Accumulation of protoporphyrin IX and Zn protoporphyrin IX in *Cyanidium caldarium*

(tetrapyrrole/biosynthesis/fluorescence)

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ABSTRACT *In vivo* fluorescence studies of *Cyanidium caldarium* mutants grown in the dark in dextrose-containing media have shown that these organisms accumulate protoporphyrin IX. In the dark the accumulated protoporphyrin IX is gradually turned into a metalloprophyrin, Zn protoporphyrin. In the light, in the chlorophyll-lacking mutant GGB, both compounds are degraded and phycobiliproteins are formed. These results implicate protoporphyrin IX *in situ* as the general precursor to tetrapyrrole pigments and Zn protoporphyrin IX as a possible intermediate or regulator in the biosynthesis of phycobilins.

Cyanidium caldarium, a red alga, synthesizes both phycobiliproteins and chlorophyll *a* when grown in the light but lacks these pigments when grown heterotrophically in the dark (1). Its mutant, GGB, is incapable of forming chlorophyll *a* and contains only phycobiliproteins, the light-absorbing chromophore being phycocyanobilin, a linear tetrapyrrole structurally related to mammalian bile pigments (2, 3). Because mammalian bile pigments are formed via cleavage of the tetrapyrrole ring of heme (4-6), a similar mechanism has been suggested for phycocyanobilin formation—i.e., degradation of a metalloprophyrin precursor (7, 8).

Protoporphyrin IX and other probable intermediates have not been isolated from *Cyanidium* cells actively synthesizing phycocyanin (8). However, ¹⁴C-labeled CO and phycocyanobilin were produced from labeled δ -aminolevulinic acid in equimolar quantities by wild-type and phycobiliprotein-containing mutants of *C. caldarium*, indicating that protoporphyrin IX or its metal complex may be a direct precursor of phycocyanobilin (9). Action spectra of phycocyanin synthesis in GGB pointed to the possible involvement of a heme compound as precursor (10).

The present study demonstrated that protoporphyrin IX is indeed synthesized in GGB cells without addition of exogenous precursors and is converted into a metalloprophyrin, Zn protoporphyrin, in the dark. In the light, both compounds disappear gradually with the onset of phycocyanobilin synthesis.

METHODS

C. caldarium was grown in darkness in Allen's medium (11) as modified (7) and supplemented with 1% dextrose. For growth in the light, dextrose was omitted from the medium; the cultures were put on a revolving orbital shaker and illuminated by two fluorescent light tubes at a distance of 40 cm. For all fluorescence measurements as a function of time the cultures were maintained in 1-cm (inner diameter) glass tubes (Kimax no. 99447). The sealed tubes maintained sterility of the cultures and allowed sequential fluorescence measurements on the same

culture. These cultures were kept dilute to avoid the effect of reabsorption of fluorescence. Cultures for producing material for chromatography were grown in large (1.5 liters) cotton-stoppered flasks. They were checked before use to make sure that, upon dilution, their spectral properties were similar to those of cultures grown in tubes and used for time-course measurements.

For chromatography the cells were centrifuged and washed twice with doubly distilled water. The pellet was resuspended in 5 ml of acetone/H₂O/0.1 M NH₄OH, 9:2:1 (vol/vol), and sonicated in a sonicator (Lab-Line Ultratip Labsonic system) for six 20-sec bursts to break the cells. Porphyrins were extracted as described (12) for plastid metalloprophyrins. The broken cell fragments were centrifuged at 39,000 $\times$ *g* for 10 min. The 75% acetone extracts were extracted with an equal volume of hexane and then with a 1/3 volume of hexane. The hexane-extracted acetone fractions were transferred to ether by adding to it 1/17 of its volume of saturated NaCl and 1/30 of its volume of 0.2 M KH₂PO₄ and extracting consecutively with peroxide-free diethyl ether. The ether extracts were combined and concentrated under vacuum before chromatography.

Zn protoporphyrin IX was synthesized by an adaptation (12) of the method recommended by Falk (13). Protoporphyrin IX (Porphyrin Products, Logan, UT) was dissolved in 2 ml of chloroform and treated, at 100°C, with 0.1 ml of glacial acetic acid saturated with zinc acetate. The reaction was deemed complete when the protoporphyrin IX emission maximum at 637 nm was no longer detectable by fluorimetry.

Chromatography was on thin layers of silica gel H in benzene/ethyl acetate/ethanol, 8:2:2 (vol/vol), as described (14). The regions that fluoresced red under UV light were scraped into 1 ml of methyl alcohol/acetone, 4:1 (vol/vol). The supernatant after centrifugation was used for spectroscopic determinations.

Fluorescence emission and excitation spectra were recorded on a Perkin-Elmer spectrofluorimeter (model MPF-44A). Both excitation and emission slit half-band-widths were set at 6 nm. Spectra were not corrected for the spectral sensitivity of the apparatus and the spectral radiance of the exciting light source because only comparative measurements were involved.

RESULTS AND DISCUSSION

When grown in the dark on dextrose-supplemented medium, wild-type and mutant *Cyanidium*, accumulated a compound that was fluorescent *in vivo*. The fluorescence maximum for whole cells was at 636 nm. Mutants GGB and GGBY, the latter incapable of forming either phycobiliproteins or chlorophyll *a*,

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FIG. 1. Fluorescence spectra of a GGB culture growing in the dark. Numbers 1–5 designate spectra at 5, 7, 9, 11, and 17 days after the start of the culture, respectively. The excitation wavelength was 410 nm.

contained relatively more of this compound than did the wild type.

The gradual development of the fluorescence band at 636 nm in whole cells of the GGB mutant, as a function of incubation time in the dark, is shown in Fig. 1. Further incubation resulted in a decrease of this band and a concurrent emergence of a fluorescence band at 585 nm. Fluorescence excitation spec-

FIG. 2. Fluorescence (A) and fluorescence excitation (B) spectra of a GGB extract before chromatography (curve a), of the two separated components (curves b and c), and of two reference compounds, Zn protoporphyrin IX (curve d) and protoporphyrin IX (curve e), after chromatography. The fluorescence excitation wavelengths were 410 nm for curves a, c, and e and 417 nm for curves b and d. The observation wavelengths for fluorescence were 632 nm for curves c and e and 645 nm for curves b and d.

tra of these two bands *in vivo* indicated that the former is probably protoporphyrin IX; the latter is a metalloprophyrin. We stress that the fluorescence measurements were carried out in the glass tubes in which the algae were growing and that the suspending medium was free of these tetrapyrroles.

The two compounds were extracted and separated by thin-layer chromatography. Fig. 2A shows the fluorescence spectra of the GGB extract before chromatography and those of the two components after separation. Dashed lines indicate the spectra of two reference compounds, commercially available protoporphyrin IX and artificially synthesized Zn-protoporphyrin IX. Correspondence was good between the fluorescence spectra (Fig. 2A) and between the fluorescence excitation spectra (Fig. 2B) of the natural extracts and the reference compounds, indicating that protoporphyrin IX is synthesized in GGB cells grown in the dark. As seen in Fig. 1, protoporphyrin IX is converted into Zn protoporphyrin IX. The fluorescence spectrum of Mg protoporphyrin IX indicates that this compound has a corrected emission at 597 nm versus 592 nm for the Zn protoporphyrin IX (12), and similar fluorescence spectra in these studies produce a 6-nm difference between the Zn and Mg compounds. There is little difficulty in distinguishing between Zn and Mg protoporphyrin IX by room temperature fluorescence emission spectroscopy.

Studies with a series of 12 interference filters to provide monochromatic illumination (10) have shown that the spectral response curve of phycocyanin synthesis in GGB has maxima at 420 nm and at 550–600 nm. Nichols and Bogorad (10) suggested that the "primary photoreceptor (or precursor) may be an unidentified heme compound." Although the possibility of a magnesium or cobalt protoporphyrin was also mentioned (8), the features of the fluorescence excitation spectrum of Zn protoporphyrin correlate well with the spectral response curve for phycocyanin synthesis.

Although protoporphyrin IX and other possible intermediates in phycobilin synthesis have not been detected in cells actively synthesizing phycocyanin in the light (8), the presence of both protoporphyrin IX and Zn protoporphyrin IX in cells growing in the dark suggests that they probably are precursors of phycobiliin synthesis. They would accumulate because this synthesis involves a light-dependent step, as implied by the

FIG. 3. Fluorescence spectra of a GGB culture grown in the dark, resuspended in sugar-free medium, and growing under illumination. The numbers indicate hours elapsed since resuspension. The excitation wavelength was 410 nm.

FIG. 4. Fluorescence spectra of dark-grown cultures of GGB resuspended in sugar-free (—) and sugar-containing (---) media. The numbers indicate hours elapsed since resuspension and commencing of illumination. The excitation wavelength was 410 nm.

action spectrum of phycocyanin synthesis (10). *C. caldarium* cells treated with δ -aminolevulinic acid can synthesize phycobilin in the dark (9), but the phycobilin thus formed is excreted into the medium, suggesting that the synthetic processes may be different from those without added exogenous precursors.

Resuspension of GGB cells in sugar-free culture medium in the light results in a decrease of the fluorescence band of protoporphyrin IX, followed by the emergence of a wide fluorescence band at 665–670 nm, attributable to fluorescence from phycobiliproteins (Fig. 3). When the Zn protoporphyrin IX fluorescence band was also present at the onset of illumination, it disappeared much faster than the protoporphyrin IX fluorescence band (not shown). These rates were dependent on the intensity of illumination.

The small fluorescence band at 593 nm in the fluorescence spectrum at 48 hr is due to the accumulation of excreted coproporphyrin III in the culture medium and is most probably related to the slow down of phycocyanin synthesis. Excretion of coproporphyrin III is a common attribute of many photosynthetic and nonphotosynthetic microorganisms. An inverse

relationship between excreted coproporphyrin III and the formation of bacteriochlorophyll in photosynthetic bacteria has been noted (15).

In a further experiment, a dark-grown culture was divided into two halves which were resuspended in sugar-containing and sugar-free media, respectively. Both cultures were then grown under illumination. The culture in the sugar-free medium lost its protoporphyrin IX and Zn protoporphyrin IX and synthesized phycobiliproteins, which fluoresce at longer wavelengths (Fig. 4). The culture in the sugar-containing medium lost and subsequently resynthesized the two compounds responsible for the fluorescence bands at 585 and 636 nm. It also formed fluorescent phycobiliproteins, although to a much lesser extent than the control culture.

The apparently complementary nature of protoporphyrin IX and phycobiliprotein synthesis indicates that the former is indeed a precursor of the latter, with Zn protoporphyrin IX a possible intermediate. We propose that Zn protoporphyrin IX, the only fluorescent metalloporphyrin shown to be actually present in *C. caldarium* *in situ*, may be the hitherto unidentified metalloporphyrin which has been invoked as responsible for the published (10) action spectrum of phycocyanin formation. The possibility that it assumes a regulatory role in phycocyanin synthesis is not excluded.

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