INTERACTIONS BETWEEN CHLOROPHYLLS AND CAROTENOIDS IN LIGHT-HARVESTING COMPLEXES OF PHOTOSYSTEM II AND IN MODEL SYSTEMS

Ph.D. Thesis

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Introduction

Photosynthesis is undoubtedly one of the most important processes on planet Earth. Almost all of the life-forms rely on the products of photosynthetic organisms such as algae, higher plants and certain bacteria. These organisms are able to capture energy directly from the solar radiation and utilize it for the synthesis of energy-rich chemical compounds, which can serve as food for other life-forms. The whole course of photosynthesis can be divided into two distinct parts. The initial steps, which require the presence of light, involve the charge separations in the reaction centers, the process of water splitting (oxygen evolution) and the production of energy-rich ATP and NADPH. These compounds are utilized in the second part of photosynthesis, which consists of a series of biochemical processes such as carbon fixation, and can be performed without the presence of light. We focussed our interest to the beginning of photosynthesis, which starts with the absorption of a photon in the antenna complexes, and the capability of higher plants to safely and efficiently utilize the excitation energy created in such a way.

The chlorophyll *a/b* containing light-harvesting pigment-protein complex (LHCII) associated with photosystem II in the chloroplasts of higher plants is one of the most abundant proteins in the biosphere. It alone binds roughly half the total amount of chlorophyll (Chl) content of plants. *In vitro* LHCII appears to be aggregates of trimeric complexes of 25-27 kDa monomers, whose structure has been resolved to 3.4 Å with the aid of electron crystallography of two-dimensional crystals. Within each monomer subunit 12-14 Chl's and 2-4 xanthophylls (Xan's) are non-covalently bound to the LHCII polypeptide. Seven Chl's which are in van der Waals contact with the two central Xan's — identified as luteins (Lut's) — were tentatively assigned as chlorophyll *a* (Chl*a*) molecules to allow efficient triplet transfer from Chl*a* to Xan.

In LHCII, any excited Xan or chlorophyll *b* (Chl*b*) molecule can transfer its singlet excitation energy to a Chl*a* pigment within picoseconds — all the other, unimolecular decay processes being too slow to compete with energy transfer (ET). From a Chl*a* molecule the excitation can be forwarded to the reaction center, where the primary charge separation takes place, or, when the reaction center is already occupied, it can be dissipated in three different ways: (*i*) by internal conversion to the ground state; (*ii*) by emitting a light quantum

(fluorescence); or (*iii*) by undergoing intersystem crossing to the triplet manifold. The formation of triplet Chl*a* ($Chl a^{\dagger}$), however, should be avoided because it can react with ground state (triplet) oxygen creating deleterious singlet oxygen. Xan's, having a low lying triplet state, can efficiently prevent this process either directly, accepting triplet excitation from Chl*a*, or indirectly, by quenching singlet oxygen. The excitation from a triplet state of a Xan is harmlessly dissipated to the environment as heat.

Apart from light-harvesting and quenching of $Chl a^{\dagger}$, Xan's are thought to have two other important roles, namely (*i*) stabilizing the structure of LHCII and (*ii*) quenching of singlet excited Chl*a* ($Chl a^{*}$) when the reaction center is closed (under excess illumination). Two mechanisms were proposed to explain this latter process. In the first one it is assumed that the S_1 state of Xan's, which has a very weak oscillator strength and does not manifest itself in the absorption spectrum, might be the key in quenching of $Chl a^{*}$ molecules. Based on model calculations on polyenes, they suggested that, under excess illumination, the reversible de-epoxidation of violaxanthin (Vio) to zeaxanthin (Zea) lowers the S_1 energy level of the latter below that of Chl*a* allowing a back transfer from Chl*a* to Zea, where the excitation is dissipated through internal conversion. Recent results, however, do not support this "molecular gear-shift" model. The other explanation does not involve the S_1 level of different Xan's but, instead, suggests that ΔpH throughout the thylakoid membrane and the xanthophyll cycle can induce structural alterations in LHCII, which result the quenching of $Chl a^{*}$. It is proposed that high ΔpH or the interconversion of Vio to Zea enhances the formation of quenching centers through, hitherto unidentified, Chl-Chl or Chl-Zea interaction while Vio inhibits that. In thylakoid membranes, due to its high self-aggregation capability, LHCII has been shown to be found in large ordered arrays. Thylakoids and lamellar aggregates of LHCII, when exposed to excess illumination, have also been shown to be capable of undergoing reversible structural changes and quenching of $Chl a^{*}$.

Major aims of this work

The present study was undertaken to investigate the disparate photophysical behaviour of LHCII at different levels of molecular organization, in suspensions of (*i*) isolated aggregates, (*ii*) intact trimers, and (*iii*) perturbed trimers, as well as (*iv*) in native thylakoid membranes. The different state of aggregation can be achieved by using non-ionic detergent. It cannot be ruled out, and indeed seems likely, that as monomers assemble into trimers, and these go on to form oligomers, additional interactions, intra-trimer (involving exterior pigments of one monomer and the interior pigments of another monomer within the same trimer) as well as extra-trimer (entailing the peripheral pigments belonging to different trimers) come into play. Our main goal was to find a connection between the extent of quenching and the changing interactions between the chromophores in LHCII and to find clues for the physical mechanism of quenching. The importance of our investigations derives from the fact that, *in vivo*, LHCII occurs in the form of oligomers, and the process of non-photochemical quenching plays an important role in protecting plants against excess illumination.

Since one of the most prominent features of aggregation is a noticeable change in the absorption spectrum compared to that of the trimers, we started our investigations with a thorough analysis of the absorption spectra of aggregates and trimers of LHCII. In order to counteract with the severe spectral distortions, due to the effects of selective scattering and the non-random distribution of trimers in the aggregates, we applied numerical correction methods to obtain the true absorption spectrum of the aggregates.

Carrying out nanosecond laser photolysis experiments on aggregated and trimeric forms of LHCII, as well as on detergent perturbed complexes, we found that the triplet formation yield of Chl*a* decreases in parallel to its fluorescence yield. Similar results were observed earlier in artificial caroteno-porphyrin dyads, which was explained by suggesting that Car's are able to enhance the internal conversion rate of a nearby Chl-like molecule. Based on this similarity, we hypothesize that the same process, which has been termed as 'catalysed internal conversion' (CIC), also operates in natural antenna complexes. The influence of a Car on a nearby Chl*a* molecule can be detected as a bleaching signal in the Q_y region of Chl*a*, which is associated with the triplet state of its Car neighbour.

We demonstrate that, in LHCII, as pigments are torn apart from each other and the influence of a nearby Car weakens, Chl*a* suffers less quenching.

We also recorded the triplet absorption spectra of a few Car's in various organic solvents in order to find a clue for the locations of their singlet and triplet absorption peaks in LHCII. It also helped us to rationalize the differences in the triplet-minus-singlet (TmS) spectrum of trimers and aggregates.

As a possible explanation for the dissipation of excitation energy of Chl*a*, reverse energy transfer from Chl*a* to Xan — which has been proposed though not yet observed — cannot be ruled out on the basis of the energy levels of the chromophores. Similarly, in the first generation caroteno-porphyrin dyads the S_1 energy level of the Car moiety was lower than that of its (Chl-like) partner molecule, thus allowing a direct quenching via the transfer of excitation energy from the partner molecule back to the Car. In order to show the generality of catalysed internal conversion, we investigated the photophysical properties of two caroteno-pyropheophorbide dyads, where the energy levels were unfavourable for *draining away excitation from the pyropheophorbide molecule by the Car moiety*. A Car-induced decrease in the fluorescence of its partner molecule must entail a process other than energy transfer.

The bleaching signal in the Q_y region of Chl*a* (or a Chl-like) molecule has been observed in a wide range of natural as well as man-made systems, where Car's are in close proximity to their partner molecule. The absence of a bleaching signal in the TmS spectrum of chloroplasts, as it was reported earlier, would imply that a prerequisite of catalysed internal conversion does not hold in green plants. This peculiarity urged us to reinvestigate this vexed issue.

Materials and methods

Chloroplasts and LHCII were isolated from leaves of two-week-old pea (grown in the greenhouse) or fresh spinach from a local market. The isolation procedures for chloroplasts and LHCII are described in the Thesis. The basic procedure applied for LHCII isolation yields four different types of preparations, depending on the concentration of detergent used for the solubilization of thylakoid membranes. These products differ from each other

in their macrostructure, lipid content and composition, content of minor antenna complexes as well as in their spectroscopic features. In our investigations we used samples of loosely stacked lamellar aggregates (type II) or large three-dimensional aggregates with long-range chiral order (type IV).

The pigment composition of the LHCII preparations used in this study was determined by applying the spectrum-reconstruction method. We found that each LHCII monomer in our samples contains 7 molecules of Chl*a*, 6 of Chl*b*, 2 of Lut, 1 Neo and a sub-stoichiometric amount (1/4–1/3) of Vio.

Two caroteno–pyropheophorbide dyads (**F-Φ** and **P-Φ**, where **Φ** stands for methyl pheophorbide-*a* and the Car moiety being fucoxanthin in **F-Φ** and peridinin in **P-Φ**) served as model systems of the light-harvesting antenna complexes in some experiments.

Lycopene and β-carotene were obtained from tomato fruits. Aggregated LHCII or petals of daffodil served as the source of lutein, neoxanthin, and violaxanthin. The carotenoids were separated by means of thin-layer chromatography (TLC).

Two different spectrophotometers were used for recording absorption spectra. One was a commercial double-beam instrument (Shimadzu 160A), which was connected to a PC for data processing. The other instrument was a single-beam fiber-optic spectrometer with an optical multichannel analyzer where at the detection side we used two different disperser-sensor systems: (*i*) an identical pair of a concave holographic grating and a 512-element diode array or (*ii*) a triple-grating monochromator with a gated and intensified diode array.

In case of large aggregates obtaining the true absorption spectra is handicapped by two effects: (*i*) selective light-scattering and (*ii*) mutual shadowing of trimers within a single aggregate. We applied numerical methods for correcting the measured absorption spectrum of the aggregates for selective scattering and for the non-random distribution of trimers in the sample.

Triplet-minus-singlet (TmS) spectra were recorded by means of a multichannel nanosecond laser flash spectrometer with right-angle geometry, as it is described in more details in the Thesis. The excitation pulse (with a duration of about 7 ns and an energy around 10 mJ) came from the tunable output of an optical parametric oscillator.

A 250 W xenon arc lamp and a mechanical chopper provided the analysing beam. The delay between the excitation pulse and the gating pulse was varied between 20 ns and 40 μs, and the gate width was held at 50 ns. For approaching oxygen-free conditions, high-purity argon was bubbled through the sample.

Fluorescence excitation spectra were recorded by using a commercial Spex Fluorolog 2 spectrofluorimeter. We operated the instrument in single beam mode and applied the *in situ* measurement of excitation intensity in order to correct the spectra for the spectral distribution of the excitation light source.

All measurements were carried out at ambient temperature (ca. 295 K).

Results

When allowance is made for scattering and sieving, the absorption spectrum of LHCII aggregates turns out to be almost identical with that of the trimers. Intertrimer interactions due to aggregation exert little influence on the absorption spectrum of the trimers. It implies that the rate constant for radiative relaxation of Chl*a** is not affected by aggregation. As a byproduct of our investigations we have the possibility to estimate the size of the aggregates. Our analysis of the trimer–aggregate system provides the first experimental evidence for the applicability of Duysens' method for the correction of the absorption spectra of turbid samples (Naqvi *et al.*, 1997a).

Our data have revealed that, when aggregates disassemble into trimers, the peripheral Xan's cease to contribute to the TmS spectrum, thereby narrowing the positive absorption band associated with Car†. Consequently, the bleaching signal in the Q_y region of Chl*a* — due to an adjacent Car† — is less pronounced in isolated trimers than that in the aggregates. Although the 100% efficiency of triplet ET from Chl*a* to Car and the lifetime of Car† are not affected by aggregation, the triplet formation yield decreases significantly. Taking into account the parallel decrease of the fluorescence yield we hypothesize that Car's enhance the internal conversion rate of the nearby Chl*a* molecules. The process of Car-mediated catalysed internal conversion (CIC) provides a channel for the non-photochemical dissipation of excitation energy of Chl*a* (Naqvi *et al.*, 1997c).

Detergent induced perturbation of the chromophoric organization of the complex severs the contact between the chromophores to such an extent that Chl*b*† starts to form, as a deactivation product of Chl*b**, competing with singlet ET from Chl*b* to Chl*a*. The triplet yield of Chl*a* continues to increase and the efficiency of triplet ET from Chl*a* to Car decreases. These results can be grasped if we assume, as we did in the case of intact trimers, that the internal conversion in Chl*a** is faster (slower) in the presence (absence) of a Car neighbour (Naqvi *et al.*, 1999).

Triplet absorption spectra of some Car's revealed only one vibronic progression in the 250-900 nm region, which belongs to the same electronic transition. The 0-0 peak was found to be the largest in contrast with the singlet absorption spectrum, where the 0-1 peak is the most intense. It overlaps but is red-shifted with respect to the singlet absorption progression and the two spectra move by almost the same amount, as the solvent changes (Jhutti *et al.*, 1998; Jávorfi & Naqvi, 1999). These results do not coincide with the previously proposed assignments of the location of Xan triplet absorption peaks in LHCII.

In caroteno-pyropheophorbide dyads, where the relative energy levels are not favourable for singlet ET back to the Car moiety, quenching in the pheophorbide fluorescence was observed. Depending on the Car moiety and the solvent, the dyad adopts different conformations. Though fluorescence quenching was observed regardless of the conformation, Car†-induced bleaching signal in the Q_y region of the partner molecule is present only if the conformation of the dyad allows fast (within about 100 ns) transfer of triplet excitation, i.e. considerable overlap of the π-electrons between the chromophores. These results indicate that the mechanism of catalysed internal conversion may still provide a relaxation channel even if the energy levels are not suitable for transfer of excitation energy. It supports our previous hypothesis that one should consider indirect effects, such as the catalysed internal conversion, when trying to explain non-photochemical quenching in natural antenna systems (Osuka *et al.*, 1999).

Our investigation on the TmS spectra of intact chloroplasts and thylakoid membranes revealed — in contrast what was published earlier — a bleaching signal in the Q_y region of Chl*a*, similar to those found in a wide variety of natural antenna complexes as well as in artificial systems, where a Chl (or a Chl-like molecule) and a Car was in intimate contact (Jávorfi *et al.* 1999). This result implies that the mechanism of catalysed internal conversion also operates in green plants.

List of publications

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- The results published under these titles have not been included in the Thesis.