Femtosecond fluorescence depolarization study of photosynthetic antenna proteins: observation of ultrafast
energy transfer in trimeric C-phycocyanin and Allophycocyanin

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ABSTRACT

C-phycocyanin (CPC) and Allophycocyanin (APC) are pigment-protein complexes isolated from
antenna systems in cyanobacteria. The crystal structure of CPC has been solved recently1-3 and APC has a
similar structure. CPC and APC have a trimeric structure, monomeric subunits are composed of an α and β
polypeptide chain, each has a tetrapyrrole chromophore chemically bound to position 84. In CPC and APC
trimers, the α84 and β84 chromophores in adjacent monomers are in close proximity, forming relatively
strong coupled pairs. Calculation of pairwise energy transfer rates using Förster theory has suggested an
extremely fast transfer (> 1 ps⁻¹) between the α84 and β84 pair in CPC.4 We have constructed a
femtosecond fluorescence up-conversion apparatus which achieves subhundred femtosecond time
resolution. Using this technique, we have experimentally observed the fast energy transfer process
between the α84 and β84 pair in both CPC and APC. We also observed a wavelength dependence of the
fluorescence depolarization kinetics which is inconsistent with Förster inductive resonance energy transfer
theory.

1. INTRODUCTION

In the initial steps of photosynthesis, electronic excitation of pigments by light absorption in light
harvesting antenna complexes is rapidly transferred among the pigments and trapped in photosynthetic
reaction centers where electron transfer occurs with high efficiency. Without knowledge of the
intermolecular distances and relative orientations of chromophores, it is often difficult to investigate the
mechanism of energy transfer from a chemical physics point of view. Recently, a few structures of
antenna proteins have become available at atomic resolution.1-3,5-8 This offers an opportunity to study the
mechanism of energy transfer in a photosynthetic system in a detailed fashion.

C-phycocyanin (CPC) and Allophycocyanin (APC) are isolated phycobiliproteins from
phycobilisomes, an antenna protein complex in cyanobacteria.9,10 Both CPC and APC have chemically
identical chromophores, the open chain tetra-pyrroles (phycocyanobilins). However the absorption
spectrum of APC is red shifted from that of CPC as a result of stronger excitonic coupling. The crystal structures of CPC from three different organisms have been solved\textsuperscript{1,2,7} and recently refined to 1.66 Å.\textsuperscript{3} CPC is composed of α and β polypeptide chains. Tetapyrrole chromophores are covalently bound via thioether bonds to cysteines, one on the α chain and two on the β chain, denoted as α84, β84 and β155 respectively. One α and one β chain form a subunit. X-ray structure analysis reveals that three monomeric subunits (αβ) form a trimer (αβ)\textsubscript{3}, as sketched in Figure 1. A C\textsubscript{3} symmetry axis passes through the center and is perpendicular to the disk like structure. In the trimers, the phycocyanobilins α-84 and β-84 of adjacent monomers are located close together, forming a strongly coupled pair with center to center distance of 20 Å. Spectral studies of isolated subunits indicate that β155 has the highest absorption maximum whereas β84 has the lowest, possibly resulting from different protein environments of the bilins.\textsuperscript{11,12} The crystal structure of APC has not been obtained. However the sequence homology between APC and CPC suggests that APC adopts a similar tertiary structure to CPC.\textsuperscript{13} A major difference in APC is the absence of β155 due to the deletion of ten residues in the β chain in the region of the β155 binding site.\textsuperscript{13} The considerable red shift of absorption and emission spectra in APC compared to CPC is believed to be due to stronger excitonic interaction between α84 and β84.\textsuperscript{14}

Figure 1. Schematic arrangement of chromophores, α84, β84 and β155 in trimeric CPC according to Ref 3-7. See text for details.

Sauer and Sheer have carried out calculations of excitation transfer rates among the chromophores of CPC using the Förster inductive resonance transfer mechanism and the coordinates for the position and orientations.\textsuperscript{4,12} This Förster calculation indicated that the fastest component for the (αβ)\textsubscript{3} trimer should have a time constant less than 1 ps, corresponding to the pairwise transfer between α84-β84. The short interchromophore distances and favorable orientation factors of α84 and β84 not only produce in the large Förster rate, but also a large value (56 cm\textsuperscript{-1}) of the exciton interaction energy. This led the authors to suggest that mechanisms other than Förster's should be incorporated. In particular, internal conversion from the upper exciton level to the lower one is proposed. The calculated Förster rate is much faster than the reported experimental value of 20 to 36 ps from fluorescence\textsuperscript{16} or the values of 21 to 48 ps obtained from transient absorption measurement.\textsuperscript{17,18} Clearly this indicates the need for experiments with femtosecond time resolution.
We have recently constructed a fluorescence upconversion apparatus with subhundred femtosecond time resolution. In investigating electronic excitation energy transfer processes, time resolved fluorescence has several advantages over transient absorption (ground state recovery) in that it is free of complications from coherent artifact, excited state absorption and stimulated emission. Time resolved fluorescence depolarization measurements have been explored both experimentally and theoretically as an approach for studying energy transfer in condensed phase. As will be demonstrated later, the application of such an approach to photosynthetic antenna is particularly informative about the rate of energy transfer, given the fact that the photosynthetic pigments have heavily overlapped spectra.

Time resolved spectroscopies have been extensively used to study the energy transfer in phycobilisome and isolated phycobiliproteins. Previous time resolved fluorescence studies on CPC and APC utilized time correlated single photon counting, streak camera and phase-shifting approaches, all with time resolution of at best several picoseconds. These studies were mostly concerned with the decay kinetics of the total fluorescence at different wavelengths, and are less sensitive to energy transfer than fluorescence depolarization. Transient absorption experiments with better time resolution (1-2ps) have been reported on intact phycobilisomes, and isolated phycobiliproteins, CPC and APC. Time dependent linear dichroism measurements have also been employed. However, insufficient time resolution in these studies is often reflected by the lack of observation of 0.4 for the initial anisotropy. Recently Beck et al. have carried out picosecond linear dichroism experiments on APC. Their results reveal an ultrafast (< 2ps) depolarization process which they attributed to the internal conversion between two excitonic states of the strongly coupled pair of a84 and b84. Depolarization experiments on these systems with femtosecond resolution have not appeared in literature.

In this paper, we present our preliminary experimental result on fluorescence depolarization in CPC and APC trimers. The data of CPC is compared to the prediction of Förster theory. We have found that Förster theory can not describe the energy transfer between a84 and b84 on the two adjacent monomers in CPC. An even faster depolarization is observed in trimeric APC.

2. EXPERIMENTAL

Fluorescence up-conversion technique serves as the best approach for achieving subpicosecond luminescence time resolution and has undergone rapid developments in recent years. In building our up-conversion apparatus, care has been taken to obtain both high time resolution and good sensitivity. The experimental details and some design considerations are briefly described in this section.

The cavity dumped antiresonant ring dye laser used in the experiment has been described in detail. This laser typically provides pulses shorter than 75fs FWHM, 2nJ in energy and had a center frequency around 605nm. When using 605nm light as excitation source, the dye laser is cavity dumped at 1 MHz, and its output is group velocity compensated by a pair of SF-10 prisms and then directly split by a 50% beam splitter for excitation and gate beams.

When using 630-660nm as excitation source, the dye laser is cavity dumped at 100KHz and the pulses are amplified by a two pass dye amplifier pumped by the frequency doubled output from a 100KHz Nd:YAG regenerative amplifier. A detailed description of this regenerative amplifier has appeared. The amplified dye pulses have 200 nJ pulse energy, and are group velocity compensated by a pair of SF-10 prisms in a double pass retroreflecting geometry, 10% of the beam is split to serve as gating pulses, the rest is focused onto a flowing water cell to generate broad continuum. Band pass interference filters (10nm FWHM) are used to select the desired excitation wavelength. The continuum is then amplified by a
single pass dye amplifier (DCM) pumped by 10% (35nJ) of the regenerative amplifier output. The amplified continuum is precompensated by another pair of SF-10 prisms in a double pass retroreflecting geometry. The cross correlation of 605nm and amplified continuum is 100-120fs FWHM.

![Figure 2. Schematic of the femtosecond up-conversion apparatus (the case of 605nm excitation).](image)

The fluorescence upconversion apparatus (the 605nm excitation case) is shown in Figure 2. The excitation beam is passed through an optical delay line, a half wave plate, focused by a 7.5 cm focal lens onto a sample cell with 1mm path length. The focal spot is smaller than 0.3mm, the excitation intensity is such that each CPC or APC trimer is excited by at most one photon. This avoids annihilation problems.

The key to achieving high time resolution is to use reflective optics to collect the fluorescence. While the group velocity dispersion induced by dispersive optics in the pump and the probe beam, such as lenses and half wave plates can always be precompensated by prism pairs, the group velocity dispersion of fluorescence light is very difficult to compensate. In choosing the reflective optics, we need to collect as much fluorescence light as possible and to focus it on the mixing crystal as tight as possible. A Cassegranian arrangement was used in previous designs. We use an elliptical mirror (Melles Griot) made of electrochemically deposited Rhodium. The elliptical mirror is free from spherical aberration and astigmatism. Although it does not give a focus as tight as a Cassegranian can (due to coma), it has a larger solid angle of collection and is easier to align. The distance between two foci is 10cm. Sample emission from the left focus has a 1:1 image on the right one, where the mixing beam is focused and overlapped onto a mixing crystal in noncollinear geometry. The lens used in the mixing beam has 7.5 cm focal length. A half wave plate in the beam makes the beam vertically polarized.

A 0.5-1mm thick Type I LiIO₃ crystal is used for sum frequency generation. The advantage of Type I mixing over Type II mixing is that the former has less group velocity mismatch (both beams polarized along o axis) when the two beams have similar wavelengths. Therefore a relatively thick crystal can be used for Type I mixing to obtain a larger signal. For 1mm Type I LiIO₃, the group velocity mismatch for 600nm and 650nm light was estimated to be 40fs. The disadvantage of Type I mixing is that the second harmonic of the mixing beam often results in a very large background signal. A double monochromator (Instrument SA, DH-20, F = 4.2) is used to suppress the background and extract the signal. The emission
detection wavelength is selected by angle tuning the crystal around a vertical axis to the appropriate phase-matching angle and setting the monochrometer to the corresponding sum frequency. Mounting the LiIO3 crystal in such a way assures the best stray light rejection of the monochrometer. The up-conversion bandwidth of the experiment is about 8 nm.

The signal is detected by a photon counting PMT (EMI, 9789B) and a gated photon counter (Stanford Research System model 400). The intensity of excitation pulses are monitored with a PMT and boxcar average combination. The intensity fluctuation is normally within a few percent and normalization of the raw data was not carried out. A 486 compatible computer is used for controlling optical delay line (Micro-Control, 1μm resolution) and data acquisition. Data was collected by repetitive scanning with 1s/pt counting time. Decay curves for parallel and perpendicular polarization with respect to the excitation polarization were obtained on alternate scans of the delay line by rotating the half waveplate in the excitation beam. Individual scans within a data set were added.

Care has been taken to assure an accurate depolarization measurement. No polarizer is used after the sample in order to maintain high time resolution and sensitivity. Only the vertically polarized fluorescence component is up-converted with the vertically polarized mixing beam when the LiIO3 crystal (o + o → e) is carefully aligned. It is necessary to reduce the solid angle of fluorescence collection by masking the elliptical mirror in order to prevent scrambling of s and p polarization by the mirror. We have verified the initial anisotropy of crysyl violet and diluted chlorophyll solution are both 0.4±0.02. Biological samples usually generate some scattering, however, we found that scattering does not affect the initial anisotropy significantly in our apparatus.

The excited state population decay \( K(t) \), and the anisotropy, \( r(t) \), are related to the deconvoluted emission curves by

\[
K(t) = I_{\text{para}}(t) + 2 I_{\text{perp}}(t) \quad (1)
\]

\[
r(t) = [I_{\text{para}}(t) - I_{\text{perp}}(t)] / [I_{\text{para}}(t) + 2 I_{\text{perp}}(t)] \quad (2)
\]

The measured parallel and perpendicular data were analyzed by simultaneous iterative reconvolution and nonlinear least-squares fitting of both data as described by Cross and Fleming.28 The data could in general be fit using one to three exponential functions for \( K(t) \) and \( r(t) \). The instrumental response function used in fitting was taken to have similar shape of the autocorrelation or cross correlation but 10 to 50 fs broader to account for the group velocity mismatch which varies depending on the emission wavelength and the crystal length.

C-Phycocyanin from *Aphanotheca halophytica* (Suspension in 50% ammonium sulfate containing 0.15M Tris buffer, pH7.4) was purchased from Sigma. It was dialyzed in 0.1M tris buffer, pH 8.0 and was filtered with glass wool. Allophycocyanin from *Spirulina sp.* (lyophilized powder) was purchased from Sigma. It was dialyzed in 0.1M phosphate buffer, pH 7.0 and was filtered with glass wool. The O.D. of the samples was between 0.5-0.8 for 1mm path length. The samples were stirred during the experiments.

3. RESULTS

3.1 CPC trimer

The absorption and emission spectra of CPC trimer from *Aphanotheca halophytica* are shown in Figure 3. The absorption maximum and shape are similar to the CPC trimer from *Mastigocladus laminosus*.11 According to molecular orbital calculations, the transition in this region is \( S_0 \rightarrow S_1 \).29 The conformation of the tetrapyrrole chain has a considerable effect on the transition energy. Also shown in
Figure 3 are spectra of the excitation pulses and up conversion bandwidths for the two experiments described below.

![Excitation Pulse and Up Conversion Bandwidth](image)

**Figure 3.** The absorption and emission spectra of trimeric CPC with the spectra of excitation pulses (centered at 605nm and 640nm) and the corresponding up-conversion windows (centered at 650nm and 720nm, respectively) used in two the experiments.

**a)**

- **Para**
- **Perp**

**Counts**

**Delay Time (fs)**

0 1000 2000 3000 4000

**r(t)**

0 0.2 0.4 0.6 0.8 1.0

**Delay Time (fs)**

0 1000 2000 3000 4000

**Figure 4.** a) Time resolved fluorescence data of CPC trimer with excitation pulses centered at 605nm. Fluorescence was probed at 650nm. The signals with parallel and perpendicular polarization are plotted (dots). The fitted curves (solid lines) give: \( K(t) = -0.22 \exp(-t / 4.7\text{ps}) + 1.22 \), and \( r(t) = 0.13 \exp(-t / 0.80\text{ps}) + 0.21\exp(-t / 20\text{ps}) + 0.07 \). A 150fs instrument response time was used to fit the data. b) The corresponding measured (dots) and fitted (solid line) \( r(t) \) are plotted, clearly showing a fast depolarization.

First we show the time resolved fluorescence data with 80fs excitation pulse centered at 605nm. The fluorescence signals probed at 650nm with parallel and perpendicular polarizations are plotted in Figure 4a. The corresponding anisotropy is shown in Figure 4b. The parameters for the fitted curves are explained in the figure caption. The fluorescence signal at the magic angle (not shown) has only very small level change in this delay time range. However the fluorescence anisotropy measurement clearly demonstrates an ultrafast depolarization process which occurs within a picosecond or two. If the rapid
depolarization results from energy transfer it must correspond to the fastest pairwise transfer or relaxation processes in the CPC trimer, which would be expected to involve the \(\alpha 84\) and \(\beta 84\) pair. We note this fast component has a considerable weight even though at least one third of photons in the 605nm excitation pulses are absorbed by \(\beta 155\), according to Sauer and Sheer's isolated spectrum.\(^{12}\) We also note that an initial anisotropy of \(-0.4\) is observed. This is consistent with the fact that the absorption and emission transition dipoles of the tetrapyrrole chromophore are parallel. Gillbro \textit{et al} have observed an \(r(0)\) of 0.4 in their transient absorption study on monomeric CPC.\(^{17}\) However, previous studies on CPC trimer have neither observed this short time behavior nor an \(r(0)\) of 0.4.

We now evaluate the data with excitation pulses centered at 640nm, the red edge of the trimeric CPC spectrum. The fluorescence is detected at 720nm, and is mostly contributed by the \(0 \rightarrow 1\) transition. The \(0 \rightarrow 1\) transition is expected to have the same transition dipole moment direction of \(0 \rightarrow 0\) transition due to the strong oscillator strength of \(S_1 \rightarrow S_0\). The parallel and perpendicular signals are plotted in Figure 5a. The corresponding anisotropy is shown in Figure 5b. The parameters for the fitted curves are explained in the figure caption. The initial anisotropy is about 0.38 and remains almost constant up to 50ps (longer run data not shown). At 640nm excitation wavelength, very little \(\beta 155\) can be excited according to the isolated spectra.\(^{12}\) The lack of \(\beta 155\) emission should make the depolarization due to the \(\alpha 84\)-\(\beta 84\) transfer, if this occurs, more obvious, as compared with the 605nm excitation case. Yet the fluorescence has no significant amount of depolarization at this excitation wavelength. This fact is intriguing and will be discussed in the next section. This result is consistent with the steady state excitation depolarization spectra which show less depolarization at the red edge.\(^{11}\)

![Figure 5](http://proceedings.spiedigitallibrary.org/proceedings.spiedigitallibrary.org/)  
**Figure 5.** a) Time resolved fluorescence data for CPC trimer with excitation pulses centered at 640nm. Fluorescence was detected at 720nm. The signals with parallel and perpendicular polarization are plotted (dots). The fitted curves (solid lines) give: \(K(t) = 0.07 \exp(- t / 0.83\text{ps}) + 0.93\), and \(r(t) = 0.04 \exp(- t / 0.35\text{ps}) + 0.34\). A 150fs instrument response time was used to fit the data. b) The corresponding measured (dots) and fitted \(r(t)\) (solid line) are plotted, showing no significant depolarization.

2. APC TRIMER

The room temperature absorption and emission spectra of trimeric APC from \textit{Spirulina sp.} are shown in Figure 6. These spectra are similar to those of APC from other organisms.\(^{10}\) The characteristic 650nm band is formed upon trimer formation. The substantial spectral change from CPC is explained in terms of excitonic coupling between \(\alpha 84\) and \(\beta 84\) by MacColl and coworkers.\(^{14}\) The coupling strength
$|V_{ab}|$ is estimated to be 105 cm$^{-1}$. A definitive assignment for this spectrum is not available. Also shown in Figure 6 are spectra of the excitation pulses and up-conversion bandwidths for the two experiments described below.

\[ \text{Figure 6. The absorption and emission spectra of trimeric APC with the spectra of} \]
\[ \text{excitation pulses (centered at 605nm and 640nm) and the corresponding up-conversion} \]
\[ \text{windows (centered at 650nm and 730nm, respectively) used in the two experiments.} \]

\[ \text{Figure 7a shows the time resolved fluorescence data with the excitation pulse centered at 605nm.} \]
\[ \text{Fluorescence was detected at 650nm. The signals with parallel and} \]
\[ \text{perpendicular polarization are plotted (dots). The fitted curves give:} \]
\[ K(t) = -0.4 \exp(-t / 0.43\text{ps}) + 1.4, \quad \text{and} \]
\[ r(t) = 0.23 \exp(-t / 0.36\text{ps}) + 0.15. \]
\[ \text{A 150fs instrument response time was used to fit the data.} \]
\[ \text{b) The corresponding measured and fitted r(t) are plotted, clearly} \]
\[ \text{showing an extremely fast depolarization.} \]

\[ \text{Figure 7a shows the time resolved fluorescence data with the excitation pulse centered at 605nm.} \]
\[ \text{Fluorescence signals probed at 650nm in both parallel and perpendicular polarizations are plotted.} \]
\[ \text{The parallel curve remains constant after rising with the instrument response, while the} \]
\[ \text{perpendicular curve rises within 1ps. This behavior results from both a spectral red shift and} \]
\[ \text{a depolarization. The} \]
corresponding anisotropy is shown in Figure 7b. The parameters for the fitted curves are explained in the figure caption. The depolarization is even faster than in trimeric CPC due to the stronger coupling between α84 and β84 in trimeric APC.

Figure 8a shows the time resolved fluorescence data obtained with an excitation pulse centered at 640nm. The fluorescence signals probed at 730nm in both parallel and perpendicular polarization are plotted. The 730nm emission band is due to the \(0 \rightarrow 1\) transition of tetrapyrrole, which should have the same transition dipole moment direction of \(0 \rightarrow 0\) transition. The corresponding anisotropy is shown in Figure 8b. The parameters for the fitted curves are explained in the figure caption. Again, a much slower depolarization rate is observed when exciting at the red portion of the absorption spectrum. This rate is still faster than 640nm data of trimeric CPC due to stronger coupling in trimeric APC. Similar results are obtained when exciting at 650nm and 656nm.

Figure 8. a) Time resolved fluorescence data for APC trimer with excitation pulses centered at 640nm. Fluorescence was detected at 730nm. The signals with parallel and perpendicular polarization are plotted (dots). The fitted curves give: \(K(t) = 0.12 \exp(-t/0.69ps) + 0.88\), and \(r(t) = 0.16 \exp(-t/2.7ps) + 0.19\). A 120fs instrument response time was used to fit the data. b) The corresponding measured and fitted \(r(t)\) are plotted.

4. DISCUSSION

Sauer and Sheer's calculation using Förster theory showed an extremely fast Förster rate (> 1ps \(^{-1}\)) between the α84 and β84 pair in CPC and thus raised the question whether Förster theory is adequate to describe the energy transfer between α84 and β84.\(^4\) Using the femtosecond fluorescence up-conversion technique, we have experimentally observed this fast energy transfer process between the α84 and β84 pair in both CPC and APC. We are now in a position to experimentally address the issues about whether or not Förster theory is valid in describing these systems, if not, for what reason it fails, to what extent it fails, and what alternative mechanisms are.

The Förster inductive resonance mechanism\(^30\) is adequate to describe the rate of energy transfer in the case of weak coupling. In this regime the interaction between chromophores does not significantly alter the absorption spectrum. In the case of very strong coupling, strong enough that an excitonic splitting is visible in absorption or CD spectra, eigenstates of a "super molecule" are delocalized within the
entire molecular complex, and spectral evolution may result from internal conversion between excitonic states. The intermediate coupling case is not well understood.

4.1. CPC TRIMER

Although there are nine chromophores in the CPC trimer, the situation is simplified when we are only interested in the short time dynamics. Only the energy transfer in the α84 and β84 pair makes a significant contribution to the depolarization in the first several picoseconds. According to Sauer and Sheer's calculation, the fastest pairwise transfer involving β155 (β155 → α84) has a time constant of 417 ps, therefore the fluorescence contribution from β155 should be almost constant on the time scale of interest and can be subtracted from the total fluorescence. Furthermore, the calculated transfer times between two the strongly coupled pairs (β84 → β'84, α84 → α'84, α84 → β84) are also longer than 100 ps. Therefore the three α84 and β'84 pairs can be considered independent at short times. In addition, at our excitation intensity, only one of the three pairs can be excited. The problem reduces to energy transfer between α84 and β84 pairs randomly oriented in solution. The effect of level kinetics of a system with two excited levels on fluorescence anisotropy has been treated by Cross et al. and is directly applicable to this case. A schematic representation of this model is shown in Figure 9. This two state model does not imply a single vibrational level on each chromophores, but rather rapid vibrational relaxation within the chromophores. To simulate the experimental data, we use the spectra of isolated α84, β84 and β155 obtained by Sauer and Sheer to determine the excitation and detection ratios for the different chromophores. These spectra have been used in their Förster calculation.

![Two State Model Diagram](image-url)

Figure 9. The two state model. The radiative rates \( k_{ar} \) and \( k_{br} \) and nonradiative decay rates \( k_{anr} \) and \( k_{brn} \) of both states are in the nanosecond range and make no contribution to the measured kinetics on the time scale of interest.

Figure 10 shows the fluorescence contribution from α84 and β84 to the data in Figure 4. 45% of the total fluorescence is assumed to be β155 emission. This value is estimated from the relative amplitudes of the extinction coefficients in the isolated spectra. The ratio of parallel and perpendicular signals from β155 is kept at 3:1 and is subtracted from the data in Figure 4. The solid line in Figure 10 is a simulated curve using the method of Cross et al. The instrumental response function is taken as a 150 fs gaussian pulse and used to convolute the simulated data. The angle between α84 and β84 is taken as 65°, the value obtained from the crystal structure. A 2:1 excitation ratio for α84 and β84 is used according to the isolated spectra. The detection ratio for α84 and β84 is assumed to be 4:6. The best fit is generated with \( k_{a84\rightarrow b84} \) and \( k_{b84\rightarrow a84} \) equal to 1 ps\(^{-1}\) and 0.8 ps\(^{-1}\), respectively. These values are very close to the corresponding calculated Förster rates, 1.53 ps\(^{-1}\) and 1.16 ps\(^{-1}\). The ratio of the forward and backward transfer rates used in the simulation is determined by the temperature (297K) and the energy gap between...
α84 and β84. Given the uncertainty in the calculation, such as the perturbation of isolated spectra upon trimer formation, the dielectric constant used, the discrepancy between experiment and calculation is not large. Therefore the Förster mechanism is not inconsistent with the data for 605nm excitation.

Figure 10. The trimeric CPC data of Figure 4 (605nm excitation) with the β155 contribution subtracted (dots) and simulated curves using a two state model (solid line), see text for the parameters used in the simulation.

An alternative mechanism responsible for the fast depolarization is internal conversion between two delocalized excitonic states. Although the two states are orthogonal to each other, they can be mixed by vibronic coupling, or interaction with phonons in the protein matrix. The observed depolarization might result from such an excited state relaxation. In principle, there is an experimental way of approaching this question: the angle between the two transition dipole moments of the exciton states should generally be different from that between the two original transition dipoles. Knowing the angle between the transition dipole moments of two excitonic states, we can use the two state model to simulate the data again. The "two states" now represent the two excitonic states, rather than the two monomers.

According to standard excitonic theory, an excitonically coupled dimer with components a and b (which may be dissimilar) has wave functions for the two excitonic states which can be written as

\[
\phi_+ = \cos \alpha \, \phi_a \phi_b + \sin \alpha \, \phi_a \phi_b',
\]

\[
\phi_- = \sin \alpha \, \phi_a \phi_b - \cos \alpha \, \phi_a \phi_b',
\]

(3)

where \(\phi_a\) and \(\phi_b\) are wave functions for the ground state monomers, the primes denote the excited states. The parameter \(\alpha\) can be obtained from

\[
tg 2\alpha = \frac{2 \, V_{ab}}{W_{a'b} - W_{ab'}}, \quad 0 \leq \alpha \leq \pi / 2
\]

(4)

Here \(V_{ab}\) is the coupling matrix element, \(W_{a'b}\) is the energy of the configuration \(\phi_a \phi_b\), and \(W_{ab'}\) has a similar meaning. The eigenvalues of \(\phi_+\) and \(\phi_-\) are

\[
E_+ = \frac{1}{2} \left( W_{a'b} + W_{ab'} + 2 V_{ab} \right), \quad E_- = \frac{1}{2} \left( W_{a'b} + W_{ab'} - 2 V_{ab} \right)
\]
\[ W_\pm = \frac{1}{2} ( W_{ab'} - W_{ab} ) \pm V_{ab} / \sin 2\alpha \]  

(5)

The transition dipoles of \( \phi_+ \) and \( \phi_- \) states are

\[ \mu_+ = \cos \alpha \mu_a + \sin \alpha \mu_b \]

\[ \mu_- = \sin \alpha \mu_a - \cos \alpha \mu_b \]  

(6)

In the weak coupling case, \( 2V_{ab} \ll |W_{ab} - W_{ab'}|, \alpha \approx 0 \), the excitation is essentially localized either in one or the other chromophore. In the strong coupling case, \( 2V_{ab} \gg |W_{ab} - W_{ab'}|, \alpha \approx \pi / 4 \), and the two monomers equally mix to give delocalized states with orthogonal transition dipoles.

Sauer and Sheer's calculation gives \( |V_{ab}| = 56 \text{ cm}^{-1} \) for the \( \alpha84-\beta84 \) pair in trimeric CPC. Such a magnitude of \( V_{ab} \) does not result in a noticeable excitonic splitting in the absorption spectrum. However, a difference CD spectrum of the CPC trimer and monomer indicates the excitonic nature of the \( \alpha84 \) and \( \beta84 \) pair. According to the crystal structure, the orientation of \( \alpha84 \) and \( \beta84 \) results in a negative dipole-dipole interaction energy. So \( V_{ab} \) has a negative sign and the \( \phi_+ \) state has a lower energy. The "a" and "b" here should denote \( \beta84 \) and \( \alpha84 \), respectively, in order to keep \( 0 < \alpha < \pi / 2 \) in Equation 4. We take \( W_{ab} - W_{ab'} \) approximately to be -130 cm\(^{-1}\) (the separation of the two absorption maxima 617 nm and 622 nm, for isolated \( \alpha84 \) and \( \beta84 \) respectively). These values result in \( \alpha = 20^\circ \). With the angle between \( \alpha84 \) and \( \beta84 \) transition dipoles (65°) known from crystal structure, the angle between the two excitonic states, \( \theta \), can be calculated by

\[
\cos \theta = \frac{\mu_+ \cdot \mu_-}{|\mu_+| |\mu_-|} = \frac{(\cos \alpha \mu_a + \sin \alpha \mu_b)(\sin \alpha \mu_a - \cos \alpha \mu_b)}{\cos \alpha \mu_a + \sin \alpha \mu_b| \sin \alpha \mu_a - \cos \alpha \mu_b|} \]

(7)

The ratio of extinction coefficients in Sauer's isolated spectra \( \alpha84 \) and \( \beta84 \) is about 5:3. Assuming \( \mu_a^2 : \mu_b^2 = 3:5 \), we get \( \theta = 118^\circ \). Here we want to emphasize that this angle between the two transition dipole moments of two excitonic states is not 90°, as often believed. The standard excitonic theory predicts a 90 degree angle only in the case of very strong coupling (\( |V_{ab}| \gg 0 \)) or in the case of a symmetric dimer with identical monomers (\( W_{ab} - W_{ab'} = 0 \)). The complementary angle of 118°, 62° is very close to the angle between \( \alpha84 \) and \( \beta84 \) monomer transition dipoles (65°). Complementary angles should result in the same depolarization kinetics and can not be experimentally distinguished. The current signal to noise ratio does not allow discrimination between 65°, and 118° (62°) fits. Therefore the 605 nm data cannot discriminate between the two possible mechanisms. The fitting results (not shown) indicate that if the internal conversion between the two excitonic states is responsible for the depolarization, the internal conversion rates \( k_{+} \) and \( k_{+} \) would be very similar to the Förster rates, \( k_{ab} \) and \( k_{ba} \). However, these two rates originate from two different coupling matrix elements.

We now turn to examine the data with 640 nm excitation. Difficulties arise when using the above two state model to simulate 640 nm data. While keeping \( k_{ab} \), \( k_{ba} \) and the angle between the transition dipoles the same as used for 605 nm data, it is impossible to simulate the 640 nm data by varying the excitation and detection ratio. Figure 11 shows several of these simulations with parameters given in the figure caption. The existence of both fast forward and backward transfer always results in more depolarization than is...
the Förster prediction when excited at the red edge of the CPC spectrum. A simple two state model requires an equilibrium between the two monomers and does not predict a wavelength dependence of the energy transfer rates.

![Simulations of CPC 640nm data with the two state kinetic model. Simulated signal in parallel and perpendicular polarizations are plotted. The angle between two transition dipoles, a and b is taken to be 65° according to the crystal structure. The forward rate (k\textsubscript{ba}), the backward transfer rate (k\textsubscript{ab}), the excitation ratio (b:a) and emission detection ratio (b:a) are listed in the inserts.](image)

Förster theory assumes fast vibrational relaxation on the excited state of the donor, in the spirit of calculating the energy transfer rate by the overlap integral of the donor emission and the acceptor absorption spectra. On the time scale of interest, vibrational relaxation time might not be instantaneous. If vibrational relaxation is comparatively slow, the "uphill" energy transfer (k\textsubscript{ba}) may be influenced more than the "downhill" transfer (k\textsubscript{ab}). The reasoning is as follows. Energy transfer from b to a occurs from unrelaxed levels of b. However, the couplings are likely to be relatively insensitive to the levels and the effect of slow relaxation may not be obvious. On the other hand, use of the microscopic reversibility relation between k\textsubscript{ab} and k\textsubscript{ba} requires that Boltzmann equilibrium be maintained in a. If the upper levels of a (i.e. those that are isoenergetic with b) are depleted by transfer to b on a short time scale the average "uphill" rate may be substantially slower than expected on the basis of the equilibrium model. Thus the
long wavelength excitation data may be consistent with a model involving slow vibrational relaxation compared to energy transfer.

Another obvious reason for the break down of Förster theory is the strong coupling. The Simpson and Peterson criterion, $|V_{ab}|$ much smaller than the electronic bandwidth, is often taken as a criterion for the validity of Förster theory.30,33 In the case of trimeric CPC, the $|V_{ab}|$ of 56 cm$^{-1}$ is compared to the whole electronic band width of $\sim$2100cm$^{-1}$, not in strong violation of this criterion. However, the intermediate coupling regime needs to be better understood. In this regime and in the strong coupling regime, the role of exciton coherence has long been discussed.32,34,35 However direct experimental investigations with ultrafast pulses have only become possible recently.36

Depolarization of fluorescence as a result of energy transfer has been studied phenomenologically for a dimer by Rahman et al.37 The inclusion of off-diagonal density matrix elements and the use of the stochastic Liouville equation resulted in predictions which are independent of the representation chosen (site, or delocalized representation). The fluorescence polarization for arbitrary coupling strength was obtained. However this treatment did not include the optical preparation step for the density matrix. In general, the evolution of the system is determined by how the system is prepared and detected (the spectral and temporal profile of pump and probe pulses). For example, excitation pulses much longer than $V_{ab}/\hbar$ will prepare the system in excitonic eigenstates (with $V_{ab}$ diagonalized). Pulses much shorter than $V_{ab}/\hbar$, on the other hand, will result in localized excitation. When the excitation pulse width is shorter than the dephasing time between these two sites, coherent excitation can occur.

4.2 APC TRIMER

Previous experiments on APC trimer have indicated a fast depolarization process.20,21 In this work, we are able to observe the depolarization directly.

The X-ray structure of APC has not been obtained. A clear assignment of the absorption spectrum in Figure 6 has not appeared. The assignment of the spectrum is dependent on whether the trimer can be treated as three identical pairs and whether the red shift upon trimer formation is due to the excitonic interaction of $\alpha$84 and $\beta$84. It is quite possible that the spectral shifting is due to interacting with protein, such as hydrogen binding and/or electrostatic interaction with charged residues.38 Although such interactions might be responsible for the spectral shift, it should not result in a fast depolarization. At the very least, our result of ultrafast depolarization in trimeric APC suggests that tetrapyrrole chromophores belonging to different monomers are brought together in close proximity. This provides additional evidence for the symmetric trimer structure.

The internal conversion between two delocalized excitonic states of $\alpha$84 and $\beta$84 pair was first postulated by MacColl and coworkers and has been adapted in several recent studies on APC20 and Phycocyanin 645.21 However, the fact that $|V_{ab}|$ is 105 cm$^{-1}$ is smaller than the electronic bandwidth ($\sim$1800cm$^{-1}$ for the whole electronic band and $\sim$900cm$^{-1}$ for the 650nm band)39 still places APC trimer in the intermediate coupling regime. The $|V_{ab}|$ of APC is about twice as large that of CPC. In the 605nm data of APC, it is reasonable to relate the faster depolarization to APC's stronger dipole-dipole interaction. On the other hand, it is not obvious why APC should have a faster internal conversion rate between its two excitonic states. The 640nm data of APC shows a similar wavelength dependence to that observed for CPC, again raising the possibility of slow vibrational relaxation. These two systems with different dipole-dipole coupling provide an interesting opportunity to explore the intermediate coupling regime of energy transfer in photosynthetic systems.
5. CONCLUSION

We have constructed a femtosecond up-conversion apparatus, making it possible to study the ultrafast energy transfer in photosynthetic antenna proteins. Using the apparatus we have directly observed very rapid fluorescence depolarization in trimeric CPC and APC. The simplest model for our observations is ultrafast energy transfer between the a84 and b84 pairs in the trimeric proteins with a rate being faster in APC due to the stronger coupling. However, the excitation wavelength dependence of the observed dynamics cannot be rationalized with a two state model assuming very fast vibrational relaxation. Our study indicates the need for a better understanding of the influence of finite vibrational relaxation rate and for a clearer understanding of the intermediate coupling regime.

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7. REFERENCE

15. N. F. Scherer; M. Du; A. J. Ruggiero; H. Gutman; G. R. Fleming, to be submitted.