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Excitation transfer and trapping kinetics in plant Photosystem I probed by two-dimensional electronic spectroscopy

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Abstract

Photosystem I is a robust and highly efficient biological solar engine. Its capacity to utilize virtually every absorbed photon's energy in a photochemical reaction generates great interest in the kinetics and mechanisms of excitation energy transfer and charge separation. In this work, we have employed room-temperature coherent two-dimensional electronic spectroscopy and time-resolved fluorescence spectroscopy to follow exciton equilibration and excitation trapping in intact Photosystem I complexes as well as core complexes isolated from *Pisum sativum*. We performed two-dimensional spectroscopy measurements with low excitation pulse energies to record excited-state kinetics free from singlet-singlet annihilation. Global lifetime analysis resolved energy transfer and trapping lifetimes closely matches the time-correlated single photon counting data. Exciton energy equilibration in the core antenna occurred on a timescale of 0.5 ps. We further observed spectral equilibration component in the core complex with a 3–4 ps lifetime between the bulk Chl states and a state absorbing at 700 nm. Trapping in the core complex occurred with a 20 ps lifetime, which in the supercomplex split into two lifetimes, 16 ps and 67–75 ps. The experimental data could be modelled with two alternative models resulting in equally good fits – a transfer-to-trap-limited model and a trap-limited model. However, the former model is only possible if the 3–4 ps component is ascribed to equilibration with a “red” core antenna pool absorbing at 700 nm. Conversely, if these low-energy states are identified with the P₇₀₀ reaction centre, the transfer-to-trap-model is ruled out in favor of a trap-limited model.

Keywords: excitation energy transfer, light harvesting, light-harvesting complexes, multidimensional spectroscopy, ultrafast spectroscopy

Introduction

Photosystem I (PSI) of green plants encompasses more than 200 pigments and cofactors, bound in a 650 kDa pigment-protein complex consisting of up to 18 protein subunits, performing a photocatalytic reaction with a quantum efficiency of nearly unity (Nelson and Junge 2015). It possesses two physically distinct structural moieties. The core complex, which is strongly conserved among all oxygen-evolving photosynthetic organisms, binds 98 chlorophyll (Chl) *a*, including the reaction centre (RC) Chls, 22 β -carotene molecules and all carriers of the electron-transport chain between the external electron donor and acceptor, plastocyanin (or cytochrome) and ferredoxin (Qin et al. 2015; Mazor et al. 2015). The second moiety is the peripheral antenna, light-harvesting complex I (LHCI), consisting of four Chl *a/b*-binding proteins, Lhca1–4.

A distinct feature of PSI of almost all organisms is the presence of long-wavelength Chl spectral forms – “red” Chls – that have lower energy levels than the RC Chl P₇₀₀ (Karapetyan et al. 2006). To utilize the energy absorbed by the “red” Chls, excitations must be transferred energetically uphill to the RC (Jennings et al. 2003). Despite that uphill excitation energy transfer (EET) slows down the overall trapping, the quantum efficiency of PSI remains very high (Gobets and van Grondelle 2001). Depending on species, the PSI core complex of cyanobacteria contains several such “red” Chls. However, in plants they are located primarily, or exclusively in the peripheral antenna,

LHCI, showing broad absorption and emission bands with maxima around 715 and 735 nm, respectively (Croce et al. 1998; Croce et al. 2002).

The processes of EET and trapping in PSI of plants, photosynthetic bacteria and algae are extensively studied by various time-resolved spectroscopy techniques, including fluorescence and femtosecond transient absorption. Briefly, spectral equilibration in the core antenna has been found to occur on a timescale from 100–150 fs to several ps. Several studies have resolved spectral changes with lifetimes in the range of 2–6 ps in cyanobacteria (Savikhin et al. 1999; Savikhin et al. 2000; Gobets and van Grondelle 2001; Andrizhiyevskaya et al. 2004; Melkozernov et al. 2000b) as well as green algae (Gibasiewicz et al. 2001; Gibasiewicz et al. 2007; Melkozernov et al. 2004), ascribed to EET between the bulk Chls in the core and “red” Chls. Overall trapping in the core complex occurs by one major phase around 20 ps but the lifetime depends on the amount and energies of the “red” Chls (Gobets and van Grondelle 2001). In the PSI–LHCI complex of green plants, trapping shows an additional phase between 50 and 130 ps (Croce et al. 2000; Ihalainen et al. 2002; Ihalainen et al. 2005; Jennings et al. 2003).

The excited-state kinetics is described in the literature by two alternative models, differing from each other by the assignment of the rate-limiting step. According to the trap-limited model, excitations are rapidly equilibrated between the antenna and the RC and the kinetics is limited by charge separation. (Melkozernov et al. 1998; Müller et al. 2003). The current prevalent view, however, is that, because of the spatial separation of the RC Chls, EET from the antenna to the RC is slow, hence the kinetics is transfer-to-trap-limited (Valkunas et al. 1995; Savikhin et al. 2000; Savikhin et al. 2001; Gobets and van Grondelle 2001).

The complexity of the PSI antenna, the large number of pigments and spectral overlap makes it difficult to unambiguously interpret the spectroscopy data and distinguish between different kinetic models. Two-dimensional electronic spectroscopy (2DES) has distinct advantages in following the kinetics of EET. 2DES is a third-order nonlinear optical spectroscopic technique that measures a frequency–frequency correlation map linking the electronic excited state frequencies of coupled transitions. We have recently shown that exciton equilibration can be explicitly resolved in the excited-state kinetics of light-harvesting complex II by room-temperature 2DES (Akhtar et al. 2017), reducing the uncertainty in ascribing kinetic lifetimes to EET processes. Previously, 2DES was performed on PSI complex from cyanobacteria demonstrating the dynamics of fast EET (Anna et al. 2012) on the timescale up to several hundred femtoseconds. However, no 2DES studies have been reported on PSI from eukaryotic organisms and only very limited femtosecond spectroscopy data overall are available for higher plant PSI.

The high resolution crystal structures of PSI from *Pisum sativum* (pea) by Nelson’s and Shen’s groups (Qin et al. 2015; Mazor et al. 2015) reveal new structural information about the green plant PSI supercomplex, including specific interactions between the core complex and LHCI. These, in combination with detailed ultrafast kinetics data might improve our understanding of the processes of EET and to draw a physical link between the EET function and the available structure. With this in mind, we performed room-temperature 2DES and time-resolved fluorescence studies of isolated PSI core complexes and intact PSI–LHCI complexes from *Pisum sativum* to extract information on the dynamics of EET and charge separation in plant PSI from the femtosecond to nanosecond timescales. Analysis of the 2DES results confirmed, with high degree of accuracy, lifetimes and spectral features of PSI obtained by a host of independent time-resolved spectroscopy experiments while providing new additional information. We were able to unequivocally resolve uphill and downhill spectral equilibration components in the core complex on timescale of 0.5–4 ps and slower (tens of ps) EET associated with LHCI. Finally, we performed kinetic model fitting in terms of the two prevailing models in the literature and show that while the kinetics can be interpreted with both a trap-limited and a transfer-to-trap-limited model, the latter model contradicts with the fact that “red” Chls in plant PSI are located in the peripheral antenna.

Materials and methods

PSI preparation

Starting material for the isolation was PSI-enriched stromal membrane vesicles isolated from pea leaves according to the protocols of Peters et al. (1983). The preparations having chlorophyll (Chl) *a/b* ratio of 9–10 were further purified by the solubilization with dodecyl- β -maltoside (β -DDM) and sucrose density gradient ultracentrifugation in Akhtar et al. (2016) to obtain PSI-LHCI complexes. PSI core complexes was isolated by further solubilisation

of purified PSI–LHCI suspensions at about 0.3 mg/ml Chl with 1% β -DDM and 0.5% Zwittergent-16 and density gradient centrifugation. The gradient was prepared by repetitive freezing and thawing of 0.4 M sucrose, 10 mM tricine buffer (pH 7.8) and 0.06% β -DDM. The samples were centrifuged in a swing-out rotor at 200 000 g for 25 h. Fractions of the gradient containing the PSI core were collected, concentrated with 30 kDa cutoff Amicon filters (Millipore) and stored at -80°C until use. The chlorophyll content of the PSI-LHCI and PSI preparations was determined from acetone extracts using the coefficients of Porra (1989). PSI-LHCI preparations had Chl *a/b* ratio of 12.8 (Online Resource Table S1), which closely matches the pea crystal structure of Qin et al. (2015). The purity of the PSI core preparations was evaluated by their Chl *b* content ($< 0.4\%$) and Lhca1 content (western blot analysis, Online Resource Figure S1). From the analysis, we estimate a stoichiometry ratio of < 0.2 LHCI subunits per PSI core.

Both samples PSI–LHCI and PSI core used for 2D experiments exhibited 0.3 optical density at the Chl *a* Q_y band (680 nm) in a 1 mm path length and contained 20 mM sodium ascorbate and 60 μM phenazine methosulfate to ensure rapid recovery of the RC. The samples were continuously circulated during the measurement to avoid the encounter of a complex with the same laser pulse twice. The absorption was recorded before and after the experiment to control the sample integrity.

Time-resolved fluorescence

Fluorescence decays were recorded at room temperature by time correlated single-photon counting (TCSPC) with a FluoTime 200 instrument (PicoQuant, Germany) equipped with a microchannel plate detector (Hamamatsu, Japan) and a PicoHarp 300 TCSPC system (PicoQuant). Excitation pulses at 633 nm with 20 MHz repetition rate, 6 ps temporal width, and 0.1 pJ pulse energy were generated by a WhiteLase Micro supercontinuum laser (Fianium, UK). Fluorescence emission was detected through a monochromator at wavelengths between 672 and 744 nm with 8-nm steps. The sample was continuously circulated through a flow cell with 1.5 mm path length. The optical density of the sample was adjusted to 0.02 at the excitation wavelength. Fluorescence decays collected at different detection wavelengths were analyzed by a global lifetime fitting routine using a kinetic model and convolution with the measured IRF and the analysis routines were written in MATLAB.

2D spectroscopy

Fourier-transform femtosecond 2DES was carried out in a partially collinear “pump-probe” geometry using an experimental laser system described earlier (Zhang et al. 2012) with the following modifications. The first two interaction pulses (excitation pulses) were tuned to a central wavelength of 680 nm with 18 nm bandwidth, matching the Q_y absorption band of Chl *a* in PSI, 45 fs temporal width and energy of 0.5–0.8 nJ per pulse. An acousto-optic programmable dispersive filter pulse shaper (Dazzler, Fastlite) was used to scan the coherence time delay t_1 from 0 to 150 fs with 3 fs increments in a 1×2 phase-cycling scheme.

White-light supercontinuum generated in a 2 mm sapphire window was used as the third interaction pulse (probe) and local oscillator. The probe beam is spatially overlapped with the pump beam on the sample and sent to a spectrometer (Acton SP2300, Princeton Instruments) equipped with a CCD detector (Pixis 100B, Princeton Instruments) to spectrally resolve the signal. The probe light was plane-polarized at the magic angle (54.7°) with respect to the polarization of the excitation pulses. The time delay between the second excitation pulse and the probe pulse (population waiting time T_w) was varied from -0.3 to 600 ps in a logarithmic progression.

Results and discussion

Fluorescence kinetics

The excited-state decay kinetics of PSI core complexes and PSI–LHCI complexes in the tens of picoseconds to nanoseconds range was followed by TCSPC boasting high dynamic range and accuracy. The fluorescence decays, recorded at different wavelengths over a 4-ns time period were subjected to global analysis with a five-exponential model. The kinetics of PSI–LHCI complexes exhibited lifetimes of 16 ps, 71 ps, 172 ps, 2.2 ns and 5 ns (Fig. 1a), virtually identical to recently published data (Akhtar et al. 2016). The core complex fluorescence decayed with lifetimes of 22 ps, 41 ps, 215 ps, 2.3 ns and 5.6 ns (Fig. 1b). Overall, the lifetimes and decay-associated emission

spectra (DAES) of both types of preparations show a high degree of similarity with the fluorescence kinetics of *A. thaliana* PSI reported by Wientjes et al. (2011b). Decay components with >1 ns lifetime can be excluded from further analysis as they originate from negligible amounts of LHCI unattached to the core (2.2 ns, $<1\%$ amplitude) and free Chls (5 ns, $<3\%$), which is evident if the spectra are compared with those of isolated LHCI (Fig. 1c). We shall also exclude from further analysis lifetime components of very small amplitudes, viz. 41 ps and 215 ps in PSI core and 172 ps in PSI-LHCI. Although components of very similar lifetime and DAES have been reported in several studies (Slavov et al. 2008; van Oort et al. 2008; Wientjes et al. 2011b) it is possible that they represent minor subpopulations of complexes. Then, the PSI core fluorescence decay is described with a single 22 ps lifetime and the PSI-LHCI kinetics is biexponential with lifetimes of 16–17 ps and 70–75 ps.

Evidently, the 22 ps DAES of the isolated core has a very similar spectral shape with the 16 ps component of PSI-LHCI, representing the overall excitation trapping in the core complex. Notably, the trapping lifetime is slightly longer in PSI-LHCI than in the isolated core. The 71 ps component shows dominating emission from low-energy “red” Chls (Engelmann et al. 2006; Ihalainen et al. 2000; Wientjes et al. 2011b) and its spectral shape is nearly identical to the emission spectrum of LHCI (Fig. 1c). Thus, the core complex and the peripheral antenna retain their spectral identities in the DAES.

The fluorescence kinetics of PSI-LHCI can be described with a simple two-compartment model encompassing energy equilibration between the “red” states in the antenna and the bulk Chls (Online Resource, Fig. S2). If the effective trapping rate constant is fixed to $(22 \text{ ps})^{-1}$, the best-fit forward and reverse red-bulk equilibration rate constants are 18 and 13 ns^{-1} . The inverse of their sum is a proxy for the equilibration time – 32 ps. The overall equilibration time is the sum of several EET times – the equilibration time between bulk and red Chls in LHCI (Lhca3/4), EET time between LHCI subunits (e.g. between Lhca1 and Lhca4), and EET from LHCI subunits to the PSI core complex. The two-compartment model cannot discriminate between these EET processes. Wientjes et al. (2011a) have shown that the timescale of red-bulk equilibration in isolated LHCI is about 16 ps. Since the overall equilibration time in PSI-LHCI is double (32 ps), it follows that the timescale of EET from the bulk LHCI Chls to the core is also in the range of 10–20 ps.

Two-dimensional spectra

The 2DES experiments were performed with excitation pulses centred at 680 nm that spectrally overlap the Q_y absorption region of Chl *a* (Online Resource, Fig. S3) to reveal the dynamics of excitation energy redistribution in PSI-LHCI and PSI core on a femtosecond–nanosecond timescale. The maximal transient absorbance signal (at 680 nm) for all experiments was no more than 2 mOD. Based on the excitation pulse energies, as well as on the magnitude of the transient signal, we estimate, on average, less than one excitation in a PSI complex per pulse. Therefore, singlet-singlet annihilation has negligible effect on the kinetics.

Representative purely absorptive 2D spectra of PSI core complexes and intact PSI-LHCI for selected waiting times T_w are plotted in Fig. 2. The vertical axis represents the excitation wavelength λ_1 and the horizontal axis represents the detection wavelength λ_3 . Spectra were recorded at different delay (waiting) times T_w following the dynamics of the system after the initial excitation. At detection wavelengths longer than 670 nm the transient absorptive signal is contributed mainly by Chl *a* ground state bleaching (GSB) and stimulated emission (SE). In principle, the intensity of the signal at coordinates (λ_1, λ_3) reflects the concentration of excited states absorbing at λ_3 , which have been populated via EET from initially excited states absorbing at λ_1 . In the absence of EET or spectral diffusion, the 2D signal is found along the diagonal line, $\lambda_1 = \lambda_3$. On the other hand, excitonic coupling and EET between two states give rise to cross peaks at λ_1 and λ_3 corresponding to the donor and acceptor state transition energy, respectively. The rise kinetics of the cross-peak signal can be examined from traces at selected λ_1/λ_3 (Online Resource, Fig. S4). In addition to EET, cross-peak signals can also originate from spectral diffusion, i.e. fluctuations in the excited-state energy levels. Due to the large spectral overlap and very small energy gaps in the Chl *a* Q_y region, cross peaks are not resolved in the 2D spectra. Nevertheless, the time evolution of the 2D spectra holds information regarding the pathways and dynamics of EET.

In a system of interacting chromophores with varying excitation energy levels, EET will occur from higher-energy to lower-energy states, a thermodynamically favourable direction causing a shift of the 2D signal to longer detection wavelengths λ_3 , i.e. to the left of the diagonal line. This fact is very well represented by 2D spectra of PSI-LHCI (Fig. 2). Inspecting the 2D spectrum at $T_w = 0.15$ ps, it is clear that within this time a part of the energy

is already transferred from initially excited states absorbing at $\lambda_1 = 675\text{--}685\text{ nm}$ to lower energy states at $\lambda_3 = 690\text{--}700\text{ nm}$. The evolution of the 2D spectrum from 0.15 ps to 5 ps shows the progressive shift to long-wavelength states ($>700\text{ nm}$), concomitant with a decrease in the overall signal intensity due to trapping of excitations by the RC. The 2D spectrum at short waiting times (T_w) shows pronounced correlation of excitation and detection wavelength but at longer waiting times, as the system becomes thermally equilibrated, this correlation is progressively lost and the spectral shape becomes independent from excitation wavelength. Because of this relationship, 2DES gives a definitive time course of thermal equilibration, which appears to be virtually complete within 5 ps in both PSI core and PSI-LHCI. The most obvious difference between the spectra of the two preparations is the extended tail at $\lambda_3 > 700\text{ nm}$ in the PSI-LHCI supercomplex, which is due to the additional “red” states in the peripheral antenna. The appearance of these long-wavelength components in the supercomplex is clearly visible within a picosecond, signifying a rapid EET to the “red” Chls in LHCI.

Fig. 3 shows the waiting time dependence of the signal at fixed excitation wavelengths (λ_1) of 675 nm, 682 nm and 690 nm. Hence, the plots represent the dynamic spectral evolution, on a logarithmic time scale, for the case when initial excitation is on the high, middle, or low energy side of the Q_y absorption band, respectively. Excitation on the blue side ($\lambda_1 = 675\text{ nm}$) leads to gradual spectral shift to longer wavelengths (downhill EET), whereas excitation in the red tail ($\lambda_1 = 692\text{ nm}$) is followed by a blue shift (uphill EET). The peak position converges for both excitation wavelengths at times longer than 10 ps. The spectral shifts reveal exciton equilibration in the Chl *a* manifold (both downhill and uphill) taking place on the time scale of hundreds of fs to about 10 ps. The same downhill/uphill EET processes are illustrated by the selected time traces in the bottom panel of Fig. 3. The time courses show energy redistribution occurring in the first few ps after excitation – the diagonal traces (675/675, etc.) rapidly decay (Fig. S4), whereas off-diagonal traces show a lag-phase (e.g. 675/690) or rise of the bleaching signal amplitude (682/700) that follows the population of states via EET. Note that EET can also be uphill (e.g. 690/675 trace). The plots also show that a component absorbing at $\sim 700\text{ nm}$ is populated at times 1–10 ps and decays afterwards.

Global analysis

For a quantitative description, the transient 2DES data were subjected to global lifetime analysis, as previously described (Wells et al. 2014). Good fit of the PSI core data in the time window 0.15–100 ps was obtained with three unconstrained exponential decay components (with lifetimes 0.49, 3.5 and 19 ps) and a non-decaying component. An overlay of the fit and measured data is shown in Fig. S4. The lifetimes are in good agreement with those obtained from pump-probe spectroscopy experiments of cyanobacteria and green algae (Andrizhiyevskaya et al. 2004; Dashdorj et al. 2005; Gibasiewicz et al. 2001; Gibasiewicz et al. 2007; Gobets et al. 2001; Melkozernov et al. 2000a; Melkozernov et al. 2004; Savikhin et al. 1999; Savikhin et al. 2000).

The pre-exponential amplitudes at different λ_1/λ_3 form two-dimensional decay-associated spectra (2D DAS), plotted in Fig. 4 for isolated core complexes. Respective one-dimensional DAS spectra obtained by integrating the 2D DAS over λ_1 can be found in Online Resource (Fig. S5). The 2D DAS amplitude shows if a 2D cross peak rises or decays with a certain lifetime and is useful to identify EET pathways. Generally, EET from state A to B is reflected in the 2D DAS as a positive (rise) cross peak at (λ_A/λ_B) and a corresponding negative (decay) diagonal peak at (λ_A/λ_A) . Conversely, the reverse EET produces a cross peak at (λ_B/λ_A) and a diagonal peak at (λ_B/λ_B) , if the excitation pulse can access λ_B . Thus, the symmetric pairs of cross/diagonal peaks in the 2D DAS can identify donor-acceptor pairs coupled by reversible (energetically downhill/uphill) EET (Akhtar et al. 2017). The first 2D DAS on Fig. 4 (0.49 ps lifetime) features a positive cross peak above the diagonal at excitation wavelengths 670–680 nm and a symmetric cross peak below the diagonal at 680–690 nm. Therefore, the 2D DAS strongly suggests that exciton equilibration between states in the 670–690 nm wavelength region occurs on a 0.5 ps timescale. Furthermore, the slanted ellipsoid shape of the cross peaks shows that multiple states of different energies are engaged in EET. In this respect, exciton equilibration in PSI is reminiscent of spectral diffusion of an inhomogeneously broadened band, which would produce the same characteristic 2D DAS shape (Akhtar et al. 2017).

The second 2D DAS (3.5 ps lifetime) exhibits a negative peak around $\lambda_3 = 685\text{ nm}$ and a broad positive cross peak at $\lambda_3 = 704\text{ nm}$. This component can describe well the net downhill EET from the bulk Chls to a pool of Chl *a* with an absorption band around 700 nm. Because the excitation pulse spectrum does not extend to 700 nm, it is not

possible to observe uphill cross peaks in this region. EET processes with a very similar spectral signature and lifetimes in the range of 2–3 ps been observed in PSI from cyanobacteria (Andrizhiyevskaya et al. 2004; Hastings et al. 1995; Savikhin et al. 1999; Gobets and van Grondelle 2001; Du et al. 1993; Dashdorj et al. 2005) and *C. reinhardtii* (Gibasiewicz et al. 2001; Melkozernov et al. 2004; Gibasiewicz et al. 2007). The spectral changes were usually attributed to equilibration between bulk antenna excited states and low-energy “red” Chls. Here we show that the plant PSI core, which contains less, if any, “red” Chls (Nelson and Junge 2015; Croce et al. 1998), exhibits the same kinetic behaviour. The 2D DAS spectrum is not conservative, i.e. the negative peak amplitude is larger than the positive peak amplitude. The stronger decay amplitude suggests loss of excitations on this timescale. We can thus argue that initial trapping already occurs on the timescale of 3 ps. The negative peak is slanted along the diagonal (inhomogeneously broadened or slow spectral diffusion), whereas the positive cross peak lacks any such correlation, which means, first, that the exciton energies are not (yet) randomized and, second, that there is minimal correlation between the donor and acceptor state energies.

The 19 ps 2D DAS has only negative amplitudes, except at detection wavelengths under 670 nm, where it shows Chl excited state absorption. The majority of excitations in the PSI core decay on this timescale. In contrast to the previous 2D DAS, the peak shape of the trapping component is completely independent from excitation wavelength, which can be confirmed by comparing horizontal slices of the 2D DAS at different λ_1 (Online Resource, Fig. S6). Therefore, we can conclude that the 19 ps component reflects trapping of excitations from a fully equilibrated antenna pool. The final 2D DAS corresponds to a state that does not decay within our experimental T_w range. It shows two distinct bleaching peaks – around 680 and 700 nm. It represents the spectrum of the oxidized RC (P_{700}^+) and probably long-lived excitations in a small number of uncoupled Chls. Horizontal slices of the 2D DAS (Fig. S6) are very similar to the P_{700}^+ transient absorption spectrum, reported for example in (Gibasiewicz et al. 2003). The non-decaying spectrum is of significantly lower amplitude than the rest, indicating that some RC remain closed between excitation cycles. From a comparison of the relative DAS amplitude, or the relative non-decaying signal amplitude at 700 nm (Fig. S4) to earlier published data on open and closed RCs (Savikhin et al. 2000), we estimate that the majority of RCs in this work must be in an open state. The non-decaying amplitude can also be reduced because of singlet-singlet annihilation and other quenching processes, for example singlet quenching by PMS (Wientjes and Croce 2012). For these reasons, the amplitudes of the DAS, and consequently SAS, are not an accurate estimate of the concentrations of the respective species.

Global analysis of the 2DES kinetics of PSI–LHCI required five components – four decay lifetimes (0.51, 3.2, 14 and 53 ps) and a non-decaying component. The resulting 2D DAS are plotted in Fig. 5. The first two (0.5, 3 ps) and the final (non-decaying) 2D DAS are essentially the same as the respective 2D DAS for the isolated PSI core. As with TCSPC, the main excitation decay lifetime was consistently found to be shorter (14–16 ps), compared to the isolated PSI core (18–20 ps). An additional 53 ps lifetime, not present in the core, is associated with the decay of “red” Chls in the peripheral antenna, absorbing at wavelengths 700–710 nm. The 14 and 53 ps 2D DAS show that trapping in the PSI supercomplex, as in the isolated core, occurs from a thermally equilibrated antenna. A comparison of the spectral shape of the main decay components for PSI–LHCI (14 ps) and PSI core (19 ps) reveals a marked difference on the long-wavelength side. A similar difference is noticeable in the fluorescence DAES of the core and the supercomplex (Fig. 1a, b) but is even more pronounced in the 2D DAS. It can be concluded that the 14 ps lifetime in PSI–LHCI reflects both trapping of excitations and equilibration with “red” Chls in the peripheral antenna. Population of “red” states results in reduced net DAS amplitude in the far-red range.

In summary, global analysis of the 2DES data of isolated PSI core complexes and the intact supercomplexes reveals spectral equilibration in the main Chl *a* Q_y band with a 0.5 ps and equilibration with “red” spectral forms at 700 nm on a 3–4 ps timescale. The main trapping lifetime in the core complex is 20 ps and additional 53 ps lifetime is associated with “red” Chls in the peripheral antenna. There is close similarity between the 2DES and TCSPC data in terms of lifetimes and spectral features.

Kinetic modelling

To examine the physical processes that determine the spectral changes and obtain the relevant EET and electron transport rate constants we performed kinetic model fitting (target analysis) of the 2DES data. In the following analysis, the spectra were integrated over a window of excitation wavelengths 670–690 nm, essentially converting the 2DES spectra to transient absorption spectra. The approach is warranted by the fact that the spectral changes

at longer delay times are independent from excitation wavelength. Subpicosecond kinetic components were excluded from the kinetic scheme. The target analysis allows us to assess whether the kinetic models produce physically realistic rate constants and species-associated spectra (Holzwarth 1996; van Stokkum et al. 2004).

Two kinetic model schemes were tested. In both cases the model comprised of two compartments for the PSI core and one for the peripheral antenna, LHC. Due to the increased number of free parameters, fitting this type of models often can end in different local χ^2 minima depending on the starting values of the fit parameters. To counter this, we performed the analysis in two steps. First, we fitted the core model (only two compartments) to the data for isolated PSI cores. Next, the full model (with LHC compartment) was set up with initial rate constants obtained from the first fit and 30% of initial excitations placed on LHC. After fitting the models to the PSI–LHCI data, the best-fit rate constants for the core compartments were found the same (within 10% margin) as they were for the isolated core complex.

The first model (Fig. 6a), which we will refer to as “red antenna” model, incorporates “red” states in the core antenna, represented by the “Core Red” compartment. Charge separation is modelled as a single decay pathway from the “Bulk” compartment. The scheme is equivalent to the model of Wientjes et al. (2011b). The rate constants, DAS and species-associated spectra (SAS) for the PSI–LHCI supercomplex are plotted in Fig. 6a. We should note that the same set of rate constants of equilibration and charge separation resulted in different apparent trapping lifetimes for the isolated core (19 ps) and the supercomplex (16 ps), in agreement with the global analysis (*vide supra*). The DAS for the supercomplex showed, as expected from the global analysis, energy equilibration occurring on a 3–4 ps timescale, followed by decay of bulk excitations with 16 ps lifetime and finally decay of “red” states in LHC with 60 ps lifetime. The LHCI–PSI equilibration time is 32 ps, almost identical to the one obtained from target analysis of TCSPC data. The SAS are single bleaching/stimulated emission bands centred at 684, 702 and ~710 nm for the bulk Chls, core red Chls, and LHC red Chls, respectively.

Thus, target analysis with this kinetic scheme produced a physically realistic model, if one assumes the existence of “red antenna” Chls in the core complex, equilibrating on a 3–4 ps timescale, and monoexponential (14 ps time constant) trapping kinetics. The kinetic scheme could well be interpreted in line with a “transfer-to-trap-limited” model, assuming that the 14 ps trapping time is limited by EET to the RC and intrinsic charge separation is very fast, so that the excited RC cannot be detected in the spectra due to its very low transient concentration. However, there are arguments against this interpretation. Note that the 3–4 ps equilibration time here has not been reported before for plant PSI. The “red antenna” states here cannot be identified with red states in other plant PSI studies. For example Wientjes et al. detected red Chls emitting around 720 nm, with 40–50 ps equilibration time. In fact, our TCSPC results are in perfect agreement with this component (note the 41 ps red-shifted DAES in Fig. 1b). Hence, to explain the 3–4 ps equilibration, we must assume the existence of additional red antenna states absorbing around 700 nm. A rough estimation of the number of Chls in the “LHC red” and “Core red” compartments is given by the thermodynamic parameters of the equilibration calculated from the ratio of forward and reverse rate constants and the SAS peak wavelengths (Online Resource Table S2). From the kinetic model it follows that both the core and LHCI must contain about 4 red Chl forms. However, the PSI core should contain fewer red antenna Chls than LHCI. According to Croce et al. (1998), PSI core complexes from maize contain at most two red forms. The reported absorption spectra are virtually identical to our PSI core preparations (Fig. S3), hence we can rule out the presence of additional red forms in the PSI core antenna. It is then reasonable to identify the “core red” compartment with the P₇₀₀ RC.

The second kinetic scheme (Fig. 6b) will be referred to as “red RC” model. It separates the core complex into a “bulk” and a “RC” compartment, the latter responsible for trapping. Note that the only difference between the kinetic schemes of the “red antenna” and “red RC” model is the location of the de-excitation (trapping) channel. Fitting resulted in essentially identical lifetimes and DAS (hence equal goodness of fit) as the “red antenna” model, although the rate constants were markedly different. The equilibration time between LHC and the core was about the same. The SAS were almost unchanged – the RC SAS matched the “Core red” spectrum’s peak position (702 nm) and half-width (19–23 nm) but with a smaller amplitude. This, and the high ratio of forward to reverse core–RC rate constants (1.6), make the model physically unlikely if the RC compartment is to represent Chl excited states. However, “RC” can be interpreted as a charge separated state (oxidized RC), which then implies a fast antenna–RC exciton equilibration, similar to Holzwarth’s trap-limited model of PSI (Müller et al. 2003; Holzwarth et al. 2005; Slavov et al. 2008). Thus, we have shown here that the same experimental kinetics of PSI can be described equally well with both alternative models. Based on the experimental data alone it is not possible to

judge which model is correct. Evidence for and against both models has been put forward and discussed extensively in the literature (Croce and van Amerongen 2013). Our results seem to favor the “red RC” model, because it can explain the kinetics without relying on the presence of multiple “red” states in the PSI core antenna.

The “red RC” model scheme is incomplete in the sense that it implies fast exciton equilibration between the antenna and RC Chls. On the other hand, the 2D DAS clearly show that energy equilibration on a 0.5 ps timescale involves the spectral region around 690 nm. Based on this we performed target analysis of the 2DES data with an extended model that includes antenna-RC exciton equilibration explicitly. The kinetic scheme consists of three compartments (antenna, excited RC, radical pair) and is identical to the PSI core model of Slavov et al. (2008). The model was fitted to the PSI core data, selecting a window of excitation wavelengths (λ_i) on the blue side of the absorption band (675 ± 5 nm). The results can be found in Online Resource (Fig. S7). The lifetimes (0.64, 4, and 19 ps) and rate constants closely matched the values reported by Slavov et al. as well as the recent model of Santabarbara et al. (2017). The SAS of the excited RC peaks slightly above 690 nm, in line with the results of Gibasiewicz et al. (2001; 2002) and the radical pair shows bleaching at 700 nm, as expected for P_{700}^+ . The model and species spectra are also in good agreement with the target analysis of transient absorption data of *Chlamydomonas* PSI, reported in (Müller et al. 2003). The excited RC is populated on a timescale of 640 fs (reported by the positive SADS amplitude at 690 nm, Fig. S7) by exciton equilibration with the antenna states. This is followed by reversible charge separation with an intrinsic rate constant of $(2.3 \text{ ps})^{-1}$ and a secondary electron transfer rate of $(10 \text{ ps})^{-1}$.

We must stress that the model was obtained by fitting spectral changes resolved along the detection wavelength only. The full 2D DAS spectra unequivocally show that spectral equilibration on 0.5 ps timescale involves a multitude of exciton states, therefore the lifetime of antenna-RC equilibration and the SAS of the “RC” are probably not accurately resolved. What is clear from the 2DES data is that direct excitations at 690 nm (not necessarily the RC) are transferred uphill in 0.5 ps. This is also well illustrated by fitting the same kinetic model to the full 2DES kinetics and examining the 2D SAS (Online Resource, Fig. S8). The 2D SAS of the “RC” compartment has pronounced symmetric antidiagonal shape that reflects spectral diffusion among a manifold of exciton states on this time range.

Conclusions

The 2DES measurements of plant PSI, reported here for the first time, allowed us to probe the exciton energy transfer and charge separation kinetics in a subpicosecond-to-nanosecond time range. Owing to the very low excitation energies used, we were able to record the excited-state dynamics without distortions from singlet-singlet annihilation. Consequently, fitting the TCSPC and 2DES transients resulted in almost the same kinetic parameters. Therefore, we have demonstrated that detailed artifact-free kinetics of the most complex systems, such as PSI, can be reliably extracted from 2DES experiments, in parallel with additional unique information that the 2D spectra provide.

Probably one of the strongest advantages of 2DES is its ability to resolve intraband exciton relaxation and identify downhill and uphill energy pathways. Our data unequivocally identify exciton relaxation in the PSI core antenna occurring mainly on a timescale of 500 fs but minor intraband equilibration (or spectral diffusion) in the 680–690 nm region is also observed on a timescale of 3 ps. Conversely, the 2D spectra can readily report if all exciton states are in thermal equilibrium. The latter is possible thanks to highly purified PSI samples (97% on Chl basis) and the reliable 2D lineshapes measured in “pump-probe” geometry 2DES. From comparison of the lineshapes along the excitation wavelength axis we conclude that thermal equilibration in the Chl Q_y band (<690 nm) is complete in about 5 ps, i.e. well before the main trapping phase.

The most significant feature of the plant PSI kinetics, which has not been reported previously, is the 3–4 ps equilibration with a 700 nm spectral component. In this respect, the spectral evolution and trapping kinetics of PSI are very similar among all groups of oxygen-evolving photosynthetic organisms, despite the fact that they differ in the Chl exciton energies, specifically regarding the so-called “red” states. Kinetic model fitting (target analysis) showed that the data can be described equally well by assigning the 700 nm component to an antenna state or to the RC. In the former case, the kinetics could be interpreted according to a “transfer-to-trap” limited model; in the latter case, the data are compatible with the trap-limited models put forward in (Holzwarth et al. 2005; Slavov et al. 2008). While we cannot unequivocally determine which model is correct, our data distinctly show Chl spectral

forms absorbing around 700 nm populated on a 3–4 ps timescale, and thus a transfer-to-trap limited model, which excludes a sizeable population of excited P_{700} , is only possible if multiple “red” Chls with different equilibration times are present in the PSI core complex.

In the PSI–LHCI supercomplex, trapping is kinetically limited by EET from LHCI, largely due to the presence of “red” states in LHCI, as has been shown by Croce and others (Croce et al. 2000; Jennings et al. 2003). In good agreement with previous works, we find that in PSI–LHCI, the overall equilibration between LHCI and the core takes place in about 30 ps. With the constraint that equilibration between the “blue” and “red” LHCI forms occurs on a time scale of 14–16 ps (Wientjes et al. 2011b), we must conclude that EET is preferentially transferred from the “blue” LHCI (Lhca1, Lhca2) to the core on a timescale of 10–20 ps, i.e. comparable with the trapping time in the core. This relatively slow EET is predicted by the latest crystal structure of PSI–LHCI (Mazor et al. 2015; Qin et al. 2015).

Taking into account all modelling results for isolated core complexes and intact supercomplexes, we can summarize the kinetics of PSI–LHCI, extending the schematic model of Croce and van Amerongen (2013). The extended scheme in terms of the “red RC” model including equilibration with LHCI is depicted in Fig. 7.

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Figure legends

Fig. 1 Decay-associated fluorescence emission spectra of isolated PSI complexes obtained from global analysis of fluorescence decays measured by TCSPC. a – intact PSI–LHCI supercomplex; b – PSI core complex; c – LHCI complexes.

Fig. 2 Representative purely absorptive 2D spectra of PSI recorded in the Chl Q_y region with excitation pulses at 680 nm at the indicated waiting times (T_w). a – PSI core; b – PSI–LHCI. The spectra are plotted in terms of excitation wavelength λ_1 and detection wavelength λ_3 . Contour lines mark areas of equal absorptive signal intensity given in the color scale bars. All 2DES are plotted on the same intensity scale.

Fig. 3 Waiting time dependence of the 2D signal at three fixed excitation wavelengths $\lambda_1 = 675$ nm, 682 nm and 690 nm. The plots can be understood as analogous to imaginary pump-probe experiments with pump wavelengths corresponding to λ_1 – i.e. on the blue and red side of the Q_y absorption band of PSI–LHCI.

Fig. 4 2D decay-associated spectra of PSI core, resulting from a four-exponential fit of the transient 2D signals from 150 fs to 300 ps with best-fit lifetimes of 0.47 ps, 3.5 ps, 19 ps and a non-decaying component. Negative amplitudes (red) represent decay and positive amplitudes – rise of the bleaching signal.

Fig. 5 2D decay-associated spectra of PSI–LHCI, resulting from a five-exponential fit of the transient 2D signals from 150 fs to 300 ps with best-fit lifetimes of 0.51 ps, 3.2 ps, 14 ps, 53 ps and a non-decaying component.

Fig. 6 Target analysis of PSI–LHCI – kinetic scheme, decay-associated and species-associated absorption difference spectra. a – “red antenna” model; b – “red RC” model. The rate constants (indicated in the scheme in ns^{-1}) and spectra were determined by fitting to the PSI–LHCI 2DES data, integrated over excitation wavelength. Subpicosecond decay components as well as the final, non-decaying trap component are omitted for the sake of brevity.

Fig. 7 Energy transfer and charge separation kinetics in PSI–LHCI. A combined scheme from modelling of fluorescence and 2DES data. The main energy and electron transfer pathways between compartments are represented by double-headed arrows with indicated effective time constants. Blue and maroon arrows represent EET between LHCI and the core, green – exciton equilibration within the core antenna, yellow – equilibration between the core antenna and RC, red – charge separation and secondary electron transfer. RP_1 and RP_2 represent oxidized states of the RC.

Delay time (ps)

$\lambda_1 = 675$ nm

$\lambda_1 = 682$ nm

$\lambda_1 = 690$ nm

a **b**