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ON THE ORIGINS OF 718 nm FLUORESCENCE FROM *PORPHYRIDIUM CRUENTUM* AT 77 K

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Summary

Emission spectra and transient behavior of fluorescence in *Porphyridium cruentum* have been studied in search of the pathway of excitation energy from the phycobilisome to Photosystem I (PS I) of photosynthesis. For activating light at 436 nm, absorbed almost entirely by chlorophyll, fluorescence is dominated by the 718 nm band generally attributed to chlorophyll of PS I. Activating light at 550 nm, absorbed mostly by the phycobilisome, gives rise to the distinctive fluorescence band of PS II chlorophyll at 696 nm but also gives a large component at 718 nm. Analysis depends critically upon the source of emission at 718 nm under 550 nm activation: does it arise from PS I or PS II? Ley and Butler (Ley, A.C. and Butler, W.L. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 3956—3960) have proposed that the 718 nm arises mostly from PS I, to which it is transferred by spillover from PS II. We suggest a different proposition: that under 550 nm activation most of the 718 emission arises from PS II. Analysis shows that this proposition provides an alternative explanation. Using the small change in fluorescence yield observed under 436 nm activation as a monitor of excitation in PS I, we provide evidence that under 550 activation most of the 718 nm fluorescence arises from PS II.

Introduction

A special problem in energy transfer arises in the phycobilisome-containing algae. Of the excitation energy in the phycobilisomes, fractions approaching

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Chl, chlorophyll; PS, photosystem; A^{436}, activation at 436 nm.

1/2 must be transferred to each of the two kinds of reaction centers for photosynthesis [1,2]. We are seeking evidence for pathways of this transfer by observations of low-temperature fluorescence. Initially our study was based upon the blue-green alga, *Anacystis nidulans*, for which we had action spectra for the two photoreactions [3]. Subsequently we turned to the red alga, *Porphyridium cruentum*, which has the advantage of greater spectral discrimination between chlorophyll (Chl) and phycobilisome absorption.

Ley and Butler (Refs. 1, 2, and personal communications) have analyzed the low temperature fluorescence of *P. cruentum*. They have derived a model, simplified from the tripartite model [4], which describes the pathway of excitation energy: phycobilisome → Chl II → Chl I [1,2]. Most of our observations confirm those of Ley and Butler [1,2] but have led to recognition that their model is not a unique explanation of the data. Herein we will present our observations, interpret them in a simple framework, and then present some evidence which supports our framework in preference to that of Ley and Butler [1,2].

Materials and Methods

Our microcomputer-controlled fluorometer was constructed from two Bausch & Lomb 500 mm grating monochromators arranged for 90° excitation-emission. For scanning of emission spectra the wavelength was advanced by motor drive at 1 nm steps with pause at each step for 1 s of photon counting by a cooled photomultiplier. For fluorescence transients initial sampling time intervals were chosen at one of the following: 1/60, 2/60, 4/60, up to 32/60 s; sampling times were doubled after every 40th interval and a total of 152 sampling intervals used for each transient. All data were stored locally and later transferred to a central computer for analysis.

For measurements at 77 K the excitation beam (8.0 nm HBW) was deflected upward upon the sample through the optically flat bottom of a Dewar flask. Fluorescence from the front surface was deflected by a second mirror to the emission monochromator (3.3 nm HBW).

Porphyridium cruentum, strain UTEX 161 [5], was grown in an ASP-2 medium [6] in 20 mm test tubes, aerated with 2% CO_2 in air, held at 20°C, and illuminated by cool white fluorescent lamps to give a specific growth rate of about 0.3 per day. Whole cell absorption spectra were similar to those reported by Ley and Butler and had absorbance ratios A_{678}/A_{550} close to one. A culture was harvested at desired absorbance (A = 0.3 to 0.6 at 550 nm), concentrated, and mixed with an equal volume of growth medium containing 20% poly(ethylene glycol) (Carbowax mol. wt 4000, extracted with diethyl ether to remove quinone impurities). A drop of this cell mixture was placed on the flat underside of a thin-wall aluminum can. A piece of nylon mesh was added as a spacer and the drop then was covered with a film of polypropylene to give a 0.3 mm layer of cell suspension. The final absorbance of the sample (per 0.3 mm) was estimated at 0.07 for 678 nm. (Both the nylon mesh and polypropylene were soaked in 5% disodium EDTA for 24 h before use.) The aluminum can was placed in the Dewar flask with sample facing downward and held above the bottom by a metal ring. The sample was then held in darkness

at room temperature for 5 min to give dark-adapted cells. On pouring liquid nitrogen into the aluminum can the sample was frozen to 77 K in less than 10 s as estimated by the time course of the fluorescence increase [7]. As judged by fluorescence intensities, reproducibility of cell quantity in replicate samples was about ±10%.

Results and Discussion

Fig. 1 shows fluorescence emission spectra for 550 and 436 nm activation (A^{550} and A^{436}) obtained at the F_M level after several minutes of actinic light. Actual wavelength positions of the peaks varied a few nm from day to day and we use nominal wavelength designations. Small phycocyanin (644 nm) and allophycocyanin (663 nm) bands are evident only at A^{550} where phycoerythrin of the phycobilisome is the major absorber. We take the 696 nm band (693—696 nm) as a component of Chl II and 718 nm band (715—718, A^{436}) as a component of Chl I. A considerable literature [1,7,8] supports the designation of the 718 and 696 nm bands as qualitative markers for Chl I and Chl II fluorescence. However, for present purposes we wish to estimate densities of excitation energy in Chl I and Chl II in terms of fluorescence yields. Hence, we need additional information to estimate purity of the two components or, ideally, to deconvolute the spectra into their components.

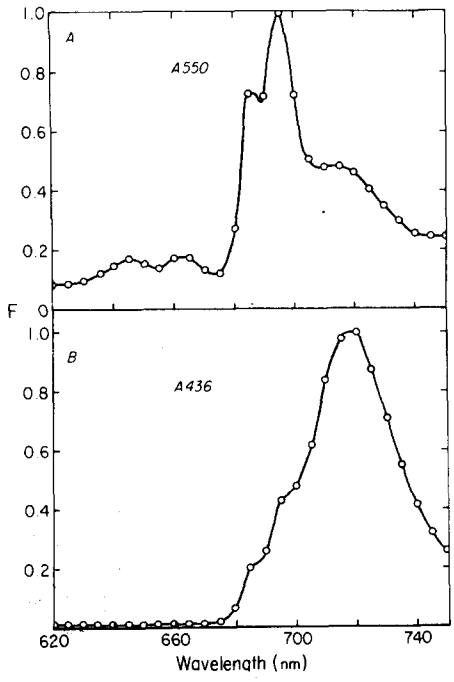

Fig. 1. Fluorescence emission spectra of *Porphyridium cruentum* obtained with cells frozen to 77 K after 5 min in darkness and then illuminated to give the F_M level. Exciting light was 550 nm (A) and 436 nm (B). Emissions measured as photon counts were corrected for spectral response to give normalized fluorescence intensities on a quantum basis. Spectra show data points at 5 nm intervals but were constructed from data at 1 nm intervals.

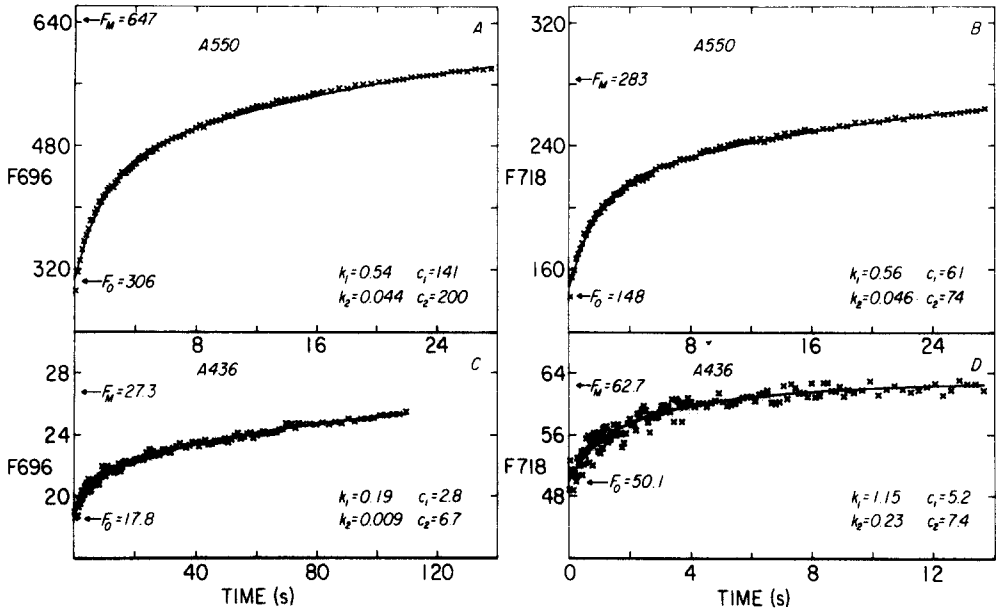

Fig. 2. Fluorescence transients observed at 696 and 718 nm under 550 and 436 nm excitation of cells frozen to 77 K and 5 min darkness. Each transient was analyzed by the equation $F = F_0 + c_1[1 - \exp(-k_1 t)] + c_2[1 - \exp(-k_2 t)]$. The variables F_0, c_1, k_1, c_2, k_2 were adjusted by computer program to give least sum of squares of errors between data points and the curve. Fluorescence was measured in 10^3 photon counts per s and was not adjusted for instrumental spectral response.

Additional information about the fluorescence components can be obtained from the fluorescence transients observed at 77 K (Fig. 2). Our analysis in terms of two exponentials is entirely operational and implies no assumption as to mechanism. It is sufficient to provide reasonable fit and comparisons in terms of the several parameters. For A^{550} the transients of F_{696} and F_{718} are closely similar in both time constants and relative magnitude of the c_1 and c_2 components. (Any preillumination before freezing increases the time constants under A^{550}.) Certainly the simplest interpretation is that for A^{550} most of the F_{718} and F_{696} arise from a single emitter which monitors Chl II. Conversely, at A^{436} the F_{696} and F_{718} have very different transient parameters. Evidently some of the F_{696} arises from a source (in Chl II) other than the emitter which monitors Chl I at F_{718}.

In order to compare the A^{550} transients more carefully, we constructed Fig. 3 from two data sets of Fig. 2 to give a plot similar to that of Ley and Butler (Ref. 2, Fig. 2). The plot describes F_{718} vs. F_{696} as fluorescence is increased from the F_0 to the F_M level by closing of PS II and PS I traps. There is some uncertainty in absolute slope since F_{718} and F_{696} were measured on replicate samples subject to about 10% variability in cell quantity. However, the features of Fig. 3 which we shall use are not affected by this uncertainty.

The small initial curvature of the F_{718} vs. F_{696} plot (Fig. 3) is ascribed to a small increase in fluorescence yield of Chl I discovered by Ley and Butler [2] and attributed by them to the closing of PS I traps. The curvature may be eliminated by a prior exposure to A^{436} (as in Fig. 2D). In Fig. 3 the effect is

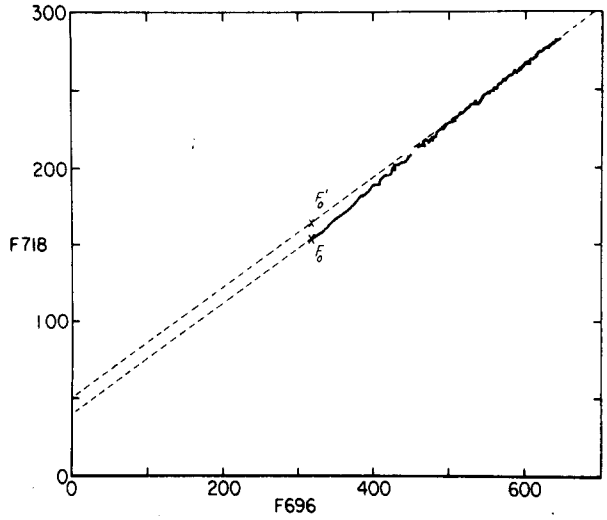

Fig. 3. F_{718} vs. F_{696} under A^{550}. Computed from the data sets of Fig. 2A and 2B. See text.

eliminated by extrapolation; this gives an intercept for F_{718} of 50 units compared to 283 units at F_M. The simplest interpretation is that the intercept value of 0.18 F_M represents a Chl I component and that the remaining 0.82 F_M represents the Chl II component at 718 nm.

Table I sets forth our analysis of the spectra of Fig. 1 for the marker wavelengths 696 and 718 nm. Our basic assumption is that under A^{436} (or any other wavelength) the variable fraction of F_{696} measures the fraction of F_{696} arising from Chl II. We start from the approximation (later to be reexamined) that under A^{550} the total F_{696} arises from Chl II. From Fig. 2A for F_{696} under A^{550} we take $F_v/F_M = 0.53$. For F_{696} under A^{436} (Fig. 2C) $F_v/F_M = 0.35$. Hence, under A^{436} a fraction $0.35/0.53 = 0.66$ of F_{696} arises from Chl II. For F_{718} under A^{550} we use the result of Fig. 3 to ascribe a fraction 0.82 to Chl II. Of the 48 relative units of F_{718} under A^{550} (Table I) 39 units are assigned to Chl II. Chl II fluorescence is now described by a ratio $F_{718}/F_{696} = 0.39$ (close to the slope, 0.37, for the upper curve of Fig. 3). The same ratio should describe the Chl II components under A^{436} ($F_{718}/F_{696} = 0.39 = 11/29$); this gives a fraction 0.11 of F_{718} ascribed to Chl II. The ratio $F_{718}/F_{696} = 6$, derived for Chl I under

TABLE I

ANALYSIS OF THE SPECTRA OF FIG. 1

Relative contributions from Chl I and Chl II are derived for the marker wavelengths 696 and 718 nm under 550 and 436 nm activation.

	A^{550}		A^{436}	
	F_{696}	F_{718}	F_{696}	F_{718}
Chl I	(1.5)	9	15	89
Chl II	(98.5)	39	29	11
Total	100	48	44	100

A^{436}, may be reiterated to F_{696} under A^{550}; the resulting values in parentheses are so close to the initial approximation (Chl I = 0, Chl II = 100) that no further iteration appears warranted.

Except on one point (see below) our experimental data are in essential agreement with those of Ley and Butler [Refs. 1, 2 and personal communications]. Further, the data can be interpreted equally well by their model or by our analysis (Table I). However, the essential assumptions are very different. Ley and Butler do not accept the assumption that almost all of the variable fluorescence at 718 nm under A^{550} arises from Chl II. On the contrary, they hold that under A^{550} the F_{718} arises mostly (as 70%) from Chl I. Their model proposes that (1) at A^{550} almost all (>90%) absorbed quanta are absorbed by the phycobilisome, (2) that energy is transferred from the phycobilisome exclusively to Chl II, and (3) that there is an energy transfer Chl II → Chl I with a probability $\phi_{T(II\to I)}$. Their spillover probability, $\phi_{T(II\to I)}$ must be sizeable (roughly 0.5) at the F_o level when PS II traps are open and must increase to a much higher value (as 0.9) at the F_M level when all PS II traps are closed.

A second area of difference between the two models lies in the implied fluorescence yields. The Ley and Butler model requires that the fluorescence yield for F_{718} in Chl I is substantial and not less than about 1/2 that of F_{696} in Chl II (the actual F_{718}/F_{696} ratio under A^{550} as in Fig. 1A). On the contrary our model requires that the yield of F_{718} in Chl I is only about 1/10 of the yield of F_{696} in Chl II.

Up to this point we have sought to show that for explanation of F_{718} our model provides an alternative to that of Ley and Butler. We now consider further the small increase in fluorescence yield caused by closing of PS I traps and use it to obtain evidence in support of our model. In the Ley and Butler model the increase in fluorescence yield of Chl I at F_{718} caused by a short A^{436} exposure should give a nearly proportional increase in F_{718} under a subsequent A^{550}. In our model a short A^{436} exposure will affect only the part (about 1/3) of the F_{718} arising from Chl I under A^{550}. We cite two kinds of experiments.

In the experiment of Fig. 3 a prior exposure to A^{436} gives rise to a linear plot which extrapolates to the same intercept (cf. also Ref. 2, Fig. 2). In effect the A^{436} exposure raises the initial point from a value of F_o to a higher value, F'_o (designations of Ley and Butler). For Fig. 3 the $F'_o/F_o = 165/155 = 1.07$. Extrapolation of both F_o and F'_o to zero F_{696} gives a $F'_o/F_o = 50/40 = 1.25$. (Actually the extrapolation should be made to the line $F_{718}/F_{696} = 6$, characteristic of Chl I, but the difference is trivial.) It is only the extrapolated value which matches $F_M/F_o = 1.26$ observed for F_{718} at A^{436} (Fig. 2D). These results require that of the F_{718} under A^{550} only a small (about 1/3 at the F_o level) fraction arises from Chl I.

In a second kind of experiment we observed the effect of a short A^{436} exposure upon the F_{718} under A^{550} (Fig. 4). A fluorescence transient for F_{718} was segmented into a series of exposures to A^{436} or A^{550}. Each exposure lasted 9 s and there was a 2.5 min dark time between exposures. Fig. 4A shows the time course of F_{718} under three successive exposures to A^{436}. In order to estimate extent of back reactions during the 2.5 min dark periods, the A^{436} was left on after the third exposure until the F_M level was reached. Thereafter five successive exposures were obtained, each preceded by 2.5 min dark; averaged

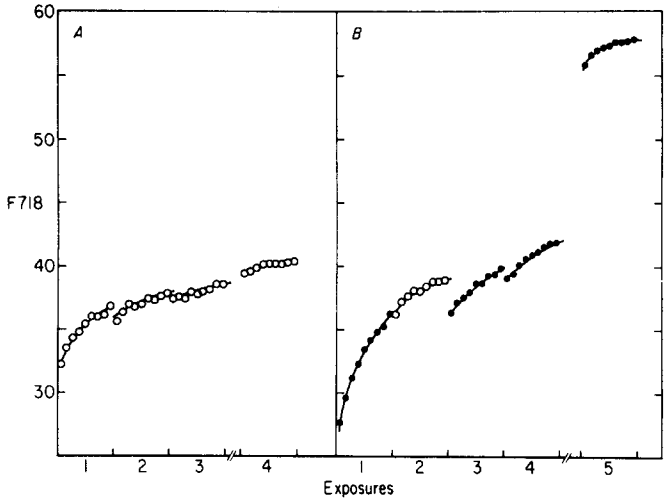

Fig. 4. The fluorescence transient at 718 nm observed in segmented exposures to A^{436} (oooo) and A^{550} (●●●●) as described in the text. Exposures lasting 9 s were separated by 2.5 min dark periods. Intensities of A^{436} and A^{550} were preadjusted to give about equal F_{718} after one A^{550} exposure. After reaching the F_M level, a series of 9 s exposures were given following dark periods of 2.5 min (A) or 5 min (B); data shown as averages of 5 repetitions provide a measure of dark decay. Units of the ordinate are in 10^3 photon counts per s.

values are shown as a fourth segment. The 2.5 min dark decay was estimated as 1.0 unit or 10% of the total F_v of 9.4 units. When this correction is applied, data for the first three exposures of Fig. 4A give a single continuous transient. Fig. 4A establishes that the first A^{436} exposure is sufficient to induce about 1/2 of the total F_v attainable under A^{436}.

Fig. 4B shows the time course of F_{718} under one A^{550} exposure, then a A^{436} exposure, and then two A^{550} exposures. The dark decay of F_{718} for 5 min darkness is shown as a fifth segment; it was estimated as 2.0 units or 7% of the total F_v of 30.2 units. The A^{436} exposure has almost no effect on the A^{550} transient. However, we must consider whether this could be a trivial result of the procedure. A small loss due to dark decay after the first A^{550} exposure is estimated at 0.6 units (7% of the 8.7 units F_v). There also should be some gain due to the fraction of F_{718} under A^{550} which arises from Chl I. During the A^{436} exposure the F_{718} increase of 7% ($\Delta F/F$) is attributed entirely to Chl I. If, as viewed by A^{550}, 1/3 (9 units) of the F_o level arises from Chl I, then the gain to be expected from the A^{436} exposure is 0.6 unit. The expected gains and losses are small and so nearly equal that the F_{718} transient is blind to an intercalated A^{436} exposure. Again the result requires that under A^{550} the F_{718} arises mainly from Chl II and only a small fraction arises from Chl I.

Conclusion

Most of our observations and those of Ley and Butler are consistent with their spillover model. The data are also equally consistent with our simple assumption that almost all of the variable fluorescence at 718 nm controlled by PS II traps arises from Chl II.

Observations are not in agreement on the effect of an increase in fluorescence yield of Chl I brought about by closing of PS I traps. Ley and Butler found the effect (F'_o/F_o for an unmonitored far-red light exposure) was independent of activating wavelength. They also found [9] that, for cells treated with DCMU and NH_2OH, the oxidation of *P*-700 in a frozen sample was more rapid if the sample had been illuminated before freezing. Both of these observations were taken as evidence for the controlled spillover feature of their model.

We have explored more carefully the variable fluorescence at 718 nm and have used the F'_o/F_o effect to measure delivery of excitation energy to PS I. We found the F'_o/F_o effect much smaller for A^{550} compared to A^{436}. Our result speaks against any large spillover of excitation energy PS II → PS I controlled by photochemical trapping in PS II.

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