Proc. Natl. Acad. Sci. USA
Vol. 73, No. 6, pp. 1969–1973, June 1976
Biophysics

Responses of *Phycomyces* indicating optical excitation of the lowest triplet state of riboflavin

(tunable laser/action spectrum)

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Contributed by Max Delbrück, March 15, 1976

ABSTRACT **Phototropic and light growth responses of the sporangiophore of *Phycomyces* have been elicited using tunable laser stimulation from 575 to 630 nm. The growth response shows additional components of the action spectrum with a sharp peak at 595 nm, a sharp cut-off at 585 nm, and a tail extending beyond 630 nm. The integral over the electronic transition (*f*-value) is 1.5×10^{-9} times that at 455 nm. These parameters indicate a direct transition from the ground state to the lowest triplet state of riboflavin.**

Green plants, fungi, and bacteria show a variety of physiological responses to light (other than photosynthesis) with an action spectrum having peaks suggestive of riboflavin (1, 2). In the case of *Phycomyces*, the absolute extinction coefficient of the receptor pigment has been estimated by modeling the kinetics of bleaching and regeneration and has been found to match that of riboflavin (p. 1028 of ref. 3). In spite of numerous efforts, however, the receptor pigment has never been positively identified. One reason for this failure is its low concentration, too low to permit "seeing" it by absorption spectrophotometry. All organisms use riboflavin as a prosthetic group for a great variety of enzymatic functions, and the receptor pigment would constitute a very small fraction of the totality of flavoproteins in the cell. Light-induced absorption changes might be more discriminating between the receptor and other flavins. Such changes, showing reduction of a cytochrome *b* and produced with an action spectrum closely fitting riboflavin, have been reported for *Neurospora* (4) and for a particulate extract of *Neurospora* (5). Similar changes, though less well characterized, have also been seen in *Phycomyces* (6). Whether these absorption changes constitute part of the normal photoresponse, or whether they constitute a spurious photochemical event brought about at high intensities by action on free riboflavin or on a flavoprotein not involved in the growth responses, is still an open question. At the output end of the light growth response pathway, regulation of chitin synthetase activity induced by light has been looked for. Indeed, in crude *Phycomyces* extracts a 30% increase in chitin synthetase activity in the presence of high intensity light has been noted by Jan (7), with similar doubts attaching to its physiological significance.

The flavin moiety of riboflavin is a photochemically active molecule. It may act by dehydrogenating a variety of external hydrogen donors or even its own ribityl side chain, as well as by ligating other groups. At neutral pH the dehydrogenations occur from the lowest triplet state, since the yield can be cut by adding triplet quenchers (8). These findings suggest that the photochemistry involved in the light responses of *Phycomyces* (and other organisms) might also be due to riboflavin, involve dehydrogenation, and happen from the triplet state. If they happen from the triplet state, it should be possible to obtain the same physiological responses by direct optical excitation of the triplet state. Here we report experiments supporting this conjecture.

MATERIALS AND METHODS

All experiments were performed with the mutant strain C2 [genotype *carA5*(−)] of *Phycomyces* (9). This is an albino mutant containing very little β-carotene, but having normal light growth responses of the sporangiophore (10).

The light growth response action spectrum measurements used the null response procedure described in detail by Delbrück and Shropshire (11). The sporangiophore is exposed during alternate 5 min periods to a standard (blue) light source and to a test source of variable wavelength. If the intensities of these two sources are adjusted so that they are equally effective in controlling the growth rate, the specimen will grow at a uniform rate. If they are not balanced, a periodic variation in growth rate will be seen.

The blue source was a tungsten filament 3 V lamp with a 455 nm interference filter (half-width 10 nm). This source was located 15 cm from the specimen and was used without optics. Its intensity was adjusted until a growth response null was attained or closely approached. The null was assessed by interpolation, with an error of about ±5% on the linear scale (±0.02 on the log scale).

The test source was the output of a Coherent Radiation model 480 tunable dye laser (with rhodamine 6G), pumped by a Coherent Radiation model 52G 2 W argon ion laser. The beam profile of the dye laser was measured using a 10 μm diameter pinhole. It was approximately gaussian in both the vertical and horizontal directions, with half-widths of 0.6 and 0.5 mm, respectively. The laser beam impinged on the middle of the growing zone of the specimen, 1 mm below the sporangium (Fig. 1). It is known that the growing zone is of fairly uniform sensitivity in the region between 0.6 and 1.5 mm below the sporangium (12). Both the blue source and the laser beam impinged at an angle of about 30° above the horizontal. This angle optimizes straight growth of the specimen. The laser beam was linearly polarized in the vertical direction.

Absolute intensities of both sources were obtained with calibrated silicon diodes (United Director Technology, Santa Monica, Calif.).

The specimen, growing in a small vial on potato dextrose agar, was placed on an *x,y* stage sitting on a turntable rotating at 2 rpm. This insures symmetric illumination around its axis. The *x,y* stage permits accurate centering of the specimen on the axis of rotation. The turntable was mounted on a sturdy *x,y,z* manipulator permitting accurate positioning of the specimen relative to the laser beam. The specimen was observed against a red background light through a 40× telescope equipped with a filar micrometer. At time zero of each 5 min interval the upper meniscus of the sporangium was set to the midline of the micrometer using the *z* motion of the manipulator; at 4 min 50 sec the movable hairline of the micrometer was set to the meniscus, thus measuring the growth during the interval. At 5 min

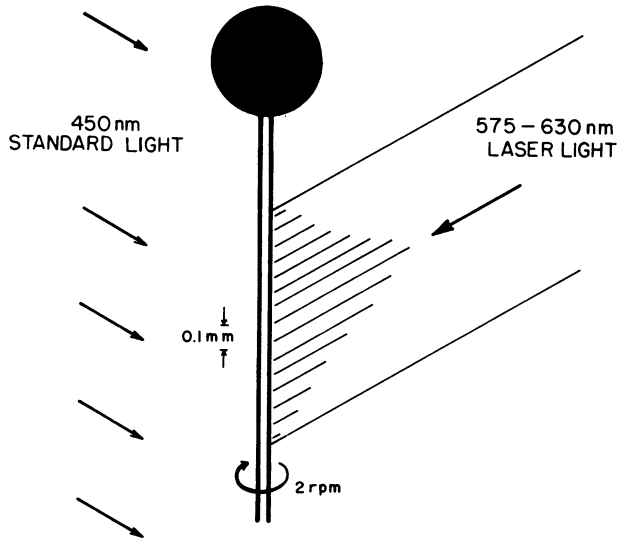

FIG. 1. Schematic diagram of sporangiophore, sporangium, and light sources. The standard blue light (450 nm) and the tunable dye laser light (575–630 nm) are impinging during alternate 5 min intervals. The blue light is a broad beam. The laser light is a narrow beam, about 0.6 mm in width, centered on the center of the growing zone, 1 mm below the sporangium. The specimen is rotated at 2 rpm to make both illuminations effectively symmetric around the axis of the specimen. Average growth of the specimen, in the area of laser illumination, is about 0.1 mm during each 5 min interval. The measured growth at the sporangium is about 0.2 mm during such an interval. Light growth responses in our set-up consisted of periodic variations of up to 20% of the growth per 5 min interval.

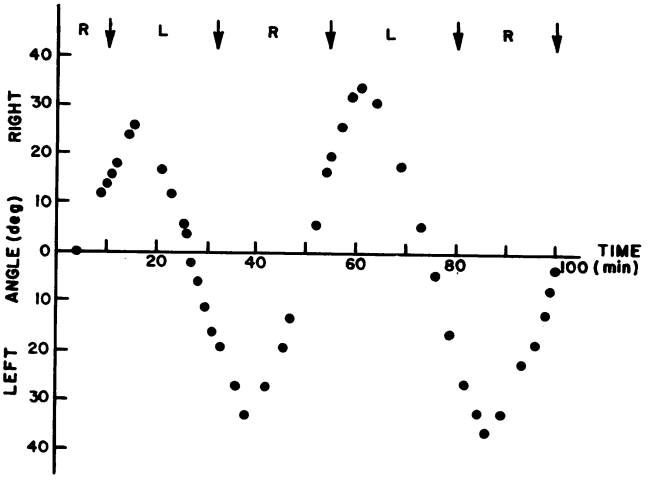

FIG. 2. Phototropic responses to 620 nm light. The specimen was pre-adapted for 1 hr by rotating at 2 rpm·in the presence of 5 nW/cm^2 blue light alternating in 5 min intervals with the equivalent 620 nm laser light of 5 W/cm^2. At time zero rotation was stopped, the blue light was switched off, the laser light was left on continuously, and the angle of tilt was measured as a function of time. Whenever the tilt became appreciable the laser light was switched to the opposite side (actually, the specimen was rotated 180°). The letters R and L between arrows indicate the intervals during which illumination was from right or left. Latency of response (about 6 min) and rate of tilt (about 4°/min) are the same as those for phototropic responses to blue light at the equivalent intensity.

(≡ 0 min) the specimen was moved back to the midline. During each 5 min interval growth measured at the sporangium amounts to about 0.2 mm, and in the middle of the growing zone (at the laser beam) about 0.1 mm.

The intensity of the blue source ranged from 2 to 100 nW/cm^2 and that of the laser beam, from 2 to 13 W/cm^2. Since the laser beam intensities are high, the question arises whether the effects observed are linearly proportional to the light intensity, or not. Therefore the effectiveness of the dye laser stimulation of *Phycomyces* was tested as a function of intensity at several wavelengths. It was found that changes in laser intensities could be matched by proportional changes in the blue light.

Controls. Because of the very low sensitivity of *Phycomyces* to the test wavelengths, precautions were taken to exclude rigorously contamination of the dye laser light with scattered blue light from the argon ion laser. We inserted two 2 mm diaphragms in the light path, one at the dye laser exit and one a few centimeters from the specimen, and inserted a yellow filter (Corning 3-70) between the two diaphragms. The beam reflected by the yellow filter fell on a silicon diode and was used to monitor the dye laser intensity.

The spectral purity of the beam emerging from the second diaphragm was checked in two ways. (*i*) It was viewed through a stack of three 5 mm Corning 5-61 blue filters. This stack represented an $A_{620} = 10.5$ and an $A_{450} = 0.3$. The stack transmitted faintly visible 620 nm light. Blue contamination of the beam was therefore less than 10^{-10} of the test wavelength. (*ii*) *Phycomyces* was used directly to test for blue contamination. A rotating sporangiophore was equilibrated with 5 nW/cm^2 blue, alternating with 5 W/cm^2 laser light at 620 nm. When a blue filter ($A_{450} = 0.1$, $A_{620} = 3.5$) was inserted into the laser beam, a periodic growth response equivalent to total elimination of the laser source was observed, showing that the 620 nm component was the actinic one in the laser beam. These controls prove that the nominal test wavelength is indeed the actinic light in the laser beam.

Another control showed that 620 nm light does produce regular phototropic responses. A specimen was equilibrated as described above. Rotation was stopped, the blue source was eliminated, the laser beam was admitted continuously, and the specimen was rotated 180° each time the phototropic response was well expressed. Fig. 2 shows a typical series of responses. Latency and tropic rates correspond closely to those observed with blue light.

Several reasons conspire to make the assignment of an intensity/cm^2 ambiguous for the laser beam: (*i*) the laser beam is spatially nonuniform in intensity; (*ii*) it covers only about half of the growing zone; (*iii*) due to growth of the specimen, the beam moves about 0.1 mm back and forth on the growing zone during each 5 min interval; (*iv*) the beam impinges at an angle of 30° from the horizontal. For these reasons the effective intensity of the laser beam, relative to that of the broad blue beam, has an intrinsic uncertainty of about a factor of 2. Fitting of our equilibrium values to those obtained with broad beams must therefore allow for a possible scaling factor of this order.

EXPERIMENTAL

Fig. 3 shows three combined light response action spectra for the albino strain C2 in the region 445–630 nm. The values from 445 to 560 nm refer to tropic response measurements carried out by Foster (13). Tropic response and growth response action spectra are known to agree if internal screening is irrelevant (11). In the tropic response measurements, broad beams and the wavelength of the test source were selected with a Bausch and Lomb monochromator. The measurements were done in part at a light level of the standard of 40 nW/cm^2 (445–495 nm), and in part at a light level of 40 pW/cm^2 (480–560 nm). The values from 575 to 630 are our present growth response

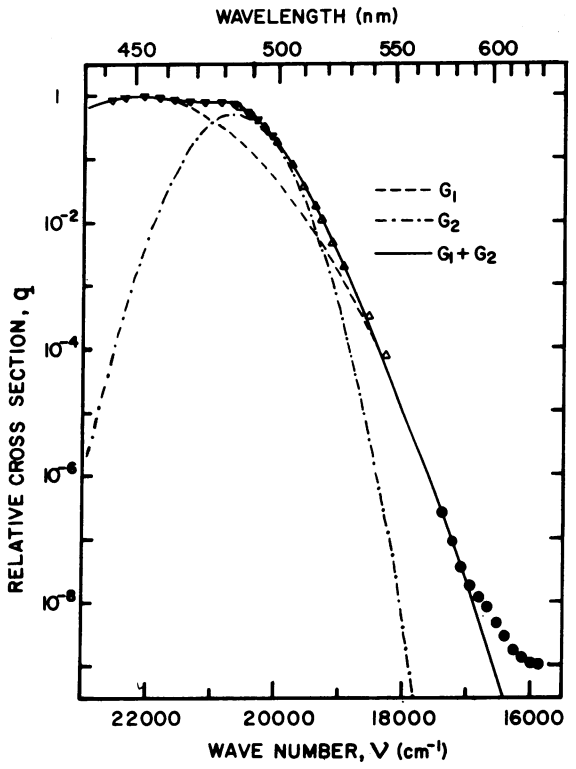

FIG. 3. Light response action spectrum. Letters and symbols used are: q, photon action cross section relative to 450 nm; ▽▽▽, tropic response equilibria matched with blue light (450 nm) of 40 nW/cm^2; △△△, tropic response equilibria matched with blue light (450 nm) of 40 pW/cm^2; and ●●●, growth response equilibria matched with blue light (450 nm) of 2-100 nW/cm^2.

measurements. The solid line is the graph of an equation developed by Foster (13) to fit his two sets of data:

$$q = G_1 + G_2 = \exp\left[-\frac{1}{2}\left(\frac{\nu - 22{,}222}{877}\right)^2\right] + 0.5\exp\left[-\frac{1}{2}\left(\frac{\nu - 20{,}704}{447}\right)^2\right] \quad [1]$$

This equation expresses the photon action cross section as the sum of two gaussians, centered at 450 nm (22,222 cm^{-1}) and 483 nm (20,704 cm^{-1}). The gaussian belonging to the 450 nm peak is broader and dominates for wavelengths longer than 530 nm.

It is seen that the present measurements fit Eq. 1 perfectly from 575 to 585 nm. As explained earlier, the fit in absolute height is somewhat arbitrary and was in fact slightly forced by scaling the experimental values upward by a factor of 1.7 on the linear scale. In view of the excellent fit in slope and the approximate fit in absolute value, it seems reasonable to trust Eq. 1 further out as representing the long wavelength tail of the strong transitions of the receptor pigment. The experimental values beyond 585 nm deviate conspicuously from Eq. 1. They represent the sum of the allowed cross sections and an exceedingly weak new mechanism. Subtracting the allowed cross section (Eq. 1), we isolate the new mechanism. The difference is plotted linearly in Fig. 4. It shows a single peak at 595 nm, a sharp cut-off on the blue side at 585 nm, and a tail on the red side, extending beyond 630 nm, the longest wavelength available to us. The integral over this transition (its "f-value") is about 1.5×10^{-9} of that over the sum of the two gaussians representing the allowed transitions.

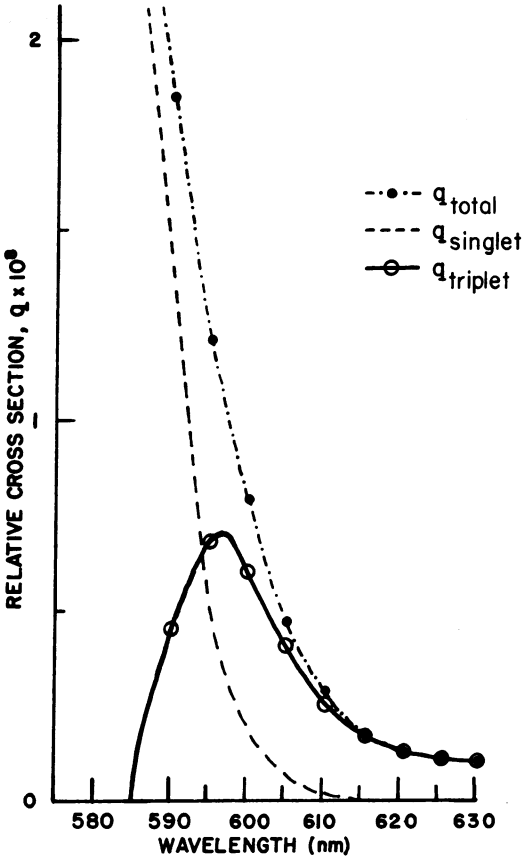

FIG. 4. Action spectrum peak at 595 nm, where q_{total} is the experimental cross section, relative to 450 nm; $q_{singlet}$ is a plot of Eq. 1, the extrapolation formula for the experimental values of the phototropic action spectrum in the region 450–560 nm (13); and $q_{triplet} = q_{total} - q_{singlet}$.

DISCUSSION

Our experiments demonstrate that the light growth response action spectrum of *Phycomyces*, after dropping off sharply according to a precise gaussian law from its peaks at 450 and 483 nm, shows a superimposed new action band between 585 and 630 nm. The peaks at 450 and 483 nm have long been adduced as evidence for riboflavin as the receptor pigment. They correspond to peaks of riboflavin, known to represent the lowest $^1\pi \rightarrow {}^1\pi^*$ transition; the peak at 450 nm represents the Franck–Condon favored transition group and the peak at 483 nm represents the O → O band. The new peak at 595 nm is entirely consistent with the lowest triplet state of riboflavin, since phosphorescence data on riboflavin in ethanol at 77 K gave an emission maximum at 600 nm (14, 15). The f-value of the new band, 1.5×10^{-9} of the allowed transition, is about a factor of two higher than that estimated, with considerable uncertainty, from luminescence measurements on riboflavin in ethanol at 77 K (ref. 15, and P.-S. Song, personal communication). Together, these findings strongly support the candidacy of riboflavin as the receptor pigment for the blue light effects in *Phycomyces*, and, by extension, in other fungi, plants, and bacteria. In addition, they show that the physiologically relevant photochemistry of these responses can occur with a high quantum yield from the triplet state.

It is reasonable to assume that the excited riboflavin mediates a one- or two-electron transfer from a donor to an acceptor. One would like to know then, whether it is the oxidized donor or the reduced acceptor which carries the signal forward in the

stimulus-response chain, or whether, alternatively, the charge separation across a membrane is the relevant event. Here we must focus, to start with, on the fact that near threshold the triggering photoevents involve a small number of absorbed quanta, as they do in vertebrate vision. The calculations to substantiate this point (ref. 1 p. 135) can now be refined in the light of our more definite knowledge of the receptor pigment and in the light of the system analysis for low intensities (3), but the result is essentially the same: we must be dealing with a small number of primary photoevents, each subject to a secondary amplification which is inversely related to the average prevailing light intensity. A plausible mechanism, in analogy to well-known electronic devices, would be for the light signal to control the conductance of a membrane, with the conductance depending exponentially on the membrane voltage. This type of situation is, in fact, found in the alamethicin model (16–18) of a membrane whose conductance channels are gated by small changes of the applied potential. One could thus envisage the primary photoevent as electrogenic across a membrane, and in turn controlling a large number of gates for membrane current. Such a mechanism is consistent with the finding that the receptor pigment is rigidly bound to some macroscopic structure, since the pigment has a defined orientation relative to the sporangiophore cylinder, with the dipole moment of the allowed transition in the blue oriented equatorially (19).

In the case of light-induced carotenogenesis (another typical blue-light effect) in the fungus *Fusarium aquaeductuum*, Rau and Theimer, in a series of elegant investigations, have sought to establish that it is the *oxidized donor* that mediates the induction of the synthesis of the enzymes involved in carotene synthesis (20). They showed that: (*i*) induction by light is prevented by the addition of dithionite, even if the dithionite is added several minutes after the light stimulus; (*ii*) H_2O_2 addition substitutes for light, whereas dithionite addition does not. These findings suggest that the relevant trigger is an oxidation rather than a reduction. However, they are also open to the alternative interpretation that the primary photoevent causes an electron transfer inward across the plasma membrane. The donor would then be on the outside and would be accessible to oxidation by H_2O_2 and to re-reduction, after the photoevent, by dithionite.

Recently Lang-Feulner and Rau (21) showed that in *Fusarium* carotenogenesis could be induced by *red* light if the mycelium was exposed to methylene blue or toluidine blue. These dyes absorb in the red, with the lowest excited singlet state corresponding to 660 nm. The triplet state must lie at a still lower energy level. These dyes, therefore, could not excite the triplet state of riboflavin by energy transfer. Instead, as the authors suggest, one may surmise that these dyes directly photo-oxidize the same donor that in the undoped mycelium is photo-oxidized by the receptor pigment. Again, we may be dealing with a donor located on the outside of the plasma membrane.

Our findings suggest that it should also be possible to obtain the physiological response by sensitized excitation of the triplet state, or to prevent it by infiltration with triplet quenchers such as KI (22, 23). Further, it should be possible to enhance or diminish the height of the triplet peak by introduction of suitable riboflavin analogues into the organism. Eventually, such an approach may permit specific labeling of the receptor pigment and thereby its isolation.

It is worth noting that the detection of a secondary action peak 10^9 times lower than the main peak is probably unique in photobiology, and rare in photochemistry. The experiment would hardly be feasible without the spectral purity, high intensity, and tunability of the dye laser, as well as the high sensitivity and low noise level of the organism. The sensitivity of *Phycomyces*, in fact, did not have to be strained. The sporangiophore shows light growth responses at intensities three orders of magnitude below the ones here used.

The experiments were performed in the laboratory of Prof. A. Yariv of the Electrical Engineering Department of the California Institute of Technology. The authors wish to express their appreciation to Professor Yariv for putting the laser facilities at their disposal, and to Messrs. P. Agmon, D. Armstrong, and H. W. Yen for indefatigable help with laser troubleshooting. The work was supported by National Science Foundation Grant no. BMS 75-11316.

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