Replacement of riboflavin by an analogue in the blue-light photoreceptor of *Phycomyces*

(roseoflavin/photobiology)

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ABSTRACT   Under suitable conditions, roseoflavin [7-methyl-8-dimethylamino-10-(1'-D-ribityl)isoalloxazine] replaces riboflavin to about 80% in the photoreceptor of *Phycomyces*. The substitute-bearing photoreceptor functions with an efficiency of about 0.1% of that of the normal receptor. The substitution is proven by (i) a decrease of the effective light flux by a factor of 4.7, expressed as a corresponding increase in threshold, and (ii) an increase of the effectiveness of 529-nm light relative to 380-nm light. It has also been shown that roseoflavin is taken up by the mycelium, translocated to the sporangiophore, and effectively phosphorylated by the riboflavin kinase of *Phycomyces*.

The action spectra for the growth and tropic responses of *Phycomyces* sporangiophores to light suggest that the primary photoreceptor is riboflavin (1). The absolute extinction coefficient of the receptor pigment has been estimated and has been found to match that of riboflavin (2). It has also been demonstrated that the action spectrum of the growth response to light of *Phycomyces*, in addition to its general resemblance to the absorption spectrum of riboflavin, has a shoulder corresponding to a small peak at 595 nm, consistent with respect to location and intensity to the transition from the ground state to the lowest triplet state of riboflavin (3). If a flavin moiety does form the chromophore of the photoreceptor, it might be possible to replace it by a flavin analogue. If this analogue had absorption peaks different from those of riboflavin and if the new receptor were functional, one could expect changes in the action spectrum as compared to undoped controls and could thus distinguish substitution in the photopigment from substitution in other enzymes. In this paper we present evidence for such changes when the analogue roseoflavin is added to the growth medium.

Roseoflavin [7-methyl-8-dimethylamino-10-(1'-D-ribityl)isoalloxazine], an antibiotic produced by a *Streptomyces* strain (4, 5), has a strong absorption maximum in aqueous solutions at 505 nm ($\varepsilon = 31,000$) compared to the much weaker ones of riboflavin at 480 nm ($\varepsilon = 8,000$) and 445 nm ($\varepsilon = 12,500$). In addition, roseoflavin has an absorption minimum near 380 nm, where riboflavin has a maximum (Fig. 1). Therefore, if roseoflavin substitutes for riboflavin to form a functional receptor, one would expect a considerable red shift in the blue maximum and an abolition of the 380-nm maximum of the normal wild-type action spectrum.

The idea of replacing riboflavin in the photoreceptor of *Phycomyces* by an analogue was originated by J. L. Reissig 6 years ago in this laboratory, and several analogues were tested by Reissig at that time. The list was extended by one of the present authors in subsequent years. The suggestion to try roseoflavin, a natural product, was made by A. Bacher, and this compound, both unlabeled and ¹⁴C-labeled, was very kindly supplied by

Kunio Matsui (Research Institute for Atomic Energy, University of Osaka). Careful studies on phototropic balance and on the light-growth null response of undoped and doped wild-type sporangiophores (unpublished data) showed shifts indicative of substitution and weak activity but the results were poorly reproducible, suggesting an erratic amount of substitution. These variable results probably were due to variations in the amount of roseoflavin present in the cell where it has to compete with endogenously synthesized riboflavin. The roseoflavin concentration is determined by the extent of transport into the cell by riboflavin permease. This permease, in turn, is subject to wide fluctuation in concentration, due to frequent spontaneous mutations in the *dar* gene (6), a gene which controls the riboflavin permease.

For these reasons it was eventually decided to repeat the whole series of experiments with a riboflavin auxotroph. In such a strain the internal ratio of riboflavin to roseoflavin is determined solely by the external ratio. This approach led to the reproducible, unambiguous results reported in this paper.

MATERIALS AND METHODS

Riboflavin Auxotroph. The strain C322, *rib B3(-)*, used in these studies was obtained from *Phycomyces* wild-type NRRL 1555 by UV mutagenesis by N. K. Wischhusen in this laboratory. Several recycling steps applied to the mutagenized spores ensured the isolation of mutants with healthy and strong sporangiophores, well suited for physiological measurements. Complementation studies showed that the riboflavin biosynthesis in C322 is blocked before riboflavin synthase, the last enzyme in riboflavin biosynthesis.

Uptake of Roseoflavin into *Phycomyces* and Translocation into Sporangiophores. *Phycomyces* spores were plated on Sutter's minimal medium (7) containing L(-)-asparagine·H₂O (2 g/liter) and varying amounts of [2-¹⁴C]-roseoflavin. The plates were kept in darkness until sporangiophores appeared; then they were illuminated from above with incandescent light (0.004 W/m²). Sporangiophores were cut 1 cm from the base, with case taken to avoid contamination from the medium. The sporangiophores were solubilized in Nuclear Chicago Solubilizer (1 ml, 30 hr, 50°C); then, 12 ml of Aquasol-2 (New England Nuclear) was added and radioactivity was determined after chemoluminescence had ceased (2 days in the dark at 5°C). Quenching within each sample was determined with [2-¹⁴C]-toluene (Amersham) as internal standard.

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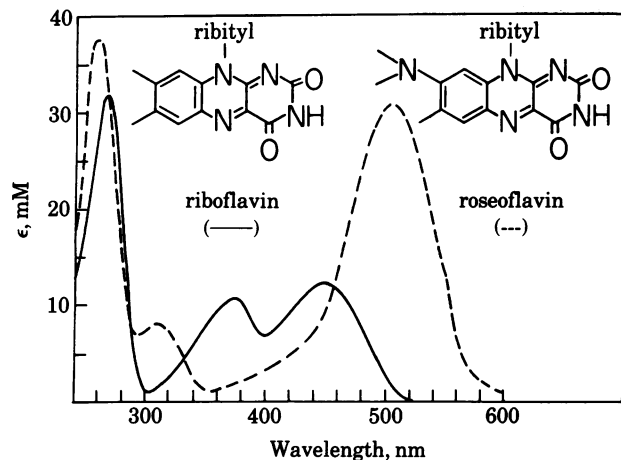‡ To whom reprint requests should be addressed.

FIG. 1. Absorption spectra of aqueous solutions of riboflavin and roseoflavin. At 380 nm, roseoflavin has only 1/10th the absorbance of riboflavin; at 530 nm, it has 180 times the absorbance.

Culture Conditions. Spores of C322 were inoculated (after heat shock for 15 min at 48°C) at the center of petri plates containing complete medium (10 spores per plate). Small pieces of mycelium from the growing edge of the mycelial mat were transferred to shell vials containing Sutter's minimal medium supplemented with roseoflavin (7 μg/ml) and riboflavin (0.5 μg/ml). The controls were supplemented with riboflavin alone (0.5 μg/ml). The inoculated vials were left in the dark till the first sporangiophores appeared. They were then transferred to a box illuminated from above with incandescent lamps (0.004 W/m²) and left uncovered. On medium free of roseoflavin, the sporangiophores appeared 3–4 days after inoculation. For the doped specimen the concentration ratio roseoflavin:riboflavin had been chosen such that the growth rate of the mycelium (progression of the mycelial front) was reduced to half the normal rate. It then took nearly 10 days for the appearance of sporangiophores. These sporangiophores were of normal size and grew at near-normal rates (>75%).

Threshold Measurements. The threshold for the phototropic responses of roseoflavin-doped and normal sporangiophores was measured in a threshold box containing 10 cubicles covering a large intensity range, as described by Bergman *et al.* (8). The light beam passed through a heat-reflecting filter and a 441-nm interference filter before it entered the first cubicle. After exposure to light for at least 8 hr, the angle of photogeotropic equilibrium of each sporangiophore was measured. An image of the sporangiophore was projected onto a ground glass screen and the maximal angle from vertical was measured with a goniometer. Absolute intensities were measured with a calibrated planar diffused silicon PIN 10 DP photodiode (United Detector Technology).

Measurement of Phototropic Equilibrium. The experimental set-up for light stimulation consisted of a specimen box equipped with two variable light sources placed opposite each other, 1 m apart. One source was a Bausch and Lomb high-intensity monochromator (set at 380 nm) with a tungsten lamp. The light passed successively through a 15% CuSO₄ solution (1-cm pathlength), the monochromator, and a ground glass diffuser before entering the specimen box. The other source was a 125-V (15-W) tungsten lamp from which light passed through a heat filter [15% CuSO₄ solution (1-cm pathlength)], a 529-nm interference filter, and a ground glass diffuser before falling on the specimen. Six shell vials, each containing not more than two straight stage IV sporangiophores, were placed in a 50-cm rack

in a sufficiently staggered manner to prevent mutual shading. The rack was placed within the specimen box aligned in the light path. At any given position, the light intensity across the rack did not vary more than 5%. The relative humidity inside the box was maintained at 65–70% by moistening the cloth cover of the box with a regulated flow of water. The temperature was kept constant at 20 ± 1°C. After exposure to both lights for 10–12 hr, the angle from vertical (as described under threshold measurements) and the relative position of each sporangiophore were measured. The intensities of the lights were regulated such that the point of phototropic neutrality was close to the center of the box.

RESULTS

Roseoflavin can compete effectively with riboflavin in various reactions of riboflavin metabolism in *Phycomyces* and is therefore a good candidate for incorporation into the photoreceptor. This is apparent from the following: (*i*) roseoflavin is taken up into the mycelium by a flavin specific permease (apparent K_m = 1 μM) as determined by uptake of [2-¹⁴C]-roseoflavin; (*ii*) roseoflavin is translocated into the sporangiophores, reaching micromolar concentrations; (*iii*) it is phorphorylated efficiently *in vitro* by purified riboflavin kinase; (*iv*) 20 μM roseoflavin in the medium inhibits growth of wild-type *Phycomyces* completely, indicating interaction with essential flavoenzymes (the background and data for these points will be published elsewhere).

The results of the threshold measurements with a 441-nm source are shown in Fig. 2. Each symbol represents the average of at least 15 specimens whose photogeotropic equilibrium angle was determined at the given intensity. In each case the doped specimen showed a smaller tropic response than did the undoped one. The curve for the undoped specimens extrapolates to a threshold of 10^{-9} W/m², the same as for wild type (data not shown). To evaluate the change in sensitivity, the horizontal displacement between the linear parts of the two curves (corresponding to a bending angle of 20° to 70°) was determined. It corresponds to a 4.7-fold increase in the threshold of the doped specimen.

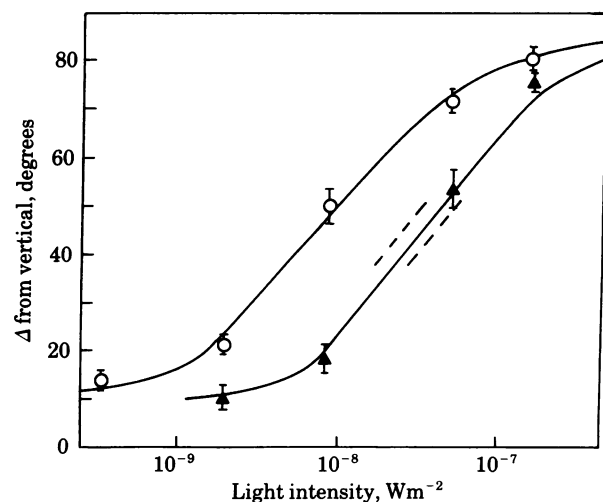FIG. 2. Threshold measurements at 441 nm for sporangiophores grown on riboflavin alone (O) or doped with roseoflavin (▲). The ordinate gives the angle from the vertical of sporangiophores exposed for 8 hr to the intensity given by the abscissa. Each data point is the mean of at least 15 specimens, and the error bars give the standard deviation of the mean. The roseoflavin curve is displaced relative to the riboflavin curve, indicating a diminution of effective flux by a factor of 4.7. Displacements by factors of 3.7 or 5.7, indicated as broken lines, would seem to cover the extreme range of possible displacements.
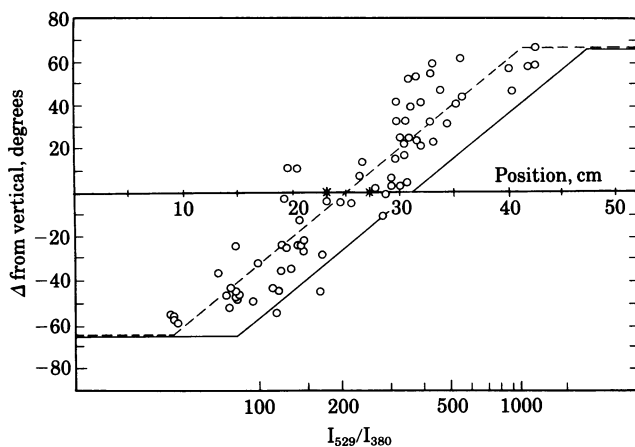
FIG. 3. Phototropic equilibrium bending at various intensity ratios of 529 nm and 380 nm light (I_{529}/I_{380}). Bending toward 529 nm or 380 nm light was taken as positive or negative, respectively; 380-nm light was positioned at the left side. The center abscissa corresponds to distances of sporangiophores from the end of the rack nearest to the 380 nm light. The solid line is the least squares fit for undoped specimens; the broken line is that for the doped specimens. Data points are shown only for the doped sporangiophores. The intensity ratio for doped specimens is 208. An estimated extreme error range in this ratio (175 and 250) is indicated by asterisks.

Fig. 3 summarizes the phototropic equilibrium data. The solid line represents the least squares fit of the undoped sporangiophores (individual points not shown) and the broken line is that for the doped specimen. Phototropic neutrality results when a specimen sees both light sources as equally bright and therefore would not bend toward either source. The intensity of either light source was sufficient to produce by itself a saturating response along the entire length of the rack. The bottom abscissa gives the ratio of the two intensities. At the equilibrium point for undoped specimens, 31 cm from the left end of the rack, this ratio is 310 ($I_{529} = 6.0 \times 10^{-5}$ W/m^2; $I_{380} = 1.67 \times 10^{-7}$ W/m^2). At the equilibrium point for doped specimens (25 cm from left end) it is 208. For the doped specimens the ratios 175 and 250 (indicated by asterisks) would seem to be a reasonable estimate for the possible range of error. The actual shift in the equilibrium position amounted to 6 cm toward the 380-nm source. Thus, the doped sporangiophores see the 529-nm light better than do the undoped ones.

Interpretation of the Results. Indirect evidence suggests that the blue light photoreceptor pigment in *Phycomyces* is riboflavin. Our experiments were aimed at providing more direct evidence for the identity of the receptor by attempts to replace the chromophore by the riboflavin analogue roseoflavin. Experiments using radioactive roseoflavin have shown that it is taken up by the mycelium and translocated into the sporangiophores and that roseoflavin is a substrate for riboflavin kinase. Thus, an observed red shift in the phototropic action spectrum of roseoflavin-doped specimens may be attributed with confidence to the substitution in the photoreceptor.

Our data may be analyzed as follows. Light striking the sporangiophore is absorbed by the photopigment. This, in turn, leads by as yet obscure processes to the corresponding physiological responses. The effective flux, $F_{eff}, = I \cdot \varepsilon \cdot \phi$ depends on the intensity I of incident light, the absorption properties of the photopigment described by the extinction coefficient ε (a function of wavelength), and the efficiency of the photoresponse, described by ϕ, the quantum yield.

If riboflavin is indeed the chromophore of the photopigment, we may assume that, upon doping, a fraction of this chromophore may be replaced by roseoflavin. Call this fraction c. Then, the effective flux consists of contributions of both pigment species:

$$F_{eff} = I(\varepsilon_{rif} \cdot \phi_{rif}(1 - c) + \varepsilon_{rof} \cdot \phi_{rof} \cdot c)$$

where the subscripts "rif" and "rof" refer to riboflavin and roseoflavin, respectively. Depending on how extinction coefficient and quantum yield of roseoflavin compare with the corresponding values of riboflavin, the F_{eff} may increase or decrease upon doping. Experimentally, we find that we need a 4.7-fold increase in intensity at 441 nm to obtain the same physiological response in doped sporangiophores compared to undoped specimen. Thus, doping reduced the effectively absorbed flux by about 80%. The extinction coefficient of roseoflavin at 441 nm is 1.82 times less than that of riboflavin, not enough by itself to account for the observed factor of 4.7. The quantum efficiency of roseoflavin must be considerably lower than that of riboflavin.

Quantitatively, one can assess both substitution and quantum yield of roseoflavin by equating effective fluxes received by doped and undoped specimens when they give the same physiological response. Thus, setting $\phi_{rif} = 1$, we write

$$F = I(\varepsilon_{rif}(1 - c) + \varepsilon_{rof} \cdot c \cdot \phi_{rof}) = \frac{1}{4.7} I\varepsilon_{rif}$$

and obtain $\phi_{rof} = -\dfrac{1.44}{c} + 1.82,$ [2]

a linear relationship between ϕ_{rof} and $1/c$. Only the part when $0 < c < 1$ and $\phi_{rof} \geq 0$ is physically meaningful. It shows that the substitution must be at least $c = 0.79$, corresponding to the case of zero quantum yield, and that the quantum yield must be less than 0.38, corresponding to the case of $c = 1$.

These conclusions leave open the possibility that roseoflavin is not active at all in the photopigment ($\phi_{rof} = 0$), thus raising doubts as to whether it can get into the photoreceptor or whether the observed effect might be spurious, possibly due to interference of roseoflavin with the general riboflavin metabolism. To decide this issue we turn to the phototropic balance experiments.

The conditions for these balance experiments had been such that a very small quantum efficiency of roseoflavin should be strongly expressed. The doped and undoped specimens were placed between lights of $\lambda_1 = 380$ nm and $\lambda_2 = 529$ nm wavelength. At λ_1, riboflavin absorbs 10 times better than riboflavin, whereas at λ_2 roseoflavin is favored by a factor of 180. Even a small quantum efficiency of roseoflavin may yield a significant contribution to the effective λ_2 flux under such conditions. The experiment indeed shows that doped sporangiophores can see λ_2 light better than undoped specimens can. This clearly shows that roseoflavin is active as photopigment.

For evaluating this experiment quantitatively we compare effective fluxes under balance conditions. From the undoped balance, $I^{\lambda_2}/I^{\lambda_1} = 360$, we obtain an estimate of the extinction ratio of riboflavin at the two wavelengths, $(\varepsilon^{\lambda_1}/\varepsilon^{\lambda_2})_{rif} = 360$, which agrees well with the value obtained from the phototropic action spectrum [$I^{\lambda_2}/I^{\lambda_1} = 478$ (9)] measured with a caroteneless *Phycomyces* mutant. The flux balance for the doped specimen is theoretically

$$F^{\lambda_1} = I^{\lambda_1}[\varepsilon_{rif}^{\lambda_1}(1 - c) + \varepsilon_{rof}^{\lambda_1}\phi_{rof} \cdot c]$$
$$= I^{\lambda_2}[\varepsilon_{rif}^{\lambda_2}(1 - c) + \varepsilon_{rof}^{\lambda_2}\phi_{rof} \cdot c]$$
$$= F^{\lambda_2},$$

and, experimentally, $I^{\lambda_2}/I^{\lambda_1} = 208$. The extinction ratios of ri-

boflavin:roseoflavin at λ_1 and at λ_2 and the extinction ratio of riboflavin at λ_1 and λ_2 have been given above. Making these substitutions, we get a second relationship between degree of substitution and quantum yield:

$$\phi_{rof} = 0.004\left(\frac{1}{c} - 1\right). \qquad [3]$$

This relationship between ϕ_{rof} and $1/c$ is again linear. We can combine Eqs. 2 and 3—i.e., the results of the two basic experiments—and derive ϕ_{rof} and c individually: $c = 0.79$ and $\phi_{rof} = 0.001$.

In conclusion then we can say that about 79% of riboflavin in the photoreceptor is replaced by roseoflavin. The latter is active as photopigment but only at 0.1% the rate of riboflavin.

To estimate the limits of error in our determinations of the degree of substitution and of the quantum yield we assume a maximal range of error of 3.7 and 5.7 (instead of 4.7) (see Fig. 2) in the threshold shift and of 175 and 250 (instead of 208) (see Fig. 3) in the equilibrium intensity ratio for the doped specimen. These values lead to extreme values of $c = 0.73$ and 0.82 and $\phi_{rof} = 0.0029$ and 0.0005, a rather narrow range in both cases.

DISCUSSION

We have shown that roseoflavin, an analogue of riboflavin in which the methyl group in the 8 position is replaced by a dimethylamino group, can substitute for riboflavin in the blue-light photoreceptor of *Phycomyces*. Specifically, we have shown that roseoflavin is taken up by the mycelium and is translocated to the sporangiophores and that sporangiophores doped in this way show a diminution of phototropically effective light flux at 441 nm and an increase in sensitivity to 529-nm light relative to 380-nm light, corresponding to differences in the absorption spectrum of roseoflavin relative to riboflavin. The quantitative reproducibility of the findings depended on the use of a riboflavin auxotroph, which allowed control of the riboflavin:roseoflavin input ratio. These results strongly reinforce the conclusion that riboflavin is the chromophore of the photoreceptor.

The interpretation of these observations hinges on a particular model for the effect of substitution: it is assumed that the observed photophysiological effects are the direct result of the replacement of riboflavin by roseoflavin in the photopigment and are not caused indirectly through the effects on other flavoproteins. Furthermore, for the quantitative evaluations, we have assumed that the light effects are strictly linear. With these model assumptions we arrive at estimates for the degree of substitution (79%) and for the quantum yield (relative to undoped photoreceptor) of 0.1% with reasonably small error ranges.

Both of these quantities are noteworthy. The degree of substitution is high, almost as high as the input ratio of roseoflavin:riboflavin, 14:1 or 94%. We doubt that many of the other flavoproteins will have undergone that high a substitution. It will be fascinating to pursue this matter further and especially to compare substitutions in FAD and FMN enzymes. It is clear at this point that the photoreceptor cannot be a flavoprotein in which the flavin is linked covalently to the protein through the 8 position, as in succinic dehydrogenase and in monoaminoxi-

dase. Roseoflavin has a dimethylamino instead of a methyl group in the 8 position.

The quantum yield is even more noteworthy. It is small ($\approx 0.1\%$) but definitely not zero. This statement can be made with confidence because the spectral characteristics of roseoflavin show a strong red-shift relationship to riboflavin. This red shift permits one to assign some of the activity seen at 529 nm to the doping species. For no other flavoprotein-catalyzed reaction could such a small activity be separated *in vivo* from that of riboflavin. What, in fact, happens to these other flavoprotein functions upon replacement of riboflavin by roseoflavin? At present we know little of the quantitative redox or hydride transfer chemistry of roseoflavin or of the photophysics of this molecule. We do not even know the position of the lowest triplet state of roseoflavin or whether it is involved in its redox chemistry. It is quite likely that the redox chemistry of roseoflavin is sufficiently disturbed to make the yield of such reactions practically zero under physiological conditions. The functioning of the organism under conditions of high substitution would then be entirely dependent on the residual moiety still operating with riboflavin.

The main difficulty with the further characterization of the blue-light receptor has been the unavailability of *in vitro* assay of the receptor. The light-induced absorbance changes (reduction of a cytochrome *b*) are of doubtful value because they occur also in the HeLa cell, in which there is no known photophysiology (10). Recently, the activation of the enzyme nitrate reductase by light in various organisms has drawn much attention (11–13). In this system, activation of the enzyme appears to be the primary output, both *in vivo* and *in vitro*. The activation (*Neurospora*) is not accompanied by reduction of cytochrome *b*, even though the enzyme contains two units of cytochrome *b* in addition to two units of FAD and one atom of molybdenum. The activation by light shows a typical blue-light action spectrum but no light-induced absorbance changes. It would be of great interest to see whether roseoflavin can be substituted and be active in this system.

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