Biochimica et Biophysica Acta, 501 (1978) 246–256

BBA 47447

PICOSECOND TIME-RESOLVED ENERGY TRANSFER IN *PORPHYRIDIUM CRUENTUM*

PART II. IN THE ISOLATED LIGHT HARVESTING COMPLEX (PHYCOBILISOMES)

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(Received June 28th, 1977)

Summary

The transfer of excitation energy between phycobiliproteins in isolated phycobilisomes has been observed on a picosecond time scale. The photon density of the excitation pulse has been carefully varied so as to control the level of exciton interactions induced in the pigment bed. The 530 nm light pulse is absorbed predominantly by B-phycoerythrin, and the fluorescence of this component rises within the pulse duration and shows a mean 1/e decay time of 70 ps. The main emission band, centred at 672 nm, is due to allophycocyanin and is prominent because of the absence of energy transfer to chlorophyll. Energy transfer to this pigment from B-phycoerythrin via R-phycocyanin produces a risetime of 120 ps to the fluorescence maximum. The lifetime of the allophycocyanin fluorescence is found to be about 4 ns using excitation pulses of low photon densities (10^{13} photons $\cdot$ cm^{-2}), but decreases to about 2 ns at higher photon densities. The relative quantum yield of the allophycocyanin fluorescence decreases almost 10 fold over the range of laser pulse intensities, 10^{13}–10^{16} photons $\cdot$ cm^{-2}. Fluorescence quenching by exciton-exciton annihilation is only observed in allophycocyanin and could be a consequence of the long lifetime of the single exciton in this pigment.

Introduction

In red algae, such as *Porphyridium cruentum*, the light harvesting accessory pigments of photosynthesis are water soluble phycobiliproteins contained within distinct prolate structures. These structures, first observed by Gantt and Conti [1] have been named phycobilisomes and are seen to be attached to the outer surface of the thylakoid membranes, which in turn contain the

chlorophyll *a* and carotenoids of the two photosystems, Photosystem I and Photosystem II. The transfer of excited state energy from the phycobilins to the reaction centre chlorophylls and the mechanism of the control of its distribution to Photosystems I and II presents a challenging problem, which we have undertaken to investigate on a picosecond time scale (see part I).

Gantt and Lipschultz were the first to isolate native phycobilisomes from *P. cruentum* and show that they retained their in vivo structure [2]. The isolation of phycobilisomes from another red alga, *Rhodella violacea* has also recently been reported [3].

After detachment from the thylakoid membrane the phycobilisomes are found to fluoresce intensely with a maximum near 675 nm [4]. This emission is almost certainly caused by the interruption of energy transfer from the accessory pigments to chlorophyll and represents the fluorescence of the last components of the energy transfer sequence within the light harvesting complex. The identity of the fluorescing species has been discussed by Gantt et al. [5], but their suggestion that a fluorescent form of aggregated allophycocyanin is present would seem less attractive than the suggestion by Glazer and Bryant [6] that allophycocyanin B, found in cyanobacteria and fluorescing at 680 nm, is also present in red algae together with allophycocyanin.

The use of ultra-short pulses of controlled intensity to excite fluorescence from photosynthetic systems gives chlorophyll fluorescence lifetimes which agree well with quantum yields measured under the same conditions [7,8]. The validity of the technique is further demonstrated in this and the accompanying paper, although care must be taken to avoid high photon densities which give rise to exciton-exciton interactions in the pigment bed [9,10,11]. In the present communication we extent our studies with intact *P. cruentum* cells (part I) by an investigation of energy transfer in isolated phycobilisomes.

Materials and Methods

P. cruentum cultures were grown, as previously described (see part I), under continuous moderate white light illumination in a sterile artificial sea water medium [12] kept agitated by bubbling with air. The cells were harvested by centrifugation at 3000 $\times g$ for 2 min. 10—12 day old cultures were used unless otherwise stated.

Phycobilisomes were isolated at 0—4°C by a procedure based on that of Gantt and Lipschultz [2] using 0.5 M sodium potassium phosphate buffer, pH 6.8, and 1% Triton X-100 (Sigma). Two modifications were introduced:

(a) the cells were ruptured by sonication (3—4 times for 20—30 s at 0°C on power setting 5 of a Dawe Soniprobe type 7530A) and spun at 3000 $\times g$ for 2 min to clear unbroken cells and large debris. 1% Triton X-100 was added to the supernatant.

(b) The sucrose gradient step was omitted. The phycobilisomes were isolated as red-purple pellets by centrifugation of the 27 000 $\times g$ supernatant in the 10 $\times$ 10 ml Titanium rotor of the MSE Superspeed 65 at 55 000 rev./min (290 000 $\times g$) for 1 h.

The pellet was washed with the phosphate buffer to remove traces of free phycobiliproteins and then redissolved in the minimum volume of phosphate

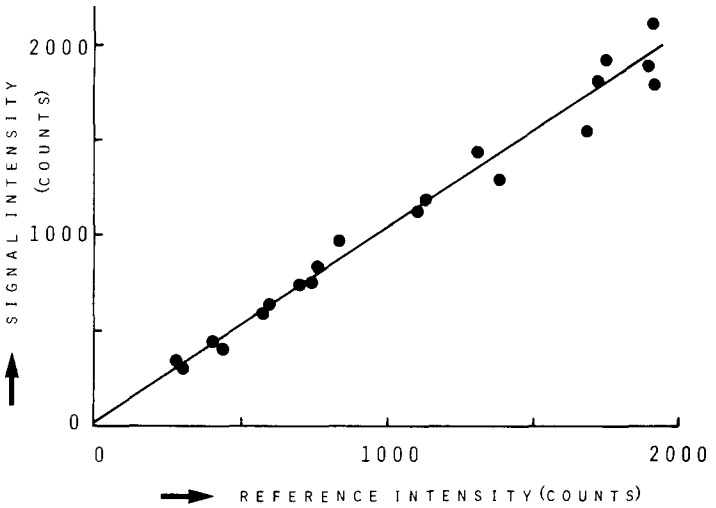

Fig. 1. The linearity of the detection system over the range of integrated intensities used (0—2000 counts/pulse) on the optical multichannel analyser. The signal intensity was varied by means of calibrated neutral optical density filters and the reference intensity corrected for the transmittance of the filter to maintain a signal: reference ratio of unity. The scatter at higher pulse intensities is caused by the inefficiency of the laser beam diffuser, whilst the small residual signal at zero reference intensity is due to thermal noise.

buffer to give a clear nonscattering suspension. The phycobilisomes were stable in the dark at 0°C as a concentrated suspension. All experiments were carried out in the same phosphate buffer (0.5 M sodium potassium phosphate pH 6.8). Phycobiliprotein concentrations were measured using the extinction coefficients of Gantt and Lipschultz [13].

Fluorescence excitation and emission spectra were measured on a Perkin Elmer MPF 3 fluorescence spectrophotometer without applying corrections for photomultiplier (Hamamatsu R446 S) sensitivity, monochromator response or xenon lamp emission. Absorption spectra were measured on an Aminco-Chance DW2 spectrophotometer in the split beam mode.

The lay-out of the picosecond laser apparatus [14] and the experimental arrangement for the observation of relative quantum yields [7] have been described earlier. Single 530 nm pulses, or a mini-train of about 10 pulses, were selected out of the full pulse train, generated by a frequency doubled, mode-locked Nd^{3+} glass laser oscillator, by means of a Pockels cell (see part I). Integration under the fluorescence peaks to obtain relative quantum yields could be carried out by the optical multichannel analyser (OMA). The linearity of the detection system was ±3% between 30 and 3000 counts in any channel of the OMA memory. Fig. 1 shows the excellent linearity of the detector.

Results

Preparation and characterisation of phycobilisomes

The procedure of Gantt and Lipschultz [2] has been modified to make the preparation time shorter without reducing the purity of the preparation. Indeed the minor chlorophyll *a* contamination of the original protocol is

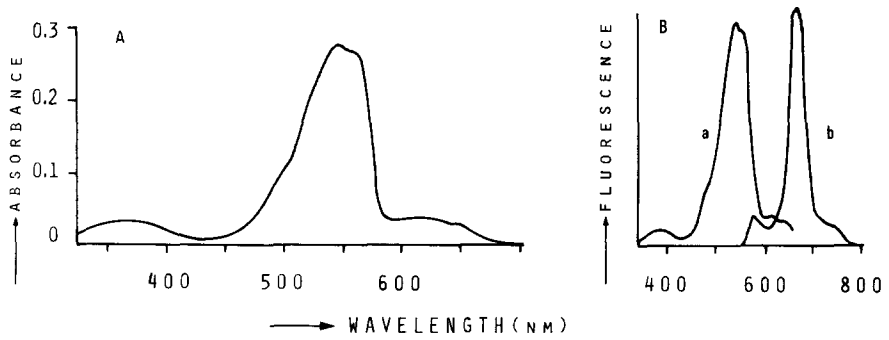

Fig. 2. The spectral characterisation of *P. cruentum* phycobilisomes. (A) The absorption spectrum after dilution into 1 mM sodium potassium phosphate buffer, pH 6.8. (B) The excitation spectrum for emission at 670 nm (a), and the emission spectrum on excitation at 530 nm (b). The concentrated sample was contained in a 1 mm pathlength cuvette and fluorescence was measured by reflection off the front face at 90° to the excitation beam. The excitation and emission bandwidths were 2 nm and 5 nm in (a), and 4 nm and 2 nm in (b), respectively. Fluorescence intensity is on an arbitrary linear scale, and no correction was made for lower photomultiplier response at longer wavelengths.

eliminated as shown by the absence of chlorophyll *a* absorption bands (Fig. 2A). The value of A_{545}/A_{620} was found normally to be about 7.7 indicating a high degree of integrity [2].

Absorption peaks at 545 and 561 nm and the shoulder at 490 nm are attributed to B-phycoerythrin; the peak at 622 nm is due to R-phycocyanin and that at 651 nm is due to allophycocyanin. The existence of b-phycoerythrin in native phycobilisomes [13] does not appear firmly established [15], and therefore the phycoerythrin content will be denoted as B-phycoerythrin in this paper. From the absorption spectrum the proportion of the three pigments can be calculated using the extinction coefficients of Gantt and Lipschultz [13]. B-phycoerythrin is the major pigment comprising 84% of the protein content of the phycobilisomes, whereas R-phycocyanin varied between 10% for 3 day old cultures and 8% for 12 day old cultures. The proportion of allophycocyanin varied between 6% (3 day) and 8% (12 day).

The fluorescence excitation spectrum for emission at 670 nm (Fig. 2B, a) corresponds well with the absorption spectrum indicating that all pigments present can transfer excitation energy to the main fluorescing species.

The uncorrected fluorescence emission spectrum (Fig. 2B, b) shows two peaks, the major emission centred near 672 nm, and a minor emission at 579 nm. This minor band is attributed to B-phycoerythrin. A shoulder at 730 nm is apparent in all preparations even on excitation at 435 nm in contrast to *Rhodella* phycobilisomes [3]. The ratio of the emission peaks (F_{672}/F_{579}) is dependent upon the concentration of the phycobilisome suspension, being 7 to 8 in a concentrated sample. After dilution 100 fold in 0.5 M sodium potassium phosphate buffer pH 6.8, there is a time dependent decrease in this ratio to less than unity, together with a shift of the 579 nm emission maximum to 575—576 nm. As this result indicated a disaggregation of the B-phycoerythrin on dilution of the phycobilisome suspension all fluorescence lifetimes were obtained with concentrated samples.

The main emission, normally seen at about 672 nm, may consist of two over-

lapping bands centred at about 660 and 680 nm and of approximately equal intensities. Some evidence for this is the observation that the emission maximum was variable from preparation to preparation between 669 and 679 nm. An emission at 660 nm is observed in the intact alga (see part I) and is assigned to allophycocyanin, whereas a 680 nm emission could tentatively be attributed to allophycocyanin B [6], or aggregated allophycocyanin [4]. The emission maximum is sometimes found to shift slightly to longer wavelengths on storage of the phycobilisomes at 4°C, and this is particularly noticeable for phycobilisomes prepared from young cell cultures.

The phycobilisomes were studied for fluorescence polarisation using the Perkin Elmer MPF3 with HN22 polarisers, but zero polarisation was found over the main emission band.

Fluorescence risetimes, decays and yields in phycobilisomes

For measurement of fluorescence lifetimes (Fig. 3) the concentration of the phycobilisome suspension was adjusted immediately prior to the experiment to give about 30% transmission at 530 nm in the 1 mm pathlength cuvette. The

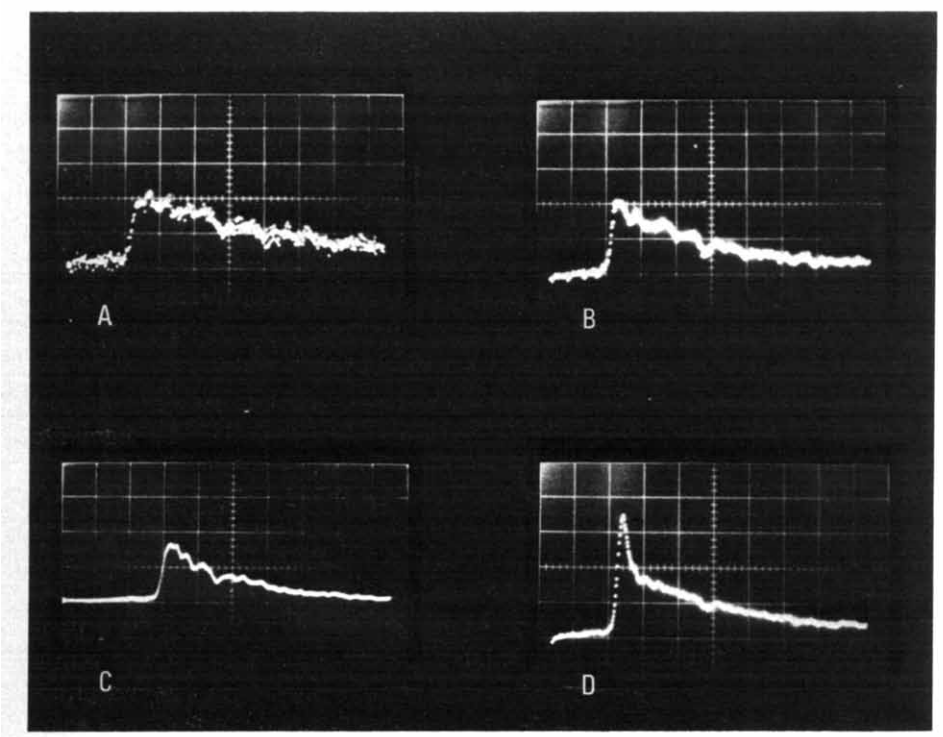

Fig. 3. The decay profiles for the main fluorescence emission of phycobilisomes on excitation by 6 ps laser pulses of varying intensity: (A) $2 \cdot 10^{13}$, (B) $8 \cdot 10^{13}$, (C) $4 \cdot 10^{14}$ and (D) about 10^{15} photons · cm^{-2}. The first three can be fitted within the experimental error to single exponentials with lifetimes of (A) 4.15 ns, (B) 3.27 ns and (C) 1.96 ns. The wavelength selection filter (Scott RG645, 4 mm) has 50% transmission at 650 nm. An upward deflection on the trace represents a fluorescence increase and the calibration of the time scale is 880 ps · cm^{-1} (each major division is 1 cm).

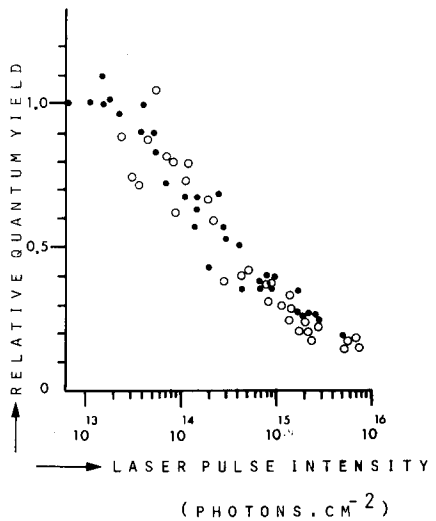

Fig. 4. The dependence of the fluorescence relative quantum yield of phycobilisomes on the laser pulse intensity. The relative quantum yield (the integrated fluorescence intensity expressed relative to the excitation pulse intensity) is taken arbitrarily to be unity at the lowest photon densities. The pulse intensity was varied using calibrated neutral absorbance filters, and further neutral absorbance filters were inserted in the excitation pulse monitoring beam to maintain the excitation and fluorescence peaks on the oscilloscope screen approximately equal in intensity. Closed circles and open circles represent values found for the first and second excitation pulses, respectively. A Schott RG645, 4 mm wavelength selection filter was used.

absorption at 530 nm can be calculated to be 94—96% due to B-phycoerythrin, 3—5% due to the phycoerythrobilin chromophore of R-phycocyanin, and less than 1% due to direct absorption by allophycocyanin. The allophycocyanin emission is therefore predominantly excited by energy transfer from the other pigments.

Fluorescence decay curves obtained for allophycocyanin over a wide range of excitation intensities are shown in Fig. 3. At the lowest intensity employed ($2 \cdot 10^{13}$ photons $\cdot$ cm^{-2}), the decay followed an exp $-kt$ decay law with a 1/e lifetime of about 4 ns (see Fig. 3(A)). For excitation intensities up to $5 \cdot 10^{14}$ photons $\cdot$ cm^{-2} the decay remained exponential, although the fluorescence lifetime decreased by a factor of about two over this range (compare Fig. 3(A—C)). Above $5 \cdot 10^{14}$ photons $\cdot$ cm^{-2}, the curves could not be described by a simple exponential decay law owing to the presence of an extremely rapid initial decay component, as shown in Fig. 3(D) for an excitation intensity of 10^{15} photons $\cdot$ cm^{-2}. A strong dependence of fluorescence lifetime on laser pulse intensity has also been found previously for *Chlorella* [7] and spinach chloroplast Photosystems I and II fractions [8].

A plot of the change in relative quantum yield as a function of the excitation intensity is shown in Fig. 4; this clearly demonstrates the marked changes observed in the fluorescence lifetime with increasing intensity. Over the range of 10^{13} to 10^{16} photons $\cdot$ cm^{-2} the quantum yield decreased by 85—90% from its initial value, and does not appear to reach a constant lower limit at the maximum intensity used in these experiments. Quantum yield values found for the first and second pulses in the train are indistinguishable at the same photon

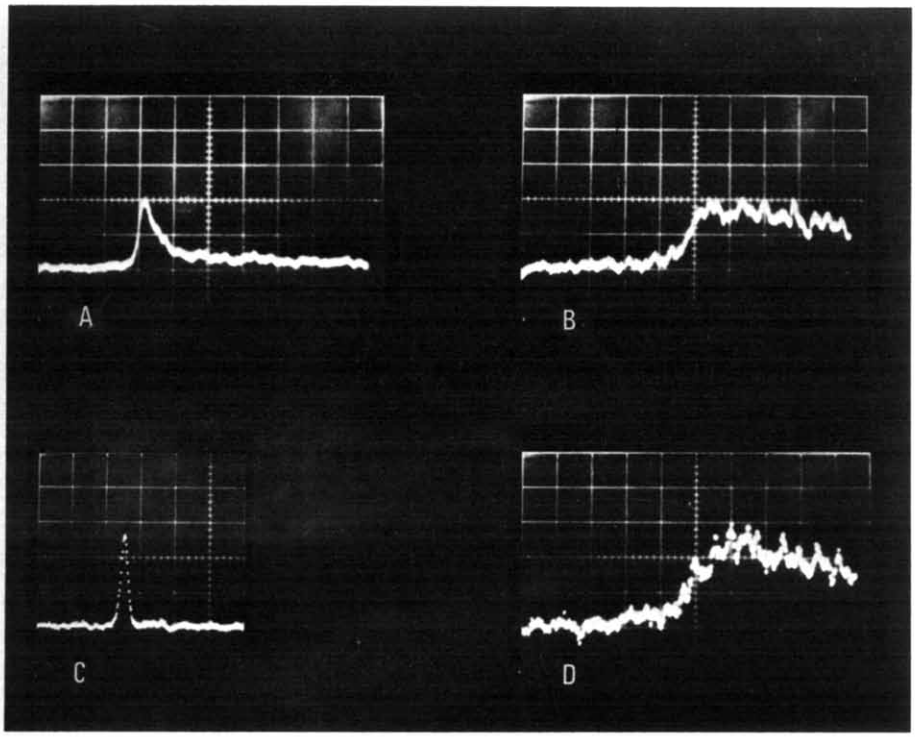

Fig. 5. The real-time observation of energy transfer from B-phycoerythrin to allophycocyanin in phycobilisomes. (A) The rise and decay of B-phycoerythrin fluorescence observed with a Balzer B-40 576 nm wavelength selection filter; (B) The rise of allophycocyanin fluorescence; (C) The laser pulse profile; and (D) the rise of allophycocyanin fluorescence on a shorter time scale. (B) and (D) were obtained with a Schott RG645, 4 mm wavelength selection filter. (A) and (B) were taken on a time scale of 120 ps · cm^{-1}, and (C) and (D) were taken on a timescale of 60 ps · cm^{-1} (each major division is 1 cm). An upward deflection of the trace represents a fluorescence increase.

density, indicating that, unlike chlorophyll *a* in *Chlorella* [7], there is no apparent formation of long-lived quenchers, such as triplet excitons, by the first excitation pulse.

The fluorescence emission from B-phycoerythrin was sufficiently separated from the allophycocyanin emission to be wavelength resolved (Fig. 2B, b). However, any emission from R-phycocyanin would be weak and obscurred by the strong allophycocyanin band which precluded a time-resolved study of this emission. At an excitation intensity of 10^{14} photons · cm^{-2} the fluorescence decay of B-phycoerythrin had a mean 1/e decay time of 70 ps (see Fig. 5(A)). Taking the fluorescence lifetime of B-phycoerythrin in vitro to be 7.1 ns [16] the transfer of energy to the other pigments can be estimated to be 99% efficient (see also part I).

Unlike energy transfer between chlorophyll molecules in green algae or higher plants, energy transfer in phycobilisomes results in a significant change in the emission wavelength as each pigment in the energy transfer sequence receives excitation energy. The time required for energy to be transferred from B-phycoerythrin to allophycocyanin is therefore observed as a risetime in the

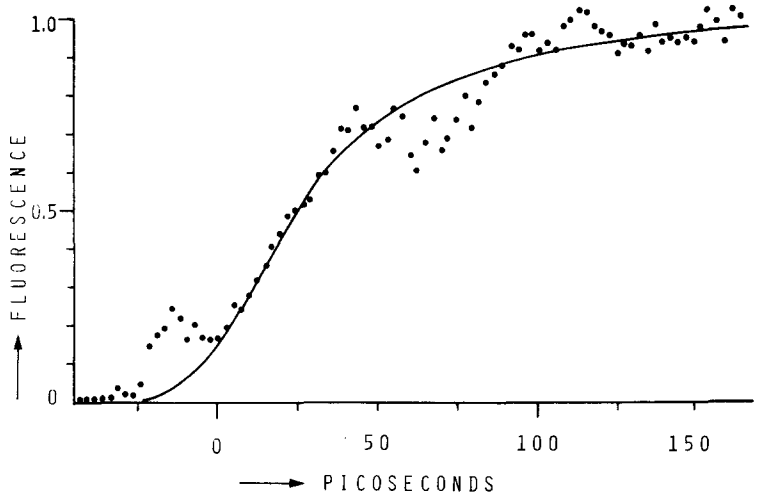

Fig. 6. The analysis of the rise time of allophycocyanin fluorescence in phycobilisomes. The experimentally determined fluorescence intensity in each channel of the optical multichannel analyser is shown by the closed circles, and the solid line is a theoretical curve described in the main text. The asymptote of the fluorescence intensity curve is arbitrarily given a value of unity. The wavelength selection filter was a Schott RG645, 4 mm cut-off filter.

fluorescence emission of the latter pigment. Fig. 5(B) shows the risetime of the allophycocyanin fluorescence emission and the initial portion of the decay at 12 ps time resolution. The risetime is seen more clearly at the maximum streak speed in Fig. 5(D) (6 ps time resolution) and contrasts with the excitation pulse profile shown in Fig. 5(C), recorded at the same streak speed. These traces were obtained at excitation intensities of less than 10^{14} photons $\cdot$ cm^{-2}; at higher excitation pulse intensities the risetime was much shorter, but this simply results from a marked reduction in the fluorescence lifetime of allophycocyanin at these intensities (see discussion).

In Fig. 6 an analysis is presented of the rise time of allophycocyanin fluorescence shown in Fig. 5(B). The solid line is the normalised curve of the following function after convolution with the streak camera resolution function.

$$L^*(t) \simeq \frac{A_1 \cdot A_2 \cdot J_0^*}{A_2 - A_1}\left[\frac{(1 - \exp - 2A_1 t^{1/2})}{A_1} - \frac{(1 - \exp - 2A_2 t^{1/2})}{A_2}\right] \cdot \exp - kt$$

Where L* (t) is the allophycocyanin excited state population at time t, J_0^* is the initial excited state population of B-phycoerythrin at $t = 0$, and A_1 and A_2 are rate constants for energy transfer from B-phycoerythrin to R-phycocyanin and from R-phycocyanin to allophycocyanin respectively, and k is the exponential decay constant of the unquenched allophycocyanin fluorescence. The values of these constants were found to be: $2A_1 = 0.26$ ps$^{-1/2}$, $2A_2 = 0.48$ ps$^{-1/2}$ and $k = 2.5 \cdot 10^{-4}$ ps^{-1} (see part I). The above expression was derived from Eqn. 3 of part I by setting the rate constant A_3 to zero. This will apply when allophycocyanin can no longer transfer energy to chlorophyll a so that it can only lose energy by fluorescence or intersystem crossing. This approximation will give a reasonably accurate description of the function L* (t), provided that the fluo-

rescence lifetime of allophycocyanin is much longer than those of the preceding pigments.

The experimental points represent the number of counts per channel on the 500-channel multichannel analyser, and it is seen that in spite of the scatter the experimental values conform well to the theoretical curve.

Discussion

Light harvesting pigments in photosynthetic organisms are able to funnel light energy in the form of excited singlet states of pigment molecules to the reaction centres of the photosynthetic units. In order to perform this function efficiently the pigments must be in a highly ordered array which will allow energy transfer by inductive resonance but preclude significant energy losses due to concentration quenching (see for example ref. 17).

In the red algae, past work has indicated that the phycobilins form a light harvesting pigment array passing excited state energy very efficiently via allophycocyanin to the photoreaction centre chlorophylls in the chlorophyll *a*/carotenoid pigment bed of the thylakoid membranes (see for example refs. 18 and 19). In contrast to green plants, fluorescence emission from intact *P. cruentum* can be spectrally resolved into the components which represent the energy transfer sequence, and consequently it is possible to analyse each transfer step (see part I).

When the intact light harvesting structures of this organism are isolated from the thylakoid membranes allophycocyanin fluorescence is no longer quenched by energy transfer to chlorophyll and an intense emission results. We have shown that the 1/e lifetime of this emission is about 4ns when observed with picosecond laser pulses of low photon densities. However, in the intact algal cell (part I), the decay of the 660 nm emission due to allophycocyanin is much faster and can be fitted by an exponential with a 1/e lifetime of 118 ps. The shortening of the fluorescence lifetime of allophycocyanin emission in vivo compared to that in isolated phycobilisomes would indicate a high efficiency of energy transfer from allophycocyanin to chlorophyll *a* in the intact alga.

In contrast to allophycocyanin, B-phycoerythrin shows the same rate of fluorescence decay ($\tau_{1/e}$ = 70 ps) in both phycobilisomes (Fig. 5A) and in intact algal cells (part I). This is evidence for the integrity of the energy transfer sequence in the phycobilisomes isolated by our procedure.

The fluorescence lifetime of allophycocyanin in isolated phycobilisomes (4 ns) is probably close to the value to be expected for allophycocyanin in dilute aqueous solution (as yet not reported) because lifetimes for the other phycobiliproteins, phycoerythrin and phycocyanin, have been reported to be 7.1 ns (quantum yield 0.85) and 1.8 ns (quantum yield 0.53) respectively [16]. This would indicate the virtual absence of concentration quenching for this pigment. Therefore phycobilisomes appear to meet two essential criteria for a light harvesting system: absence of concentration quenching and efficient energy transfer. Structural studies by Gantt and co-workers [5,20] have already begun to demonstrate how the phycobiliproteins aggregate in a highly specific manner to form the phycobilisome.

It is interesting to compare these results for phycobilisomes with data for

the accessory light harvesting pigment complex of green plants. The spinach chloroplast accessory pigment complex, free of reaction centres, has been isolated as the F_{III} fraction of Wessels [21] and has been found to have a room temperature fluorescence lifetime of 4 ns (Barber, Porter, Searle and Tredwell, unpublished results). This is close to the lifetime of a dilute solution of chlorophyll *a* (5.5 ns, ref. 16) indicating that these chlorophyll molecules are also organised so as to minimise concentration quenching. The fluorescence decay curves of F_{III} appear to follow an exp $-kt$ decay law, and the 1/e lifetime shortens as the laser pulse intensity is increased. Allophycocyanin in phycobilisomes and chlorophyll in F_{III} therefore have similar fluorescence decay characteristics, which appear to be related to their roles in light harvesting pigment systems.

The intensity dependence of the relative quantum yield of allophycocyanin in phycobilisomes appears to be analogous to the changes previously observed in *Chlorella pyrenoidosa* [7]. If singlet exciton annihilation were a significant factor in determining the decay kinetics and quantum yield of energy transfer in B-phycoerythrin and R-phycocyanin, the quantum yield of fluorescence from allophycocyanin should show a marked decrease, but no change should be observed in the fluorescence lifetimes. In practise the allophycocyanin fluorescence lifetime decreases with the quantum yield, implying that within the intensity range of 10^{13} to 10^{15} photons $\cdot$ cm^{-2} singlet exciton annihilation in the preceeding pigments is not a significant factor. This is confirmed by the observation that the mean fluorescence lifetime of B-phycoerythrin ($\tau_{1/e}$ = 70 ps) remains constant within this range. It appears that only a few migrating jumps are required to transfer energy from one pigment to the next, thus reducing the probability of exciton-exciton interaction within these pigments. However, once the excitation energy is transferred to allophycocyanin, which has a long fluorescence lifetime, singlet excitons must make many migrating jumps until the energy is emitted as fluorescence or quenched by some other process. Consequently the probability of exciton-exciton annihilation in this pigment should be much higher.

Although strongly quenched by exciton-exciton annihilation, the fluorescence decay law of allophycocyanin remains exponential within experimental error up to 50% quenching (see Fig. 3(A, C), and it is only past this point that the decay law becomes more complex (see Fig. 3(D)). Similar observations have been made for chlorophyll *a* fluorescence in liposomes and vesicles [22]. At low excitation intensities the concentration of excitons is such that a very large number of migration jumps would be required to bring two excitons within the interaction distance. Consequently, the non-exponential transfer kinetics are time averaged and will not be apparent in the fluorescence decay kinetics. As the excitation intensity is increased the migration distances between excitons would be decreased and the non-exponential behaviour is then observed (see Fig. 3(D)). Upon generating a large exciton population, annihilation will proceed rapidly as shown by the initial decay rate in Fig. 3(D), but as the exciton population is depleted the decay rate would slow down. Eventually the exciton population will be comparable to that of Fig. 3(C) and the decay law will become exponential. The suggestion by Katz and co-workers [23] that an initial rapidly decaying component could be explained by lasing of photosyn-

thetic pigments at high photon densities must be considered unlikely as this would not result in the observed decrease in relative quantum yield noted both here in phycobilisomes and previously in *Chlorella* [7].

From the fluorescence trace shown in Fig. 5(D), the risetime of the allophycocyanin emission to its maximum intensity is seen to be approximately 120 ps. Since the fluorescence lifetime of allophycocyanin is so long, this rise corresponds to the time required for all of the energy to be transferred from B-phycoerythrin, via R-phycocyanin, to allophycocyanin. The time for the rise to the maximum fluorescence will be determined by two factors. Energy transfer from B-phycoerythrin and R-phycocyanin determines the rate at which excitons are delivered to allophycocyanin, and fluorescence emission principally determines the rate at which the exciton population is depleted. At high excitation intensities the rise time appears more rapid (not illustrated), as a result of the marked increase in the fluorescence decay rate of allophycocyanin under these conditions. The derivation of the kinetic equation used above is based upon Birks' treatment of donor:acceptor fluorescence emission functions [24], although it is still not clear whether the form of these equations indicates the validity of Förster-type energy transfer [25] in these systems.

Acknowledgements

This work was supported by the E.E.C. Solar Energy Research and Development Programme, the Science Research Council and by the award of a Ministry of Defence fellowship to C.J.T. The technical assistance of Elizabeth Dibb is greatly appreciated.

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