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ENERGY TRANSFER IN TRIMERIC C-PHYCOCYANIN STUDIED BY PICOSECOND FLUORESCENCE KINETICS

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Abstract—The excited state kinetics of trimeric C-phycocyanin from *Mastigocladus laminosus* has been measured as a function of the emission and excitation wavelength by the single-photon timing technique with picosecond resolution and simultaneous data analysis. A fast decay component of 22 ps (C-phycocyanin with linker peptides) and 36 ps (C-phycocyanin lacking linker peptides) is attributed to efficient energy transfer from sensitizing to fluorescing chromophores. At long detection wavelengths the fast decay components are found to turn into a rise term. This finding further corroborates the concept of intramolecular energy transfer. Previous reports on the conformational heterogeneity of the chromophores and/or proteins in C-phycocyanin are confirmed. Our data also provide indications for the importance of the uncoloured linker peptides for this heterogeneity.

INTRODUCTION

C-Phycocyanin is the major light harvesting pigment of many cyanobacteria (blue-green algae). *In situ*, it is highly aggregated and organized together with other phycobiliproteins and colorless linker peptides in the supramolecular complexes called phycobilisomes. They constitute the antenna to (mainly) photosystem II (Gantt, 1981; Scheer, 1982; Glazer, 1983; Wehrmeyer, 1983a; Wehrmeyer, 1983b). *In vitro*, in particular in the absence of linker peptides, the aggregation is limited to trimers (Hefferle *et al.*, 1984a) or hexamers (see MacColl and Berns, 1981, for leading references) which are believed to be the building blocks for phycobilisomes (Wehrmeyer, 1983a; Glazer, 1983; Gantt, 1980) and phycocyanin crystals (Schirmer *et al.*, 1985a; Schirmer *et al.*, 1985b).

The phycobilisomes collect efficiently light in the green and orange spectral range and transfer it with quantum efficiencies approaching 100% to the reaction centers (Porter *et al.*, 1978). Several groups have recently begun to study the kinetics of this process by picosecond time-resolved spectroscopy of entire phycobilisomes and their constituent phycobiliproteins (Hefferle *et al.*, 1983a; Hefferle *et al.*, 1983b; Porter *et al.*, 1978; Searle *et al.*, 1978; Kobayashi *et al.*, 1979; Pellegrino *et al.*, 1981; Holzwarth *et al.*, 1982; Holzwarth *et al.*, 1983b; Suter *et al.*, 1984; Hefferle *et al.*, 1984a; Hefferle *et al.*, 1984b; Gillbro *et al.*, 1983; Mimuro *et al.*, 1984; Yamazaki *et al.*, 1984; Switalski and Sauer, 1984; Wong *et al.*, 1981; Holzwarth, 1985; Wehrmeyer *et al.*, 1985; Gillbro *et al.*, 1985; Mimuro *et al.*, 1985; Glazer *et al.*, 1985; Hanzlik *et al.*, 1985; for recent reviews see Karukstis and Sauer, 1983; Scheer, 1985). These studies support a general scheme, where the excitation energy flows from the highest energy components (generally the phycoerythrins) *via* the intermediate phycocyanins to the lowest energy components, e.g. the allophycocyanins and *in vivo* to the chlorophylls. From these studies there remained nonetheless considerable differences regarding the laws governing the deactivation kinetics (multi-exponential or non-exponential), the number of components, and the physical interpretation of the derived time constants. These differences are, at least in part, due to the application of experimental techniques differing in their time resolutions and their capabilities for multicomponent resolution. Further differences derive from pigment systems differing in their parent species, aggregation states, and preparation procedures. In order to correlate the data obtained in different laboratories, and to compare critically the potential of the experimental methods, parallel investigations using different techniques are therefore useful. Here, we wish to report the fluorescence kinetics of trimeric C-phycocyanin from *Mastigocladus laminosus* measured by the single-photon timing technique. We also apply for the first time the simultaneous data analysis technique to such a complex pigment system with energy transfer. This technique drastically improves the capability for multicomponent resolution of complex decay data. The results will be compared with polarized fluorescence data obtained previously on the same pigment with a repetitive streak camera (Hefferle *et al.*, 1984a). C-Phycocyanin from this alga has been characterized earlier using biochemical methods by Binder *et al.* (1972) and Byfield and Zuber (1972).

MATERIALS AND METHODS

C-Phycocyanin was isolated from *Mastigocladus laminosus* as reported earlier (Hefferle *et al.*, 1984a). One of the preparations was ≥ 80% pure with respect to the C-phycocyanin biliprotein and contained colorless peptides as

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analyzed by SDS-PAGE (*cf.* Fig. 1, lane b). Two of these polypeptides (22.2 and 25.4 kDa) are associated with phycocyanin in the phycobilisomes and are also isolated with phycocyanin upon dissociation of the phycobilisomes (unpublished results, Scheer, 1985). The relative staining intensities of $\alpha + \beta$, the 22.2 and 25.4 kDa subunits are 25 : 1 : 2. All other peptides (> 26 kDa) are contaminants due to the isolation procedure and are not found in phycobilisomes. The other preparation was freed from polypeptides other than the two C-phycocyanin subunits as shown in Fig. 1 (lane c). All samples were prepared in potassium phosphate buffer (80 m*M*, pH 6.0) containing sodium azide (3.0 m*M*). They were kept on ice until the measurement which was carried out within 48 h. For the measurements the sample was diluted to a chromophore concentration of $< 5 \times 10^{-6}$ *M* corresponding to an absorbance $A_{620}^{1\,cm} < 0.5$. For fluorescence measurements a pathlength of 1.5 mm was used in order to avoid selfabsorption effects. The aggregation state of these solutions was determined to be trimeric (sedimentation constant $S_{20,w} = 5.6$) for both preparations by analytical ultracentrifugation (see Hefferle *et al.*, 1984a).

Picosecond fluorescence decays have been measured by the single-photon counting technique as described (Holzwarth *et al.*, 1982; Holzwarth *et al.*, 1983a; Holzwarth *et al.*, 1983b). The apparatus function was ≤ 120 ps wide (full-width at half maximum). This system provides a time-resolution of about 10 ps by deconvolution. The detection wavelength range was selected by a monochromator (Jobin Yvon DH 10) with a bandwidth of 4 nm. All measurements have been carried out at ambient temperature (22–25°C) under magic angle polarization, i.e. the fluorescence kinetics free from anisotropy effects has been detected.

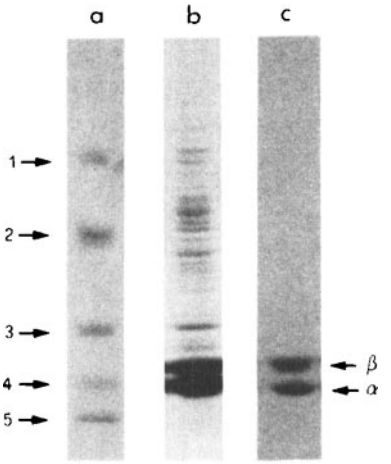

Figure 1. SDS polyacrylamide gel electrophoresis of (a) standard proteins (1 bovine serum albumin mol wt = 66 kDa, 2 egg albumin mol wt = 45 kDa, 3 trypsinogen mol wt = 24 kDa, 4 β-lactoglobulin mol wt = 18.4 kDa and 5 Lysozyme mol wt = 14.3 kDa), (b) phycocyanin preparation containing additional polypeptides (gel overloaded to show the latter more clearly) and (c) phycocyanin preparation without such polypeptides. The phycocyanin subunits are marked α and β.

DATA ANALYSIS

The data analysis has been carried out using two different methods. The first one involves the more conventional deconvolution of single fluorescence decays in terms of a sum-of-exponentials model (Holzwarth *et al.*, 1982; Holzwarth *et al.*, 1983b). The second method is a simultaneous analysis procedure based on a global optimization algorithm similar to the one used by Knutson *et al.* (1983) (Holzwarth *et al.*, to be published). Instead of analyzing individual decay curves recorded at one wavelength, all decay curves recorded at different wavelengths are analyzed simultaneously in a single run. The algorithm is based on the assumption that the decay constant (lifetime) of a particular decay component should be independent of wavelength while the preexponential factors vary. The fluorescence decay is described by a sum of exponential functions

$$F(t,\lambda) = \sum_{i=1}^{n} A_i(\lambda) \exp(-t/\tau_i)$$

If the lifetimes are independent of emission wavelength the dimensionality of the fitting problem can be reduced from $2 \times N \times n$, to $N \times n + n$ by applying the global analysis. Here, N denotes the number of independent measurements at different wavelengths and n the number of decay components. We have recently derived analytical expressions for the improvement in the errors of the extracted parameters as compared to an independent analysis of the individual decay curves (to be published). The reduction in the dimensionality of the free parameters leads to a dramatic improvement both in the accuracy of the extracted parameters as well as in the capability for multi-component resolution. The quality of the fits was judged by a global χ^2-value, individual χ^2-values, and plots of the weighted residuals. The iteration procedure applied in our program is a semi-linear Marquardt algorithm (Marquardt, 1963). The feasibility and reliability of the four-exponential analysis has been tested on a number of simulated data sets with three and four decay components, which mimicked the experimental data. Poissonian noise had been added to these simulated decay data. These tests confirmed the expected drastic improvement of the global data analysis as compared to the conventional single-decay analysis. In all these simulations the theoretical parameters (amplitudes and lifetimes) were recovered very closely which makes us confident that the present analysis is both reliable and accurate.

RESULTS AND DISCUSSION

All measurements were carried out on the two different C-phycocyanin preparations. Both were trimeric as determined by ultracentrifugation, but differed by the presence and absence, respectively, of additional polypeptides other than the α- and β-subunits of C-phycocyanin. Of these additional polypeptides, two are isolated as integral components of the phycobilisomes, from which they dissociate together with C-phycocyanin upon incubation with buffers of decreased ionic strength (John and Scheer, unpublished results). These two

polypeptides (22.2 and 25.4 kDa, see experimental part) are therefore considered as linker peptides, which have been characterized in detail for other species (see Glazer *et al.*, 1983, for leading references). In *M. laminosus* phycobilisomes, the detailed function and the stoichiometry of the different linker polypeptides is still unclear. If judged from the relative staining intensities of the bands (see experimental part) and the trimeric nature of the C-phycocyanin, a heterogeneous population of trimers in the sample which contained different linkers is likely.

Fluorescence decays of both trimeric C-phycocyanin preparations have been measured at various excitation/emission wavelength pairs. At wavelengths $\lambda_{exc} \leqslant 605$ nm the s-chromophores should be preferentially excited, whereas at ≈ 620 nm the f-chromophores† contribute the largest part to the absorption (Teale and Dale, 1970; Glazer *et al.*, 1973; Switalski and Sauer, 1984). The residual plots resulting from a simultaneous analysis of the fluorescence decays from the preparation that included the linker peptides are shown in Fig. 2. Three- and four-exponential model functions have been used for the analysis presented in Figs. 2a and 2b, respectively. These data clearly indicate the requirement for a four-exponential model to describe the fluorescence decays. By the same criteria the preparation that did not contain linker peptides required also four components but the amplitude ratios and lifetimes were different. All lifetimes and amplitudes for the decay components of the two phycocyanin preparations are presented in Table 1.

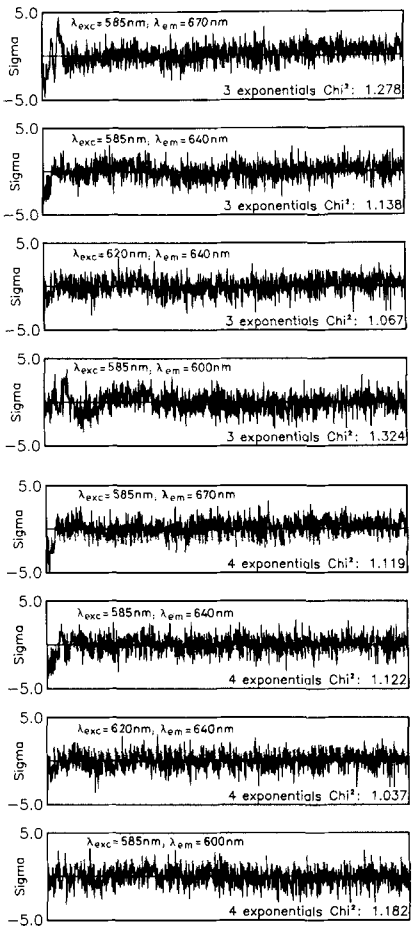

Figure 2. Residual plots resulting from a simultaneous analysis of the fluorescence decays of trimeric C-phycocyanin from *Mastigocladus laminosus* including linker peptides. Three-exponential (a) and four-exponential (b) model functions have been applied (for the kinetic data see Table 1). Note the systematic deviations in the residuals of the three-exponential analysis. The decays have been analyzed over a time range of ≈ 10 ns (*cf.* Fig. 3).

Short-lived fluorescence components

In both preparations we observe a very short-lived fluorescence decay, $\tau_1 = 22$ and 36 ps, respectively, at short excitation and emission wavelengths. Its ampli-

Table 1. Lifetimes (τ) and amplitudes (A) of the fluorescence decay components from trimeric C-phycocyanin as analyzed by the simultaneous deconvolution procedure

λ_{exc} (nm)	λ_{em} (nm)	A_1*	A_2*	A_3*	A_4*	τ_1†	τ_2†	τ_3†	τ_4†
585‡	600	1	0.087	0.15	0.21				
585‡	640	−0.017	0.043	0.18	0.24	36	203	807	1420
620‡	640	−0.15	0.075	0.24	0.33				
585§	600	1	0.12	0.13	0.14				
585§	640	−0.43	0.063	0.16	0.20	22	120	800	1490
620§	640	−0.086	0.026	0.095	0.12				

*All amplitudes have been normalized to the largest decay component of each sample; a negative amplitude denotes a rise-term. Absolute amplitudes can not be deduced from these data because different neutral density filters have been used for the measurements at different wavelength pairs in order to compensate for the largely differing fluorescence intensities.

†All lifetimes in ps; the maximum errors for amplitudes and lifetimes are ± 10%.

‡C-phycocyanin without linker peptides.

§C-phycocyanin including linker peptides.

†*Abbreviations*: f-chromophore(s), fluorescing chromophore(s); s-chromophore(s), sensitizing chromophore(s).

tude is very prominent under these conditions (see Table 1). The short-lived decay turns into a fluorescence rise-term (negative amplitude) in both preparations when the fluorescence is detected at or above 640 nm. This finding provides direct evidence in favor of ultrafast energy transfer between chromophores whose absorption and emission spectra are shifted against each other, i.e., s → f transfer. A similarly fast fluorescence decay also turning into a rise-time at long wavelengths, has been observed recently with B-phycoerythrin aggregates (Wehrmeyer *et al.*, 1985) and phycocyanin 612 (Hanzlik *et al.*, 1985). We should like to note that the basic features of the fluorescence decay of C-phycocyanin, i.e. a fast decay at short wavelength and a rise-term at long wavelength, appear already in a conventional single decay analysis. However, the statistical errors in both lifetimes and the amplitudes are much reduced by the simultaneous analysis and the distinction between three and four components is much more pronounced. An example of a single decay analysis is provided in Fig. 3. This example shows that a conventional analysis of a decay recorded at one wavelength is not able to resolve the details of the fast energy transfer components in this case.

Previous picosecond studies on isolated phycobiliproteins revealed only fast decay components without rise-terms (Kobayashi *et al.*, 1979; Holzwarth *et al.*, 1983b; Pellegrino *et al.*, 1981) which were interpreted in terms of s → f energy transfer (Kobayashi *et al.*, 1979; Holzwarth *et al.*, 1983b) or exciton annihilation (Pellegrino *et al.*, 1981). The latter complication can be definitely excluded for the data presented here since we worked at photon densities well below 10^{11} photons/cm^2/pulse. It shoud be pointed out that the observation of a corresponding risetime component for the fast decays constitutes a prerequisite for an unequivocal assignment of the fast (τ_1) fluorescence component to intramolecular energy transfer. This result is in excellent agreement with the observation of similarly fast decay components of fluorescence, transient absorption, and transient absorption anisotropy in the C-phycocyanin rods of phycobilisomes from *Synechococcus 6301* (Suter *et al.*, 1984; Gillbro *et al.*, 1983; Gillbro *et al.*, 1985) and in isolated C-phycocyanin aggregates (Holzwarth, 1985) from the same cyanobacterium.

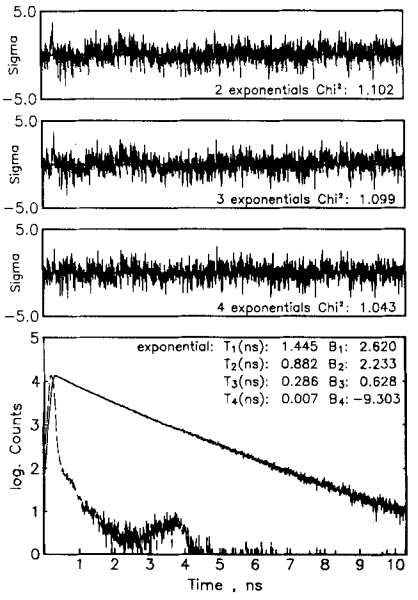

Figure 3. Conventional single decay analysis of the fluorescence kinetics of C-phycocyanin including linker peptides; λ_{exc} = 585 nm, λ_{em} = 640 nm. Lower panel: Excitation function (dashed) and fluorescence decay (full line) on a semilogarithmic scale. The inset gives the lifetimes (T_i) and the absolute amplitudes (B_i). Note the negative amplitude B_4 which indicates a riseterm in the fluorescence kinetics. Upper panels: Weighted residuals plots for model functions of two, three, and four exponentials, respectively. The different kinetic models are hardly distinguishable in this single decay analysis in contrast to the simultaneous analysis (Fig. 2).

The very short isotropic decay component found here by single-photon timing should be compared to the fast fluorescence anisotropy decay measured recently on the same pigment with a synchronous streak camera (Hefferle *et al.*, 1984a) (fast component of the difference function ≈ 62 ps, depolarization time estimated to ≈ 70 ps) which has been attributed mainly to s → f energy transfer. Taking into account the lower signal/noise ratio and lower dynamic range of the streak camera, which allowed only analysis in terms of a double-exponential model, it can be concluded, that the fluorescence anisotropy decay (Hefferle *et al.*, 1984a) is in agreement with the results reported here. The fast decay and rise components had not been observed in the isotropic decay, however. This might be due to the fact that broadband filters had been used to select the fluorescence in contrast to the narrow band detection (4 nm bandwidth) used in this work. A similar conclusion applies to the picosecond absorption measurements of Kobayashi *et al.* (1979). Given the limited capability for multicomponent resolution of that method, their results ($\tau \approx 56$ ps for trimeric aggregation) agree favorably well with ours. It should be noted, however, that their C-phycocyanin had been isolated from a different organism. Due to the overlap of the spectra of 's'- and 'f'-chromophores the relative amplitude of the fast components at λ_{em} = 640 nm depends strongly on the excitation wavelength. This indicates that this wavelength (λ_{em} = 640 nm) is close to the wavelength where the zero-crossing occurs, i.e., the amplitudes of the fast decay term and the fast riseterm compensate. We estimate that this zero-crossing occurs in the wavelength range between 630 and 640 nm. A similar observation has been made for phycocyanin aggregates from *Synechococcus 6301* (Holzwarth, 1985). This behaviour provides a further explanation for the failure of some previous investigators to detect the isotropic fast component. If broadband detection is used, the amplitude of the fast component will always be reduced to a varying

extent due to compensation effects. In unfavourable cases this component could thus be missed completely. Similarly the fast component will not be detectable if narrow-band detection near the zero-crossing wavelength is used as the only detection wavelength. We should like to point out that in all the phycobiliproteins which we have investigated so far, we have observed fast isotropic decays in the order of a few tens of picoseconds and in most cases also fast riseterms (Holzwarth *et al.*, 1983b; Wehrmeyer *et al.*, 1985; Holzwarth, 1985). This finding is in line with the results from other groups (Hanzlik *et al.*, 1985; Kobayashi *et al.*, 1979) and also with the observation of fast anisotropy decays (Hefferle *et al.*, 1983a,b; Hefferle *et al.*, 1984a,b). Different results have been reported recently for isolated R-phycoerythrin and allophycocyanin (Glazer *et al.*, 1985). Both of these phycobiliproteins have not been studied by us so far, however.

While the fast decay components (τ_1 = 22 and 36 ps) of the two preparations (with and without linker peptides) can be clearly interpreted in terms of intramolecular energy transfer, the situation is more difficult for the τ_2 components (200 and 120 ps). Their contribution to the total amplitude is relatively small. They might nevertheless also originate from energy transfer processes. One possibility would be energy transfer from pigments with somewhat distorted apoproteins and/or prosthetic groups. Both of these short decay components show variations with the two preparations which may be significant with respect to the functions of the colorless linker peptides. The function of these colorless polypeptides is, however, presently less well understood for *M. laminosus* than for other species (Glazer *et al.*, 1983). Both τ_1 and τ_2 are reduced in the preparation containing these peptides. This effect may indicate an increased coupling between s and f-chromophores induced by the linker peptides.

The long-lived decay components

We observe in each of the two preparations two long-lived decay components with lifetimes in the range of $\tau_3 \approx 800$ ps and $\tau_4 \approx 1450$ ps. Both of these decays have very similar amplitudes (see Table 1). The long-lived decay is attributed to the emission from the f-chromophores and one would *a priori* expect a single lifetime. The two decays of about equal amplitude indicate a basic heterogeneity in these pigments. Heterogeneous chromophore populations have already been inferred for integral C-phycocyanin (Switalski and Sauer, 1984) and for isolated subunits (Switalski and Sauer, 1984; Hefferle *et al.*, 1984a) from the observation of two long-lived fluorescence components. The longest lifetime reported by Switalski and Sauer (1984) for integral C-phycocyanin from *Anabaena variabilis* was similar to the τ_4 component while the other was substantially shorter. These differences can be explained readily by the analysis in terms of two (Switalski and Sauer, 1984) and four exponentials (this work). Otherwise our results are in good agreement with theirs and confirm the heterogeneity in the f-chromophores. It is interesting to note that, using different isolation procedures and different organisms, very similar results are obtained with respect to this heterogeneity. Apparently it is not caused by a modification of the proteins introduced during the isolation procedures but might rather be related to the aggregation state, the specific interaction with linker peptides and/or variations in the chromophore geometries. In fact a fluorescence study of C-phycocyanin from *Synechococcus 6301* indicates a much less pronounced heterogeneity in the hexameric aggregation state (Holzwarth, 1985). The τ_2 components of 200 and 120 ps observed here could perhaps be explained in terms of this heterogeneity. Our data suggest that the linker peptides and aggregation state play a more pronounced role than do any species dependent differences (Seibert and Connolly, 1984). Since C-phycocyanin contains two 's'- and one 'f'-type chromophore, another possible explanation for this second fast component would be energy transfer from the second 's'-chromophore. This point deserves further investigations.

CONCLUSIONS

The fluorescence decays of trimeric C-phycocyanin show a complex pattern of rise- and decay terms which requires a four-exponential model function. For the analysis of this complex fluorescence kinetics the simultaneous data analysis procedure turned out to be of great importance. The very fast decay and rise components found in this study can be rationalized by intramolecular energy transfer. Given the more limited time-resolution and/or component-resolution of previous time-resolved studies of the excited state kinetics of C-phycocyanin, our data are in agreement with these results. Differences seem to be present in the fast energy transfer kinetics between the preparations with or without additional colorless peptides. These differences may be relevant with respect to the function of the linker peptides as modulators for the chromophore conformation and/or chromophore/- protein interaction in phycobiliproteins.

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