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Light Harvesting in *Anacystis nidulans* Studied in Pigment Mutants[1, 2]

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ABSTRACT

Spontaneous pigment mutants of *Anacystis nidulans* were self-selected for improved growth in far red light (> 650 nanometers). Questions were asked about those features of the light-harvesting mechanism which altered to give the mutants improved photosynthetic performance in far red. Answers were sought by comparing pigment and reaction center concentrations for the parent and six mutants grown in gold fluorescent and in far red light. Three significant results emerged. The ratio of reaction centers for photoreactions I and II (RC1/RC2) varied by a value of about 2.1 for all clones grown in gold and a value of about 1.1 for all clones grown in far red. Alteration of the ratio was not evident in any of the mutants.

Phycobilisome alterations were evident as decreased phycocyanin content in all mutants. In three mutants, allophycocyanin became the major remaining phycobilisome component. Action spectra for photoreactions I and II allowed estimates of chlorophylls serving each of the two reaction centers. Ratios of chlorophylls to reaction centers within each photosystem were chlorophyll I/RC1 = 118 ± 11 and chlorophyll II/RC2 = 52 ± 9 for all seven clones grown in both gold and far red light. Remarkable constancy of these ratios, in spite of wide variation in cell material, supports an hypothesis that in *A. nidulans* there are two chlorophyll proteins, each bearing a reaction center and chlorophylls in fixed ratio.

For functional studies of photosynthetic pigments, we have used the cyanobacterium (cyanophyte) *Anacystis nidulans*. A useful feature of the organism is that its pigment composition can be manipulated to obtain wide variations. Pigment mutants were obtained by nitrosoguanidine treatment which had either lowered Chl or lowered PC[3] content (20, 23). Unfortunately, these proved to be unstable and all have been lost. Pigment variation also is obtained in FR light (> 650 nm) in which *A. nidulans* grows poorly and develops a low Chl content (8, 12). During continued culture over many generations in FR light, we observed increases in growth rate and in the Chl/PC ratio, usually occurring in a concomitant and stepwise fashion. From such cultures, we were able to establish clones which differed from the parent in pigment composition. We have described six clones, which are presumed to be spontaneous mutants selected under the photosynthetically restrictive condition of FR illumination (13). As compared to the parent, all mutants showed better performance (growth) under FR light for which Chl is the principal absorber but poorer performance under gold fluorescent light for which PC is the principal absorber.

For the report presented here, we have used the parent and six mutants grown both in gold fluorescent and in FR light to provide a wide range of comparative material. To this material, we apply a functional analysis of pigments based upon three kinds of measurements: (*a*) cellular concentrations of Chl and PC, (*b*) cellular concentrations of reaction centers for photoreactions I and II, and (*c*) action spectra for photoreactions I and II.

MATERIALS AND METHODS

Cell Material. *A. nidulans*, strain Tx20 of our laboratory, is considered identical to *Synechococcus* sp. PCC6301, ATCC27144 (18). Six putative mutants of Tx20, cloned from selection cultures grown for many generations in FR light, have been described (13). Cells for study were grown in test-tube cultures at 39 C in a modified medium D under 1% CO_2 in air and harvested during exponential growth (12). Illumination for growth was provided by two sources: (*a*) GE gold fluorescent lamps described as having about 500 to 750 nm total bandwidth with a HBW of about 80 nm centered at 590 nm or (*b*) tungsten-halogen lamps with Corning No. 2030 filters to give a FR > 650 nm. The ratio of probabilities of absorption (PC/Chl) is about 3.5 for gold and about 0.06 for FR. Intensities of the two sources gave about equal growth rates for Tx20. We emphasize that, for purposes at hand, FR has the special meaning that it does not contain significant energy below 650 nm. For example, even light obtained via an interference filter of 10 nm pass band centered at 650 nm will not significantly distort the pigmentation of Tx20 (12).

Pigment Concentrations. Pigment contents referred to cell volumes were obtained from the following measurements. Cell volume was determined by centrifuging an aliquot of cell suspension at 2,500*g* for 1 h in tubes with lower section of 1 mm bore capillary. Chl was extracted in cold 80% acetone and estimated spectrophotometrically using an absorption coefficient of 82.0 $(g/l)^{-1}$ cm^{-1} at 663 nm. Whole cell spectra were obtained with a Cary 14 spectrophotometer using 3-mm light-scattering plates of translucent Plexiglas (Rohm & Haas 7328) inserted in 10-mm cuvettes. *A* so obtained were corrected by subtraction of residual *A* at 720 nm. We did not apply any further correction for particle flattening which, because of the small cell size, would not have been greater than 1.08 at peak wavelengths (17). PC and Chl were calculated from corrected *A* at 625 and 678 nm by the simultaneous equations (12):

$$A_{625}^{PC} = 1.0162\,A_{625} - 0.2612\,A_{678}$$

$$A_{678}^{Chl} = 1.0162\,A_{678} - 0.0630\,A_{625}$$

PC content was estimated from A_{625}^{PC} in terms of the phycocyanobilin chromophore by absorption coefficient 111 mM^{-1} cm^{-1} (4). Chl was estimated from A_{678}^{Chl} by an absorption coefficient of 68

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[2] To our friend, Bessel Kok, whose genius left an impact upon our discipline which was remarkable in that it was both conceptual and experimental.

[3] Abbreviations: PC, phycocyanin; FR, far red; HBW, half-band width.

mM^{-1} cm^{-1} previously determined by calibration against Chl by extraction (12). The redundancy of two independent measurements of Chl is purposeful and both estimates will be cited.

Reaction Centers. Concentrations of reaction centers for photoreaction I (RC1) were obtained from measurements of P700 by a dual wave length spectrophotometer designed by B. Kok and made by G. Johnson. P700 was estimated from change in A of a whole cell suspension at 702 *versus* 735 nm caused by 30 w/m^2 of 443-nm actinic light (HBW, 65 nm) in the presence of 2 μM DCMU and use of absorption coefficient 70 mM^{-1} cm^{-1} (6). Appropriate checks were made to guard against fluorescence artifacts, actinic effects of measuring light, or insufficient intensity of actinic light.

Concentrations of reaction centers for photoreaction II (RC2) were obtained from flash yields of O_2 evolution measured at 39 C by a Clark-type O_2 electrode. The method was modified from that of a previous report (11). The flash tube was powered by improved circuitry to give flash contours of 3.6 μs to one-third and 8.0 μs to one-tenth peak intensity; its effective intensity was increased by a backing mirror behind the measuring cuvette. Measurements were made with and without a protecting yellow filter (Tiffen No. 12, >500 nm). Some cell material gave higher observed rates without the filter; other cells gave gradually decaying rates without the filter. The routine test of flash saturation was that attenuation by a 25% screen did not reduce flash yield to less than 0.63, *i.e.* $1 - (1/e)$; hence, each flash provided at least 4 quanta/reaction center. Most measurements were made at a flash rate of 20/s with frequent checks that equal yields were obtained at 10/s.

Action Spectra. Action spectra for photoreactions I and II were obtained by a method previously described in detail (22, 23) and summarized as follows. Rates of O_2 evolution were measured as the modulated amperometric signals produced by a thin cell layer on a platinum rate-measuring electrode under modulated light.

In a first series of measurements, the light beam from a monochromator (M1) was chopped at 53 Hz and used to obtain a conventional action spectrum. An absorption spectrum was obtained, using a shallow chamber to mimic the 2 to 5 μm cell layer used on the electrode. The absorption and action spectra allowed calculation of a spectrum for relative quantum yield (*cf.* Fig. 1). The maximum for the quantum yield spectrum was taken as the neutral wavelength, the wavelength at which the two photoreactions have equal actions (22).

In a second series of measurements, the same cell preparation on the electrode was illuminated by actinic beams from two monochromators, M1 and M2. The measuring beam from M1 (3 nm HBW) was chopped at 53 Hz and then combined with a constant background beam (6 nm HBW) from M2 set at the neutral wavelength. The combined beam was modulated at 73 Hz by a second chopper. Amperometric signals were recovered by two lock-in amplifiers, each tuned to one of the chopping frequencies. The signal at 73 Hz measured the total rate of O_2 evolution contributed by the background beam (I_b = 2 to 7 w/m^2 peak to peak) and the increment in total rate caused by addition of the weak measuring beam ($I_m < 0.05\ I_b$). The small increment in total rate was called $\Delta(AC)_b$. The 53-Hz signal measured the direct effect of I_m; after scaling (to units equivalent to those of the 73-Hz signal), it was called $\overline{AC}$.

Our analysis rests on the proposition that action spectra for photoreactions I and II can be obtained by separation of the direct and indirect effects involved in enhancement. The $\overline{AC}$ signal is the direct effect of I_m and, when observed *versus* wave length, gives a photoreaction II spectrum as $\overline{AC}/I_m$. The increment in total rate, $\Delta(AC)_b$, includes both the direct effect and also the indirect effect of I_m in causing a small perturbation in reaction center conditions. For the particular condition of background light at the neutral wavelength, action of photoreaction I is estimated as $[2\Delta(AC)_b - \overline{AC}]/I_m$. The method properly scales the two action spectra to each other and removes contribution of the state I-state II phenomenon (22).

RESULTS

Our results describe the parent Tx20 and six mutants grown in gold and in FR light. Of the 14 sets of spectral data, we present six for illustration of important features. Data derived from action spectra are presented together with other analytical data in Table I.

Action Spectra. Figure 1 shows spectra for relative quantum yield (ϕ). Each was computed from the modulated rate of O_2 evolution divided by incident quantum intensity and by fractional absorption measured on a mimicking cell layer. Maxima for ϕ were used to estimate neutral wavelengths at which there are equal fractions of open reaction centers for each photoreaction. The maxima were not always well-defined at short wavelength, leading to some uncertainty in choice of neutral wavelength, especially for Tx20 grown in gold.

Also shown in Figure 1 are spectra for α' which are obtained from the spectra of Figure 2 as action II/actions (I + II). We have defined α' as the fraction of total electrons transferred by both photoreactions which would be transferred by photoreaction II under the hypothetical condition that all centers are open (22). Under actual conditions, we do not expect to find any wavelength at which all reaction centers are open (7). However, we argue that there is likely to be some wavelength at which there are equal fractions of open reaction centers for each photoreaction ($\alpha' = 0.5$). Quantum yield ϕ will be maximum at this neutral wavelength and ϕ will become less than maximum as α' departs from 0.5.

For the parent Tx20 grown in gold, ϕ is relatively flat over the 600- to 650-nm region where α' remains close to 0.5. However, when grown in FR, Tx20 develops a characteristic low-Chl syndrome. The effect is seen in Figure 1 in terms of an elevated α' and depressed ϕ across the 600- to 650-nm region and a neutral wavelength displaced to 675 nm. As compared to Tx20, all of the mutants grown in FR light showed smaller departures from $\alpha' = 0.5$ across the 600- to 650-nm region of high PC absorption.

Figure 2 presents action spectra normalized to the measured fractional absorptions at the respective neutral wavelengths where actions I and II are equal. We consider first the spectra for Tx20 grown in gold. Action II shows a major PC component at 625 nm and a minor Chl component at 678 nm. Action I shows a major Chl component but also a significant PC component.

It should be noted that actions I and II, and their sum (I + II), are obtained under the special conditions of background light at neutral wavelength which holds reaction center conditions constant and equally open for both photoreactions. The spectrum for summed action (I + II) is different from that of a conventional photosynthetic action spectrum. In the latter case, the contribution of action I is likely to be underestimated as is evident in the pioneer work of Haxo and Blinks (5). We consider it a test of our method that (unless there is inactive pigment absorption) actions (I + II) should match approximately the fractional absorption curve.

Under more careful scrutiny, it will be noticed that, for Tx20 grown in gold (also for 30Y grown in gold), the match between absorption and actions (I + II) is incomplete and asymmetric with respect to the 625- and 678-nm peaks. We have learned from practical experience with the method that such small and opposite errors may arise from improper choice of neutral wavelength. In the present case, it is likely that the neutral wavelength chosen (635 nm) was too short, leading to overestimate for the 678-nm band and underestimate for the 625-nm band. For other spectra of Tx20 grown in gold, 650 nm was chosen as the neutral wavelength. Actually, spectra for Tx20 were not significantly different for cells grown in gold or in tungsten illumination (23).

When Tx20 is grown in FR light, its low Chl condition leads to

Table I. *Description of Parent and Six Mutants*

Clone	Growth Rate	Reaction Centers			Pigments			Distribution from Action Spectra		Pigments to Reaction Centers				
		RC1	RC2	RC1/RC2	Cell Spectra PC	Cell Spectra Chl	Extract Chl	PC in I	Chl in I	PC/RC2	Chl/RC1	Chl/RC2	Chl I/RC1	Chl II/RC2
	μday^{-1}	μM	μM	ratio		mM		%				ratio		
Grown in gold light														
Tx20	2.3	57.3	22.7	2.5	4.71	8.10	8.17	32	86	207	143	360	123	50
30Y	2.5	63.8	29.0	2.2	5.50	8.34	8.92	34	87	190	140	308	122	40
69Y	1.4	64.9	37.8	1.7	(0.83)	9.48	9.53	(37)	78	(22)	147	252	115	55
85Y	1.3	67.4	31.1	2.2	(1.23)	9.50	9.64	(35)	82	(40)	143	310	117	56
49B	2.2	41.3	23.7	1.7	4.80	5.30	5.41	27	80	203	131	228	105	46
59G	1.6	62.8	25.9	2.4	2.32	8.61	8.92	30	84	90	142	344	119	55
19Y	1.0	56.3	31.5	1.8	(0.63)	8.74	8.67	(29)	80	(20)	154	275	123	55
Averages				2.1					82		143	297	118	51
Grown in far red light														
Tx20	2.3	9.1	9.3	1.0	6.33	1.61	1.69	19	60	681	186	182	111	73
30Y	4.6	19.0	17.5	1.1	2.68	3.47	3.16	35	77	153	166	181	128	42
69Y	4.6	25.4	22.4	1.1	(1.15)	4.20	3.60	(42)	70	(51)	142	161	99	48
85Y	5.1	22.8	19.9	1.1	(1.35)	4.10	3.50	(42)	72	(68)	154	176	111	49
49B	3.0	13.3	11.4	1.2	5.38	2.80	2.61	37	71	472	196	229	139	66
59G	3.1	18.4	18.8	1.0	2.25	3.60	3.05	36	74	120	166	162	123	42
19Y	3.5	26.8	23.5	1.1	(0.67)	4.75	4.43	(25)	73	(29)	165	189	121	51
Averages				1.1					71		168	183	119	53

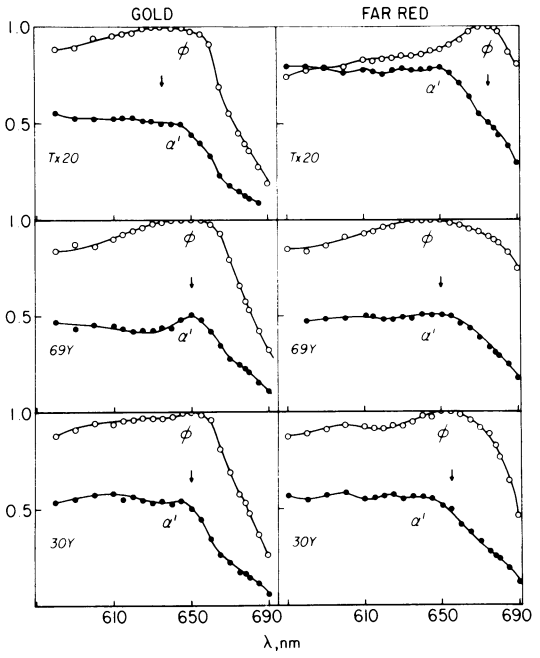

FIG. 1. Spectra for relative quantum yield ϕ (○——○) and the distribution factor α' (●——●). Values for ϕ are scaled to 1.0 at the maximum. Values of α' are calculated as action II/actions (I + II) from Figure 2 and are not scaled. Vertical arrows show the wavelengths selected as neutral wavelengths from maxima for ϕ. The six sets of spectra are for the parent Tx20 and mutants 69Y and 30Y grown in gold and in FR light.

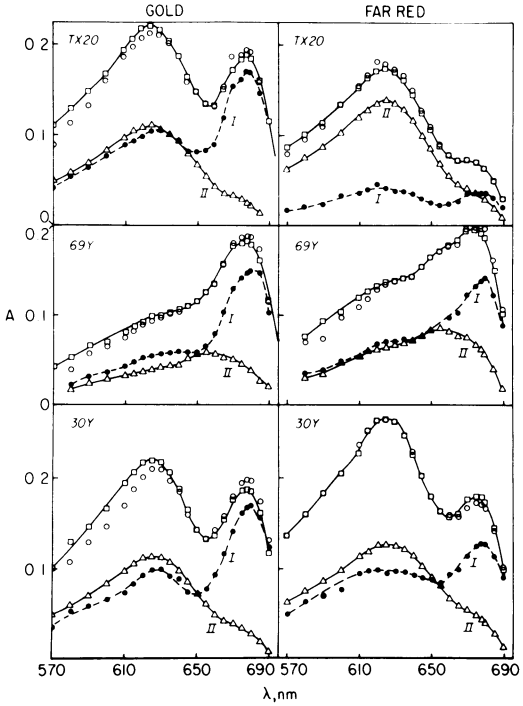

FIG. 2. Action spectra for photoreaction I (●- - -●), for photoreaction II (△——△), and for photoreactions (I + II) (○——○). Also shown are spectra for fractional absorption (□——□) measured on a mimicking cell layer. Actions are normalized so that actions (I + II) = absorption at the neutral wavelength. The six sets of data correspond to those of Figure 1.

a very low action I which becomes greater than action II only at wavelengths longer than 675 nm. As compared to Tx20, all mutants grown in FR show increased Chl and decreased PC (Table I). Yet none of them escape the limitation that, at 678 nm, action II is smaller than action I. The spectra for 69Y demonstrate a feature (found also in 19Y and 85Y) and anticipated from absorption spectra (13): a new pigment component, presumably allophycocyanin, becomes evident. This is seen most clearly in action II and displacement of its maximum to 650 nm.

Table I displays the data on reaction centers, pigments, and distribution of pigments as viewed by photoreactions I and II. Specific growth rates observed in gold and in FR light (13) demonstrate that the six mutants are better adapted than the

parent to use of FR and are generally less well adapted to use of gold light.

Reaction Centers. Estimates for concentrations of reaction centers RC1 and RC2 and of pigments PC and Chl were made together on aliquots of a harvested sample. Values cited are averages of data obtained on at least two independent cell samples which usually differed by less than 10%. Although reaction centers usually are counted in terms of Chl, we have chosen to express both reaction centers and pigments in terms of cell volume, *i.e.* as concentrations.

The ratio of reaction centers (RC1/RC2) for all seven clones grown in gold light varies around an average of 2.1. We have observed much the same range of values in various laboratory cultures of Tx20 grown under tungsten or fluorescent lights. For the seven clones grown in FR light, the RC1/RC2 ratio decreases and shows small variance around an average 1.1.

Pigment Concentrations. Concentrations derived from cell spectra are clouded by known optical errors of a scattering system. Further, the data were obtained via simultaneous equations which assume that PC and a single Chl are the only absorbing components. Hence we expected some systematic errors, especially in PC for clones 19Y, 69Y, and 85Y in which a presumed allophycocyanin component became evident. In these we examined the problem further in terms of the phycobilin spectra left after exhaustive extraction (two to four times) in 80% acetone at 0 C. We found that, in clones 19Y, 69Y, and 85Y, allophycocyanin is the dominant phycobilin. Figure 3 shows spectra for extracted cells of 85Y and Tx20. The A_{625}/A_{650} ratio for the phycobilin of 85Y is 0.73, not far from the value for pure allophycocyanin (1). Estimates of PC for 19Y, 69Y, and 85Y in Table I are shown in parentheses because they obviously are in error.

Chl concentrations used in subsequent calculations were obtained by acetone extraction and are our most precise estimates. However, we also estimated Chl from whole cell spectra, using the simultaneous equations and an absorption coefficient derived from a previous comparison of cell spectra with extracts (12). Hence, agreement in absolute values (Table I) is expected and trivial. We made the comparison only to see if systematic errors would appear for the three clones with evident allophycocyanin. There were no evident systematic deviations. We take agreement in the two sets of data as sufficient validation of our analysis for Chl from cell absorption spectra which we shall apply also to the action spectra.

Our estimates for pigment concentrations (and also reaction centers) in Tx20 are close to those obtained in a previous study (12). In FR light, Tx20 develops low concentrations of Chl and reaction centers. Continued growth in FR light for many generations led to increases in Chl and in growth rate which allowed isolation of the several mutant clones. Clones 49B, 59G, and 19Y represent what we have called a hierarchy of mutants isolated after 190, 360, and 390 generations; they show progressive increases in growth rate and in Chl and reaction centers and progressive decrease in PC.

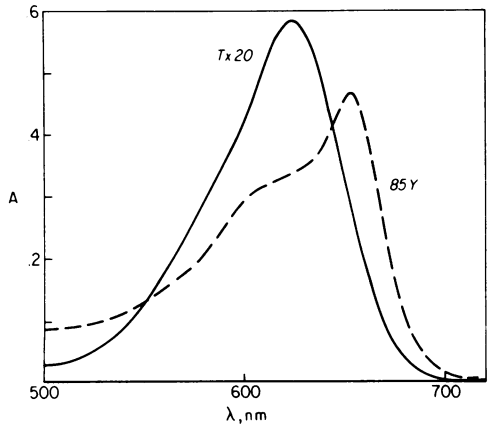

FIG. 3. Absorption spectra for the phycobilins remaining in cells from which Chl was extracted with 80% acetone at 0 C. After extraction, the cells were suspended in 0.2 M acetate buffer (pH 5.5). Tx20 was grown in gold light and mutant 85Y was grown in FR. The spectra are adjusted to zero *A* at 720 nm but are not scaled to each other (actual amount of cell material was much higher for 85Y than for Tx20).

Distribution of Pigments. The distribution of pigments between photoreactions I and II has been calculated from actions at 625 and 678 nm and via the same simultaneous equations used for absorption. For example, of the total Chl of Tx20 grown in gold, 86% is assigned to I and 14% to II. The formal meaning of this assignment is that, for a quantum absorbed by Chl and delivered to a reaction center as an excitation, the probability of delivery to RC1 is 0.86 and the probability of delivery to RC2 is 0.14. Our estimates for Chl distribution will be in error if the averaged absorption coefficients for the several Chl I species is different from that for the several Chl II species. However, such an error should be consistent in that it will affect the absolute, but not the relative, values estimated for Chl distribution.

For the seven clones treated as a group and compared for cells grown in gold and in FR, there is no significant change in distribution of PC. However, we draw no conclusions about PC because of uncertainties in analysis noted above. For Chl, there is a significantly greater distribution to photoreaction II in cells grown in FR. Further, the distribution of Chl to II in FR is greater by a factor of 1.6, about the same as the factor (1.5) for the greater fraction of reaction centers for II. This relationship is considered explicitly below.

Pigments/Reaction Centers. We relate pigments to reaction centers in two ways (Table I). First, we count reaction centers in terms of total amounts of light-harvesting pigments Chl or PC. Our estimates of total Chl/RC1 and total Chl/RC2 for all seven clones grown in gold fall within the range of values previously reported for *A. nidulans* (9, 16). However, there are significant shifts in these ratios for cells grown in FR. Values of the PC/RC2 ratio are cited to make evident that this parameter is not at all constant, even if clones 19Y, 69Y, and 85Y are neglected.

We also use the per cent distribution of Chl obtained from action spectra to estimate values for Chl I/RC1 and Chl II/RC2. These values turn out to be remarkably constant, a finding which we consider our most significant result: Chl I/RC1 = 118 ± 11 (SD) and Chl II/RC2 = 52 ± 9.

DISCUSSION

Our selection process for mutants with improved performance in FR light was not exhaustive. Advantageous alterations contributing very small increases in growth rate might not have been selected out in our time frame of less than 600 generations. Nor have we exhaustively characterized all the qualities which did provide the mutants with up to a 2-fold increase in growth rate under FR. However, our exploration does provide a discrimination: in *A. nidulans*, some features of the photosynthetic apparatus are readily varied, whereas other features are relatively invariant or conserved.

We select three of our results for further discussion.

First, there is the ratio of reaction centers RC1/RC2, often assumed to be about 1 in chloroplasts and shown to be consistently >1 in several cyanophytes (9). Values of 1.5 to 2 4 have been reported for *A. nidulans* (9). As noted in Table I, we observe only a small variation (1.7 to 2.5) in the RC1/RC2 ratio for Tx20 and its six mutants grown in gold light. Hence, we consider remarkable the ratio of about 1.1 found in Tx20 and the six mutants grown under FR. Evidently some important change in the thylakoid apparatus occurs in response to FR *versus* gold light. However,

when viewed as a feature, alteration of which might provide selective advantage in FR, the RC1/RC2 ratio is relatively invariant.

Second, there is the phenomenon of large decrease in PC and appearance of allophycocyanin in spectra of mutants. In the phycobilisomes of *A. nidulans* PC is the predominant pigment, accounting for about 95% of *A* at 621 nm and a considerable fraction of the cellular protein content (24). Allophycocyanin is a smaller component, about 12% of phycobilisome weight; and there is a still smaller component of allophycocyanin B (24). A phycobilisome of this composition becomes an almost vestigial organelle in FR light. Selective advantage should result from decrease in size or in number of phycobilisomes or by relative increase in allophycocyanin as a longer wavelength absorber. Decrease in PC content and relative increase in allophycocyanin is indeed observed. In the context of selection for advantageous mutations, phycobilisome assembly evidently has some relatively variant features. This conclusion is not surprising in view of variations in phycobilisome composition observed in different species (1). The question of possible variance in phycobilisome number can be cast in terms of the hypothesis of a constant (1:1) phycobilisome/RC2 ratio (3, 10). In mutants 19Y, 69Y, and 85Y, the phycobilisomes seem to have become stripped down close to their presumed allophycocyanin cores (2). The possibility of an invariant phycobilisome/RC2 feature might be revealed by study of the allophycocyanin/RC2 ratio.

Third, there are two different treatments of data on the relation of Chl to reaction centers (Table I). Ratios of total Chl to reaction centers are conventional measures often called photosynthetic units. Both the ratios Chl/RC1 and Chl/RC2 have reasonably constant, but not equal, values for all clones grown in gold light. An equivalent statement may be made for all clones grown in FR light. However, there are significant differences both in Chl/RC1 and in Chl/RC2 between gold and FR light. In conventional terms, one would have to say that the photosynthetic unit size changes between these two growth conditions.

A further simplification emerges if there is associated with each reaction center only that fraction of the Chl assigned by the corresponding action spectrum. This procedure gives the ratios Chl I/RC1 and Chl II/RC2 cited in the last two columns of Table I. The obvious result is that each of the ratios is sensibly constant for all clones grown in both gold and FR light. From all values, the Chl I/RC1 is 118 ± 11 and the Chl II/RC2 is 52 ± 9. We consider these ratios remarkably constant in view of the fact that they are obtained from a range of cell material which varied 2-fold or more in Chl and reaction center concentrations, in Chl/PC ratios, and in RC1/RC2 ratios. Estimates of Mimuro and Fujita (10) for similar values in *Anabaena variabilis*, using different methods, were Chl I/RC1 = 130 and Chl II/RC2 = 20. Because of known sources of difficulty in our analytical data, we cannot ascribe to our derived Chl I/RC1 and Chl II/RC2 ratios accuracies as great as is implied by the standard deviations for random variation. However, we cannot think of any consistent error which would give an erroneous appearance of constancy in either ratio. As features, alteration of which might provide selective advantage in FR light, the Chl I/RC1 and Chl II/RC2 ratios are relatively invariant.

Simply because we find no other explanation, we take the constant values of Chl I/RC1 and Chl II/RC2 as evidence that such reaction center is actually linked to a stoichiometric number of Chl. Hence, we propose the following hypothesis: the thylakoid membranes of *A. nidulans* contain two Chl-bearing proteins, a Chl I protein-bearing RC1 and Chl I in a fixed ratio, such as 1/120, and a Chl II protein bearing RC2 and Chl II in a fixed ratio, such as 1/50.

Our hypothesis is neither supported by nor inconsistent with the literature on Chl-protein complexes of cyanophyte thylakoids. The P700-Chl *a*-protein with a ratio of 40 Chl/P700, described as the "heart of photosystem I," was obtained in a deliberate attempt to isolate a particle stripped down toward the guts of a reaction center (21). A "third Chl *a* protein" was proposed as an auxiliary light-harvesting Chl I component (21). Our proposal is merely that, if there are such distinguishable components *in vivo*, they occur in a ratio stoichiometric to P700. One of the early isolates of membrane particles from a cyanophyte was a "photosystem I fragment," isolated under mild conditions of low Triton concentration and accounting for 80% of the Chl at an estimated ratio 100 Chl/P700 (15). Two more recent reports on Chl proteins from cyanophytes support our notion that there are only two *in vivo* components (14, 19).

In a more speculative sense, we now consider the question: why is it that none of the mutants developed effective light-harvesting by Chl for photoreaction II? *A priori*, it would seem that no other alteration could be so effective in providing selective advantage in FR light. Failure to find this alteration in any of the mutants is, of course, a negative result. However, we consider the result as if it were evidence that such alteration is very unlikely or forbidden. According to our hypothesis, Chl II and RC2 are tied together. Increase in Chl II to as much as 50% of total Chl would require an RC1/RC2 ratio of less than 0.5. The principle governing the RC1/RC2 ratio in thylakoid membranes is not known. We presume that there is some constraint which prevents a low RC1/RC2 ratio and, therefore, also prevents large increase in Chl II. We are led to think that alterations in Chl of the thylakoid system are achieved by limited variation in the proportions of two highly conserved Chl proteins.

Our speculations have been limited to the particular cyanophyte *A. nidulans*. We suspect that they are applicable also to other cyanophytes and we suggest that it would be prudent to examine more generally assumptions made about the RC1/RC2 ratio and about what is called the photosynthetic unit.

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