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ACTIVATION OF PHYCOMYCES ADENOSINE 3', 5' - MONOPHOSPHATE PHOSPHODIESTERASE BY BLUE LIGHT

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SUMMARY. Cyclic AMP phosphodiesterase can be extracted from sporangiophores of Phycomyces blakesleeanus. Activity is enhanced by 1-10 μM FAD and FMN but not by riboflavin. Moderate intensity of blue light also activates the enzyme, especially in the presence of 1mM GTP. The enzyme must be extracted and stored in the absence of blue light for this result. Forty times the intensity of red light has no effect. This finding is consistent with the very sudden transient drop in cyclic AMP level upon light stimulation in the intact sporangiophore.

The sporangiophore of Phycomyces blakesleeanus transiently increases its growth rate upon moderate blue light stimulation. Detection of this stimulus is almost certainly achieved by a flavoprotein (1, 2); the subsequent molecular mediation of the blue light photo-response system is unknown. We have shown earlier (3) that immediately after a stimulus and well before the growth response, a sharp transient 60% dip in cyclic AMP level occurs. Sporangiophores immersed in perfluorotributylamine or water exhibit a 10 min decrease in growth rate when dibutyryl cyclic AMP is added. These observations implicate cyclic nucleotides in the light stimulus-response system. Indeed, throughout the living kingdom, cyclic nucleotides seem to be involved in nearly every sensory system examined. The most dramatic and best worked out is the cyclic GMP monophosphate phosphodiesterase system of vertebrate rod outer segment (4); its light activation corresponds to the action spectrum of rhodopsin. Still the role of cyclic nucleotides in sensory transduction is unknown. Demonstrated here are two pertinent properties of cyclic AMP phosphodiesterase activity (EC 3.1.4.17) isolated from Phycomyces sporangiophores: its enhancement by certain flavins, and its specific activation by low intensity blue light especially in the presence of GTP.

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MATERIALS & METHODS

ENZYME ISOLATION. Phycomyces blakesleeanus NRRL1555(-) was grown under room fluorescent lights on commercial instant potatoes supplemented with 0.1% yeast extract (Difco) and 0.25 ppm thiamine HCl in large trays 30cm x 50cm. Sporangiophores initiate after about 50 h; developmental synchrony was achieved by chilling at 4° in the dark overnight and then exposing the trays to room light and temperature (24°). After about 20 h the mature (stage IVb) sporangiophores were returned to the cold room for 30 min either in the dark or under light conditions. The extraction and isolation procedures occur at 4° and either in the light ("light extracted") or in the dark aided when necessary by a dim light equipped with a Kodak #1 red filter ("dark extracted"). Sporangiophores were harvested and cut into 3mm pieces into a mortar containing an equal volume of 150 mM NaCl, 10 mM tris·HCl buffer (pH 7.4). The material was ground with a pestle and squeezed through 3 layers of cheesecloth. These steps were repeated. The filtrate was centrifuged at 1100 xg for 10 min, and the supernatant solution centrifuged again at 31,000 xg for 30 min. The remaining supernatant solution was made up to 40% saturation with ammonium sulfate, then allowed to incubate at room temperature for 10 min. The precipitate was pelleted at 7700 xg for 5 min and dissolved in 10 mM tris·HCl (pH 7.4). This material was dialyzed 4 6 h in 500 volumes of the same buffer, diluted and frozen at -20° in 1 ml aliquots. Portions were assayed for protein using the Lowry method (5). Enzymatic activity reported here was determined within 2-3 weeks but full phosphodiesterase activity remained after more than 2-3 months storage.

STANDARD PHOSPHODIESTERASE ASSAY. The determinations were done in plastic scintillation vials. The reaction was run in a total volume of 110 µl which contained 4 µM [^{3}H]- cyclic AMP (10^5 cpm), 50-300 µg phosphodiesterase extract, 30 µg snake venom (Crotalus atrox, Sigma), 500 µg bovin serum albumin and additives in 5 mM $MgCl_2$, 40 mM tris·HCl (pH 8.0). After thermal equilibration, the reaction was started by the addition of substrate and the mixture equilibrated 30 min. The reaction was stopped by the addition of 0.8 ml of a slurry (60% settled volume) of AG1-X2, 200-400 mesh anion-exchange resin in water. The mixture was allowed to equilibrate 10 min, 10 ml of scintillation fluid added (125g naphthalene, 7.5 g PPO, and 0.4 g dimethyl-POPOP per 1 p-dioxane) and the samples counted. Blanks were as assay, including additives, but were stopped immediately by resin. Quenching was accounted for by internal standard calibration (6).

Snake venom, which provides 5'-nucleotidase activity, was incorporated in the assay because the Phycomyces extract contains high but variable endogenous nucleotidase activity much as found in Neurospora crassa (7). Phosphodiesterase activity was negligible in the snake venom.

EFFECT OF LIGHT ON PHOSPHODIESTERASE ACTIVITY. Concentrations of assay components were as above except the total volume was 200 µl. Mixtures were preincubated in 5 mm pathlength quartz microcells without substrate for 20 min in the dark, in red light, or in blue light. Labeled cyclic AMP was added and the light conditions simultaneously altered to one of the three regimes indicated. After a 20 min incubation, the mixture was quantitatively transferred to a scintillation vial with 0.8 ml 60% settled volume of resin, incubated 10 min; and the cocktail counted. All measurements were in triplicate. Temperatures, 24°-25°, were measured with a black tipped mercury thermometer and did not vary more than 0.5° during any experiment.

LIGHT CONDITIONS. The light source was a tungsten incandescent lamp (Sylvania CZA). An Eastman heat filter was employed to absorb infrared. "Blue light" was obtained using a Corning 5-61 filter plus a 1.0 absorbance neutral density

TABLE I Effect of Flavins on Phosphodiesterase Activity
Activities in pmole·min^{-1}·mg^{-1}, and percent control
with standard deviations (N=4)

	Concentration (μM)				
Flavin	1	2	3	5	10
Riboflavin	6.59±0.22 (103±3)	6.59±0.26 (101±4)	7.00±0.21 (107±3)	6.66±0.14 (102±2)	6.53±0.08 (100±1)
FAD	7.93±0.21 (122±3)	7.68±0.04 (117±6)	7.91±0.13 (122±2)	7.56±0.53 (116±7)	7.15±0.31 (110±4)
FMN	7.51±0.05 (115±1)	7.20±0.17 (111±3)	8.55±0.38 (131±4)	7.97±0.21 (122±3)	7.36±0.24 (113±3)

CONTROL 6.51±0.22 (100±3)

filter (Bausch and Lomb). The 5-61 is a broad band filter centered at 400-450 nm. An RG-610 cut-off filter (610 nm, Schott & Gen., Main) was positioned to provide the red light. A lucite diffuser plate was placed between the source and the microcells. The distance between source and sample was 90 cm. A Keithley electrometer, Model 610 C, equipped with a Schottky-Barrier UV-enhanced PIN photodiode (United Detector Technology) was used to measure light intensity. A yellow Springs Instrument Radiometer was used to calibrate the instrument. This in turn was checked for linearity using a standard incandescent lamp. Light intensity did not vary more than 10% over the 30 cm chamber used for the experiments. In this report blue light intensity was 6 μwatts·cm^{-2} at the sample, and red light intensity was 250 μwatts.cm^{-2}. When necessary a dim light equipped with a Kodak #1 filter was employed for a few seconds to facilitate the addition of solution to the microcells.

RESULTS

Phosphodiesterase extracted in the light and tested under ordinary fluorescent room lights was activated by 1-10 μM FAD or FMN, but not by riboflavin up to 10 μM. The effect is small, 10-31%, but significant and reproducible (Table I). While not proving a direct relation, this finding is consistent with the action spectrum for the growth response of *Phycomyces* which indicated that the photodetector protein contains a bound flavin (1, 2).

The effects of light on dark extracted phosphodiesterase are shown in Table II. Samples were preincubated in microcuvettes for 20 min in the dark, in high intensity red light (250 μwatt·cm^{-2}) or in moderate intensity blue light (6 μwatt·cm^{-2}), labeled cyclic AMP added and the enzyme incubated 20 min in the presence of one of the light regimes mentioned above. In experiment I,

TABLE II Effect of Light on Phosphodiesterase Activity

Experiment	Program*	Additive	Activity (pmole·min^{-1}·mg^{-1})	%
I	DD	none	7.24±0.18	100±2
	RD		7.48±0.36	103±5
	RR		7.63±0.13	105±2
	RB		8.51±0.10	117±1
	BR		8.32±0.17	115±2
II	DD	none	8.72±0.52	100±5
	DB		10.29±0.07	118±1
	BB		9.80±0.43	112±4
	BD		9.53±0.18	109±2
III	DD	1 mM GTP	3.42±0.19	100±5
	DB		5.24±0.13	153±2
	BB		4.69±0.20	137±4
	BD		3.47±0.02	102±1
IV	DD	1 mM GTP	2.67±0.17	100±6
	DR		2.83±0.18	106±6
	RR		2.82±0.03	106±1
V	DD	1 mM GTP +	3.57±0.26	100±8
	DB	2.5 μM FAD	5.24±0.02	147±0
	BB		4.98±0.11	139±2
	BD		4.76±0.09	133±2

* D = dark, R = red (250 μwatt·cm^{-2}), B = blue (6 μwatt·cm^{-2})
First letter refers to 20 min preincubation period, second letter to 20 min incubation. Average of triplicate determinations, with standard deviations. See text for details.

red light has no effect. However, moderate blue light either during the preincubation or incubation period or both significantly affected phosphodiesterase activity (Exp. I and II). The effect is, however, small, 9-18%.

The activation of phosphodiesterase by incubation in blue light is considerably enhanced by 1mM GTP. Up to 53% enhancement by blue light is observed while red light has no effect (Exp. III and IV). The effect is very similar when done at pH 7.2 (DB, 119 ± 3%, BB, 130 ± 0%, both relative to dark incubation) instead of 8.0. One cycle of freezing and thawing the enzyme preparation has no adverse consequences. However, phosphodiesterase isolated under room light conditions does not respond to light. Experiments not shown here

indicated that 0.2 mM and 0.5 mM GTP are not sufficient to enhance the light activation. It should be noted that 1 mM GTP has a net inhibitory action on phosphodiesterase. The further addition of 2.5 μM FAD in Experiment V does not modify the effect of GTP with the possible exception of the blue light preincubated sample assayed in the dark.

DISCUSSION

We emphasize that these findings do not prove that the blue absorbing photodetector and the phosphodiesterase activity reside on the same molecule. The enzyme may be a part of a heterogeneous protein aggregate or may be bound to a membrane fragment which contains a photoreceptor. Furthermore, the enhanced activity in the presence of FAD or FMN may be due to blue light sensitization and energy transfer. On the other hand, the results in Table I and Experiment V in Table II are consistent with the speculation that blue light absorption may cause the release of a flavin moiety; addition of exogenous flavin would thus have no effect on dark extracted receptor protein.

The considerable enhancement of the light activation of phosphodiesterase by GTP should be compared to the enhancement of cyclic GMP phosphodiesterase in rod outer segment by GTP and ATP (4). GTP may be acting directly on a light sensitive phosphodiesterase in Phycomyces. However, 1 mM GTP inhibits total phosphodiesterase activity by about 50% and the effect may be in part due to a possible differential inhibition of a separate phosphodiesterase activity, which is not light sensitive. The activation of phosphodiesterase seems specific to blue light since 6 μwatts·cm^{-2} is effective while 40 times the intensity in red light is ineffective. (The blue light intensity is about that found at ambient laboratory light conditions.)

A temptation is to attempt to compare these findings to the blue light effects commonly found in fungi and higher plants. However, only some of the effects on proteins are due to a relatively straight forward interaction of a bound chromophore or a photodetector protein. For example, the blue light activation of phenylalanine ammonia-lyase and the flavoprotein nitrate reduct-

ase are via enhanced protein synthesis. In contrast, blue light, in the presence of FMN, inhibits directly the flavoproteins lactate dehydrogenase and glycolate oxidase while it activates a glycine oxidase (8).

Could the observation be due to a temperature effect? Temperatures were measured before and after each experiment with a black tipped mercury thermometer. The largest temperature increase recorded was 0.5°C. Nevertheless, the temperature coefficient for activity was determined in separate experiments and was only 5.9% per degree at 22-27°. Also in several repetitions of the experiments in Table II, the order of the incubations was changed randomly with essentially equivalent results.

The effect of light on phosphodiesterase activity may or may not be direct. Nevertheless, the purification and characterization of the system may provide insight into the ubiquitous presence of cyclic nucleotides and associated proteins in sensory processes. Initial characterization of phosphodiesterase enzymatic properties have been submitted elsewhere for publication. Reconstitution of light activated phosphodiesterase activity from wild type and mutant Phycomyces seems especially promising.

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