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EXCITON ANNIHILATION IN THE ISOLATED PHYCOBILIPROTEINS FROM THE BLUE-GREEN ALGA *NOSTOC* SP. USING PICOSECOND ABSORPTION SPECTROSCOPY

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Abstract—Phycobilisomes from the blue-green alga *Nostoc* sp. contain the phycobiliproteins: *c*-phycoerythrin (*c*-PE), *c*-phycocyanin (*c*-PC) and allophycocyanin (APC). The depletion and the recovery of the ground states for the individual phycobiliproteins were measured using picosecond (ps) absorption spectroscopy. In all cases the depletion time was $\leqslant 10$ ps. The recovery was found to be non-exponential which could be fitted to a single exponential ('fast' component) and a second component with a relaxation time of >300 ps. The recovery times of the fast component were found to be *intensity dependent* and for *c*-PE, *c*-PC and APC were measured to be 19, 27 and 35 ps, respectively, at intensity (I) $\sim 7 \times 10^{20}$ photons/m^2 and increased to 54, 55 and 67 ps, respectively, at $I \sim 8 \times 10^{19}$ photons/m^2. The ps absorption data support the assignment of the 'fast' component to singlet–singlet exciton annihilation.

INTRODUCTION

The primary step in photosynthesis involves the absorption of sunlight by specialized harvesting pigments and the transfer of the absorbed energy to the reaction centers. The sequence of events takes place in picoseconds (ps) and has been the subject of considerable work by ps absorption and fluorescence techniques (Campillo and Shapiro, 1978; Kobayashi *et al.*, 1979; Breton and Geacintov, 1980). In the blue-green algae the antenna pigments consist of the phycobiliproteins: *c*-phycoerythrin (*c*-PE)‡; *c*-phycocyanin (*c*-PC) and allophycocyanin (APC) (Gantt, 1975). The absorbed energy follows the path: *c*-PE → *c*-PC → APC → Chl *a* (Gantt and Lipschultz, 1973; Porter *et al.*, 1978). Phycobiliproteins contain a chromophore which is an open chain tetrapyrrole that is covalently bound to an apoprotein (Crespi *et al.*, 1968; Schram and Kroes, 1971). Most phycobiliproteins are made up of two subunits α and β present in equal amounts. The number and the chemical nature of the chromophores depend on the origin and spectroscopic class of the phycobiliproteins. In *c*-PE, the α subunit contains 2 chromophores and the β subunit contains 4 chromophores. In *c*-PC, the corresponding number of the chromophores is 1 and 2, respectively. In APC, each subunit contains one chromophore (Glazer, 1977). The protein can be found in different states of aggregation, e.g. $(\alpha\beta)$, $(\alpha\beta)_3$, $(\alpha\beta)_6$. The degree of aggregation depends on the ionic strength, protein concentration, pH and other factors (Glazer, 1977).

This paper is the second in a series of studies on the relaxation kinetics of the blue-green algal photosynthetic system. The first (Wong *et al.*, 1981) and the third (Pellegrino *et al.*, 1981) papers deal with the relaxation kinetics of isolated phycobiliproteins and intact phycobilisomes of *Nostoc* sp., respectively. The purpose of this work is to complement the data presented in part I by Wong *et al.* (1981). They have shown, using ps time resolved fluorescence spectroscopy that singlet–singlet exciton annihilation occurs in the isolated phycobiliproteins: *c*-PE, *c*-PC and APC. We present here the first direct exciton annihilation measurements in photosynthetic pigments using ps absorption spectroscopy.

The importance of this type of work is two-fold. First, it shows that nonlinear effects may be predominant at the excitation intensity required for ps absorption measurements. Second, the study of exciton annihilation coupled to recent theoretical advances (Paillotin *et al.*, 1979) could provide additional information on the topology of the pigments (e.g. the molecular domain over which the singlet exciton migrates).

MATERIALS AND METHODS

Nostoc sp. was grown in a New Brunswick Scientific fermentor on medium CG-10 (Ingram and Van Baalen,

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‡*Abbreviations:* APC, allophycocyanin; *c*-PC, *c*-phycocyanin; *c*-PE, *c*-phycoerythrin.

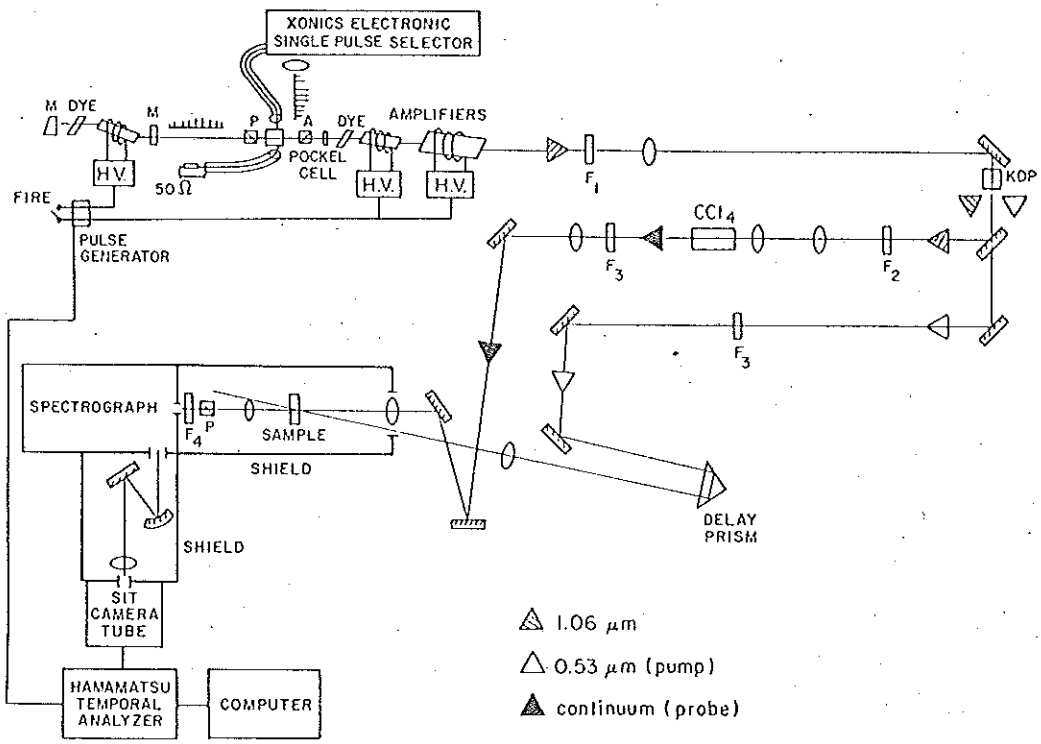

Figure 1. Scheme of the double beam picosecond absorption apparatus.

1970) according to the method of Rusckowski and Zilinskas (1980). Phycobilisomes were isolated as described by Zilinskas *et al.* (1978), following a modification of the procedure of Gray and Gantt (1975). Phycobilisomes were the starting material from which each of the phycobiliproteins were isolated. Details of phycobiliprotein isolation have been given elsewhere (Wong *et al.*, 1981). The spectral form of allophycocyanin, called APC II, which is the most abundant APC form in the phycobilisomes and has been described in detail elsewhere (Zilinskas *et al.*, 1978, 1980) was the APC used in this study. The isolated phycobiliproteins were lyophilized and kept in the refrigerator until ready to use. *c*-Phycoerythrin and APC were dissolved in 0.1 *M* potassium phosphate buffer, pH 7.0. *c*-Phycocyanin was dissolved in 0.1 *M* potassium phosphate buffer, pH 8.0. Under these conditions all three phycobiliproteins form trimers, as determined from the sedimentation coefficients (Wong *et al.*, 1981).

The experiments were performed in a double beam ps absorption spectrophotometer shown in Fig. 1. A single pulse from a Nd^{3+}-glass laser is selected and amplified to 30 mJ. Approximately 5% of the pulse is converted to the second harmonic at 530 nm and 6–8 ps pulse duration. The 1060 nm pulse is reflected by a dielectric mirror and focused into a 15 cm long CCl_4 cell. This produces a pulse of broad-band spectral continuum covering the whole visible and near infrared of approximately 10 ps pulse width (Alfano and Shapiro, 1971; Madge and Windsor, 1974). At the sample site, the probe beam is divided into two pulses of approximately equal intensity designated, $I^e(t)$ and $I^r(t)$. $I^e(t)$ is transmitted through the same area of the sample which is excited by the 530 nm pulse. The intensity of the transmitted probe beam depends of course, on the changes initiated by the exciting pulse. $I^r(t)$ is the reference beam and is transmitted through a different part of the sample. Both pulses, $I^e(t)$ and $I^r(t)$ are measured by a spectrograph coupled to a Hamamatsu SIT camera (C-1000) and temporal analyzer (C-1098).

The change in optical density (OD) for a given delay time is obtained as

$$\Delta OD(t) = -\log\left[\frac{I^e(t)}{I^r(t)} \times \frac{I^r_0}{I^e_0}\right], \qquad (1)$$

where the ratio I^r_0/I^e_0 is the normalization factor for the two beams before the sample. Typically, a $\Delta OD \approx 0.02$ can be measured. The zero time was determined using a CS_2 optical Kerr shutter at the sample site (Duguay and Hansen, 1969). The position zero is accurate to ± 5 ps. The resolution of the apparatus is ~ 10 ps.

The samples were placed in a cuvette of 2 mm pathlength with OD of 1.0–1.5 at the absorption maximum. The sample was stirred after every shot. Each point represents the average of 10–15 shots. Experiments were performed at two excitation energy levels, namely, $\sim 800\,\mu$J and $100\,\mu$J. The pulse was focused to an area of 0.03 cm^2 on to the sample. Under these conditions, the pulse intensity was 6.8×10^{20} photons/m^2 and 8.5×10^{19} photons/m^2, respectively. The high excitation intensity is required in the kinetic absorption experiments in order to obtain measurable OD changes. The intensity of the pulse was monitored before and after the sample so that the number of photons absorbed by the sample could be measured. The number of photons absorbed, measured in this way, agreed closely with the value calculated from the absorbance of the sample. The agreement of the two values eliminated the possibility of multiple excitation which could cause quenching of the ground state recovery.

RESULTS

c-Phycoerythrin (c-PE)

Figures 2 and 3 display the absorption change of *c*-PE following the excitation by a single pulse. The

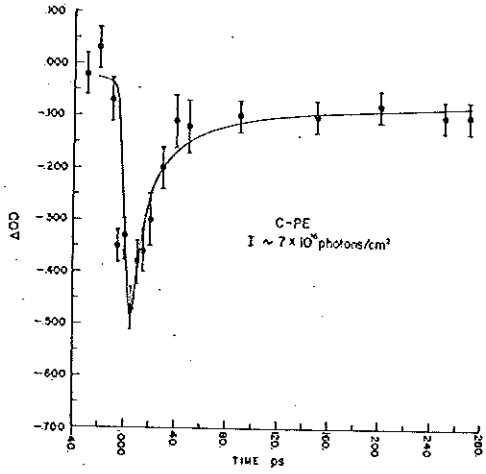

Figure 2. Depletion and recovery of the ground state of c-PE measured at 550 nm, induced by a single pulse of average intensity 7×10^{20} photons/m^2. Solid line represents the plot of Eq. 2, as explained in the text.

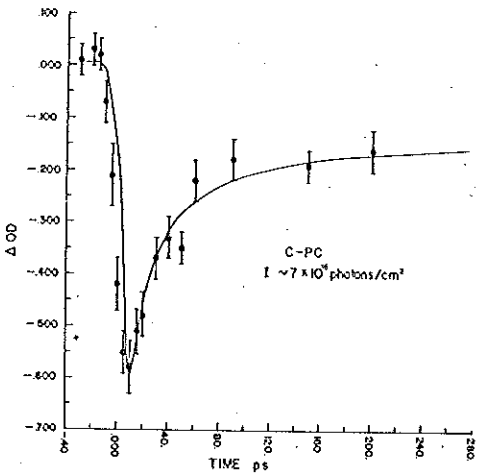

Figure 4. Depletion and recovery of the ground state of c-PC measured at 620 nm, induced by a single pulse of average intensity 7×10^{20} photons/m^2. Solid line represents the plot of Eq. 2, as explained in the text.

depletion of the ground state is found to occur in less than 10 ps. Recovery of the ground state absorption is non-exponential. It can be approximated by a single exponential ('fast' component) followed by a second component which is too slow (>300 ps) to be measured in this absorption apparatus. The 'fast' component is found to be intensity dependent with 1/*e* decay times of 19 and 54 ps for average intensities of 7×10^{20} photons/m^2 and 8×10^{19} photons/m^2, respectively. In addition, the ratio of the initial amplitude of the 'fast' component to that of the 'slow' component decreases from 4.4 at 7×10^{20} photons/m^2 to 1.2 at 8×10^{19} photons/m^2. Our ps absorption results are in good agreement with the fluorescence relaxation kinetic measurements by Wong *et al.* (1981), where at high intensities they observed a non-exponential fluorescence decay.

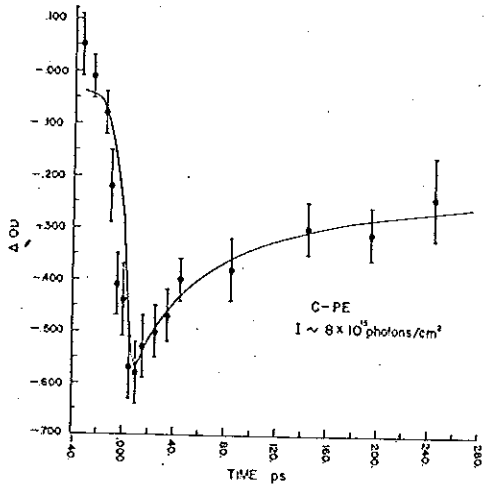

Figure 3. Depletion and recovery of the ground state of c-PE measured at 550 nm, induced by a single pulse of average intensity 8×10^{19} photons/m^2. Solid line represents the plot of Eq. 2, as explained in the text.

c-*Phycocyanin* (c-*PC*)

Figures 4 and 5 display the absorption changes of c-PC induced subsequent to the optical excitation. The onset of the absorption occurs in less than 10 ps. c-Phycocyanin also shows a non-exponential recovery of the ground state. The 'fast' component of the recovery kinetics can be fitted to a single exponential with a 1/*e* decay time of 27 ps at an average intensity of 7×10^{20} photons/m^2 and 55 ps at 8×10^{19} photons/m^2. The ratio of the initial amplitude of the 'fast' component to that of the 'slow' component of the recovery decreases from 2.5 at 7×10^{20} photons/m^2 to 1.6 at 8×10^{19} photons/m^2.

The fluorescence kinetic measurements (Wong *et al.*, 1981) have shown only an intensity dependent single exponential decay. However, these measurements were performed at intensities lower by at least an order of magnitude than the measurements presented here. In fact, recent measurements at high intensities ($\sim 2 \times 10^{20}$ photons/m^2) show that the fluorescence relaxation becomes non-exponential.

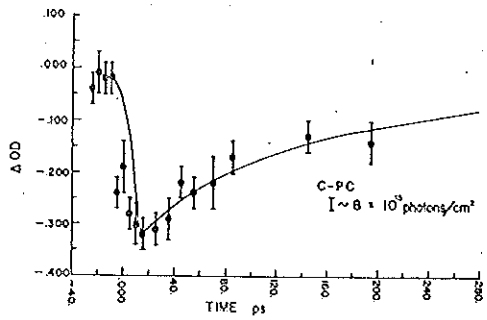

Figure 5. Depletion and recovery of the ground state of c-PC measured at 620 nm, induced by a single pulse of average intensity of 8×10^{19} photons/m^2. Solid line represents the plot of Eq. 2, as explained in the text.

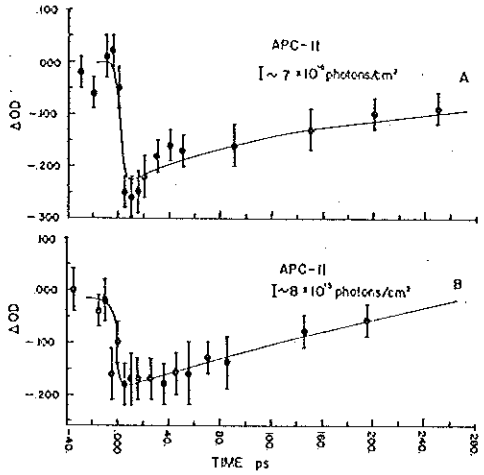

Figure 6. (A) Depletion and recovery of the ground state of APC measured at 630 nm, induced by a single pulse of average intensity 7×10^{20} photons/m^2. (B) Depletion and recovery of the ground state at APC measured at 630 nm, induced by a single pulse of average intensity 8×10^{19} photons/m^2. Solid lines in (A) and (B) represent the plot of Eq. 2, as explained in the text.

Allophycocyanin (APC II)

Figures 6A and B display the absorption changes of APC induced by the optical excitation. The onset of the absorption occurs in less than 10 ps. Allophycocyanin also shows a non-exponential recovery of the ground state. However, the effect is weaker than in *c*-PE and *c*-PC. The recovery can also be fitted to a 'fast' single exponential and a second component too slow to be measured in the present apparatus. The $1/e$ decay times of the 'fast' component are measured to be 35 and 67 ps at average intensities of 7×10^{20} photons/m^2 and 8×10^{19} photons/m^2, respectively. The ratio of the intensity of the 'fast' component to that of the 'slow' component of the recovery changes from 1.6 to 1.0 as the intensity decreases from 7×10^{20} photons/m^2 to 8×10^{19} photons/m^2. It should be noted that the recovery times estimated here are approximate due to the few points used in the exponential fits; however, they are accurate enough to demonstrate the dependence of the ground-state recovery time on the intensity of the exciting pulse.

To eliminate the possibility of stimulated emission, we measured the depletion and the recovery of the ground state of Rh-6-G at a concentration of 0.2 m*M*. As can be seen in Fig. 7, the recovery of ground state absorption of Rh-6-G at an intensity of 7×10^{20} photons/m^2 shows no stimulated emission even though the fluorescence quantum yield of Rh-6-G is much greater than the quantum yield of any of the phycobiliproteins (Wong *et al.*, 1981).

DISCUSSION

The data presented here show a definite dependence of the recovery time of the 'fast' component on the intensity of the excitation pulse, in agreement with the fluorescence measurements of Wong *et al.* (1981). In addition, the fact that the quenching becomes progressively weaker in the pigments *c*-PE, *c*-PC and APC, following the decrease in absorbance at the exciting wavelength, supports the assumption of singlet–singlet exciton annihilation. Kobayashi *et al.* (1979) have assigned the 'fast' component in their *c*-PC measurements to the transfer of the excitation energy from the 'sensitizing' chromophore to the 'fluorescing' chromophore in the *c*-PC unit. Following the work of Glazer *et al.* (1973) the α and β subunits are assigned to the 'fluorescing' and 'sensitizing' chromophores, respectively. The ps absorption data presented here, in conjunction with the ps fluorescence measurements by Wong *et al.* (1981) argue against the interpretation of the 'fast' component as energy transfer from the 'sensitizing' to the 'fluorescing' chromophore.

To further test the exciton annihilation model, we will use the parameters calculated from the fluorescence kinetic measurements by Wong *et al.* (1981) to fit our absorption kinetic data. The rate equation governing the singlet population and including singlet–singlet annihilation (Campillo *et al.*, 1977) is

$$\frac{dn}{dt} = \alpha I(t) - \gamma_1 n - \tfrac{1}{2}\gamma_2 n^2, \qquad (2)$$

where n is the singlet density, $I(t)$ is the laser intensity, α is the local absorption coefficient, γ_1 is the rate constant of the fluorescence relaxation, and γ_2 is the rate of the singlet–singlet annihilation. A discussion of these parameters is given in part I (Wong *et al.*, 1981).

We have assumed a Gaussian pulse of $\tau \approx 8$ ps FWHM (full width at half maximum)

$$I(t) = I_0 \exp\left[-\frac{4 \ln 2 t^2}{\tau^2}\right], \qquad (3)$$

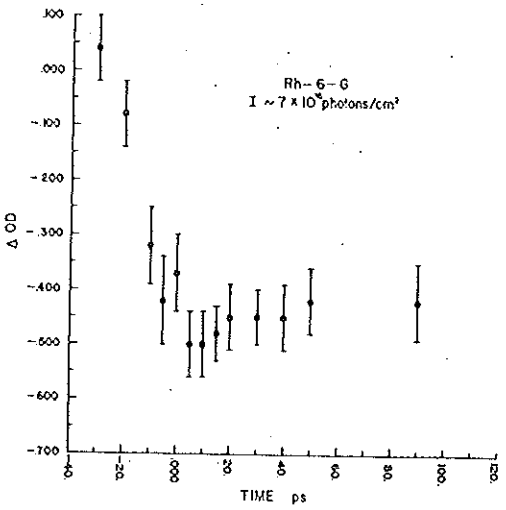

Figure 7. Depletion and recovery of the ground state of Rh-6-G measured at 490 nm, induced by a single pulse of average intensity 7×10^{20} photons/m^2. The recovery of the ground state absorption shows no induced emission.

Table 1. Values of the parameters γ_1, γ_2 and α used in Eq. 2 (Wong *et al.*, 1981)

	γ_1 (s^{-1}) ($\times 10^8$)	γ_2 ($cm^3 s^{-1}$) ($\times 10^{-9}$)	α (cm^{-1})
c-PE	5.45	1.44	2988
c-PC	4.74	6.3	298
APC	5.36	1.6	86

where I_0 is determined so that the integrated intensity is equal to the intensity of the exciting pulse. Equation 2 was solved numerically using the fourth order Runge Kutta method (Ralston, 1965), in the range from -20 to 270 ps.

The singlet density $n(t)$ must be correlated with the changes of the OD. If N is the number of ground state molecules of phycobiliproteins per cm^3, the OD difference as a function of time is equal to

$$\Delta OD(t) = -\left[\epsilon \frac{N}{N_A} l - \epsilon \frac{N - n(t)}{N_A} l\right]$$

$$= -\epsilon \frac{n(t)}{N_A} l = -An(t) \quad (4)$$

where ϵ is the molar extinction coefficient in $M^{-1} cm^{-1}$, N_A the Avogadro number normalized for 1 cm^3 and l is the pathlength in cm. The change in the optical density $\Delta OD(t)$ at a given delay time is proportional to the density of the singlet states, with ϵ, N_A and l being constants.

Before we attempted to fit the theoretical curves of ΔOD vs. time to the data we studied the solutions of the rate equation for four intensities, namely 3.3×10^{20} photons/m^2, 1.7×10^{20} photons/m^2, 3.3×10^{19} photons/m^2 and 1.7×10^{18} photons/m^2. The values for the parameters α, γ_1 and γ_2 were taken from Table 1 (Wong *et al.*, 1981) (for *c*-PE). Figure 8 shows the theoretical recovery of the ground state population as a function of time. For convenience, the integrated area for each curve has been normalized to one. The effect of exciton annihilation quenching on the excited state population at high intensities is very clear.

Consequently Eq. 2 was solved using the values of α, γ_1 and γ_2 for all the three pigments in Table 1 and for the two laser intensities used in the experiment, namely 7×10^{20} photons/m^2 and 8×10^{19} photons/m^2. Thus, $n(t)$ was determined as a function of time. In principle, one has only to substitute the values of the constants in Eq. 3 and calculate the $\Delta OD(t)$. In reality, however, the proportionality constant is not known. In the experimental apparatus the two beams (530 nm and probe) cross inside the sample so that the overlap region is not precisely known. It should be noted here that the measured decay times are not dependent on the degree of the overlap of the two beams. The absolute value of ΔOD, of course, will be affected. The absolute value of ΔOD, however, is not important in these experiments.

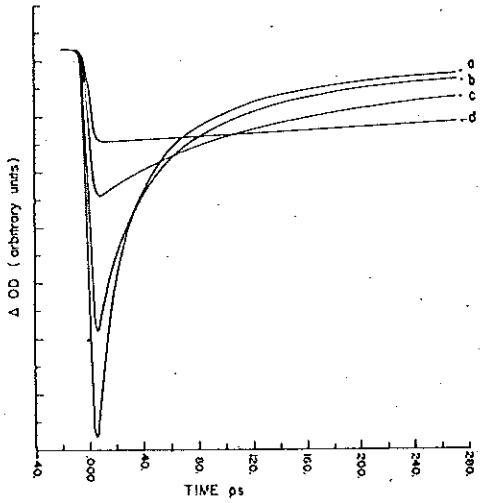

Figure 8. Theoretical recovery curves of the ground state of *c*-PE at four excitation intensities (a) $I = 3.3 \times 10^{20}$ photons/m^2; (b) $I = 1.7 \times 10^{20}$ photons/m^2; (c) $I = 3.3 \times 10^{19}$ photons/m^2; (d) $I = 1.7 \times 10^{18}$ photons/m^2. For convenience, the integrated area for each curve has been normalized to one.

The coefficient A was determined by a least-square fit of the values $n(t)$ to the experimental data points. It was found that the best fit was obtained when the data points were fitted to $-An(t) - B$, where B is a constant. The value of B ranges from a few percent at high intensities to 15% at low intensities. It is possible that B represents some slow process not included in Eq. 2 such as triplet annihilation, diffusion, or even protein denaturation. The solid lines in Figs. 2, 3, 4, 5, 6A and B show the theoretical fit of the ground state recovery using Eq. 2 with the parameters of Table 1. The fact that these parameters, determined independently from the fluorescence experiments of Wong *et al.* (1981), fit the absorption measurements provides further evidence that singlet–singlet exciton annihilation is the predominant mechanism of relaxation at high intensities.

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