PICOSECOND FLUORESCENCE OF CRYPTOMONAD BILIPROTEINS

Effects of Excitation Intensity and the Fluorescence Decay Times of Phycocyanin 612, Phycocyanin 645, and Phycoerythrin 545

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ABSTRACT The fluorescence of purified biliproteins (phycocyanin 645, phycocyanin 612, and phycoerythrin 545) from three cryptomonads, *Chroomonas* species, *Hemiselmis virescens*, and *Rhodomonas lens*, and C-phycocyanin from *Anacystis nidulans* has been time resolved in the picosecond region with a streak camera system having ≤2-ps jitter. The fluorescence lifetimes of phycocyanins from *Chroomonas* species and *Hemiselmis virescens* are 1.5 ± 0.2 ns and 2.3 ± 0.2 ns, respectively, regardless of the fluence of the 30 ps, 532-nm excitation pulse. (Fluence [or photons/cm^2] = $\int$ intensity [photons/cm^2 s]dt.) In contrast, that of C-phycocyanin is 2.3 ± 0.2 ns when the excitation fluence is 8.2×10^{11} photons/cm^2 and decreases to a decay approximated by an exponential decay time of 0.65 ± 0.1 ns at 7.2×10^{16} photons/cm^2. The cryptomonad phycoerythrin fluorescence decay lifetime is also dependent on intensity, having a decay time of 1.5 ± 0.1 ns at low fluences and becoming clearly biphasic at higher fluences ($>10^{15}$ photons/cm^2). We interpret the shortening of decay times for C-phycocyanin and phycoerythrin 545 in terms of exciton annihilation, and have discussed the applicability of exciton annihilation theories to the high fluence effects.

INTRODUCTION

In three phyla of lower plants, Cryptophyceae, Cyanophyceae, and Rhodophyceae, the algae contain arrays of accessory pigments in addition to chlorophylls, carotenes, and xanthophylls. These pigments, known as phycobiliproteins, have covalently bound tetrapyrrole groups, which gather light in the spectral regions with low chlorophyll absorption. The chromophores transfer this energy stepwise to chlorophyll *a* (Gantt, 1975, 1981). There are many different types of phycobiliproteins and their properties have been reviewed (Gantt, 1981; Cohen-Bazire and Bryant, 1982).

Previous studies have analyzed the transfer of the excitation energy by picosecond time-resolved techniques in whole algae (Porter et al., 1978; Brody et al., 1981*a*, *b*), isolated phycobilisomes (Pellegrino et al., 1981; Hefferle et al., 1983*a*; Holzwarth et al., 1982; Suter et al., 1984; Searle et al., 1978), and isolated phycobiliproteins (Holzwarth et al., 1983; Hefferle et al., 1983*b*; Doukas et al., 1981; Wong et al., 1981; Kobayashi et al., 1979). Cyanophytes and Rhodophytes have been the models presented in most of the literature. Cryptomonads, which differ from the other two phyla in that their phycobiliproteins have not yet been found to organize into phycobilisomes, have been used on a very limited basis for such studies (Kobayashi et al., 1979; Holzwarth et al., 1983).

Cryptomonad phycobiliproteins have been studied very little compared with other phycobiliproteins, and the primary focus of this study is to further elucidate the basic properties of these unusual phycobiliproteins. Recently, biochemical experiments have established the chromophore content of two cryptomonad phycocyanins 612 and 645 (MacColl and Guard-Friar, 1983*a*, *b*) and they each are isolated as dimeric proteins (consisting of two α and two β subunits) with each protein having a total of eight tetrapyrrole chromophores. This knowledge of the structure of these proteins is quite necessary to understand the results of photophysical measurements.

It has been shown for certain noncryptomonad phycobiliproteins, which differ from cryptomonad proteins both in types and numbers of their chromophores, that exciton annihilation occurs at high fluences (Wong et al., 1981). The important biological phenomenon we wish to study is excitation energy transfer among the tetrapyrroles (Dale and Teale, 1970) and, since annihilation may alter or obscure such events, it must, therefore, be fully understood. We have found in these studies that two cryptomonad phycocyanins 612 and 645 apparently do not show annihi-

lation effects up to very high intensities. Phycoerythrin 545, after a fluence independent region, dramatically exhibits annihilation. Rationales for these observations are presented and, in general, a firm basis is laid for future studies on the energy transfer properties of these proteins. An analysis of these results shows that a simple continuum model is not appropriate for describing annihilation in these small systems and that the number of chromophores per protein unit affects the degree of annihilation significantly.

EXPERIMENTAL

Cultures of *Rhodomonas lens* (*R. lens*) (phycoerythrin 545), *Hemiselmis virescens* (*H. virescens*) (phycocyanin 612), *Chroomonas* species (phycocyanin 645), and *Anacystis nidulans* (*A. nidulans*) (C-phycocyanin) were grown and harvested, and the respective phycobiliproteins were purified, as described previously (MacColl et al., 1983; MacColl and Guard-Friar, 1983*a*, *b*). Purified protein was dialyzed exhaustively into distilled water, lyophilized, and stored at 4°C. Both steady state and time-resolved fluorescence measurements were performed on phycobiliproteins dissolved in pH 6.0, 0.10 ionic strength, sodium phosphate buffer.

The steady state measurements utilized solutions with 0.05 OD (1-cm light path) at the maximum absorbance wavelength characteristic of each phycobiliprotein and were recorded on a fluorescence spectrophotometer (MPF44a; Perkin-Elmer Corp., Norwalk, CT) at ambient temperature.

Time-resolved studies were performed with instrumentation of the Subpicosecond Biological Physics Facility at the Laboratory for Laser Energetics at the University of Rochester (Rochester, NY). The samples were excited with single, 30 ps, 532-nm pulses from frequency-doubled, amplified, active-passive mode locked Nd^{3+}:YAG (neodymium^{3+}:yttrium aluminum garnet) laser at a 0.5-Hz repetition rate. Fluorescence was detected at 90° to the excitation with a low-jitter (≤2 ps) streak camera-optical multichannel analyzer system (Knox and Mourou, 1981; Knox, 1984). The average energy of excitation was measured with an Rj-7200 Energy Ratiometer (Laser Precision Corporation, Utica, NY). Pulse-to-pulse fluctuations were ±10% or better. The intensity was varied by adjusting a 16-cm focal length spherical lens and by the use of glass neutral density filters in front of this lens. A 532-nm interference filter placed over the sample chamber entrance window ensured that the sample was excited by only the laser pulses. The sample cuvette was mounted with the normal to its front surface ~30° to the excitation beam. Fluorescence was filtered through Schott cut-off filters (Schott Glass Technologies, Inc., Duryea, PA) as follows: for C-phycocyanin, phycocyanin 612 and 645, an OG 590, 2 mm thick; for phycoerythrin 545, an OG 550 and OG 570, both 2 mm thick.

The detection system gain was adjusted to yield the best signal-to-noise ratio for the lowest level of excitation and remained at these settings for all measurements. The wide range of excitation level required the use of neutral density filters to control the fluorescence collected at the higher excitation intensities to prevent the signal from saturating the system. This is why the signal-to-noise ratio of the data presented does not increase dramatically as the excitation level increases. All data have been corrected for nonlinearity in the system's response to signal intensity and nonuniformity in the channel-to-channel response of the detector. The time axis is calibrated using the multiple reflections from a 125-ps Etalon (Newport Corp., Fountain Valley, CA) (Knox, 1984). The deviation from the average number of optical multichannel analyzer channels per 125-ps interval is ≤15% and typically ≤5% during the first four-fifths of a full scan. The curves represent averages of 100–200 shots. To minimize the problem of background drift during averaging a data acquisition program (Knox and Forsley, 1983) takes a background measurement between each shot and subtracts it automatically.

At its slowest setting, our streak camera can scan up to a 600 ps block of time. To extend this limit, an adjustable optical delay line was used to change the timing of the trigger to the streak camera relative to the excitation pulse hitting the sample. This allowed us to measure blocks of 600 ps along the time course of the sample's fluorescence. This technique was successfully used to extend our measurements to ~1 ns, enabling better estimates of long fluorescence lifetimes.

The phycobiliproteins were studied at room temperature in a 1-mm light path glass cuvette. The absorbances in a 1-mm light path were 0.2 at 545 nm for phycoerythrin 545, 0.2 at 645 nm for phycocyanin 645, 0.2 at 612 nm for phycocyanin 612, 0.2 at 620 nm for C-phycocyanin. Phycoerythrin 545 and phycocyanin 645 were also studied at a 530-nm absorbance of 0.02, and this is the same OD as C-phycocyanin has at 530 nm when its OD is 0.2 at 620 nm. The optical density at 530 nm was kept ≤0.15 to ensure uniform intensity of excitation throughout the sample.

RESULTS

The absorption and emission profiles are presented in Fig. 1. The absorption spectra are presented on an arbitrary scale but the absorptivities for 1 g/l solutions in 1-cm light paths are: C-phycocyanin, 6.0 at 620 nm; phycocyanin 645, 11.4 at 645 nm; phycoerythrin 545, 12.6 at 545 nm; phycocyanin 612, not determined. The fluorescence from phycocyanin 612 can be approximated within the noise of our measurements with an exponential lifetime of 2.3 ± 0.2 ns over an excitation range of 3.0×10^{11} photons/cm^2 to ≥ 1×10^{17} photons/cm^2 (Fig. 2 *a*). The phycocyanin 645 fluorescence decay time is also fluence independent, with an apparent lifetime of 1.5 ± 0.2 ns over the range 8.2×10^{11} photons/cm^2 to 7.2×10^{16} photons/cm^2 (Fig. 2 *b*). The decay time of C-phycocyanin varies with excitation fluence (fluence [photons/cm^2] = $\int$ intensity [photons/cm^{2}5] dt). It appears exponential at 8.2×10^{11} photons/cm^2 with a lifetime of 2.3 ± 0.2 ns (Fig. 3). With each increase in fluence the decay time decreases such that at 2.3×10^{14} photons/cm^2 it can be approximated by an

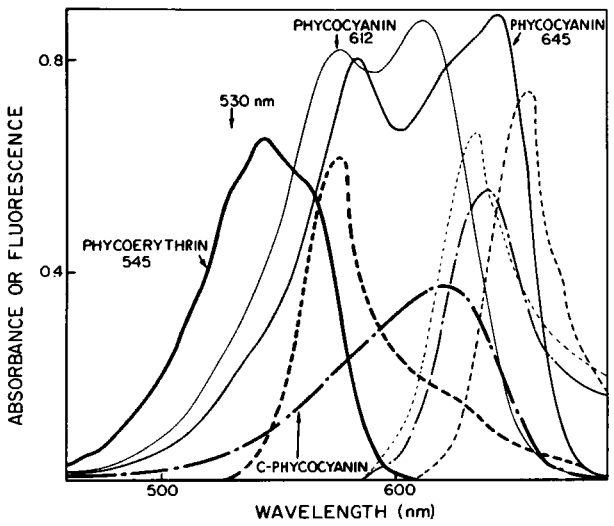

FIGURE 1 Absorption (—) and fluorescence emission (---) spectra of phycoerythrin 545 from *R. lens*, phycocyanin 612 from *H. virescens*, phycocyanin 645 from *Chroomonas* species, and C-phycocyanin from *A. nidulans* in pH 6.0 phosphate buffer. The fluorescence spectra are shown as dashed lines of corresponding thickness.

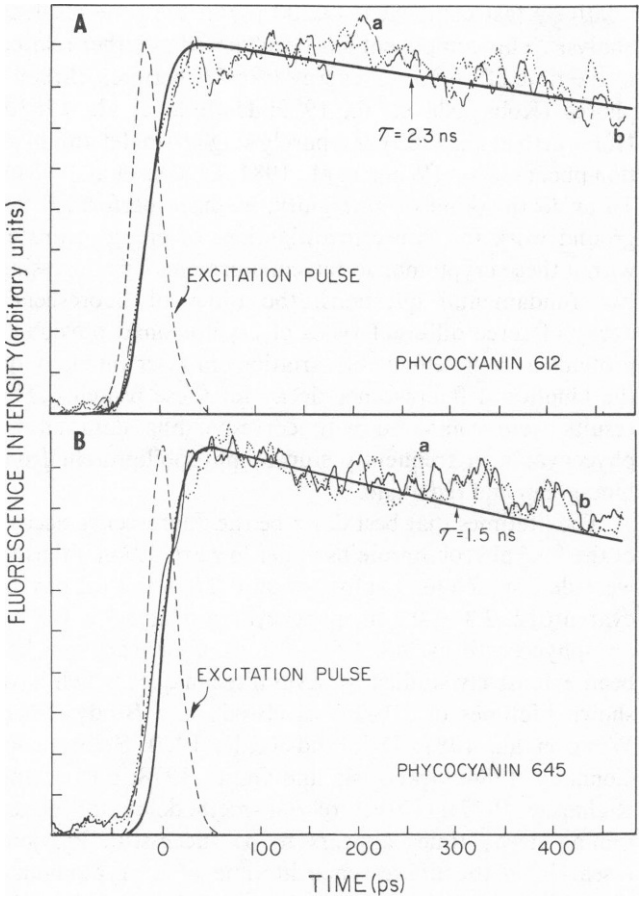

FIGURE 2 Time-resolved emission data for phycocyanins 612 (*A*) and 645 (*B*) at high and low excitation fluences. The theoretical curve is a convolution of the measured 532-nm excitation pulse using Eq. 1 with the parameter given in Table I. Lines *Aa* and *Ab* represent data from fluences of 3×10^{11} photons/cm^2 and 1.4×10^{17} photons/cm^2, respectively. Lines *Ba* and *Bb* depict data from fluences of 8.2×10^{11} photons/cm^2 and 5.3×10^{16} photons/cm^2, respectively.

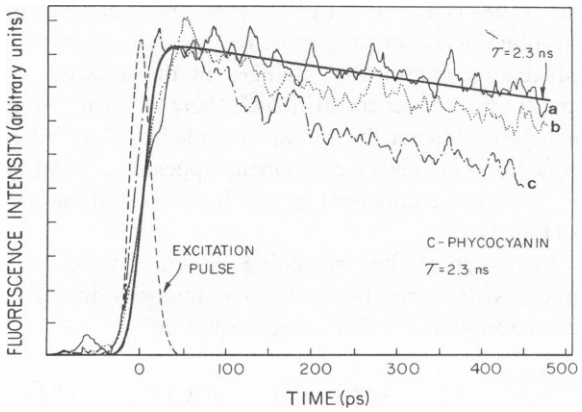

FIGURE 3 Time-resolved fluorescence data for C-phycocyanin at three excitation fluences: line *a*, the lowest fluence, 8.2×10^{11} photons/cm^2; line *b*, 2.3×10^{14} photons/cm^2; line *c*, the highest fluence, 7.2×10^{16} photons/cm^2.

exponential lifetime of 1.3 ± 0.2 ns, and at 7.2×10^{16} photons/cm^2 a lifetime of 0.65 ± 0.1 ns. The decay of fluorescence from phycoerythrin 545 is also excitation-intensity dependent, but behaves very differently compared with the results for C-phycocyanin. At low intensities it is approximately exponential, with a lifetime of 1.5 ± 0.2 ns (Figs. 4 and 5). From a fluence of 2.3×10^{13} photons/cm^2 up to 4.9×10^{15} photons/cm^2 very little effect on the decay time is seen. At 4.7×10^{16} photons/cm^2 a prominent, very short-lived decay component appears (Fig. 4). The fluorescence decay has a dramatic biphasic appearance that resembles a 100 ± 10 ps lifetime during the first 50 ps of decay and a 1.5 ± 0.2 ns lifetime during the last 250 ps (Fig. 6).

For clarity only a few curves representing the excitation intensity dependence are shown. Typically the intensity of excitation was increased by approximately factors of 10 over the ranges given. Neither stimulated Raman scattering of the excitation pulse nor fluorescence from the buffer was detected in these measurements.

The theoretical results shown in Fig. 2–6 are convolutions of the excitation pulse profile, measured using scattered 532-nm excitation light from the sample, using the

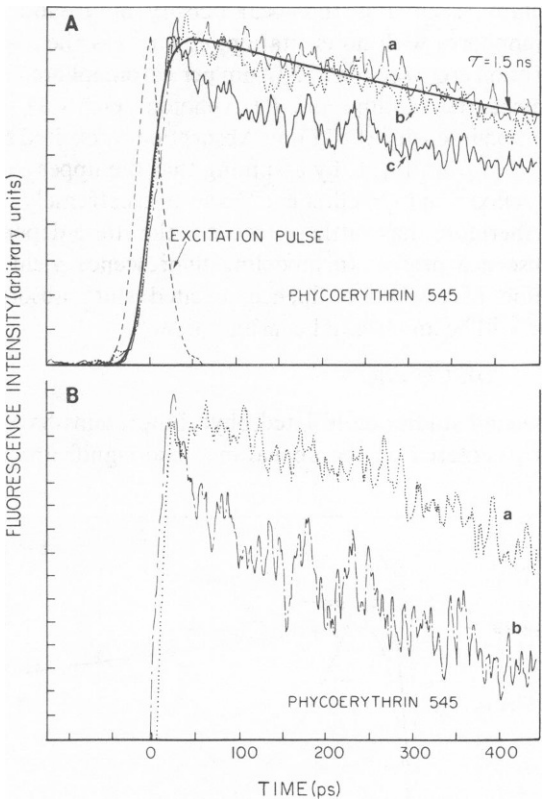

FIGURE 4 Time-resolved emission data for phycoerythrin 545 at three excitation fluences (*A*), and in the log emission vs. time scale (*B*). These results were the same at both phycoerythrin concentrations studied. Lines *Aa*, *Ab*, and *Ac* represent data from fluences of 2.4×10^{13} photons/cm^2, 4.9×10^{15} photons/cm^2 and 4.7×10^{16} photons/cm^2, respectively. Lines *Ba* and *Bb* result from fluences of 2.4×10^{13} photons/cm^2 and 4.7×10^{16} photons/cm^2.

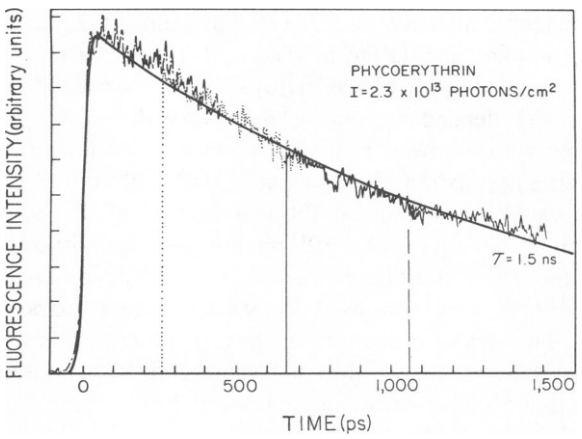

FIGURE 5 Time-resolved emission data for phycoerythrin 545 plotted on extended time axis. Vertical lines segregate decay profiles into the overlapped time blocks.

kinetic equation

$$\frac{dN_1(t)}{dt} = [N_o - N_1(t)]\,\sigma_o I(t) - \frac{1}{\tau} N_1(t), \qquad (1)$$

where $N_1(t)$ (number/cm^3) is the local density of chromophores within the protein in their first excited singlet state, N_o (number/cm^3) is the local density of ground state chromophores with no excitation, σ_o (cm^2) is the average absorption cross section at 532 nm per chromophore, $\tau(s)$ is the measured lifetime and $I(t)$ (photons/cm^2 · s) is the excitation intensity at 532 nm. Absorption by excited states is omitted from Eq. 1, by assuming that the upper excited state relaxation to the first excited state is extremely rapid, and therefore has little effect on the time-dependent fluorescence profile. In modeling fluorescence yield as a function of excitation fluence, excited state absorption would still be an essential consideration.

DISCUSSION

Picosecond studies on isolated phycobiliproteins have previously centered on the appearance and significance of a relatively fast component (<200 ps) in the time-resolved analysis. This component has been viewed as either related to the time of excitation energy transfer between chromophores (Kobayashi et al., 1979; Hefferle et al., 1983*a*; Holzwarth et al., 1983) or a purely singlet-singlet annihilation phenomenon (Wong et al., 1981; Doukas et al., 1981). To avoid this kind of ambiguity, we have performed the ground work for future investigations of energy transfer within these cryptomonad phycobiliproteins by addressing two fundamental questions: the times of fluorescence decay of three different types of cryptomonad phycobiliproteins and the effects of variations in laser intensity on the kinetics of fluorescence decay for these proteins. The results were compared with corresponding data on C-phycocyanin, a frequently studied phycobiliprotein from blue-green and red algae.

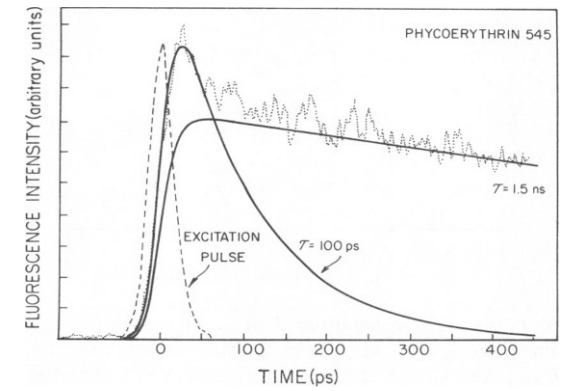

FIGURE 6 Time-resolved emission data for phycoerythrin 545 at 4.7 × 10^{16} photons/cm^2. Data compared with curves having decay times of 1.5 ns and 100 ps.

The lifetimes that best describe the fluorescence decays of the four phycobiliproteins under low excitation intensity were determined as: C-phycocyanin, 2.3 ± 0.2 ns; phycocyanin 612, 2.3 ± 0.2 ns; phycocyanin 645, 1.5 ± 0.2 ns; and phycoerythrin 545, 1.5 ± 0.1 ns. C-phycocyanin has been extensively studied by several techniques which have shown lifetimes of 1.1–2.3 ns (Brody and Brody, 1961; Wong et al., 1981; Dale and Teale, 1970; Seibert and Connolly, 1984; Grabowski and Gantt, 1978; Barber and Richards, 1977). Therefore our methodoloy for determining decay times appears to be successful. Previous research on the fluorescence lifetime of a cryptomonad phycobiliprotein was on phycocyanin 645, whose lifetime was determined as 1.4 ns when carefully studied by picosecond spectroscopy, 1.6 ns by phase shift, and 1.4 ns by modulation (Jung et al., 1980; Holzwarth et al., 1983) which is, again, in excellent agreement with our result.

The effect of laser fluence at 532 nm is different for the cryptomonad phycobiliproteins and for C-phycocyanin from a blue-green alga. For C-phycocyanin the fluorescence decay times steadily decreased over the entire range as the intensity of excitation increased (Fig. 3). A similar result has been reported by Wong et al. (1981) and Doukas et al. (1981). For the two cryptomonad phycocyanins, excitation fluence varying from 10^{11} to $\geq 10^{17}$ photons/cm^2 produced no measurable changes in fluorescence decay (Fig. 2). For phycoerythrin 545 there is little intensity effect until above a fluence of 10^{15} photons/cm^2 where a significant short-decay component appears in addition to the slow-decay component seen at lower excitation intensities (Fig. 4).

We interpret the shortening of decay time, or lack thereof, with increasing excitation intensity in terms of exciton annihilation. The kinetic equation

$$\frac{dN_1(t)}{dt} = [N_o - N_1(t)]\,\sigma_o I(t) - \frac{1}{\tau} N_1(t) - \gamma_{ss}[N_1(t)]^2, \qquad (2)$$

where γ_{ss}(cm^3/s) is the bimolecular annihilation rate, describes the phenomena of exciton annihilation in the continuum limit (large number of chromophores) for a

homogeneous system (see, for example, Swenberg et al. [1976]). The relative importance of annihilation, for a given γ_{ss}, depends primarily on the density of excitons in the system. This density depends not only on the excitation intensity, but also on the optical cross section at the excitation wavelength. It is therefore inappropriate to compare the degree of annihilation in systems with different excitation cross sections solely in terms of excitation intensity. The density of excited states created is the variable to consider. The constant γ_{ss} also determines the degree of annihilation and depends on a few intuitive parameters; the natural radiative lifetime, the pairwise energy transfer rate between an excited and ground state molecule that controls the exciton diffusion rate and the pairwise energy transfer rate between two excited state molecules (Rahman and Knox, 1973).

These results illustrate that in these small systems the degree of annihilation not only depends on the continuum-model parameters of lifetime, density of excitons created, chromophore density and emission-absorption overlaps, but also is affected by the number of chromophores present in the protein unit (Gülen et al., 1984; Gülen and Knox, 1984). The salient observation is that both phycocyanin 612 and phycocyanin 645 have less chromophores than the other phycobiliproteins we studied. The major problem with using Eq. 2 is the assumption that the system is large, which our results illustrate is not appropriate for these proteins. The formalism of Paillotin et al. (1979) also fails to predict our fluorescence decay measurements, because in the small domain limit, this theory predicts exponential behavior that does not characterize C-phycocyanin or phycoerythrin 545 at high intensities. This disagreement is likely caused in part by the assumption of a delta-function excitation in the derivation.

The chromophore content of phycoerythrin 545, which is a dimer, is not yet completely known, but the 5:1 ratio of phycoerythrobilins to cryptoviolin indicates an estimate of ~11–12 bilins per dimer (unpublished results, based on data from Guard-Friar and MacColl, 1984). The density of chromophores is larger than that of phycocyanin 612 and 645 (dimers = 8 chromophores) and the 532-nm cross section is about three times greater (see Table I). For a given excitation intensity an average of three times as many chromophores per protein will be excited in phycoerythrin 545 as compared with phycocyanin 612 or 645 and five times as many compared with C-phycocyanin (18 chromophores).

C-phycocyanin exists mainly as hexamers at pH 6.0 when purified by ammonium sulfate fractionation (MacColl et al., 1971), and therefore has 18 chromophores, quite a few more than the other proteins. C-phycocyanin has a smaller cross section and chromophore density relative to the other proteins, particularly in relation to phycoerythrin 545, the only other protein exhibiting any intensity dependence. Therefore the only known variable that could account for annihilation in C-phycocyanin over the other proteins is the larger number of chromophores per protein unit. An earlier ps paper on C-phycocyanin (Kobayashi et al., 1979) studied hexamers (18 chromophores), trimers (9 chromophores), and monomers (3 chromophores). Their hexamer results were then probably affected by annihilation, but the monomer data was no doubt completely free of this effect and was in fact the first measure, 85 ps, of energy transfer on an isolated biliprotein.

The failure of the simple continuum theory to predict the measured fluorescence decay curves is most clearly illustrated by the high fluence behavior of phycoerythrin 545. At 4.7×10^{16} photons/cm^2, the fluorescence decay is approximated as a 100 ± 10 ps exponential decay during the first 50 ps and a 1.5 ± 0.2 ns decay during the last 250 ps (Fig. 6). These values give a simple representation of the data but have no physical significance because exciton annihilation is a nonlinear process. Notice how rapidly the fluorescence returns to a decay resembling a 1.5 ns lifetime (Fig. 6). This unusual behavior in fluorescence decay vs. excitation intensity can be appreciated in comparison to C-phycocyanin (Fig. 3) and a set of theoretical curves based on the continuum model (Eq. 2) where a range of annihilation rates have been used (Fig. 7). Fig. 7 dramatically illustrates the inability of a simple model of exciton annihilation to describe the small system of phycoerythrin 545. Using the parameters of Table I and annihilation constants in the range of 0 to 10^{-10} cm^3 s^{-1}, the theoretical curves in Fig. 7 illustrate steadily decreasing decay times with an increase in γ_{ss}, which is typical of annihilation in a

TABLE I
SUMMARY OF DATA FROM FLUORESCENCE TIME-RESOLVED EXPERIMENTS COMPARED WITH DATA ON CHROMOPHORE CONTENT OF THE PHYCOBILIPROTEINS

Item	Aggregate state	Molecular weight (gram/mole protein)	No. of chromophores	532-nm cross section	Volume	Density (chromophores/cm^3)	Lifetimes
				cm^2	*cm^3*		*ns*
C-Phycocyanin	$\alpha_6\beta_6$	2.5×10^5	18	3.8×10^{-17}	2×10^{-19}	9×10^{19}	2.3
Phycocyanin 612	$\alpha_2\beta_2$	5.5×10^4	8	6.4×10^{-17}	6.7×10^{-20}	1.2×10^{20}	2.3
Phycocyanin 645	$\alpha_2\beta_2$	5.5×10^4	8	6.1×10^{-17}	6.7×10^{-20}	1.2×10^{20}	1.5
Phycoerythrin 545	$\alpha_2\beta_2$	5.5×10^4	11–12	1.9×10^{-16}	6.7×10^{-20}	1.8×10^{20}	1.5

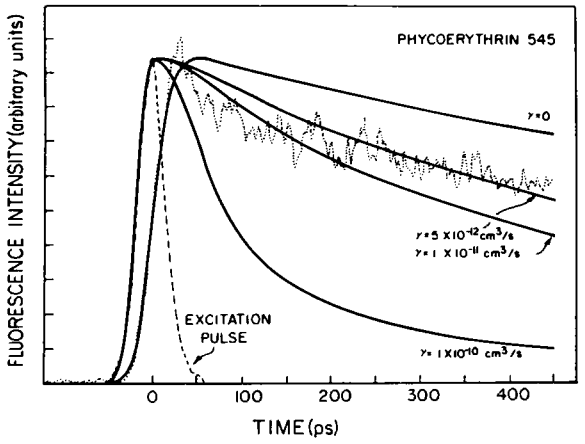

FIGURE 7 Continuum model annihilation theory predictions compared with time-resolved emission of phycoerythrin 545 at 4.7×10^{16} photons/cm^2. Parameters used in Eq. 2 are given in Table I. A range of values for the annihilation constant, γ_{ss}, were chosen to illustrate the failure of this theory to describe the experimental results.

large system made up of a single component. It is clear that no value of γ_{ss} in Eq. 2 could explain the abrupt changes in the fluorescence decay with increasing excitation intensity. Another problem is the rapid rise times of the theoretical curves in comparison with experimental results. The fast rise times are caused by a significant depletion of the ground state population with the beginning of the excitation pulse due to a large absorption cross section at 532 nm.

We believe a major reason for the threefold diversity of response of these phycobiliproteins to varying excitation intensity is simply the number of chromophores per protein unit. Nordlund and Knox (1981) came to a similar conclusion for a chlorophyll protein after finding that a difference in protein size strongly changed the response to excitation intensity. An important consideration is that neither their work nor ours has examined fluorescence yield vs. excitation intensity. Annihilation may occur in phycocyanin 612 and 645 but because these systems are so small, annihilation happens so quickly that it occurs totally within the excitation pulse and little change in the lifetime of decay is detected (Paillotin et al., 1979; D. Gülen, private communication). Another possibility is that the cross section of the first excited state and the lifetimes of the upper excited states are not small and fast enough, respectively, to ignore their effect on the time-dependent kinetics, particularly over the duration of the excitation pulse.

This research has clearly established that three distinct classes of decay by different phycobiliproteins exist in response to variations in laser intensity (no effect, continuous effect, threshold). In cases where the decay profiles are clearly independent of intensity, the decay times have been measured. Future ultrafast kinetic studies in the no effect class can focus on measurement of the rates of excitation energy transfer between chromophores apparently without concern for the presence of annihilation effects. However, the interesting and perhaps complicating possibilities of very rapid annihilations occurring within the pulse time are also raised by these results.

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