Excitation Dynamics in the Core Antenna of PS I from *Chlamydomonas reinhardtii* CC 2696 at Room Temperature

Krzysztof Gibasiewicz, V. M. Ramesh, Alexander N. Melkozernov, Su Lin, Robert E. Blankenship, and Andrew N. Webber

Department of Plant Biology, Department of Chemistry and Biochemistry, and Center for the Study of Early Events in Photosynthesis, Arizona State University, Tempe, Arizona 85287-1601, and Institute of Physics, Adam Mickiewicz University, ul. Umultowska 85, 61-614 Poznań, Poland

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Photosystem I particles from a eukaryotic organism, the green alga *Chlamydomonas reinhardtii* CC 2696, were studied by transient hole-burning spectroscopy at room temperature. Global analysis of the spectra recorded after excitation of chlorophyll *a* molecules in Photosystem I at selected wavelengths between 670 and 710 nm reveals excitation dynamics with subpicosecond, 2–3 ps, and 20–23 ps components. The subpicosecond and 2–3 ps components are ascribed to energy equilibration within the core antenna, whereas the 20–23 ps component is ascribed to energy trapping by the reaction center. Energy equilibration components describe both uphill and downhill energy transfer depending of the excitation wavelength. The initial transient absorbance bands after direct excitation of the red tail of the *Q*<sub>y</sub> transition band of chlorophyll *a* (at 700, 705, and 710 nm) are 25 nm wide and structured, revealing strongly coupled excited states among a group of molecules, most likely reaction center chlorophyll molecules. Excitation at shorter wavelengths (670, 680, and 695 nm) results in only 5–7 nm wide initial absorbance bands originating from photobleaching and stimulated emission of antenna chlorophyll molecules. The results are compared to the excitation dynamics of Photosystem I from the cyanobacterium *Synechocystis* sp. PCC 6803. The most significant difference is that the 2–3 ps phase describes internal excitation dynamics within higher-energy antenna chlorophyll molecules in the algal PS I system rather than between bulk and red chlorophylls, as observed in cyanobacterial PS I. No indications of core antenna red pigments absorbing above 700 nm were found in the preparation from *Chlamydomonas*. Independent of excitation wavelength, after at most a few picoseconds, all excitons are distributed over the same pool of chlorophyll molecules centered at ~682 nm.

**Introduction**

Photosystem I (PS I) is a membrane protein complex that uses light energy to initiate electron transfer across the photosynthetic membrane (reviewed in refs 1–4). It is composed of more than 13 subunits. The two largest subunits, PsaA and PsaB, form a heterodimeric core which binds approximately 100 chlorophyll *a* (Chl *a*) molecules and the electron-transfer cofactors P<sub>700</sub>, A<sub>0</sub>, A<sub>1</sub>, A<sub>x</sub>, and F<sub>X</sub>. A. There are two quasisymmetrical branches of A, A<sub>0</sub>, and A<sub>1</sub> secondary electron acceptors termed A<sub>1</sub>, F<sub>X</sub>, F<sub>A</sub>, and F<sub>B</sub>. P<sub>700</sub> and A<sub>0</sub> are Chl *a* molecules (P<sub>700</sub> is a dimer of Chl *a*), A<sub>1</sub> is a phylloquinone, and F<sub>X</sub>, F<sub>A</sub>, and F<sub>B</sub> are iron–sulfur clusters. Between P<sub>700</sub> and A<sub>0</sub>, there is another Chl *a* molecule termed A. There are two quasisymmetrical branches of A, A<sub>0</sub>, and A<sub>1</sub> molecules. It is still being debated whether only one or both of these potential electron-transfer chains is active.

It is generally believed that PS I is structurally and functionally similar in all oxygenic photosynthetic organisms. So far, a detailed 4 Å resolution structure has been solved by X-ray crystallography of PS I complexes from cyanobacterium *Synechococcus elongatus*. A total of 83 antenna Chl *a* molecules and 6 electron-transfer Chl *a* molecules were identified. Eighty-one of the antenna Chl *a* molecules are grouped into four clusters. These clusters are somewhat removed from the electron-transfer cofactors. The two remaining antenna Chl *a* molecules are symmetrically positioned very close to the electron-transfer cofactors, one ~13 Å and the other ~15 Å from their respective A<sub>0</sub> molecules, and are often thought of as connecting Chls linking the rest of the antenna Chl molecules to the P<sub>700</sub>. The availability of this structural model of PS I encourages investigation of the excitation energy transfer pathways in the core antenna system and the role of connecting Chls. In particular, the structural data suggests that excitation...
energy may reach the primary donor through a bottleneck of the connecting Chls. Site-directed mutants in PsA and PsB of *Chlamydomonas reinhardtii* that disrupt the connecting Chls are a useful tool to investigate this hypothesis. However, the energy transfer processes in wild type PS I from eukaryotic organisms are poorly defined.

To date, excitation dynamics has been studied mainly in PS I core complexes from cyanobacteria$^7,13,15,25,31$ and to a limited extent from green algae$^1,6,10,14,17,32,33$ and higher plants.$^6,10,17,20,36$ In some of these studies, in addition to the 20–30 ps trapping time, faster phases have been resolved. From fluorescence depolarization measurements on PS I from *C. reinhardtii* strain CC 2696 (previously named A4-d), Du et al.$^{35}$ estimated an upper limit to the time of a single hop between two Chl $\alpha$ molecules of 150–300 fs. This hopping time was independent of excitation wavelength. In addition, they found a 5 ps phase in isotropic fluorescence decay attributed to spectral equilibration within the antenna. Similar phases of 1.5–12 ps were resolved in cyanobacterial PS I particles containing about 100 Chls/PSII and were attributed to energy equilibration between bulk antenna Chl molecules and red Chls absorbing at wavelengths longer than 700 nm.$^7,10,12,13,15$ Also, in a spinach PS I preparation, a 5.8–7.5 ps component was ascribed to energy transfer from bulk antenna Chl molecules to red Chls.$^{10}$ In recent papers, downhill and uphill energy transfer on a subpicosecond time scale has been reported in PS I from cyanobacterium *Synechocystis* sp. PCC 6803.$^{12,13,15}$ So far, no hole burning studies of PS I core particles from eukaryotes have been performed.

Neither the nature nor location and number of red Chls is clearly known. It has been proposed that their long-wavelength absorption bands originate from interactions between closely located monomers constituting Chl $a$ dimers.$^{15,27}$ Some authors have suggested a close proximity of the red pigments to the RC$^15,19,24,37,38$ while others have favored a peripheral location.$^{20,29,39}$ It is probable that number and location of red pigments is species dependent. It was proposed that there are one or two red Chls in the PS I from *Synechocystis* with a maximum absorbance at 708 nm,$^{27}$ whereas four or five red Chls with a maximum absorbance at 708 nm plus five or six Chl molecules absorbs at 719 nm are found in PS I from *S. elongatus*.$^{30}$ On the basis of time-resolved fluorescence measurements and simulations of absorbance spectra, one or two red Chls with a maximum absorbance at 703 nm, located close to the RC, were proposed for PS I from *C. reinhardtii* CC 2696.$^{33,34}$

Excitation dynamics in the PS I core antenna from *C. reinhardtii* have been studied mainly by single photon counting methods with a time resolution of about 10 ps (instrument response function of 50–60 ps), which allows trapping components to be resolved but not energy equilibration.$^6,10,14,17,32,33$ Comparative studies on PS I particles from *Synechocystis* sp. PCC 6803, *C. reinhardtii* CC 2696 and spinach by transient absorbance measurements with femtosecond excitation have resolved a 3.7–7.5 ps phase attributed to energy transfer from Chls of the bulk antenna to red pigments$^{10}$ in addition to a 19–24 ps trapping component. These results were interpreted in terms of the similarity of the energy transfer processes in PS I from different organisms.

In this paper, we report the results of transient spectral hole-burning applied to eukaryotic PS I particles to characterize energy transfer with subpicosecond resolution. In particular, we consider whether distinct populations of Chl $a$ molecules (perhaps corresponding to the groups of clustered Chl molecules) can be detected, and we determine the contribution of the red pigments to the energy transfer process. We have found that the excitation dynamics in PS I from *Chlamydomonas* has several important differences compared to that reported from cyanobacterial PS I. In particular, no red pigments with a maximum absorbion beyond 700 nm were observed in *Chlamydomonas*. In line with this, a several picoseconds phase, ascribed in cyanobacteria to energy equilibration between bulk and red Chls, is assigned in our preparation to energy transfer between different spectral forms of higher energy Chl molecules. In addition, broad absorbance changes at a very early time after excitation at $\geq 700$ nm are observed indicating strong coupling of excited states. Such broad initial transient bands were not reported for cyanobacteria, although coupling of red pigments, $P_{570}$ and monomeric Chls in cyanobacterial PS I core was discussed in Melkozernov et al.$^{40,41}$

**Materials and Methods**

**Preparation of PS I Particles.** PS I was prepared from thylakoid membranes using a mild detergent isolation procedure essentially as described.$^6,25$ Thylakoid membranes were suspended in buffer A (40 mM Hepes, pH 7.2, 5 mM CaCl$_2$, 5 mM MgCl$_2$, 20% glycerol and 0.03% β-dodecyl maltoside) at a Chl concentration of 0.5 mg/mL. A 10% stock solution of dodecyl maltoside (Sigma) was added dropwise to this suspension of thylakoid membranes to give a final concentration of 0.6% detergent. Extraction of PS I particles from thylakoid membranes proceeded in the dark for 15 min on ice with gentle stirring. The suspension was then centrifuged at 40 000 rpm in a Beckman 50.2 Ti rotor for 20 min at 4 °C. The supernatant was loaded onto a DEAE Toyopearl 650 S column (2.2 × 23 cm, a weak anion exchanger, TosO Haas) previously equilibrated with 500 mL of equilibration buffer A. The column was washed with 6 column volumes of equilibration buffer A and then with 5 column volumes of buffer B (40 mM Hepes, pH 7.2, 10 mM CaCl$_2$, 5 mM MgCl$_2$, 20% glycerol (v/v) and 0.03% dodecyl maltoside). The PS I complex was eluted with buffer C (buffer B + 100 mM MgSO$_4$) in a step gradient (5%, 8%, 10%, 13%, 15%, 18%, and 20% buffer C). Fractions with Chl absorbance maxima between 675 and 677 nm were pooled and concentrated. The number of Chl molecules per RC was estimated on the basis of the ratio of absorption of antenna Chls at 675 nm and $A_{600}$ due to oxidation of $P_{700}$ after saturating flash. Assuming a molar extinction coefficient of 60 m$^{-1}$ cm$^{-1}$ for Chl $a$ molecules$^{43}$ and a differential molar extinction coefficient of 64 m$^{-1}$ cm$^{-1}$ for $P_{570}$, 44 85 Chls/RC was calculated for this preparation. This number is higher than those in other PS I preparations from *C. reinhardtii* CC 2696 (usually 40–63 Chls/RC) by$^{10,33}$, most probably due to the very mild procedure of isolation.

**Femtosecond Transient Absorption Measurements.** The sample was diluted with buffer C with 10 μM phenazine methosulfate (PMS), and 20 mM sodium ascorbate added to ensure efficient rereduction of oxidized primary donor. Additionally, the sample was placed in a transparent wheel rotating with a rotation frequency of ~2 Hz so that each laser flash excited a fresh sample. The OD of the sample was typically between 1 and 1.2 at 675 nm. To avoid annihilation effects, excitation pulse intensity was kept at a level of ~1 photon per RC, as monitored by the maximal transient absorbance signal ($\Delta A$ below 0.01).

The experimental pump/probe setup was described earlier.$^{15,45}$ Spectrally narrow (fwhm of ~5 nm) laser pulses with a ~150 fs duration at 670, 680, 695, 700, 705, and 710 nm were used to excite the sample with repetition rate of 1 kHz. The probe
beam was a white continuum pulse generated in a cuvette with flowing water. The polarization of the pump and probe beams was set at the magic angle with respect to each other. Transient absorbance spectra in the region between 630 and 750 nm were collected on two time scales: from −1 to 5 ps with a step size of 54 fs and from 5 to 100 ps with a step size of 2 ps. The spectral resolution of the spectrometer was about 0.14 nm per channel. Data were averaged over 2 nm intervals for all spectra recorded. Decay-associated spectra (DAS) were calculated from global fitting accounting for deconvolution of the recorded signals with the instrument response function using locally written software (ASFIT, web address: www.public.asu.edu/~laserweb/asfit/asfit.html). The instrument response function was modeled by a Gaussian function with a width of ~0.3−0.4 ps. In addition, the time versus wavelength absorbance change surfaces were corrected for the spectral dispersion of the probe beam.

**Time-Correlated Single-Photon Counting.** Fluorescence measurements were performed with time resolution of about 5 ps as described earlier.

**Millisecond Transient Absorption Measurements.** The millisecond resolution instrument used for transient absorption measurements of the primary donor oxidation with 532 nm excitation has been described previously by Kleinherenbrink et al.

**Results**

**Absorbance Change Measurements.** PS I core complexes were excited with ~150-fs-duration light pulses at six different wavelengths covering the maximum and red tail of the Chl a Qy absorbance band: 670, 680, 695, 700, 705, and 710 nm (Figure 1). The spectral width (fwhm) of excitation pulses was about 5 nm. Time evolution of absorbance changes between 640 and 730 nm induced by these excitation pulses at several representative pump−probe delay times is shown in Figure 2.

Excitation at 670 nm (Figure 2A) initially induces a very narrow photobleaching/stimulated emission band with a spectral width of the excitation pulse and centered at about the excitation wavelength (trace marked 0.160 ps). There is an additional small but broad shoulder on the red slope of the band. As the pump−probe delay time increased, the width of absorbance changes gradually increases (compare traces 0.540, 2.38, and 11 ps). These changes reflect spectral broadening due to excitation equilibration over a distribution of different spectral forms of Chl a molecules (see Discussion). The gradual broadening is asymmetric and proceeds mostly toward longer wavelengths. The maximum of the trace marked 2.38 ps is red shifted about 8 nm compared to the earlier traces, which indicates downhill energy transfer among spectrally inhomogeneous Chl a molecules on a subpicosecond and/or picosecond time scale. Some contribution to this shift may come from stimulated emission from vibrationally relaxed Chl a molecules.

Excitation at 680 nm (Figure 2B) again causes very narrow excitation-spectral-width-limited initial absorbance changes (trace 0.160 ps) centered close to 680 nm. These changes are followed by a gradual symmetric broadening of the signal (compare traces at 0.540, 2.11, 12 ps), which, in contrast to excitation at 670 nm, shifts only very little to the red with time.

The initial absorbance photobleaching/stimulated emission band after excitation at 695 nm (Figure 2C, trace 0.170 ps) is again very narrow, but the maximum is slightly blue shifted (693 nm) in relation to the excitation wavelength. This may be due to the steep slope of the absorption spectrum at this wavelength (Figure 1). Consequently, absorbance changes are bigger under the blue side of excitation pulse profile than under the red side. An additional relatively small initial absorbance decrease can be seen at 677 nm. With time, asymmetric broadening mainly toward the blue takes place (compare traces at 0.440, 1.95, and 11 ps). An approximately 8 nm blue shift of the maximum is observed when comparing traces at 1.95 and 0.170 ps, indicating uphill energy transfer.

In contrast to shorter excitation wavelengths, excitation at 700 nm and longer wavelengths causes broad and structured absorbance change bands at very early times (Figure 2D−F; compare traces marked 0.160 ps). In addition to more pronounced negative absorbance changes in the region between 670 and 705 nm, there are also broad positive bands between 640 and 670 nm that can be attributed to excited-state absorption of Chl a molecules (this is particularly evident when 710 nm excitation is used, Figure 2F). For the sake of comparison, initial transient spectra were normalized to the same maximal amplitude (Figure 3B). The common feature of all these traces is their maximum at about 687−689 nm, significantly blue-shifted from the excitation wavelengths. There are also additional, less pronounced peaks at 697 and 675 nm in spectra measured with excitation at 700 and 710 nm, respectively. Time evolution of the spectra leads to slightly broader blue-shifted bands with less structure. The small blue shifts observed when comparing traces 0.430 and 1.7 ps (Figure 2D), 0.370 and 2.2 ps (Figure 2E) as well as 0.320 and 2.16 ps (Figure 2F) may indicate some uphill energy transfer. Additional bands at 705 nm in Figure 2E and at 710 nm in Figure 2F are artifacts resulting from light scattering and instability of excitation pulse energy. Finally, no clear bands centered at wavelengths longer than 697 nm were observed after excitation at any wavelength.

After about 100 ps, absorbance changes have two maxima, at 677 and 691 nm, independent of excitation wavelength (Figure 2). However, the amplitude of the 691 nm band is systematically increasing relative to the 677 nm band with increasing excitation wavelength.

**Global Analysis.** Kinetic behavior of the spectra was analyzed by global exponential fitting resulting in decay associated spectra (DAS) presented in Figure 4. Good fits were obtained with four (670 and 680 nm excitation data sets) or three (excitation at 695 nm, 700, 705, and 710 nm) kinetic components. Independently of excitation wavelength, three similar kinetic phases were found (Figure 4A−F): a subpico-
second component (0.180–0.560 ps), a 20–23 ps component, and a nondecaying component (on the 100 ps time scale). Excitation at 670 and 680 nm induces an additional 2.2–2.9 ps phase (Figure 4A,B).

The negative bands of the subpicosecond DASs extracted from data recorded after excitation at 670, 680, and 695 nm (Figure 4A–C; traces 0.30, 0.30, and 0.49 ps, respectively) are very narrow and centered very close to the excitation wavelengths, resembling the initial absorbance changes (compare to traces 0.160 and 0.170 in Figure 2A–C and in Figure 3A). Positive bands of subpicosecond DASs after excitation at 670 and 680 nm are found on the red and both on the red and (less pronounced) blue sides of the negative bands, respectively. After excitation at 695 nm, there is a pronounced positive band of 0.49 ps DAS centered at about 670 nm.

Figure 2. Transient absorption spectra of the PS I core antenna from C. reinhardtii CC 2696 measured using excitation at (A) 670, (B) 680, (C) 695, (D) 700, (E) 705, and (F) 710 nm. Delay times between pump and probe pulses and excitation wavelengths are indicated. Additional bands at 705 nm in Figure 2E and at 710 nm in Figure 2F are artifacts resulting from light scattering and instability of excitation pulse energy.

Subpicosecond DASs obtained with excitation at 700, 705, and 710 nm (Figure 4D–F; 0.56, 0.35, and 0.18 ps, respectively) are broad and structured both in their negative and positive bands, similar to the respective initial absorbance changes (Figure 2D–F). The negative band of the subpicosecond spectrum after excitation at 700 nm (trace 0.56 ps in Figure 4D) is relatively the narrowest, and its maximum at 697 nm agrees well with the respective band in the transient absorbance spectrum in Figure 2D (trace 0.16 ps).

Excitation at 670 and 680 nm results in 2.9 and 2.2 ps components, respectively. Only negative amplitudes with these lifetimes were found. The bands of the spectra with these lifetimes are broader than the respective subpicosecond bands and, in the case of 670 nm excitation, slightly red shifted.

Independent of excitation wavelength, a 20–23 ps exponential component was found (Figure 4). The DAS of this component has essentially the same shape for all excitation wavelengths (Figure 5A) with a flat positive band at \( \lambda < 660 \) nm and a dominating negative band centered at about 682 nm.

The shapes of the nondecaying (ND) components obtained in a global analysis are essentially the same as those of transient spectra recorded 100 ps after excitation (Figure 2) for respective excitation wavelengths. To compare the shapes of the ND
rapid energy transfer, strong electron–phonon coupling, or strong excitonic interaction between a group of molecules. The first phenomenon seems unlikely. Energy transfer within 300 fs, which is the time proposed for single energy hop between two Chl $a$ molecules in PS I,\textsuperscript{15} was clearly resolved in this study upon excitation at 670 and 680 nm. To our knowledge, no significantly faster components in energy transfer were observed in PS I. Another explanation of the very broad initial $\Delta A$ bands could be direct excitation of a homogeneously broad form of Chl $a$. However, the broadest spectral forms of Chl $a$ in PS I proposed in the literature do not exceed 20 nm,\textsuperscript{15,27,30,34} As this value accounts for heterogeneity of the particular spectral forms among an ensemble of PS I core antenna, one could expect that a spectrally narrow femtosecond excitation will select molecules characterized by absorbance bands even narrower than 20 nm.

In fact, in a previous study, Melkozernov et al.\textsuperscript{15} have shown that 5 nm wide 150 fs pulses induce initial absorbance changes only 5–10 nm wide when exciting cyanobacterial PS I core antenna at 693 and 710 nm. Probably the most likely explanation of wide initial transient bands after excitation at ≥700 nm is excitonic interaction between a group of Chl $a$ molecules. Obvious candidates for this interaction are the six Chl $a$ molecules of the RC core which are close enough to each other (distances between the closest neighbors are less than 10 Å\textsuperscript{24}) to be strongly excitonically coupled, as was modeled by Beddard.\textsuperscript{68} Thus, the structure of the initial bands may result from overlapping of several exciton levels of the six interacting electron transfer cofactors. It is interesting to notice that the whole structure of these bands is spectrally overlapped with the transient spectrum attributed to P$\textsubscript{700}^{+}$ (compare traces 0.160 and 100 ps in Figure 2D–F; see below), indicating that P$\textsubscript{700}^{+}$ may be at least in part responsible for the wide bands induced by excitation at ≥700 nm. It is less probable that the group of interacting molecules belong to the antenna, as no such groups were identified in PS I core antenna structure. Instead, several pairs of closely located Chl $a$ molecules which were proposed to be red Chls\textsuperscript{15,27} were found in the vicinity of the RC. However, transient spectra of the red Chls are only 5–10 nm wide.\textsuperscript{15}

It should be mentioned that due to low absorption to the red of 700 nm, the light intensity used for excitation at wavelengths greater than or equal to 700 nm was significantly higher compared to excitation intensities used at 670 and 680 nm. This was necessary in order to give comparable signals in both wavelength regions. Moreover, pump pulses at 695 nm, where absorption is already low (~20% of maximum, see Figure 1), apparently excite mainly a spectrally distinct pool of Chl $a$ molecules, different from that excited at ≥700 nm, although both pools are spectrally overlapped (compare trace 0.170 ps in Figure 2C with traces 0.160 ps in Figure 2D–F). A small shoulder in the region between 675 and 683 nm accompanying the main band generated by 695 nm pump pulse (Figure 2C; trace 0.170 ps) is the only indication of weak excitation of the broad band dominating after using longer excitation wavelengths. All of this indicates a very low number of molecules per P$\textsubscript{700}$ involved in the transient spectrum induced by excitation at ≥700 nm.

### Dynamics of the Excited States

To quantify the dynamics of the excited states, an exponential model for relaxation of Chl $a$ excitation has been assumed, and global analysis of kinetics has been performed in the spectral region of 630 nm–710 nm, yielding decay associated spectra for all excitation wavelengths (Figure 4).
The negative amplitudes of DASs with subpicosecond lifetimes in Figure 4A–C reflect decay of photobleaching and stimulated emission (SE) caused by initial excitation of spectrally narrow pools of Chl a molecules in the inhomogeneously broad absorption band of the PS I core antenna. This decay is most probably due to energy transfer to neighboring spectrally shifted Chl a molecules. This energy transfer proceeds toward mainly red (downhill energy transfer), both toward red and blue, and toward mainly blue (uphill energy transfer) after excitation at 670, 680, and 695 nm, respectively, as can be seen as the positive amplitudes of DASs. The directions of energy transfer revealed by positive amplitudes of DASs agree with the directions of apparent shifts of initial transient spectra in Figure 2A–C (compare traces marked 0.160 and 0.170 ps with spectra recorded ~0.5 ps and ~2 ps after excitation). Another effect of subpicosecond energy transfer is fast spectral broadening of initially narrow ΔA bands (compare 0.160–0.170 ps vs 0.440–0.540 ps spectra in Figure 2A–C). An alternative explanation of the subpicosecond kinetic component could be conformational diffusion, a process of interconversion of different nuclear conformations in a pigment’s neighborhood taking place due to thermal energy at room temperature. Consequently, interactions between the pigment and surrounding protein are continuously modulated. As an effect, spectral properties of the pigment are also changing with time, and a shift and/or broadening of transient spectra can be than observed. Conformational diffusion has been proposed in bacterial RCs.49,50 However, in our case, this process plays probably a minor role as at 10 K, when nuclear movement and conformational diffusion are frozen, we still observe subpicosecond component similar to that at RT (unpublished). The lifetime for subpicosecond energy transfer of 300 fs after excitation at 670 and 680 nm is similar to that found by Du et al.35 for a single hop between two Chl a molecules. It suggests that as little as one energy transfer hop is enough to observe significant spectral broadening. It means that initially excited Chl a molecules are in direct contact with a variety of different spectral forms of these pigments.

In contrast to excitation at longer wavelengths, excitation at 670 and 680 nm induces an absorbance change component.
characterized by a lifetime of 2.2–2.9 ps. Comparison of transient spectra in Figure 2A,B recorded 0.540 ps versus 2 ps after excitation pulse shows that during this time interval, further spectral broadening and, in the case of excitation at 670 nm, red shift of the spectrum take place. These features suggest continuation of energy equilibration process started in the subpicosecond time domain. It is not clear why 2.9 and 2.2 ps DASs (Figure A,B) do not have clearly pronounced positive amplitudes in addition to negative ones, as would be expected in the case of energy transfer. One explanation may be that appearance of the photobleaching, which should manifest itself by positive amplitudes in DAS, is masked by simultaneously appearing excited-state absorption, normally manifested by negative amplitudes in DAS. Alternatively, energy transfer on several picoseconds time scale may be a nonexponential process not properly described by the assumed model. Neither transient spectra (Figure 2A,B) during the first picoseconds after excitation nor 2.2 and 2.9 ps DASs (Figure 4A,B) show indications that the several picoseconds process resolved in this work could be assigned to energy transfer to red pigments absorbing above 700 nm. This is opposite to the case of PS I from Synechocystis sp. PC 6803,10–13,15 where clear energy transfer with lifetimes of 2.0–6.5 ps occurred from bulk Chl molecules to red pigments with an absorption band above 700 nm. Similar assignment of several picosecond DASs was done in case of the PS I core from Synechococcus sp.7,31 Also, in C. reinhardtii CC 2696, a 4.6 ps DAS has been resolved and interpreted in terms of downhill energy transfer (see Figure 1B in ref 10). However, in PS I from Chlamydomonas, the 4.6 ps downhill energy transfer leads to excitation of more blue shifted Chl molecules peaking at about 696 nm. Moreover, one could expect that this energy transfer should lead to contribution of 696 nm peaking Chl molecules to the equilibrated excited-state spectrum, manifested by negative amplitudes of DAS in this region, as observed in cyanobacteria (Figure 1A in ref 10). In fact, it is not the case, and the equilibrated excited-state spectrum (22.4 ps DAS in Figure 1B in ref 10) has a small positive amplitude at 696 nm. Taking into account these remarks, in our work, the 2.2–2.9 ps phase is attributed instead to an equilibration process between different spectral forms of Chl a molecules within a relatively narrow spectral region.

Global analysis of spectra recorded after excitation at 700, 705, and 710 nm reveals the subpicosecond excitation decay of an initially excited group of molecules (see above). Positive bands in subpicosecond DASs (Figure 4D–F) between 640 nm and 675 nm are attributed partly to a decay of excited-state absorption which is initially very pronounced (see traces 0.160 ps in Figure 2D–F and Figure 3B) and partly to uphill energy transfer. When exciting at 695 nm and above, no 2–3 ps phase is observed, in contrast to shorter wavelength excitations. This indicates that equilibration is achieved in a subpicosecond time domain after longer wavelength excitations. Independent of excitation wavelength, a 20–23 ps DAS of similar shape was found (Figure 5A) that indicates that excitation energy always equilibrates over the same pool of bulk Chl molecules with maximum $\Delta A$ at $\sim 682$ nm. It is interesting, however, that this equilibration is achieved later, after 2–3 ps, when the excitation is at 670 or 680 nm than when exciting at longer wavelengths, likely directly into RC Chl molecules. This difference is expected to result from the structural organization of PS I. One possible explanation may be that final equilibration...
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occurs not over the whole PS I core antenna but over a smaller pool of Chl molecules located close to the RC, similar to that proposed for PS I core from *spinach* and from cyanobacteria. This would be consistent with a red shift of the 682 nm AA equilibrated excited-state band (Figure 5A), which could be due to excitation of the proposed smaller pool of Chl molecules, relative to the steady-state absorption maximum at 675 nm (Figure 1) contributed by all antenna Chl molecules. The smaller pool could be one or two of the four Chl $a$ clusters found in structural studies. An alternative explanation of this shift may be a significant contribution of uncoupled blue shifted Chl molecules in the steady-state spectrum (seen in ND component of DASs in Figure 6; see below) and/or significant contribution of stimulated emission of vibrationally relaxed bulk Chl $a$ molecules in the 20–23 ps DAS. It is interesting to note that the 20–23 ps DAS which describes the decay of the equilibrated excitation in the antenna has no negative amplitudes beyond 695 nm (Figure 5A). This indicates that in this equilibrium no antenna pigments absorbing above 695 nm contribute confirming that the most red-shifted antenna pigments in *C. reinhardtii* CC 2696 absorb clearly below 700 nm. This conclusion is further supported by the observation of the broad transient spectra induced at ≥700 nm, ascribed to Chls transporting electrons, apparently not covered by spectrally overlapping red antenna Chls.

The spectrum of the equilibrated state may also be visualized without referring to the exponential model-dependent global analysis. Figure 5B presents normalized to the same maximal amplitude, transient spectra from Figure 2A–F recorded ~11 ps after excitation pulses of all six wavelengths used. After that time, full energy equilibration has already taken place, and the spectra presented are contributed by excited bulk Chl molecules (centered at ~682 nm; see above), uncoupled excited Chl molecules (centered at ~677 nm; see below), and oxidized primary donor (centered at ~691 nm; see below). To extract spectra from the equilibrated excited-state only, the ND spectra (Figure 2A–F), containing contribution from uncoupled Chl molecules and oxidized primary donor, were subtracted from the respective ~11 ps spectra. The result of this subtraction is shown in Figure 5C: the equilibrated excited-state spectrum has the same shape and the same peak position for all excitation wavelengths, perfectly agreeing with spectra in Figure 5A. A shoulder on the blue slope of the band induced by excitation at 670 nm (Figure 5C) may suggest some minor contribution of more blue-shifted forms of Chl $a$ in the equilibrium. A similar, although very slightly pronounced shoulder is present in the 670 nm DAS in Figure 5A.

The spectra of the nondecaying component (on the time scale of 100 ps) from Figure 4A–D, normalized to the same amplitude at 691 nm, are shown in Figure 6 together with millisecond difference spectrum of P$_{700}$–P$_{700}$. Depending on the excitation wavelength, there is a different relative contribution of the 677 nm band. The highest contribution is after excitation at 670 nm and then at 680 nm. Using excitation at 695 nm or longer, this contribution is stabilized to a level of about 50% of the 691 nm band amplitude. The long excitation wavelength induced ND spectrum (excitation at ≥695 nm) is ascribed to P$_{700}$–P$_{700}$, as charge separation in the RC takes place on the 20 ps time scale and is completed by 100 ps. The shape of P$_{700}$–P$_{700}$ spectrum agrees well with that in the literature. However, the peak position of the main band is usually not below 695 nm, although it is preparation-dependent (see for example$^{51,52}$). In our P$_{700}$–P$_{700}$ spectrum recorded ~1 ms after excitation (Figure 6), the maximum position of this band is at 696 nm. The origin of this 5 nm blue shift at early time is not clear. Schaffer and Junge$^{51,52}$ put forward the hypothesis that the position of the P$_{700}$–P$_{700}$ absorption change includes an electrochromic response of neighboring antenna molecules. One could suggest that this response is time dependent in line with the idea of protein relaxation following charge transfer.

The reason for the variable amplitude of the 677 nm band is most probably a different contribution of excited states of uncoupled Chls. Single-photon counting (SPC) measurements were performed to detect the presence of uncoupled Chl $a$ molecules in our preparation. Indeed, apart from 20–24 ps phase agreeing perfectly with the trapping time found in transient absorption measurements, a significant contribution of a ~5 ns phase characteristic of uncoupled Chls was observed in fluorescence kinetics. PS I particles from *C. reinhardtii* often show the presence of a relatively high amount of uncoupled Chls.$^{6,13,33}$ Also, a similar excitation wavelength dependence of the ND spectra with a ~670 nm band assigned to uncoupled Chls was observed in *Synechocystis* sp. PCC 6803 PS I particles.$^{15}$ Exciting at longer wavelengths apparently avoids excitation of uncoupled Chls.

Assuming that 700 nm and longer wavelength photons excite mainly RC Chl molecules, it is interesting to speculate on the energy distribution between the immediate excitation trapping by charge separation, which is expected to dominate according to diffusion-limited model, and its escape to the antenna which should dominate if the trap limited model is a better description of the trapping process. In the recent paper of Kumazaki et al.$^{20}$ they excited directly P$_{700}$ in solvent extracted PS I particles from *spinach* containing 12–13 Chl $a$ molecules. They found that about 50% of initially excited RCs decay in 0.8 ps by direct charge separation and the rest of the energy is equilibrated over the antenna Chls. This result does not favor any of the two models mentioned above. However, a relatively high contribution of immediate charge separation may be caused by structural modification after harsh treatment of the PS I core. In our experiments, most of the energy seems to escape from the RCs to antenna Chls. This may be judged from similar amplitudes of the 20–23 ps DAS component relative to the P$_{700}$–P$_{700}$ band in ND component (Figure 4) independently of excitation wavelength. Apparently, the same relative amount of energy is equilibrated over the bulk antenna Chl $a$ molecules independently if one excites preferentially the RC or the antenna pigments. Thus, our results favor a trap limited model of the PS I core. To better determine the rate constant of direct charge separation from the primary donor, one could construct a mutant lacking connecting Chls. If the connecting Chls indeed play a role of a bottleneck between the core antenna and RC Chl molecules, their loss should manifest itself by uncoupling the RC from the antenna and the appearance of prompt charge separation after direct excitation of P$_{700}$.

Finally, we would like to address the question of the content of red Chls absorbing above 700 nm in the PS I core antenna from *C. reinhardtii*. Although their presence in PS I core from cyanobacteria is well documented, there is no strong evidence in the literature for red Chls in the PS I core from *C. reinhardtii*. In our preparation, we did not see any indication of Chl $a$ molecules with absorption maximum above 700 nm. In particular, no Chl $a$ molecules absorbing mainly at 703 nm, as proposed by Jia et al.$^{34}$ were observed in this study.

In summary, while excitation dynamics in PS I from *C. reinhardtii* CC 2696 is similar to that of PS I from *Synechocystis* sp. PC6803, with kinetic components of 0.18–0.56, 2.2–2.9,
and 20–23 ps, the spectral changes from *Chlamydomonas* PS I core are limited to a much narrower blue-shifted region. The 2.2–2.9 ps energy equilibration component resolved using 670 and 680 nm excitation is not observed upon direct excitation of the red tail of the Chl a Q s absorption band when instead energy equilibrates on the subpicosecond time scale. The difference may come from the presence of a separate pool of Chl a molecules with maximum absorbance at 682 nm located close to the RC, over which the excitation is finally equilibrated. Excitation of the red tail results in very broad and structured absorbance changes consistent with strong excitonic coupling between several Chl species, likely from the RC. Very narrow initial absorbance bands after excitation by shorter wavelength pulses demonstrate a variety of different spectral forms of Chl a molecules and their nearest neighbors—randomly distributed other spectral forms of Chl molecules.

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References and Notes