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# STUDIES ON CHROMOPHORE COUPLING IN ISOLATED PHYCOBILIPROTEINS

## I. PICOSECOND FLUORESCENCE KINETICS OF ENERGY TRANSFER IN PHYCOCYANIN 645 FROM *CHROOMONAS* sp.

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**By applying the single-photon timing method the fluorescence kinetics of phycocyanin 645 from *Chroomonas* sp. has been measured as a function of both the excitation and emission wavelength using low-intensity excitation. The fluorescence kinetics were found to be dominated by a fast (15 ps) and a slow (1.44 ns) decay component. The relative yields and amplitudes of these components depended strongly on both the excitation and emission wavelengths. A component with a small relative amplitude and a lifetime ($\tau$) in the range of 360–680 ps has been found as well. The fast decay component is attributed to intramolecular energy transfer from sensitizing to fluorescing chromophores. Our results are discussed in relation to a chromophore coupling model suggested previously (Jung, J., Song, P.-S., Paxton, R.J., Edelstein, M.S., Swanson, R. and Hazen, E.E. (1980) Biochemistry 19, 24–32).**

## Introduction

Phycobiliproteins contain open-chain tetrapyrrole chromophores which are bound covalently to apoproteins [1,2]. In Cyanophyceae, Rhodophyceae and Cryptophyceae they serve as light-harvesting pigments of the photosynthetic apparatus. Phycobiliproteins of both Rhodophyceae and Cyanophyceae form higher aggregates of both homogeneous and heterogeneous types. Aggregates with a highly organized structure, the so-called phycobilisomes, are present in vivo in these algae [3]. Cryptomonad phycobiliproteins, on the other hand, do not aggregate above dimeric states and do not form any phycobilisomes [2]. Even in their protomeric form phycobiliproteins are complex molecules which, in general, contain more than one chromophore. They can either differ or not in molecular structure and/or environmental type. Because of their close distance and often strong spectral overlap energy transfer between different chromophores should be a favourable process [4]. Based on fluorescence spectra and stationary fluorescence polarization, Dale and Teale [5] developped the concept of 'sensitizing' ('s') and 'fluorescing' ('f') chromophores for the prosthetic groups present in phycobiliproteins.

From kinetic studies in the picosecond range there is now a great deal of direct evidence available – although not understood in detail so far – for energy transfer between different phycobiliproteins in phycobilisomes of blue-green and red algae [6–12]. Similar attempts to follow the energy transfer in isolated phycobiliproteins of both blue-green and cryptomonad algae led to contradicting results [13–15]. Kobayashi et al. [13] found both a short-lived (picosecond) and a long-lived (nanosecond) decay component in phycocyanin 645 and in different aggregation states of C-phycocyanin upon excitation with picosecond pulses from an Nd-YAG laser at 530 nm. These authors took the short-lived component as evidence for intraprotein energy transfer between different bilinoid prosthetic groups. Fluorescence and absorption kinetic measurements as a function of pulse intensity, on the other hand, led to the interpretation that, in phycobiliproteins of blue-green algae, the short decay component was caused by singlet-singlet annihilation effects [14]. The latter results would thus argue against the validity of the concept of s and f chromophores [5]. Therefore, the question as to whether intraprotein energy transfer occurs between different chromophores within phycobiliproteins had to be considered open. Clarification of this problem is of the utmost importance for a better understanding of the energy-transfer kinetics in higher aggregates such as phycobilisomes.

It will be essential for such studies to work in an excitation intensity range where singlet-singlet exciton annihilation does not occur. While both singlet-singlet annihilation and energy transfer can give rise to a short-lived decay component, the two phenomena differ in their intensity dependence.

This paper deals with the picosecond fluorescence kinetics of cryptomonad phycocyanin 645 upon low-intensity excitation. Compared to rhodophycean and cyanophycean phycobiliproteins, phycocyanin 645 is special and advantageous in the sense that it occurs in a dimeric aggregation state only [16–19]. An important aspect of our present work is the evaluation of the fluorescence kinetics as a function of both emission and excitation wavelength by selective excitation of the various groups of different chromophores. Using the method of single-photon timing combined with mode-locked laser excitation, both picosecond and nanosecond components can be measured in the same experiment simultaneously and with high accuracy.

The structure and subunit composition of phycocyanin 645 from *Chroomonas* have been studied earlier [16,17,19]. An $\alpha\alpha'\beta_2$ substructure is now generally accepted for phycocyanin 645 [17,19]. An energy-transfer study as well as a spectral lineshape analysis [18,19] have also been published.

## Materials and Methods

The cultivation of the freshwater cryptomonad *Chroomonas* sp. was carried out as described earlier [17]. Cell suspensions were harvested by low-speed centrifugation and phycocyanin 645 extracted by either three cycles of freeze-thawing of the cells [16], or by a treatment with 0.02% (w/v) benzalkonium chloride in phosphate buffer, pH 6.9, for 5 min (Morisset and Wehrmeyer, unpublished data). The supernatant obtained after centrifugation at $40\,000 \times g$ for 5 min at 5°C was used for measurements. It is characteristic of cryptophytes that they contain only one biliprotein in any species, i.e., either phycocyanin or phycoerythrin, and never allophycocyanin. We should like to point out that we intentionally avoided ammonium sulphate precipitation during the preparations in order to keep the phycobiliproteins in a native state. Nevertheless, an $A_{645}/A_{280}$ absorbance ratio of 3.3 was obtained in our preparations.

Sedimentation velocity experiments of the biliprotein sample were performed on the analytical ultracentrifuge Model E equipped with an AN-F Ti rotor and a 12 mm double-sector centerpiece (Beckman, Munich) at 20°C following the method described by Kranz and Schmidt [20]. For the rapidly extracted phycocyanin 645 the high percentage of 94% corresponds to a sedimentation coefficient $s_{20,w} = 4.5 \cdot 10^{-13}$ S. This is in line with values reported by MacColl et al. [16,21] and indicates a far-reaching homogeneity of the samples. Moreover, the extreme constancy of the lifetime values of the long-lived component ($\tau_3$) together with the fact that the relative amplitude of the unassigned middle component ($\tau_2$) never exceeds 8.7% also indicate a high degree of purity and integrity of the sample. For the data presented phycocyanin 645 was dissolved in phosphate buffer 20 mM, pH 6.9, with 2 mM 2-mercaptoethanol added. Both preparation methods gave identical results. Measurements in 750 mM and 10 mM phosphate buffer solution agreed within the error limits (cf. Table II) with the 20 mM buffer. The protein concentration was in the range 0.1–0.3 mg/ml. All experiments were carried out within less than 30 h after isolation of the pigment.

Absorption spectra have been measured on a Perkin Elmer PE 320 absorption spectrometer.

Corrected fluorescence and excitation spectra have been recorded in 1-mm cuvettes (front face arrangement) on a computer-controlled Spex Fluorolog fluorescence spectrometer [22]. For the emission spectra the bandwidth was 2.5 and 5.0 nm in the emission and excitation monochromator, respectively. For the excitation spectra the slits were reversed. In the wavelength range above 600 nm the exciting lamp intensity has been determined by a radiometer (EG + G model 550) calibrated against NBS standards.

For fluorescence decay measurements we used the single-photon timing technique with picosecond resolution. The source of picosecond pulses was a synchronously pumped mode-locked and cavity-dumped laser system (Spectra Physics) with an $Ar^+$ laser as the pumping source. The dye laser was operated with rhodamine 6G (tuning range 570–650 nm). This apparatus has been used previously for kinetic studies [11,23,24]. For the lifetime measurements the sample was pumped slowly through a flow cuvette, in order to exclude any effects arising from light-induced degradation during the measurement. Fluorescence was selected by a double monochromator with 4 nm bandwidth. Polarizers were used in both the excitation and emission paths. Their relative orientation was 54.7° (magic angle) in order to exclude transient polarization effects. The width of the apparatus function, as measured by single-photon timing, was approx. 230 ps (full-width at half-maximum) using excitation pulses of approx. 10–15 ps width. Our apparatus is at present capable of measuring fluorescence lifetimes as short as 10 ps. This time resolution has been confirmed inter alia by measuring the lifetimes of various cyanine dyes [24] and comparing the data with those obtained by independent measuring techniques.

The single-photon timing technique features very high sensitivity with unsurpassed signal-to-noise ratio. These properties are combined with a high dynamic range for both the intensity and time scales. Laser pulse intensities in our measurements were in the range $10^{10}$–$10^{12}$ photons/cm$^2$ pulse with a repetition rate of 800 kHz, i.e., at least one order of magnitude lower than the lowest intensity used by other authors [14] for the study of phycobiliprotein fluorescence decay.

Approx. 20 000 counts were routinely collected in the peak channel. Lifetimes according to a decay law:

$$I(t)=\sum_{i=1}^{n} R_i \exp(-t/\tau_i)$$

were calculated by an iterative convolution method. The quality of the fits was judged by both a reduced $\chi^2$ criterium and a plot of weighted residuals [25].

## Results and Discussion

The absorption, fluorescence and excitation spectra of phycocyanin 645 in 20 mM phosphate buffer solution of pH 6.9 are shown in Fig. 1. The corresponding spectra in 750 and 10 mM buffer solutions of the same pH are virtually identical with those displayed in Fig. 1. All maxima of the absorption, excitation and emission spectra are compiled in Table I. A pronounced difference between the absorption and excitation spectrum,

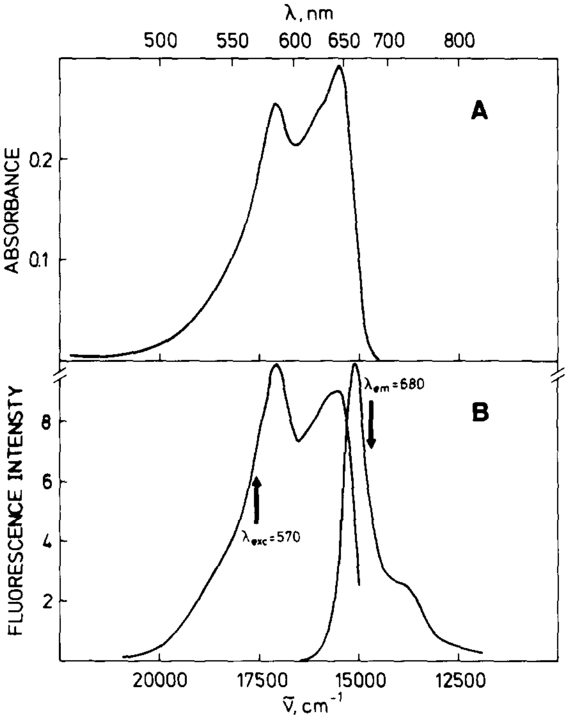

Fig. 1. Absorption (A), fluorescence excitation and emission spectra (B) of phycocyanin 645 in 20 mM phosphate buffer, pH 6.9. The path length for recording the absorption spectrum was 2 mm.

TABLE I

MAXIMA OF ABSORPTION, FLUORESCENCE AND FLUORESCENCE EXCITATION SPECTRA OF PHYCOCYANIN 645 IN 20 mM PHOSPHATE BUFFER

sh, shoulder.

| $\lambda_{abs}^{max}$ (nm) | Excitation | | Emission | |
|---|---|---|---|---|
| | $\lambda_{em}$ (nm) | $\lambda_{exc}^{max}$ (nm) | $\lambda_{exc}$ (nm) | $\lambda_{em}^{max}$ (nm) |
| 644, 620 (sh), 585, 370 | | | | |
| | 680 | 642, 585, 370 | | |
| | 720 | 643, 586, 370 | | |
| | | | 570 | 662, 719 (sh) |
| | | | 620 | 662, 720 (sh) |

regarding the intensity ratio of the two visible absorption bands, is revealed in Fig. 1. Self-absorption can be excluded as the cause of this difference, since the absorbance of the sample was sufficiently low. This result has been obtained repeatedly, using several independent preparations with both procedures described in Materials and Methods. The difference is certainly beyond the maximum possible error made in correcting for the exciting lamp intensity as a function of wavelength (±5%). The excitation spectrum published by MacColl et al. [16] seems to be in better agreement with the absorption than in our case, although it does not show very well the shoulder around 620 nm, which is clearly present in the absorption. No conclusive explanation is available at present for this difference. Polarization effects might play some role, although not to a sufficient extent. A possible rationalization, not in disagreement with our kinetic data, would be the presence of several f chromophores with somewhat displaced emission spectra.

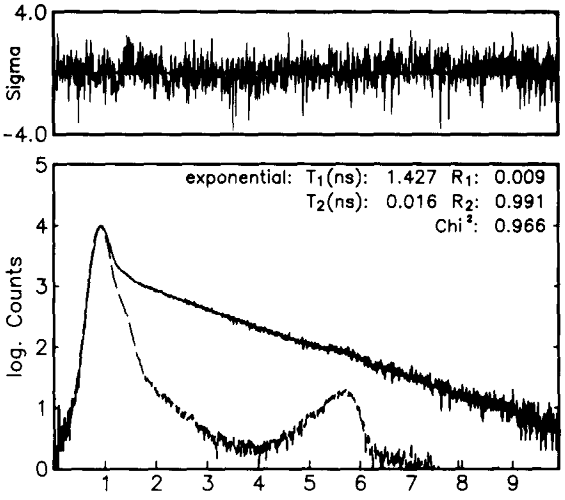

Fig. 2. Semilogarithmic plot of fluorescence decay of phycocyanin 645 in 20 mM phosphate buffer, pH 6.9. $\lambda_{exc} = 569$ nm, $\lambda_{em} = 585$ nm. Note (Figs. 2–4): Superimposed on the experimental decay is the fluorescence decay (thin line) calculated from the best-fit kinetic parameters (cf. inset). Also shown is the semilogarithmic presentation of the exciting pulse (— — —). In the inset are given the lifetimes ($\tau_1 \cdots \tau_n$) and amplitudes ($R_1 \cdots R_n$) of the decay components. The frame in the middle (on top for Fig. 2) shows a weighted residuals plot, indicating the deviations between the computer-fitted and experimental decay curves. The frame on top (not shown for Fig. 2) gives the weighted residuals plot for an evaluation of the same experiment in terms of $n-1$ decay components. Also shown in the residuals plots are the reduced $\chi^2$ values from the statistical analysis.

Fluorescence decay curves for various excitation and emission wavelengths of phycocyanin 645 are given in Figs. 2–4. At all wavelength pairs the decays deviate from a single exponential, requiring

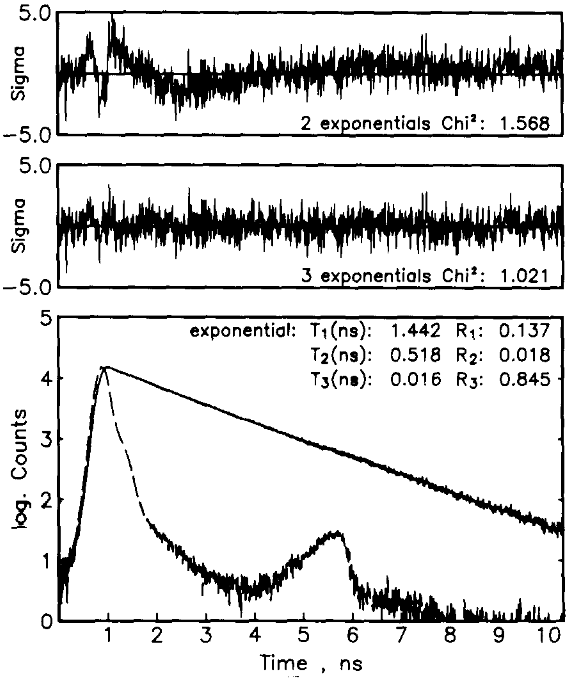

Fig. 3. Semilogarithmic plot of fluorescence decay of phycocyanin 645 in 20 mM phosphate buffer, pH 6.9 (cf. Note, Fig. 2). $\lambda_{exc} = 590$ nm, $\lambda_{em} = 605$ nm.

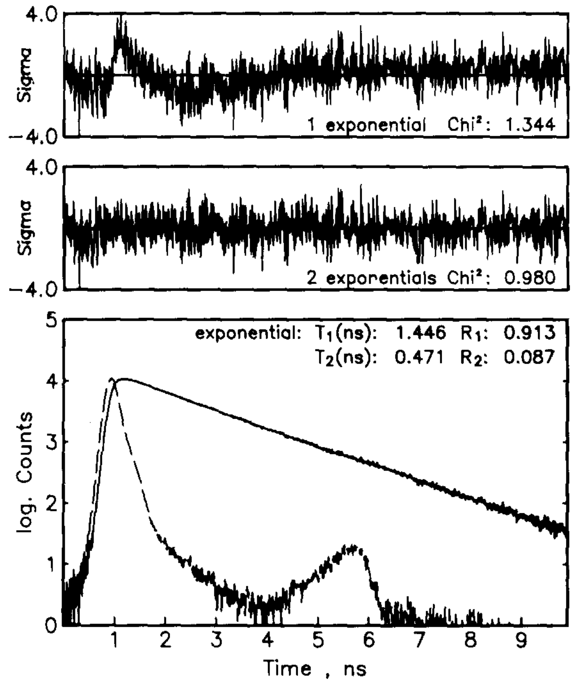

Fig. 4. Semilogarithmic plot of fluorescence decay of phycocyanin 645 in 20 mM phosphate buffer, pH 6.90 (cf. Note, Fig. 2). $\lambda_{exc} = 617$ nm, $\lambda_{em} = 662$ nm.

up to three exponential components for a good fit. At $\lambda_{em} = 585$ nm an excellent fit with only two decay terms was obtained (cf. Fig. 2). These two decay terms comprise the characteristic features of the fluorescence kinetics of phycocyanin 645. At all excitation/emission wavelength pairs examined the middle component ($\tau_2 = 300$–$500$ ps) has very small amplitudes only. One of the major results from these measurements is that the contribution of the short-lived decay component with $\tau_1 = 15 \pm 3$ ps is strongly dependent on both the emission and excitation wavelengths. Its relative yield is highest at $\lambda_{em} = 585$ nm (54%) (cf. Fig. 2 and Table II) and decreases to 2% at $\lambda_{em}$ 662 nm (cf. Table II) for $\lambda_{exc} = 569$ nm. It is an important finding that only for excitation into the short-wavelength absorption band ($\lambda_{exc} \leqslant 600$ nm) can the short-lived $\tau_1$ component (15 ps) be observed. Excitation into either the long-wavelength absorption maximum or the shoulder around 620 nm does not give rise to this picosecond decay component. All lifetimes, relative amplitudes and relative yields derived from the decay experiments are compiled in Table II.

Since our excitation pulse intensities are well below the values where singlet annihilation could occur, we take the shortest-lived decay component as evidence for the presence of a genuine short-lived excited state of one of the chromophores. The most reasonable explanation for this finding is intramolecular energy transfer between individual chromophores of different environmental or molecular type within one phycocyanin 645 dimer.

Dimeric phycocyanin 645 contains most proba-

TABLE II

LIFETIMES, RELATIVE AMPLITUDES AND RELATIVE YIELDS OF THE FLUORESCENCE DECAY COMPONENTS OF PHYCOCYANIN 645 IN 20 mM PHOSPHATE BUFFER, pH 6.9

Lifetime values deviate from the numbering in Figs. 2–4, lifetimes are ordered according to increasing value. Amplitudes and yields are normalized to 100%. Errors for the picosecond components are ±3 ps when the relative yield of this component is above 5%. For lower yields the error may be twice as large. The long-lived component has an error of approx. 1%.

| $\lambda_{exc}$ | $\lambda_{em}$ | Lifetime (ps) | | | Relative amplitude (%) | | | Relative yield (%) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| (nm) | (nm) | $\tau_1$ | $\tau_2$ | $\tau_3$ | $R_1$ | $R_2$ | $R_3$ | $Q_1$ | $Q_2$ | $Q_3$ |
| 569 | 585 | 16 | – | 1430 | 99.1 | – | 0.9 | 53.7 | – | 46.3 |
| 569 | 620 | 10 | 374 | 1430 | 74.4 | 2.4 | 23.2 | 2.0 | 2.6 | 95.4 |
| 569 | 662 | 25 | 593 | 1450 | 52.1 | 4.0 | 43.9 | 1.9 | 3.6 | 94.5 |
| 590 | 600 | 19 | 686 | 1450 | 91.7 | 0.9 | 7.4 | 13.8 | 4.7 | 81.5 |
| 590 | 605 | 16 | 510 | 1440 | 84.5 | 1.8 | 13.7 | 6.1 | 4.1 | 89.8 |
| 590 | 620 | 13 | 417 | 1440 | 61.3 | 3.6 | 35.1 | 1.5 | 2.8 | 95.7 |
| 590 | 662 | 18 | 392 | 1440 | 38.6 | 5.4 | 55.9 | 0.8 | 2.6 | 96.6 |
| 617 | 630 | – | 438 | 1430 | – | 8.1 | 91.9 | – | 2.6 | 97.4 |
| 617 | 640 | – | 360 | 1440 | – | 7.5 | 92.5 | – | 2.0 | 98.0 |
| 617 | 662 | – | 471 | 1450 | – | 8.7 | 91.3 | – | 3.0 | 97.0 |

bly altogether six chromophores in the α- and β-subunits. They form at least three groups which differ in their absorption spectra [19,21]. At least two of the chromophores should be termed s chromophores. Jung et al. [19] analyzed the complete absorption spectrum of intact phycocyanin 645 in terms of even five spectral components, including a term due to β-β exciton coupling. Considering the short relative distance and strong spectral overlap of some of these chromophores, Förster energy transfer is very likely to occur. We interpret the 15 ps decay component as the time required for energy transfer from the group of s chromophores to the f chromophores. This model implies that the emission spectrum of the f chromophores is red shifted relative to the one of the s chromophores. Thus, the relative contribution of the decay component originating from the initially excited short-wavelength chromophores is expected to decrease with increasing emission wavelength. This expection is in excellent agreement with our experimental findings (cf. Table II).

Our results essentially support the interpretation of the absorption kinetic measurements of Kobayashi et al. [13]. We find the transfer time to be somewhat longer (15 ps) as compared to their value (8 ps), however. Thus, a partial contribution of exciton annihilation, which would lower the apparent transfer time, cannot be fully excluded in their work. This could be due to the much higher pulse intensities required for picosecond absorption detection as compared to our fluorescence single-photon timing measurements. Furthermore, the data for the long-lived decay of the terminal emitter (1.44 ± 0.01 ns) are determined much more accurately in our work than in Ref. 13, where only an approximate lower limit has been given. The constancy of this lifetime over the whole emission band (up to 725 nm) is highly remarkable. This long-lived decay time agrees with that obtained in Ref. 19 by both phase shift and streak camera detection. No short-lived component was reported in that work.

It is tempting to identify the various decay components with the spectral components analyzed by Jung et al. [19]. In order to test this band analysis and assignment the excitation and emission wavelengths presented in Table II have been chosen. In the following we refer to this analysis. The relative contribution $R_1$ of the short-lived (15 ps) component at $\lambda_{em} = 620$ nm is higher for $\lambda_{exc} = 569$ nm than for $\lambda_{exc} = 590$ nm. This would be in agreement with fast energy transfer from the short-wavelength s chromophores in the β-subunits to f chromophores in either the α- or β-subunits. If this model applies, $R_1$ for $\lambda_{exc} = 590$ nm should decrease, since upon preferential excitation into the upper exciton absorption band the actual relaxation process should be internal conversion to the lower exciton state rather than energy transfer. This process might proceed in a time considerably shorter than 15 ps and would thus not be detected in our measurement. Our findings thus do not disagree with the suggested exciton splitting and band assignment. From the fact that for excitation above 600 nm no short-lived decay can be observed, we conclude that these chromophores do not transfer excitation energy among each other in the isolated phycocyanin 645. Since these chromophores are most probably non-isoenergetic, as is suggested from both the absorption spectrum and the analysis of Ref. 19, we are left with several classes of slightly energy-shifted f chromophores. With respect to this finding a partial revision of the model of Jung et al. [19] seems to be in order. The differences between the absorption and excitation spectra could thus arise from this 'inhomogeneous' emission. The most surprizing result, however, consists of the fact that these different f chromophores should posess identical fluorescence lifetimes as evidenced from the constancy of the long-lived decay time across the whole emission band. Although we would not be able to resolve slightly different lifetimes, such differences should show up, if at all existent, in a continuous change of the average lifetime across the emission spectrum. This is obviously not the case.

While the interpretation of both the short-lived (15 ps) and long-lived (1.44 ns) decay components is clearly possible, the middle component (360–680 ps) poses more problems. While its relative amplitude is always small (maximum 8%) its lifetime varies. For these reasons it is not considered as an energy-transfer component. We believe, rather, that it arises from a small amount of environmentally perturbed chromophores. Strong dependence of the fluorescence properties of both biliproteins and tetrapyrrole chromophores on the environmental conditions is known from the literature [22,24,26–28].

In the case of intramolecular energy transfer from s chromophores with higher energy to f chromophores with lower energy, a rise term (negative amplitude) should occur in the long-lived fluorescence from the f chromophores upon short-wavelength excitation ($\lambda_{exc} \leqslant 600$ nm). The rate constant of this rise term should equal that of the directly measured decay term from the s chromophores. Such rise terms have been found in the fluorescence kinetics of, e.g., isolated phycobilisomes by Searle et al. [7], Pellegrino et al. [8] and by us [11], but not in phycobiliproteins [13,14]. Despite some indications for such a rise term being present in some of our fits, no clear experimental proof for their presence was possible. It should be kept in mind, however, that fast rise terms are more difficult to trace experimentally than decay terms of the same rate constant. This fact alone might be sufficient to explain our failure to detect the expected rise component. A further complication arises from the assumed exciton coupling in phycocyanin 645 between chromophores in the $\beta$-subunits (cf. discussion above). This coupling is expected to result in fast internal conversion and would thus lead to an almost instantaneous rise of part of the long-lived fluorescence. Taking all these complications into account, the expected effects may lie within the assumed error limits of our apparatus.

In the light of the recent results reported by Alfano and coworkers [14,15], who interpreted short-lived components as arising from singlet-singlet annihilation and not from energy transfer, it is of considerable interest to perform similar measurements on cyanophycean and rhodophycean biliproteins at low light intensities. Such measurements have recently been carried out by Hefferle et al. [28] using pulses of approx. $10^{13}$ photons/cm$^2$. They also find short-lived components in the fluorescence decays of phycocyanin from *Spirulina platensis* in various aggregation states. Their short-lived decay times, which are interpreted as energy-transfer times, are in the range of approx. 300 ps at room temperature. Preliminary measurements from our research group carried out at still lower intensities indicate that, in C-phycocyanin isolated from *Anacystis nidulans*, energy transfer proceeds on a time scale of tens of picoseconds.

## Conclusions

We conclude that our wavelength- and time-resolved fluorescence data proove fast internal energy transfer within phycocyanin 645. They are also gratifying in the sense that they are in full agreement with the expected emission and excitation wavelength dependence of the relative yields and amplitudes of the decay components. Thus, the general concept of s and f chromophores, as introduced by Dale and Teale [5], is supported by our results. Our interpretation of the short-lived fluorescence decay component as the time required for intramolecular energy transfer is in general agreement with that of the transient absorption data by Kobayashi et al. [13]. No rise term has been found for the long-lived fluorescence from the f chromophores upon excitation of the s chromophores. This rise term should be searched for in the future using appropriate methods.

Nothing definite can be said in favour of the mechanism of interaction, however. Although Förster resonance transfer is the most probable one to occur, alternative mechanisms, e.g., exciton coupling, cannot be excluded at present.

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