MICROSPECTROPHOTOMETRY AND THE PHOTORECEPTOR OF *PHYCOMYCES* I

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ABSTRACT

By applying microspectrophotometry to the sporangiophore of *Phycomyces blakesleeanus* wild-type and the albino car-10(−) type II, absorption spectra were obtained for 1- to 5-day cultures. Spectra in the growing-zone of the wild-type during Stage IVb, taken from 0.1 to 3 mm below the base of the sporangium, show two distinctly different spectra: one is more characteristic of a carotene, the other of a flavin. Combined, these absorption spectra reproduce closely the action spectrum. For the albino car-10(−), which is deficient in carotenes, only the spectrum characteristic of lumichrome or a reduced flavin was found. A *c*-type cytochrome was isolated from both strains which, if coupled with a flavin, could permit a photoreversible oxidation-reduction system. Birefringent crystals were observed to be aligned in the growing zone in which the photoreceptor is believed to lie. Microspectrophotometry of these crystals shows absorption peaks similar to those of riboflavin crystals.

INTRODUCTION

Phycomyces blakesleeanus has long been the object of research concerned with the sensitivity of the single cell sporangiophore to light as measured by the phototropic response and by the light growth response (4, 7, 9, 18). Light is not essential for growth, but functions as a signal to alter the growth in either space or time (18). To date, however, no photoreceptor structure has been identified, and it is not known what pigment or pigment system is responsible for these light responses. A recent review by Bergman et al. (2) summarizes the many physical, biochemical, and photobehavioral studies of *Phycomyces*.

Action spectra show absorption peaks around 280, 365–385, 420–425, 445–455, and 475–485 nm (5, 8, 20). The similarity of the action spectrum to the absorption spectrum of β-carotene in the visible part of the spectrum indicates that β-carotene could be one of the light receptor molecules (8, 17). However, the absorption peaks in the ultraviolet near 280 and 370 nm of the action spectrum and in the visible around 450 nm would suggest a flavin or flavoprotein (3) to be one of the photoreceptor molecules. The action spectrum may also indicate that more than one pigment participates in the process.

To learn more about this phenomenon and the meaning of the action spectra, the following studies were undertaken: electron microscopy of the sporangiophore in search of a photoreceptor structure, extraction of the sporangiophore in attempts to isolate a pigment or pigment system, and microspectrophotometry to obtain absorption spectra in the growing zone. This report is concerned mainly with the microspectrophotometry of the sporangiophore and the crystals found in the growing zone.

MATERIALS AND METHODS

Phycomyces blakesleeanus wild-type (±) strains and a mutant, albino car-10(−) type II, which is deficient in carotenes, particularly β-carotene, were used in

these studies. The fungi were grown in a temperature-controlled growth chamber, at 22°C and a relative humidity near 50%, under continuous fluorescent light of about 65 ft-c or in darkness. *Phycomyces* used for microspectrophotometry and electron microscopy were grown on Sabouraud potato dextrose agar (Difco Laboratories, Detroit) in Petri dishes 100 × 20 mm. For chemical extraction, they were grown in a liquid nutrient medium, according to Carlile (3), in large bottom, wide-mouth Erlenmeyer flasks.

Electron Microscopy

Phycomyces sporangiophores during Stage IVb were fixed with 1% OsO_4 (pH 4.6) for 1 hr at 5°C and were washed with distilled water. They were then dehydrated with a series of graded alcohols and flat embedded in a mixture (1:1) of Epon and Araldite so as to increase bonding between the tissue and the plastic. After polymerization, the sporangiophores were cut out and remounted in order to obtain the desired orientation for sectioning. Thin sections were cut with a Porter-Blum microtome, mounted on grids, and scanned with a Phillips 200 EM.

Microspectrophotometry

The sporangiophores develop through four distinct stages (18) and, for microspectrophotometry, they were plucked from the mycelia during Stage IVb, mounted on a quartz microscope slide in air, and covered with a quartz cover slip to avoid crushing them. Cytoplasmic streaming continued during the measurements.

All absorption spectra were obtained with the recording microspectrophotometer M-5. This instrument is designed to rapidly record absorption spectra in a single sweep at relatively low light levels, from the ultraviolet, 230 nm, through the visible to 800 nm in the infrared. The design of the optical system, electronics, the sensitivity and performance of this instrument are described by Wolken et al. (25). Absorption spectra from cell organelles and particles as small as 0.5 μ in diameter are obtainable. In these analyses, areas from 1 μ^2 to 8 μ^2 were used to scan the sporangiophore.

RESULTS AND DISCUSSION

The general appearance of the light-sensitive sporangiophore, Stage IVb, is a nearly transparent cylindrical filament supporting a spherical black sporangium (18). Absorption spectra of Stage IVb of the wild-type sporangiophore, from the sporangium through the growing zone (within 3 mm below the base of the sporangium), are shown in Fig. 1. The spectrum of Fig. 1 *a* is typical of what is found from 0.1 mm to slightly beyond 1 mm below the sporangium. Scanning down the sporangiophore, from 1 mm below the sporangium to just beyond the growing zone, the absorption spectrum gradually shifts from the spectrum shown in Fig. 1 *a* to that of Fig. 1 *b*. The spectrum of Fig. 1 *a* is similar to a carotene, and its absorption peaks are very near to those of β-carotene at 430, 460, and 480 nm. This was the major pigment found by Zankel et al. (27). On the other hand, the spectrum of Fig. 1 *b* with its major absorption

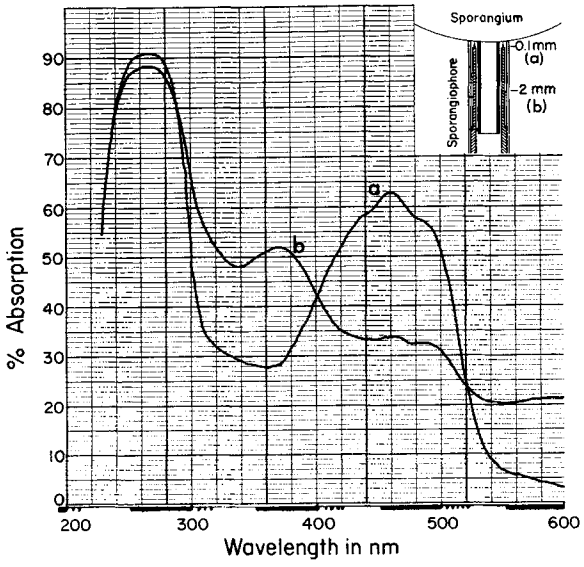

FIGURE 1 Spectra of *Phycomyces* sporangiophore Stage IVb, wild-type. *a*, Spectrum taken at 1 mm below the sporangium (typical of spectra taken from 0.1 mm–1 mm). *b*, Spectrum taken at 2 mm below base of sporangium. Taken with M-5 microspectrophotometer.

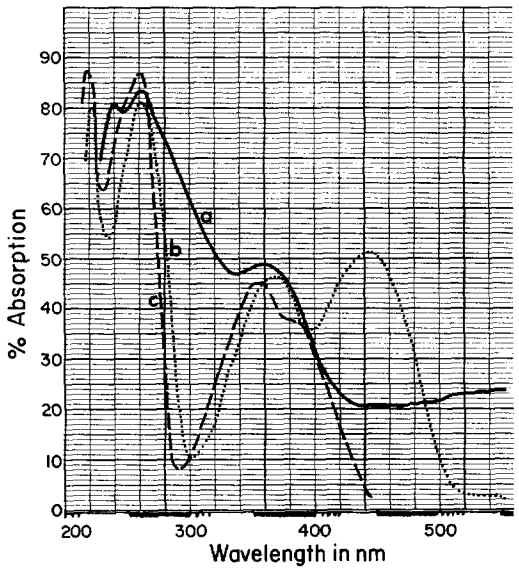

FIGURE 2 Comparison of spectra: *a*, *Phycomyces* sporangiophore Stage IVb car-10(−), typical of that found throughout growing zone and below growing zone; *b*, riboflavin in distilled water; *c*, lumichrome, (reference **1**, Fig. 1, p. 23). Taken with M-5 microspectrophotometer.

peaks near 280 and 370 nm would indicate another substance. It is interesting to note that the absorption peaks of Fig. 1 *a* and 1 *b* together give peaks at 280, 370, 435, 460, and 485 nm, which are found in the complete action spectrum (5, 8).

The *Phycomyces* car-10(−) mutant, which responds as well as the wild-type to light stimuli, is deficient in carotenes, particularly β-carotene, and its spectra are more informative. Meissner and Delbrück (17) estimated that the wild-type contains 220 μg β-carotene per gram dry weight of sporangiophore, whereas the car-10(−) contains only 0.1 μg or less of β-carotene per gram dry weight of sporangiophore. The absorption spectrum within the growing zone of the car-10(−) (Fig. 2 *a*) shows absorption peaks near 230, 267, and 370 nm, and is a typical spectrum obtained in scanning throughout the growing zone of the sporangiophore. It is also similar to the spectrum of the wild-type shown in Fig. 1 *b*. No absorption peaks typical of a carotene were found in the car-10(—). It is important, then, to reconcile these spectral findings to see more precisely the meaning of our absorption spectra.

Even though no detectable β-carotene spectra were observed by microspectrophotometry in the growing zone of car-10(−), can β-carotene be ruled out as the photoreceptor pigment? It was estimated that if all the β-carotene found in the sporangiophore were concentrated in the growing zone, there would be 1.2×10^{10} molecules (17). This is more than sufficient when compared to chlorophyll in chloroplasts and to rhodopsin in the retinal photoreceptors which contain from 10^6–10^9 pigment molecules (24). If such a concentration were present in the growing zone it would have been detected by our microspectrophotometer (25), but no such concentration of β-carotene was observed. Therefore, whatever β-carotene is present is most likely spread throughout the sporangiophore and would not function as the primary photoreceptor molecule. What role β-carotene does perform in the photoreceptor process is not clear, for it can act as a filter to screen or shade in the wavelength range of 400–500 nm, can prevent photo-oxidation (destruction) at high light intensities, or it can participate as an accessory pigment molecule in the energy transfer process.

What then is the photoreceptor molecule or molecules? Riboflavin or a flavoprotein has been previously suggested (3, 11, 13, 14). Riboflavin is yellow and in solution has absorption peaks in the oxidized state around 221–227, 265–270, 365–370, and near 445 nm (14, 22). Upon ultraviolet excitation, it fluoresces green (520–560 nm). It can be reversibly reduced and oxidized; the reduced form is colorless.

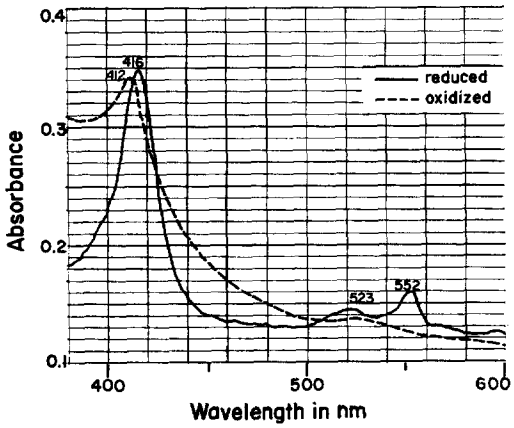

FIGURE 3 Spectra of cytochrome isolated from both wild type and car-10(−) strains: reduced—, oxidized ---. Taken with Cary 14 spectrophotometer.

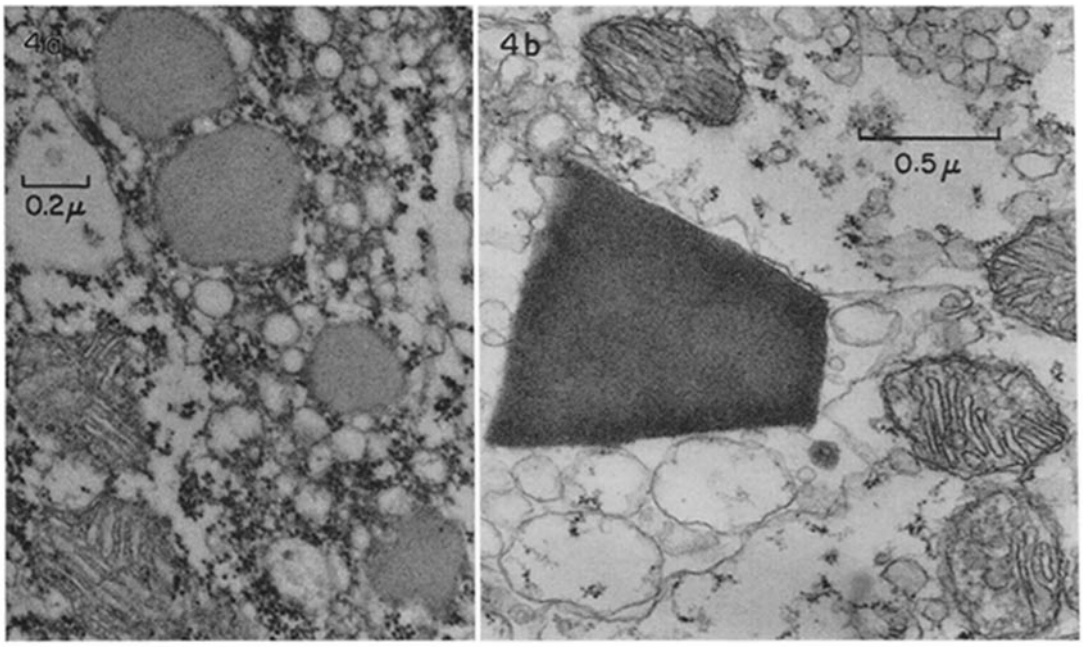

FIGURE 4 Electron micrograph of cross-section of sporangiophore Stage IVb. *a*, Note lipid globules and ferritin-like particles in cytoplasm (× 43,000). *b*, Single crystal from growing zone. Note relation to surrounding membrane and mitochondria (× 36,500).

To reversibly reduce and oxidize riboflavin, a likely molecule would be a cytochrome (23). Chemical fractionation of the sporangiophores and column chromatography, according to the method of Margoliash (15), were used to isolate from both light-grown wild-type and car-10(−) a *c*-type cytochrome in the reduced state (26). The reduced spectrum shows peaks at 280, 416, 523, and 552 nm; when oxidized, the absorption peaks shift to 412 and 525 nm (Fig. 3).

Riboflavin is light sensitive in neutral or acid solutions, and forms *lumichrome* with absorption peaks near 223, 260, and 360 nm (1, 14). The spectral data Figs. 1 *b* and 2 *a* would support the hypothesis that a flavin or flavoprotein is one of the photoreceptor molecules which becomes reduced to lumichrome (Fig. 2 *c*) upon irradiation (3).

For investigating the possibility that a recognizable cytoplasmic structure could serve as the photoreceptor, sections of fixed sporangiophores were examined with the electron microscope. The electron micrographs showed numerous mitochondria, electron-opague granules in various aggregated states, and lipid globules (Fig. 4 *a*). None of these structures at present can be identified as the photoreceptor (21). However, during Stage IVb, there are crystals from 1 to 2 μ wide and from 5 to 10 μ in length (Figs. 4 *b* and 5), which are aligned near the vacuole, within 1 mm of where the maximum light growth response occurs and where the photoreceptor is believed to lie (13, 27). These crystals were found in both the wild-type and car-10(−); they are easily seen under the phase microscope, and with polarizing optics they are birefringent (Fig. 5 *a*). In the wild-type the crystals are relatively large and yellow, while in the car-10(−) they appear to be smaller, less numerous, and more sensitive to photodestruction.

In addition to *Phycomyces* (21), electron microscopic studies have revealed similar intracellular crystals in a wide range of plants, for example, the blue-green alga *Anabaena*, *Avena* coleoptiles, carrot tissue, and tobacco leaves (10, 19, 21). In animals, crystals have been found in almost all the phyla from protozoa to mammals (16). Of particular interest are the crystals isolated from the retinal tissue of fish and mammal eyes which have been identified as a flavin (6).

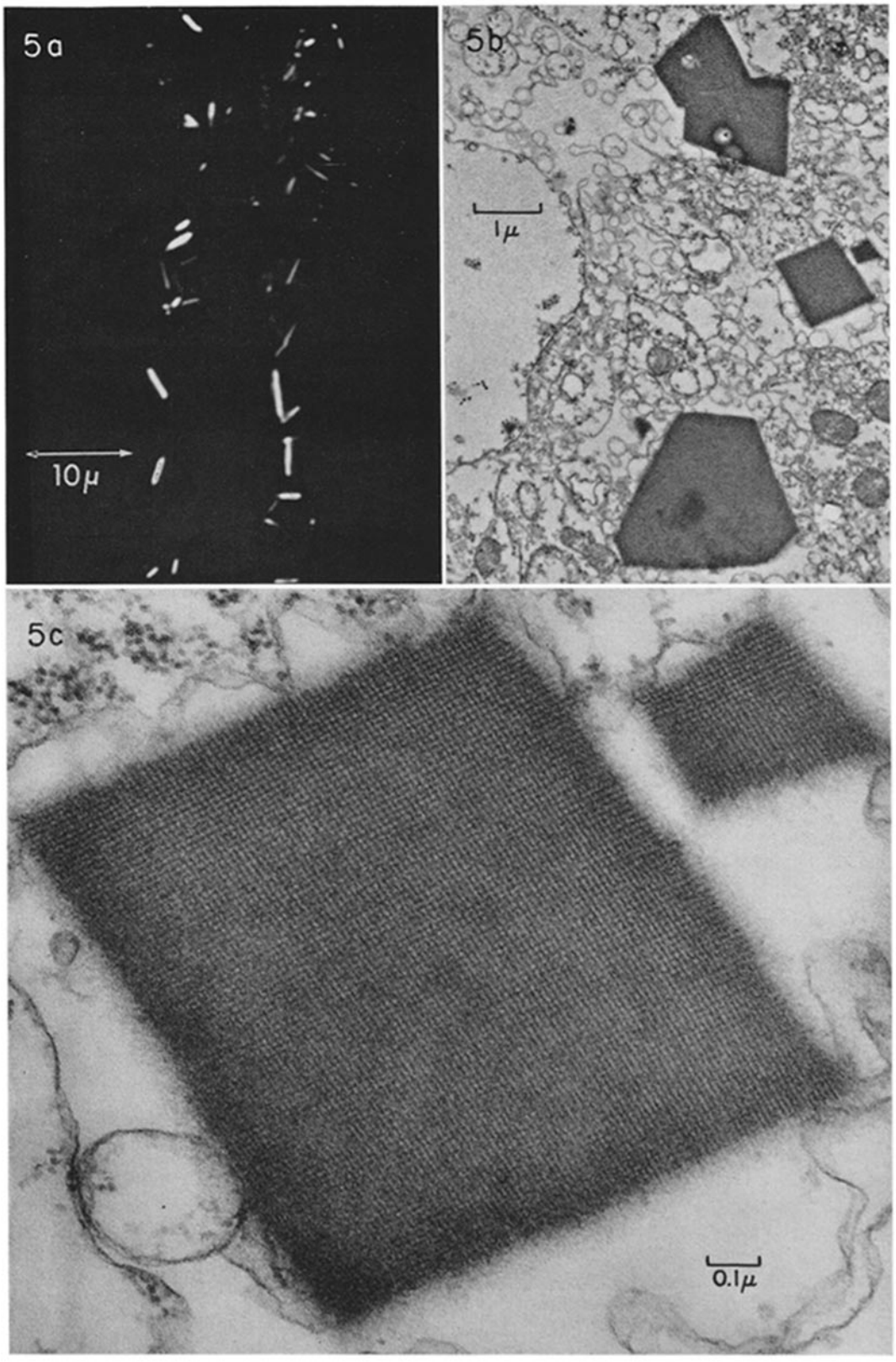

FIGURE 5 *a*, Crystals observed in polarized light (× 1,700). Note birefringence and location along vacuole. *b*, Electron micrograph of cross-section through growing zone showing cytoplasmic particles, mitochondria, and crystals near vacuole (×10,500). *c*, Highly magnified area from *b* (× 83,000). Note crystal lattice (~ 100 A).

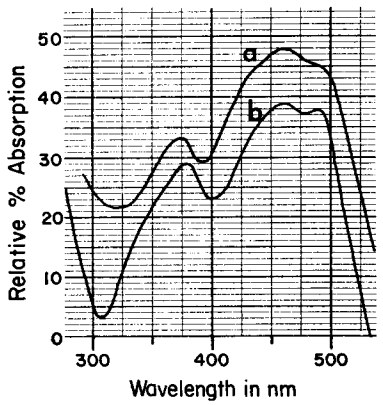

FIGURE 6 *a*, Absorption spectrum of single crystal isolated from wild-type *Phycomyces* sporangiophore. Typical of spectra of *in situ* crystals. *b*, Absorption spectrum of riboflavin crystal. There is strong absorption in the uv, but scatter and optical effects prevented proper resolution of the exact peaks. Taken with M-5 microspectrophotometer.

Since the crystals in *Phycomyces* are located primarily in the growing zone during Stage IVb, and within 1 mm of where the maximum light response occurs (13), it becomes necessary to isolate and chemically identify them.

In the crystals identified by microscopy, microspectrophotometry of both *in situ* and isolated crystals showed strong absorption in the ultraviolet, 260–290 nm, peaks near 370 and 460 nm, a shoulder near 430, and a smaller peak near 490 nm (Fig. 6 *a*). This spectrum compares remarkably well with the absorption spectrum of riboflavin crystals (Fig. 6 *b*) (22), to a molecular dispersion of riboflavin in caster oil (12), and to the absorption spectra of the sporangiophore (Fig. 1 *a* and *b*).

Even if these crystals are not the photoreceptor, spectra of the growing zone (Figs 1 and 2 *a*) and of the isolated crystals (Fig. 6 *a*) bear a striking spectral resemblance to a flavin or flavoprotein (Figs. 2 *b* and 6 *b*). This information, together with the isolation of a cytochrome (Fig. 3), suggests a possible scheme which would provide a photoreversible system, for example, from the oxidized, yellow riboflavin, via cytochrome and light, to the reduced, colorless form (23).

Since this phenomenon is a time-limited response, there is also the possibility of conversion of riboflavin in light and acid pH to lumichrome (3). Compare the spectra of Figs. 1 *b* and 2 *a* to that of lumichrome in Fig. 2 *c*. What is observed in these spectra is probably the production of lumichrome upon irradiation.

Therefore, β-carotene does not appear to be the primary photoreceptor molecule. β-carotene, or one of the other carotenes present (17), most likely functions as an accessory pigment molecule and thus complicates interpretation of the action spectra obtained for the process (2). However, additional action spectra, especially for the albino mutants, and chemical analysis are required in order to specifically demonstrate that the crystals in the growing zone are a *flavin* and that they are associated with the photoreceptor system of *Phycomyces*.

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