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THERMAL DENATURATION OF MONOMERIC AND TRIMERIC PHYCOCYANINS STUDIED BY STATIC AND POLARIZED TIME-RESOLVED FLUORESCENCE SPECTROSCOPY

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Abstract—C-Phycocyanin (PC) and allophycocyanin (APC), as well as the α-subunit of PC, have been isolated from the blue-green alga (cyanobacterium), *Spirulina platensis*. The effects of partial thermal denaturation of PC and of its state of aggregation have been studied by ps time-resolved, polarized fluorescence spectroscopy. All measurements have been performed under low photon fluxes ($\leq 10^{13}$ photons/pulse × cm^2) to minimize singlet–singlet annihilation processes. A complex decay is obtained under most conditions, which can be fitted satisfactorily with a bi-exponential (τ_1 = 70–400 ps, τ_2 = 1000–3000 ps) for both the isotropic and the polarized part, but with different intensities and time constants for the two decay curves. The data are interpreted in the framework of the model first developed by *Teale* and *Dale* [*Biochem. J.* **116**, 161 (1970)], which divides the spectroscopically different chromophores in (predominantly) sensitizing (*s*) and fluorescing (*f*) ones. If one assumes temperature dependent losses in the energy transfer from the *s* to the *f* and between *f* chromophores, both the biexponential nature of the isotropic fluorescence decay and the polarization data can be rationalized. In the isotropic emission (corresponding to the population of excited states) the short lifetime is related to the $s \rightarrow f$ transfer, the longer one to the "free" decay of the final acceptor(s) (= *f*). The polarized part is dominated by an extremely short decay time, which is related to $s \rightarrow f$ transfer, *as well as* to resonance transfer between the *f*-chromophores.

INTRODUCTION

Phycobiliproteins are light harvesting pigments in cyanobacteria, red and cryptophyte algae. In the former two, they are organized in microscopic complexes, the phycobilisomes, which are to date probably the best understood photosynthetic antenna system (Gantt, 1980; Glazer, 1980; MacColl and Berns, 1981; Scheer, 1981). The chromophores of native biliproteins display some remarkable properties which are crucial for their efficient light absorption and energy transfer.

The current understanding of the light-harvesting process in biliproteins is mainly based on polarized or kinetic fluorescence data. Studies with conventional light sources (Brody and Brody, 1961; Brody and Rabinowitch, 1957; Dale and Teale, 1970; Dimitrievski *et al.*, 1957; Frackowiak *et al.*, 1981; Grabowski and Gantt, 1978 a,b; Latimer, 1956; MacDowall and Walker, 1968; Barber and Richards, 1977; Zickendraht-Wendelstadt *et al.*, 1980) led to a model, in which the chromophores can be classified in predominantly sensitizing ("s"-type) and fluorescing ("f"-type) chromophores (Teale and Dale, 1970). In isolated phycobiliproteins, the former transfer energy efficiently by a Förster type mechanism to the latter, which in turn emit the excitation energy with fluorescence yields ≥50%. The number of "s"-type chromophores increases and the energy transfer becomes increasingly complex with an increased aggregation of biliproteins, ranging from monomers containing 2–6 chromophores up to intact phycobilisomes containing hundreds of chromophores. Following the early work of Merkelo *et al.* (1969), mode-locked lasers have been used by several groups (see Breton and Geacintov, 1980; Brody, 1981; Holzwarth *et al.*, 1982; Searle *et al.*, 1978, 1979; Pellegrino *et al.*, 1981; Kobayashi *et al.*, 1979) to obtain a more detailed picture on the excitation migration and fluorescence in biliproteins. Earlier picosecond studies have been reviewed by Breton and Geacintov (1980) and in a monograph edited by Alfano (1982). Besides a higher precision in the determination of the fluorescence lifetimes, for the first time these measurements allowed direct investigation of the energy transfer kinetics. There are, nonetheless, considerable discrepancies regarding the details of the process. These are related, on the one hand, to the complexity of the biological system and the differences in sample preparation (e.g. different organisms, different aggregation states, presence of varying amounts of colorless "linker" proteins). On the other hand, they are due to specific limitations of the measuring equipment (e.g. too high light intensities which lead to singlet–singlet annihilation), excitation at a non-optimum wavelength and also ambiguities in the interpretation (multiexponential *vs* non-linear exponent, e.g. $t^{\frac{1}{2}}$). Some of these problems have been critically dis-

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cussed in the reviews by Breton and Geacintov (1980) and in Alfano (1982).

We are presently engaged in a systematic study on the fluorescence properties of isolated biliproteins and small aggregates. Detailed knowledge of the excited state kinetics should contribute to a better understanding of the basic steps in the energy transfer chain and of the chromophore–protein interaction. The latter are essential for the low rates of intramolecular radiationless deactivation and thereby for efficient excitation transfer in these pigments.

In this paper, we present results on isolated biliproteins, especially the influence of temperature on excited state life time, the time-resolved polarisation of the fluorescence and its yield. The time resolved measurements were performed with a tunable mode-locked cw dye laser for excitation in combination with a repetitively working streak camera as the detection system. In spite of the low excitation intensity [$\leq 10^{13}$ photons/(cm^2 × pulse)], the sampling technique gave a sufficiently high signal/noise ratio to allow the recording of polarized fluorescence decay curves. The latter information is important in view of the fact that the high fluorescence yield of biliproteins is abolished by complete unfolding of the proteins, but there is already a pronounced decrease below the actual melting point of the polypeptide chain. There is independent evidence from absorption (Scheer and Kufer, 1977; Kufer and Scheer, 1979) and circular dichroism data (Lehner and Scheer, 1983) for intermediates in the unfolding process. Such partially unfolded biliproteins have recently gained considerable interest for their photochromic properties (Ohki and Fujita, 1979; Ohad *et al.*, 1979; de Kok *et al.*, 1981). The underlying mechanisms are hitherto only poorly understood. The knowledge of the fluorescence kinetics under these partially denaturing conditions should help to distinguish between the several possible causes of fluorescence quenching upon thermal denaturation.

MATERIALS AND METHODS

Isolation of biliproteins. During the preparation of biliproteins, all solutions were kept on ice and manipulations were done at 4°C. Algae, solutions at the final stages of isolation and lyophilized samples were stored at −20°C. All buffers contained NaN_3 (3 m*M*) and Na_4 EDTA (1 m*M*) if not mentioned otherwise. Biliproteins were isolated from *Spirulina platensis* by the method of Kufer and Scheer (1979). The final purification was achieved by chromatography on a brushite-column (Ø 1.7 cm, l = 15 cm), equilibrated with phosphate buffer. C-phycocyanin (C-PC)* was removed almost entirely by elution with 400 mℓ of the starting buffer, APC was then eluted with a linear gradient (400 mℓ buffer, 5–50 m*M* potassium phosphate, flow rate = 50 mℓ/h). Two fractions were collected: C-PC ($\epsilon_{620}/\epsilon_{280} > 3.6$; $\epsilon_{620}/\epsilon_{350} \approx 6.8$) and APC ($\epsilon_{650}/\epsilon_{280} > 3.9$; $\epsilon_{650}/\epsilon_{620} = 1.5$; $\epsilon_{650}/\epsilon_{350} = 6$–7). Pooled APC and PC fractions were precipitated with 60% ammonium sulfate, dialyzed against distilled water and lyophilized. Yield after dialysis: 4.5 μmol of C-PC and 0.8 μmol of APC from 30 g (wet weight) of frozen cells.

APC and C-PC were each free of the other according to gel electrophoresis, but contained minor amounts of uncolored peptides. Subunits of PC from *S. platensis* were isolated according to Glazer and Fang (1972). Ten to fifteen milligrams of PC were dissolved in 1 mℓ aqueous acetic acid (pH 3.0) containing urea (7 *M*). The solution was loaded on a Biorex 70 column (Biorad, CA, 1.5 × 5 cm, sodium form, minus 400 mesh, equilibrated with the same buffer, linear velocity 1.4 cm/s). The subunits were separated with a linear gradient of urea (7.5–9 *M*, 250 mℓ) and eluted at 8.1 *M* (α) and 8.5 *M* (β). The pooled fractions were freed from urea and acid without delay on a P2 column (Biorad, CA, 5 × 25 cm), equilibrated with potassium phosphate buffer (80 m*M*, pH 6.0). The α-subunit was free of other proteins and especially of the β-subunit according to SDS-PAGE. Yield: $A_{622}^{1\,cm} = 0.3$ (0.07 μmol) were obtained from 7.9 mg PC (0.18 μmol). Spectral properties: Absorption: $\lambda_{max} = 622$, 580 (shoulder), 370 nm; $E_{622}/E_{350} = 4.8$; fluorescence emission: $\lambda_{max} = 645$ nm.

The β-subunit eluting after the α-subunit from the column is protein-chemically pure according to SDS-PAGE, but unsuitable for fluorescence measurements. This was due to (partly) irreversible denaturation, oxidation of the chromophores, and to precipitation in solution, partly because it remains much longer on the column than the α-subunit.

Chemicals and Methods. All chemicals used were analytical reagent grade unless stated otherwise. UV-vis spectra were recorded in a model 320 (Perkin-Elmer, Germany) or a model DMR 22 spectrometer (Zeiss, Germany). For the static fluorescence experiments the DMR 22 was equipped with a fluorescence attachment consisting of a M4Q III monochromator (Zeiss, Germany) and a 450 W Xe Lamp (Osram, Germany) for excitation. The cell holders were thermostated. In the fluorescence experiments, the temperature was measured by a model P5 Pt 100 resistor (Degussa, Germany) and recorded simultaneously with the fluorescence (see Fig. 1). Below ambient temperature, the sample compartment was flushed with dry air. The samples for fluorescence measurements were dissolved in potassium

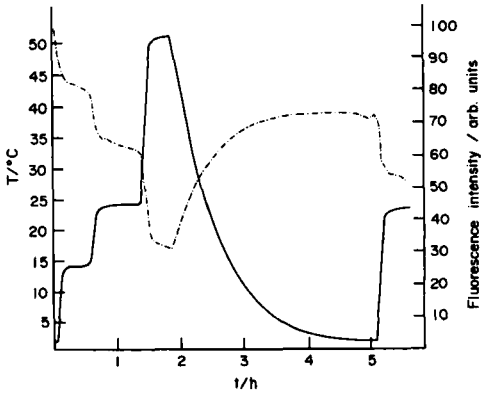

Figure 1. Thermal quenching and recovery of fluorescence under continuous irradiation. Typical trace of the simultaneous recording of T (——) and the relative fluorescence intensity (–·–·–·–) given in percent of the initial fluorescence at T = 0°C. The data of such experiments are summarized in Table 2. The trace shown corresponds to the PC trimer from *S. platensis*.

**Abbreviations:* C-PC = C-phycocyanin, APC = Allophycocyanin, Na_4 EDTA: sodium salt of ethylenediamintetraacetic acid, tris = tris-hydroxymethyl aminomethane, PAGE = polyacrylamide electrophoresis, SDS = sodium dodecylsulfate.

Table 1. Uncorrected sedimentation coefficients for aggregated biliproteins and for the monomers obtained after dissociation with 1 *M* NaSCN

Pigment and state of aggregation†	Concentration (μg/mℓ)	Sedimentation coefficients (Svedberg units)	Remarks
APC aggregated $(\alpha\beta)_3$	43	4.7 (2)	Value in brackets not accurately determinable due to smearing, approx. 20% of total
APC disaggregated $(\alpha\beta)$	43	2.4	
PC aggregated $(\alpha\beta)_3$	38	4.3	In runs with higher concentrations ($E_{620} = 0.75$) and at pH 7.0 a minor band (~15%) with S = 9.5 was discernible
PC disaggregated $(\alpha\beta)$	38	2.0	At higher concentrations and at pH 7.0 a second band (~35%) with S = 9.5 was observed

All measurements were performed in phosphate-buffer (pH 6.0, I = 0.1). The concentrations correspond to an absorption of 0.3 at the red maximum (λ_{max} = 650 nm for aggregated APC, and 620 nm for all other samples). NaSCN (1 *M*) was included in the gradients for the measurement of the monomers. Test runs showed that the sedimentation pattern of PC was unchanged after storage of the solutions for 10 days in the refrigerator.

phosphate buffer (ionic strength = 0.1, pH 6), containing 1 *M* NaSCN (MacColl *et al.*, 1981) in the case of monomeric (α,β_1) biliproteins. The maximum extinction of the solutions in the red spectral range was kept below 0.75. For the degradation experiments, two samples were measured simultaneously with one of them being continuously in the excitation beam and the other in the dark except for the actual measuring time (≤1% of the total time). The excitation bandwidth was 28 nm and the emission bandwidth was 8 nm. The incident fluence rate in the static fluorescence experiments was 15 Wm^{-2}.

All experiments (sedimentation, static and kinetic fluorescence, and photobleaching) have been done with aliquots of the same sample, at the same concentrations, The extinction at the red absorption maximum was kept constant at 0.3/cm (≡ 38 μg PC/mℓ), and was raised to a maximum of 0.75 (95 μg/mℓ) only to obtain a better S/N in a few experiments, without a significant change of the data.

Analytical methods. Polyacrylamid gel electrophoresis (PAGE) was performed with the system of Maurer (1968) (7.5% gel) with vertical slabs (13 × 16 × 0.15 cm). SDS-PAGE was performed in the same system, but with 0.2% SDS in the gels. Samples were dissolved during 2 h at 50°C in the presence of 0.2% SDS and 0.2% 2-mercaptoethanol (Blaich, 1978). The gels were stained with Coomassie brilliant blue G-250 (Serva, Germany) and documented by photography. Sedimentation coefficients were determined in a model E analytical ultracentrifuge (Beckmann, Germany) at 20°C with the scanner wavelength set at 620 nm. An AN-F rotor was used at 52000 or 56000 rpm. Sample concentrations were similar to the ones used for the kinetic fluorescence studies ($\epsilon_{620}^{1\,cm} = 0.3$). The buffers used for the sedimentation runs were identical to the ones in the fluorescence experiments (potassium phosphate, pH 6.0, ionic strength = 0.1 for the aggregated pigments, and the same buffer containing 1 *M* NaSCN for the dissociated pigments). The S-values (Table 1) decreased generally with increasing run lengths and were extrapolated to time zero. They are otherwise uncorrected. Test runs showed, that the sedimentation pattern of PC is unchanged after storage of the solutions for 10 days in a refrigerator.

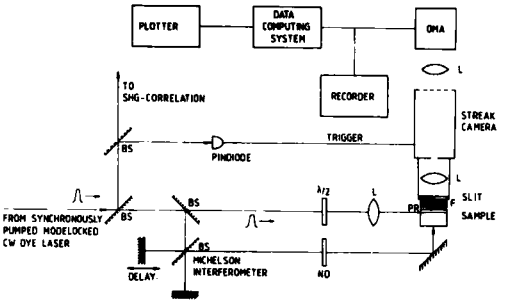

Figure 2. Experimental set up for recording polarized fluorescence decay curves (BS = beamsplitter, ND = neutral density filter, PR = polarizing film, F = filter, L = lens).

Gel-filtration was carried out on agarose gels (Biogel A 0.5 m, 200–400 mesh, Bio Rad, California; 90 × 1.5 Ø). The columns were equilibrated and developed with either potassium phosphate buffer (ionic strength 0.1, pH 6.0) for the aggregated pigments, or with the same buffer containing in addition 1 *M* NaSCN for the free pigments. Alternatively, an Ultropac TSK G, 3000 SW column (LKB, Sweden, 60 × 0.75 cm Ø) was used in one experiment. The thermal stability of the biliproteins was tested either by their fluorescence (see above), or by heating the samples for 15 min at defined temperatures and subsequent filtration over Biogel P2 (BioRad, CA) to remove coagulated pigments.

Fluorescence decay curves. The experimental set-up used for recording the fluorescence decay curves is illustrated in

†A_0 has been provided to take care of any reactions leading to a constant background. This could principally include a remaining ^{1}s-population as well as triplet or radical formation, although the latter are expected to be negligible (Land, 1979; Berns, 1976; Ilani and Berns, 1971; Friedrich *et al.*, 1981).

Fig. 2 (Hefferle *et al.*, 1983). The source of excitation is a continuous wave dye laser (Spectra Physics, model 375; tuning range with rhodamine 6G: 575 nm $< \lambda <$ 625 nm) synchronously pumped by an acoustooptically mode-locked Ar ion laser (Spectra Physics model 171) with 80 MHz repetition rate. The dye laser beam is divided into three parts. Two parts are used to trigger the streak camera and to calibrate the time scale. The third and main part is directed onto the sample cell after passing a polarizer and an appropriately oriented polarization rotator ($\lambda/2$ plate).

Fluorescence is monitored at 90° to the excitation beam with a synchroscan streak camera (Hadland photonics Imacon 675). Cut-off filters reduced the scattered excitation light intensity. Polarized emission is obtained by insertion of an additional thin polarizing film. The streaked image is detected and digitized with an optical multichannel analyser (PAR model 1205). Data is then transferred to a minicomputer for data storage and subsequent manipulation.

From the decay curves with the polarization analyser being parallel [$I_{\parallel}(t)$] and perpendicular [$(I_{\perp}(t)$] to the polarization of the exciting laser beam, the expressions $I(t) = I_{\parallel}(t) + 2I_{\perp}(t)$ and $D(t) = I_{\parallel}(t) - I_{\perp}(t)$ are calculated. These two functions give information on the decay of the excited state population [$I(t)$] and on the difference function [$D(t)$], which is related to the anisotropy of the fluorescence, respectively [see Appendix for the discussion of $D(t)$].

The sample cell is thermostated in order that sample temperature variations resulting from the different illumination conditions (laser powers, integration time of detection) are minimized. In all experiments, the wavelength of excitation (λ_{ex}) was 600 nm. At this wavelength, the dye laser produces the most stable train of ps light pulses. According to the autocorrelation trace, the FWHM is less than 4 ps. Due to the jitter in the interpulse separation and/or the limited resolution of the synchroscan streak camera–optical multichannel analyser system, the profile of the excitation pulse is recorded with an apparent FWHM of about 25 ps (5 channels of the OMA-system).

The theoretical decay curves are computed by convolution of the assumed decay law (mono- or biexponential) with this apparently broadened pulse shape. As discussed below, the true functional dependence of the decay law can be derived only if the complete scheme of all primary photophysical- and photochemical processes would be known. The adoption of a mono- or biexponential decay law is mainly based upon the fact that the (four) parameters of this approximation can be determined with reasonable effort and may be used to characterize the dominant features in the experimental decay curves. But even for a truly biexponential decay

$$I(t) = \int_{-\infty}^{+\infty} E(t-t')\,[A_1 \exp(-t'/\tau_1) + A_2 \exp(-t'/\tau_2)]\,dt' + A_0$$

the derivation of the fitting parameters A_1, A_2, τ_1, τ_2 and A_0^* may be difficult [$E(t)$ is the apparent excitation profile of A_0 a constant background]. Their precision does not only depend on the S/N ratio, but also on the relative magnitudes of the fit parameters (Linde *et al.*, 1977).

On the basis of parameter variation tests, we estimate that the uncertainty in the values of the fit parameters is about ± 10%. Because of competing energy transfer processes, the decay law may be even more complex. In spite of all these limitations, the results discussed below show, however, such clear trends, that the essential conclusions are independent of the details in the evaluation. Furthermore, it should be mentioned here that the streak camera was tested to prove the linearity in the sweep. Measurements of the fluorescence decay of organic dye stuffs with an exponential decay law were recorded to check for any irregularities.

RESULTS

Static fluorescence measurements

These measurements were performed to establish the state and stability of the samples and to determine the proper conditions for the kinetic experiments. An increase in sample temperature causes the fluorescence intensity of isolated biliproteins to decrease without any measurable delay with respect to the heating rate of the thermostat. This is illustrated in Fig. 1 for the case of trimers $(\alpha\beta)_3$† of C-PC. For each of the three pigments studied the decrease of fluorescence intensity (relative to that at 0°C) is dependent on the state of aggregation (see Tables 1 and 2) and on the nature of the biliprotein. In particular, trimeric APC $(\alpha\beta)_3$ is more stable than monomeric APC ($\alpha\beta$) and than tri- and monomeric PC from the same species, *Spirulina platensis*. The latter effect has been studied by a simple method up to fully denaturing temperature (70°C). The APC/PC mixture was held at well defined temperature for 5 min in a hot water bath, then quenched on ice and immediately filtered over a short Biogel P2-column, or centrifuged at 3000 ***g*** for 5 min. The filtrate and supernatant were analysed by UV-vis absorption. The PC-yield dropped sharply above 52°C, whereas APC was stable [as a trimer, if judged from its UV-vis absorption (MacColl *et al.*, 1981)] up to 60°C. It may be noteworthy that this allows a rather efficient separation of large amounts of APC and C-PC of this alga.

The thermally induced process, which causes the fluorescence quenching, is only in part reversible. The longer the sample is kept at elevated temperature, the lower is the fluorescence recovery. As can be seen from Fig. 1, steady denaturation of the sample occurs at slow, but measurable speed when the temperature is kept constant around 50°C. The recovery is better for the trimeric than for the monomeric pigments, in particular with APC. If the sample is kept at room temperature (24°C), an irreversible photoinduced reduction in fluorescence yield can be observed which is accompanied by a decrease in absorption (Table 3). The effect is again more pronounced for monomers ($\alpha\beta$) than for trimers $(\alpha\beta)_3$. In contrast to the thermal stability, APC is photobleached more easily than is C-PC.

Picosecond time resolved fluorescence decay curves

The results of time-resolved fluorescence measurements on C-PC monomers ($\alpha\beta$) are shown in Fig. 3. Due to the reduced fluorescence intensity at 51.8°C, the signal-to-noise ratio is slightly reduced in Fig. 3c compared to that of Figs 3b and 3a, respectively. Nevertheless, this figure shows that the

Table 2. Temperature effect on the fluorescence intensity of the phycobiliproteins, C-PC and APC, in different aggregation states

Sample	Temperature	Relative fluorescence intensity %	
	T°C	Trimer	Monomer
C-PC‡	0	100	100
from *Spirulina platensis*	14	81	81
	24	62	67.5
	50‖	31	36
	0*	72	64
	24†	53	54
APC§	0	100	100
from *Spirulina platensis*	14	—	81
	24	82	67
	50‖	52	28
	0*	83	61
	24†	71	45

*Fluorescence "recovery" after cooling the thermally treated sample to 0°C. †Sample newly heated to 24°C. ‡Trimer kept a total of 65 and 25 min above ambient temperature and 48°C, respectively. The corresponding times for the monomer are 90 and 50 min. §Trimer kept a total of 70 and 25 min above ambient temperature and 48°C, respectively. The corresponding times for the monomer are 75 and 30 min. ‖The 50° value has a considerable error, because at the highest measuring temperature, a continuous denaturation of all samples has been observed except for the APC trimer (Fig. 1). All values have been given relative to the fluorescence intensity at the beginning of the experiment (0°C). A typical trace for C-PC from *Spirulina platensis* is given in Fig. 1. Excitation wavelength 600 nm, except for 610 nm with APC trimer. Emission set at fluorescence maximum (approx. 645 nm for monomeric and trimeric C-PC and for the AP monomer, 665 nm for APC trimer).

Table 3. Illumination effect on the fluorescence and absorption of C-PC and APC from *Spirulina platensis* at 20°C

Sample	Duration of illumination (h)	Wavelength of illumination (nm)	Relative fluorescence intensities (%)			Relative absorption (%)		
			Irradiated	Dark	Q_f	Irradiated	Dark	Q
C-PC trimer	20	600	98	98	1.00	100	100	1.00
C-PC monomer	28	600	66	91	0.73	82	100	0.82
APC trimer	22.5	620	88	96	0.92	100	100	1.00
APC monomer	21	610	52	94	0.55	65	100	0.65

All values are relative with respect to the fluorescence or absorbance at the beginning of the experiment. The emission wavelength was adjusted to the fluorescence maximum (approx. 645 nm for the monomer and trimer C-PC and for the monomer of APC, and 665 nm for APC trimer). The irradiation wavelength is given in the table. The effect of photobleaching decreases with shorter wavelengths. Relative absorption was measured at the absorption maxima (650 nm for the trimer of APC and 620 nm for the others). The excitation wavelength was chosen at points of similar extinction coefficients and slightly below the absorption maximum to avoid interference from the excitation beam and the detected fluorescence.

deactivation kinetics have changed at 51.8°C. From the plot of $I(t)$ in Fig. 3c, we conclude that the contribution of the long-lived component is increased and the deviation of the biexponential least square fit is greater than at lower temperatures. More drastic, however, is the influence of temperature on the deactivation as manifested in the decay of the fluorescence difference function $D(t)$ (Fig. 3d–f).

At 18.6 and 39.2°C, the fluorescence decay curves can be fitted by essentially the same parameters. This indicates that the decay kinetics of the emitting species remain unchanged, despite a decrease in the fluorescence intensity of about 50% in going from 18.6 to 39.2°C (see Fig. 1). At 51.8°C both the rapidly and the slowly decaying components are fitted by markedly longer lifetimes.

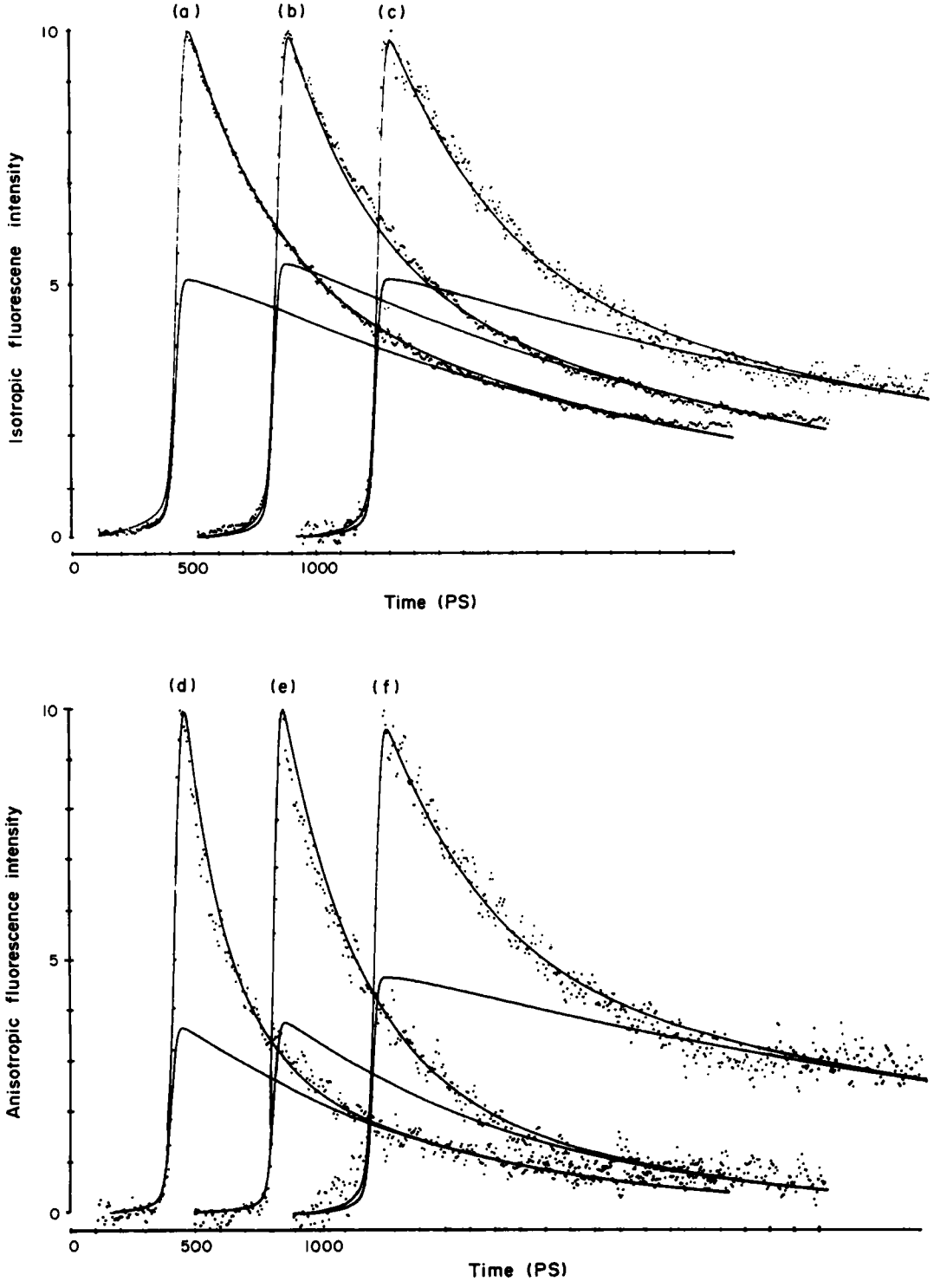

Figure 3. Isotropic ($I_{\parallel} + 2I_{\perp}$) (curves a–c) and difference functions ($I_{\parallel}-I_{\perp}$) (curves d–f) of the fluorescence kinetics of monomeric C-PC from *Spirulina platensis* at different temperatures (T) after laser (-pulse) excitation. The figures show the curves constructed from the experimentally obtained profiles (dots), and the biexponential fits and their long-lived components (solid lines). The curves are shifted arbitrarily on the time (=x) axis for display. The fits were obtained with the following parameters (τ_1, τ_2 = lifetimes, A_1, A_2 = maxima of the intensities of the long- and short-lived components, respectively): Isotropic fluorescence: (a) T = 18.6°C; τ_1 = 2200 ps, A_1 = 51%, τ_2 = 300 ps, A_2 = 49%; (b) T = 39.2°C; τ_1 = 2200 ps, A_1 = 54%, τ_2 = 290 ps, A_2 = 46%; (c) T = 51.8°C; τ_1 = 3200 ps, A_1 = 51%, τ_2 = 400 ps, A_2 = 49%. Difference function of fluorescence: (d) T = 18.6°C; τ_1 = 1050 ps, A_1 = 37%, τ_2 = 150 ps, A_2 = 63%; (e) T = 39.2°C; τ_1 = 1000 ps, A_1 = 38%, τ_2 = 250 ps, A_2 = 62%; (f) T = 51.8°C; τ_1 = 3500 ps, A_1 = 47%, τ_2 = 380 ps, A_2 = 53%.

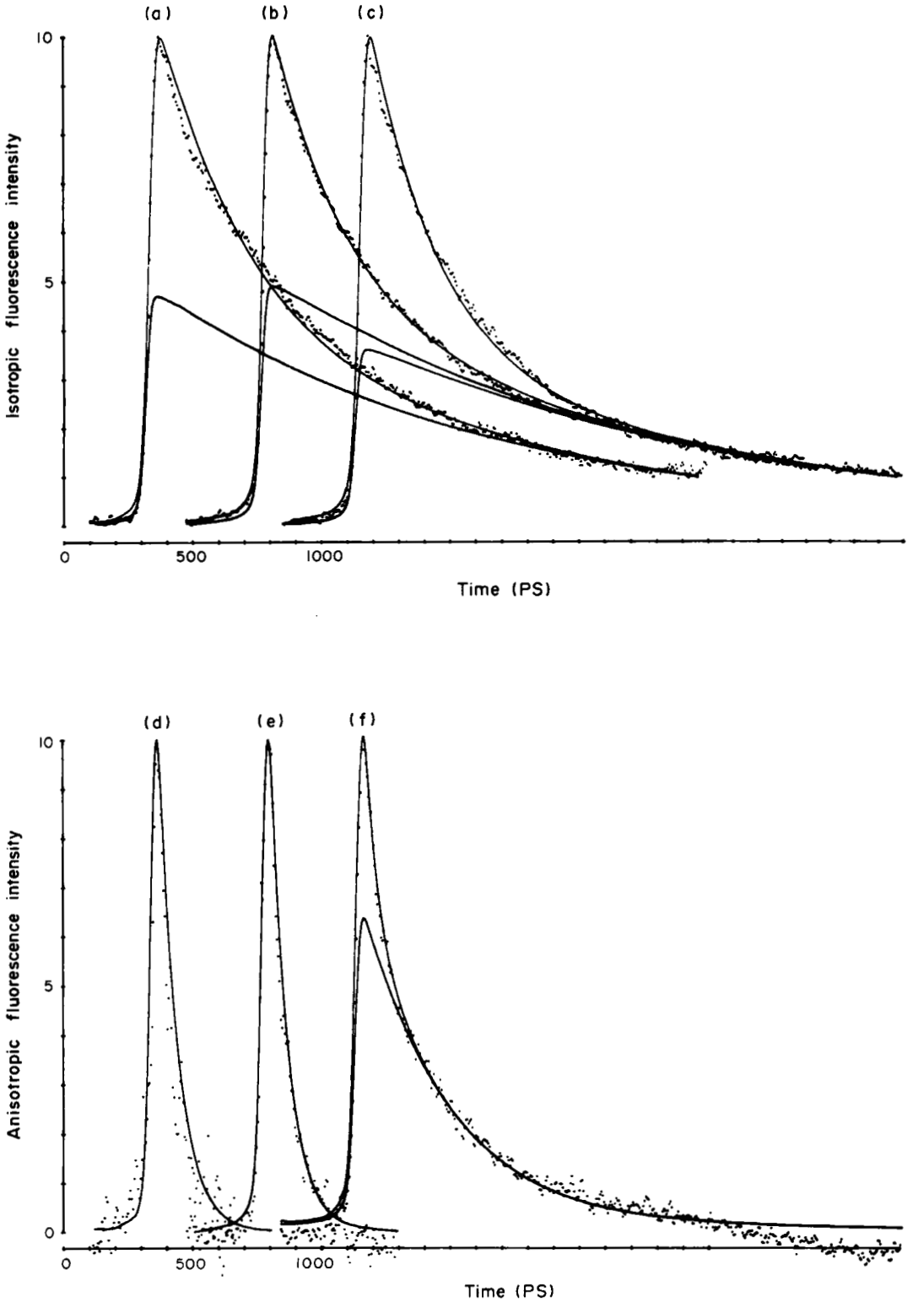

Figure 4. Isotropic ($I_{\parallel} + 2I_{\perp}$) (curves a–c) and difference function ($I_{\parallel} - I_{\perp}$) (curves d–f) fluorescence kinetics of C-PC trimer from *Spirulina platensis* at different temperatures (T). Display as in Fig. 3. The fits (solid lines) were obtained with the following parameters: Isotropic fluorescence: (a) T = 18.6°C; τ_1 = 1300 ps, A_1 = 47%, τ_2 = 320 ps, A_2 = 53%; (b) T = 39.2°C; τ_1 = 1500 ps, A_1 = 49%, τ_2 = 270 ps, A_2 = 51%; (c) T = 51.8°C; τ_1 = 1600 ps, A_1 = 36%, τ_2 = 260 ps, A_2 = 64%. Difference function of fluorescence: (d) T = 18.6°C; τ_2 = 70 ps, A_2 = 100% (no long-lived component); (e) T = 39.2°C; τ_2 = 70 ps, A_2 = 100% (no long-lived component); (f) T = 51.8°C; τ_1 = 350 ps, A_1 = 54%, τ_2 = 70 ps, A_2 = 46%.

To fit the function $D(t)$ one must also assume a biexponential decay law at 18.6°C and 39.2°C. The lifetime of the slow decaying contribution of $D(t)$ is estimated to be 1000 ps or about ½ of the long decay time of $I(t)$. At 51.8°C, however, the decay time of $D(t)$ is increased to approximately 3500 ps and, therefore, of the same size as the electronic lifetime [$I(t)$]. The short-lived component of $D(t)$ shows a similar behaviour. Its decay time is half of that of the fast component of the isotropic fluorescence decay curve $I(t)$ at the lowest temperature (18.6°C) and approaches the isotropic fluorescence lifetime at 51.8°C (see legend Fig. 3).

The time dependence of the fluorescence decay of C-PC trimers $(\alpha\beta)_3$ (Fig. 4) under identical conditions differs from that of the corresponding monomers in two essential points:

(i) The decay of the function $I(t)$ describing the relaxation of the excited state population is only slightly dependent on temperature. The contribution of the short-lived component is higher than in the case of the monomer.

(ii) At all three temperatures, the function $D(t)$ decays very rapidly to zero. The corresponding lifetimes of the fluorescence anisotropy are in the order of 70 ps at 18.6 and 39.2°C. At 51.8°C, a biexponential decay with $\tau_2 = 70$ ps and $\tau_1 = 350$ ps results in the best fit.

The fluorescence decay curves of the α-subunit of C-PC are shown in Fig. 5. Again a biexponential decay must be assumed in order to get a good fit of the experimentally observed decay. Parameters used are: $\tau_2 = 250$ ps and $\tau_1 = 1300$ ps. Unfortunately, no polarized emission spectra could be recorded and the intensity recorded for Fig. 5, therefore, corresponds to $I_{\parallel} + I_{\perp}$.

DISCUSSION

Phycobiliproteins have their chromophores covalently attached to the apoproteins. The thioether bond is stable even at elevated temperature at neutral pH, and the changes of the chromophores upon thermal denaturation are thus fully due to the uncoupling of non-covalent chromophore–protein and chromophore–chromophore interactions. In C-PE, a comparative analysis by fluorescence, circular dichroism and absorption spectroscopy has shown a differential sensitivity of these methods (Langer *et al.*, 1980). In a titration with the denaturant urea, the fluorescence has been shown to be most sensitive to small perturbations of the protein. The same is true for the controlled thermal denaturation of C-PC (Table 2) if the rather uniform decrease of fluorescence described above is compared with earlier absorption (Scheer and Kufer, 1977), and circular dichroism (Lehner and Scheer, 1983) measurements on this process.

Static fluorescence studies provide a simple method to compare the thermal (Table 2) and

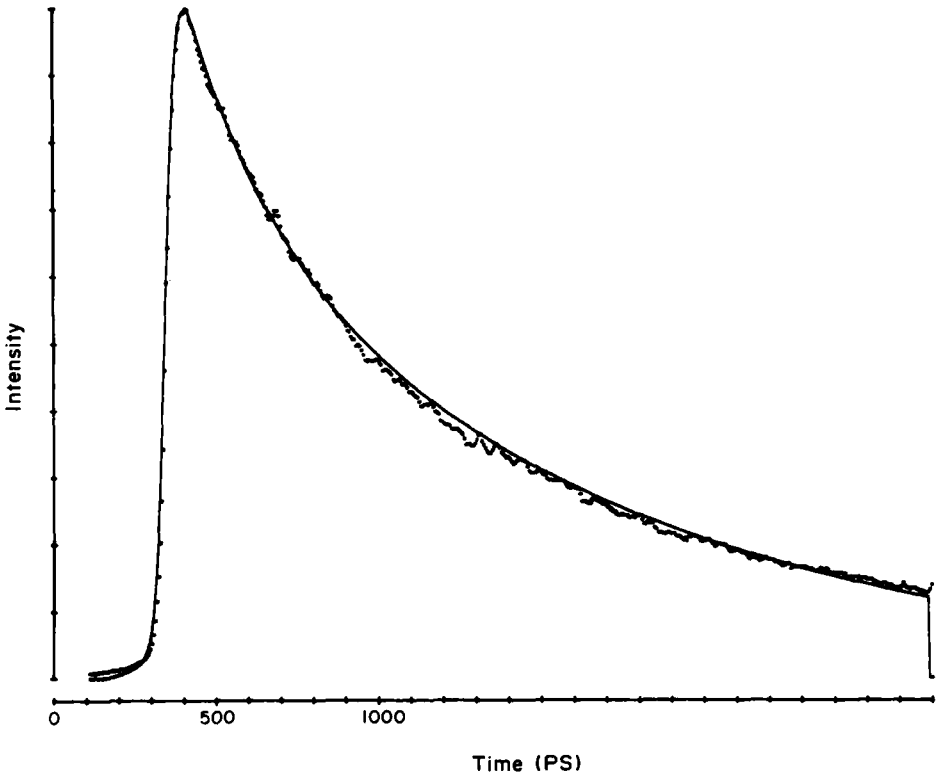

Figure 5. Fluorescence profile (dotted) of the α-subunit of C-PC from *Spirulina platensis*. Display as in Fig. 3. Data for the biexponential fit (solid line): $\tau_1 = 1300$ ps, $A_1 = 73\%$, $\tau_2 = 250$ ps, $A_2 = 27\%$.

photochemical (Table 3) stablity of different biliproteins and aggregation states*.

The data indicate, that the thermal stability is similar for monomeric ($\alpha\beta$) and trimeric $(\alpha\beta)_3$ PC, as well as for monomeric ($\alpha\beta$) APC, but is considerably better for trimeric $(\alpha\beta)_3$ APC. The APC trimer does not disaggregate up to about 60°, if judged from its characteristic absorption (MacColl *et al.*, 1981). Chen *et al.* (1977) have provided evidence for hydrophobic interactions in biliprotein aggregation, which would explain this thermal stability. Both PC and APC are stabilized towards photobleaching by the aggregation to trimers (Table 3), which is in agreement with results of Abeliovich and Shilo (1972) on C-PC from different sources.

A crucial point in thermal denaturation studies is the lability of biliprotein chromophores uncoupled from the protein, especially in the presence of light. The resulting (photo-)oxidation products very often have a higher fluorescence yield than the free chromophores. The reversibility of the thermally induced fluorescence intensity decrease (Table 2) and of the variation in the shape of the fluorescence emission and absorption spectra provides a test for such processes. If judged from these criteria, short ($\leq$10 s) exposures to temperatures up to 70°C are fully reversible. For exposure times sufficiently long to collect experimental data, the temperatures should be kept below 50 and 55°C for C-PC and APC, respectively. To minimize interference of this kind, the time-resolved fluorescence studies have been restricted to this temperature range.

The first result derived from the decay curves in Figs 3 and 4 is that the underlaying decay law is at least biexponential in nature. This can be explained as fluorescence from two different molecules or conformations, or from the same molecules in a different environment. It is well established that C-PC possesses three phycocyanobilin chromophores, two in the β-chain and one in the α-subunit. The difference in excitation energy of the two chromophores in the β-subunit of C-PC from *Spirulina platensis* is in the order of 15 nm ($\hat{=}$ 400 cm^{-1}). The excitation energy of the chromophore in the α-subunit coincides with that of the one in the β-subunit which absorbs at the red part of the absorption spectrum (see Scheer, 1981, for references). The latter were called fluorescing chromophores, because they should predominantly emit the commonly observed fluorescence (Teale and Dale, 1970).

The second chromophore in the β-subunit is called a sensitizing chromophore because its electronic relaxation is dominated by energy transfer to the fluorescing chromophores (see e.g. Grabowski and Gantt, 1978).

On the basis that the perturbation of the electronic eigenstates of the chromophores is small, the oscillator strength, and therefore, the radiative transition rate, k_r, should be approximately equal for the three chromophores present in similar conformations. Furthermore, if the rate of intramolecular radiationless transition, k_{nr}, is about the same, then a change in the excited state lifetime must be attributed to a radiationless intermolecular transition, in particular to energy transfer [the rate for photoinduced reactions is assumed to be negligible in comparison to the fast non-reactive deactivation channels (Friedrich *et al.*, 1981)].

In view of the above-described situation, we could interpret the results of the time-resolved fluorescence measurements as follows: The long decay time derived from the function $I(t) = I_{\parallel}(t) + 2\,I_{\perp}(t)$ corresponds to the "averaged" lifetime of the excited chromophores. If the lifetimes of the two fluorescing chromophores were the same, the decay function at sufficiently long times should be a simple exponential; due to small differences, the experimental curve should correspond to a non-resolvable superposition.

The short-lived fluorescence component in the monomeric ($\alpha\beta$) and trimeric $(\alpha\beta)_3$ pigments is interpreted as leakage fluorescence from the sensitizing chromophore in the β-subunit. The intensity ratio of the emission from sensitizing and fluorescing chromophores which would be recorded in a *cw*-experiment, can be estimated from the decay curves as: $R = I_2 \times \tau_2/I_1 \times \tau_1$. For C-PC monomer (α, β) at temperatures of 18.6 and 39.2°C, the ratio is: $R = 0.5 \times 300/0.5 \times 2200 \approx 13\%$. At higher temperature, this ratio is nearly the same (12.5%). Since we do not know what the extinction coefficients of the three chromophores are at the excitation wavelength, nor how the intramolecular rates change with temperature, we cannot derive the yields for energy transfer. More information, we believe, is provided by the decay of the fluorescence difference function $D(t)$. For the decay time of this function, Perrin's formula for the degree of fluorescence polarization in the presence of orientational relaxation leads to the expression

$$\frac{1}{\tau'} = \frac{1}{\tau_{ef}} + \frac{1}{\tau_{dep}}$$

where τ_{ef} is the electronic lifetime derived from the function $I(t)$ and τ_{dep} is the depolarization time. In the original formulation, τ_{dep} is the rotational relaxation time τ_{or}. Since it seems unlikely that a

*We are aware of the different aggregation numbers measured by us (n=3) and other authors (n=6) (see MacColl and Berns, 1981, for leading references). Most of the more detailed studies have, however, been done with PC from other organisms (see MacColl, *et al.* 1971, for some data on *S. platensis*). The aggregation state may also be crucially dependent on the details of the isolation procedure. Lundell *et al.* (1981) and others have recently shed some light on the function of the colorless "linker" peptides in promoting biliprotein aggregation. It is then conceivable that different (small) amounts of these peptides in the preparations are responsible for the observed aggregation differences.

chromophore bound to the protein has an orientational lifetime of less than 100 ps, we believe that fluorescence depolarization is caused in part by (resonance) energy transfer. Since reliable biexponential fitting is rather difficult to perform (*vide supra*) we cannot draw unequivocal conclusions.

At 18.6°C the dominating part of the monomer decay curve corresponds to a decay time of about 150 ps. If this value is combined with the short-lived component of the isotropic emission (Fig. 3a) τ_{dep} is calculated as 300 ps. In combination with the long lifetime (2200 ps), τ_{dep} results as 160 ps. At 39.2°C, the combination of the two corresponding (short) lifetimes yields a τ_{dep} of 1810 ps. An analogous consideration of the result at 51.8°C (Fig. 3c) suggests that no depolarization mechanism is active at this temperature. One possible explanation for this behaviour is that at a higher temperature the protein chain starts to unfold, with the distance between the chromophores being consequently increased and the relative orientation of the chromophores changed. Both effects would result in a decrease of the energy transfer rate. The excited chromophore is also the emitting chromophore and depolarization by rotational relaxation should still be negligible due to the binding of the chromophores to the apoprotein.

In the case of the aggregated biliproteins (trimers = $(\alpha\beta)_3$), the basic mechanisms are the same, but the temperature effects are smaller due to the higher thermal stability (*vide supra*). The shortened electronic lifetime of the fluorescing chromophore in the trimers is probably a consequence of modified interactions between chromophore and apoprotein. The importance of chromophore–protein interaction for the intramolecular radiationless processes is amply verified. It has been found that the reported lifetimes strongly depend on aggregation (Searle *et al.*, 1978), the sample preparation (Kobayashi *et al.*, 1979) and the age of the sample (Hefferle, 1982).

At low temperature the lifetime of the short-lived component of the trimer is about the same as that of the monomer. This observation seems reasonable, when the energy transfer process is assumed to take place within a monomer unit.

In contrast to the results described above for the monomer, the anisotropy of the trimer fluorescence is extremely short-lived at all temperatures studied. This probably means that the resonance transfer among the fluorescing chromophores (between 6 and 9 per trimer depending on the pigment) is very effective. Due to the quasirandom orientational distribution of the chromophores, the orientational anisotropy of excited molecules is destroyed very rapidly. Energy transfer from *s*- to *f*-chromophores has, of course, the same effect, when *s*- and *f*-chromophores are not aligned parallel by the apoprotein.

Since the protein conformation is thermally more stable in the trimer, essentially no changes are observed upon raising the temperature to 39.2°C. At 51.8°C, unfolding of the protein starts, the transfer rates change, although less dramatically than in the monomer.

Since there is only one chromophore in the α-subunit, the biexponential nature of its fluorescence decay cannot be understood except on the following grounds:

(i) There is an equilibrium between monomers and higher aggregates ($n \times \alpha \Leftrightarrow (\alpha)_n$). Previous results have shown that aggregation occurs between isolated subunits, in the case of the β-subunit (Glazer *et al.*, 1973, Cohen-Bazire *et al.*, 1977).

(ii) A single chromophore exists in a microheterogeneous environment which is either intrinsic or the result of an incomplete refolding of the apoprotein after removal of the denaturing agent. This in turn could cause a variety of chromophore configurations, e.g. some chromophores are present in the extended form as in the native biliprotein, and others in the semi-extended or helical form as in the case of denatured biliproteins or in free bile pigments.

In the limit of complete uncoupling between chromophore and apoprotein, one would expect a fast component with a lifetime in the range of several hundred picoseconds. It can be estimated from the following: (i) The natural lifetime of the uncoupled chromophore is increased by a factor of 5–7, which is derived from a similar decrease in absorption (Scheer and Kufer, 1977). (ii) The fluorescence yield is decreased by about three orders of magnitude. The two factors combined would be compatible with the above mentioned 250 ps, compared to 1300 ps of the long-lived component. The lifetime should be further shortened by the non-planarity of the helical form together with the possibility of intramolecular hydrogen bonding; two factors which in general enhance the rate for radiationless transitions. Preliminary measurements on PC-peptides yielded fluorescence decay times of approximately 70 ps.

CONCLUDING REMARKS

The results presented here show that time-resolved emission spectroscopy including the information on the fluorescence polarization provides a valuable tool to study the details of energy transfer in the light harvesting pigments of blue-green algae. In order to distinguish between relaxation by energy transfer and non-linear effects like singlet-singlet annihilation as discussed by Wong *et al.* (1981), the intensity dependence of the lifetimes must be kept in mind. The latter processes are unlikely in the small aggregates and with the low excitation photon flux (10^{13} photons/pulse × cm^2) used in this work.

There remain nonetheless several problems which are currently worked on. The first concerns the status of the sample, which can not yet be described

unequivocally. Processes like aging or the influence of colorless proteins on the aggregation (Lundell *et al.*, 1981) are hitherto only partly understood.

Secondly, we cannot exclude at the moment the possibility that the decay functions are governed by a more complex decay law (e.g. triexponential or nonexponential). The latter can be expected (Blumen and Manz, 1979) whenever energy transfer is of importance. An analysis of the fluorescence decay curve of intact phycobilisomes by Holzwarth *et al.* (1982) showed that an approximation of the data with a biexponential decay law resulted in an equally good fit as one using an exp $(-A \times t^{\frac{1}{2}})$ term. To distinguish between these possibilities the decay function would have to be monitored with a range of deflection speeds of the streak camera in order to get a sufficiently high number of points for each part of the decay curve. At the same time, the concentration dependence of the decay curves must be tested (aggregation) as well as the influence of complete unfolding and refolding of the apoprotein.

Furthermore, it will be important to vary excitation and emission wavelengths. The lifetime data presented in this paper demonstrate that the decrease in fluorescence intensity upon (partial) thermal denaturation can not simply be explained by a decrease in fluorescence quantum efficiencies due to faster nonradiative deactivation channels. One possible explanation could be that one part of the chromophores is transformed via a reversible reaction into a metastable product with a very low fluorescence quantum yield and/or with an emission in a wavelength regime, which is outside of the presently accessible range. Work along these lines is in progress.

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APPENDIX

From the fluorescence decay curves monitored with the polarization filter oriented parallel [$I_{\parallel}(t)$] and perpendicular [$I_{\perp}(t)$] to the polarization of the exciting laser beam, the expressions

$$I(t) = I_{\parallel}(t) + 2I_{\perp}(t) = \int e(t') \times i(t-t')\mathrm{d}t' \tag{1}$$

$$D(t) = I_{\parallel}(t) - I_{\perp}(t) = \int e(t') \times \mathrm{d}(t-t')\mathrm{d}t' \tag{2}$$

$$R(t) = \frac{I_{\parallel}(t) - I_{\perp}(t)}{I_{\parallel}(t) + 2I_{\perp}(t)} \neq \int e(t') \times i(t-t') \times r(t-t')\mathrm{d}t' \tag{3}$$

are calculated. These functions give information on the decay of the excited state population as well as on the polarization of the fluorescence emission. The problem in the interpretation arises from the fact that the recorded responses $I_{\parallel}(t)$ and $I_{\perp}(t)$, respectively, are the convolution of the response to a δ-function excitation (expressed by lower case letters) with the excitation pulse profile $e(t')$. Although this convolution generates no problem for the evaluation of $I(t)$ and $D(t)$, respectively, the quotient of the convoluted functions $D(t)$ and $I(t)$ must not be equal to the convolution of the function $r(t) = \mathrm{d}(t)/i(t)$. We, therefore, concentrated in general on the generation of the functions $I(t)$ and $D(t)$, and show one example only for the function $R(t)$. The function $i(t)$ corresponds to the true decay law of the excited state population. The time-dependent fluorescence polarization anisotropy $r(t)$ is directly related to the correlation function for the second Legendre polynomial of the transition dipole reorientation angle (Fleming *et al.*, 1976).

$$r(t) = \frac{2}{5}\langle P_2[\hat{e}(0) \times \hat{e}(t)]\rangle \tag{4}$$

if at time zero the emission dipole is parallel to the absorption dipole. Its determination would yield information on the processes that give rise to the depolarization of the fluorescence, i.e. rotation of the excited molecule and (resonance) energy transfer to neighbouring molecules with different orientation. In the simplest case, where the molecule undergoes Brownian rotation as an Einstein sphere, the correlation function (4) reads

$$r(t) = \frac{2}{5}\exp(-6\,Dt) = \frac{2}{5}\exp(-t/\tau_{or}) \tag{5}$$

The orientational relaxation time τ_{or} is related *via* the rotational diffusion constant D to the effective volume of the solute molecule V, the solvent viscosity η and temperature T

$$\tau_{or} = \frac{1}{6D} = \frac{V\eta}{kT} \tag{6}$$

If in addition, the excited state population decays as single exponential

$$i(t) = i_0 \times \exp(-t/\tau_{el}) \tag{7}$$

then $\mathrm{d}(t)$ can also be expressed as an exponential of the form

$$\mathrm{d}(t) = \frac{2}{5} \times \exp\,(-t/\tau_{or} - t/\tau_{el}) = \frac{2}{5}\exp(-t/\tau') \tag{8}$$

with

$$\frac{1}{\tau'} = \frac{1}{\tau_{or}} + \frac{1}{\tau_{el}} \tag{9}$$

By a least square fit procedure of functions calculated according to Eqs. 1 and 2 to those determined experimentally, the parameters τ_{or} and τ_{el} can be evaluated. Since for the actual systems studied, the origin of the fluorescence depolarization can not unambiguously be described, we use the assumption outlined above (correlation function is approximated by an exponential) to deduct characteristic numbers for the speed of the fluorescence depolarization independent of the underlying mechanism. If one assumes that molecular reorientation of the chromophores bound to the protein is much slower, then the characteristic time derived under this assumptions must be seen in context with resonance energy transfer, although this number can not *a priori* be set *equal* to an energy transfer time. An attempt to calculate the correlation function (4) for the case of energy transfer within a nonisotropic distribution of chromophores (as in the phycobilisomes) has not been undertaken in the past. Current theoretical investigations by Blumen (unpublished) show that the correlation function (4) can be approximated reasonably good by an exponential under circumstances present in aggregated phycobiliproteins. The time constant of this exponential corresponds to the angle and distance averaged rate constant for energy transfer.