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FLUORESCENCE RELAXATION KINETICS AND QUANTUM YIELD FROM THE ISOLATED PHYCOBILIPROTEINS OF THE BLUE-GREEN ALGA *NOSTOC* SP. MEASURED AS A FUNCTION OF SINGLE PICOSECOND PULSE INTENSITY, *I*

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Abstract—Phycobilisomes from the blue-green alga *Nostoc* sp. are known to contain the phycobiliproteins: *c*-phycoerythrin (*c*-PE), *c*-phycocyanin (*c*-PC) and four forms of allophycocyanin (APC I, II, III, and B). We have made a detailed study of the effects of the intensity of a single 6 ps excitation pulse on the decay kinetics and the yield of fluorescence in the individual isolated phycobiliproteins at pH 7 and 23°C. The risetime of the fluorescence of *c*-PE, *c*-PC and APC was <12 ps. We found that the decay of the fluorescence was exponential at intensities of $\lesssim 10^{14}$ photons/cm^2 in all the phycobiliproteins; the lifetimes being 1552 ± 31 ps for *c*-PE, 2111 ± 83 ps for *c*-PC, 1932 ± 165 ps for APC I, 1870 ± 90 ps for APC II, 1816 ± 88 ps for APC III, (1869 ± 62 ps for the averaged APC's I, II, and III), and 2667 ± 233 ps for APC B. We also found that the fluorescence decay became non-exponential in *c*-PE at excitation intensities $>10^{14}$ photons/cm^2, but was exponential for all the other phycobiliproteins even at a pulse intensity of 10^{15} photons/cm^2. The relaxation times of *c*-PE and *c*-PC decreased with excitation intensity above 10^{14} photons/cm^2. For *c*-PE and *c*-PC the relative fluorescence vs excitation intensity was readily described by a relationship derived for a model in which exciton–exciton annihilation occurs. In APC the fluorescence yield and relaxation time were only slightly dependent on the excitation intensity. The results are interpreted to indicate the occurrence of singlet–singlet annihilation intramolecularly among the several phycobilin chromophores within the individual phycobiliprotein molecules in solution. The *s* to *f* transfer time is less than 12 ps in *c*-PC.

INTRODUCTION

Photosynthetic organisms have evolved a number of light-harvesting antenna systems for the primary purpose of absorbing sunlight and transferring the absorbed energy to the photochemically active centers (Sauer, 1975; Gantt, 1975; Prezelin *et al.*, 1978). In red and blue-green algae, the light-harvesting system consists of an aggregation of phycobiliproteins: phycoerythrin, phycocyanin, and allophycocyanin, forming the phycobilisomes (Gantt, 1975). The pathway of energy transfer in the phycobilisome is phycoerythrin (PE) → phycocyanin (PC) → allophycocyanin (APC) (Gantt *et al.*, 1973), in which the energy transfers occur on a ps time-scale (Porter *et al.*, 1978; Grabowski and Gantt, 1978). Time-resolved ps fluorescence techniques have been used for some time in the study of photosynthetic systems (Campillo and Shapiro, 1978). In a recent study, Searle *et al.* (1978) have shown that energy transfer in phycobilisomes, isolated from the red alga *Porphyridium cruentum*, can be traced by measuring the fluorescence kinetics. Interestingly, it is also found that quenching of the red fluorescence occurs even with single-pulse excitation of intensity 10^{14} photons/cm^2. In order to better understand this fluorescence quenching in phycobilisomes, it is necessary to study the fluorescence kinetics from the isolated phycobiliproteins. The isolated phycobiliproteins *c*-PE, *c*-PC and APC each contain different amounts of bilin chromophores (i.e. *c*-PE contains 18 chromophores in the trimer, *c*-PC contains 18 chromophores in the hexamer form, and APC contains 6 chromophores in the trimer form). The bilin chromophores are covalently attached to the apoproteins which are 120Å in diameter by 30Å thick in the basic trimer unit of these biliproteins. Therefore, because of this close packing of chromophores, one should expect to observe fluorescence quenching due to singlet–singlet excitation annihilation within each phycobiliprotein.

In this paper we report on the first ps time-resolved fluorescence kinetic measurements from phycoerythrin, phycocyanin, and the allophycocyanins isolated from phycobilisomes of the blue-green alga *Nostoc* sp. The effects of excitation pulse intensity on the decay kinetics and the yield of fluorescence are reported. Our results are consistent with the occurrence of singlet–singlet excitation annihilation among the bilin prosthetic chromophores in the phycoerythrin and phycocyanin units. In the allophycocyanins only a slight fluorescence quenching was observed over the range of 10^{13} to 10^{15} photons/cm^2.

MATERIALS AND METHODS

Materials

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

Phycobilisomes from several isolations which had been stored at 4°C in pelleted form were suspended in a minimal volume of 0.75 *M* potassium phosphate buffer, pH 7.0, and dialyzed against multiple changes of 0.01 *M* potassium phosphate buffer, pH 7.0, over a 3 h period. After dialysis, these partially dissociated phycobilisomes were centrifuged at 40000 *g* for 30 min to remove any contaminating membrane material. The resulting supernatant was applied to 2.5 × 30 cm brushite column which had been equilibrated with 0.01 *M* potassium phosphate buffer, pH 7.0, containing 0.1 *M* NaCl and 0.02% NaN_3 (brushite equilibration buffer). Brushite was prepared according to the procedure of Siegelman and Firer (1964). A mixture of *c*-PE and *c*-PC was eluted from this column with 500 mℓ of brushite equilibrating buffer. The APC fraction from the dissociated phycobilisomes remained adsorbed at the top of the brushite column. Allophycocyanin I, II, III and B were separated from this allophycocyanin pool according to the method of Zilinskas *et al.* (1978) with modifications described by Troxler *et al.* (1980).

The mixture of *c*-PC described above was concentrated on an Amicon ultrafilter cell (PM-10 filter), dialyzed exhaustively against water, and lyopholized. This mixture [equal absorbance at 620 nm (PC) and 570 nm (PE)] was suspended in a minimal volume of 1 m*M* potassium phosphate buffer, pH 7.0, and layered on a 2.5 × 60 cm brushite column equilibrated with the same buffer, containing 100 m*M* NaCl and 0.02% NaN_3. The column was developed with a linear gradient of potassium phosphate from 1 to 20 m*M*, containing 100 m*M* NaCl and 0.02% NaCl (700 mℓ total volume). Fractions with a 560/620 nm absorbance ratio $\geq$ 1.5 were pooled as PE-enriched; fractions with a 620/560 nm ratio $\geq$ 1.85 were pooled as PC-enriched.

The PE-enriched pool was brought to 35% saturation with $(NH_4)_2SO_4$ at 20°C according to the procedure of Bennett and Bogorad (1971). The pellet, greatly enriched in PE, was resuspended in 10 mℓ of 10 m*M* potassium phosphate buffer, pH 7.0, and was brought to 35% saturation with $(NH_4)_2SO_4$. This was repeated once more, and the final pellet, suspended in 100 m*M* potassium phosphate, pH 7.0, showed no absorbance beyond 600 nm and is considered pure *c*-PE.

The PC-enriched pool was similarly brought to 35% saturation with $(NH_4)_2SO_4$. Surprisingly, the pellet was considerably enriched in PC ($A_{620}/A_{560} = 2.5$), and the supernatant was enriched in PE ($A_{620}/A_{560} = 1.6$). After three such repeated fractionations in 35% saturated $(NH_4)_2SO_4$, the final pellet was resuspended in 100 m*M* potassium phosphate, pH 7.0. Absorption and fluorescence spectra indicated that the sample was free of all PE. The anomalous behavior of PE and PC with respect to the 35% $(NH_4)_2SO_4$ fractionation described above might be explained by: (1) the PC pelleted by 35% saturated $(NH_4)SO_4$ may be different from the PC contaminant removed from the PE-enriched pool (e.g. it must obviously have different surface charges), or (2) if rather than a simple mixture of individual PE and PC molecules in the PE- and PC-enriched pools, one is instead dealing with complexes of PE and PC such as have been recently described (Koller *et al.*, 1978), surface charges and consequent salting-out effects may differ from those seen in proteins not existing in complexes.

An alternate purification procedure for PC was also developed. The PE–PC lyophilized mixture was suspended in a small volume of 100 mℓ potassium phosphate buffer, pH 7.0, and was layered on a 2.5 × 60 cm Sephacryl S-200 gel filtration column equilibrated with the same buffer. PC-enriched fractions eluted slightly ahead of the PE-enriched fractions. The PC-enriched fractions were pooled, concentrated on an Amicon PM-10 ultrafilter and reapplied to the Sephacryl S-200 column and PC-enriched fractions again collected. This was repeated once more and the resultant PC was applied to a 1.5 × 30 cm brushite column equilibrated with 1 m*M* potassium phosphate buffer, pH 7.0, containing 100 m*M* NaCl and 0.02% NaN_3. The column was developed with a linear gradient of potassium phosphate buffer, pH 7.0, from 1–20 m*M*, containing 100 m*M* NaCl and 0.02% NaN_3. Phycocyanin fractions which were free of all phycoerythrin as determined by absorption and fluorescence spectra were pooled and concentrated.

All biliprotein samples were stored at 4°C in 100 m*M* potassium phosphate buffer, pH 7.0, containing 0.02% NaN_3. In most cases, ps measurements were made within a week of isolation. Lyophilized biliproteins were also used on occasion and no differences were detected in measurements of these lyophilized samples and fresh samples.

Sedimentation constants ($s_{20,w}$) were determined by centrifugation in a Beckman SW40 rotor at 40000 rpm for 16 h using a linear sucrose gradient of 0.15–0.8 *M* sucrose in 0.1 *M* potassium phosphate, pH 7.0, total volume 13 mℓ. The approximate $s_{20,w}$ values reported here were calculated by the numerical integration of the centrifuge equation and also by estimation according to McEwen (1967). Two hundred $\mu\ell$ of sample, 0.5-2 mg protein/mℓ, were layered on each gradient.

Methods. Absorption spectra were recorded at room temperature in a Cary 17 D recording spectrophotometer. Fluorescence spectra were measured in an Aminco-Bowman spectrofluorimeter equipped with a R446S (Hamamatsu TV) photomultiplier tube. The band pass on the excitation and emission sides was 1.1 and 2.8 nm, respectively, for excitation spectra and the reverse for emission spectra. The spectra were uncorrected for lamp output and emission grating–phototube efficiency. Relative quantum yields of fluorescence were measured on the samples and standards adjusted to an absorption at the excitation wavelength of 0.1 (540 nm for rhodamine B and PE and 600 nm for cresyl fast violet and PC) with fluorescence yields for rhodamine B and cresyl fast violet in ethanol of 0.94 and 0.98, respectively (Dale and Teale, 1970).

The experimental arrangement (Yu *et al.*, 1977) used in the fluorescence kinetics measurements is shown in Fig. 1. In the experiment a single pulse is first selected from the output of a Nd:glass laser consisting of a train of ~100 pulses. The pulses in the beginning of the train are ~9 ps in duration and are spaced 7 ns apart. The single pulse selection is achieved by applying a 5 ns, 8 kV pulse from a laser triggered spark gap to a Pockels cell situated between crossed polarizers. The selected pulse at 1.06 μm is then passed through a phase matched Potassium Di-Hydrogen Phosphate crystal where the second harmonic of the laser at 0.53 μm and of 6 ps duration is generated. This pulse is used both to excite the sample and calibrate the detection apparatus. The exciting beam is collimated to an elliptical spot size of 2 × 1 mm of uniform intensity at the sample position. The sample is frontally excited and the fluorescence is collected with *f* 1.25 optics and focused onto the entrance slit of a Hamamatsu streak camera. A 0.53 μm prepulse is used to provide a reference mark on the time axis for signal averaging. In the streak camera, photoelectrons emitted from a S-20 photocathode by light incident on the detector surface are deflected vertically by applying

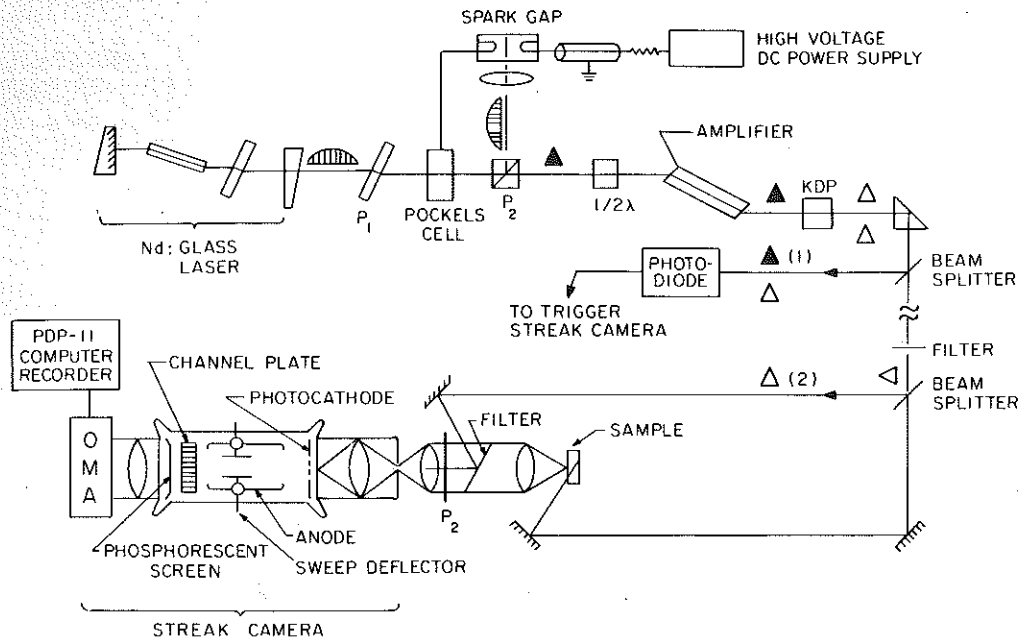

Figure 1. Scheme of ps fluorescence apparatus.

a linearly decreasing voltage to a pair of deflection plates situated between the photocathode and anode. If the voltage ramp is linear, the temporal horizontal separation between photoelectrons in the streak tube will be mapped onto a linear vertical spatial separation. In the Hamamatsu camera, the vertically swept photoelectrons are passed through a microchannel plate which multiples the photoelectrons before they strike the phosphor screen at the rear of the streak tube. The output from the phosphor is imaged onto a Video detector model 1205B Optical Multi-channel Analyzer (OMA) from Princeton Applied Research. The streak trace is digitized and subsequently stored and processed in a Declab PDP 11/03 minicomputer. After normalization both in time and in intensity for non-linearities in the streak rate, the data are analyzed through appropriate use of signal averaging and curve fitting techniques. The time resolution of the laser-streak camera system is $\lesssim 12$ ps. The intensity of excitation at the sample site was determined by monitoring the height of a calibrating pulse with a Hadron 105C biplanar photodiode and Tektronix 519 oscilloscope. The photodetector and oscilloscope were calibrated with a Laser Precision energy probe (RKP 335) position at the sample site and a Laser Precision (LP 3230) energy meter.

RESULTS

Phycoerythrin (c-PE)

The absorption and fluorescence emission and excitation spectra of *c*-phycoerythrin in 0.1 *M* potassium phosphate buffer, pH 7.0, are shown in Fig. 2. Ultracentrifugation experiments indicated that under these conditions at a protein concentration of 0.5–2 mg/m/ the phycoerythrin exists as a trimer (5.3 s). The relative quantum yield of fluorescence of *c*-phycoerythrin from *Nostoc* sp. with rhodamine B in ethanol as standard was 0.62 ± 0.05. Recent work on aggregations is discussed by Glazer (1976, 1979).

The risetime of phycoerythrin fluorescence subsequent to a single 6 ps, 530 nm pulse excitation was found to be within the instrument resolution time of $\lesssim 12$ ps. The decay kinetics of the fluorescence were found to be dependent on the excitation intensity, I (intensity in this paper is defined as photons/cm^2). At low pulse intensities ($I < 10^{14}$ photons/cm^2), the fluorescence relaxation was exponential with a 1/e time of 1552 ± 31 ps (average of 12 single-shots, each with I between 2×10^{13} and 8×10^{13} photons/cm^2). A typical low and high intensity fluorescence decay are displayed in Figs 3a and 3b, respectively. At higher excitation intensities, the relaxation kinetics were

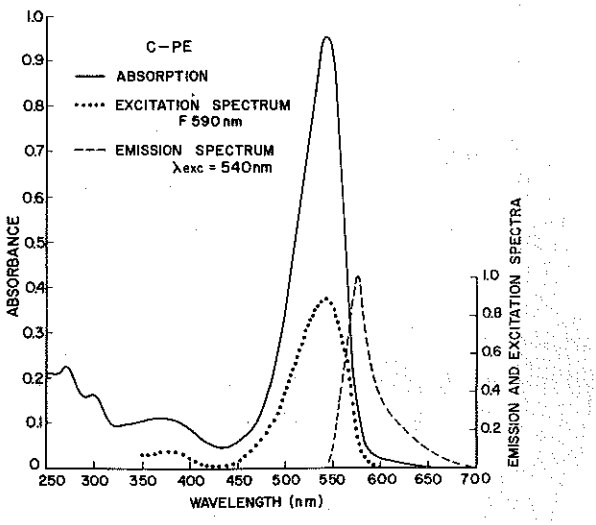

Figure 2. *C*-PE absorption spectrum, relative fluorescence emission and excitation spectra.

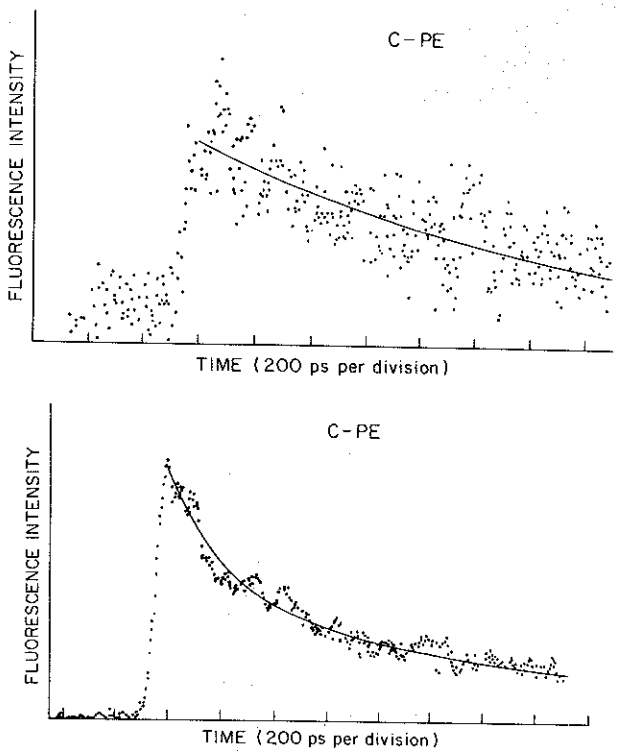

Figure 3. Decay profiles of *c*-phycoerythrin fluorescence excited by 6 ps, 530 nm laser pulse. Dots denote the experimental trace and solid lines show the simulated fits. (A) At excitation intensity of 2.57×10^{13} photons/cm^2, the decay was fitted with a single exponential curve with a lifetime of 1437 ps. (B) At excitation intensity of 1.07×10^{15} photons/cm^2, the decay was fitted with the curve: $F = 0.48 \exp(-t/184) + 0.52 \exp(-t/1434)$. Fluorescence was detected through a Corning CS 3-67 glass filter and an Optical Coating Laboratory Cyan Dichroic filter. *c*-Phycoerythrin was suspended in 100 m*M* phosphate buffer at pH 7, OD = 0.08 at 530 nm in a 2 mm cuvette; temperature was 23–25°C.

non-exponential, but could be satisfactorily approximated by the sum of two exponentials with a 1/e time of 130 ± 23 ps for the fast component (average of 26 single-shots) and 1417 ± 29 ps for the slow component. The ratio of the relative initial amplitude of the 'fast' phase to that of the 'slow' phase of the decay varied from 0.6 at $I \sim 2 \times 10^{14}$ to 1.0 at $I \sim 2 \times 10^{15}$ photons/cm^2. It is interesting to note that while the overall 1/e time for the fluorescence decay of PE decreases to 0.3 of its initial value, the individual fast and slow components decrease to only 0.75 over the intensity range of 3×10^{13} to 2×10^{15} photons/cm^2 (Fig. 4). The normalized fluorescence yield, Φ, defined as the ratio of the relative quantum yield at intensity I to the relative quantum yield at low intensities, declined significantly at $I \sim 10^{14}$ photons/cm^2 (Fig. 5).

Phycocyanin (c-PC)

The absorption and fluorescence emission and excitation spectra of *c*-phycocyanin in 0.1 *M* potassium phosphate at pH 7.0 are shown in Fig. 6. Excitation of phycocyanin at either 540 or 600 nm resulted in identical fluorescence emission spectra with no detectable fluorescence at 575 nm, characteristic of phycoerythrin contamination. Ultracentrifugation studies of *c*-PC at pH 7.0 in 100 m*M* phosphate at a protein concentration of 0.5–2 mg/m/ showed the phycocyanin to exist predominantly as the trimer (5.7 s) with approximately 25% in the hexameric (10.5 s) aggregation state. The existence of these aggregation states of phycocyanin is well documented in the literature (see Berns, 1971; Glazer, 1976, 1979; Bryant *et al.*, 1979). The relative quantum yield of fluorescence based on cresyl fast violet in ethanol was 0.75 ± 0.06.

In Fig. 7 the rise of the *c*-PC fluorescence in time at pH 5 and pH 8 subsequent to single pulse excitation is compared with that from Rhodamine 6G. All of the risetimes were found to be within the temporal solution of our experimental system, $\lesssim 12$ ps.

The decay kinetics of *c*-PC fluorescence at pH 7, unlike that for *c*-PE, were found to be exponential (see Fig. 8) for excitation intensities in the range 10^{13} to 10^{15} photons/cm^3. In Fig. 9 the intensity dependence of the relaxation time is shown. For a single pulse of $I \sim 3 \times 10^{13}$ photons/cm^2, the fluorescence lifetime, τ, was 2111 ± 83 ps (10 single-shots). The lifetime declined with increasing excitation intensity giving $\tau = 1376 \pm 24$ ps (6 single shots) at $I = 1.3 \pm 0.2 \times 10^{15}$ photons/cm^2. As in the case of *c*-PE, the quantum yield for *c*-PC decreases with intensity as shown in Fig. 10. Measurements on *c*-PC obtained by two different purification procedures (see Materials

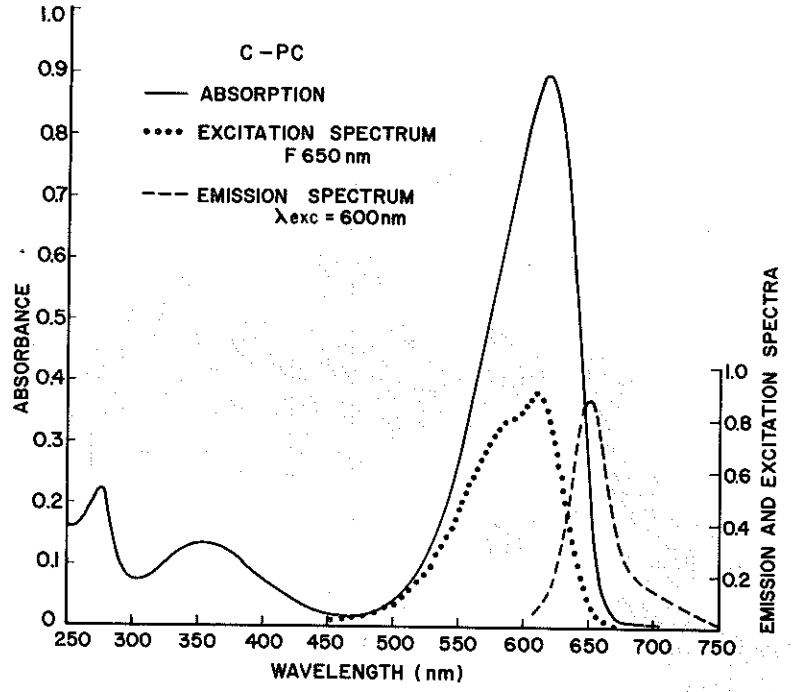

Figure 6. *c*-PC absorption spectrum, relative fluorescence emission and excitation spectra.

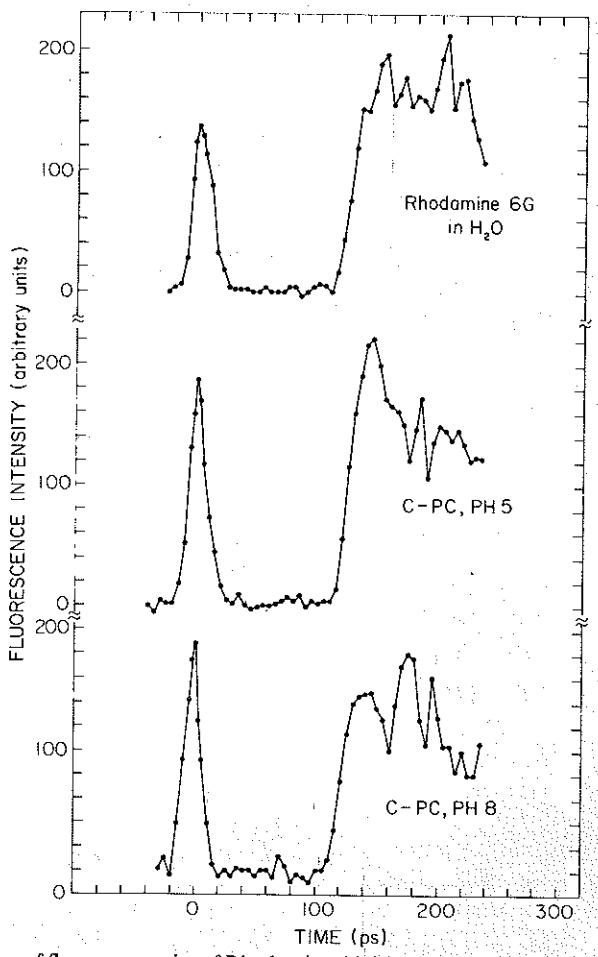

Figure 7. Comparison of fluorescence rise of Rhodamine 6G in water with that of *c*-phycocyanin at pH 5 and pH 8. *C*-phycocyanin was suspended in 100 m*M* phosphate buffer. OD = 0.07 at 530 nm, and the rhodamine 6G concentration was 0.2 m*M*. Fluorescence was detected through a combination of a Corning CS 3-67 filter and an Optical Coating Laboratory Meganta Dichroic filter; temperature was 23–25°C.

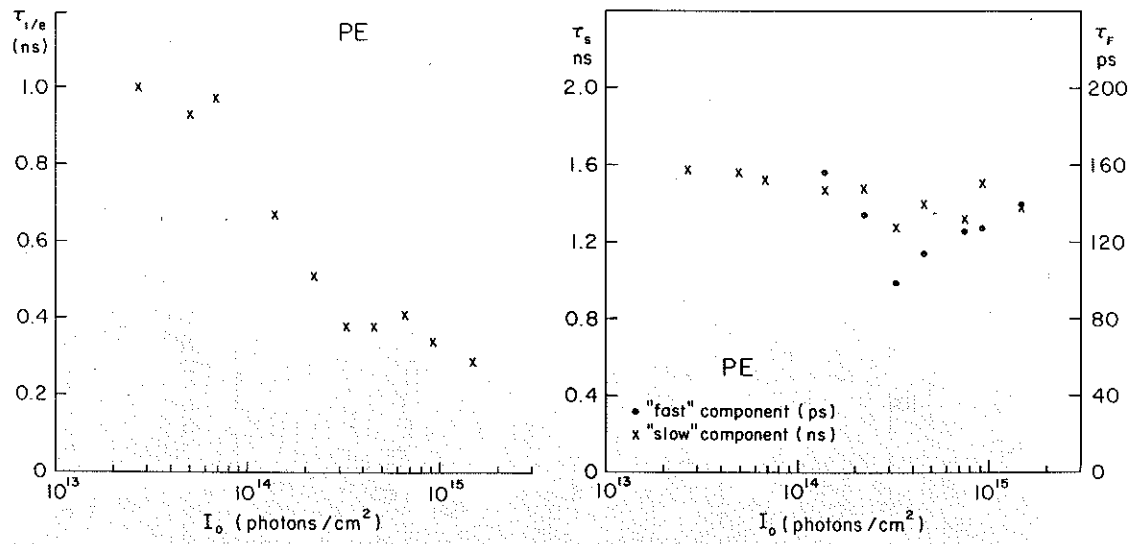

Figure 4. Plot of *c*-phycoerythrin relaxation times as a function of excitation pulse intensity: the $1/e$ times of the fluorescence decay profile is shown in (a). A double exponential fit to the fluorescence decay profile yielded a fast and slow decay component of different amplitudes which are plotted in Fig. b.

and Methods section) gave similar results. No significant difference was observed in the fluorescence kinetics between *c*-PC samples at pH 5 and at pH 8.

Allophycocyanin (APC)

The sedimentation and spectral properties of allophycocyanin I, II, III and B were as described previously in detail (Zilinskas *et al.*, 1978; Zilinskas *et al.*, 1980; Troxler *et al.*, 1980).

The individual fluorescence kinetics of the APC forms I, II, III and B (Zilinskas *et al.*, 1978) were measured separately. The fluorescence rise was within the instrument resolution time. The fluorescence decay curve in each sample was readily fitted with a single exponential at single pulse excitation intensities of 10^{13} to 10^{15} photons/cm^2 (see Fig. 11). The relative fluorescence yield and lifetime for APC I, II, III and B are displayed in Figs. 12–15, respectively. For I between 10^{13} and 10^{14} photons/cm^2, the fluorescence lifetime was 1932 ± 165 ps for APC I (average of 11 single shots, $1870 \pm$ ps for APC II (average of 17 single shots) and 1816 ± 88 for APC III (average of 14 single shots). The average of APC forms I, II and III, was 1869 ± 62 ps (27 single-shots) and for APC B

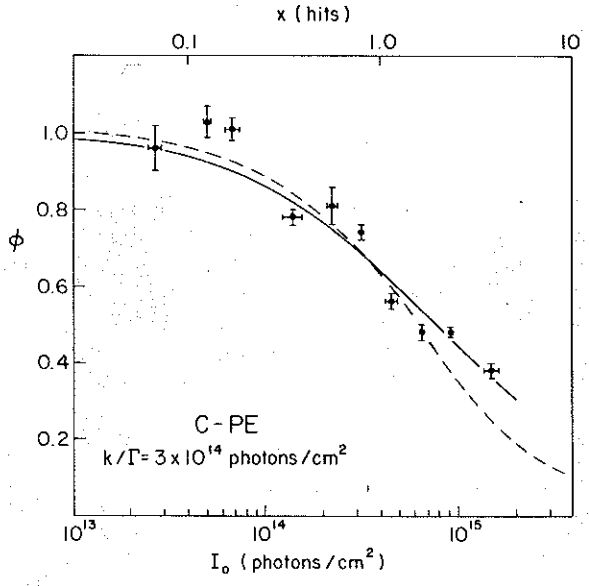

Figure 5. Relative fluorescence yield of *c*-phycoerythrin vs. excitation pulse intensity. The relative fluorescence yield was calculated as the total integrated area under the best-fit curve. Other experimental conditions were as given in the legends of Fig. 3. Error bars denote 1 SD. The solid curve is a plot of Eq. 1 and the dashed curve is a plot of Eq. 2 fitted to the data.

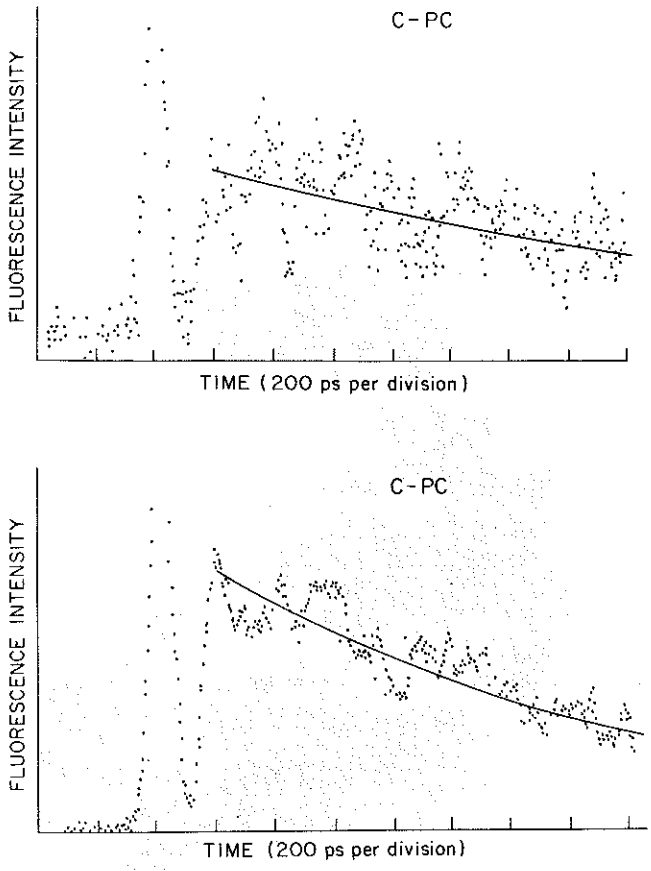

Figure 8. Decay profiles of *c*-phycocyanin fluorescence at different single pulse excitation intensities I. Both experimental traces (dotted curves) were fitted with single exponential functions (solid curves), (A) $I = 2.82 \times 10^{13}$ photons/cm^2, $\tau = 2432$ ps; (B) $I = 1.94 \times 10^{15}$ photons/cm^2, $\tau = 1433$ ps.

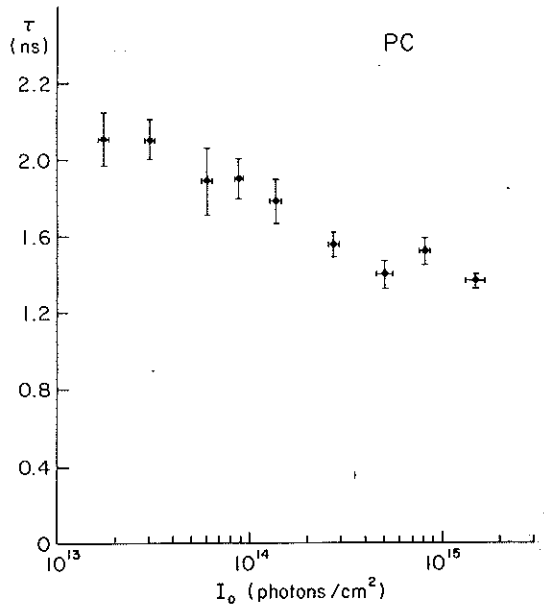

Figure 9. Plot of *c*-phycocyanin relaxation lifetime vs. the excitation pulse intensity. Error bars denote 1 SD. Other experimental details were as given in Fig. 7.

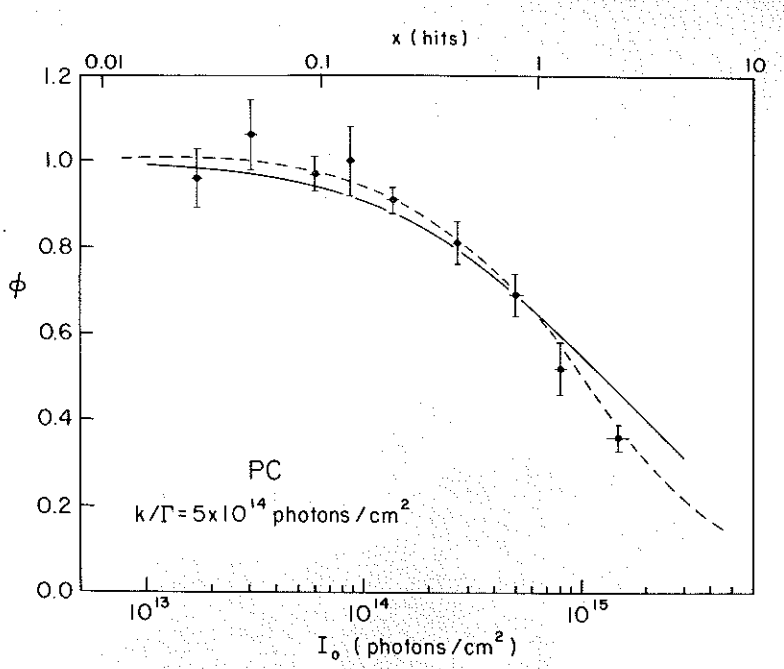

Figure 10. Relative fluorescence yield of *c*-phycocyanin vs. excitation pulse intensity. The relative fluorescence yield was based on the total integrated area under the best-fit exponential. Error bars denote 1 SD. The solid curve is a plot of Eq. 1 where $k/\Gamma = 5 \times 10^{14}$ photons/cm^2. The dashed curve is a plot of Eq. 2 fitted to the data.

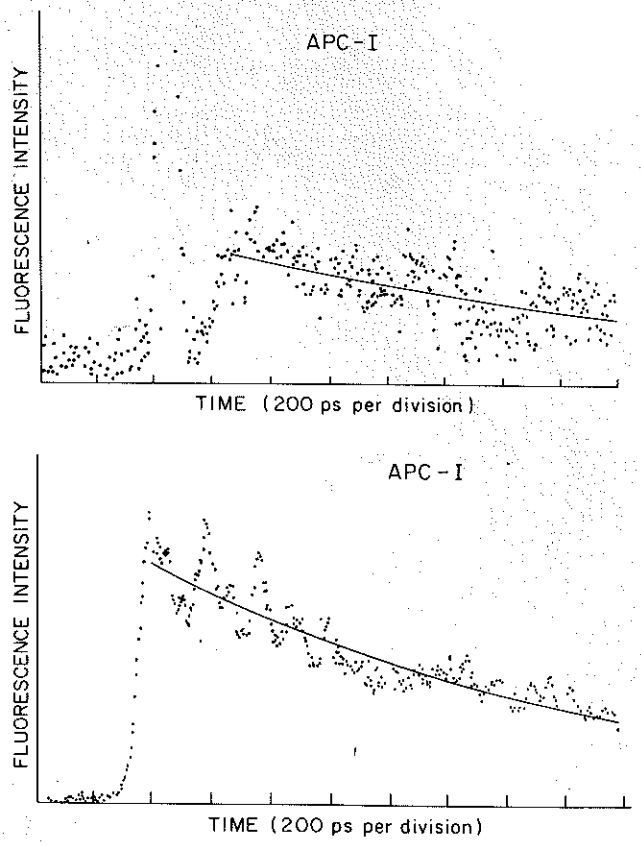

Figure 11. Decay profiles of allophycocyanin I at two excitation pulse intensities. Both decays (dotted curves) were fitted to single exponential functions (solid curves). (A) $I = 3.98 \times 10^{13}$ photons/cm^2, $\tau = 1759$ ps; (B) $I = 1.68 \times 10^{15}$ photons/cm^2, $\tau = 1512$ ps. Allophycocyanin was suspended in 100 m*M* phosphate buffer at pH 7, OD = 0.03 at 530 nm in 2 mm cuvette.

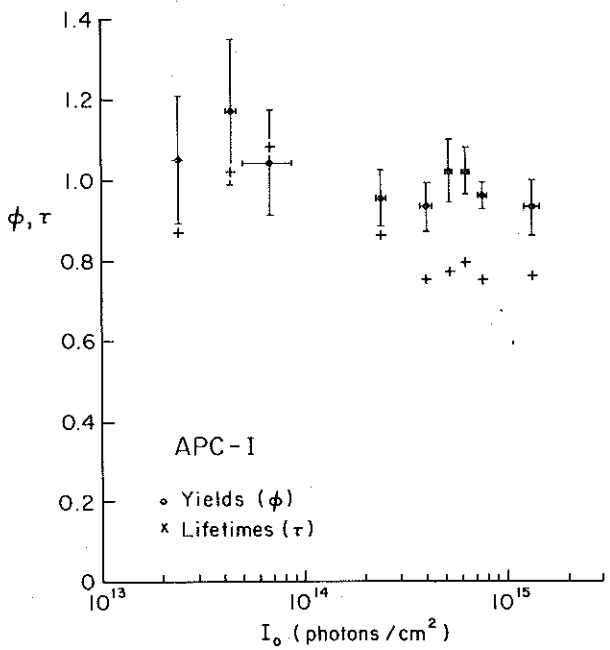

Figure 12. Relative fluorescence yield (.) and relaxation decay lifetime (+) vs. the excitation pulse intensity of APC I. Experimental conditions were as given in the legends of Fig. 9. By extrapolating this data to higher intensities, we have estimated the onset of quenching to occur at value of $k/\Gamma > 8 \times 10^{15}$ photons/cm^2. The decay times are normalized to a value of unity at 1933 ps.

2577 ± 121 ps (average 20 single-shots). Although the lifetimes were found to be ~20% shorter at the higher excitation intensities, the fluorescence quantum yield remained constant throughout the intensity range used.

DISCUSSION

In this paper, we have reported on the first detailed ps time-resolved investigation of the fluorescence from the different biliproteins (*c*-PE, *c*-PC and the APC's) isolated from *Nostoc* sp. The fluorescence

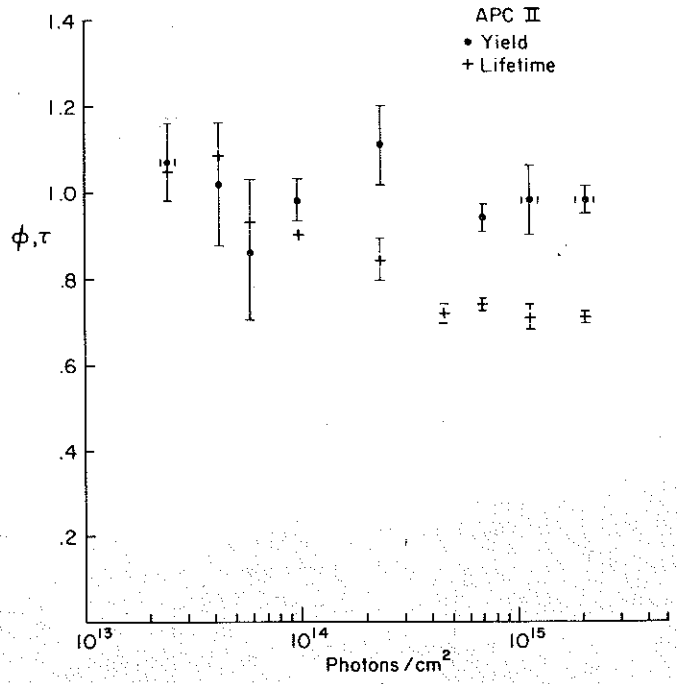

Figure 13. Relative fluorescence yield (.) and relaxation decay lifetime (+) vs. excitation pulse intensity for APC II. The decay times are normalized to a value of unity at 1900 ps.

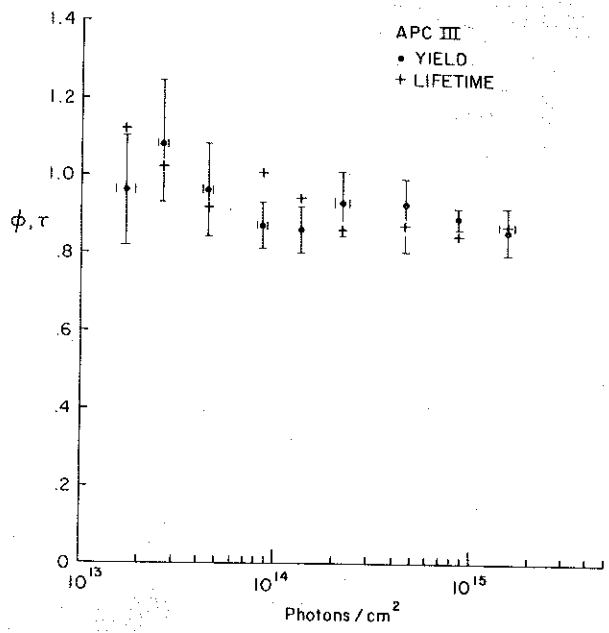

Figure 14. Relative fluorescence yield (.) and relaxation decay lifetime (+) vs. excitation pulse intensity for APC III. The decay times are normalized to a value unity at 1862 ps.

decay kinetics measured as a function of the excitation intensity of a single 6 ps pulse have shown: (1) *c*-PE fluorescence decayed exponentially for $I < 10^{14}$ photons/cm^2 and non-exponentially for higher intensities; (2) *c*-PC and the APC forms I, II, III and B decayed exponentially up to the highest intensities used in this study ($I \sim 2 \times 10^{15}$ photons/cm^2); (3) the risetimes of the fluorescence were found to be within the time resolution of the detection apparatus, $\leqslant 12$ ps. These results directly demonstrate that at low excitation intensities, the fluorescence relaxation of the emitting chromophores in each of the biliproteins isolated from *Nostoc* sp. is exponential.

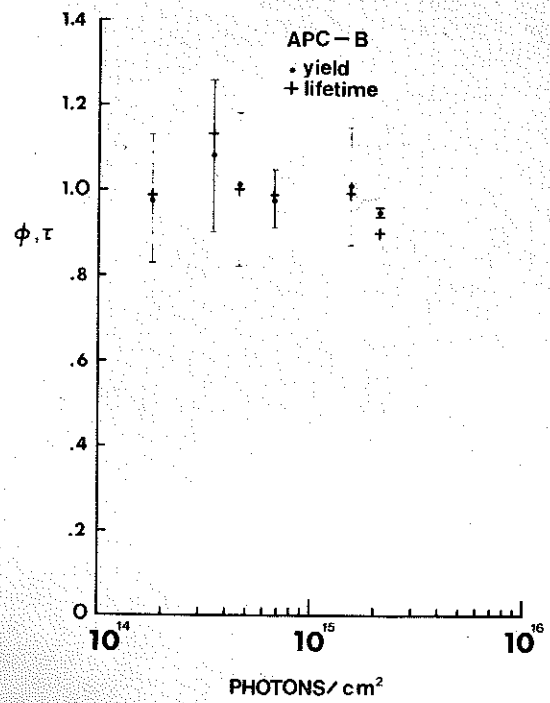

Figure 15. Relative fluorescence yield (.) and relaxation decay lifetime (+) vs. excitation pulse intensity for APC B. The decay times are normalized for a value unity at 2577 ps.

Reports in the literature have shown much variation in the lifetime of *c*-PE fluorescence: 3.5 ± 0.25 ns by Dale and Teale (1970), 3.1 ± 0.1 ns by Barber and Richards (1977), 2.6 ± 0.2 ns for *c*-PE–I and 2.8 ± 0.1 ns for *c*-PE–II by Grabowski and Gantt (1978), and 1.6 ± 0.1 ns by Wong *et al.* by the phase shift method (unpublished). Our measurements here gave 1552 ± 31 ps for the lifetime of *c*-PE. We do not know the reason for the discrepancy in the measured lifetimes; however, a possible explanation for the difference can be the difference in the aggregation states of the samples used in each study. In contrast, our measured lifetime of 2111 ± 83 ps for *c*-PC is in excellent agreement with previous reports in the literature (Barber and Richards, 1977; Zilinskas *et al.*, 1978) on the biliprotein. The lifetime of APC isolated by chromatography has been measured previously by Grabowski and Gantt (1978) who reported a value of 2.7 ± 0.1 ns for APC, and Wong *et al.* (unpublished) who obtained 2.5 ± 0.1 ns for all four forms of APC. Our values of 1869 ± 62 ps for the average lifetime for APC I, II and III, and 2577 ± 121 ps for APC B are taken to be in reasonable agreement with previous results. All the above lifetimes for APC are significantly shorter than the 4 ns for APC in red alga, *Porphyidium cruentum*, reported by Searle *et al.* (1978). We do not speculate on the significance of this difference since the biochemical identity of the species con-

taining the fluorescent chromophore was not ascertained in that work (Searle *et al.*, 1978) and APC was extracted from a different alga.

The relation between the fluorescence yield at intensity $\Phi(I)$ and excitation intensity I (photon/cm^2), in the presence of exciton-exciton annihilation, is given by the expression:

$$\Phi(I)/\Phi(0) = \frac{k}{\Gamma I}\ln\left(1+\frac{\Gamma I}{k}\right) \quad (1)$$

where k is the inverse of the lifetime of fluorescence at low intensities, Γ is an experimentally accessible parameter related to the bimolecular rate constant for singlet–singlet annihilation γ_{ss} ($\Gamma = \gamma_{ss}\alpha/2$), and α is the local absorption coefficient (see references Campillo *et al.*, 1976; Swenberg *et al.*, 1976; Beddard *et al.*, 1977; Campillo *et al.*, 1977; Geacintov *et al.*, 1977; Mauzerall, 1978, for general model and derivation). This relation, plotted as a solid line in Figs 5 and 10, satisfactorily fits the fluorescence yield data for the parameters $(k/\Gamma)_{PE} = 3 \times 10^{14}$ photons/cm^2 and $(k/\Gamma)_{PC} = 5 \times 10^{14}$ photons/cm^2.

In the case of APC, exciton annihilation may occur at a substantially higher intensity than was covered in this experiment. By extrapolating a fit to the Φ vs I curves shown in Fig. 12, we have estimated the onset of quenching to occur at a value of $k/\Gamma < 8 \times 10^{15}$ photons/cm^2. In the case of the isolated phycobiliproteins exciton annihilation must occur within the chromophore pool of the individual phycobiliprotein units rather than between the isolated separate phycobiliprotein units in solution (i.e. the 18 phycocyanobilins in chromophores in a *c*-PC hexamer and not between separate *c*-PC and *c*-PC hexamers in solution). In order to calculate the singlet–singlet annihilation coefficient, it is necessary to know the local absorption coefficient of the phycobiliproteins. Using the molar extinction coefficients of the individual biliproteins (Glazer and Hixon, 1975; Cohen-Bazire *et al.*, 1977), the number of chromophores in each phycobiliprotein and the protein volumes (Glazer, 1976, 1979), one can calculate the local absorption coefficient at 530 nm for the phycobiliprotein units—$\alpha_{PE} \sim 2988$ cm^{-1}, $\alpha_{PC} \sim 298$ cm^{-1} and $\alpha_{APC} \leqslant 86$ cm^{-1}. The local absorption coefficient can alternatively be calculated from the multiple excitation theory of Mauzerall (1978) based on a Poisson distribution of 'hits' per phycobiliprotein units, which is presented below. The values of the absorption coefficient calculated by these two methods can differ by as much as a factor of 4. This difference can arise from uncertainties in the values used for the reported extinction coefficients in the literature, and the exact state of aggregation used in the experiment (Glazer, 1976, 1979). Since the applicability of the multiple excitation theory is not well established, we have chosen the values of α as obtained through the use of the extinction coefficient (Glazer and Hixson, 1975) and the molarity (Glazer, 1979) (for example, see Geacintov *et al.*, 1977; Campillo and Shapiro, 1978).

For the phycobiliproteins *c*-PE, *c*-PC and APC I, the singlet–singlet exciton annihilation coefficient, γ_{ss} can be calculated from the Γ's obtained from the fluorescence quenching curves of Figs. 5, 10 and 12, respectively. By using the measured low intensity fluorescence decay rates and k/Γ from quenching data we obtain:

$$\Gamma c\text{-PE} = 2.15 \times 10^{-6}\ \text{cm}^2\ \text{s}^{-1},$$
$$\Gamma c\text{-PC} = 9.47 \times 10^{-7}\ \text{cm}^2\ \text{s}^{-1}, \text{ and}$$
$$\Gamma \text{APC I} \leqslant 6.7 \times 10^{-8}\ \text{cm}^2\ \text{s}^{-1}$$

These values show that the threshold for the onset of exciton annihilation, which depends on the value of the local absorption coefficient and the singlet–singlet exciton annihilation rate, is different in the different phycobiliproteins. The calculated values for γ_{ss} are

$$\gamma_{ss}(c\text{-PE}) \sim 1.44 \times 10^{-9}\ \text{cm}^3\ \text{s}^{-1}$$
$$\gamma_{ss}(c\text{-PC}) \sim 6.3 \times 10^{-9}\ \text{cm}^3\ \text{s}^{-1}, \text{ and}$$
$$\gamma_{ss}(\text{APC I}) \leqslant 1.6 \times 10^{-9}\ \text{cm}^3\ \text{s}^{-1}$$

The calculated singlet–singlet annihilation coefficients are comparable in magnitude. These values are similar to the value measured for chlorophyll in a chloroplast (Mauzerall, 1978; Geacintov, 1977). Although these singlet–singlet annihilation coefficients indicate a higher annihilation rate in *c*-PC over *c*-PE and APC, it is the higher local absorption of *c*-PE at the wavelength of excitation which allows exciton annihilation to manifest itself earlier in that phycobiliprotein.

When excitons are confined to a localized domain, Mauzerall (1978) has shown that the fluorescence yield can be described in terms of the number of excitations per domain by the Eq.

$$\Phi/\Phi(0) = \frac{1-e^{-x}}{x}, \quad (2)$$

where x is the number of hits per domain. This equation also provides a satisfactory fit to the measured fluorescence quantum yield data as shown by the dashed lines displayed in Figs. 5 and 10. From these fits the following effective optical cross-sections are estimated: $\delta_{PE} = 2.5 \times 10^{-15}$ cm^2, $\delta_{PC} = 1.7 \times 10^{-15}$ cm^2 and $\delta_{APC} < 1.2 \times 10^{-16}$ cm^2.

The important aspect of our present work is the simultaneous evaluation of fluorescence lifetime and relative yield as a function of the excitation pulse intensity. An analysis of these data shows clear differences in the three groups of biliproteins. First, the APC's showed small intensity dependence of both the lifetime or relative yield. Second, *c*-phycocyanin showed a slight dependence of the lifetime, but a strong dependence of the relative yield on excitation intensity. Summarizing, our results above for PC revealed that the lifetime decreased 10–30% as the excitation intensity in a pulse increased from 10^{13} to 10^{15} photons/cm^2, the fluorescence decay remaining exponential, but the relative yield changes, on the other hand, corresponded well with a model of exciton annihilation in the isolated *c*-PC, presumably in

the monomers and trimers (Glazer, 1976, 1979). We note that our fluorescence yield results for PC appear contradictory to those recently published by Kobayashi *et al.* (1979), where no intensity dependence of the yield was observed. We point out, however, that a proper comparison between the two studies is not possible; without the beam cross-sectional area, the intensity region investigated by Kobayashi *et al.* (1979) is undefined. Third, *c*-PE showed strong dependence of the fluorescence decay and relative yield on the excitation intensity. The change of the decay curve from exponential to non-exponential with increasing intensity and the relative yield vs. intensity changing according to Eq. 1 are consistent with the occurrence of exciton–exciton annihilation in *c*-PE and *c*-PC. Finally, we favor exciton annhilation as the explanation for the above results for PE, PC and APC.

Our observation that the fluorescence from the isolated biliproteins, in particular PC at pH 5 and pH 8, rise as fast as from rhodamine 6 G (Fig. 7) suggests that the fluorescent chromophores were excited in less than 12 ps. Our results argue against the concept of fluorescence from '*f*' chromophores excited by energy transfer from the '*s*' chromophores, if it is taken that energy transfer from '*s*' to '*f*' occurs in 32 to 85 ps (Kobayashi *et al.*, 1979). But, if the '*s*' to '*f*' energy transfer (Teale and Dale, 1970) does take place, it must occur in <12 ps. From our results, it also appears that the fast absorption component in *c*-PC measured by Kobayashi *et al.* (1979) arises from singlet–singlet annihilation and not from the '*s*' to '*f*' transfer.

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