

PICOSECOND TIME-RESOLVED FLUORESCENCE OF PHYCOBILIPROTEINS: SUBUNITS OF PHYCOCYANIN FROM *Mastigocladus laminosus*

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(Received April 19, 1989; accepted July 13, 1989)

Keywords. Cyanobacteria, photosynthesis, phycobiliproteins, phycocyanin, subunit, aggregation, fluorescence polarization, fluorescence kinetics, energy transfer.

Summary

The α - and β -subunits of C-phycocyanin from *Mastigocladus laminosus* were prepared according to revised procedures. Both subunits are isolated as dimers, which can be dissociated into monomers with detergent mixtures. The fluorescence decay kinetics are similar for the respective monomers and dimers. In no case could they be fitted by only one (α -subunit) or two exponentials (β -subunit) which are predicted by theory for samples with a unique chromophore-protein arrangement containing one and two chromophores, respectively. It is suggested that there exists a heterogeneity among the chromophores of the subunits, which may persist in the highly aggregated complexes present in cyanobacterial antennas.

1. Introduction

Cyanobacteria and rhodophytes contain special light-harvesting complexes, the phycobilisomes, which are situated at the cytoplasmic surface of the thylakoid membranes [1 - 6]. They are complex structures containing phycobiliprotein aggregates (phycocyanin (PC), phycoerythrin, phycoerythrocyanin, allophycocyanin) and so-called linker peptides. All phyco-

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biliproteins and some of the linkers contain covalently bound bile-pigment chromophores. The phycobilisomes comprise highly ordered arrays of some 400 - 2000 chromophores which collect light and transfer the absorbed energy efficiently to photosystem II.

The phycobilisomes are a unique system in which energy transfer in a photosynthetic antenna can be studied. They can be reversibly dissociated into well-defined fragments [1 - 8]. The crystal structures of several phycobiliproteins have been determined with atomic resolution [9, 10], and the primary structures are known for a variety of phycobiliproteins and linker polypeptides [11, 12]. Much interest has consequently been focused on the spectroscopy and excited-state kinetics of such fragments with the aim to understand the energy transfer mechanism of the entire system [see 1 - 6 and 13 - 17 for leading references].

Rather unexpectedly, even the most simple of such complexes, *e.g.* PC α -subunits, showed multicomponent decay patterns [18, 19]. The α -subunit is the smallest PC fragment carrying only a single phycocyanobilin chromophore. In time-resolved fluorescence measurements a monoexponential decay is therefore expected for a structurally homogeneous sample. One possible origin for the observed multiexponential decay is a natural microheterogeneity of the biliprotein chromophore sites [20 - 22]. Another possibility is that this decay pattern is artefactual (*i.e.* due to the formation of homo-aggregates) and hence of no relevance to the situation in phycobilisomes. Since subunit isolation in C-phycocyanin (C-PC) involves a complete unfolding/separation/refolding sequence [23 - 25], another possible source for artefacts is that the (under such conditions) labile bile-pigment chromophores [26] or the polypeptides are damaged in the process of separation. In order to understand the origin for the complex decay kinetics, we have reinvestigated the aggregation and fluorescence decay kinetics of both the α - and β -subunits of PC prepared under protective conditions, from *Mastigocladus laminosus*.

2. Materials and methods

Mastigocladus laminosus was cultivated in Castenholz medium [27] in 300 l batches at the Gesellschaft für Biotechnologische Forschung, Stöckheim (F.R.G.), and stored deep frozen.

The preparation of PC followed the method of Füglistaller *et al.* [24]. The subunits of PC were obtained by an improved isoelectric focusing procedure in 7 M urea. The modifications of the previously used procedure [25] were as follows. The dextran gel was presaturated with argon, and the electrofocusing done at 2 °C under argon and in darkness. After elution from the gel, the subunits were renatured by dialysis against potassium phosphate buffer (100 mM; pH 7.0) first at room temperature for 15 h and subsequently at 4 °C for 6 h. A further purification of the β -subunit was achieved by two subsequent ammonium sulfate precipitations (40% each).

For dissociation of the homodimers obtained under these conditions (*vide infra*), solution with an absorbance in the red band of about 2.5 (α -subunit) and 1.5 cm^{-1} (β -subunit) were treated with a mixture of the detergents, R-AMDML and T-DF12 (Rewo and Steinau, F.R.G.) (0.2 and 0.5% each, respectively, for the α - and β -subunits). The native state of the subunits was controlled by circular dichroism and the relative amplitudes of the red and near-UV absorptions, which are very sensitive to chromophore geometry [28 - 30].

The aggregation state of the subunits was studied by sucrose density gradient centrifugation [31] in a model Ultraspinn 55 ultracentrifuge (LKB, Munich, F.R.G.) at $238\,000 \times g$. Trimeric PC from *Mastigocladus laminosus* (106.1 kDa), horse-heart myoglobin (17.5 kDa) and cytochrome c (12.4 kDa) were used as reference. The colored bands were withdrawn and quantitated by absorption spectroscopy.

Absorption spectra were recorded on a Lambda 2 (Perkin-Elmer, Überlingen, F.R.G.) or a model 8451 A spectrophotometer (Hewlett-Packard, Corvallis, OR, U.S.A.). Fluorescence polarization spectra were determined in a model 221 fluorimeter (Spex, München, F.R.G.).

For picosecond time-resolved fluorescence measurements, the samples were excited at low intensity (about 10^{13} photons per pulse) by the pulse train of a mode-locked picosecond dye-laser (pulsewidth less than or equal to 3 ps) at a repetition rate of 82 MHz. The fluorescence decay traces were recorded with a synchroscan streak camera (Hamamatsu Photonics, Hamamatsu, Japan) with polarizers parallel (I_{\parallel}) or orthogonal (I_{\perp}) to the polarization of the exciting radiation. Alternatively, measurements were made with the analyzer under 54° (magic angle) to eliminate the effects of chromophore reorientation or energy transfer on the isotropic decay. The monitored fluorescence was spectrally selected by interference filters with a bandwidth of 6 - 8 nm. The experimental decay curves were analyzed by means of a nonlinear least-squares fit routine on the basis of a multi-exponential decay law [32, 33].

3. Results and discussion

3.1. Subunit preparation

The subunit preparation followed essentially the procedure of Köst-Reyes *et al.* [25], but involved several additional safeguards. The original procedure generally yielded subunits which on SDS-PAGE showed one or more bands moving ahead of the main colored band. This may arise in part from protein degradation. Since the phycocyanobilin chromophores are very labile when uncoupled from the protein [26], oxidative degradation is a likely cause for this heterogeneity. Degradation was particularly critical for the β -subunit, which gave colored and uncolored extra bands moving faster than the β -subunit on SDS-polyacrylamide gels. According to their relative mobilities, and the chromophore:protein ratios (which were determined by staining intensity *vs.* absorption before staining), these additional bands

carried one or no chromophore. They are therefore believed to originate from sequential oxidative degradation of the two chromophores in the β -subunit. Effective protective measures were: (1) to run the electrofocusing gel under argon in a suitably modified apparatus (addition of ascorbate to the gel was less reliable); (2) to maintain low temperature (2 °C), which required a concomitant decrease of the urea concentration to 7 M and a carefully controlled renaturation.

3.2. Aggregation state of subunits

When investigated by the sedimentation velocity method [31], both PC subunits proved dimeric. Attempts to dissociate these dimers by chaotropic salts, KSCN or LiClO₄, were unsuccessful. The two PC subunits have very similar three-dimensional structures [9], which may lead to homo- rather than hetero-aggregation in the absence of the correct partner. In the integral PC, chaotropic salts are also ineffective in dissociating heterodimers ("monomers") into the subunits, but rather dissociate higher aggregates into such heterodimers.

Further attempts to obtain monomeric subunits concentrated on the use of detergents. The ones tested fell into two classes. They were either ineffective, or they were too 'strong' and led not only to monomerization, but to (partial or complete) unfolding of the subunits at the same time, as judged from the relative intensities of the two absorption bands (details will be published separately). A more successful approach was to use mixtures of detergents of either class. A mixture of the nonionic T-DF12 and the zwitterionic detergent R-AMDML (0.2 and 0.5% each for the α - and β -subunits, respectively) dissociated the subunits into monomers. However, even under these optimum conditions the absorption spectra indicated already a partial unfolding (decrease of $Q_{\text{red/UV}}$, the relative absorptivities in the red and near-UV maxima), which increased upon prolonged standing.

3.3. Fluorescence polarization

Additional evidence for aggregation of subunits to homodimers comes from fluorescence anisotropy measurements in the presence and absence of detergents. The fluorescence and polarization spectra (Fig. 1) are similar to those reported for the subunits of this pigment [16]. The polarization spectra of the α -subunit are rather independent of the excitation wavelength, a fact which indicates that emission takes place from the excited chromophore only. The polarization spectra of the β -subunit show a pronounced step. This is expected for the β -subunit with two or more non-equivalent chromophores coupled by energy transfer. Polarization reaches a maximum value at long-wavelength excitation, which is higher than for the α -subunit. In view of the chromophore heterogeneity and aggregation, we have not attempted to evaluate an angle between excitation and emission transition dipole moments from these data.

The polarization spectra do not change significantly when the subunits were treated with increasing amounts of a 1:1 mixture of the two aforemen-

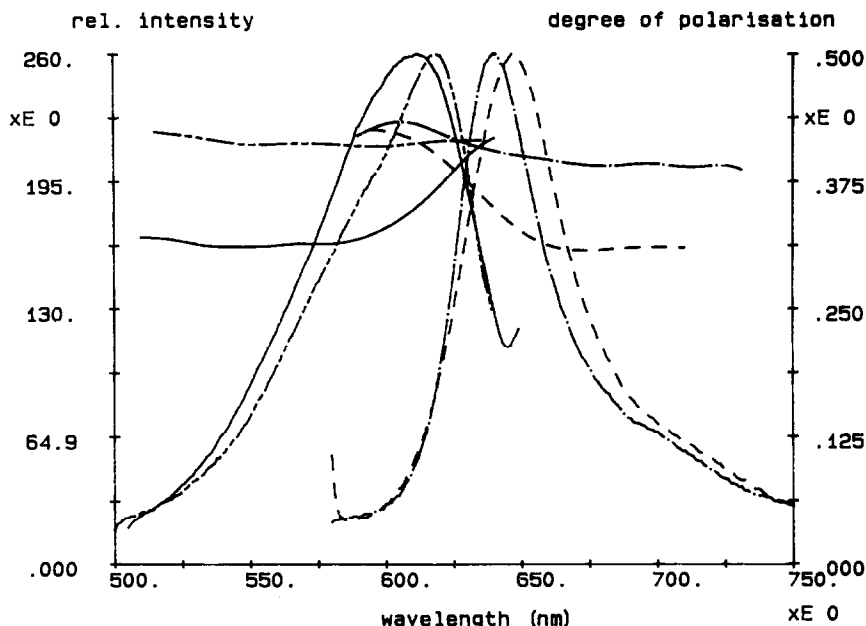


Fig. 1. Fluorescence excitation, emission and polarization spectra of C-PC subunits (homodimer) from *M. lamosus* α -subunit: excitation and excitation polarization (---) ($\lambda_{\text{det}} = 650$ nm); emission and emission polarization (-·-) ($\lambda_{\text{exc}} = 580$ nm). β -subunit: excitation and excitation polarization (—) ($\lambda_{\text{det}} = 650$ nm); emission and emission polarization (- - -) ($\lambda_{\text{exc}} = 560$ nm). The excitation and emission spectra are normalized to equal maximum intensity.

tioned detergents. However, there is an initial increase of the degree of polarization, followed by a plateau at higher detergent concentrations (Fig. 2). The emission polarization of the α -subunit reaches 0.36 and that of the β -subunit, in case of long-wavelength excitation, almost the theoretical maximum of 0.5. The changes at low detergent concentrations can be due to a decreased rotational relaxation, conformational changes, or a decreased energy transfer. Addition of detergents are likely to increase rotational relaxation. Pronounced conformational changes should also be seen in the absorption spectra. We therefore take the decreased energy transfer as the most likely cause, which is in line with a disaggregation of subunit homoaggregates.

Since addition of increasing amounts of detergents leads at the same time to progressive denaturation of the subunits (decrease of the relative band intensities, $Q_{\text{red/UV}}$ not shown), there is only a narrow gap at which native monomeric subunits can be obtained. Even under these conditions, the samples tend to degrade slowly and are much more unstable than the homo-dimeric subunits, a fact which imposed restrictions on the measurements with such samples.

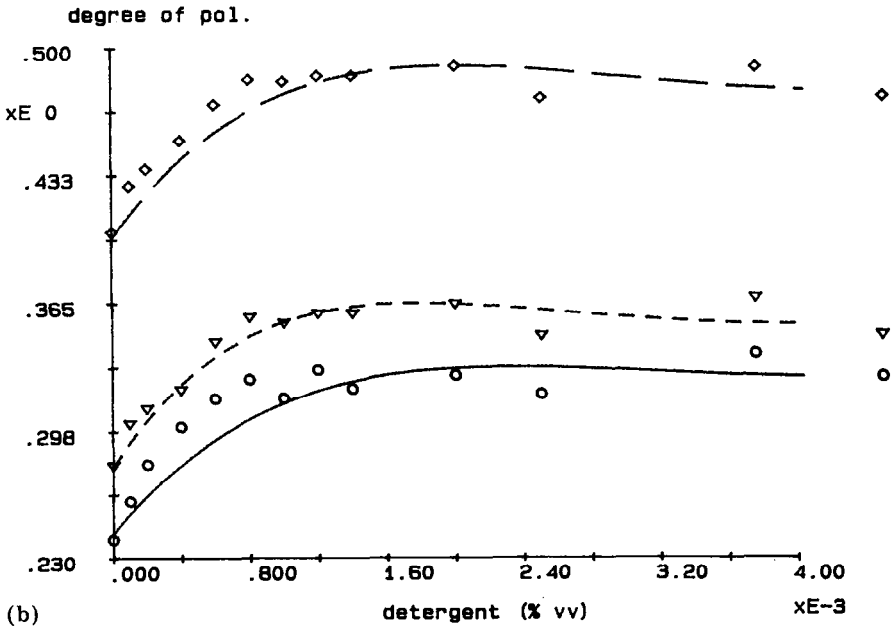
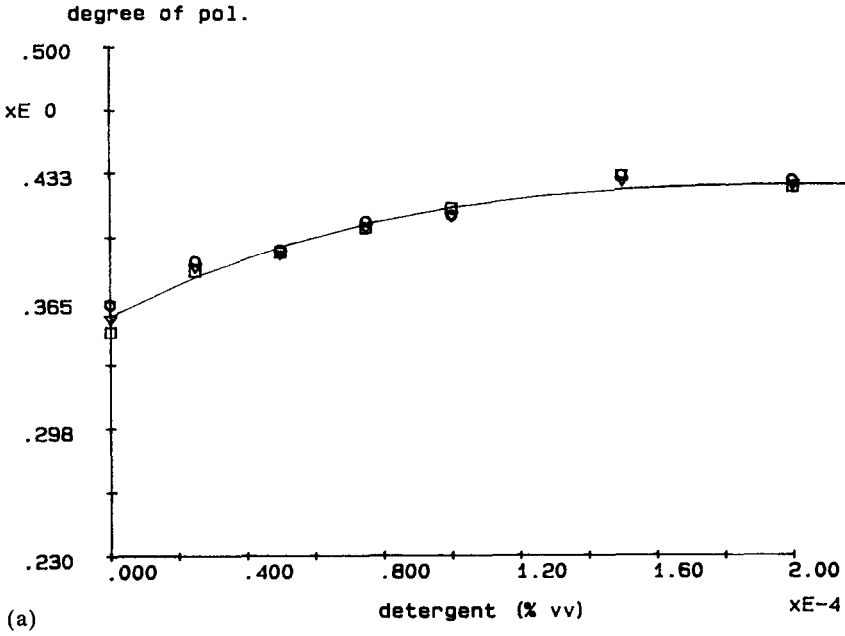


Fig. 2. Effect of detergent concentration on fluorescence polarization of PC subunits (1:1 mixture of a R-AMDML and T-DF12). (a) α -subunit: $\lambda_{det} = 645$ nm; $\lambda_{exc} = 540$ nm (\square), 580 nm (∇) and 616 nm (\circ). (b) β -subunit: $\lambda_{det} = 650$ nm, $\lambda_{exc} = 598$ nm (\circ), 614 nm (∇) and 640 nm (\diamond).

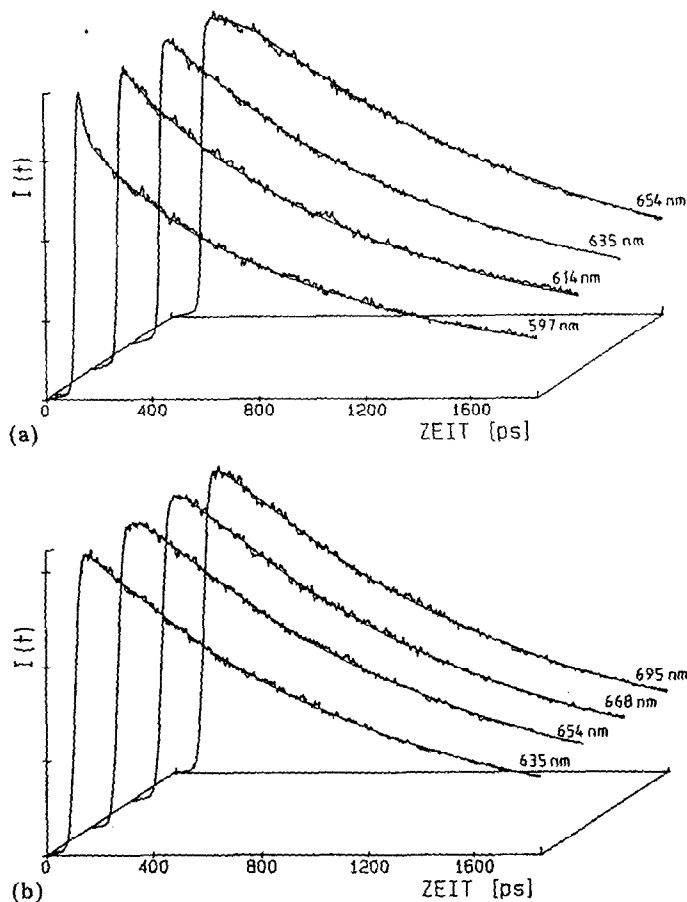


Fig. 3. Fluorescence decay of α -subunit (homodimer) in dependence on the detection wavelength. (a) $\lambda_{exc} = 580$ nm; (b) $\lambda_{exc} = 620$ nm.

3.4. Fluorescence decay kinetics: α -subunit

Fluorescence decay measurements were performed for different excitation and detection wavelengths (Fig. 3). The fit parameters, *i.e.* lifetimes and amplitudes, are summarized in Table 1. It should be pointed out here that the absolute amplitudes of different measurements are not directly comparable, because of experimental conditions the decay curves are not uniformly normalized.

In all cases, the observed fluorescence decay of the α -subunit is not monoexponential. The decay traces are clearly dominated by a long-lived component with a lifetime of 1.1 - 1.2 ns, but additional components are found. Especially at 580 nm excitation and 597 nm detection, a very fast component with only 16 ps lifetime is evident. By additional cut-off filters in detection it was ascertained that there was no scattered excitation light, but only emission from the sample. At long-wavelength excitation (620 nm), rising fluorescence components (negative amplitudes) with comparable life-

TABLE 1

Lifetime (τ) in picoseconds with amplitude (A) in parentheses, for the α - and β -subunits, at various wavelengths of excitation (λ_{exc}) and detection (λ_{det})

λ_{det} (nm)	λ_{exc} (nm)		
	580	600	620
<i>α-Subunit</i>			
597	16 (210)		
	180 (85)		
	1130 (320)		
614	160 (130)		
	1160 (660)		
635	200 (220)		— —
	1210 (1720)		1120 (2790)
654	95 (−100)		25 (−510)
	1230 (1510)		1150 (3050)
668			15 (−1180)
			1130 (3110)
695			10 (−1060)
			1140 (2940)
<i>β-Subunit</i>			
597	26 (2780)		
	120 (1040)		
	790 (450)		
614	26 (2770)	28 (2360)	
	130 (1190)	180 (760)	
	800 (1390)	820 (1060)	
635	25 (1170)	32 (1150)	
	160 (1560)	220 (1350)	140 (890)
	800 (1390)	860 (1190)	780 (990)
654	200 (910)	230 (1810)	210 (1620)
	820 (1060)	910 (1420)	860 (1840)
668			240 (1630)
			880 (1700)
695	190 (1880)	220 (1310)	210 (1530)
	790 (1890)	890 (1130)	820 (1520)

times are found. These short-lived components could be due to either energy transfer or a fast photoinduced process like an isomerisation of a part of the excited chromophores. The latter process is likely because isolated subunits show, in contrast to integral PC, a low but distinct photoreversible absorption change [34]. Photochemistry may also be one cause for the comparably low degree of fluorescence polarization in the α -subunit. A third component with a lifetime of typically around 200 ps and low relative amplitude is detected for short wavelength excitation (580 nm), but not observed for 620 nm excitation.

It is not possible to decide at present whether these additional decay components are inherent also to "native" α -subunit contained in integral PC, or whether they are due to heterogeneities present only in the isolated sub-

unit. In view of the denaturation/renaturation sequence, and the time (approximately 4 h) required for separation during which the subunits are unfolded, small deviations of the protein–chromophore arrangement from the “native” state can not be excluded. Theoretical estimates of the effects of such variations on the absorption and circular-dichroism spectra are given by Scharnagl *et al.* [35].

Despite these heterogeneities, the fluorescence decay of the α -subunit is definitely dominated by the long-lived component, which contributes more than 95% of the total fluorescence. This is much improved over our previous preparations [18], and indicates that at least part of the heterogeneity observed earlier is a result of preparation artefacts. Based on these results, a lifetime of 1.1 - 1.2 ns can be assigned to those α -84 chromophores which are embedded in the protein in a manner which compares with the native PC system (lifetime of fluorescing chromophores is about 1.2 ns) [14 - 17].

3.5. Fluorescence decay kinetics: β -subunit

Figure 4 shows the time-resolved fluorescence of dimers of the β -subunit for 580 nm and 620 nm excitation and different detection wavelengths. The fit parameters of all measurements are summarized in Table 1. In Fig. 5, a plot of the analyzed lifetimes and amplitudes in dependence on the detection wavelength is given. Measurements were performed also with monomers prepared by detergent treatment (*vide supra*). The derived lifetimes and amplitudes were so close to those obtained for the dimers that only the results obtained with the much more stable dimers are discussed.

The β -subunit contains two chromophores between which energy transfer occurs *via* dipole–dipole interactions. Therefore, two exponentials should be necessary and sufficient to describe the time course of the fluorescence. One lifetime is related to the energy transfer and the second one to the lifetimes of the chromophores, if they are long compared with the energy transfer time (*vide infra*). The experimental decay curves, however, are characterized in most cases by a three-exponential decay. Only in some cases is a description with two exponentials sufficient (see Table 1).

For excitation with 580 nm and 600 nm light and for detection wavelengths up to 635 nm, a component with a lifetime of 25 - 30 ps is clearly apparent. For detection above 635 nm, this short-lived decay is not observed, nor a corresponding rising component. The lifetime of the “intermediate” component varies between 120 and 240 ps with a tendency to increase with longer detection wavelengths. In all measurements this intermediate component occurs with significant amplitude. The third observed lifetime (800 - 900 ps) is significantly shorter than the corresponding lifetime in the α -subunit.

Since the chromophores of the β -subunit are connected by energy transfer, two decay rates ($k_{a,b}$) are expected which are related to those of the individual chromophores (k_1° for β -155, k_2° for β -84) and the

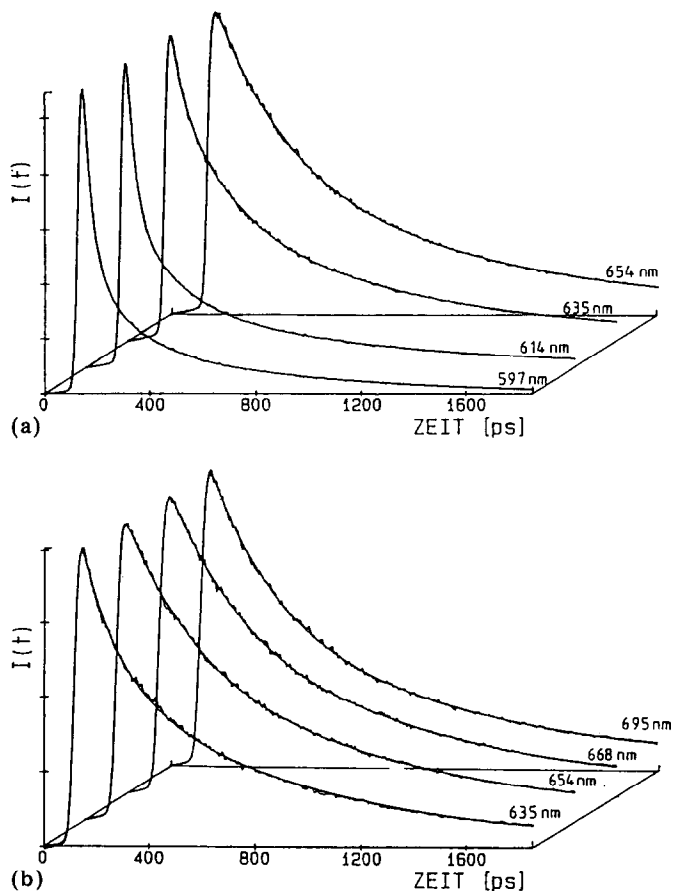


Fig. 4. Fluorescence decay of β -subunit (homodimer) in dependence on the detection wavelength. (a) $\lambda_{exc} = 580$ nm; (b) $\lambda_{exc} = 620$ nm.

rates of energy transfer (k_{12} and k_{21} for transfer from and to β -155, respectively) via eqn. (1):

$$k_{a,b} = \frac{1}{2} (k_s \pm \sqrt{k_s^2 - 4(k_{12}k_2^\circ + k_{21}k_1^\circ + k_1^\circ k_2^\circ)}) \quad (1)$$

$$\text{where } k_s = k_1^\circ + k_2^\circ + k_{12} + k_{21} \quad (2)$$

When the theoretical values assigned to k_{12} and k_{21} are correct, then the inequity $k_{12} + k_{21} \gg k_1^\circ + k_2^\circ$ holds. In that case, eqn. (1) reduces to

$$k_a = k_{12} + k_{21} \quad (3a)$$

$$k_b = \frac{k_{12}k_2^\circ + k_{21}k_1^\circ}{k_{12} + k_{21}} \quad (3b)$$

Since the 25 ps component is mainly observed at short wavelength excitation, *i.e.* predominant excitation of the β -155 chromophore [36, 37], this

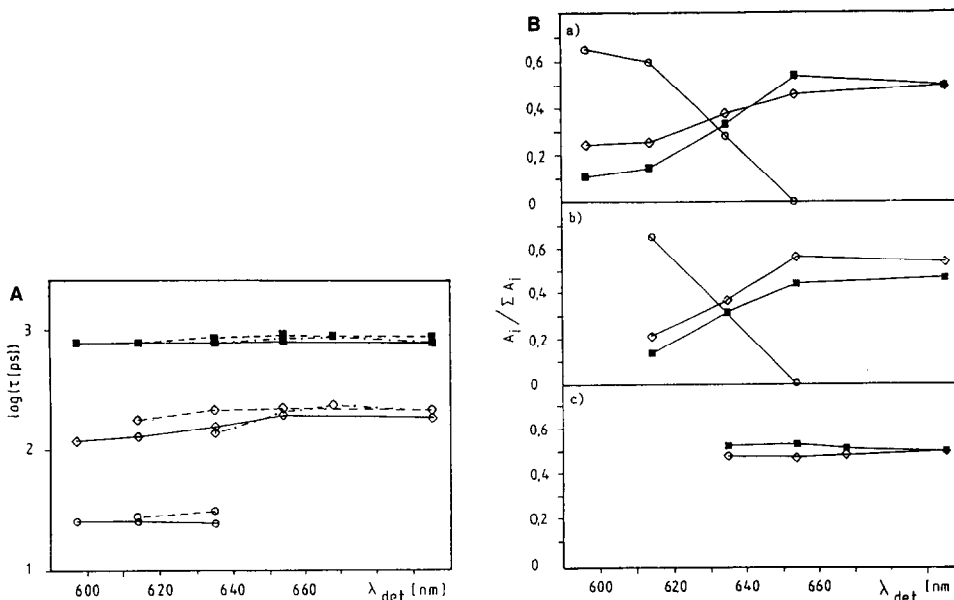


Fig. 5. Fluorescence decay fit parameters of the β -subunits. Dependence of components: fast (\circ), medium (∇), slow (\blacksquare) on excitation and detection wavelengths. A) lifetimes with $\lambda_{\text{exc}} = 580$ nm (—), $\lambda_{\text{exc}} = 600$ nm (---), $\lambda_{\text{exc}} = 620$ nm (- · -); B) relative amplitudes with $\lambda_{\text{exc}} = 580$ (a), 600 (b), 620 nm (c).

time constant can be assigned to the decay of this chromophore. According to eqn. (3a), the decay time is governed by the energy transfer rate to and from the β -84 chromophore k_{12} and k_{21} respectively. From theoretical calculations [13] it was concluded that both these transfer times are much shorter than the lifetimes of the individual chromophores $(k_1^0)^{-1}$ and $(k_2^0)^{-1}$ respectively. It is then surprising that there is no fluorescence rise observed at long detection wavelengths, which would prove the indirect excitation of β -84 chromophores via energy transfer. A compensation occurs between the contributions of the fast decay of “donor” fluorescence and the correspondingly rising “acceptor” fluorescence; this may be due to spectra overlap of the two chromophores, but other mechanisms cannot be ruled out.

Additional evidence for the interpretation of the fast decay time as energy transfer time is found in the difference curve $D(t) = I_{\parallel}(t) - I_{\perp}(t)$ (not shown). A fit produces a major component with a lifetime of about 30 ps. Since orientational relaxation of the protein to which this chromophore is attached is much slower, the fluorescence depolarization must be connected to energy transfer, or fast local motions. The latter is improbable in the native binding situation.

The lifetime of the third component (800 - 900 ps) is considerably shorter than the longest lifetime in the α -subunit. This is in accordance with the smaller steady-state fluorescence quantum yield of the β -subunit [38].

According to eqn. (3b) the longer one of the two lifetimes corresponds to a weighted average of the lifetimes of the two chromophores. Unless they

have equal decay times (usually assumed in discussions on this topic), the lifetime of the chromophore with the shorter energy transfer time dominates in the evaluation of eqn. (3). Spectral studies have shown that the energy gap between the two chromophores is small enough that the back transfer (β -84 to β -155) is not negligible [13, 16, 39 - 41]. In this case, the observed decay time will be intermediate between the individual lifetimes of the two chromophores.

Two extreme cases result if one assigns a lifetime of 1.1 - 1.2 ns (*e.g.* that of the α -chromophore) to either one of the two β -chromophores, and takes $k_a = 0.83 - 0.91$ ns and $k_b = 37$ ps [13]. If β -155 is the longer-lived chromophore, then β -84 would have a lifetime of 790 - 810 ps; in the case of β -84 being the longer-lived one, then β -155 would have a lifetime of 400 - 450 ps. X-ray analysis has shown that the chromophore arrangements of β -84 and α -84 are rather similar, and that both are close to the protein surface in monomers and subunits [9]. β -155 is much more twisted, and may be the one to become uncoupled first from the protein. It is then tempting to assume that its lifetime is significantly reduced compared with β -84 because conformational mobility is a cause for the rapid internal conversion in bile pigments [5, 42]. However, β -84 seems to be involved in reversible photochemistry in partly denatured PC, and in conformational changes upon binding of linker polypeptides, which would favor this as the chromophore with a reduced lifetime. Clearly, more work is necessary to decide between the two possibilities.

More speculative is the assignment of the intermediate "200 ps" component. The relative amplitudes of this component are in general too high to be due to small fractions of deteriorated sample. Furthermore, the relative amplitude of this component shows a dependence on the detection wavelength, as does the amplitude of the long-lived component. This means that the emission spectrum of the species from which the 200 ps component originates corresponds fairly well to the β -84 chromophore. A possible explanation is that β -84 can adopt (at least) two different states (*e.g.* conformations, geometries or the like); one of them has a lifetime of about 800 ps, the second one a lifetime of about 200 ps. Since the relative amplitudes of both components increase with longer wavelengths of detection, the "second type" chromophore with the shorter lifetime must also act as an acceptor for energy being transferred from the β -155 chromophore.

The proposed explanation also seems to be reasonable on grounds of the information provided by X-ray analysis. The β -84 chromophore is not completely embedded by the surrounding protein. Therefore, the conformation of this chromophore is probably not as well fixed by the protein as it is for the α -84 and the β -155 chromophores. The conformation of this chromophore may become more rigid in the intact antenna system because there it is fixed not only by the interaction with the α -subunit, but also with the neighboring β -subunit and with linker polypeptides. The weaker stabilization of the β -84 chromophore results in different, nearly isoenergetic conformations in the isolated subunits with characteristically different lifetimes.

Components with lifetimes of about 200 ps were also found in earlier measurements on chromopeptides. In these, the chromophores are not held by the protein in extended geometries as judged from their absorption and circular dichroism spectra [35]. In light of this explanation, the lifetime of 800 - 900 ps must be assigned to the β -84 chromophore in a conformation which is close to the "native" one.

The complex decay kinetics are (at least in part) a manifestation of heterogeneities in the chromophore-protein arrangements of biliproteins. Their presence in the simplest form, *e.g.* the α -subunit in its monomeric state, should be kept in mind when interpreting the fluorescence decay of higher aggregates with models based on perfect symmetry and/or identical arrangements of the individual chromophores (*e.g.* α -84, β -84, β -155 in C-phycoerythrin).

Acknowledgments

This work was supported by the Deutsche Forschungsgemeinschaft, 53 Bonn, F.R.G. (SFB 143, projects A1 and B1) and also by the Fonds der Chemischen Industrie. Mass culture of cells by Dr. G. Reng (Gesellschaft für Biotechnologische Forschung, 3301 Stöckheim, F.R.G.) is acknowledged. We also wish to thank Hamamatsu Photonics Europe for the loan of the synchroscan streak camera.

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