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Picosecond study of energy-transfer kinetics in phycobilisomes of *Synechococcus* 6301 and the mutant AN 112

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Excitation-energy-transfer kinetics in isolated phycobilisomes from the cyanobacterium *Synechococcus* 6301 (*Anacystis nidulans*) and the mutant *AN 112* (rods containing one hexameric C-phycocyanin unit only) was investigated by picosecond absorption and fluorescence techniques. The different chromophores in the phycobilisomes were selectively excited. A lifetime component of about 10 ps was found for both C-phycocyanin and allophycocyanin in both types of phycobilisomes. We assign these signals to a transfer of excitation energy from sensitizing ('s') to fluorescing ('f') chromophores within C-phycocyanin and allophycocyanin units. A 10 ps component was also observed in the anisotropy relaxation measurements. The anisotropy decay is attributed mainly to differently oriented transition dipole moments of 's'- and 'f'-chromophores and partially to 'f' → 'f' transfer. An absorption recovery signal of $\tau \approx 90$ ps at $\lambda \leq 630$ nm in phycobilisomes of *Synechococcus* 6301 is reduced to 40–50 ps in *AN 112* phycobilisomes. This is rationalized in terms of a decreased rod → core transfer time in the shorter rods of *AN 112*. The 40–50 ps lifetime of fluorescence and absorption recovery in *AN 112* phycobilisomes is assigned mainly to a rate-limiting transfer step between C-phycocyanin and the allophycocyanin core. A decay component of allophycocyanin $\tau \approx 50$ ps was observed both in absorption recovery measurements and in fluorescence decay. It is assigned to energy transfer to the terminal chromophores. The final emitter(s) of the phycobilisomes from *AN 112* have fluorescence lifetimes of 1.9 and 1.3 ns. We find a good correlation in the fluorescence kinetics between the decay times of phycocyanin and allophycocyanin and the fluorescence risetimes of the terminal emitters.

Introduction

Most of the antenna pigments of cyanobacteria and red algae are located in protein complexes called phycobilisomes, which are attached to the outer surface of the thylakoid membrane [1]. The building blocks of the phycobilisomes are disc-shaped aggregates (hexamers or trimers) of phycobiliproteins [2–9]. They carry covalently bound bilinoid pigments as chromophores [10]. Each disc generally contains one kind of phycobilin, i.e., either phycoerythrin, phycocyanin or allophycocyanin. The discs composed of phycoerythrin or phycocyanin are stacked in rods, which are connected to a core of allophycocyanin discs [8,11]. The phycocyanin discs are situated closest to allophycocyanin. The various discs are held together by colourless linker peptides [5,12].

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Abbreviations: PBS, phycobilisome(s); *S* 6301, *Synechococcus* 6301; *AN 112*, *Synechococcus* mutant.

The energy collected by phycobilisomes is transferred preferentially to chlorophyll *a* of Photosystem II in the thylakoid membrane of these organisms [7,13]. The transfer of excitation energy within a phycobilisome is known to occur in the sequence phycoerythrin → phycocyanin → allophycocyanin, i.e., from highest to the lowest excitation energy [7]. Its kinetics has been studied by picosecond fluorescence spectroscopy [14–20,21–23], picosecond absorption spectroscopy [17,19,24] and time-resolved anisotropy [18,19,24]. As a further proof of fast energy transfer within phycobilisomes Grabowski and Gantt [25] found that the 680 nm emission from the final emitter allophycocyanin-B or the 75 kDa polypeptide [8,9] was almost completely depolarized, i.e., the emitting chromophore(s) have little memory of the polarization state of the exciting light. The aim of this work was to explore further the energy transfer between phycocyanin chromophores, from phycocyanin to allophycocyanin, and within the allophycocyanin core, using picosecond spectroscopy. In order to obtain maximum information on the energy-transfer processes we have used both picosecond absorption and fluorescence spectroscopy, since these methods give complementary information. The picosecond absorption technique generally has a better time resolution, and is convenient for the study of anisotropy relaxation. The fluorescence technique using single-photon counting has a higher sensitivity and a considerably higher dynamic range which allows an evaluation of more complicated kinetics (3–4 exponential decays) in particular at times longer than about 20 ps [17,19,20,23].

We have chosen phycobilisomes of the cyanobacterium *Synechococcus* 6301 (*Anacystis nidulans*) for energy-transfer studies for the following reasons: their structure and biochemical composition is known [26–29], their peripheral units contain only C-phycocyanin [7–9], and a mutant *AN 112* is available with rods containing one hexameric C-phycocyanin unit only [29,30]. By comparing the energy flow in *S* 6301 with that in *AN 112* it should be possible to draw some further conclusions about the kinetics of energy migration in phycobilisomes. It must be kept in mind, however, that the rod length of *S* 6301 phycobilisomes is variable, depending on the growth conditions [31]. Studies on absorption recovery and anisotropy decay as well as fluorescence kinetics in phycobilisomes of *S* 6301 have been published by us earlier [20,24].

Materials and Methods

Preparation of phycobilisomes

S 6301 (*Anacystis nidulans*) and *AN 112* were grown under continuous white light illumination at approx. 17 $\mu E \cdot m^{-2} \cdot s^{-1}$ at 37°C in an aerated medium prepared according to Siva et al. [32]. The cells were harvested after 10–14 days and the phycobilisomes were isolated on a continuous sucrose gradient as described previously [20]. This protocol follows, with slight modifications, the procedure described in Ref. 26.

The breakage of the cells was achieved by two different methods. In the first method 1 g (wet weight) of cells were suspended in 8 ml of 0.75 M potassium phosphate buffer (pH 7.0), and then passed twice through a French pressure cell ($1.4 \cdot 10^8$ Pa). Triton X-100 was added at a concentration of 1% (vol/vol) and the mixture was incubated for 45 min. In the second method 1 g (wet weight) cells was suspended in 15 ml of the same buffer with 3% (vol/vol) Triton X-100 and 0.3% (weight/vol) lysozyme. The mixture was then stirred for 2 h. The subsequent steps were identical for both methods [20]. For fluorescence lifetime measurements only phycobilisomes isolated by the French pressure cell breakage were used. The temperature for cell breakage was room temperature (22–25°C) in both methods.

The integrity of the phycobilisomes preparations was checked by measuring the fluorescence and fluorescence excitation spectra (cf. Fig. 1) at different wavelengths. As judged from these criteria the phycobilisomes had a high degree of integrity.

In phycobilisomes of *AN 112* the 624-to-654 nm absorbance ratio was 1.23. According to Yamanaka et al. [30] values in the range 1.3–1.2 are appropriate for phycobilisomes of *AN 112*. The corresponding ratio in phycobilisomes of *S* 6301 used in the picosecond absorption recovery experiments was 2.8. This indicates that these phycobilisomes contained about three hexameric units of C-phycocyanin per rod [31]. Our recent picosecond fluorescence work [20] used slightly

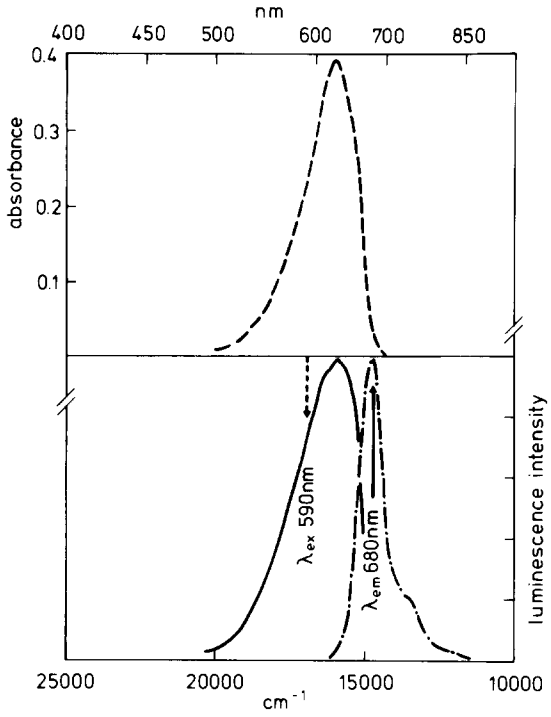

Fig. 1. Absorption (- - - -), fluorescence emission (– · – · –), and fluorescence excitation (————) spectra of *AN 112* phycobilisomes in 0.75 M phosphate buffer (pH 7.0).

larger phycobilisomes with about 3.5–4 hexameric units per rod. The emission spectra showed a maximum at 675 nm, which indicates an efficient energy transfer between C-phycocyanin and allophycocyanin (cf. Fig. 1). Most of the picosecond experiments were performed on fresh samples. Measurements on samples that had been stored for several days at room temperature, however, gave identical results.

Picosecond absorption measurements

The excitation source for both the picosecond absorption and fluorescence measurements was a cavity-dumped synchronously pumped dye laser [17,33] which typically gives pulses 5–15 ps long with an energy of a few nanojoules. For the absorption measurements, the pump-and-probe technique was used. With this technique the time resolution is only limited by the excitation pulse width, which in our experiments was 5–10 ps, as measured by the autocorrelation technique.

The picosecond absorption measurements were all carried out at room temperature (23°C) in a 0.5 mm flow cuvette with an absorbance of approx. 0.4 at 622 nm. In order to avoid the accumulation of possibly long-lived photoproducts, the sample flow was effected by applying a gentle air pressure on the sample reservoir. Experiments performed at different photon densities ($2 \cdot 10^{14}$–$1 \cdot 10^{13}$ photons/cm^2) by placing neutral density filters in the laser beam gave identical results within the experimental error, except for allophycocyanin excitation at wavelengths of at least 650 nm at the upper limit of the intensity range. The high photon density data will not be discussed here.

In some picosecond absorption experiments we used a newly developed technique, where the wavelength of the analyzing light can be varied independently of the excitation light (two-wavelength experiment). Since this technique has been described in detail elsewhere [34] we will only give a schematic description of the apparatus here. The analyzing light is derived from a CW dye laser. In order to reduce the average power of this beam it is chopped into 20-ns-long pulses which are synchronized to the picosecond excitation pulses. Both beams are focused onto the same spot in the sample. The analyzing light pulses thus contain kinetic information on the excited states produced by the pump beam. The kinetic information is then extracted by mixing the analyzing light with a small part of the picosecond pulses in a $LiIO_3$ non-linear crystal. The intensity of ultraviolet light produced is proportional to the intensity of the analyzing light after having passed the sample. The delay of the picosecond pulses relative to the analyzing light pulses gives the time resolution in a similar manner as in an ordinary pump-probe experiment.

The functions $I_{\parallel}(t)$ and $I_{\perp}(t)$ were obtained by recording the signal intensity at two different polarizations of the excitation light; either parallel ($I_{\parallel}$) or perpendicular ($I_{\perp}$) to the analyzing light polarization [19]. The polarization of the excitation beam was set by a Soleil-Babinet compensator. Rotation-free signals were obtained with the polarization angle set at 54.7°, the so-called magic angle.

The polarization anisotropy ($r(t)$ is defined as:

$$r(t) = \frac{I_{\parallel}(t) - I_{\perp}(t)}{I_{\parallel}(t) + 2I_{\perp}(t)} = \frac{D(t)}{S(t)} \qquad (1)$$

When there are two decay components, each exhibiting its own anisotropy decay, the sum, $S(t)$, and difference, $D(t)$, functions have to be evaluated separately [19].

Fluorescence measurements

Steady-state fluorescence measurements were performed on a computer-controlled Spex Fluorolog instrument [35]. The bandwidth was 5 nm in the excitation monochromator and 2.5 nm in the emission monochromator for emission spectra, and vice versa for excitation spectra. Emission and excitation spectra of the highly concentrated phycobilisomes samples were measured in 1 mm cuvettes in order to avoid self-absorption and other nonlinear effects. Maxima are accurate to ± 2 nm. All spectra were corrected for wavelength dependence of photomultiplier sensitivity and lamp intensity. Absorption spectra were measured on a Perkin Elmer Model 320 UV/VIS spectrometer in a 2 mm cuvette.

The detection system for time-resolved fluorescence was a single-photon timing apparatus capable of measuring lifetimes below 20 ps [19,20,36,37]. Fluorescence was selected by a double-monochromator with slits set to give a 4 nm bandwidth. The width of the apparatus function was approx. 130 ps (full-width at half-maximum) and 400 ps (full-width at tenth-maximum). The high sensitivity of this technique enabled us to use photon densities for excitation in the range 10^{10}–10^{11} photons/cm^2, working at a repetition rate of 400 or 800 kHz. Between 20 000 and 50 000 counts were collected in the peak channel. Fluorescence was excited with the vertically polarized laser beam, and detected through a sheet polarizer set to the magic angle of 54.7° for measuring the isotropic decay function. Fluorescence lifetimes according to multiexponential kinetics were calculated by an iterative convolution method. The quality of fits was judged by both a reduced χ^2 criterion and a plot of weighted residuals [38]. The necessary requirements allowing to carry out decay analyses in terms of three and four exponentials are discussed in part in Ref. 37, and will be fully described in a forthcoming publication (Wendler, J. and Holzwarth, A.R., unpublished data). All measurements were carried out at room temperature (23–25°C) in flow cells of either 1.5 or 3 mm optical path-length (flow, approx. 0.5 ml/min) by applying air pressure on the sample reservoir.

Results

Absorption recovery kinetics

Fig. 2a and b show the recovery kinetics of *AN 112* phycobilisomes at 620 nm with the relative polarization of the analyzing light set to the magic angle. We thus observe the decay kinetics free of processes that may alter the direction of the transition dipole moments of the molecules studied, such as rotational motion of the chromophores or energy transfer among identical molecules with non-parallel transition dipole moments. The transfer from C-phycocyanin to allophycocyanin will lead to a recovery of the absorbance at 620 nm as shown in Fig. 2a and b. This signal can be analyzed in terms of two exponentials with lifetimes of approx. 7 and approx. 37 ps, respectively. For comparison the semilogarithmic plot of a corresponding experiment on phycobilisomes of *S* 6301 at the same wavelength is shown in Fig. 2c. We note that the short lifetime is the same within the experimental error for both types of phycobilisome (see also Table I). The long lifetime, however, is substantially longer in phycobilisomes of *S* 6301 than in phycobilisomes of *AN 112*. When the same pump-probe experiment is performed at 657 nm (Fig. 3) the ground state recovery looks different. In addition, a long-lived component is observed. The absorption recovery at 657 nm is best described as the sum of three exponentials with average values of $\tau_1 = 10$ ps, $\tau_2 \approx 50$ ps and $\tau_3 \approx 1.1$ ns (see Table I). The longest lifetime is only approximate due to the poor signal-to-noise ratio at times longer than 1 ns and the limited length of the optical delay (approx. 30 cm). In addition to pump-probe measurements with an identical wavelength, we have performed such experiments with different wavelengths of the analyzing and exciting light [34,39]. Results obtained for phycobilisomes of *S* 6301 at a fixed analyzing wavelength ($\lambda_a =$ 650 nm) using different excitation wavelengths ($\lambda_{exc} = 587$ and $\lambda_{exc} = 618$ nm) are shown in Fig. 4. The absorption recovery lifetimes obtained at different wavelengths are collected in Table I together with the anisotropy decay data.

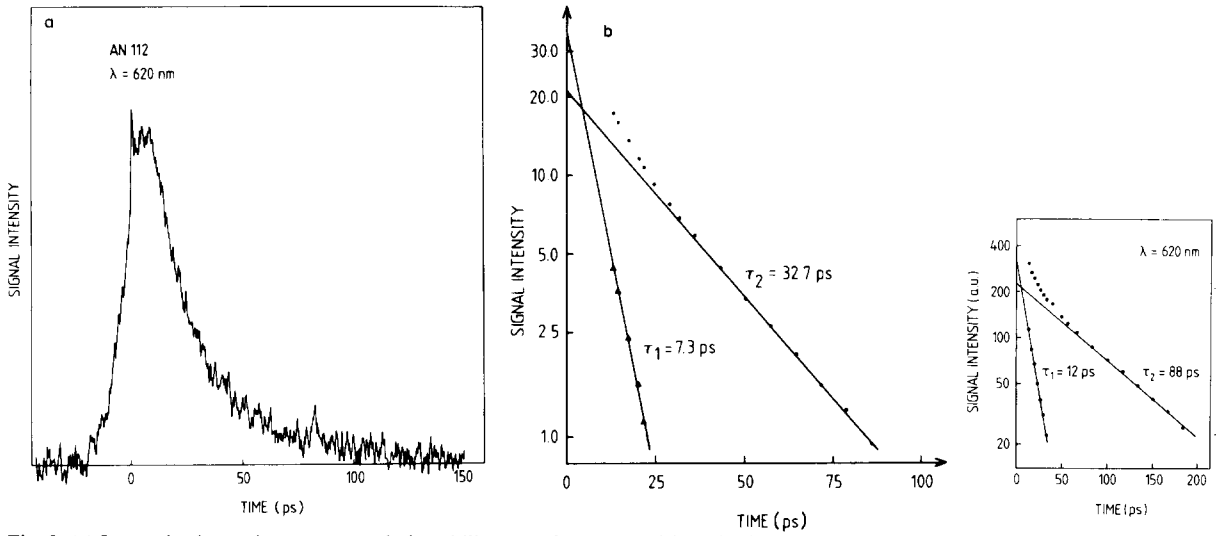

Fig. 2. (a) Isotropic absorption recovery of phycobilisomes of *AN 112* with excitation and analyzing wavelength at 620 nm. (b) Shows a semilogarithmic plot of the same signal. (c) Semilogarithmic plot of the absorption recovery signal of phycobilisomes from S 6301 at $\lambda_{exc} = \lambda_d = 620$ nm.

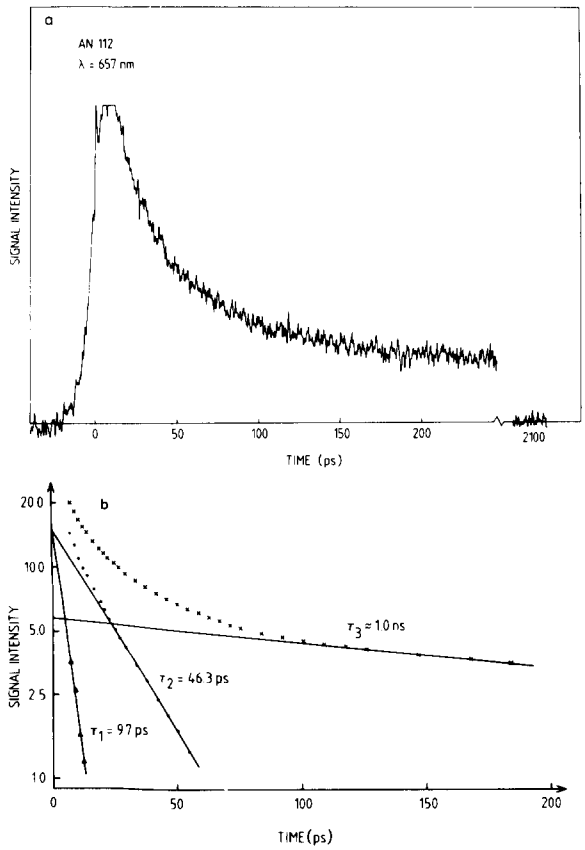

Fig. 3. (a) Isotropic absorption recovery signal of phycobilisomes from mutant *AN 112* with excitation and analyzing light at 657 nm; (b) shows a semilogarithmic plot of the same signal.

Absorption anisotropy

In a previous publication on phycobilisomes of *S* 6301 we presented data on the relaxation of the absorption anisotropy in the wavelength range 585–630 nm [24]. We now obtained very similar results for phycobilisomes of *AN 112* in the range 609–630 nm (Table I). An anisotropy relaxation measurement for allophycocyanin excitation at 657 nm is shown in Fig. 5. Since the anisotropy relaxations involve at least two components, sum and difference function should be analyzed separately, as noted above. Because of the low signal-to-noise ratio at times above approx. 50 ps we have chosen instead to fit our data according to Eqn. 2, i.e., to

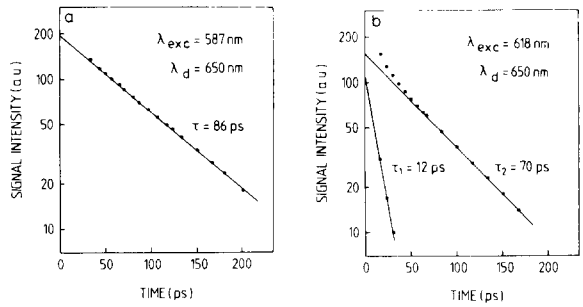

Fig. 4. (a) Isotropic absorption recovery of phycobilisomes of *S* 6301 excited at $\lambda_{exc} = 587$ nm and analyzed at $\lambda_d = 650$ nm. (b) The same as above, but excited at $\lambda_{exc} = 618$ nm.

TABLE I

ABSORPTION RECOVERY ($\tau_{1,2}$) AND ANISOTROPY DATA τ_r, $r(\infty)$ OF PHYCOBILISOMES OF *S* 6301 AND *AN 112* AT DIFFERENT WAVELENGTH INTERVALS

Average values are given for all data. The data for *S* 6301 were taken from Ref. 24. The λ_{exc} data were taken from the isotropic two-wavelength experiment on *S* 6301 phycobilisomes (cf. Materials and Methods); detection at $\lambda_d = 650$ nm.

	τ_1 (ps)	τ_2 (ps)	A_1/A_2 [a]	τ_r (ps)	$r(\infty)$ [b]
S 6301					
585 nm $\leqslant \lambda \leqslant$ 630 nm	12 ±3	84±8	1.0±0.5	10±2	0.07 ±0.02
λ_{exc} = 587 nm	–	86			
λ_{exc} = 618 nm	12	70			
AN 112					
609 nm $\leqslant \lambda \leqslant$ 630 nm	8.0±1.5	37±5.0	1.8±0.4	11±2	0.084±0.025
λ = 657 nm	9.9±1.4	51±5	0.8±0.2	11±2	0.084±0.025

[a] Amplitude ratio of short-lived and long-lived components.
[b] For evaluation and interpretation of the $r(\infty)$ value, see text.

a sum of a constant term and an exponential function:

$$r(t) = \{r(0) - r(\infty)\}\, e^{-t/\tau_r} + r(\infty) \qquad (2)$$

Our procedure is justified by the relatively large difference between the short and long relaxation lifetimes. This allows us, within the experimental error, to consider the anisotropy decays at $t \leq 20$ ps and at $t \geq 50$ ps separately. Attempts to fit the difference function $D(t)$ to two exponentials gave very large errors in the long lifetime, but confirmed the presence of two relaxation phases. Following the simplified procedure we found a satisfactory fit to the experimental data with $r(\infty) = 0.08$ and $\tau_r = 8.5$ ps. The value for $r(0)$ was close

TABLE II

FLUORESCENCE LIFETIMES (τ) AND AMPLITUDES (R, RELATIVE, AND B, ABSOLUTE) OF PHYCOBILISOMES OF *AN 112* AT DIFFERENT EXCITATION (λ_{ex}) AND EMISSION (λ_{em}) WAVELENGTHS

λ_{ex} (nm)	λ_{em} (nm)	τ_1 (ps)	R_1 (%)	τ_2 (ps)	R_2 (%)	τ_3 (ns)	R_3 (%)	τ_4 (ns)	R_4 (%)
582	595			34	89	0.55	3	1.73	8
582	595	12	79	51	15	0.66	2	1.77	4
585	600			39	92	0.67	1	1.83	7
581	680			42 [a]	−237 [b]	1.44	31 [b]	1.98	125 [b]
620	680			51 [a]	−311 [b]	1.39	42 [b]	1.98	176 [b]
660	680			60 [a]	−49 [b]	1.12	30 [b]	1.96	228 [b]
582,585 [c]	595,600,620			32–39		0.61±0.12		1.85±0.04	
580,585,620 [d]	640			43–45		0.65±0.19		1.87±0.04	
580,620 [e]	680			45±6 [a]		1.37±0.12		1.95±0.05	
650,660 [f]	675,680			50±9 [a]		1.13±0.47		1.97±0.07	

[a] Fluorescence risetime; negative amplitude.
[b] Absolute amplitude of the decay component.
[c] Data from 8 experiments.
[d] Data from 18 experiments.
[e] Data from 18 experiments.
[f] Data from 3 experiments.

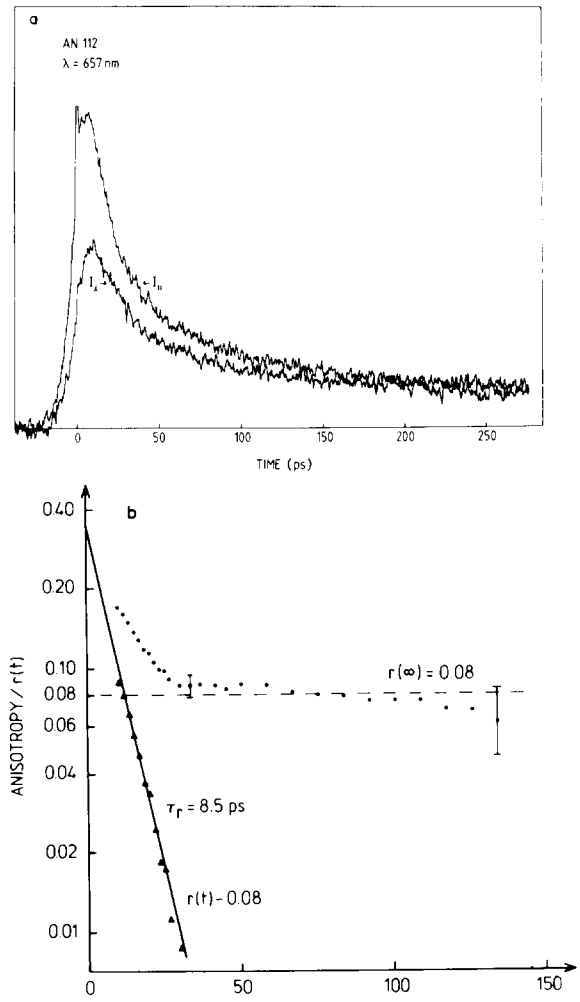

Fig. 5. Absorption recovery of phycobilisomes of *AN 112* with (a) analyzing light parallel ($I_{\parallel}$) and perpendicular ($I_{\perp}$) to the excitation light $\lambda_{exc} = \lambda_b = 657$ nm). (b) shows the calculated anisotropy function, $r(t)$. Note the Discussion section for the evaluation and interpretation of $r(t)$ and $r(\infty)$.

to the theoretical value of 0.4. These results are similar to those of Ref. 24 for *S* 6301 phycobilisome at 630 nm, i.e. $r(\infty) = 0.07$ and $\tau_r = 10 \pm 2$ ps. The value of $r(\infty)$ is more accurate for the measurements at 657 nm than at 630 nm, since the amplitudes of $I_{\parallel}(t)$ and $I_{\perp}(t)$ at longer times are much larger at 657 nm. It should be noted, however, that our analysis does not exclude the possibility of a long-lived relaxation of the 'constant' anisotropy, $r(\infty)$, at times longer than approx. 100 ps. Owing to the small signal intensity our data are less accurate in that time range.

Fluorescence kinetics

TIme-resolved fluorescence measurements on *AN 112* phycobilisomes generally showed complex kinetics which varied with the excitation and analyzing wavelength. The excitation wavelengths used in these experiments were 581–660 nm, while the fluorescence kinetics were studied in the range 595–685 nm. In Fig. 6 a typical fluorescence decay at 595 nm for *AN 112* phycobilisomes excited at 585 nm is shown. The residuals plot on top clearly

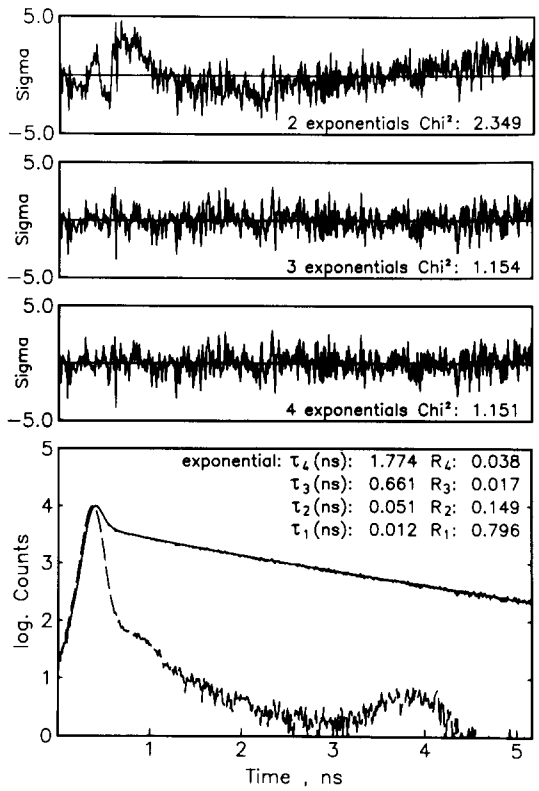

Fig. 6. Semilogarithmic plot of the isotropic fluorescence decay of phycobilisomes from *AN 112* detected at 595 nm, $\lambda_{exc} = 582$ nm.

Note to Figs. 6 and 7: Superimposed on the experimental decay is the fluorescence (thin line) calculated from the best fit parameters (cf. inset). Also shown is the semilogarithmic presentation of the exciting pulse (dashed). In the inset are given the lifetimes ($\lambda_1 \ldots \lambda_n$) and relative ($R_1 \ldots R_n$) or absolute ($B_1 \ldots B_n$) amplitudes of the exponential decay components. The subscripts coincide with those in Table II. The frames above the main figures show the weighted residual plots obtained for different kinetic model functions. The residuals indicate the deviations between the computer-fitted and experimental decay curves. Also shown in the residuals plots are the reduced χ^2-values from the statistical analysis.

indicates that more than two exponential functions are needed in order to describe the experimental kinetics properly. In a three-exponential fit of the decays with $\lambda_{em} = 595–605$ nm a fast component with a lifetime of 36 ± 3 ps is dominating the decays ($88 \pm 2\%$ amplitude). The second component with a lifetime of approx. 550 ps only represents 3–4% of the total amplitude. The longest lifetime of 1.75 ± 0.06 ns contributes 7–8%. There is a slight systematic deviation, near the maximum of the exciting pulse, in the residuals plot of the decays with short emission wavelengths ($\lambda_{em} =$ 595–605 nm). A fit with four exponentials generally gave only a small improvement in χ^2, but abolished the deviation in the residuals plot. The four exponential fit essentially resolved the 36 ps component into two exponentials with lifetimes of 12 ± 5 and 51 ± 5 ps (Table II). At $\lambda_{em} = 595$ nm the 12 ps component carries nearly 80% of the total intensity while the second component contributes only 15%. This four-exponential analysis is very close to the resolution limit, however, and we take it only as an indication for the presence of a decay component with lifetime up to 20 ps. Table II shows the results of all the fluorescence decay measurements performed in different excitation and fluorescence spectral regions. The fluorescence kinetics between 600–640 nm is similar when the excitation wavelength is in the range 580–620 nm (cf. Table II, lower part). However, there is some systematic increase in the decay time of the shortest-lived component (τ_2) obtained from the three-exponential fits when the emission wavelength is increased from 595 nm to 640 nm (cf. Table II). This may be taken as another indication that the short lifetime in a three-exponential fit may in fact represent a mixture of two components.

At 675–680 nm the fluorescence is clearly delayed relative to the instrumental response function when excited at $\lambda_{exc} \leqslant 660$ nm. The fluorescence kinetics have a component with negative sign of the preexponential factor. A negative sign corresponds to a growth term which indicates an indirect excitation via energy transfer. The best fit yielded two decay terms and one growth term, as illustrated in Fig. 7. It should be pointed out here that in our data analysis program neither the amplitudes nor the lifetimes are bound to any limits. It is noted that the risetime of approx. 45 ps found at long emission wavelength upon phycocyanin excitation is in good agreement with the short decay time at $\lambda_{em} = 640$ nm (40–45 ps).

Discussion

Phycocyanin transfer kinetics

We will first discuss the results of the absorption recovery and anisotropy measurements, and then compare these results with the fluorescence lifetime data and ultimately condense our findings into a model for the kinetics of energy transfer in phycobilisomes of *S* 6301.

Table I summarizes the data of the ground state recovery and anisotropy decay for PBS of *S* 6301 (from Ref. 24) and *AN 112*. The most striking difference between the two phycobilisomes is the increase of the long lifetime, τ_2, from approx. 40 ps in *AN 112* to approx. 90 ps in *S* 6301 at wavelengths shorter than 630 nm, where contributions from allophycocyanin to τ_2 are thought to be of minor importance (see below for a more detailed discussion of this problem). The size of the phycobilisomes rods increases from one hexameric C-phycocyanin unit in *AN 112* to about three to four in the *S* 6301 phycobilisomes used in these experiments. It is thus reasonable to consider τ_2 as a measure of the average time it takes for the excitation energy to reach the allophycocyanin core. The same conclusion may be drawn from a comparison of the fluorescence kinetic data of *AN 112* phycobilisomes (Table II) with those of *S* 6301 phycobilisomes [20]. The fluorescence decay and risetimes due to rod → core transfer are approx. 130 ps and approx. 50 ps in phycobilisomes of *S* 6301 and *AN 112*, respectively. It is therefore obvious that τ_2 is very sensitive to the length of the phycobilisomes rods.

The fast absorption recovery lifetime, $\tau_1 \approx 10$ ps, is the same within the experimental error in both kinds of phycobilisomes studied. This ultrafast transfer process has to take place between close-lying chromophores, provided that the Förster mechanism is in operation [40]. In order to be able to detect a fast isotropic absorption recovery signal the chromophores involved in the energy transfer must have different absorption spectra. According to Ref. 41 the 's'-chromo-

phores have absorption maxima at 608 nm, while the 'f'-chromophores absorb at 620 nm. We would therefore expect the amplitude ratio A_1/A_2 of the two fast decays to decrease with increasing wavelength if an assignment of the fast τ_1 signal to 's' → 'f' transfer applies. However, our pump-probe experiments at $\lambda = 609$–630 nm did not meet this expectation. Rather A_1/A_2 remained constant at $A_1/A_2 = 1.8 \pm 0.4$. As will be discussed below in connection with the allophycocyanin kinetics, this behaviour probably reflects the fact that the β-chromophores of allophycocyanin absorb strongly around 610–630 nm [9]. They might contribute to the fast absorption recovery kinetics at these wavelengths in *AN 112* and thus modify the amplitude ratio. The ratio A_1/A_2 should be similar in phycobilisomes of *AN 112* and *S* 6301 if the fast kinetics is only due to 's' → 'f' energy transfer in C-phycocyanin hexamers. (It is believed that the α-chain of C-phycocyanin contains one fluorescing 'f' chromophore and that the β-chain contains one 'f' and one sensitizing 's' chromophore.) However, this ratio (at $\lambda \leqslant 630$ nm) is only 1.0 ± 0.5 in phycobilisomes of *S* 6301, which indicates that the fast kinetics is not just due to 's' → 'f' transfer in C-phycocyanin hexamers, but must contain also other components, e.g., $\beta \rightarrow \alpha$ transfer in allophycocyanin. The relative content of allophycocyanin is about three times larger in *AN 112* than in *S* 6301, which qualitatively explains the higher A_1/A_2 ratio in phycobilisomes of the former. Another complication arises from the inhomogeneous absorption spectra of phycocyanin along the rods of *S* 6301 phycobilisomes [9]. All these factors influence the amplitude ratio A_1/A_2, and thus impede an unequivocal quantitative analysis at present.

Our fluorescence kinetic measurements also support an assignment of the fast τ_1 lifetime to 's' → 'f' transfer. Even under the difficult conditions encountered in *AN 112* the decays at short emission-wavelength (cf. Fig. 6) indicate the presence of a fast component ($\tau \lesssim 20$ ps) at short detection wavelength, as is more clearly the case also in *S* 6301 phycobilisomes [20]. In a three-exponential analysis, where the two fast subcomponents in the fluorescence kinetics are not resolved the short-lived average decay time increases with increasing detection wavelength [19,20]. Similar findings indicate the presence of two short-lived components with different emission spectra also in phycobilisomes of *AN 112* (Table II). The faster of the two subcomponents is associated with fluorescence and absorption spectra peaking at shorter wavelength than those of the longer-lived component. Furthermore, the longer-lived of the two fast subcomponents is in better agreement with the risetime in the allophycocyanin emission observed in both *S* 6301 [20] and *AN 112* (Table II).

Using a three-exponential analysis we obtained values of this lifetime ranging from 32 to 45 ps (Table II) at emission wavelengths in the range 600–640 nm and excitation between 580 and 620 nm. This is in good agreement with the corresponding lifetime of the absorption measurements at similar wavelengths. It is thus clear that τ_2 describes the energy transfer from the C-phycocyanin rods to the allophycocyanin core. At longer emission wavelengths, i.e., at 680 nm, the contribution from phycocyanin emission is small [9], and we mainly observe the emission from one or both of the final emitters allophycocyanin-B and the 75 kDa polypeptide [8,9]. They have fluorescence maxima at 670–680 nm. There is an evident time delay in the emission at 680 nm as compared to the range 620–640 nm (cf. Figs. 6 and 7). The risetime of the emission at 680 nm was found to be 45 ± 6 ps when C-phycocyanin was excited at 580–620 nm. This value is identical with the fast fluorescence decay at 640 nm (44 ps) and very similar to the absorption recovery at $\lambda < 630$ nm (37 ± 5 ps). It is also in good agreement with the 50 ± 6 ps ground state recovery of allophycocyanin measured at $\lambda = 651$–657 nm (Table I). One can thus conclude that the rate of excitation energy transfer from the innermost trimer of C-phycocyanin to allophycocyanin-B is the rate-determining step as was suggested previously [20].

The long fluorescence lifetime at 600–640 nm of 1.85 ± 0.04 ns is most likely due to the emission from C-phycocyanin that has been disconnected from the phycobilisomes core either as hexameric discs or as free C-phycocyanin rods [17,19,20,23]. The rather high intensity of this emission, e.g., $13 \pm 5\%$, as compared to that of other phycobilisomes [17,19,20,42] is at least partly due to the fact that the intensity of the 10–20 ps component is not fully accounted for in the fluorescence mea-

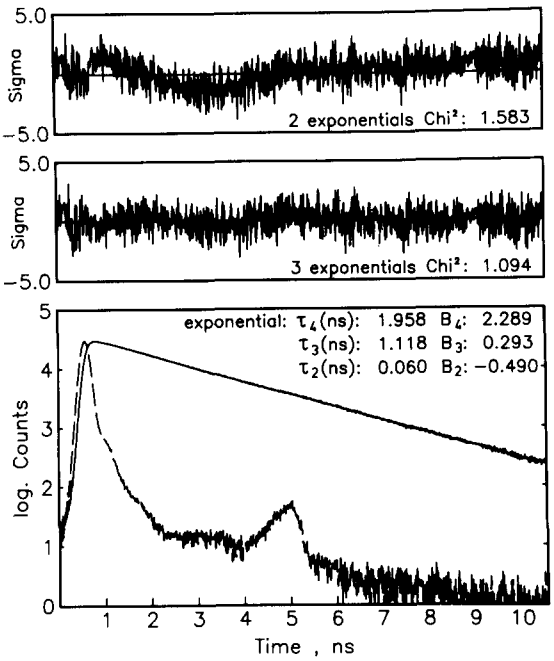

Fig. 7. Semilogarithmic ppot of the fluorescence kinetics of indirectly excited allophycocyanin in phycobilisomes from *AN 112* detected at 680 nm; $\lambda^{exc} = 660$ nm. The $B_1 \ldots B_4$ values in the inset are absolute coefficients. A negative sign indicates a risetime (cf. Note Fig. 6).

surements. In most cases the latter has a relative yield which is too small to be resolved by our instrument. From the absorption recovery measurements we know that at least 50% of the total signal is due to the fastest component. This reduces the actual contribution of the long 1.85 ns signal to $\leqslant 6\%$. The low signal-to-noise ratio in the picosecond absorption experiments (see Fig. 2) at long delays does not allow us to calculate the exact amount of any long-lived component, but we estimate it to be approx. 3% in the short-wavelength range.

The very weak contribution to the fluorescence decay signal with a lifetime of 0.65 ± 0.19 ns is more difficult to explain. It does not seem to be an artifact, however, since it was observed in all measurements in the wavelength range 600–640 nm. It has also been observed in phycobilisomes of *S* 6301 [20]. We again suggest that this signal most likely comes from phycobilisomes which have been perturbed during the isolation procedure in such a way that the energy transfer to the core is slowed down. Another possibility would be that the C-phycocyanin chromophores have been distorted by, e.g., changes in the protein structure [37,43].

The fast anisotropy decay lifetime (cf. Table I) is identical within experimental error in phycobilisomes of *AN 112* and *S* 6301, i.e., about 10 ps. It is also very similar to the lifetime of the fast isotropic decay in the systems discussed above. It seems reasonable to assign both the isotropic and anisotropic fast absorption recovery to the same process, namely energy transfer between a donor ('s'-chromophore) and a closely situated acceptor ('f'-chromophore) within the same C-phycocyanin unit. Part of the fast anisotropy decay might also be due to homo transfer between 'f'-chromophores in the C-phycocyanin pigment bed as suggested previously [24].

The remaining (slowly decaying) anisotropy ($r(\infty)$) at times much longer than τ_1, i.e. $t > 50$ ps, can be understood as a measure of the nonisotropic character of the system. Since $r(\infty)$ is only about 0.08 the angle (θ) between the transition moments of the interacting chromophores has to be substantial. An $r(\infty)$ value of 0.4 would indicate parallel [44] transition dipole moments. If the observed anisotropy relaxation would be due to a single transfer step between a well-defined pair of 's'- and 'f'-chromophores:

$$r(\infty) = r(0) = \frac{3\cos^2\theta - 1}{2}$$

Using $r(\infty) = 0.08$ and $r(0) = 0.40$ one obtains $\theta \approx 47°$ or 133°. It is more likely, however, that this angle should be regarded as a mean value resulting from different transfer steps rather than being related to a single process.

The almost constant value of $r(t)$ at $t \geqslant 50$ ps further suggsts that the energy transfer in the C-phycocyanin rods following the initial fast process(es) takes place between chromophores with almost parallel transition dipole moments. Most probably the energy is transferred along the shortest path towards the core through 'f'-chromophores situated above each other in the C-phycocyanin trimers. This is a favorable situation, since according to the Förster model [40] the energy transfer is fastest if the transition dipoles are parallel (orientation factor $K^2 = 4$). We have pro-

posed this model previously for the heterogeneous rods of *Porphyridium cruentum* phycobilisomes [19] and it is interesting that it also applies to the homogeneous rods of *S* 6301 [20], and *AN 112* phycobilisomes.

Transfer kinetics within the allophycocyanin core

It was mentioned above that allophycocyanin might contribute to the absorption kinetics even at $\lambda = 610$–630 nm, where C-phycocyanin absorbs strongly. That this is the case is demonstrated in Fig. 4a and b, which show the allophycocyanin absorption kinetics at 650 nm upon exciting the *S* 6301 phycobilisomes at 587 or 618 nm. At the lower excitation wavelength the kinetics consists of one exponential with a lifetime of 86 ps, which is close to the rod → core transfer rate in *S* 6301 (see Table I). The lack of a 10 ps component in this experiment further supports our previous conclusion that the fast isotropic decay ($\tau \approx 10$ ps) in C-phycocyanin is not due to a direct transfer to allophycocyanin. Excitation at 618 nm, however, gives a double exponential decay with $\tau_1 = 12$ ps and $\tau_2 = 70$ ps (cf. Fig. 4b). The appearance of a short lifetime of 12 ps indicates that now also part of the sensitizing chromophores of allophycocyanin are directly excited. The longer lifetime is shortened, however, because it represents the average value of the ≈ 90 ps lifetime resulting from rod → core transfer and a lifetime of 50 ps, which is due to the relaxation of directly excited allophycocyanin chromophores. The latter is directly observed in pump-probe measurements on *AN 112* phycobilisomes at 657 nm (cf. Fig. 3 and Table I). At this wavelength the excitation of the 'sensitizing' β-chromophore of C-phycocyanin, which has an absorption maximum at about 608 nm [41], should be negligible. The 10 ps lifetime found in the experiment shown in Fig. 3 is therefore most likely due to energy transfer from a sensitizing β-chromophore of allophycocyanin to an α-chromophore within an allophycocyanin trimer or hexamer unit. The fast energy transfer in allophycocyanin aggregates is thus very similar to the fast isotropic and anisotropic decay in C-phycocyanin. This similarity is further supported by the anisotropy relaxation at 657 nm, which has a fast component with a lifetime of 8.5 ps (Table I).

The 51 ± 5 ps absorption recovery lifetime at 657 nm is assigned to the transfer of excitation energy from allophycocyanin α-chromophores to one of the final emitters, allophycocyanin-B or the 75 kDa polypeptide. This component is also found in the fluorescence decays upon 650 and 660 nm excitation (Table II). The almost constant anisotropy of approx. 0.08 in the time range 30–50 ps (see Fig. 4), during which the excitation energy is still mainly localized on the allophycocyanin α-chromophores, indicates again that transfer between chromophores with differentialy oriented transition dipole moments hardly occurs after the fast 10 ps $\beta \rightarrow \alpha$-chain transfer. The anisotropy at 657 nm might, however, contain some contribution from directly excited allophycocyanin-B and 75 kDa polypeptide. From their absorption spectra and relative concentration in the core [9] we estimate that these directly excited final emitters contribute about 15% to $r(\infty)$.

The fluorescence decay at 680 nm is composed of two components. The strongest signal with a lifetime of 1.95 ± 0.05 ns is obviously due to one or both of the final emitters allophycocyanin-B [9,45] and the 75 kDa polypeptide [27,46]. Our value is significantly shorter than the lifetime of 2.7 ns reported by Grabowski and Gantt [47] for the isolated allophycocyanin, and it is longer than the fluorescence lifetime of 1.4 ns obtained for *Mastigocladus laminosus* phycobilisomes at 668 nm [18]. The difference is possibly due to the inaccuracy of the streak camera detection technique at lifetimes longer than about 1 ns. The shorter lifetimes of allophycocyanin-B as compared to allophycocyanin or free phycobiliproteins might arise from changes in the protein structure upon aggregation, which are known to cause spectral shifts in the allophycocyanin chromophores and might influence the radiative lifetime as well.

The 1.3 ns lifetime which carries about 18% of the fluorescence intensity at 680 nm is more difficult to explain. We do not assign this component to free phycobiliproteins because of its high relative intensity even when excited at 660 nm. One possible origin would be a weak coupling between the final emitters allophycocyanin-B and 75 kDa polypeptide permitting a direct energy transfer from allophycocyanin-B to 75 kDa when the transfer path from allophycocyanin-B to the thylakoid

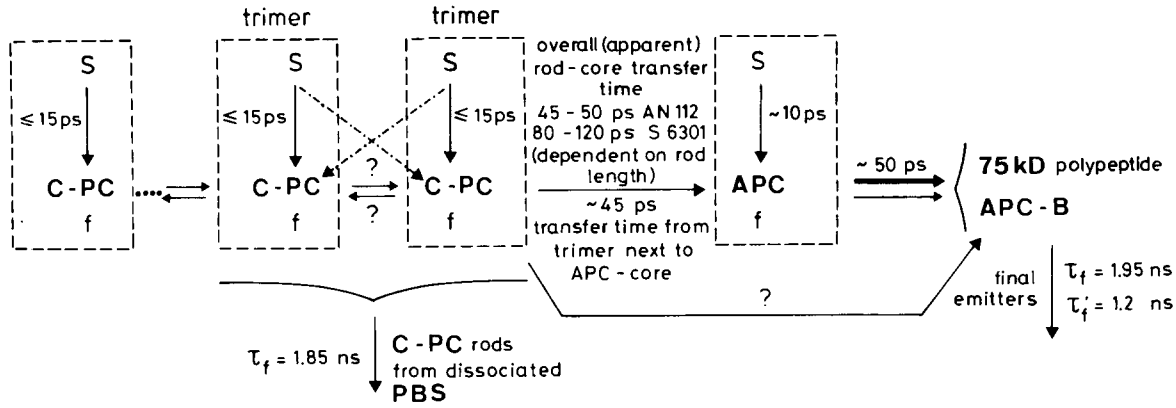

Fig. 8. Extended scheme of the various energy transfer processes in phycobilisomes of *AN 112* and *S* 6301 assigned on the basis of the measurements presented in this and the previous papers [20,24] (compare also the scheme given in Ref. 20). C-PC, C-phycocyanin; PBS, phycobilisomes; APC, allophycocyanin.

membrane is interrupted. However, since no rise-time for such a process has been observed, there is no further support for this possibility. We should like to point out that we found two long-lived components in all phycobilisomes studied so far in detail [17,19–21]. The observation of two different lifetimes also precludes the possibility that there is a fast transfer of energy back and forth between the two final emitters [9], because in this case just one long-lived decay should be observed. The question as to the individual assignment of the two lifetimes still remains open. All transfer processes in *AN 112* phycobilisomes are combined into a scheme in Fig. 8.

Summary and Conclusions

The combined picosecond absorption and fluorescence study on phycobilisomes of *S* 6301 and the mutant *AN 112* has improved our understanding of the pathways of excitation energy transfer in these photosynthetic antenna pigment systems. According to the results presented in this and the previous paper [20,24] the energy transfer within the C-phycocyanin hexamers and from the rods to the core can best be described by a double exponential function. The most striking result is the shortening of the rod → core transfer time from about 90 ps in phycobilisomes of *S* 6301 to about 40 ps in *AN 112* which reflects the shortening of the rod length from three to one hexameric units of C-phycocyanin. It is interesting that life-times in the same range (40 ps) have been found for phycocyanin → allophycocyanin transfer in phycobilisomes with heterogeneous rods containing only one phycocyanin hexamer per rod [17,19,21,23]. We observed a good correlation between the decay of C-phycocyanin excitation and the growth of the long-wavelength emission. This indicates that only a few transfer steps involving allophycocyanin chromophores are necessary, and it is consistent with the idea that the allophycocyanin-B (or 75 kDa) chromophores are situated close to the rods. Direct excitation of allophycocyanin leads to a somewhat less efficient energy transfer to allophycocyanin-B. This is indicated both by absorption recovery as well as by fluorescence measurements.

The short lifetime of about 10 ps in the absorption recovery and in the anisotropy relaxation data is found across the entire wavelength range (585–657 nm) investigated, which means that it is present in the rods as well as in the core. We propose that this component arises preferentially from transfer between closely situated 's'- and 'f'-chromophores (possibly located in different C-phycocyanin trimers) with non-parallel transition dipole moments. The anisotropy decay probably also includes a contribution from 'f' → 'f' transfer in phycocyanin. Lifetimes in the range of 10–20 ps seem to be typical for 's' → 'f' transfer in phycobiliproteins [19,20,36]. Using the Förster mechanism of energy transfer [47] we can estimate the transfer distance, R_{DA}, to be 2.0 nm when $R_0 = 4.7$ nm,

$\tau_D = 1.9$ ns, $\kappa^2 = 2/3$ and $k_{ET} = 1/\tau_1 = 1.0 \cdot 10^{11}$ s^{-1}. This distance should be regarded as a lower limit, however, since both R_0 and κ^2 are probably larger, assuming a highly efficient transfer between s- and f-chromophores of both C-phycocyanin and allophycocyanin. A lower limit of about 2.0 nm is also realistic in view of recent structural investigations on C-phycocyanin trimers [48].

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