Interfacing fast spectroscopy and molecular biology reveals mechanisms of light reactions in photosynthesis

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Introduction

The first step of photosynthesis consists of sunlight absorption by pigments (chlorophylls and carotenoids) followed by excitation energy transfer to reaction centres (RC). Light reactions take place in two supramolecular complexes, PhotoSystems (PS) I and II, embedded in the inner membrane of Chloroplasts. In each PS, a core complex is surrounded by peripheral antenna or light harvesting complexes (Lhc).

The members of the Lhc family coordinate a high number of chromophores per polypeptide, namely 8-13 chlorophylls (Chl) and 2-4 carotenoids (Car). Identification and experimental modification of the aminoacid residues involved in pigment co-ordination is difficult because of the non-covalent nature of binding. Since Lhc proteins are highly homologous, it has been possible to identify invariant residues as putative ligands for Chls. A new expression system[1] has been devised to produce recombinant proteins, modified in their chromophore content, which were studied by differential spectroscopy. The mutational approach allowed the identification and characterisation of the contribution of chlorophylls bound to the minor antenna system CP29, a member of Lhc family.[2]

Lhc proteins also play a key role in photosynthetic transfer and dissipation of energy in excess. The excitation transfer between chromophores of the antenna systems are extremely fast processes accessible exclusively by time-resolved spectroscopy, for example transient absorption spectroscopy (picosecond time scale) and fluorescent decay kinetics (nanoseconds time scale). Femtosecond pulsed lasers in, respectively, multi-colour pump-probe and single-photon counting configurations, accomplish the necessary time resolution.

Actually, the importance of Cars as major photoprotective agents in plants (xantophylls cycle) was demonstrated by time-resolved fluorescence on recombinant CP29 with modulated pigment content.[3]

Here, transient absorption is performed on CP29 at room temperature (RT), for investigating the kinetics of pigment excited states upon initial excitation of Chl-b pool and simulated in the frame of the Förster mechanism using pigment distances from an LHCII model[4] (the only Lhc structure experimentally resolved).

Steady-state spectroscopy and mutational approach

Photosinthetic antenna are spectroscopically complex objects; for example, in the red-most part of the visible (Q_y region) they show the overlap of many optical transitions associated with energetically-distinct binding sites while only two Chl species, Chl-a and b, are present.[5] Recently, some insight into this heterogeneity has been obtained by the mutational approach: all the outer antenna of PSII were

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successfully reconstructed *in vitro* after pigment mutation. Recombinant apoproteins are given by over-expressing the lhcb genes in which individual chlorophyll binding residues are substituted in bacteria (*E.Coli*) with residues unable to make co-ordination (see Figure 1). After refolding with purified pigments, proteins missing specific chromophores are obtained. The differential analysis by Absorption (A) spectroscopy, that is wild type A minus mutant A, reveals the spectral form of the missing chlorophyll in the mutated site and the associated main transition wavelength.

A further step is accomplished by coupling the mutational approach with linear dicroism spectroscopy (LD) on Lhc proteins. Included in polyacrylamide gel and oriented by compression these complexes approach their natural configuration like in a membrane plane. In principle, LD yields the azimutal angle φ, with respect to the plane normal, of each chlorophyll dipole moment whose Q_y transition is pointed out by differential spectroscopy. Theoretically,[6] the relationship between absolute LD and A signal is given by:

$$LD = \tfrac{3}{2} A\,[1 - 3 \bullet \cos^2(\varphi)] \qquad (1)$$

Recently,[7] we succeeded in directly normalising both LD and A differential spectra of all CP29 mutants: by combining the structural model of CP29 (Figure 1) with the LHCII structure, the spatial orientation of all eight Chls bound to the complex were determined. Finally, both A and LD of the native CP29 pigment–protein complex have been reconstructed by the same set of independent chlorophyll transition bands. In agreement with biochemical analysis, the existence of four mixed sites, i.e. sites with multiple affinity but different probability of binding a Chl-a or a Chl-b, was introduced for a good fitting (Figure 2).

Time-resolved absorption spectroscopy

After absorption of the solar light by their pigments, antenna systems funnel the energy toward the RC. From Cars to Chl-b and finally Chl-a and RC, the excitation steps down lower and lower energy levels: the small energy gap guarantees an irreversible flow of energy with an efficiency of more than 90% in plants. The non-radiative en-

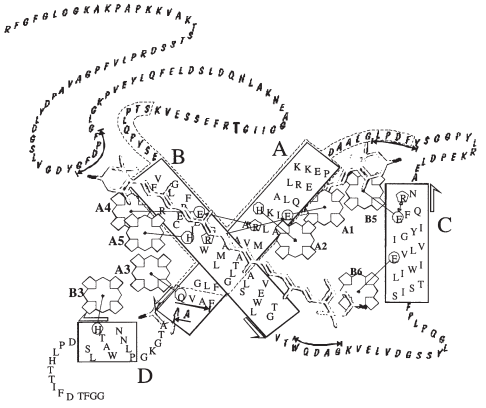

Figure 1. Model of CP29 pigment-protein complex in the membrane plane. Capital letters label α-helices (boxes). Circled letters identify side-chain ligands of chlorophylls (tetrapyrrole planes in grey) and carotenoids are long chain structures.

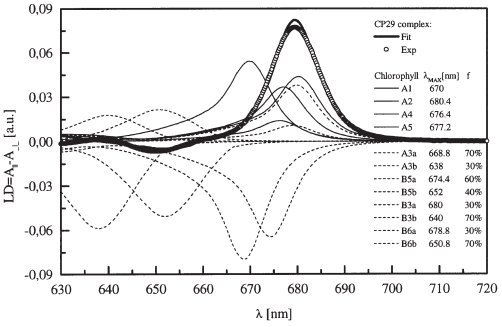

Figure 2. Linear dicroism spectroscopy of native CP29 and reconstruction by independent chlorophyll sub-bands: main absorption wavelength and chlorophyll population is reported for each binding site.

ergy transfer among Chls of Lhc systems takes place in a few picoseconds and time-resolved absorption can investigate the kinetics of excited states.

Femtosecond transient absorption was performed by a pump-and-probe laser system[8] on native CP29 at RT and initial excitation Chl-b absorption at 650 nm. Schematically, subpicosecond excitation flashes were generated by a Ti-Sapphire laser oscillator. The pulses, with a FWHM of about 70 fsec, were frequency-shifted using an optical parametric generator with nearly transform limited output. Part of the beam from the amplifier was used to generate a super-continuum white light by frequency broadening. This probe beam and the major pump pulse were relatively polarised at the magic angle to exclude kinetic depolarisation effects, while the detection was performed by a spectrograph/diode array system with a signal-to-noise ratio of 10^5. The low photon density flux of less than 10^{14} ph sec^{-1} cm^{-1} per pulse at the repetition rate of 3 kHz avoided bleaching of the sample.

In the difference A spectra, that is time-resolved A minus steady-state one, the signal at different times reflects the excited state population of chlorophylls absorbing in the detected wavelength window. Neglecting the first hundreds of fsecs, to avoid ultrafast coherent effects and detector response artefacts, the excitation of Chl-b has completely moved into a Chl-a pool (absorbing at longer wavelengths) in about 1 psecs.

Förster mechanism and transient absorption simulation

Mutational analysis coupled to differential spectroscopy gave Q_y energy levels and dipole moments associated to each Chls in the minor antenna CP29. With chromophore distances available by homology with an LHCII structure, all the necessary parameters for modelling the energy transfer within CP29 are known. Concerning the mechanism, Förster theory[9] is generally assumed[10] for chlorophylls bound to Lhc proteins when excluding coherent effects (subpicosecond scale). In brief, two Chls, far enough to be weakly coupled, interact by Coulomb (dipole–dipole) mechanism and their energy transfer rate is:

$$K_{DA} = \frac{k^2}{\tau_D}\left(\frac{R_0}{R_{DA}}\right)^6 \tag{2}$$

Notice the inverse dependence with the distance R^6. R_0 accounts for overlap integral between the donor (D) fluorescence and the acceptor (A) absorbance (or energy matching between the transitions) and τ is the free-pigment fluorescence lifetime (4 to 5 nsecs for Chls). We calculated the *ab initio* transfer rates between the eight Chls of CP29, but due to the four mixed sites the final result is a 12 by 12 element matrix which fully describes the energetic coupling in the antenna system. In essence, the excitation pathway shows that the ensemble of four pure Chl-a is easily and rapidly equilibrated (K from 10 to 50 $psec^{-1}$), and is surrounded by four additional weakly-coupled mixed sites. When populated by Chl-a these sites are also energetically equilibrated but weakly (K around 1 $psec^{-1}$), otherwise the excitation flows slowly and outwardly from them. The only exception is site B5 (see Figure 1) which is always equilibrated independently of the Chl species (K from 1 to 10 $psec^{-1}$). The excitation kinetics are fully determined on solving the Master equation,[10] i.e. the set of linear differential equations describing the time evolution of the excitation probability (p) of each i^{th} Chl:

$$\dot{p}_i(t) = \sum_j p_j(t)K_{ji} - p_i(t)\sum_j (K_{ij} + K_{diss}) \tag{3}$$

here, K_{diss} includes all de-excitation processes. The Master equation of CP29 chlorophyll-binding complex has been solved numerically by a finite difference method; for comparison with experiments, the initial condition was set to excitation probability equal to 1 for Chl-b absorbing at 650 nm. The simulated differential A of Figure 3 results from the overlap of Chl spectral forms, each weighted by its

time dependent excitation probability. In respect of the actual data, the simulations in Figure 3 reproduce most of the experimental features, in particular the rapid stepping down of energy from Chls-b to Chls-a and the wide absorption bands associated with the pools. Below 640 nm, the signal is over estimated because of the high-energy components present in the steady-state Chl-a band used.

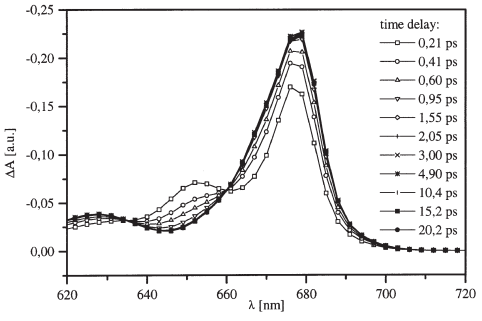

Figure 3. Simulated differential absorption of native CP29 at RT in terms of Förster transfer between chlorophylls upon excitation of chlorophyll b at 650 nm: different signs indicate different time delay between pump and probe.

Conclusions

It is shown that a mutational approach and differential analysis are powerful tools to characterise spectroscopically light harvesting complexes. For the first time, the transfer rates in an antenna protein, namely CP29, have been calculated *ab initio* on the basis of the Förster mechanism. The transient absorption was obtained by numerically integrating the master equation; the simulations agreed with the experiments by ultrafast spectroscopy on native CP29 at room temperature.

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