Optical Properties and Structure of Tetrapyrroles

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TIME-RESOLVED FLUORESCENCE DEPOLARISATION OF PHYCOCYANINS IN DIFFERENT STATES OF AGGREGATION

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Introduction

Biliproteins are the main photosynthetic light harvesting pigments in cyanobacteria, red algae and cryptophytes (1-4). In the former two organisms, they are present as highly organized structures, the phycobilisomes, which are not an integral part of the photosynthetic membrane, but rather attached to its surface (2,3,5,6). They contain up to several hundred biliproteins carrying up to 2000 chromophores in a single functional unit, but also a number of so called linker peptides which are probably responsible for the attachment and internal organization of the phycobilisomes (7-10). Excitation energy is captured efficiently by the phycobilisomes and transfered with a high quantum yield via several intermediate acceptors to the chlorophyllous reaction centers within the photosynthetic membrane. This energy transfer has been the subject of active research over the past decade, involving both the investigation of entire phycobilisomes and of fragments thereof (11-19). In view

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of the complex structure of the former, we have focused mainly on a single biliprotein, C-phycocyanin (PC), which is generally the major pigment of cyanobacterial phycobilisomes (17,19). PC has a pronounced tendency for aggregation (4), which is also strongly influenced by the different linker peptides (10). These linker peptides also seem to be important in fine-tuning of the absorption of biliproteins. In this paper we want to report on the photophysical properties of such a complex between PC and linker peptides, viz. PC 636, which has been isolated from green-light adapted cultures of the cyanobacterium Mastigocladus laminosus. The time resolved fluorescence is compared to that of common PC 618, which is free of additional linkers.

Sample Preparation

Cultures of Mastigocladus laminosus were grown as described earlier with fluorescent tubes producing only small amounts of red light. Monomers and trimers of PC were prepared as reported earlier (19). PC 636 was eluted from the DEAE columns between phycoerythrocyanin, a second pigment formed in green light, and the major PC. It was dialysed against phosphate buffer (80 mM, pH 6.0) and used without delay and without storage in the freezer.

The chromatography of a crude extract of M. laminosus on DEAE cellulose produces generally APC I, PC and APC II in order of increasing salt concentration. When the cells are grown under light of reduced intensity at $\lambda \approx 600$nm, two additional pigments are formed in small amounts. One is the well known phycoerythrocyanin (24) eluting first from the column, which is followed by a phycocyanin named PC 636 according to its extremly red shifted absorption spectrum. The main analytical difference of this pigment seems to be the pre-

sence of two colorless peptides in addition to the common PC subunits.
It has a slightly higher buoyant density on a sucrose gradient, and dissociates upon prolonged standing or freezing in buffer of low ionic strength. Details of the preparation and its properties are to be published separately.

Measurements and data analysis

The fluorescence decay curves were measured using a synchronously pumped mode-locked ring dye laser (rhodamine 6G, 80 MHz repetition rate, pulse width ≤ 1 ps) in conjunction with a repetitively working streak camera (for details see e.g. (16)). The apparent time resolution of this system is approximately 25 ps without deconvolution procedure; it allows measurements with low excitation intensities (10^{13} photons per pulse and cm^2). The fluorescence decay curves measured with the analyzer parallel, ($I_p(t)$), and orthogonal, ($I_s(t)$), to the polarization of the exciting beam are transfered to a minicomputer where, after proper correction for the systems response, the expressions $I(t) = I_p(t) + 2\ I_s(t)$ and $D(t) = I_p(t) - I_s(t)$ are calculated. I(t) measures the decay of the excited state population (electronic lifetime) and D(t) the product of the former with the correlation function of the absorption and emission dipoles (17, 20). In contrast to the anisotropy function R(t) the difference function D(t) is additive and can be evaluated if more than one emitting species is present. Lacking better information, we approximate the correlation function by an exponential. The best fits for both functions (I and D) are determined under the assumption of a biexponential response function (two emitting species) by means of a Marquardt algorithm. Depending on the S/N ratio of the recorded fluorescence decay curves and their relative magnitude, the fit parameters

derived may be subject to considerable error. We will, therefore, discuss their trends rather than their absolute magnitude.

Results and Discussion

It is found that in all cases the decay curves can be fitted sufficiently well as convolutions of biexponentials. The fit parameters, e.g. the decay times (T_1,T_2 in psec) and the relative amplitudes (A_1,A_2 in %) of the short- and long-lived component, resp., are given in the inserts in figure 1. The measurements were performed at three different temperatures, namely at 18°C (A), at 36°C (B), the temperature the algae are grown, and at 52°C (C) where irreversible thermal denaturation starts to become effective. Partial denaturation already takes place at lower temperatures. Static measurements show a drastic loss in fluorescence yield (up to four orders of magnitude), which is much larger than the decrease in absorption connected with a conformational change of the chromophore (21). The time-integrated fluorescence intensities expressed as $A_1*T_1+A_2*T_2$ also confirm the reduction at higher temperature. It is found as a general rule that the decrease is more pronounced in the alpha than in the beta subunit and larger for the monomer than for the trimer. The normalized fluorescence decay curves also show small but distinct variations with temperature. Therefore the results presented in figure 1 must be taken as evidence for an intermediate state being present during the process of thermal denaturation.

The alpha subunit of PC contains only one chromophore. If it is stabilized by noncovalent interaction with the protein to adopt only one conformation, a single exponential decay is expected with a lifetime of 1.5 to 2.5 ns (lifetime of

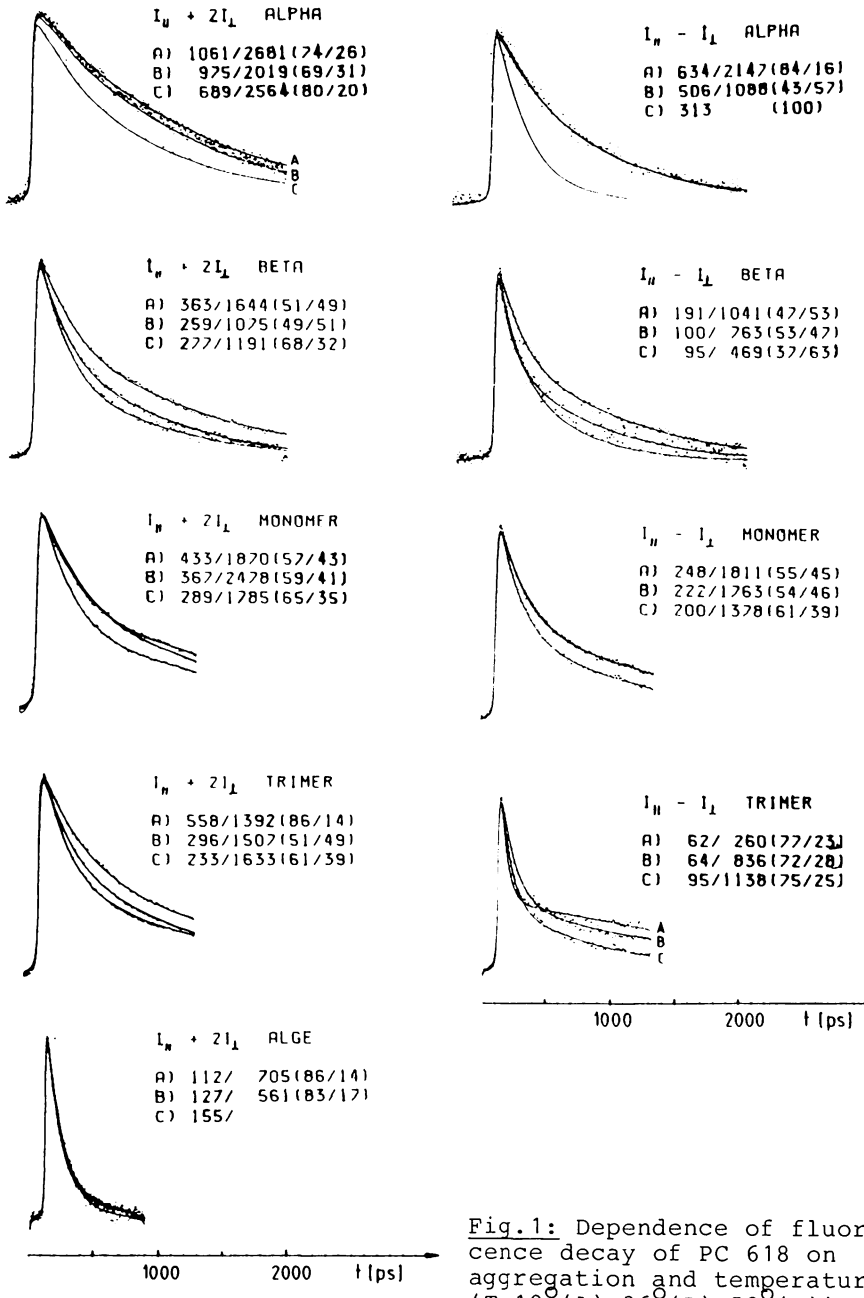

Fig.1: Dependence of fluorescence decay of PC 618 on aggregation and temperature ($T=18^{\circ}(A), 36^{\circ}(B), 52^{\circ}(C)$)

the chromophore in a native environment). Instead, an additional short-lived component is found, whose lifetime varies with temperature between 690 and 1060 psec. A similar behaviour was verified for the alpha subunit of Spirulina platensis (17) and Anabaena variabilis (22). Since aggregation of the subunits is unlikely, one must assume at least two different sets of emitting species (chromophore-protein-arrangements). The long-lived species must be close to that in native environment, whilst the short-lived form should be closer to the denatured, less interacting species. The faster decay in the difference function D(t), furthermore, signals that the faster component is subject to a depolarization mechanism with T 1500 psec. Since no acceptor molecules are present, the depolarisation should be due to orientational relaxation of the less rigidly bound chromophores.

The beta subunit contains two chromophores in different protein environment. The respective absorption maxima are separated by about 20 nm. The stationary fluorescence spectra of both subunits are essentially equal, which indicates an efficient energy transfer from the "sensitizing" to the "fluorescing" chromophore. The energy transfer is also manifested in the fluorescence decay curves. The short-lived component (T_1 300ps) is interpreted as "leakage" fluorescence from the s-chromophore, whose lifetime is shortened due to energy transfer to the f-chromophore in the same subunit. The depolarisation time of the fast component is much shorter than that of the alpha subunit and decreases with increasing temperature from 400 to 150 psec. The longer lifetime is close to the shorter one in the alpha subunit; a lifetime of 2 ns, which would be expected for the f-chromophore in a native environment is not detected, possibly for experimental reasons. An unambiguous interpretation is presently not possible, because different subsets of chromophore-protein-arrangements cannot be excluded in view of

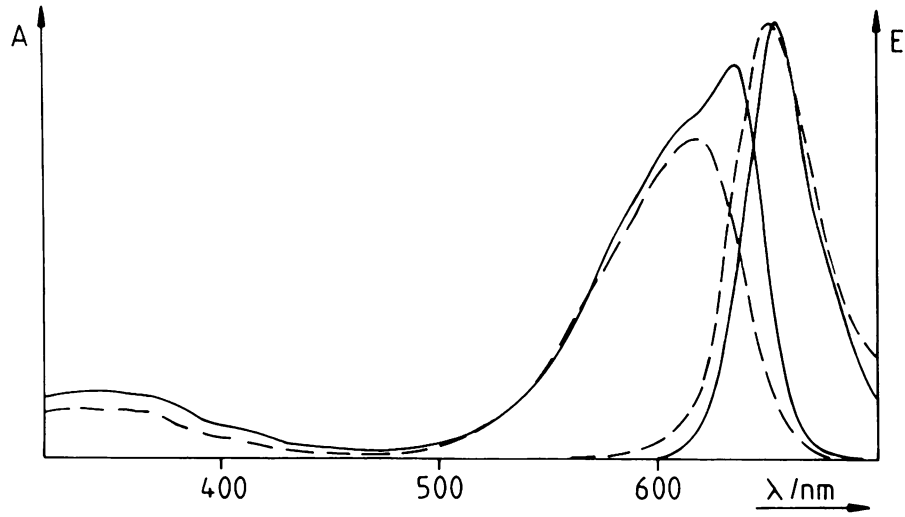

Fig.2: Absorption and emission spectra of trimers of PC-618 (— — —) and PC-636 (———)

the preparation sequence, which involves a denaturation-renaturation sequence.

The isotropic decay curves of monomers and trimers are similar to each other. The short lifetime is in the range of 200-500 psec and represents the lifetime of the s-chromophores, which are quenched by energy transfer. The longer one between 1600 and 2500 psec characterizes the terminal acceptor, i.e. the f-chromophore in the native environement. Chromophores excited via energy transfer rather than directly by photon absorption should emit a less polarized fluorescence. Only the short-lived leakage fluorescence is partly polarized, but the depolarization times are moderately short. In contrast to Spirulina platensis (17) we observe for PC from the thermophilic algae no lengthening of the depolarization time with temperature.

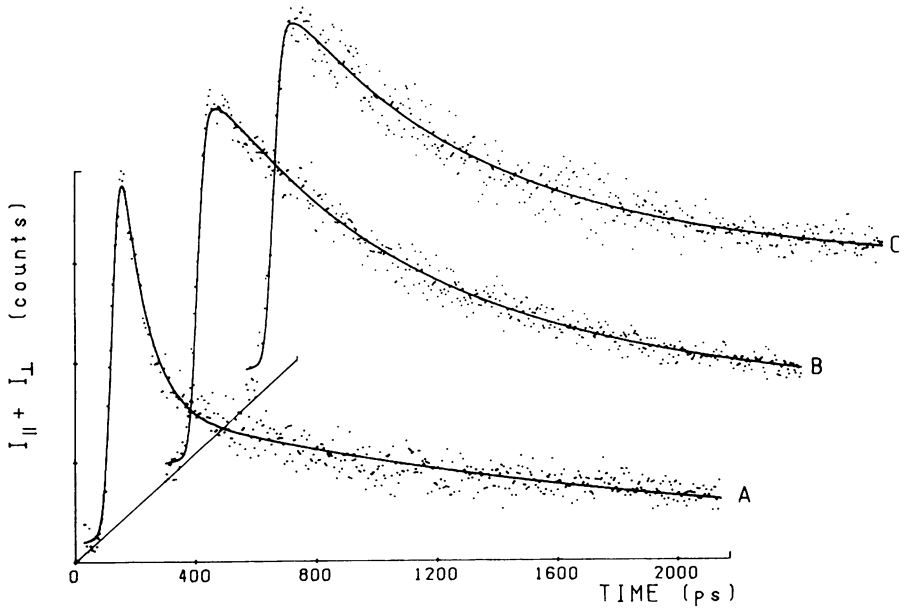

Fig.3: Fluorescence decay of PC-636 excited at 580 nm and recorded with interference filters λ_i = 614 nm (A), λ_i = 635 nm (B) and λ_i = 654 nm (C). Solid lines are calculated fit curves with parameters T_1/T_2 in psec and A_1/A_2 in % in parenthesis.

(A)	72/ 1700	(75/25)
(B)	609/ 4200	(66/34)
(C)	610/10100	(64/36)

In the intact alga, the energy is efficiently transfered to the nonfluorescing reaction center. The observed emission is only leakage fluorescence from PC (fast component with lifetime of approx. 130 psec) and Allophycocyanin (slow component). Since the fraction of emission from directly excited chromophores is small, the emission is essentially unpolarized. Furthermore, it is found that an increase in temperature does not result in a long-lived component, which would indicate a prevention of energy transfer.

In PC-618, the difference in excitation energy of s- and

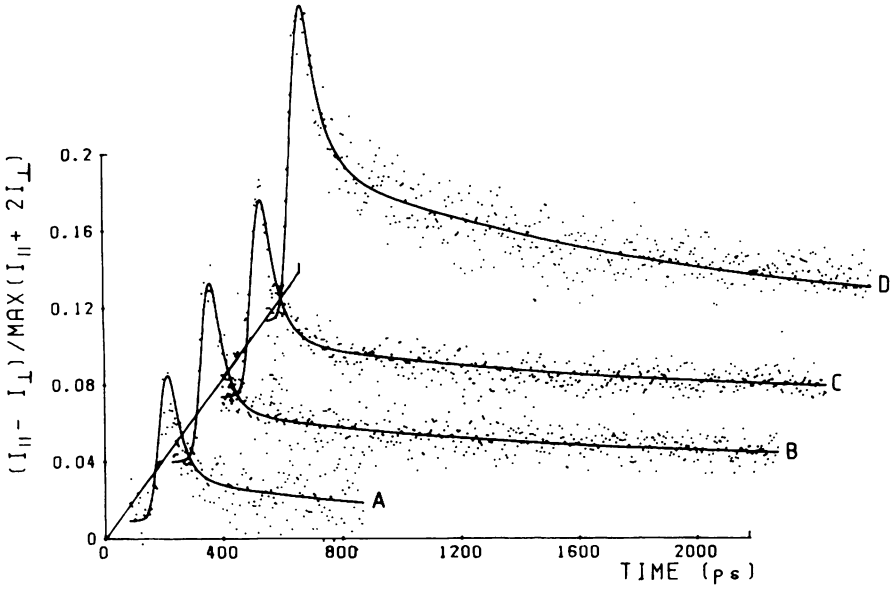

Fig.4: Calculated difference functions $I_D - I_S$ normalized with respect to the maximum of the isotropic decay curve I(t). The fluorescence was recorded with cut-off filters, whose cut-off wavelength is 20 nm larger than the excitation wavelength. Solid lines are calculated fit curves with parameters T_1/T_2 in ps and A_1/A_2 in % in parenthesis.

(A) 35/ 760 (86/14) λ_{exc} = 610 nm
(B) 36/1130 (86/14) λ_{exc} = 600 nm
(C) 42/1165 (85/15) λ_{exc} = 590 nm
(D) 53/1290 (69/31) λ_{exc} = 580 nm

f-chromphores, resp., is small (≈20 nm). Selective excitation of both chromophores is more difficult than in PC-636, in which the absorption of the f-chromophores is shifted bathochromically due to interaction with two colorless proteins (fig.2). The fluorescence decay curves of trimeric PC-636 prove to be strongly dependent on the recording wavelength (fig.3). Upon short-wavelength excitation (580 nm) the longer wavelength emission (both from s- and f-chromophores) is dominated by a fast component (T≈600 psec) as in the case of PC-618. Its lifetime could be related to the energy transfer time from s- to f-chromophores.

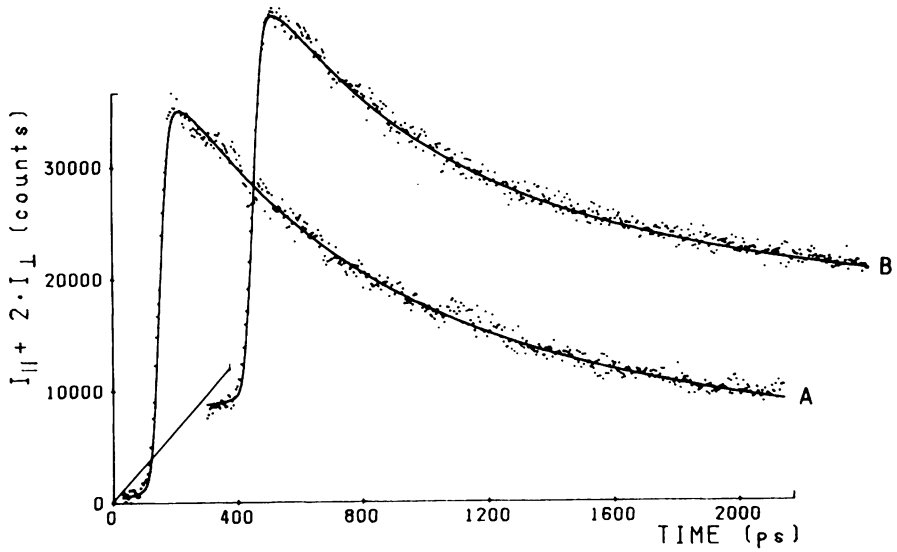

Fig.5: Isotropic fluorescence decay of trimers of PC-636. The fluorescence was recorded with cut-off filters, whose cut-off wavelength was 20 nm larger than the excitation wavelength. Solid lines are fit curves calculated with parameters T_1/T_2 in ps and A_1/A_2 in % in parenthesis.
(A) 610/3200 (63/37) λ_{exc} = 610 nm
(B) 535/4700 (55/45) λ_{exc} = 580 nm

The lifetime of the slow component (emission from terminal f-chromophores) varies somewhat with observation wavelength, a fact, which could again be indicative for a nonuniform chromophore-protein-arrangement. If the recorded fluorescence is restricted to near-resonant emission (614 nm), a much faster component dominates the decay curve. We presume that this is in part a consequence of resonant energy transfer within the s-chromophore manifold. The latter can be verified by inspection of the normalized difference curves displayed in fig. 4. Upon short wavelength excitation (s-chromophore excitation) a pronounced fraction of the short-lived emission is polarized. The decay time of $\approx$40 ps is related to the energy transfer time within the s-chromophore manifold (homo-transfer). Such a transfer does not change the number

of excited molecules of this species. Accordingly, in the isotropic decay function $I=I_p+2\ I_s$ no such extremely short-lived component can be detected (fig. 5).

Keeping the results for the alpha- and the beta-subunits in mind, one could of course argue that the fast component observed in the decay curves of monomers and trimers is due to a second chromophore-protein arrangement, which differs significantly from that in the native species. We believe, however, that an artefactial microheterogeneity can be excluded in the case of PC 636, because it requires much milder manipulations than those applied in the preparations of PC-subunits. Any heterogeneity is then expected to be inherent to the biliprotein.

From the large body of measurements denoted to the study of fluorescence decay and energy transfer within functionally intact phycobilisomes and its constituent aggregated biliproteins one may conclude that the transfer times are greatly reduced when the size of the aggregate, i.e. the number of chromophores is increased. It might be that in a monomeric unit the distance between the chromophores is larger than between chromophores of a trimer belonging to different monomers. This could also imply that in hexamers (and higher aggregates) the important "vertical" energy transfer between adjacent trimers is faster than the "horizontal" transfer within one trimer, which carries no energy in the direction of the reaction center (23).

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Discussion to this and next lecture was combined, see page 407-410.