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Original Communications

Energy Transfer within PC Trimers of *Mastigocladus laminosus* Studied by Picosecond Time-Resolved Transient Absorption Spectroscopy

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Energy Transfer, Phycocyanin Trimer, Excited State Lifetimes, Transient Absorption Recovery

The transient absorption recovery induced in phycocyanin trimers by picosecond pulses of variable wavelength (570–620 nm) has been recorded and analyzed by applying a least-squares multi-exponential fit procedure.

The results suggest that in native PC trimers the chromophores exhibit a microheterogeneity with the effect that the derived apparent lifetimes are functions of excitation and probing wavelength. It is suggested that, due to strong excitonic coupling between α-84 and β-84 chromophores, the lifetime of the terminal acceptor state is reduced to about 900 ps; the apparent energy transfer time from chromophore β-155 to α-84 and β-84 chromophores varies between 20–50 ps depending on the actual chromophore-protein arrangement (microheterogeneity).

Introduction

The light harvesting antenna complexes of cyanobacteria and red algae, the phycobilisomes (PBS), are highly organized assemblies of biliproteins and linker peptides. The extensive investigation of the fast and efficient energy transfer within the phycobilisomes and further to the reaction center located in the thylakoid membrane has led to a qualitative model for these processes [1]. Important steps in its development were the detection of the organization scheme by electron microscopy [2], the identification of the building blocks, namely trimers and hexamers of allophycocyanin, phycocyanin (PC) and phycoerythrin [1–3] and finally the determination of the structure of two C-phycocyanins from different organisms by X-ray cristallography [4, 5]. The latter proved that in monomeric PC the three phycocyanobilin chromophores experience different microenvironments: the so-called β-84 chromophore (bound to cystein 84* of the β-chain) extends in the inner core region and is, therefore, most likely subject to interaction with the linker peptides. Based on the analysis of the UV-absorption, emission and CD-spectra [6–8] it had been shown earlier that the three phycocyanobilin chromophores were spectroscopically distinct species. According to their assumed function in the energy transfer chain, they were divided into sensitizing and fluorescing chromophores. Titration experiments with PCMS (*para*-chloromercurybenzenesulfonate) [9] and crystal spectroscopic studies (T. Schirmer, private communication) gave recently the final proof that the red-most absorbing, "fluorescing" chromophore is identical to β-84.

Early picosecond time-resolved fluorescence decay studies showed decay curves which could be fit best by multi-exponentials. 2–4 decay times were obtained, which fall into 3 distinct ranges i) 1.3–1.8 ns, ii) 100–500 ps and iii) 10–80 ps.

The longest decay time has generally been interpreted as the "free" decay of the fluorescing chromophore(s). The others are due to energy transfer within the complex as shown in particular by time-resolved depolarization studies and the observation of rise-terms in fluorescence [10–15], but their physical meaning is still under debate. From the variation of amplitudes with wavelength the fastest decay component has generally been assumed to be due mainly to the s → f transfer, *i.e.* energy transfer from β-155 to β-84 and/or α-84.

* The alignment adjusted nomenclature [4] has been adopted for amino acid numbering.

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After the X-ray structure became known, which estimates the closest distance between an α-84 and a β-84 chromophore of neighbouring monomers in a trimeric unit to be only about 20 Å, the fastest decay component was assigned alternatively to the energy transfer between these two chromophores [16, 17]; the decay time of around 300 ps had to be interpreted as the lifetime of β-155, which is located at the periphery of the trimeric unit.

Theoretical considerations [16, 17] show that the energy transfer times between chromophores are sensitive functions of not only the distance, but also of the overall orientation of the tetrapyrrole chromophores. They should in addition vary with the precise values of the torsional angles at the methine bridges since direction and magnitude of the transition dipole moment change significantly with these parameters [18].

In an attempt to distinguish between the above mentioned alternative interpretations of the observed fluorescence decay patterns of isolated PC trimers we have reinvestigated this problem applying picosecond time-resolved transient absorption spectroscopy.

Materials and Methods

Preparation of PC trimers

Trimers of PC were prepared according to the procedure described previously [19]. Measurements were performed after dilution in buffer. Chromophore concentration was chosen such that OD (620 nm) ≤ 1 cm^{-1} 0.1 M phosphate buffer, pH 7.0.

Absorption spectra were recorded before and after each measurement to check for deterioration.

Picosecond time-resolved transient absorption spectroscopy

For measuring the groundstate recovery time, the pump-probe technique was used as displayed schematically in Fig. 1. The sample is excited by tunable picosecond pulses from a cavity-dumped dye laser ($t_p \approx 10$ ps, $E_p \approx 10$ nJ pulse^{-1}). The change in transmission of a weak probe pulse, which is derived from the excitation pulse by means of a beam splitter and sent through a variable optical delay line, is measured as a function of delay time between pump and probe pulse. In our set-up, the maximum delay, which can be generated without changing the exact geometrical overlap of pump and probe beam in the sample, is about 2 ns; the time window is, therefore, comparable to that of the synchroscan streak camera used in previous measurements [10–14]. In order to avoid artifacts because of thermal lensing, pulse repetition rates of about 80 kHz were chosen; under this condition average light intensities are less than 1 mW (or 10^{13} photons cm^{-2} pulse^{-1}). The induced transmission changes are less than 1% for which reason phase sensitive detection (lock-in amplifier, Ithaco model 393) is used for improving the signal/noise ratio.

The time resolution of the experiment is limited by the pulse width which is determined by recording the background free autocorrelation. It was found that the experimentally determined correlation curves

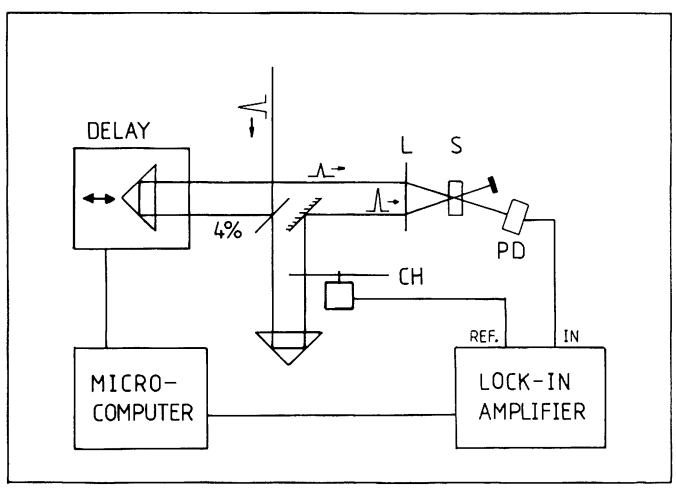

Fig. 1. Experimental arrangement for picosecond time-resolved transient absorption spectroscopy.

can be fitted very well by assuming either a $sech^2$ or gaussian pulse profile. Attempts to analyze the time course of the probe pulse transmission $T(t)$ by a least-squares fit procedure (analogous to the evaluation of the fluorescence data) as a convolution of the experimentally determined autocorrelation function (which includes also the coherence spike, if the step size in optical delay is smaller than the coherence length of the laser pulse) and a multi-exponential decay law failed. The reason is simply that the inclusion of the coherence spike results in a contribution to the response function, which is actually not real. In the response function (decay curve) the coherence spike is very often of neglegible amplitude (because of larger step sizes). Instead of suppressing the coherence spike in the experimental (noisy) autocorrelation function by data manipulation, we prefer to substitute it in the least-squares fit procedure by the analytical function which is calculated for a $sech^2$ or a gaussian pulse profile, respectively. The pulse parameters are taken from the fit of the autocorrelation function (in principle, they could be treated as additional free parameters in the fit routine of the ground-state recovery curves [21]). In contrast to examples reported in the literature [22, 23] where the lifetime are estimated by means of a semi-logarithmic plot of the decaying part of $T(t)$ only, here full use is made of the information which is buried in the onset of the transmission change. Therefore, decay times down to about ⅓ of the apparent pulse width can be evaluated with good accuracy. An additional benefit of the described procedure is that any irregularities in laser performance causing *e.g.* an increase in pulse width become immediately apparent.

Results

The changes in transmission, which are induced in a buffer solution of PC trimers by picosecond pulses of different wavelengths are shown in Fig. 2. For better comparison, all curves are normalized with respect to the maximum value of the bleaching (ΔT_{max}). Since the integration time setting of the lock-in amplifier was kept at a fixed value the S/N ratio

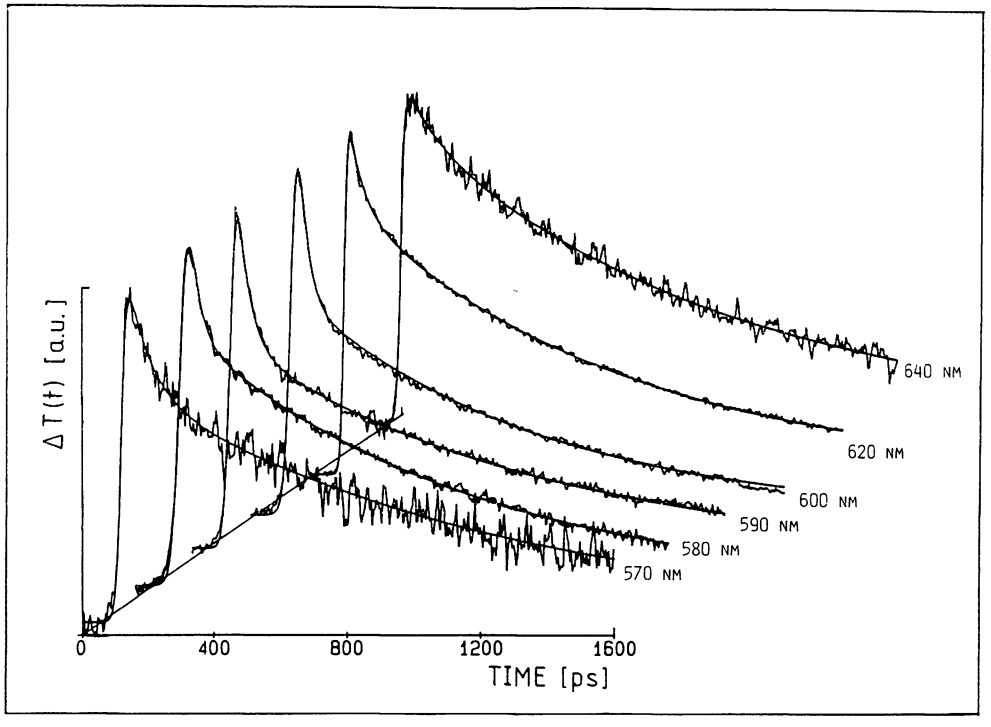

Fig. 2. Excitation wavelength dependence of groundstate recovery of native phycocyanin trimers *(Mastigocladus laminosus)*. The solid lines represent bi-exponential fits with parameters given in Table I.

appears highest for excitation pulses with $\lambda \approx 620$ nm, which yield the highest transmission change, namely 1%.

It is evident already from inspection of Fig. 2 that pulses with $\lambda_{ex} = 600$ nm produce the highest fraction of a very short-lived (excited) species. The reduction in the relative amount of the shorter-lived species seems to be more pronounced when going towards longer wavelength. Without analysis, it can, however, not be decided whether it is only the amplitude ratio, which varies with wavelength or whether the underlaying decay times are in addition dependent on the excitation wavelength. A least-squares fit based on a two-exponential decay law yields the lifetimes and amplitudes summarized in Table I. Although the distribution of the residuals does not necessarily suggest the extension of the analysis to a 3-exponential decay law, such an analysis is recommended for two reasons.

(i) Both short and long lifetime vary systematically with excitation wavelength, such that for $\lambda_{ex} = 600$ nm the shortest decay times are deduced by the fit routine. Such a behaviour is indicative for the presence of an additional component.

(ii) According to the above mentioned models, the three types of chromophores should exhibit just three different lifetimes, if a three-fold symmetry is taken into account [1, 20].

It has been pointed out in connection with the discussion of fluorescence decay curves that depending on experimental conditions the amplitude of one component can be very small and, therefore, difficult to detect unless the S/N ratio is extremely high [13, 24]. Therefore, a fit with a lower number of exponentials often yields an essentially equally good fit.

The results obtained from a least-squares fit based upon a 3-exponential decay law are summarized in Table II. In accordance with the above mentioned expectation the fast component of the 2-exponential fit splits into two components. The amplitude ratio of both components varies with wavelength, but also the lifetimes. Because of the fact that the amplitudes of these short-lived components are fairly high, the variation in lifetimes seems to indicate that the fast part of the decay is not due to a superposition of only two components with fixed lifetimes.

The decay times determined for the third component vary between 800 and 1300 ps. On one hand such a variation could be related to the fact that the observation window comprises only 1500 ps or about 1.5 decay times, for which reason no accuracy better than ± 10% can be expected. On the other hand, the average decay time $\tau_3 \approx 950$ ps is significantly shorter than the fluorescence lifetimes reported in the literature for native PC trimers (1.1–1.5 ns [13, 15]). One possible explanation for this discrepancy could be that the slow component approximates also two components with lifetimes in the range 0.5 ns to 1.5 ns, *i.e.* the least-squares fit should be based upon a 4-exponential fit. It is, however, obvious that the results of such an attempt are subject to discussion in view of the actual S/N ratio and the limitations in observation time. If one forces the fit routine to fix

Table I. Fit parameters (lifetimes and relative amplitudes) obtained for a two-exponential analysis of groundstate recovery curves displayed in Fig. 2.

λ [nm]	T_1 [ps]	A_1 [%]	T_2 [ps]	A_2 [%]
570	64	45	1220	55
580	37	53	935	47
590	36	61	996	39
600	28	66	823	34
620	48	34	898	66
640	97	20	1036	80

Table II. Fit parameters (lifetimes and relative amplitudes) obtained for a three-exponential analysis of groundstate recovery curves displayed in Fig. 2. A: free fit of all parameters; B: long lifetime kept fixed at 1200 ps.

	A		B	
λ [nm]	T_i [ps]	A_i [%]	T_i [ps]	A_i [%]
570	16	30	18	22
	86	26	75	30
	1265	44	1200	48
580	32	54	32	54
	388	7	600	18
	1042	39	1200	28
590	13	21	36	61
	41	43	910	26
	1010	36	1200	13
600	28	64		
	42	2	–	–
	829	34		
620	29	33	36	35
	194	10	549	27
	974	57	1200	38
640	98	13	86	12
	970	43	764	44
	1047	44	1200	44

the lifetime of the third component at a given value, *e.g.* τ_3 = 1200 ps, then the program does not converge in all cases (*e.g.* not for λ = 600 nm). Where it does the second component adopts decay times between 600 and 900 ps (see Table II); the fastest component(s) is (are) approximated by a single exponential with lifetimes close to those derived from the biexponential fit.

Discussion

Before entering the discussion of the above results it should be mentioned that time-resolved fluorescence measurements using single photon timing showed that a 3-exponential fit was not sufficient [15]. Using a 4-exponential global fit analysis, the set of decay curves was reproduced by exponentials with lifetimes of 1420, 807, 203 and 36 ps, respectively. The 800 ps component was assigned to denatured chromophores. In the polarized synchroscan streak camera measurements usually two components with $\tau_1 \approx 50$ ps and $\tau_2 \approx 1000-1200$ ps were found in the isotropic decay curves $I_0 = (I_p + 2I_s)$ of freshly prepared trimers [13] while in the difference function $D = I_p - I_s$ a 800 ps component was detected next to a fast one ($\tau \approx 50$ ps). When crystallized trimers [5] were dissolved again in buffer solution, both functions I_0 and D could be fit by two exponentials with lifetimes of 50 and 1300 ps, respectively [14].

This brief compilation of prior results demonstrates that the new results gained by the pump-probe technique must be discussed for each of the following three cases:

(i) The three-exponential fit yields an adequate description of the time-resolved transmission curves because there are 3 well defined types of chromophores.

(ii) The adequate fit of the set of experimental curves requires the superposition of ≥ 4 exponentials with wavelength independent decay constants, but wavelength dependent amplitudes.

(iii) The multi-exponential fit with wavelength dependent decay times is descriptive, *i.e.* the derived decay times are not directly related to lifetimes of a small number of structurally well defined chromophores but rather the average over a distribution of geometries.

case (i):

This interpretation implies that the phycocyanin trimer in solution still exhibits the three-fold symmetry which is found in the crystal [16, 17]. Assuming that the spectral features of the three chromophores are unchanged when going from the subunits to the trimer then the fastest rate should be assigned to the energy transfer between α-84 and β-84 as has already been mentioned above [16, 17]. In accordance with such an assignment would be the observation that the fastest component is predominant when the excitation wavelength falls between the absorption maxima of β-155 ($\approx$ 595 nm) and β-84 ($\approx$ 622 nm). The second fastest rate should then be governed by the lifetime of β-155. The variations in lifetimes and relative amplitudes of the two fast components of the 3-exponential fit are, however, such that one can not rationalize them as being due to the inaccuracy inherent to the fitting procedure. Large variations in the decay times when applying a 3-exponential fit to groundstate recovery measurements, have also been observed by other authors for similar preparations of PC trimers [23]. Similar decay times were found when the same excitation wavelength was used, which supports the hypothesis that these variations are not random but inherent to the system. Therefore, one has to reject model (i).

The dominance of a 800–900 ps component in the transient transmission recovery at longer wavelength indicates that upon formation of the trimeric state most of the β-84 chromophores adopt a specific geometry with such a lifetime. Chromophores with other geometries or in slightly different protein environment could have longer lifetimes and most likely larger fluorescence quantum yields, for which reason their contribution to the fluorescence decay should be more pronounced. In contradiction to the interpretation given by Wendler *et al.* [15] transient absorption measurements would suggest that the shorter-lived chromophore-protein configuration would be the dominant one in isolated trimers (without linker peptides). Changes in fluorescence properties upon deaggregation and reaggregation have also been reported for allophycocyanin [3, 25].

case (ii):

The second alternative, which is similar to a model proposed by Wendler *et al.* [15], assumes implicitly that the fast kinetics is not modified by the presence of "denatured" chromophores. *I.e.*, the modified chromophores are not involved in the energy transfer chain, but are excited directly and relax to the groundstate like isolated molecules. In this connec-

tion it is noteworthy that the long-lived component can not always be split into two components with one lifetime of 1200 ps (fixed) and another one around 600–800 ps (see Table II). This indicates again that with excitation wavelength 600 nm, that chromophore protein configuration must be predominantly excited (either directly or *via* energy transfer) which has a lifetime below 1 ns.

A completely different interpretation of case (ii) follows the lines given earlier by Schneider *et al.* [13, 14]. Based on the refined data from X-ray analysis of the structure of the chromophores in C-phycocyanin [30] we have calculated the energy transfer rates between the three types of chromophores according to Försters formula for resonant dipole-dipole transfer. Information about the spectral properties of the chromophores, *i.e.* the spectral overlap integrals were taken from Sauer *et al.* [17]. The result of these calculations is summarized Fig. 3. The transfer rates between the α-84 and β-84 chromophores of neighbouring monomers and *vice versa* are denoted as X and Y, respectively. Since the center-to-center distance between these chromophores is only about 20 Å, the calculated rates are very high, namely 916 ns^{-1} for α-84 → β-84 and 771 ns^{-1} for β-84 → α-84. Therefore, under the assumption of Förster type energy transfer a fast equilibration between these chromophores within 1 or 2 ps should be expected. On the other hand transfer times of some picoseconds are comparable to typical time-constants of vibrational relaxation of the excited molecules, *i.e.* Försters assumption of incoherent transfer steps should no longer be valid, but coherent excitonic interaction must be taken into account. In this case the α-84 and β-84 chromophores in the trimeric unit form a weak excitonic coupled system, *i.e.* their excited states form a kind of "compound state" (localized exciton). Measurable excitation transfer

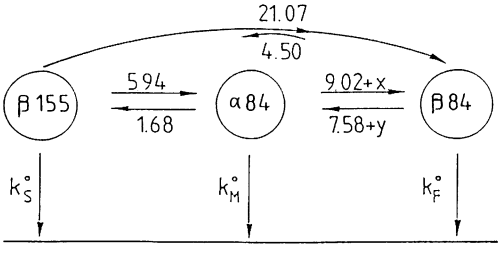

Fig. 3. Calculated energy transfer rates (in $nsec^{-1}$) between the three different types of chromophores in a C-PC trimer (for more details see text).

then takes place only between the β-155 chromophore and such a compound state. For this reason only two decay times should be found experimentally, one describing the energy transfer from β-155 to the coupled chromophore system, the second one describing the decay of this compound state.

The appearance of four fixed decay times in a global data analysis could be rationalized by dividing them into two pairs, each pair exhibiting one fast and one slow component. To be more specific, one could postulate that β-84 or α-84 exists in two (slightly) different geometries, each giving rise to a distinct coupled state with a precise lifetime. Then the energy transfer from β-155 to either one of these states should also be described by one fixed rate constant each.

The assumption that β-84 could exist in two different geometries is suggested by the fact that this chromophore appears to be most susceptible to pertubations [4, 5, 7, 9]. Microheterogeneity of α-84 is suggested by the observation that the fit of the fluorescence decay of α-subunits needs more than one exponential [11, 26].

case (iii):

This model is a straight-forward extension of the two state model described above to one with many different chromophore conformations (microscopic heterogeneity) as it is also suggested from holeburning experiments (J. Friedrich, private communication). Different geometries, *e.g.* angles of torsion around methin bridges, cause differences in excited state properties like excitation energies and lifetimes [27, 28] and they should also affect the above discussed interaction between the α-84 and β-84 chromophores. In case of weak excitonic coupling it is obvious that differences in the excited state wavefunctions cause variing coupling constants, thus giving rise to different "compound states". Since these coupled chromophores are acceptor states for the depopulation of the β-155 chromophores *via* energy transfer, a distribution of transfer rates for this step (short lifetime) as well as the observed variation of the long lifetime with wavelength (decay of different compound states) can easily be rationalized. But even in case of very weak coupling (Förster type energy transfer) a microscopic heterogeneity in the chromophore conformations should result in a distribution of transfer rates, because the probability for resonant dipole-dipole energy transfer depends

sensitive on the geometrical orientation factor $\varkappa$ between donor and acceptor molecules ($k_{D\to A} \sim \varkappa^2$).

James *et al.* [24] have demonstrated for fluorescence decay curves that whenever the recorded fluorescence is a superposition of several components with a finite distribution of lifetimes a satisfactory fit can be achieved by assuming a bi-exponential decay law. Since one is dealing with the same mathematical problem when analyzing groundstate recovery curves, an *a priori* distinction between the latter interpretations can not be given on the basis of this experimental information alone (a fact which is apparently ignored by many authors). It seems plausible that in case of a distribution of similar chromophore-protein arrangements the bi-exponential fit, where each lifetime then represents an average over the individual components, does show a fairly systematic variation of fit parameters with excitation wavelength.

In our opinion additional evidence for the assumption of coupling between α-84 and β-84 in PC is also given by the completely different fluorescence decay pattern of phycoerythrocyanin trimers [13]. There the excited state of the "modified" α-84 chromophore lies above that of β-155, *i.e.* the energetic near-degeneracy with β-84 is removed. As a consequence three fluorescence components with distinctly different lifetimes can be deduced ($\tau_1 \approx 100$ ps, $\tau_2 \approx 400$ ps, $\tau_3 \approx 1400$ ps). A similar observation can also be made for monomers; there the coupling between α-84 and β-84 is much smaller than that between chromophores belonging to different monomers in one trimeric unit (Geiselhart, unpublished results).

Conclusion

Considering the experimental results collected on biliproteins in various states of aggregation by different spectroscopic techniques we feel that the interpretation given for case (iii) is currently the only one which can explain all observations without leading to contradictions. In contrast to the widely adopted model described above we postulate that in native PC-trimers, weak excitonic coupling between α-84 and β-84 prevents the detection of Förster type energy transfer between these chromophores. The proof for this statement can come from three sources.

(i) Knowing the absorption spectra of the three types of chromophores, the time course of the induced absorbance changes can be calculated for each kinetic model and especially the variation of the amplitudes of each exponential with wavelength. Because of discrepancies between theory and experiment some models might be rejected. Work in this direction is under way in our group.

(ii) Higher resolution X-ray data could provide the basis for quantum mechanical model calculations to determine theoretically the interaction between α-84 and β-84 chromophores (assumption of excitonic coupling).

(iii) Investigation of chemically modified biliproteins, for which both kinetic (lifetime measurements) and structural information (*e.g.* CARS-spectra [29]) is available.

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Note added in proof: The work of Schirmer cited on the title page has been published: T. Schirmer and M. G. Vincent, Biochim. Biophys. Acta **893,** 379–385 (1987). An update of ref. [17] using the revised coordinates for PC [30] arrives at similar fast Förster transfer rates and excitonic couplings as discussed above (K. Sauer, H. Scheer, unpublished).

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