Photoreceptor Pigment for Blue Light in *Neurospora crassa*

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VICTOR MuñOZ\(^1\,^3\) AND WARREN L. BUTLER
Department of Biology, University of California, San Diego, La Jolla, California 92037

ABSTRACT

Irradiating the mycelium of *Neurospora crassa* with moderate intensities of blue light causes a reversible photoreduction of a b-type cytochrome. The action spectrum for the photoreduction of cytochrome b is very similar to the absorption spectrum of flavin pigments. Prolonged irradiation of the mycelium with strong blue light irreversibly bleaches flavin-like pigments and as these pigments are bleached the photoreponse of cytochrome b is lost. We conclude from these and other data that a flavin is the photoreceptor pigment for the photoreduction of cytochrome b. The close similarity between the action spectrum for the photoreduction of cytochrome b and action spectra for a number of physiological photoresponses suggests that this photoreceptor pigment controls a wide variety of photobiological processes in a wide diversity of organisms.

A number of metabolic and physiologic processes in living organisms are initiated or controlled by blue light. The similarity between the action spectra for many of these responses suggests a common photoreceptor pigment system. We sought direct evidence for this photoreceptor pigment by examining photoresponsive organisms for light-induced absorbance changes that have an action spectrum similar to the action spectrum for the physiological "blue light responses." Such absorbance changes have been found recently in cells of the slime mold *Dictyostelium discoideum* (9) and in mycelium of the fungi *Phycomyces blakesleeanus* (9) and *Neurospora crassa* (8). A photoreceptor pigment with an absorption maximum at about 460 nm (possibly a flavin) appears to mediate the photoreduction of a b type Cyt in these organisms.

Our interest in *Neurospora* derives in part from the ability of light to influence the circadian rhythm of spore formation (conidiation) in the band strain. Brief periods of irradiation shift the phase of the rhythm (12), and prolonged irradiation with very low intensities of blue light inhibit the expression of the rhythm (13). The purpose of the work reported here was to expand upon our previous preliminary report (8) and to examine cell-free extracts from the *Neurospora* mycelium for the photoresponsive pigment system.

MATERIALS AND METHODS

The mycelium of an albino band strain (al-2, bd) of *Neurospora crassa* was grown on 15-cm Petri dishes under conditions (1) in which the circadian rhythm of conidiation is expressed. This strain was isolated from a cross between the strains al-2 (FGSC No. 99) and bd (FGSC No. 1959) obtained from the Fungal Genetics Stock Center, California State University, Humboldt, Arcata, Calif. After 48 hr of growth (the first 18 hr in white fluorescent light, the last 30 hr in darkness), mycelium from the outer conidiating region was harvested under red safelight and 0.3 g of the tissue was pressed evenly onto the window of a cylindrical spectrophotometer cuvette to give a uniform sample about 2 mm thick. Sample preparation and subsequent experiments were carried out at room temperature (23 ± 1°C).

Absorption spectra were measured with a single beam spectrophotometer on line with a small computer (3). Kinetic measurements were made with a double beam spectrophotometer in which the two measuring wavelengths were defined by interference filters (4). Actinic light was obtained from a xenon lamp in conjunction with interference filters and heat-blocking filters. The measuring beams and the actinic light passed through the sample in the vertical direction from top to bottom. The difference spectrum of the light-induced absorbance change was determined by measuring the absorption spectrum of the sample before and immediately after a 1-min irradiation with actinic light, during which time the phototube shutter was closed. Twelve sec were required to open the shutter and scan the spectrum from 400 to 570 nm (i.e., the spectral region of interest) after the end of the actinic irradiation. All of the difference spectra were plotted directly from the computer to the X-Y recorder. Continuous measurements showing the extent and the kinetics of the absorbance change were made before, during, and after the actinic irradiation with the double beam spectrophotometer with blocking filters between the sample and the phototube to prevent the actinic light from reaching the phototube.

Irreversible light-induced absorbance changes due to extended periods of irradiation with strong (22 mw/cm\(^2\)) blue (475 nm) light were determined by measuring the absorption spectrum of the sample before and 10 min after the end of the irradiation period. The 10-min dark period allowed the reversible absorbance changes to decay so that only the irreversible changes remained. The irradiation periods used in such experiments were sufficiently long so that the slow oxidative processes which occur in the dark also caused absorbance changes during the course of the experiment. Thus, difference spectra were measured on control samples kept in the dark for the same periods of time to determine the extent and nature of these dark changes. The difference spectrum for the irreversible light-induced absorbance change was obtained as the difference between two difference spectra; one measured on the irradiated sample, the other on the dark control sample. The sensitivity of the reversible light-induced change as a function of the irradiation time with the strong blue light was also determined by stopping the irradiation at a given time, measuring the absorption spectrum of the sample (after a 10-min dark period) when mimicking the extent of the reversible ab-
sorbance change \( (A_w - A_m) \) due to a lower intensity (6 mw/cm²), 1-min irradiation with 475 nm light and then proceeding with the high intensity irradiation to the next point of time. Such experiments were made to correlate the extent of the reversible photoreduction of Cyt b with the degree of irreversible bleaching at 460 nm.

For the preparation of cell-free extracts, 62 g of tissue grown for 84 hr at 30°C were collected from 40 Petri dishes and frozen with liquid nitrogen. The frozen tissue was chopped in a Waring Blender (at 3 v) under nitrogen at 0°C with 180 ml of the extraction medium (0.44 m sucrose, 1 mm EDTA, and 10 mm tris, pH 7.3) which had been bubbled with N₂. The slurry was ground further with sand in a mortar under N₂ at 0°C. The pellet from a 1000g centrifugation for 10 min was discarded and the supernatant was centrifuged at 10,000g for 30 min. The precipitate was resuspended in 16 ml of the extraction medium to give the 10,000g sample and the supernatant was centrifuged at 100,000g for 2 hr. The supernatant from the latter centrifugation was used for the 100,000g sample.

**RESULTS**

The absorption spectrum of a sample of mycelium from the albino band strain of *Neurospora* (Fig. 1) shows the absorption bands of reduced Cyt: the bands at 610, 560, and 550 nm are due to the \( \alpha \) bands of \( \alpha \)-, \( \beta \)-, and \( c \)-type Cyt, respectively; the bands in the 510 to 530 nm region, to the \( \beta \) bands of the \( b \) and \( c \) type Cyt; and the strong band at 418 nm, to the combined Soret bands of the \( b \)- and \( c \)-type Cyt. The small shoulder at about 450 nm is due, at least in part, to the Soret bands of Cyt \( a \) and \( a' \). The spectrum shown in Figure 1 was measured shortly after the mycelial tissue was pressed into the cuvette, at which time the Cyt, as indicated by the magnitude of the absorption bands, are fully reduced. However, if the tissue is allowed to stand in the cuvette at room temperature the Cyt oxidize slowly. The difference spectra in Figure 2 show the absorbance changes which occur when the tissue remains in the cuvette over a period of several hours. Progressive oxidation of the \( \alpha \)-, \( \beta \)-, and \( c \)-type Cyt is apparent; in addition the broad positive band in the 460 to 490 region suggests the oxidation of flavoproteins as well. After 4 hr the Cyt are 20 to 30% oxidized.

Irradiation of the freshly prepared (i.e., fully reduced) sample of mycelium with blue light does not result in any light-induced absorbance changes. If the sample is allowed to become partially oxidized by standing in the cuvette before the irradiation treatment, light-induced absorbance changes can be observed and the extent of the changes increases as the sample becomes more oxidized. Figure 3 shows the light-minus-dark difference spectrum of a sample which had been placed in the cuvette 4 hr before the irradiation treatment. These light-induced absorbance changes clearly indicate the photoreduction of a \( b \)-type Cyt. The broad minimum in the difference spectrum at 460 nm suggests the photoreduction of a flavoprotein as well. The 4-hr incubation period in the cuvette was adopted as part of the sample preparation procedure for these investigations of the light-induced absorbance changes in the mycelial tissue.

Although all of the work reported here was carried out on the albino band strain of *Neurospora*, the same light-induced absorbance changes were also found in mycelium of the wild-type strain which had been grown in darkness to minimize the carotenoid content of the tissue.

The kinetics of the light-induced absorbance changes were
measured in the Soret band and in the α band regions of the photoresponsive Cyt b. The absorbance change at 424 nm (measured against a reference wavelength at 466 nm) induced by irradiation with a 490 nm actinic light is shown in Figure 4A; the change at 560 nm (against a reference wavelength at 578 nm) induced by two intensities of actinic light at 450 nm, in Figure 4B. Figure 4B shows that both the initial rate of change and the extent of the change at the photostationary state depend on light intensity. The rate of dark decay of the light-induced change is the same in both wavelength regions (half time of about 20 sec).

Dose-response relations were determined for the extent of the 560 nm absorbance change as a function of light intensity for a series of actinic wavelengths between 360 and 530 nm. The results obtained with 470 nm actinic light are presented in Figure 5. Linear semilogarithmic intensity-response curves were found at all actinic wavelengths. Such intensity-response curves were used to determine the quantum flux required at each actinic wavelength to produce an absorbance change of 0.003 between 560 and 578 nm. The action spectrum presented in Figure 6 is a plot of the reciprocal of this quantum flux as a function of actinic wavelength. The same features were shown previously in a less rigorous action spectrum determined by measuring the extent of the absorbance change induced by fixed quantum flux of actinic light (8). The action spectrum shows clearly that cytochrome b is not the photoreceptor pigment; rather, a pigment absorbing maximally at about 460 nm absorbs the light and mediates the photoreduction of the Cyt b.

Long term irradiation of the sample with high intensity blue light causes an irreversible bleaching in the 460 nm region. Figure 7 shows the irreversible light-induced absorbance changes (corrected for the slow absorbance changes which occur in the dark during the course of the experiment—see protocol under "Materials and Methods") due to an irradiation with 475 nm light at 22 mw/cm² for 70 min. The bleaching of this blue-absorbing pigment is accompanied by a loss of the reversible light-induced absorbance change. Figure 8 compares the extent of the irreversible bleaching at 460 nm with the extent of the reversible light-induced absorbance change at 560 nm (due to a 1-min irradiation with 475 nm light at 6 mw/cm²) after different periods of irradiation with high intensity blue light. The absorption spectrum of the pigment(s) bleached by the prolonged high-intensity irradiation with blue light is similar to that of a flavin. The absorption spectrum of the photobleached pigment(s) is compared with the absorption spectrum of FAD and with the action spectrum for the photoreduction of Cyt b in Figure 9.

Cell-free extracts of the Neurospora mycelium were also examined for light-induced absorbance changes. These extracts (obtained from supernatants from either a 10,000g centrifugation for 30 min or a 100,000g centrifugation for 2 hr) contained Cyt b and Cyt c in their oxidized states but showed no light-induced absorbance changes. If FAD or FMN was added to these extracts, however, and allowed to incubate for a few minutes, both Cyt became photoreducible. Figure 10 shows the light minus dark difference spectra measured before addition, 1.5 min after addition, and 5 min after addition of 30 μM FAD to the 10,000g fraction. Similar light-induced changes were also observed after addition of FAD or FMN to the 100,000g supernatant. Kinetic measurements indicated that the Cyt c was photoreduced prior to the Cyt b and that both Cyt were oxidized in the dark with the Cyt b being oxidized considerably more rapidly (half time of about 30 sec) than the Cyt c (half time of about 10 min).

The soluble extracts with added FAD or FMN showed a marked stimulation of O₂ uptake by light which was inhibited by azide. Oxygen consumption by the 100,000g supernatant with 30 μM FMN during irradiation with blue light is shown in Figure 11. The likely source of electrons to reduce the O₂ is the EDTA (1 mM) present in the reaction medium. The inhibitory action of azide, which is known to quench free radicals, suggests the role of superoxide in the photoreduction of the Cyt and the photostimulation of O₂ uptake. Work to

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**Fig. 4.** A: Kinetics of the light-induced absorbance change measured at 424 nm against a reference at 466 nm due to an irradiation (on at upward arrows; off at downward arrows) at 490 nm with 8 mw/cm². B: Same at 560 nm versus a reference at 578 nm due to an irradiation at 450 nm with 6.5 mw/cm².

**Fig. 5.** Extent of the light-induced absorbance change at 560 nm in mycelial samples as a function of the intensity of irradiation.

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1. Although linear semilogarithmic dose-response curves are quite common in physiological action spectra there are no theoretical reasons based on photochemistry why such curves should be obtained. Rather, a number of factors combine (including the absorbance of the tissue, the averaging of photoresponses throughout the tissue, and the kinetics of dark reactions) to give the linear semilogarithmic relationship over a particular range of the response.

2. Abbreviations: FAD: flavin adenine dinucleotide; FMN: flavin mononucleotide.
elucidate these photoreactions in the soluble extracts is continuing.

**DISCUSSION**

The nature of the photoreceptor pigment for blue light responses has been a controversy of long standing, some claiming that the action spectra indicate a flavin, others that these spectra indicate a carotene. In point of fact, the action spectra for the physiological responses are not capable of distinguishing between these two candidates because the spectral characteristics of either of these pigments in vivo may be modified by their specific protein association and local environment, and even though such photoresponses may be measured in so-called carotenoidless mutants, such as the albino strains of *Neurospora*, these strains still contain detectable amounts of carotenoid pigments. The present work on the light-induced redox change of a Cyt strongly indicates, for the following reasons, that a flavin or flavoprotein is the photoreceptor for

**Fig. 8.** Extent of irreversible bleaching as a function of the time of irradiation with 22 mw/cm² at 475 nm (---). Extent of light-induced absorbance change at 560 nm due to a 1 min irradiation with 6 mw/cm² at 475 nm as a function of the time of irradiation with the strong blue source (---). Extent of light-induced absorbance change at 560 nm with dark control sample (---).

**Fig. 9.** Top curve: absorption spectrum of pigments bleached in Fig. 7; middle curve; absorption spectrum of FAD; bottom curve: action spectrum for photoreduction of Cyt b (from Fig. 6).
this response. (a) Flavins are known to be closely associated with Cyt, especially b type Cyt (e.g., Cyt b1 and Cyt b2) and capable of mediating redox changes (14). Carotenes have never been implicated directly in redox changes. (b) Flavins in the Neurospora mycelium are photoresponsive in that they appear to be reversibly photoreduced in the light minus dark difference spectra due to short irradiation periods (Fig. 3) and irreversibly bleached by long term irradiation (Fig. 7). (c) The progressive photodestruction of the flavins by prolonged irradiation is accompanied by a progressive decrease in photoreduction of Cyt b (Fig. 8). (d) The action spectrum for the photoreduction of Cyt b is entirely consistent with a flavin being the photoreceptor (Fig. 9). (e) Photoreduction of Cyt b and Cyt c in soluble cell-free extracts of the Neurospora mycelium could be observed after adding FMN or FAD to the cell-free preparations (Fig. 10).

The action spectrum for the photoreduction of Cyt b in the mycelium of Neurospora is consistent with the effects of light on physiological responses of Neurospora. Figure 12A compares our action spectrum for the photoreduction of Cyt b (upper curve) with the action spectrum for the inhibition of the expression of conidiation as a circadian rhythm (middle curve) (this spectrum was replotted on a linear scale from the data of Sargent and Briggs (13) which were presented on a logarithmic scale) and the action spectrum for carotenogenesis (15) (lower curve) all of which are from Neurospora. Figure 12B compares our action spectrum from Neurospora with action spectra for carotenogenesis in Fusarium (10), for phototropism in Avena coleoptiles (5), for phototropism in Phycomyces sporangiophores (5) and for photoinduced O2 uptake in a carotenoidless mutant of Chlorella (7). The action spectrum for carotenogenesis in Mycobacterium (11) is also very similar, showing broad maxima at 460 and 370 nm with no action beyond 520 nm. Given the problems inherent in physiological action spectroscopy we believe that these various spectra are sufficiently similar to implicate the same type of photoreceptor pigment in all of these responses. These photobiological responses are found over a wide range of diversity and complexity from bacteria, through fungi and algae to higher plants. We believe that they also extend to animal systems and that the less precise action spectra for the photoregulation of circadian rhythms in Drosophila (6) and in Pectiniphora (2), which show a broad action through the blue with very little action at wavelengths beyond 520 nm, are consistent with the action spectra presented here.

We conclude that flavin is the photoreceptor molecule for all of these “blue light responses” and that a light-induced redox change is the primary step in the photorecontrol mechanism. It seemed reasonable to suppose that this photoreponsive pigment system appeared very early in evolution and was available, as higher forms of life evolved, as a general cellular process which could be incorporated into various photobiological control mechanisms which proved to be advantageous for survival.

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LITERATURE CITED


