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FLUORESCENCE KINETICS OF EMISSION FROM A SMALL FINITE VOLUME OF A BIOLOGICAL SYSTEM

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The fluorescence decay, apparent quantum yield and transmission from chromophores constrained to a microscopic volume using a single picosecond laser excitation were measured as a function of incident intensity. The β subunit of phycoerythrin aggregate isolated from the photosynthetic antenna system of *Nostoc* sp. was selected since it contains only four chromophores in a volume of less than 5.6×10^4 Å^3. The non-exponential fluorescence decay profiles were intensity independent for the intensity range studied (5×10^{13}–2×10^{15} photon cm^{-2} per pulse). The apparent decrease in the relative fluorescence quantum yield and increase of the relative transmission with increasing excitation intensity is attributed to the combined effects of ground state depletion and upper excited state absorption. Evidence suggests that exciton annihilation is absent within isolated β subunits.

1. Introduction

Over the past 30 years energy transfer dynamics of excited electronic states has been extensively studied and applied to a wide range of processes in biological and chemical systems [1–8]. Most of the theoretical and experimental studies have been concerned with systems composed of chromophores randomly distributed in either solutions or solids of *infinite spatial extent*. There are however, many important molecular systems where the distributions of chromophores are limited to a *small finite volume*, e.g., the chlorophyll light harvesting pigments of the photosynthetic unit of green plants, chromophores incorporated into small micellar units, as well as polymers which are constrained to volumes of microscopic dimensions. Recently, Ediger and Fayer [9] have constructed a theoretical formalism for calculating observables when electronic energy is transported among molecules confined to small volumes. Their results demonstrate that time-dependent observables can be significantly altered in small systems relative to their behavior for infinite systems. In particular the fluorescence kinetics, in the absence of bimolecular annihilation, are fluence independent and non-exponential. An ideal biological system to study the properties of electronic energy transfer in small domains is the β subunit of phycoerythrin isolated from the photosynthetic antenna system of the blue-green alga *Nostoc* sp. The phycoerythrin pigment is one component of the phycobilisomes, the well defined organelles, on the exterior surface of the thylakoid membranes of these organisms. In trimer form phycoerythrin of blue–green algae occupies a volume approximated

by a right circular disk of radius 60 Å and height 30 Å [10]. The basic monomer of the pigment consists of two dissimilar polypeptide chains to which chromophores are covalently bonded; these chains, called the α and β subunits, contain two and four chromophores and have molecular weights of 16600 and 19500 dalton respectively [11,12]. In this paper we report the first picosecond fluorescence kinetic measurements of the phycoerythrobolin chromophores in the β subunit of phycoerythrin. We present evidence that the observed decrease in the fluorescence quantum yield with increasing laser intensity arises from enhanced transmission (with possible contributions from upper excited state absorption) and not bimolecular exciton annihilation [13]. The non-exponential fluorescence kinetic profiles were found to be intensity independent and could be fitted to either a double exponential or the Green function theory of Ediger and Fayer for energy transfer in small volumes.

2. Materials and methods

Nostoc sp. (Strain Mac) was grown in a 14 ℓ fermentor with pink or cool white fluorescent light as described previously [14]. Phycobilisomes were isolated from these cells according to the protocol of Troxler et al. [15]. Phycoerythrin was obtained from dissociated phycobilisomes by use of calcium phosphate chromatography and sedimentation on linear gradients of sucrose as detailed by Zilinskas and Howell [14]. The smallest phycoerythrin aggregate so obtained (trimers $(\alpha\beta)_3$) were removed from the gradient, dialyzed exhaustively against 1 mM potassium phosphate, *pH* 5.0, containing 0.02% sodium azide, and were then lyophilized. This sample was dissolved at 10 mg/mℓ in 8.0 M urea, 0.01 M potassium phosphate *pH* 8.0, and 0.01 M β-mercaptoethanol and incubated at 37°C for 2 h. The denatured phycoerythrin was then applied to a DEAE–Sephacel column preequilibrated with the denaturation solution. The α and β subunits were eluted (in that order) with a linear gradient of increasing NaCl in equilibrating solution. The peak fractions were pooled separately, dialyzed against 0.1 M potassium phosphate, *pH* 5.0 to effect renaturation, and their identities assessed by absorption, sedimentation on linear gradients, and electrophoresis on SDS-polyacrylamide gels. Details of these procedures are as described earlier [15].

Experiments were performed on the β subunit component suspended in 0.1 M potassium phosphate at *pH* 5. The sample OD at the excitation frequency was 0.38. The effects of self-absorption are minimized because the sample is frontally excited and most of the observed fluorescence is beyond 580 nm, where the absorption of the sample is small. A frequency doubled (530 nm) 8 ps single pulse from a mode locked Nd:glass laser was used to excite the sample at room temperature. The intensity of the incident fluence was varied by placing appropriate neutral density filters in the excitation pathway. An RCA 7265 PMT was employed for relative quantum yield measurements, whereas the transmitted light was monitored with a diode located behind the sample. A streak camera and OMA system with a 12 ps resolution was utilized to record the fluorescence kinetic decays which were digitized and stored in a computer for later analysis.

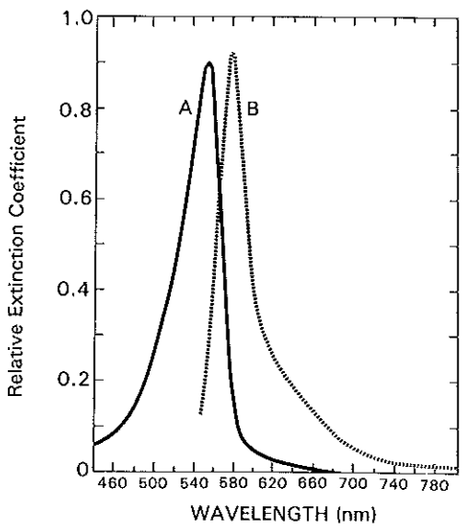
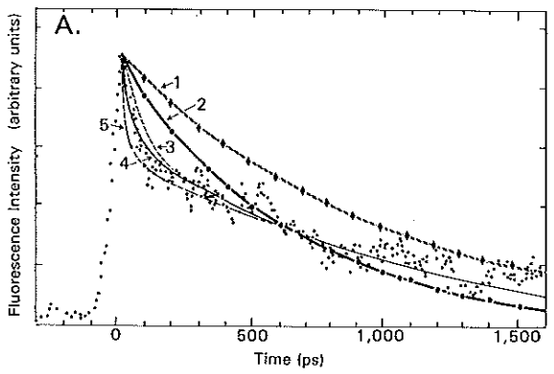


Fig. 1. (A) Relative extinction coefficient for β subunit, (max) at 550 nm = 310000 M^{-1} cm^{-1}. (B) Relative fluorescence intensity versus wavelength; excitation wavelength 530 nm, $T = 300$ K.

3. Results

Fig. 1 shows the relative extinction coefficient and fluorescence emission spectra of the β subunit. These spectra are nearly mirror images of each other and are similar to those reported by Zickendraht-Wendelstadt et al. [12] for the β subunit isolated from Pseudanabaena W1173. The fluorescence kinetic profiles (experimental points indicated by dotted curve) for low and high photon fluence (5.4×10^{13}–1.7×10^{15} photon cm^{-2}) are shown in fig. 2. The decays are highly non-exponential as can be seen on comparisons with exponential fits shown in fig. 2A (curves 1 and 2). The decays are unchanged in shape over the intensity range investigated. Emission rise times are within experimental resolution of the system, i.e. less than or equal to 12 ps. The decays reach one-third of their peak value in about 400 ps. Illustrated in fig. 3 is the relative fluorescence quantum yield (defined as the integrated fluorescence emission divided by the incident number of photons, normalized to unity at low laser intensity) as a function of pulse intensity. Its form is quite similar to quenching curves reported by other workers for complexes of photosynthetic bacteria [16] and green plant photosynthetic components [17]. Also shown in fig. 3 are the relative transmission data which are approximately a mirror image of the quantum yield curve and offset the quantum yield's apparent decrease at high intensity. The two curves break from unity at $\approx 1.75\times10^{14}$ photons cm^{-2}.



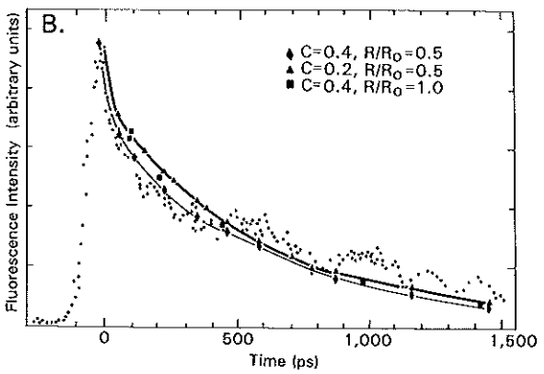


Fig. 2. Fluorescence intensity decays for single pulse excitation. Dotted curves experimental data. (A) Incident fluence 5.46×10^{13} photons cm^{-2} per pulse. Theoretical fits: (1) exponential, $k=1.0\times10^{9}$ s^{-1}, (2) exponential, $k=1.6\times10^{9}$ s^{-1}, (3) double exponential $0.65\exp(-kt)+0.33\exp(-11kt)$, $k=1.0\times10^{9}$ s^{-1}, (4) Paillotin et al. [13], exciton annihilation theory with $r=0.1$, $Z=1$ and $k=1.0\times10^{9}$ s^{-1}, [corresponds to $0.65\exp(-kt)+0.32\exp(-22kt)$], (5) double exponential $0.65\exp(-kt)+0.33\exp(-44kt)$, $k=1.0\times10^{9}$ s^{-1}. (B) Incident fluence 1.75×10^{15} photons cm^{-2} per pulse. Fits to the expression $[1+CG_s(N,V,t)]\exp(-kt)$, $k=1.72\times10^{9}$ s^{-1}, $N=4$ with ■ $C=0.4$, $R/R_0=1.0$; ▲ $C=0.2$, $R/R_0=0.5$ and ◆ $C=0.4$, $R/R_0=0.5$.

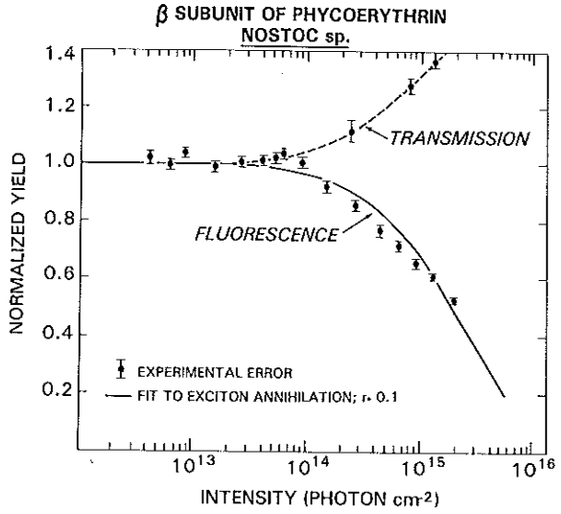


Fig. 3. Relative apparent quantum yield and transmission versus incident single-pulse fluence (photon cm^{-2}). Solid line denotes theoretical fit to exciton fusion model with $r=0.1$ [13].

4. Discussion

The kinetic curve profiles are intensity independent with an overall e^{-1} time of ≈ 400 ps (see fig. 2). They can be made to fit the exciton annihilation theory of Paillotin et al. [13] with values of $r = 0.1$, $k = 1.0 \times 10^9\ s^{-1}$, and $Z = 1.0$, where r is the ratio of twice the monomolecular decay rate to the exciton annihilation rate and $Z = y(1 + X)$, where the constant $X \leqslant 1$ and y is the average number of excitons per domain at $t = 0$. The theory predicts multiexponential expressions for the fluorescence decay curves. For these values of r and Z, the decay is approximately a double exponential of the form

$$0.65 \exp(-kt) + 0.32 \exp(-22kt).$$

The observation that the decay profiles are independent of excitation intensity however invalidates interpreting the non-exponential decay in terms of exciton fusion since a five-fold increase or decrease in Z should produce observable shape differences in the fluorescence curves. For the β subunit, $\epsilon_{550} = 310\,000\ M^{-1}\ cm^{-1}$ [12] implies $\sigma I = y = 1$ at an incident fluence of 1.3×10^{15} photon cm^{-2} and therefore variations in Z are within the experimentally probed intensity range. The extreme sensitivity of the shape of the fluorescence decays to changes in Z over the excitation intensity range investigated ensures that we would have noticed such changes if bimolecular exciton annihilation processes were responsible for the non-exponential decays. Furthermore, the experimental data in fig. 3 demonstrate that corresponding to the decrease in the apparent fluorescence quantum yield with increasing pulse intensity there is an increase in transmission. The theory of exciton annihilation as given by Paillotin et al. [13] is known to be applicable only if the transmission is intensity independent. We cannot offer a quantitative explanation for the single pulse data reported in fig. 3. However, the observation that the relative transmission (T_R) and apparent fluorescence yield (ϕ_R) are approximately mirror reflection of each other about the unit axis is strongly suggestive of ground-state depletion since under steady-state excitation a three-level system gives $\phi_R = T_R^{-1}$ provided the incident intensity is less than the saturation intensity; the saturation intensity being defined as that intensity required to obtain a fluorescence yield decrease of 50% relative to its low intensity yield. Without a quantitative theory, contributions of upper excited-state absorption to the observed decrease in fluorescence quantum yield cannot be ruled out.

It is possible to fit the fluorescence profiles to an expression of the form $\exp(-at^{1/3} - kt)$ with $k = 4.4 \times 10^8\ s^{-1}$, $a = 1.27 \times 10^3\ s^{-1/3}$; however, such a fit must be considered fortuitous since this functional behavior is predicted under assumptions that are not applicable to the β-subunit, namely energy transfer limited to two-dimensions in a system of infinite extent [2]. Our data can best be explained by ascribing the fluence independent, non-exponentiality of the fluorescence kinetics to emission arising from more than a single chromophore. A good fit to the decays can be obtained assuming a minimum two-component model (see fig. 2A, curve 3). The zero of time in figs. 2A and 2B was set at the peak of the fluorescence intensity. All theoretical curves were set equal to the maximum intensity.

It is interesting to note that Fayer's approximation for the solution to the rate equations governing excitation on a finite number of chromophore, randomly dispersed in a finite volume, also provides a reasonable quantitative fit to the kinetic profiles. Several theoretical curves are shown in fig. 2B. The key quantity in Ediger–Fayer theory [9] for electronic excited-state transport among a finite number N molecules distributed randomly in a finite volume V is the probability, $G_s(N, V, t)$, that an excitation is on the originally excited chromophore at time t in the absence of decay due to its finite lifetime. $G_s(N, V, t)$ is a function of both N, the number of chromophores in the domain and R/R_0, where R_0 is the Forster critical radius [3,4] and R is the radius of the sphere which approximates the volume of the domain (for the β subunit R is ≈ 22 Å).

In terms of the response function, $G_s(t)$, the fluorescence decay $F(t)$ we observe is given by

$$F(t) = F_{\parallel}(t) + F_{\perp}(t) \propto \exp(-kt)\left[1 + CG_s(N, V, t)\right], \qquad (1)$$

where C is a constant which depends on the initial excitation distribution and the chromophores' intrinsic anisotropy and $F_\perp$ and $F_\parallel$ denote the fluorescence emission viewed perpendicular and parallel to the incident polarization direction. As indicated in fig. 2B a value of R/R_0 on the order of 0.5 with $k = 1.72 \times 10^9\ s^{-1}$ gives good fits with $N = 4$. The neglect of the rotational diffusion in eq. (1) is justified since $\tau_{rot} = \eta V/KT \approx 10^{-6}$ s at room temperature assuming η is unity. The four chromophores of the β-subunit have definite orientations relative to each other and therefore in a strict sense violate the assumptions employed in deriving eq. (1). Either the fits to eq. (1) are fortuitous or for small R/R_0 and N, eq. (1) has a much broader range of applicability than the randomization of chromophore distances (averaging) implies. This could be rationalized by assuming that few configurations contribute for $R/R \leqslant 1$ with N small. There is also the possibility that the solvent produces slight positional shifts of the β-subunit chromophores thereby providing some degree of spatial randomness. For N fixed, eq. (1) constitutes a three-parameter k, R/R_0, and C. For example, with $R = 22$ Å (the radius of a sphere approximating the β-subunit volume), $R/R_0 \approx 0.5$ as determined from the fluorescence decay curves implies a Forster transfer distance (R) on the order of 40 Å. This is comparable to $R_0 = 21$ Å determined for the α-subunit [18].

In summary, the simplest model consistent with the experimental data reported in figs. 2 and 3 is that the domain size is too small to accommodate more than a single excitation, a view consistent with the onset of bleaching at a fluence of $\approx 2 \times 10^{14}$ photon cm^{-2} ($y \approx 0.2$). Our model suggests that after the absorption of one photon by a single chromophore the excitation energy is quickly dispersed among the other three chromophores comprising the β subunit. The time for randomization of the initial excitation is on the order of hundreds of picoseconds, the time it takes $G_s(N, V, t)$ to approach 0.25. The fast rise-time (< 10 ps) is indicative of the time for the initial emitting chromophore to begin fluorescing. The bleaching indicated by fig. 3 is interpreted in terms of the inability of 530 nm photons to connect the doubly excited state of the β aggregate to its first excited state. Hence in contrast to the non-linear process of singlet-exciton annihilation observed in larger size aggregates of the α and β subunits, for example the c-phycoerythrin [19], it appears that exciton fusion is absent in very small domains. The critical size of a domain for the inoperativeness of exciton annihilation in the general case presumably depends not only on the number of chromophores but also on the rate (proportional to R_0^{-n}, $n \geqslant 6$) at which energy is transferred among them. A general theory of when exciton annihilation is inoperative for a small aggregate of chromophores constitutes an intriguing theoretical problem still lacking a solution.

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