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LETTERS

Direct Observation of Energy Transfer in a Photosynthetic Membrane: Chlorophyll *b* to Chlorophyll *a* Transfer in LHC

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Subpicosecond fluorescence upconversion has been used to measure the rate of chlorophyll *b* to chlorophyll *a* electronic energy transfer in situ within the LHC pigment proteins of *Chlamydomonas reinhardtii* mutant C2. The time scale of energy transfer is 0.5 ± 0.2 ps as determined from the rise time of chlorophyll *a* fluorescence following chlorophyll *b* excitation. Estimates of the energy-transfer rate based on Förster weak coupling theory are discussed.

Introduction

The light harvesting system of green plants and algae is exquisitely efficient in transferring electronic excitation to the photochemical reaction centers.[1,2] The light harvesting, or antenna, system comprises a number of pigment–protein complexes assembled into arrays "surrounding" the reaction centers. The key to the efficiency of the light harvesting process is the ultrafast energy transfer that occurs between chromophores, both within and between individual pigment proteins (i.e., an excitation visits the reaction center within a fraction of the excited-state lifetime of the antenna pigments). It is not obvious that a description of energy transfer based on a weak coupling between the chromophores, such as Förster theory,[3] provides an adequate description of the system. At short distances the point dipole approximation is known to be inaccurate and it may be necessary to include exchange contributions to the coupling between the donor and acceptor chromophores.[4] The energy-transfer rate may be sufficiently fast that conventional time scale separations between electronic and vibrational relaxation do not exist.[5] In order to address these issues, both detailed kinetic and structural data are required. Present structural information with atomic resolution of antenna pigment proteins is limited to the bacteriochlorophyll *a* protein of *Prosthecochloris aestuarii*,[6] the phycobiliprotein phycocyanin trimer of *Mastigocladus laminosus*[7] and the phycocyanin hexamer of *Agmenellum quadruplicatum*.[8]

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While many kinetic investigations of the light harvesting process have been carried out, few have had the time resoltuion necessary to directly observe the elementary energy-transfer step. Perhaps the most detailed studies have been on phycobilisomes[9-14] where the observation of the energy flow is facilitated by the distinct spectra of the various phycobiliproteins and the extensive structural information available.[7,8] Sauer and Scheer have recently calculated the rates of energy transfer within the phycocyanin protein based on the most recent crystal structure. They concluded that the assumptions inherent in the Förster weak coupling limit of energy transfer may not be valid in this system.[15]

The light harvesting system in higher plants and green algae consists of chlorophyll *a* and chlorophyll *b* associated with distinct membrane proteins. The most abundant pigment–protein complex in higher plants is the LHC II complex which contains both Chl *a* and Chl *b*.[16] LHC II functions as an antenna for both photosystems, although it transfers most of its excitation to the photosystem II core antenna. LHC II has been studied extensively at the spectroscopic level[16-24] and presents an excellent system for energy-transfer studies because Chl *b* can be preferentially excited and the electronic energy transfer monitored by the appearance of excited Chl *a* molecules. Isolated LHC II has been studied previously in the picosecond time domain by Gillbro et al.[25,26] Their initial work has been discussed by Lin and Knox[27] in terms of the Van Metter–Shepanski–Knox model[22-24] of LHC II. Using 12-ps pulses Gillbro et al.[25] reported a (6 ± 4)-ps Chl *b* to Chl *a* energy-transfer time and a 20-ps transfer time between the Chl *a* molecules. These times are much longer than the Chl *a* to Chl *a* transfer times estimated by Gulotty et al.[28] and Owens et al.[29,30] from a series of photosystem I preparations. In the work of Owens et al.[29,30] the size of the Chl *a* core antenna was varied and a single-step transfer time of 0.2 ps was estimated based on the homogeneous regular lattice model of Pearlstein.[31] (By single-step transfer time we mean the coordination number multiplied by the inverse of the rate for transfer between two molecules.) There are difficulties in the interpretation of both measurements since the latter study[29,30] is clearly indirect and model dependent, while the former study[25] is complicated by the presence of the coherence spike,[32] and the relatively poor time resolution compared to the subpicosecond time scales predicted by Owens et al.[29,30] Accordingly, we felt it worthwhile to reexamine the Chl *b* to Chl *a* energy transfer in LHC with the much higher time resolution of the fluorescence upconversion technique, which also avoids the coherence artifact. In addition we have minimized the possibility of protein modification during the isolation procedure by studying a strain of *C. reinhardtii* which is devoic of both photosystem I and photosystem II. The LHC class of proteins are the only chlorophyll–proteins that are synthesized and assembled with their prosthetic groups in this mutant alga.

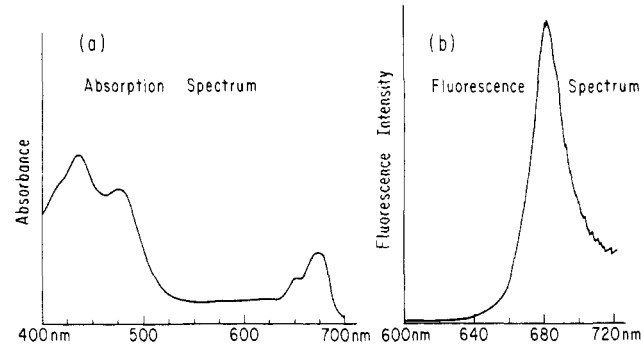

Figure 1. (a) Steady-state absorption spectrum of LHC in thylakoids of *C. reinhardtii* mutant C2. The 435- and 670-nm peaks are due to Chl *a* and the 465- and 650-nm peaks arise from Chl *b*. (b) Steady-state fluorescence emission spectrum of LHC in mutant C2 thylakoids. Excitation was in the Chl *b* Soret band 465 nm (10-nm band-pass). There is no detectable fluorescence from Chl *b* in the 650–657-nm region.

Experimental Section

Thylakoid membranes were prepared from the *C. reinhardtii* PS I–PS II double deficiency strain C2 isolated by L. Mets. The C2 strain carries a complete deletion of the chloroplast Ps*b*A gene[33,34] and is therefore missing all of the proteins of the PS II reaction center and core antenna complex.[35,36] It also carries a deletion of the chloroplast *tsc*A gene, which is necessary for the synthesis of the PS I reaction center apoproteins[33,37] and is therefore missing all of the proteins of the PS I reaction center and core antenna complex.[38] The strain retains a full complement of the peripheral antenna LHC proteins, primarily LHC II, but possibly including LHC I as well. Chl *a*/chl *b* ratios for C2 are in the range of 1.1–1.4. Cell cultures were typically grown to a cell density of 3×10^6 cells/mL in TAP medium[39] at 25 °C, 50–100 foot-candles cool white fluorescent light. After concentration by centrifugation, the cells were suspended in a sorbitol buffer (330 mM sorbitol, 2 mM EDTA, 0.1% bovine serum albumin, 2 mM $MgCl_2$, 2 mM ascorbate, 50 mM tricine, pH 7.8) and ruptured by two passes through a French press cell at 750 psi. The LHC-containing thylakoids were decanted after centrifugation at 300*g* to remove cellular debris and immediately used for the kinetic measurements. The thylakoids were flowed at a rate of 25–50 mL/min through a 1 × 1 mm cell and maintained at 4–10 °C. The integrity of the sample was monitored by CD measurements or time-correlated single photon counting measurements during the course of the experiment.

The laser system is a hybrid cavity-dumped mode-locked dye laser synchronously pumped by a CW mode-locked Nd:YAG laser.

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The dye laser generated pulses of 0.5-ps duration at 648–652 nm operaitng with a gain medium of rhodamine B and sulforhodamine 640 and a saturable absorber of DQTCI. The DQTCI was first suspended in water and then added to an ethylene glycol solution. The fluorescence signal was detected with a resolution of 66.67 fs/channel at parallel, magic, or perpendicular angle to the polarization of the excitation pulse via type II upconversion in a urea crystal. The sum frequency was detected by single photon counting. A detailed description of the apparatus has been given elsewhere.[40] In all the experiments the laser repetition rate was 3.8 MHz and the pump intensity at the sample was (0.5–5.0) × 10^{14} photons/cm^2/pulse. The autocorrelation function of the laser pulses was obtained by replacing the sample with buffer and adjusting the phase matching conditions in the urea crystal.

Data were fit to sums of exponentials by using a program based on the MINUIT package. MINUIT is available from the CERN program library Division DD, CND, CH-1211 Geneve 23, Switzerland.

Results

(*a*) *Magic Angle.* The steady-state absorption spectrum, Figure 1a, of *C. reinhardtii* C2 thylakoids shows the contribution due to Chl *b* in the Soret (465 nm) and red (650 nm) portions of the visible absorption spectrum. Figure 1a shows that excitation at wavelengths between 640 and 660 nm preferentially excites Chl *b*. At 650 nm we estimate the ratio of Chl *b* to Chl *a* absorbance is ~5:1. Thus fluorescence detected to the red of the Chl *b* emission and in the Chl *a* emission spectrum should show a rise time reflecting the kinetic step(s) of Chl *b* to Chl *a* energy transfer. Figure 2 shows typical time resolved isotropic fluorescence data collected at the magic angle for 650-nm exciation, 691-nm center wavelength detection, and 4-nm bandwidth. Nearly identical results were obtained for detection wavelengths from 682 to 700 nm. Figure 2a shows a fit to the data using a single-exponential decay convoluted with the measured instrument function. Figure 2b is a fit of the same data including a 0.67-ps exponential rise time in addition to the decay. The amplitudes of the rising and decaying terms are comparable (see figure caption). Although the deviation from "prompt" decay is small, the rising portion of the curve is clearly better fit by the inclusion of the rising component. Analysis of our data gives rise times in the range 0.3–0.7 ps, independent of detection wavelength in the 685–695 nm range. Using excitation pulses of slightly greater length (1 ps) we found no evidence for a rise time of 1 ps or longer in the emission range 680–720 nm.

The decay of the Chl *a* fluorescence in Figure 2 (50–100 ps) is clearly much shorter than the lifetime that was measured by single photon counting on the same samples (2.2 ns). That the upconversion data can be described by a single exponential of 50–100 ps is due to the higher excitation intensities used in these experiments resulting in excitation annihilation[41] and the limited dynamic range (0–20 ps). At higher incident laser intensities ((5–10) × 10^{14} photons/cm^2/pulse) the decays become noticeably nonexponential due to the excitation annihilation effects (e.g., one or two short decay components must be included in order to accurately fit the data). At the intensity used in Figure 2 (2 × 10^{14} photons/cm^2/pulse) the annihilation time scale is 100 times larger than the rise time attributed to Chl *b* → Chl *a* energy transfer. We, therefore, do not feel that the annilhilation kinetics influence our conclusion that the transfer time is 0.5 ± 0.2 ps.

To assess the sensitivity of our data analysis, we carried out a series of simulations. The simulations assume (a) an initial excitation ratio of 5:1 for Chl *b* to Chl *a*, (b) that the detected emission arises only from Chl *a*, and (c) that the excitation is transferred from Chl *b* to Chl *a* in one step. The stimulated data was convoluted with a real instrument function and Gaussian noise added before fitting. Figure 3 shows single- and double-exponential fits to simulated magic angle curves with 0.5 ps (Figure 3, a and b) and 3 ps (Figure 3, c and d) rise times. Parts a and b of Figure 3 are similar to the experimental data whereas parts c and d of Figure 3 rule out the presence of a rise time in our data of the range (6 ± 4 ps) reported by Gillbro et al.[25] A rising component could also be accurately detected in simulated curves when either the noise level was increased to double the experimental value or the initial excitation ratio was 3:1. We feel confident that the data in Figure 2 reveal the Chl *b* to Chl *a* energy transfer in LHC in situ.

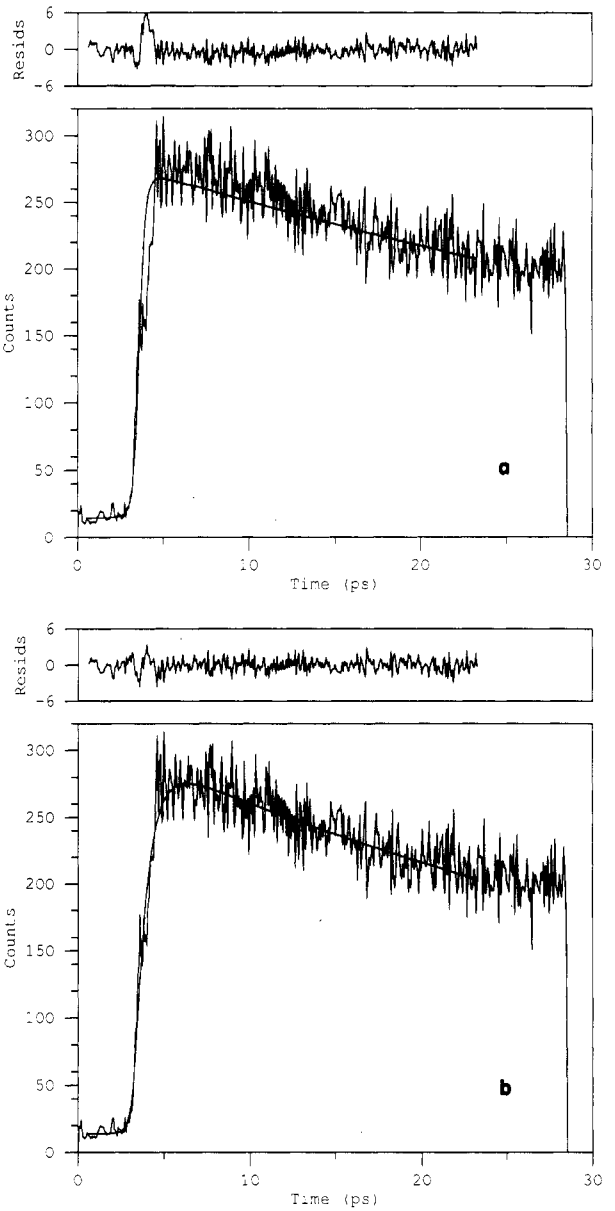

Figure 2. (a) Time-resolved isotropic (magic angle) fluorescence of LHC containing thylakoids. Excitation at 650 nm and emission at 690 nm (4-nm band-pass). The solid line is a best fit of the data to a prompt exponential decay convoluted with the instrument response function. The residuals of the fit are displayed above the decay; τ = 68 ps, χ_r^2 = 2.0. (b) Fit of the same data including an exponential rise time. The best fit parameters of a biexponential function of the form $A_1 \exp(-t/\tau_1) + A_1 \exp(-t/\tau_1)$ are $A_1 = -0.75$, $\tau_1 = 0.68$ ps, $A_2 = 1.0$, $\tau_2 = 50$ ps, $\chi_r^2 = 1.35$.

(*b*) *Parallel and Perpendicular Components.* We also measured the anisotropic signal of the in situ LHC and find an extremely small initial value (0.02) for the anisotropy in accord with the steady-state results of Van Metter[21] for excitation at 650 nm for solubilized LHC II and our own steady-state measurements of LHC in situ. This limited range for the anisotropy combined with our relatively small signal to noise ratio does not allow us to provide a firm value for the anisotropy decay time; however, it is certainly less than 1 ps. We find no evidence for a long residual anisotropy

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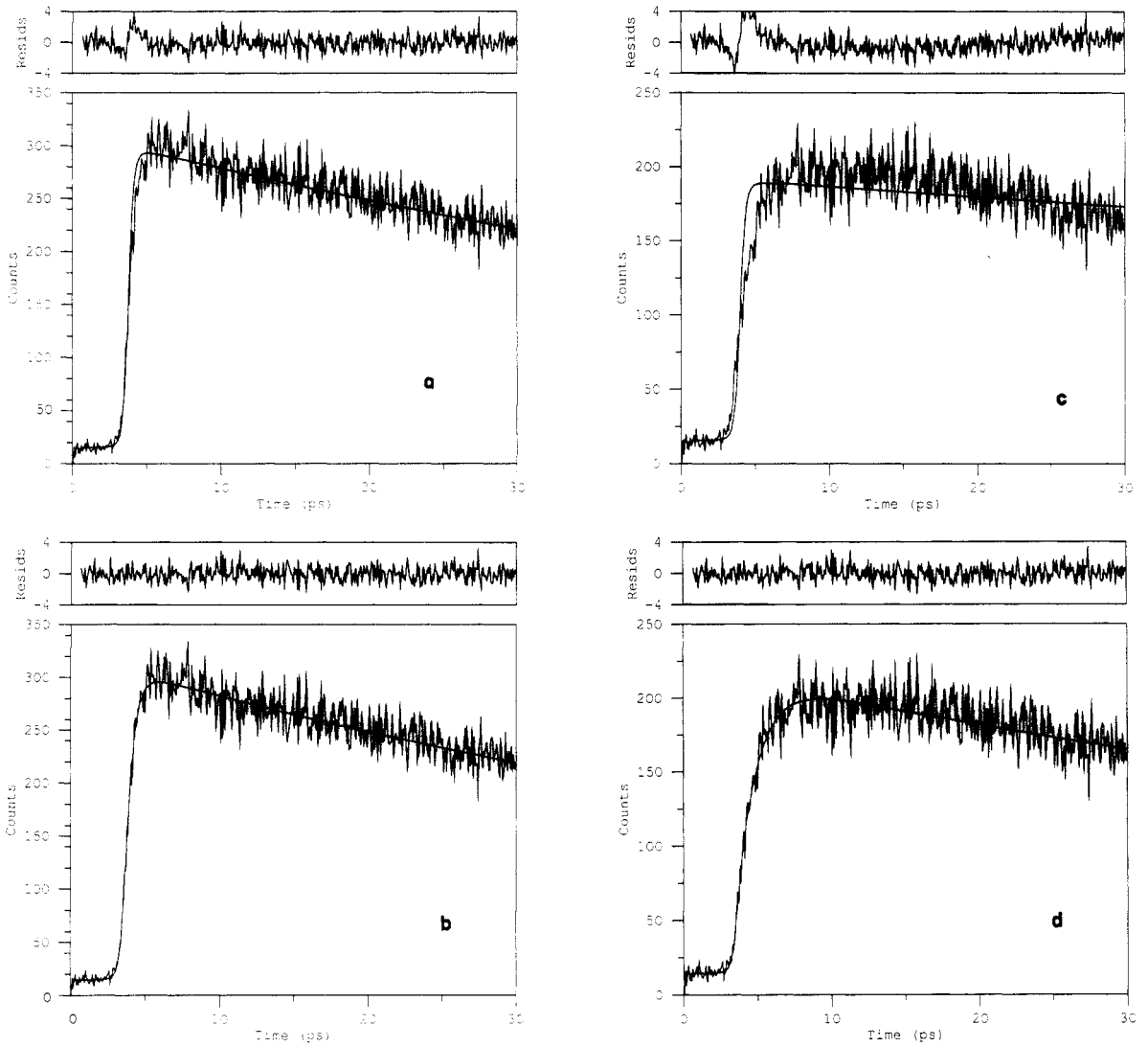

Figure 3. Simulated magic-angle fluorescence decay curves. In all cases the ratio of initial excitation was 5:1 Chl *b*:Chl *a*, and the calculated curve was convoluted with an experimental instrument response function. Gaussian noise was then added to each curve. (a) and (b) 0.5 ps Chl *b* to Chl *a* transfer time. Part a shows a fit to a single-exponential decay $\tau = 68$ ps, $\chi_r^2 = 1.15$ and part b shows a fit including a rising component. The fitted parameters were $A_1 = -0.96$, $\tau_1 = 0.41$ ps, $A_2 = 1.0$, $\tau_2 = 74$ ps, $\chi_r^2 = 0.99$. (c) and (d) 3 ps Chl *b* to Chl *a* transfer time. The fitted parameters were (c) $\tau = 244$ ps, $\chi_r^2 = 1.83$ and (d) $A_1 = -0.76$, $\tau_1 = 1.41$ ps, $A_2 = 1.0$, $\tau_2 = 94$ ps, $\chi_r^2 = 1.0$.

as reported by Gillbro et al.[25] for isolated LHC II. This may result from a much enhanced Chl *a* connectivity in our intact thylakoid samples.

Discussion

A high-resolution molecular structure for LHC is not yet available; however, the crystal structure of solubilized LHC II chlorophyll *a*/chlorophyll *b* protein has been determined at 7 Å resolution via X-ray crystallography and electron microscopy by Kuhlbrandt.[42] The crystalline protein forms 3-fold symmetric trimers. The pigment stoichiometry of the protein monomers has been reported to have various values (ranging from 6 to 20 Chl per 26 kDa peptide[16,22,43,44]) but the most recent determination by Butler and Kuhlbrandt[44] gives 8 Chl *a* and 7 Chl *b* per peptide monomer or 45 Chl per trimeric unit. The strong similarity of the circular dichroism spectra of *C. reinhardtii* mutant C2 thylakoids[38] and a microcrystalline suspension of LHC[20] suggests that the trimeric structure is also present in situ. [The CD spectrum of C2 thylakoids[45] differs significantly from that of isolated (presumably monomeric) LHC.[21,22]]

A model of energy transfer in LHC II has been developed by Van Metter and Knox and co-workers.[22-24] On the basis of CD and steady-state fluorescence polarization studies of in vitro LHC II micelles they propose that three Chl *b* molecules are strongly coupled in an excitonic trimer and that excitation in the 650-nm region prepares states of the $(\text{Chl } b)_3$ supermolecule. According to the analysis of Shepanski and Knox,[23] there are two transitions, with the state at 652 nm being doubly degenerate and carrying 88% of the total dipole strength, while a second state at 665 nm carries the remaining 12% of the dipole strength. The Chl *a* molecules are proposed to be much more weakly coupled than the Chl *b* molecules and to exist in two forms with absorbance maxima at 670 and 677 nm.

We previously showed that a weak coupling localized phonon calculation (such as the Förster very weak coupling limit) of energy transfer in the reaction center of photosynthetic bacteria produced rates that were significantly slower than the experimentally observed rates.[46] In order to assess the appropriateness of Förster theory and the Van Metter–Shepanski–Knox model of LHC II, we have calculated energy-transfer rates based on Förster theory and the assignments of Knox and co-workers. (It should be noted,

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TABLE I: Förster Theory Estimates of Energy-Transfer Times (in ps)

	Chl $b \rightarrow$ Chl a		Chl $b \rightarrow$ Chl a		Chl $b \rightarrow$ Chl a		Chl $a \rightarrow$ Chl a		Chl $a \rightarrow$ Chl a	
	(652 nm)[b]	(672 nm)	(652 nm)	(672 nm)	(665 nm)	(672 nm)	(672 nm)	(672 nm)	(672 nm)	(678 nm)
separation, Å	11.57 D²[a]	16.85 D²	15.31 D²	16.85 D²	4.08 D²	16.85 D²	16.85 D²	16.85 D²	16.85 D²	16.85 D²
10	1.4		1.06		0.62		0.17		0.14	
12	4.19		3.17		1.85		0.50		0.42	
14	10.58		7.99		4.65		1.25		1.07	
16	23.57		17.81		10.37		2.79		2.37	

[a] These are values of μ^2. [b] The wavelength in parentheses refers to the absorption maximum for the particular species.

however, that their model is based on in vitro micelle preparations of LHC whereas our results refer to LHC in intact thylakoids.)

The rate of relaxation from the 652-nm states to the 665-nm state is unknown but presumably very rapid. We calculated the energy-transfer rate between both these states and a Chl a molecule absorbing at 670 nm. Following the procedure described in ref 46, the steady-state absorption spectrum in Figure 1 was modeled as the sum of two Gaussians for each Chl species and the energy-transfer rate calculated from the following expression

$$k_{ij} = \frac{4\pi\cdot 10^{-24}}{h^2 c n^4 R_{ij}^6}\kappa^2\left(\frac{\mu_i^2\mu_j^2}{2\pi^{1/2}\sigma_i\sigma_j}\right)\times \left(\frac{1}{2\sigma_i^2}+\frac{1}{2\sigma_j^2}\right)^{-1/2}\exp\left[\frac{-\Delta^2}{4\sigma_i^2\sigma_j^2}\left(\frac{1}{2\sigma_i^2}+\frac{1}{2\sigma_j^2}\right)^{-1}\right]$$

where n is the refractive index, R_{ij} is the center to center separation in Å, κ^2 is the orientation factor,[3] μ_i^2 is the dipole strength in (debye)², σ_i is the width (fwhm = 2.34σ) of the Gaussian band, and Δ is the difference in energy (in cm^{-1}) between the peak of the donor emission spectrum and the peak of the acceptor absorption spectrum. The dipole strengths of Chl b and Chl a in solution are 11.57 and 16.85 D², respectively.[47] The spectral widths, σ, were taken as 145 and 135 cm^{-1} for Chl b and Chl a, respectively, and the refractive index was taken to be 1.33.[48] The value of Δ depends on the specific model for the transfer process as described below. In order to estimate Δ for the various cases, the solution Stokes shift (110 cm^{-1}) was assumed in all cases. We somewhat arbitrarily use a value of 1.25 for the orientation factor. Rates were calculated for separations between 10 and 16 Å. In the bacteriochlorophyll protein of *P. aestuarii*[6] the average spacing between Chl molecules is 12 Å.

The first column in Table I shows transfer times calculated with the assumption that transfer occurs from a single Chl b to a single Chl a molecule with absorption maxima at the two peaks of the spectrum in Figure 1 (652 and 672 nm). The calculated transfer time is clearly much longer than that observed experimentally. The second two columns in Table I use the Van Metter–Shepanski–Knox model. The distribution of dipole strength between the two states is taken from ref 23, with the 665 nm Chl b state having a dipole strength of $11.57 \times 3/8.5 = 4.08$ D². Although this state has a relatively low oscillator strength, it has excellent overlap with Chl a absorbing at 672 nm and has a rapid (1.85 ps) transfer time for a 12-Å separation. In the Van Metter–Shepanski–Knox model[22,23,49] all three Chl a molecules are assumed equidistant from the Chl b trimer. Two Chl a molecules are assigned to absorb at 672 nm and one at 678 nm.[23] The calculated rate of energy transfer from the Chl b 652 state to the 678-nm Chl a molecule is ~30% slower than to the 672-nm molecules, and since the Chl a (672 nm) to Chl a (678 nm) rate is very fast (see below), the appearance time of Chl a fluorescence from either the 672- or 678-nm state in the context of this model would be roughly half the values listed under the 665 → 672 nm column in Table I. Thus, for a 12-Å Chl b–Chl$a$ separation the calculated rise time (~0.9 ps) is similar to the experimental value. By contrast, the 652-nm Chl b state has a substantially lower energy-transfer rate than the experimental value at this separation. Thus, even pooling essentially all the Chl b dipole strength into the 652-nm band produces rates that are too slow unless the separation is 10 Å. At this separation (and even at 12 Å) the assumption of weak coupling seems rather dubious. In addition, the calculated rates are probably too slow to compete with a Chl b (652 nm) to Chl b (665 nm) radiationless relaxation. Thus this relaxation would be unlikely to influence the observed signal. The final columns in the table list Förster transfer times for Chl a (672 nm) to Chl a (672 nm) and Chl a (672 nm) to Chl a (678 nm) monomer transfer, respectively. Note that transfer between Chl a molecules is significantly faster than transfer from Chl b to Chl a at the same separation as a result of the higher dipole strength of Chl a.

A variant of the original Knox et al. model has recently been proposed by Gulen et al.[49,50] Based on CD data one Chl a (672 nm) is strongly coupled to the Chl b trimer. In this case our observations would relate to transfer to the remaining 672- and 678-nm Chl a molecules. We have not considered this model in detail.

Concluding Remarks

The ultrafast transfer of excitation from Chl b to Chl a in 0.5 ± 0.2 ps is consistent with our previous estimates of hopping times in light harvesting arrays.[28,29] To obtain transfer times that are consistent with our experiment from a Förster theory calculation requires either one of two arrangements of the chromophores: (a) strong coupling between the Chl b molecules such that the resulting supermolecule is coupled to several Chl a molecules and a lower energy state with improved overlap with Chl a is formed; this type of model was originally proposed by Knox and co-workers;[22,24] or (b) a large number of Chl a molecules within 12 Å of the donor Chl b molecule. However, only when a high-resolution molecular structure of LHC II is available will a definitive test of the model and of the applicability of Förster theory to the in vivo Chl b to Chl a energy transfer be possible.

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