



Determination of the excitation migration time in Photosystem II Consequences for the membrane organization and charge separation parameters

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ARTICLE INFO

Article history:

Received 6 November 2007

Received in revised form 14 February 2008

Accepted 15 February 2008

Available online 4 March 2008

Keywords:

LHCII

CP29

CP26

CP24

Excitation energy transfer

Non-photochemical quenching

ABSTRACT

The fluorescence decay kinetics of Photosystem II (PSII) membranes from spinach with open reaction centers (RCs), were compared after exciting at 420 and 484 nm. These wavelengths lead to preferential excitation of chlorophyll (Chl) *a* and Chl *b*, respectively, which causes different initial excited-state populations in the inner and outer antenna system. The non-exponential fluorescence decay appears to be 4.3 ± 1.8 ps slower upon 484 nm excitation for preparations that contain on average 2.45 LHCII (light-harvesting complex II) trimers per reaction center. Using a recently introduced coarse-grained model it can be concluded that the average migration time of an electronic excitation towards the RC contributes $\sim 23\%$ to the overall average trapping time. The migration time appears to be approximately two times faster than expected based on previous ultrafast transient absorption and fluorescence measurements. It is concluded that excitation energy transfer in PSII follows specific energy transfer pathways that require an optimized organization of the antenna complexes with respect to each other. Within the context of the coarse-grained model it can be calculated that the rate of primary charge separation of the RC is $(5.5 \pm 0.4 \text{ ps})^{-1}$, the rate of secondary charge separation is $(137 \pm 5 \text{ ps})^{-1}$ and the drop in free energy upon primary charge separation is $826 \pm 30 \text{ cm}^{-1}$. These parameters are in rather good agreement with recently published results on isolated core complexes [Y. Miloslavina, M. Szczepaniak, M.G. Muller, J. Sander, M. Nowaczyk, M. Rögner, A.R. Holzwarth, Charge separation kinetics in intact Photosystem II core particles is trap-limited. A picosecond fluorescence study, *Biochemistry* 45 (2006) 2436–2442].

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1. Introduction

Photosystem II (PSII) is a large supramolecular pigment–protein complex embedded in the thylakoid membranes of green plants, algae, and cyanobacteria. It uses sunlight to split water into molecular oxygen, protons, and electrons. PSII in higher plants can be subdivided into 1) a core, consisting of the reaction center (RC) and the light-harvesting complexes CP43 and CP47 and 2) the outer antenna complexes CP24, CP26, CP29 and light-harvesting complex II (LHCII) (Fig. 1) [1]. In the RC the excitations are used to create a charge separation (CS), and to transport the electron to quinone A (Q_A) and then further along the electron transfer pathway. The outer antennae are not only important for harvesting light, but also play essential roles in several regulation mechanisms like state transition and nonphotochemical quenching [2–4].

The quantum efficiency of the CS process depends on the rate- or time constants of various processes: 1) excitation energy transfer

(EET) from the antenna to the RC, 2) CS and charge recombination, 3) stabilization of the CS by secondary electron transfer and 4) relaxation or loss processes of the excited state: intersystem crossing, internal conversion, and fluorescence.

In a previous article [5] we provided a coarse-grained method to correlate these processes to the fluorescence kinetics of PSII membranes with open RCs, i.e. with the secondary electron acceptor Q_A being oxidized. The dimeric supercomplex of PSII (Fig. 1) forms the basic unit for this coarse-grained model. A hopping rate k_{hop} was defined for excitation energy transfer between neighboring monomeric (sub)units, indicated by the bars in the same figure. Forward and backward rates were adjusted by rescaling the single hopping rates in accordance with the differences in the number of Chl *a* molecules per monomeric unit. The outer antenna complexes all transfer their excitations to the RC via CP47 or CP43. Excitations can leave the RC again into the antenna. Charge separation in the reaction center is represented either by one irreversible charge separation step with rate k_{CS} or by reversible charge transfer to the primary acceptor followed by irreversible charge transfer to the secondary acceptor. It was concluded that the excitation diffusion to the RC contributes significantly to the overall charge separation time and that the charge separation is fast

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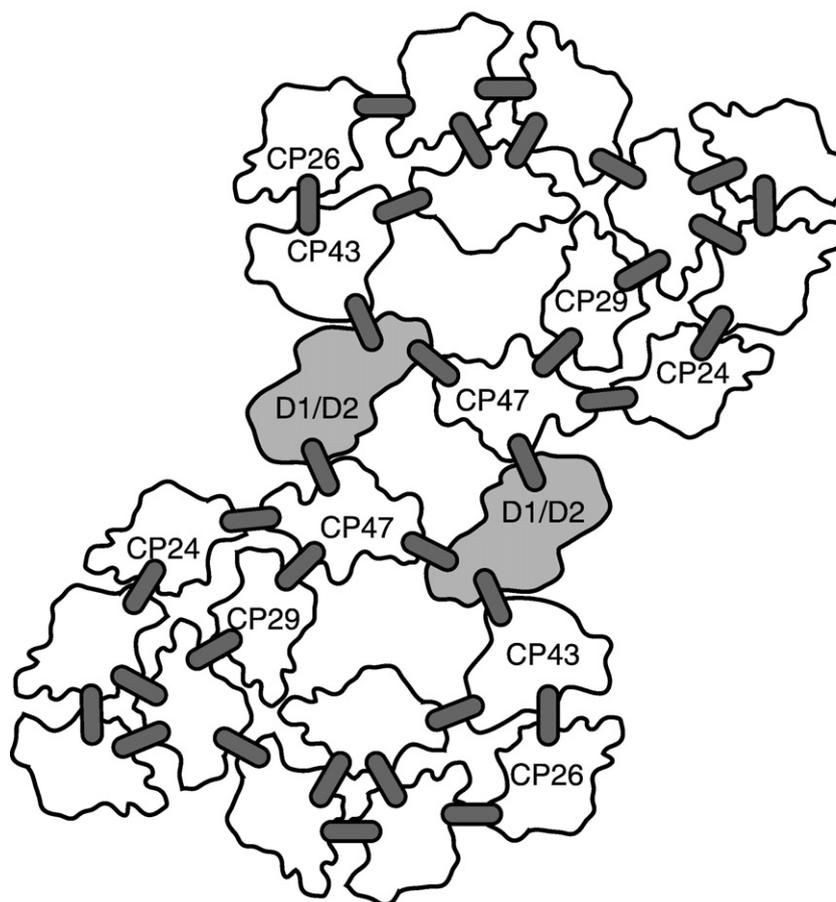


Fig. 1. Membrane organization of PS II that is used for the coarse-grained modelling. Bars represent putative energy transfer links between the light-harvesting complexes. Transfer from one complex to the other along a bar occurs with hopping rate k_{hop} . Charge separation occurs in the reaction centers (D1/D2) with rate k_{CS} .

and corresponds to a significant drop in free energy upon primary charge separation. However, the data provided a large distribution of fits of similar quality and therefore the outcome was not unique. In the present work we provide a refinement of the previous method by measuring and modelling the difference in fluorescence kinetics after preferential excitation of Chl *a* and Chl *b* in PSII membranes (so-called BBY preparations) [6]. Because there is no Chl *b* in the core, the difference in the fluorescence lifetimes is a measure for the migration time τ_{mig} , which is defined as the average time for an excitation to reach the RC for the first time (also called first passage time). Determination of the migration time is important because it also allows determination of the rate(s) of electron transfer and the drop in free energy upon charge separation in the membrane system. This allows direct comparison with values obtained for isolated RCs and cores, for which it was proposed that the drop in free energy upon charge separation occurs on a slower time scale than for more intact systems [5,7,8]. Recently, the coarse-grained model [5] was used to determine the characteristics of the charge separation of PSII at different locations in the thylakoid membranes [7]. The migration time was not known at that time and a hopping time of 17 ps was taken, reflecting excitation energy transfer from one pigment–protein complex to the other. It was concluded that the charge separation characteristics are strongly dependent on the location in the membrane. An accurate determination of the migration time will allow a refined determination of these characteristics, because it scales linearly with the hopping time. Finally, the relative contribution of the migration time to the overall trapping time is relevant for the mechanism of nonphotochemical quenching (NPQ), which protects plants from dangerous excess light conditions [5]. In the case of very fast excitation migration [9,10] one

quencher per RC may be enough to explain the quenching kinetics, whereas a higher number might be needed in the case of slow migration.

2. Materials and methods

2.1. Sample preparation

PSII membranes (BBY particles) were prepared according to Berthold et al. [6] from fresh spinach leaves. The amount of PSI (on a Chl basis) was undetectably low and at most 2%. Absorption spectra were measured with a Cary 5E spectrophotometer (Varian, Palo Alto, CA). From the absorption spectrum of the acetonic extract the Chl *a* / Chl *b* ratio was determined (See Appendix) to be 2.11 ± 0.01 and 2.04 ± 0.01 for the preparations with the fastest and slowest average fluorescence decay time. This corresponds to 2.35 and 2.55 trimers per reaction center, respectively (see Appendix). The average value for the six different preparations was 2.45.

Steady-state fluorescence spectra were measured with a Fluorolog-3.22 (Jobin Yvon-Spex, Edison, NJ) at room temperature. Time-correlated single photon counting (TCSPC) measurements were performed at magic angle (54.7°) polarization as described previously [11]. The BBY particles were diluted to an optical density of 0.08 per cm at 420 nm in a buffer of 20 mM HEPES pH 7.5, 15 mM NaCl, 5 mM $MgCl_2$, 0.0003% β -DM and 0.3 mM ferricyanide [12]. The repetition rate of the excitation pulses was 3.8 MHz and the excitation wavelength was either 420 nm or 484 nm. Sub-ps pulse energies were used with pulse duration of 0.2 ps and spot diameter of 1 mm. The samples were placed in a 3.5 mL and 10 mm light path fused silica cuvette and stirred in a temperature controlled ($13^\circ C$) sample holder. In combination with the low intensities of excitation and the use of ferricyanide this guaranteed that close to 100% of the reaction centers stayed open whereas significant build-up of triplet states was avoided [5]. The full-width at half maximum (fwhm) of the system response function was 60 ps and the kinetics were recorded with a resolution of 1 and 2 ps per channel (total 4096 channels). The dynamic instrumental response function of the setup was obtained from pinacyanol (Exciton, inc., Dayton, Ohio) in methanol with a lifetime of 10 ps. A 688 nm interference filter (Balzers, Liechtenstein model B40) was used for detection. Data analysis was performed using a home-built computer programme [13,14].

3. Results

We have measured the fluorescence decay kinetics of PSII membranes with open RCs upon excitation at 420 nm and 484 nm. At 420 nm the absorption is mainly due to Chl *a* and carotenoid molecules whereas excitation at 484 nm leads to excitation of predominantly Chl *b* (only present in the outer antenna) and carotenoids (see Appendix for further quantification). The probability to excite the outer antenna complexes is calculated to be approximately 0.74 upon 420 nm excitation and 0.89 upon 484 nm excitation (see Appendix). Excitation energy transfer from carotenoids and Chls *b* to Chls *a* in LHClI (the dominant complex of the outer antenna) has been studied extensively in the past [15–24] and occurs with an average rate that is substantially faster than 1 ps^{-1} . Therefore, energy transfer to the Chl *a* molecules within LHClI does hardly contribute to the overall migration time. The identified pigment binding sites in the minor complexes are the same as in LHClI and their steady-state spectroscopic properties point to a similar structural organization [25–29] so that also for these complexes a similar fast transfer rate should occur. This has indeed been confirmed experimentally for CP29 [30–34]. Fig. 2 shows both (420 and 484 nm excitation) fluorescence decay curves of one of the preparations. Excitation at 420 nm leads to a slightly faster decay, which is hard to see by eye because of the relatively broad instrument response function (IRF). Quantifying the difference between the two decay curves requires accurate fitting. For a good fit four decay times are needed in all cases. After excitation at 420 nm the decay is dominated by three components: 74 ps (41.7%), 175 ps (51.0%) and 377 ps (7.1%) for the decay presented in Fig. 2. The contribution of a slow component of 2.2 ns is very small (0.1%) and it is probably due to a small amount of PSII with closed RCs, free Chl or detached pigment–protein complexes and will not be used for the modelling. The exact fitting values are not directly interpreted but are used to reconstruct the fluorescence decay curves, thereby eliminating the contribution of the IRF. This reconstructed decay is then used for further modelling (*vide infra*). The decay can also be fitted with slightly different values but this leads to very similar modelling results and the deviation is insignificant for any of the conclusions drawn. The first three components correspond to a weighted average decay time of 147.0 ps which is similar to our previous results [5].

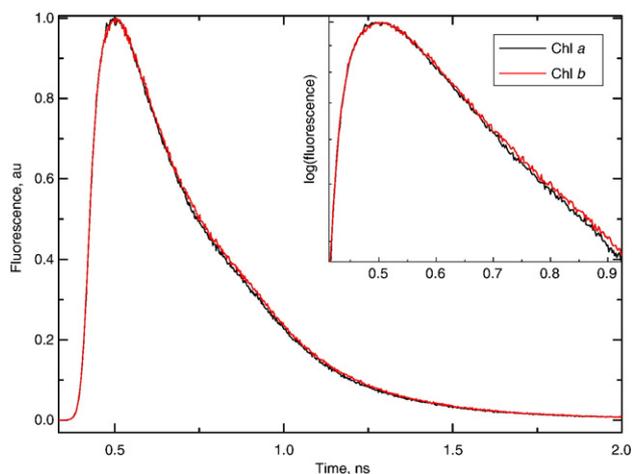


Fig. 2. Fluorescence decay curves of PSII membranes with 2.35 LHClI trimers per RC, measured at 13 °C. The black trace (Chl *a*) corresponds to excitation at 420 nm and the fitted decay times and relative amplitude are 74 ps (41.7%), 175 ps (51%), 377 ps (7.1%) and 2.2 ns (0.1%). The red trace (Chl *b*) corresponds to excitation at 484 nm detection and the fitted decay times and relative amplitude are: 81.3 ps (41.4%), 179 (51.9%), 380 ps (6.6%) and 1.8 ns (0.1%). Fluorescence is detected with a 688 nm band-pass filter. The inset focuses on the first part of the decay and the fluorescence is represented on a log scale.

Table 1

Results of simultaneous fitting of the reconstructed 420 nm and 484 nm decay curves with the coarse-grained model (Fig. 1) [5]

BBY	Average	sem ^a
Trimer/RC	2.45	0.10
Chl <i>a/b</i>	2.08	0.04
$\Delta\tau_{\text{avg}}$ (ps)	4.3	1.8
τ_{h} (ps)	3.5	0.9
τ_{CS} (ps)	5.5	0.4
ΔG (cm ⁻¹)	826	30
τ_{RP} (ps)	137	5
τ_{mig} (ps)	34.5/38.8	13.8/15.5
τ_{avg} (ps)	154.5/158.8	3.7/5.1

Six different preparations were measured and analyzed independently. The average obtained values are presented together with the standard error of the mean. All parameters are defined in the text. τ_{mig} and τ_{avg} show the separately times for the 420 and 484 nm excitation.

^a Standard error of the mean.

Also upon excitation at 484 nm the decay is dominated by the fastest three components: 81.3 ps (41.4%), 179 (51.9%), and 380 ps (6.6%). Again, there is a minor contribution from a slow component (1.8 ns, 0.1%) which will be ignored below. The weighted average of the first three decay times is 151.5 ps.

Repetition of the experiments on this preparation consistently showed a difference in the average decay times ($\Delta\tau_{\text{avg}}$), i.e. preferential excitation of the outer antenna leads to slightly slower overall trapping.

The experiments were performed for six different prepared BBY samples. The average decay time of all samples was 154.5 ps upon 420 nm excitation and on average the decay was 4.3 ps slower upon 484 nm excitation. The standard deviation of the determined lifetimes (0.3 ps) as determined by repeating the measurement on individual samples is small. Upon comparison of the six different samples the standard deviation of the mean is 1.8 ps (Table 1). Therefore, the former standard deviation of 0.3 ps can safely be neglected.

4. Discussion

In the study of Broess et al. [5] it was found that the overall trapping time of excitations in PSII BBYs can already be described rather well with a very simple coarse-grained model, describing excitation energy transfer and charge separation in PSII supercomplexes with 2 parameters: one hopping rate for excitation transfer from one complex to the other and one effective charge separation rate, when an excitation is located anywhere in the reaction center (see Fig. 1). The description with this simple model was optimal for a hopping rate of 17 ps^{-1} and a charge separation rate of 1.2 ps^{-1} . Below the terms hopping time τ_{h} and charge separation time τ_{CS} are used, being the reciprocal of the corresponding rates. The migration time τ_{mig} is linearly proportional to the hopping time [5]. The overall trapping time τ (fluorescence lifetime in this case) is the sum of τ_{mig} and τ_{trap} in this simplified approach. Here τ_{trap} is the charge separation time when the excitation is spatially/thermodynamically equilibrated over PSII (core+outer antenna). As mentioned above different combinations of τ_{h} and τ_{CS} lead to comparable description of the data, with a nearly linear relationship between the optimal values for τ_{h} and τ_{CS} [5]: larger values for the hopping time require a smaller charge separation time in order to describe the data. Since at that time no direct experimental results were available that could provide the best combination of τ_{hop} and τ_{CS} , estimates were made in an indirect way making use of earlier results from singlet–singlet annihilation experiments on isolated LHClI trimers and LHClI aggregates [35]. This led to a hopping time of 13 ps and a corresponding charge separation time of 4.1 ps. It should be noted that this model is oversimplified because reversible charge recombination is not taken into account.

In the present study the fluorescence kinetics of BBY particles were compared after excitation at 420 and 484 nm. The difference in average

lifetime scales linearly with the migration (and thus the hopping) time and the proportionality constant depends on the relative probabilities of exciting the various complexes. By determining the relative protein composition of the BBY preparations, these probabilities can be estimated rather accurately using the absorption spectra of the individual complexes (see Appendix) and the expected kinetics can be calculated within the context of the coarse-grained model. In this way τ_h and τ_{CS} can be determined. For instance, $\Delta\tau_{avg}$ is expected to be 0 ps in case of infinitely fast excitation migration ($\tau_h=0$ ps) like in the exciton radical-pair equilibrium (ERPE) model [36,37], thereby also fixing τ_{CS} . However, finite values of $\Delta\tau_{avg}$ imply that the hopping time is non-zero and consequently τ_{CS} decreases. Once the excitation reaches the RC it can move into the antenna again or lead to a charge separation but the subsequent kinetic behaviour is independent of the excitation wavelength i.e. τ_{trap} is independent of excitation wavelength (for an overview of the modelling principles see e.g. [38]).

4.1. Reversible charge separation

In general, the overall charge separation process in the RC of PSII is considered to be partially reversible [10,39–42]. Also in our preceding study on BBY particles [5] it was shown that the fitting of the data improved when reversible charge separation was included in the modelling. What is the effect of reversible charge separation on the observed kinetics? The value of τ_{mig} is determined by the hopping time and the initial probability distribution of excitations over the membrane and in any model its fitted value will be fixed by the value of $\Delta\tau_{avg}$. Upon raising the rate of charge recombination within the model, the overall fluorescence lifetime will increase when keeping the hopping time and charge separation time identical. In order to keep the modelled average lifetime in accordance with the experimentally determined one, an increased rate of back transfer needs to be compensated by an increased rate of charge separation. The ratio of these two rates is determined by the drop in free energy upon charge separation via the detailed-balance relation.

Here we modelled further electron transfer to the quinone Q_A with one irreversible electron transfer rate. Note that in general, an additional reversible electron transfer step is supposed to take place before irreversible transfer to Q_A occurs. This is often taken into account for the modelling of charge separation in PSII cores and RCs [10,39–41]. However, the BBY particles are too large to allow such detailed modelling and therefore the multi-step reversible electron transfer has to be replaced by one effective electron transfer process.

It is now possible to fit the 420 and 484 nm results simultaneously with a two-step electron transfer model and 4 parameters: the hopping time τ_h and charge separation time τ_{CS} , the drop in free energy ΔG upon primary charge separation and the secondary charge separation time τ_{RP} . The results are given in Table 1 (average of the results for six different BBY preparations) and are summarized by the values $\tau_h=3.5\pm 0.9$ ps, $\tau_{CS}=5.5\pm 0.4$ ps, $\Delta G=826\pm 30$ cm⁻¹ and $\tau_{RP}=137\pm 5$ ps, where the errors indicate the standard error of the mean (see also Fig. 3). The fitting results are not shown but they are virtually indistinguishable from the reconstructed decay curves. Whereas in our previous study there was a large uncertainty in these values, the simultaneous analysis of the 420 and 484 nm data provide relatively accurate values for all parameters because the value of $\Delta\tau_{avg}$ essentially fixes the value of τ_{mig} and thus of τ_h . As was mentioned above, the hopping time scales linearly with the migration time τ_{mig} and it is found to be 35 ps upon 420 nm excitation and 39 ps upon 484 nm excitation, i.e. around 22–24% of the average overall trapping time of 154.5 or 158.8 ps (note that both τ_{mig} and τ_{trap} are expected to increase upon increasing the number of antenna complexes, but not the values for τ_{CS} , ΔG , and τ_{RP}). Jennings et al. [8] found a percentage of 30% based on steady-state fluorescence quenching measurements and kinetic modelling but in that case the results might be influenced by the presence of some Chl that is not connected to the RC. It should be noted that the obtained number of LHClI trimers per RC

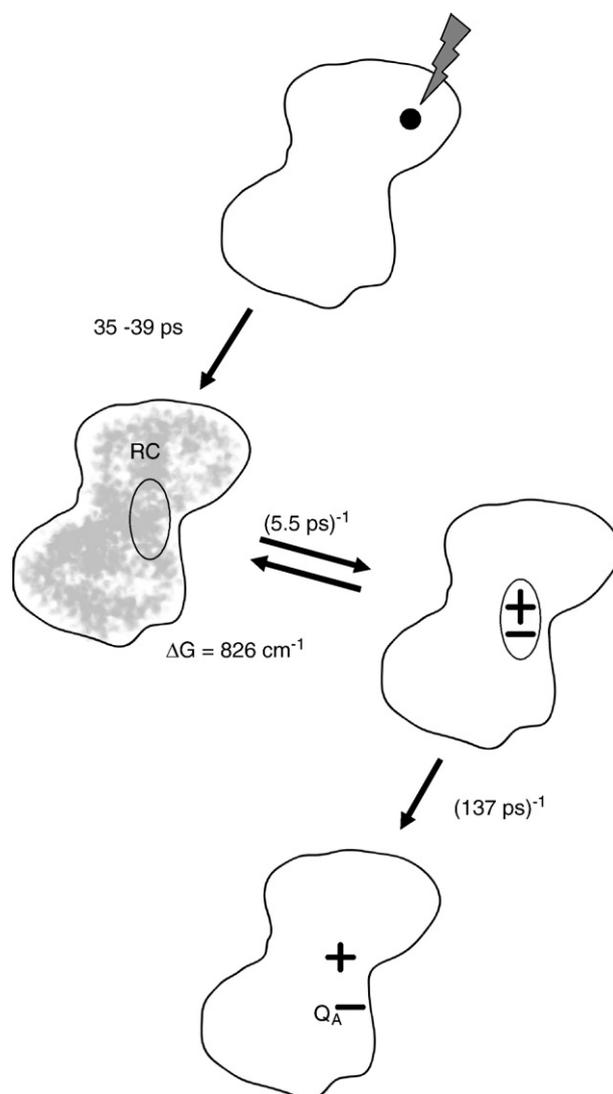


Fig. 3. Summary of the obtained results obtained on PSII membrane preparations (BBYs). Excitation of light-harvesting complex somewhere in PSII (upper figure) is followed by excitation energy transfer throughout the membrane (the grey shading in the left figure schematically represents the excitation probability distribution over the different complexes) and the average time needed to reach the RC for the first time (τ_{mig}) is 35 ps after 420 nm excitation and 39 ps after 484 nm excitation. Energy migration is followed by primary charge separation. The rate of reversible primary charge separation from the excited RC is 5.5 ps⁻¹ and it is accompanied by a drop in free energy of 826 cm⁻¹. Subsequent electron transfer to Q_A occurs with a rate constant of 137 ps⁻¹.

is higher than the number of 2 used for the modelling (Fig. 1). This obtained number may even be higher because of some uncertainty in the actual number of pigments in the minor complexes. The effect of the number of LHClI trimers per RC will be investigated in a future study in which the antenna size will be changed in a systematic way. Here we note that the preparation with the highest LHClI content showed a somewhat larger overall trapping time. However, the fitting results did not change to a large extent when either a number of 2.35 or 2.55 trimers per RC were assumed for a particular data set.

The values for τ_{CS} , τ_{RP} and ΔG obtained here for PSII preparations with outer antenna are similar to the most recent results obtained with time-resolved fluorescence by Holzwarth et al. [10] for core preparations without outer antenna. Their value of 863 cm⁻¹ [10] (drop in free energy before irreversible electron transfer to the Q_A occurs) is nearly identical to the value found in the present study: $\Delta G=826\pm 30$ cm⁻¹. The charge separation time of 5.5 ± 0.4 ps in our case is somewhat slower than the effective time of 4.5 ps given in [43]. It should be kept in mind that the cores used by Holzwarth et al.

[10,22] were prepared from *Thermosynechococcus elongatus* whereas our samples were from spinach. Two different values for the secondary charge separation time for core preparations (313 ps [10] and 175 ps [43]) were reported by Holzwarth et al. The origin of this difference is unclear. The latter value is closest to the one of 137 ± 5 ps found in the present study.

As was mentioned above, a value of $\tau_{\text{hop}} = 17$ ps was used in [7] to draw the conclusion that the PSII charge separation kinetics is substantially different in the various parts of the thylakoid membrane. In light of the much faster hopping time (3.5 ps) obtained in the present study, these conclusions should be reconsidered.

Here a migration time of 35 or 39 ps is obtained upon excitation at 420 and 484 nm, respectively. It is interesting to compare these values to the value that can be estimated from a singlet–singlet annihilation study on LHCII [35]. It was concluded in that study that the average migration time in PSII would be close to $N \times \sim 32$ ps, N being the number of LHCII trimers per reaction center and ~ 32 ps being the approximate spatial equilibration time of an excitation over an LHCII trimer in a random aggregate of trimers. For a preparation with $N = 2.45$ we might therefore expect a contribution of 78 ps, two times larger than the 35–39 ps found in the present study. In a recent study it was demonstrated that upon applying high hydrostatic pressure to LHCII trimers, the fluorescence of a large fraction of the trimers is quenched with a time constant of 25 ps, which reflects the time it takes for an excitation to reach a quencher within the trimer that is being created upon applying high pressure [44]. This value is only somewhat faster than the 32 ps mentioned above, not enough to explain the above mentioned difference of a factor of two. The most obvious explanation for the remarkably fast migration time in PSII is the presence of specific transfer pathways. The excitations in LHCII trimers tend to be localized on the outside of these oligomers [19,45]. Therefore, excitation energy transfer within the trimer, where the low-energy states of the constituting monomers are relatively far apart, might be significantly slower than energy transfer from one trimer to the other, or to a different light-harvesting complex, when the low-energy pigments of the different complexes come closer together. Future determination of high-resolution electron-microscopy maps of PSII will be invaluable to determine the relative orientation of the complexes allowing for more detailed calculations.

Acknowledgements

This work is part of the research programme of the ‘Stichting voor Fundamenteel Onderzoek der Materie (FOM)’, which is financially supported by the ‘Nederlandse Organisatie voor Wetenschappelijk Onderzoek (NWO)’. K.B. was supported by FOM. G.T. and R.C. acknowledge support (visitor grant) from the ‘Nederlandse Organisatie voor Wetenschappelijk Onderzoek (NWO)’.

Appendix

The Chl *a/b* ratio of the samples was determined by fitting the absorption spectrum of the acetone extract with the spectra of individual pigments in acetone [26] (three repetitions). The Chl *a/b* ratio

Table 2a

The number of pigments per pigment–protein complex that was used for the calculations (see Appendix)

LHCII trimer	24 Chl <i>a</i>	18 Chl <i>b</i>
CP24	5 Chl <i>a</i>	5 Chl <i>b</i>
CP26	6 Chl <i>a</i>	3 Chl <i>b</i>
CP29	6 Chl <i>a</i>	2 Chl <i>b</i>
CP47	16 Chl <i>a</i>	
CP43	13 Chl <i>a</i>	
RC	6 Chl <i>a</i>	2 Pheo <i>a</i>
Core	35 Chl <i>a</i>	2 Pheo <i>a</i>

Table 2b

Percentage of direct excitation of the individual complexes upon excitation at 420 and 484 nm (see Appendix)

	420 nm	484 nm
LHCII 2.35 trimers	58.6	73.8
CP29	4.9	4.4
CP26	5.7	5.3
CP24	5	5.4
CP47	10.8	4.5
CP43	8.5	3.4
RC	6.5	3.2

was used to estimate the number of LHCII trimers present in the preparation, while all the other complexes were considered to be present in a 1:1 ratio with respect to the reaction center, according to literature [4,46]. As an example we report the calculation for the sample with a Chl *a*/Chl *b* ratio of 2.11 ± 0.01 below. To estimate the number of complexes the pigment to protein stoichiometry of the individual complexes was used [47,48] and they are reported in Table 2a.

Per RC there is one core complex (containing 1 CP47, 1 CP43 and 1 RC), 1 CP24, 1 CP26 and 1 CP29, and an unknown number of n LHCII trimers, which can be determined in the following way. The number # of Chl *a* and Chl *b* molecules per RC depends on the number of trimers n according to: #Chl *a* = $53 + 24n$ and #Chl *b* = $10 + 18n$. For the subsequent calculations the two Pheo *a* molecules were replaced by 1 Chl *a*, which has a similar amount of absorption. The measured Chl *a*/Chl *b* ratio of 2.11 should then be equal to $(53 + 24n)/(10 + 18n)$. This leads to a number of 2.35 trimers per RC.

To determine the percentage of initial excitation of the individual complexes, the PSII spectrum was reconstructed using the spectra of individual complexes in their native state, normalized to the Chl content, and multiplied by the number of each complex in the membrane as obtained from the pigment analysis (i.e. the spectrum of the LHCII trimer was multiplied by 2.35, while all other spectra were multiplied by 1). Note that the reconstructed spectrum cannot be directly compared to the measured BBY spectrum because of sieving effects. Using the normalized spectra the percentage of excitation at 420 and 484 nm was estimated for all complexes, looking at their relative contribution at the excitation wavelengths (Table 2b). The probability to excite the outer antenna complexes was found to be 0.74 upon 420 nm excitation and 0.91 upon 484 nm excitation. Fitting the (distorted) BBY spectrum with spectra of Chls and carotenoids in protein [49] and using the known pigment and protein stoichiometries, led to the same numbers showing the robustness of the method. The main reason is that Chl *a* and Chl *b* hardly absorb at 484 and 420 nm, respectively.

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