

Oxygen-Regulated Steps in the *Rhodobacter capsulatus* Tetrapyrrole Biosynthetic Pathway

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The effect of exogenous aminolevulinic acid and porphobilinogen on protoporphyrin accumulation in *Rhodobacter capsulatus* was measured. Oxygen inhibited protoporphyrin accumulation in strain AJB456, a *bchH* mutant, even in the presence of exogenous aminolevulinic acid, suggesting that some step in the formation of protoporphyrin from aminolevulinic acid is regulated by oxygen. In contrast, in the presence of exogenous porphobilinogen, oxygen did not inhibit protoporphyrin accumulation. The results presented in this study indicate that oxygen regulates the formation of porphobilinogen from aminolevulinic acid.

The work of Cohen-Bazire et al. (6) demonstrated that oxygen inhibits bacteriochlorophyll synthesis in purple non-sulfur photosynthetic bacteria. The most widely accepted theory is that oxygen inhibits conversion of protoporphyrin to Mg-protoporphyrin monomethyl ester, which results in an increased accumulation of heme. The excess heme then feedback inhibits aminolevulinic acid synthase, thus reducing the synthesis of protoporphyrin (11). This theory would predict that cultures grown under high oxygen tensions should have much lower aminolevulinic acid synthase activity than photosynthetically grown cultures. We have been unable to detect any such changes in aminolevulinic acid synthase activity in *Rhodobacter capsulatus* (unpublished data). If, however, the *R. capsulatus* enzyme, like that of *R. sphaeroides*, is activated by trisulfides (9), it may be very difficult to get accurate measurements of aminolevulinic acid synthase activity in vivo. While the actual change in aminolevulinic acid synthase activity may be larger than measured, it is clear that transcription of the *R. capsulatus hemA* gene varies at most twofold (7, 17).

We have previously shown that oxygen regulates protoporphyrin accumulation (1). If this regulation is the result of oxygen either directly or indirectly inhibiting aminolevulinic acid formation, then the addition of exogenous aminolevulinic acid ought to result in protoporphyrin accumulation even under high-oxygen growth conditions. In this study we report on the effects of exogenous aminolevulinic acid and porphobilinogen on protoporphyrin and bacteriochlorophyll accumulation in *R. capsulatus*.

MATERIALS AND METHODS

Growth conditions. In each experiment, an overnight culture was subcultured into appropriately supplemented RCV⁺ medium (16) and grown under high-oxygen (23% oxygen) tension and low-oxygen (3% oxygen) tension as previously described (1). Because of day to day variations in the porphyrin levels of the inoculum and in the growth rate of the cultures, it is difficult to compare porphyrin levels between experiments. When porphobilinogen (Porphyrin Products, Logan, Utah) was included in the medium, it was dissolved in a minimal amount of dimethyl sulfoxide and added to cultures so that the concentration of dimethyl sulfoxide was the same as that in RCV⁺. Aminolevulinic acid (Sigma Chemical Co., St. Louis, Mo.) and porphobilinogen were added to cultures to final concentrations of 1.0 and 0.25

mM, respectively. In an *R. capsulatus hemA* mutant, these concentrations of substrates have been shown to allow production of normal levels of bacteriochlorophyll without reducing the growth rate (16).

Measurements. Bacteriochlorophyll concentration was determined by harvesting a 1-ml sample and extracting the pellet with an equal volume of acetone-methanol (7:2), as described by Cohen-Bazire et al. (6). A millimolar extinction coefficient of 76 (770 nm) was used (5).

Protoporphyrin concentration was determined by harvesting 10-ml samples, suspending the pellets in 1 ml of water, and extracting the samples with 1 ml of ethyl acetate-acetic acid (4:1). The samples were evaporated to dryness under vacuum, and the porphyrinogens were oxidized to porphyrins with benzoquinone (8). After the samples were evaporated to dryness, the porphyrins were esterified with methanol-sulfuric acid and extracted into chloroform (8). The samples were spotted on silica gel thin-layer chromatography plates and developed with benzene-ethyl acetate-ethanol (80:18:2). The protoporphyrin dimethyl ester spot was eluted with methanol-chloroform (1:1).

Coproporphyrin was extracted from 10-ml samples by the procedure of Tait (14). Fluorescence of coproporphyrin and protoporphyrin dimethyl ester was measured by using an excitation wavelength of 402 nm and an emission wavelength of 630 nm.

Mg-protoporphyrin monomethyl ester was determined by harvesting 10-ml samples and extracting them with acetone-methanol (7:2) and measuring the fluorescence by using an excitation wavelength of 402 nm and an emission wavelength of 594 nm.

Porphyrin concentrations were calculated by comparing the fluorescence values to curves constructed by using porphyrin standards (Porphyrin Products). Protein was measured as previously described (1).

RESULTS

Regulation of bacteriochlorophyll formation in a *bch*⁺ strain. Figure 1 depicts the bacteriochlorophyll concentrations of strain PAS100 (*bch*⁺ [15]) grown under high- and low-oxygen tensions. Under high oxygen, no synthesis of bacteriochlorophyll occurred, indicating that some step in bacteriochlorophyll synthesis is completely shut off in the presence of oxygen. Since cytochromes are essential for growth of *R. capsulatus*, heme synthesis must be continuing,

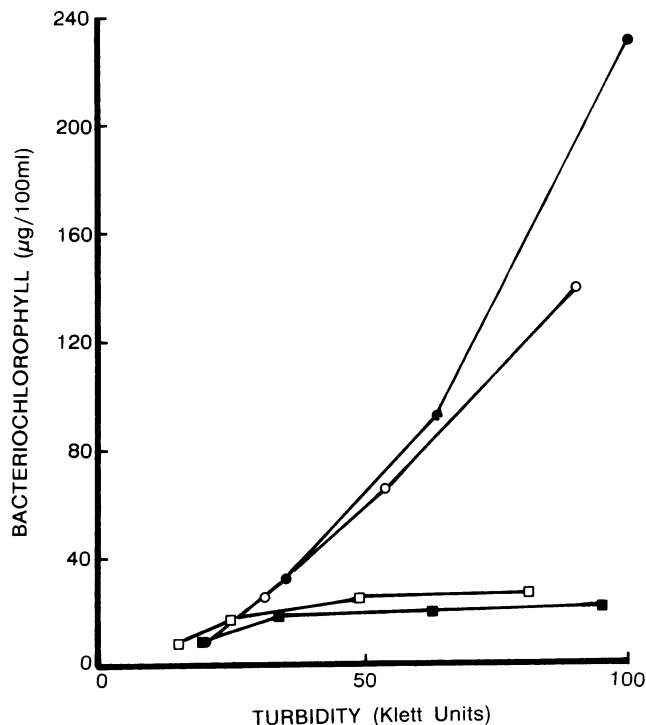


FIG. 1. Bacteriochlorophyll accumulation in the *bch*⁺ strain PAS100. The strain was grown under high oxygen (□ and ■) or low oxygen (○ and ●) in the presence of 1.0 mM aminolevulinate (open symbols) or in the absence of aminolevulinate (closed symbols).

suggesting that the on-off switch in the bacteriochlorophyll biosynthetic pathway must be somewhere in the conversion of protoporphyrin to bacteriochlorophyll (Fig. 2). The addition of exogenous aminolevulinate had no effect on bacteriochlorophyll synthesis either under low or high oxygen, indicating that aminolevulinate formation is not the only regulated step in the pathway.

Regulation of Mg-protoporphyrin monomethyl ester formation in a *bchE* strain. Mg-protoporphyrin monomethyl ester accumulation was monitored in strain BPY78 (*bchE* [B. Marrs]) grown under high and low oxygen to determine where the on-off switch was located (Fig. 3). The results indicate that the formation of Mg-protoporphyrin monomethyl ester is regulated by oxygen. Since the synthesis of protoporphyrin must not be completely shut down, these results imply that the on-off switch is in the conversion of protoporphyrin to Mg-protoporphyrin monomethyl ester.

Regulation of protoporphyrin formation in a *bchH* strain. In order to determine which step in the common tetrapyrrole pathway is regulated by oxygen, protoporphyrin accumulation in AJB456, a *bchH* mutant (1), was measured after growth under high- and low-oxygen tensions (Table 1). In the absence of any additions to the basal medium, oxygen regulated protoporphyrin accumulation 20-fold. Protoporphyrin accumulation was still regulated by oxygen even in the presence of exogenous aminolevulinate. The addition of porphobilinogen, however, caused AJB456 to accumulate protoporphyrin even under high oxygen.

The effect of exogenous porphobilinogen on coproporphyrin accumulation in strain AJB530 (2) was also measured. AJB530 overproduces and excretes coproporphyrin because of a lack of *c*-type cytochromes (2). Coproporphyrin arises

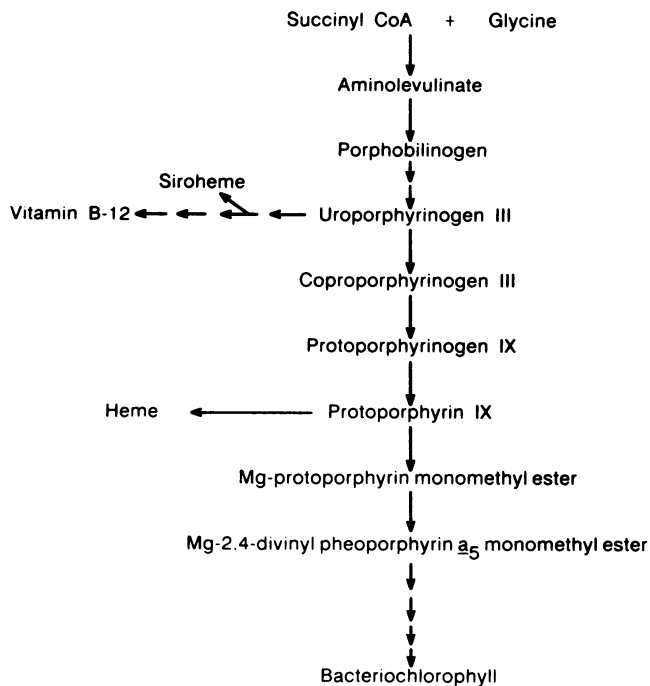


FIG. 2. Tetrapyrrole biosynthetic pathway.

by spontaneous oxidation of coproporphyrinogen. Under high oxygen, the coproporphyrin concentration in the medium was 0.1 μ M, while under low oxygen the concentration reached 0.7 μ M. In the presence of porphobilinogen, the coproporphyrin concentration in the medium was 0.5 μ M under both oxygen tensions.

DISCUSSION

The regulation of tetrapyrrole biosynthesis in *R. capsulatus* is extremely complicated. At least three factors, oxygen

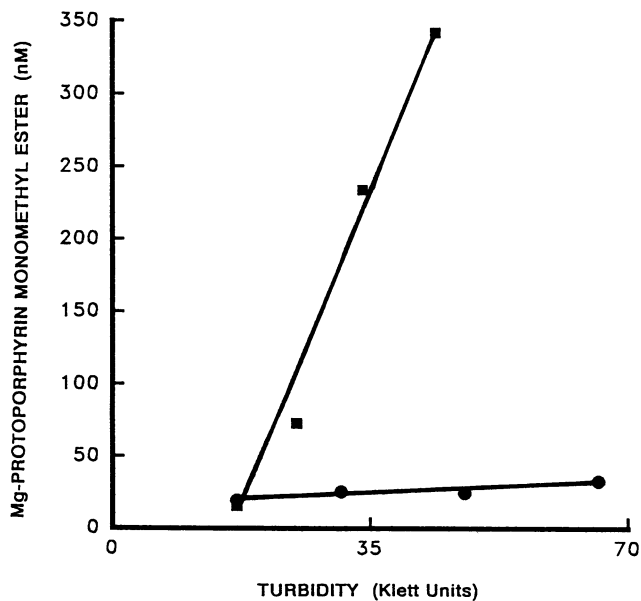


FIG. 3. Mg-protoporphyrin monomethyl ester accumulation in a *bchE* strain. The strain was grown under high oxygen (■) or low oxygen (●).

TABLE 1. Protoporphyrin formation in AJB456

Expt and addition	Oxygen tension	Protoporphyrin concn ^a
1, none	High	2.1
	Low	43.0
2, aminolevulinate	High	3.7
	Low	46.0
3, porphobilinogen	High	16.0
	Low	17.0

^a Protoporphyrin levels were determined as protoporphyrin dimethyl ester and are expressed as picomoles per milligram of protein.

(4, 6), heme (3; unpublished data) and *c*-type cytochromes (2), have major influences on the amounts of the various tetrapyrroles synthesized. The observation that a *hemA* mutant of *R. capsulatus* can use exogenous aminolevulinate for both heme and bacteriochlorophyll synthesis (16) has facilitated this study on the role of aminolevulinate in oxygen-mediated regulation of tetrapyrrole biosynthesis.

The results presented here indicate that oxygen controls the pathway leading to bacteriochlorophyll at two points. The formation of Mg-protoporphyrin monomethyl ester from protoporphyrin seems to be completely blocked in high-oxygen-grown cells. This is in agreement with the current model and suggests that this very complicated step is the major control point of oxygen-mediated regulation in the bacteriochlorophyll biosynthetic branch.

In order to prevent a buildup of protoporphyrin, one of the steps in the common tetrapyrrole pathway must be drastically inhibited in the presence of oxygen. It would seem logical that the first step in the common pathway, the formation of aminolevulinate from succinyl coenzyme A and glycine, would be the regulated step.

The observation presented here that exogenous aminolevulinate does not eliminate oxygen-mediated regulation of protoporphyrin accumulation argues that oxygen regulates some step in the common tetrapyrrole pathway after aminolevulinate formation. That exogenous porphobilinogen does eliminate oxygen-mediated regulation of protoporphyrin accumulation suggests that the conversion of aminolevulinate to porphobilinogen is a major control point in the common tetrapyrrole pathway. These results apply only to oxygen-mediated regulation of the common tetrapyrrole pathway. Other factors, such as heme and *c*-type cytochromes, may control the pathway at other sites.

It would seem unusual for the second step in a pathway to be regulated, as it would result in a buildup of the first intermediate in the pathway, in this case, aminolevulinate. This raises the possibility that porphobilinogen, and not aminolevulinate, is the first committed precursor in the common tetrapyrrole pathway. Several studies have investigated the metabolism of aminolevulinate in different organisms. Shemin's group demonstrated that in ducks and rats the δ -carbon of aminolevulinate is incorporated into purines and suggested that aminolevulinate was actually part of a succinate-glycine cycle (10, 12). Shigesada investigated the metabolism of [4-¹⁴C]aminolevulinate in washed cell suspensions of photosynthetically grown *Rhodospirillum rubrum* (13). Only 10% of the incorporated radioactivity was associated with tetrapyrroles, while the rest was found in 5-ami-

no-4-hydroxyvalerate, 2-hydroxyglutarate, 2-ketoglutarate, and glutamate, compounds closely related to aminolevulinate. Shigesada demonstrated that aminolevulinate was metabolized via a second pathway and postulated the existence of a succinate-glycine cycle in *R. rubrum* (13). These studies, along with the results presented here, indicate the need for investigations on the metabolism of aminolevulinate and porphobilinogen to determine which compound is the first committed precursor of protoporphyrin.

ACKNOWLEDGMENT

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REFERENCES

1. Biel, A. J., and B. L. Marrs. 1983. Transcriptional regulation of several genes for bacteriochlorophyll biosynthesis in *Rhodospseudomonas capsulata* in response to oxygen. *J. Bacteriol.* **156**:686-694.
2. Biel, S. W., and A. J. Biel. 1990. Isolation of a *Rhodobacter capsulatus* mutant that lacks *c*-type cytochromes and excretes porphyrins. *J. Bacteriol.* **172**:1321-1326.
3. Burnham, B. F., and J. Lascelles. 1963. Control of porphyrin biosynthesis through a negative-feedback mechanism. *Biochem. J.* **87**:462-472.
4. Clark, W. G., E. Davidson, and B. L. Marrs. 1984. Variation of levels of mRNA coding for antenna and reaction center polypeptides in *Rhodospseudomonas capsulata* in response to changes in oxygen concentration. *J. Bacteriol.* **157**:945-948.
5. Clayton, R. K. 1966. Spectroscopic analysis of bacteriochlorophylls in vitro and in vivo. *Photochem. Photobiol.* **5**:669-677.
6. Cohen-Bazire, G., W. R. Sistrom, and R. Y. Stanier. 1957. Kinetic studies of pigment synthesis by non-sulfur purple bacteria. *J. Cell. Comp. Physiol.* **49**:25-68.
7. Hornberger, U., B. Wieseler, and G. Drews. 1991. Oxygen tension regulated expression of the *hemA* gene of *Rhodobacter capsulatus*. *Arch. Microbiol.* **156**:129-134.
8. Jordan, P. M. 1982. Uroporphyrinogen III cosynthase: a direct assay method. *Enzyme* **28**:158-169.
9. Marriott, J., A. Neuberger, and G. H. Tait. 1969. Control of δ -aminolevulinate synthetase activity in *Rhodospseudomonas spheroides*. *Biochem. J.* **111**:385-394.
10. Nemeth, A. M., C. S. Russell, and D. Shemin. 1957. The succinate-glycine cycle. II. Metabolism of δ -aminolevulinic acid. *J. Biol. Chem.* **229**:415-422.
11. Rebeiz, C. A., and J. Lascelles. 1982. Biosynthesis of pigments in plants and bacteria, p. 699-780. *In* Photosynthesis: energy conversion by plants and bacteria, vol. I. Academic Press, Inc., New York.
12. Shemin, D., and C. S. Russell. 1953. δ -Aminolevulinic acid, its role in the biosynthesis of porphyrins and purines. *J. Am. Chem. Soc.* **75**:4873-4874.
13. Shigesada, K. 1972. Possible occurrence of a succinate-glycine cycle in *Rhodospirillum rubrum*. *J. Biochem.* **71**:961-972.
14. Tait, G. H. 1972. Coproporphyrinogenase activities in extracts of *Rhodospseudomonas spheroides* and *Chromatium* strain D. *Biochem. J.* **128**:1159-1169.
15. Taylor, D. P., S. N. Cohen, W. G. Clark, and B. L. Marrs. 1983. Alignment of genetic and restriction maps of the photosynthesis region of the *Rhodospseudomonas capsulata* chromosome by a conjugation-mediated marker rescue technique. *J. Bacteriol.* **154**:580-590.
16. Wright, M. S., R. D. Cardin, and A. J. Biel. 1987. Isolation and characterization of an aminolevulinate-requiring *Rhodobacter capsulatus* mutant. *J. Bacteriol.* **169**:961-966.
17. Wright, M. S., J. J. Eckert, S. W. Biel, and A. J. Biel. 1991. Use of a *lacZ* fusion to study transcriptional regulation of the *Rhodobacter capsulatus hemA* gene. *FEMS Microbiol. Lett.* **78**:339-342.