquenched state was shown to be an intrinsic property of LHCII (77). Combining ultrafast spectroscopy with Raman spectroscopy, a mechanism for qE in LHCII was identified (78): a slight conformational change rearranges the energy levels of the lowest exciton state and the S_1 level of one of the Luts in LHCII, thereby enabling excitation energy transfer from the Chls to the Lut and the subsequent rapid dissipation of this energy as heat upon relaxation of the Lut excited state.

More recently, evidence for other qE mechanisms has been proposed by different groups. One favoured proposition is that qE occurs in CP29 and arises by the formation of a Zea radical cation (79, 80). However, most models agree that the LHCII trimer is the site of qE. Quenchers that have been implicated are Zea (3) and even Chl-Chl charge-transfer states (81) or impurities (82). In an attempt to unify some of the models for qE it has been suggested that more than one mechanism may be involved (83, 84).

Although NPQ in plants has drawn the most attention, it is not restricted to plants but is also a vital process in LHCs of several other oxygenic organisms, such as diatoms (85), dinoflagellates (86), and cyanobacteria (87). The distinct compositional differences between the LHCs of these organisms and plants give rise to different fluorescence kinetics and distinct NPQ mechanisms as compared to vascular plants.

1.9. This Thesis

The aim of this thesis is to get a glimpse into the relationship between the fluorescence, structure and function of single, isolated LHCII trimers near room temperature. The fluorescence from single complexes was observed as these complexes switched between different emission states. In particular, variations in the fluorescence spectrum and fluctuations in the rate of photon emission were assessed.

As described in Sections 1.5 and 1.6, spectral changes are indicative of changes in the underlying energy level structure, which in turn describes the variations in the pigment site energies that are induced by slow protein conformational changes. Comparison between the spectral dynamics of LHCII and the structurally closely related but functionally different LHCs of PSI (Lhca) may provide insight into the functional diversity of these systems and help to explain their spectral dynamics. Obviously, this experimental approach does not reveal the specifics of the microscopic structural changes, but it instead allows for the most general and systematic description of the electronic structures associated with the different conformations of Lhca and Lhcb complexes.

Fluctuations in the photon emission rate, commonly known as fluorescence intermittency, indicate the presence of processes that enhance energy dissipation for extended periods of time. The environmental sensitivity of these fluctuations sheds light on the nature of the underlying mechanism. A primary objective of this thesis is to determine whether any relationship exists between fluorescence intermittency and the all-important processes involved with NPQ in LHCII.

In Chapter 2, the experimental setup that was used to perform single-molecule spectroscopy is described and the performance and practical limits of the setup are examined in detail. A quantitative analysis is made of the optimal signal-to-noise ratio that can be obtained for single LHCII trimers, a critical parameter in SMS.

Chapter 3 is aimed at discovering, classifying and interpreting the spectral dynamics from LHCII trimers and deals with the following questions: Can the ensemble fluorescence spectrum be deconvoluted into various spectral components at room temperature, *i.e.*, what is the extent of spectral heterogeneity and the contribution of different subpopulations? What are the frequency and rate of the spectral fluctuations? Do theory and experiment concur in the spectral diversity exhibited by single LHCII trimers? If not, how can deviating populations be interpreted in terms of structure and function?

In Chapter 4, the spectral fluctuations of closely related complexes in PSI and PSII are investigated to resolve questions such as: How much is the spectral behaviour of the different complexes related? How do the differences in spectral disorder relate to the structural and functional differences?

In Chapter 5, a new approach to resolve and analyse transitions between different intensity levels of fluorescing systems is described and demonstrated for the time-resolved fluorescence from single LHCII trimers. This chapter provides underpinning for the final two chapters.

In Chapter 6, the environmental sensitivity of the fluorescence intermittency of LHCII trimers is investigated and a simple model is suggested for the underlying mechanism.

The final chapter builds upon the results of all the previous chapters to investigate the important question of whether fluorescence intermittency serves a functional role in plants, in contrast to nonbiological systems that also display this phenomenon. In other words, can fluorescence intermittency in plants be connected to the vital regulation process of qE? If this is the case, SMS can provide valuable information about the nature of the switches into quenched states and the associated spectroscopic signatures.

References

1. Zander, C., J. Enderlein, and R. A. Keller. 2002. Single molecule detection in solution: methods and applications. Wiley-VCH, Berlin.
2. Petsko, G. A., and D. Ringe. 2004. Protein structure and function New Science Press, London.
3. Standfuss, J., A. C. Terwisscha van Scheltinga, M. Lamborghini, and W. Kühlbrandt. 2005. Mechanisms of photoprotection and nonphotochemical quenching in pea light-harvesting complex at 2.5 Å resolution. *EMBO J.* 24:919-928.
4. Dekker, J. P., and E. J. Boekema. 2005. Supramolecular organization of thylakoid membrane proteins in green plants. *Biochem. Biophys. Acta-Bioenerg.* 1706:12-39.
5. Amunts, A., O. Drory, and N. Nelson. 2007. The structure of a plant photosystem I supercomplex at 3.4 angstrom resolution. *Nature* 447:58-63.
6. Ben-Shem, A., F. Frolow, and N. Nelson. 2003. Crystal structure of plant photosystem I. *Nature* 426:630-635.
7. Croce, R., G. Canino, F. Ros, and R. Bassi. 2002. Chromophore organization in the higher-plant photosystem II antenna protein CP26. *Biochem.* 41:7334-7343.
8. Wientjes, E., I. H. M. van Stokkum, H. van Amerongen, and R. Croce. 2011. Excitation-energy transfer dynamics of higher plant photosystem I light-harvesting complexes. *Biophys. J.* 100:1372-1380.
9. Caffarri, S., R. Kouril, S. Kereiche, E. J. Boekema, and R. Croce. 2009. Functional architecture of higher plant photosystem II supercomplexes. *EMBO J.* 28:3052-3063.
10. Liu, Z., H. Yan, K. Wang, T. Kuang, J. Zhang, L. Gui, X. An, and W. Chang. 2004. Crystal structure of spinach major light-harvesting complex at 2.72 Å resolution. *Nature* 428:287-292.
11. Pan, X., M. Li, T. Wan, L. Wang, C. Jia, Z. Hou, X. Zhao, J. Zhang, and W. Chang. 2011. Structural insights into energy regulation of light-harvesting complex CP29 from spinach. *Natl. Struct. Mol. Biol.* 18:309-315.
12. Umena, Y., K. Kawakami, J. R. Shen, and N. Kamiya. 2011. Crystal structure of oxygen-evolving photosystem II at a resolution of 1.9 Å. *Nature* 473:55-60.
13. Jansson, S. 1999. A guide to the Lhc genes and their relatives in Arabidopsis. *Trends in Plant Sci.* 4:236-240.
14. van Amerongen, H., and R. van Grondelle. 2001. Understanding the energy transfer function of LHCII, the major light-harvesting complex of green plants. *J. Phys. Chem. B* 105:604-617.
15. Allen, J. F., J. Bennett, K. E. Steinback, and C. J. Arntzen. 1981. Chloroplast Protein-Phosphorylation Couples Plastoquinone Redox State to Distribution of Excitation-Energy between Photosystems. *Nature* 291:25-29.
16. Nelson, N., and C. F. Yocum. 2006. Structure and function of photosystems I and II. *Annu. Rev. Plant Biol.* 57:521-565.
17. Wollman, F. A. 2001. State transitions reveal the dynamics and flexibility of the photosynthetic apparatus. *EMBO J.* 20:3623-3630.

18. Raven, P. H., R. F. Evert, and S. E. Eichhorn. 2005. Photosynthesis, Light, and Life. *In* Biology of Plants, 7th Edition. W. H. Freeman, editor. 119-127.
19. Frank, H. A., and R. J. Cogdell. 1996. Carotenoids in photosynthesis. *Photochem. Photobiol.* 63:257-264.
20. Scheer, H. 2003. The pigments. *In* Light-harvesting antennas in photosynthesis. B. R. Green, and W. W. Parson, editors. Kluwer Academic Publisher, Dordrecht.
21. Demmig-Adams, B., and W. W. Adams. 2002. Antioxidants in photosynthesis and human nutrition. *Science* 298:2149-2153.
22. Kuhlbrandt, W., D. N. Wang, and Y. Fujiyoshi. 1994. Atomic Model of Plant Light-Harvesting Complex by Electron Crystallography. *Nature* 367:614-621.
23. Croce, R., A. Chojnicka, T. Morosinotto, J. A. Ihalainen, F. van Mourik, J. P. Dekker, R. Bassi, and R. van Grondelle. 2007. The low-energy forms of photosystem I light-harvesting complexes: Spectroscopic properties and pigment-pigment interaction characteristics. *Biophys. J.* 93:2418-2428.
24. Ihalainen, J. A., M. Ratsep, P. E. Jensen, H. V. Scheller, R. Croce, R. Bassi, J. E. I. Korppi-Tommola, and A. Freiberg. 2003. Red spectral forms of chlorophylls in green plant PSI - a site-selective and high-pressure spectroscopy study. *J. Phys. Chem. B* 107:9086-9093.
25. Morosinotto, T., J. Breton, R. Bassi, and R. Croce. 2003. The nature of a chlorophyll ligand in Lhca proteins determines the far red fluorescence emission typical of photosystem I. *J. Biol. Chem.* 278:49223-49229.
26. Romero, E., M. Mozzo, I. H. M. van Stokkum, J. P. Dekker, R. van Grondelle, and R. Croce. 2009. The Origin of the Low-Energy Form of Photosystem I Light-Harvesting Complex Lhca4: Mixing of the Lowest Exciton with a Charge-Transfer State. *Biophys. J.* 96:L35-L37.
27. Vaitekonis, S., G. Trinkunas, and L. Valkunas. 2005. Red chlorophylls in the exciton model of photosystem I. *Photosynth. Res.* 86:185-201.
28. Rivadossi, A., G. Zucchelli, F. M. Garlaschi, and R. C. Jennings. 1999. The importance of PSI chlorophyll red forms in light-harvesting by leaves. *Photosynth. Res.* 60:209-215.
29. Van Amerongen, H., L. Valkunas, and R. van Grondelle. 2000. Photosynthetic Excitons. World Scientific Publishing, Singapore.
30. Van Grondelle, R., J. P. Dekker, and V. I. Novoderezhki. 2009. Modeling light-harvesting and primary charge separation in photosystem I and photosystem II. *In* Photosynthesis in silico: understanding complexity from molecules to ecosystems, A. Laisk, L. Nedbal, and Govindjee, editors. Springer, Dordrecht. 33-53.
31. Novoderezhkin, V. I., and R. van Grondelle. 2010. Physical origins and models of energy transfer in photosynthetic light-harvesting. *Phys. Chem. Chem. Phys.* 12:7352-7365.
32. Förster, T. 1965. Delocalized excitation and excitation transfer. *In* Modern Quantum Chemistry. O. Sinanoglu, editor. Academic, New York. 93-137.
33. Redfield, A. G. 1965. *Adv. Mag. Res.* 1:1.
34. Novoderezhkin, V., J. M. Salverda, H. van Amerongen, and R. van Grondelle. 2003. Exciton modeling of energy-transfer dynamics in the LHCII complex of higher plants: A redfield theory approach. *J. Phys. Chem. B* 107:1893-1912.
35. Novoderezhkin, V. I., M. A. Palacios, H. van Amerongen, and R. van Grondelle. 2004. Energy-transfer dynamics in the LHCII complex of higher plants: Modified redfield approach. *J. Phys. Chem. B* 108:10363-10375.
36. Meier, T., V. Chernyak, and S. Mukamel. 1997. Femtosecond photon echoes in molecular aggregates. *J. Chem. Phys.* 107:8759-8780.
37. Zhang, W. M., T. Meier, V. Chernyak, and S. Mukamel. 1998. Exciton-migration and three-pulse femtosecond optical spectroscopies of photosynthetic antenna complexes. *J. Chem. Phys.* 108:7763-7774.
38. Novoderezhkin, V., A. Marin, and R. van Grondelle. Intra- and inter-monomeric transfers in the light harvesting LHCII complex: the Redfield-Förster picture. *Phys. Chem. Chem. Phys.* submitted.

39. Van Grondelle, R., and V. I. Novoderezhkin. 2006. Energy transfer in photosynthesis: experimental insights and quantitative models. *Phys. Chem. Chem. Phys.* 8:793-807.
40. Frauenfelder, H., G. A. Petsko, and D. Tsernoglou. 1979. Temperature-Dependent X-Ray-Diffraction as a Probe of Protein Structural Dynamics. *Nature* 280:558-563.
41. Frauenfelder, H., S. G. Sligar, and P. G. Wolynes. 1991. The Energy Landscapes and Motions of Proteins. *Science* 254:1598-1603.
42. Elber, R., and M. Karplus. 1987. Multiple Conformational States of Proteins - a Molecular-Dynamics Analysis of Myoglobin. *Science* 235:318-321.
43. van Gunsteren, W. F., D. Bakowies, R. Baron, I. Chandrasekhar, M. Christen, X. Daura, P. Gee, D. P. Geerke, A. Glattli, P. H. Hunenberger, M. A. Kastenholz, C. Ostenbrink, M. Schenk, D. Trzesniak, N. F. A. van der Vegt, and H. B. Yu. 2006. Biomolecular modeling: Goals, problems, perspectives. *Angew. Chem.* 45:4064-4092.
44. Zwanzig, R. 1990. Rate-Processes with Dynamic Disorder. *Acc. of Chem. Res.* 23:148-152.
45. Moerner, W. E. 2002. A dozen years of single-molecule spectroscopy in physics, chemistry, and biophysics. *J. Phys. Chem. B* 106:910-927.
46. Plakhotnik, T., E. A. Donley, and U. P. Wild. 1997. Single-molecule spectroscopy. *Annu. Rev. Phys. Chem.* 48:181-212.
47. Yanagida, T., and Y. Ishi. 2009. Single Molecule Dynamics in Life Science. WILEY-VCH, Weinheim.
48. Hofmann, C., T. J. Aartsma, H. Michel, and J. Kohler. 2003. Direct observation of tiers in the energy landscape of a chromoprotein: A single-molecule study. *Proc. Natl. Acad. Sci. U.S.A.* 100:15534-15538.
49. Brecht, M., J. B. Nieder, H. Studier, E. Schlodder, and R. Bittl. 2008. Red antenna states of photosystem I from Synechococcus sp. PCC 7002. *Photosynth. Res.* 95:155-162.
50. Brecht, M., V. Radics, J. B. Nieder, and R. Bittl. 2009. Protein dynamics-induced variation of excitation energy transfer pathways. *Proc. Natl. Acad. Sci. U.S.A.* 106:11857-11861.
51. Cogdell, R. J., and J. Kohler. 2009. Use of single-molecule spectroscopy to tackle fundamental problems in biochemistry: using studies on purple bacterial antenna complexes as an example. *Biochem. J.* 422:193-205.
52. Rutkauskas, D. 2006. The dynamic organization of the bacterial photosynthetic light-harvesting complexes: fluorescence microspectroscopy. *Ph. D. Thesis*, VU University, Amsterdam.
53. Rutkauskas, D., V. Novoderezhkin, R. J. Cogdell, and R. van Grondelle. 2005. Fluorescence spectroscopy of conformational changes of single LH2 complexes. *Biophys. J.* 88:422-435.
54. Rutkauskas, D., J. Olsen, A. Gall, R. J. Cogdell, C. N. Hunter, and R. van Grondelle. 2006. Comparative study of spectral flexibilities of bacterial light-harvesting complexes: Structural implications. *Biophys. J.* 90:2463-2474.
55. Novoderezhkin, V. I., D. Rutkauskas, and R. van Grondelle. 2006. Dynamics of the emission spectrum of a single LH2 complex: Interplay of slow and fast nuclear motions. *Biopys. J.* 90:2890-2902.
56. Novoderezhkin, V. I., D. Rutkauskas, and R. van Grondelle. 2007. Multistate conformational model of a single LH2 complex: Quantitative picture of time-dependent spectral fluctuations. *Chem. Phys.* 341:45-56.
57. Bopp, M. A., Y. W. Jia, L. Q. Li, R. J. Cogdell, and R. M. Hochstrasser. 1997. Fluorescence and photobleaching dynamics of single light-harvesting complexes. *Proc. Natl. Acad. Sci. U.S.A.* 94:10630-10635.
58. Cichos, F., C. von Borczyskowski, and M. Orrit. 2007. Power-law intermittency of single emitters. *Curr. Opin. Colloid Interface Sci.* 12:272-284.
59. Kulzer, F., and M. Orrit. 2004. Single-molecule optics. *Annu. Rev. Phys. Chem.* 55:585-611.
60. Peterman, E. J. G., S. Brasselet, and W. E. Moerner. 1999. The fluorescence dynamics of single molecules of green fluorescent protein. *J. Phys. Chem. A* 103:10553-10560.
61. Vanden Bout, D. A., W. T. Yip, D. H. Hu, D. K. Fu, T. M. Swager, and P. F. Barbara. 1997. Discrete intensity jumps and intramolecular electronic energy transfer in the spectroscopy of single conjugated polymer molecules. *Science* 277:1074-1077.

62. Ying, L. M., and X. S. Xie. 1998. Fluorescence spectroscopy, exciton dynamics, and photochemistry of single allophycocyanin trimers. *J. Phys. Chem. B* 102:10399-10409.
63. Goldsmith, R. H., and W. E. Moerner. 2010. Watching conformational- and photodynamics of single fluorescent proteins in solution. *Nat. Chem.* 2:179-186.
64. Hofkens, J., W. Schroeyers, D. Loos, M. Cotlet, F. Kohn, T. Vosch, M. Maus, A. Herrmann, K. Mullen, T. Gensch, and F. C. De Schryver. 2001. Triplet states as non-radiative traps in multichromophoric entities: single molecule spectroscopy of an artificial and natural antenna system. *Spectrochim. Acta, Part A* 57:2093-2107.
65. Loos, D., M. Cotlet, F. De Schryver, S. Habuchi, and J. Hofkens. 2004. Single-molecule spectroscopy selectively probes donor and acceptor chromophores in the phycobiliprotein allophycocyanin. *Biophys. J.* 87:2598-2608.
66. Unterkofler, S., T. Pflock, J. Southall, R. J. Cogdell, and J. Kohler. Fluorescence Blinking of the RC-LH1 Complex from Rhodopseudomonas palustris. *ChemPhysChem* 12:711-716.
67. Foyer, C. H., and J. Harbinson. 1994. Oxygen metabolism and the regulation of photosynthetic electron transport. *In* Causes of Photooxidative Stress and Amelioration of Defense Systems in Plants. C. H. Foyer, and P. M. Mullineaux, editors. Boca Raton: CRC Press. 1-42.
68. Demmig-Adams, B., W. W. Adams, U. Heber, S. Neimanis, K. Winter, A. Kruger, F. C. Czygan, W. Bilger, and O. Bjorkman. 1990. Inhibition of Zeaxanthin Formation and of Rapid Changes in Radiationless Energy-Dissipation by Dithiothreitol in Spinach Leaves and Chloroplasts. *Plant Physiol.* 92:293-301.
69. Horton, P., A. V. Ruban, and R. G. Walters. 1996. Regulation of light harvesting in green plants. *Annual Rev. Plant Physiol. Plant Mol. Biol.* 47:655-684.
70. Muller, P., X. P. Li, and K. K. Niyogi. 2001. Non-photochemical quenching. A response to excess light energy. *Plant Physiol.* 125:1558-1566.
71. Niyogi, K. K. 1999. Photoprotection revisited: Genetic and molecular approaches. *Annual Rev. Plant Physiol. Plant Mol. Biol.* 50:333-359.
72. Li, X. P., O. Bjorkman, C. Shih, A. R. Grossman, M. Rosenquist, S. Jansson, and K. K. Niyogi. 2000. A pigment-binding protein essential for regulation of photosynthetic light harvesting. *Nature* 403:391-395.
73. Walters, R. G., A. V. Ruban, and P. Horton. 1994. Higher plant light-harvesting complexes LHCIIa and LHCIIc are bound by dicyclohexylcarbodiimide during inhibition of energy dissipation. *Eur. J. Biochem.* 226:1063-1069.
74. Horton, P., A. V. Ruban, D. Rees, A. A. Pascal, G. Noctor, and A. J. Young. 1991. Control of the Light-Harvesting Function of Chloroplast Membranes by Aggregation of the Lhcii Chlorophyll Protein Complex. *FEBS Lett.* 292:1-4.
75. Horton, P., M. P. Johnson, M. L. Perez-Bueno, A. Z. Kiss, and A. V. Ruban. 2008. Photosynthetic acclimation: Does the dynamic structure and macro-organisation of photosystem II in higher plant grana membranes regulate light harvesting states? *FEBS J.* 275:1069-1079.
76. Horton, P., M. Wentworth, and A. Ruban. 2005. Control of the light harvesting function of chloroplast membranes: The LHCII-aggregation model for non-photochemical quenching. *FEBS Lett.* 579:4201-4206.
77. Pascal, A. A., Z. F. Liu, K. Broess, B. van Oort, H. van Amerongen, C. Wang, P. Horton, B. Robert, W. R. Chang, and A. Ruban. 2005. Molecular basis of photoprotection and control of photosynthetic light-harvesting. *Nature* 436:134-137.
78. Ruban, A. V., R. Berera, C. Ilioaia, I. H. M. van Stokkum, J. T. M. Kennis, A. A. Pascal, H. van Amerongen, B. Robert, P. Horton, and R. van Grondelle. 2007. Identification of a mechanism of photoprotective energy dissipation in higher plants. *Nature* 450:575-U522.
79. Ahn, T. K., T. J. Avenson, M. Ballottari, Y. C. Cheng, K. K. Niyogi, R. Bassi, and G. R. Fleming. 2008. Architecture of a charge-transfer state regulating light harvesting in a plant antenna protein. *Science* 320:794-797.

80. Avenson, T. J., T. K. Ahn, D. Zigmantas, K. K. Niyogi, Z. Li, M. Ballottari, R. Bassi, and G. R. Fleming. 2008. Zeaxanthin radical cation formation in minor light-harvesting complexes of higher plant antenna. *J. biol. chem.* 283:3550-3558.
81. Muller, M. G., P. Lambrev, M. Reus, E. Wientjes, R. Croce, and A. R. Holzwarth. 2009. Singlet energy dissipation in the photosystem II light-harvesting complex does not involve energy transfer to carotenoids. *ChemPhysChem* 11:1289-1296.
82. Barros, T., A. Royant, J. Standfuss, A. Dreuw, and W. Kuhlbrandt. 2009. Crystal structure of plant light-harvesting complex shows the active, energy-transmitting state. *EMBO J.* 28:298-306.
83. Bode, S., C. C. Quentmeier, P. N. Liao, N. Hafi, T. Barros, L. Wilk, F. Bittner, and P. J. Walla. 2009. On the regulation of photosynthesis by excitonic interactions between carotenoids and chlorophylls. *Proc. Natl. Acad. Sci. U.S.A.* 106:12311-12316.
84. Holzwarth, A. R., Y. Miloslavina, M. Nilkens, and P. Jahns. 2009. Identification of two quenching sites active in the regulation of photosynthetic light-harvesting studied by time-resolved fluorescence. *Chem. Phys. Lett.* 483:262-267.
85. Ting, C. S., and T. G. Owens. 1993. Photochemical and Nonphotochemical Fluorescence Quenching Processes in the Diatom Phaeodactylum-Tricornutum. *Plant Physiol.* 101:1323-1330.
86. Hofmann, E., P. M. Wrench, F. P. Sharples, R. G. Hiller, W. Welte, and K. Diederichs. 1996. Structural basis of light harvesting by carotenoids: Peridinin-chlorophyll-protein from Amphidinium carterae. *Science* 272:1788-1791.
87. Wilson, A., Ajlani, G., Verbavatz, J.M. 2006. A soluble carotenoid protein involved in phycobilisome-related energy dissipation in cyanobacteria. *Plant Cell* 18:992-1007.