EFFECTS OF 5-AMINOLEVULINATE AND N-METHYL- PROTOPORPHYRIN DIMETHYL ESTER ON BACTERIOCHLOROPHYLL SYNTHESIS IN AEROBIC BACTERIA

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The aerobic bacteria, *Erythrobacter longus* and *Roseobacter denitrificans*, were examined for the effects of 5-aminolevulinate (ALA) and N-methylprotoporphyrin dimethyl ester (NMPD) on bacteriochlorophyll (Bchl) synthesis. Although external ALA seemed to be incorporated into a tetrapyrrole synthetic pathway leading to Bchl synthesis, there was no increase of Bchl in either *Ro. denitrificans* or *E. longus*. With the addition of ALA, increased was protoporphyrin IX in *Ro. denitrificans*, or protoheme in *E. longus*. NMPD enhanced the Bchl synthesis in *E. longus* but not in *Ro. denitrificans*. It is likely that *E. longus* and *Ro. denitrificans* are different from each other in the regulatory mechanism of Bchl synthesis.

The regulation of bacteriochlorophyll *a* (Bchl) synthesis has been investigated extensively with the anaerobic phototrophic bacterium *Rhodobacter sphaeroides* (7, 9). Bchl is formed through the tetrapyrrole synthetic pathway which starts from the synthesis of 5-aminolevulinate (ALA) and diverges at the step of protoporphyrin IX into the magnesium (:Bchl) branch and iron (:cytochrome) branch (7). The synthesis of Bchl is regulated by ALA, and the synthesis of ALA is under a negative feed-back control by protoheme which is the first intermediate of iron branch (9).

Erythrobacter longus and *Roseobacter denitrificans* are the aerobic bacteria which synthesize Bchl (17, 18). The Bchl synthetic activity in the bacteria are enhanced at, or tolerant to, higher oxygen tension (15). This is in contrast to the anaerobic phototrophic bacteria in which Bchl synthesis is suppressed by oxygen (8). Hence, it is intriguing how Bchl synthesis is regulated in these aerobic bacteria. Although Shioi et al. (19) have already reported the effect of external ALA on the

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Bchl synthesis of *Erythrobacter* sp. OCh114, the strain is now identified as a new species of a new different genus: *Ro. denitrificans* (17). Also the concentration of administrated ALA was more than 0.5 mM, which seemed to be too high for examining the effect of ALA. The present paper compares the effects of ALA and *N*-methylprotoporphyrin dimethyl ester on Bchl synthesis between *E. longus* and *Ro. denitrificans*. The concentration of ALA was decreased to the range of 1 to 1,000 μ M.

MATERIALS AND METHODS

Bacterial strains and culture. *Erythrobacter longus* (OCh101) ATCC 33941 and *Roseobacter denitrificans* (OCh114) ATCC 33942 were both isolated from *Enteromorpha linza* (17, 18). The strains were cultured in a liquid complex medium of PPES-II (16, 21) at 20°C in the dark. The cell density was measured by determining optical density at 650 nm. The optical density of 1.0 corresponded to 0.5 mg dry cell in 1 ml of the suspension of *E. longus*, and 0.46 mg in *Ro. denitrificans*.

Effects of 5-aminolevulinic acid (ALA) and *N*-methylprotoporphyrin dimethyl ester (NMPD). ALA and NMPD were filter-sterilized, and then added into PPES-II medium at different concentrations. A 2 ml of the cell suspension in early stationary growth phase was inoculated into 50 ml of the media. The cell suspensions were vigorously shaken in the dark. After incubation overnight, the suspensions were centrifuged and the pellets were examined for the level of porphyrin compounds.

Determinations of protoheme and cytochrome *c*. After removal of bacteriochlorophyll (Bchl) and carotenoids with methanol, protoheme was extracted by suspending the cell pellets in 4% HCl/acetone and then stored at 4°C in the dark for 2 h. After centrifugation, the supernatant was mixed with one volume of diethyl ether and two volumes of 0.27 M of HCl. The separated hydrochloric acid solution was washed with one volume of diethyl ether, and then the solutions of diethyl ether were combined and dried over anhydrous sodium sulfate under the flow of nitrogen gas. Cytochrome *c* was determined with the residual cells after HCl/acetone extraction. Protoheme and cytochrome *c* were determined by using their pyridine hemochrome spectra (6, 13).

Determination of protoporphyrin and Mg-protoporphyrin compounds. The porphyrins were obtained by three serial extractions with methanol. The extracts were analyzed by a high performance liquid chromatography (HPLC) using Ultrasphere ODS column (Beckman), 70% methanol as a solvent, and tetrabutyl ammonium-phosphate as an ion pair reagent (3). Peaks were identified by comparing their retention time and characteristic fluorescent emission spectra to authentic standards. Mg-2, 4-divinyl phaeoporphyrin a_5 monomethyl ester was tentatively identified by spectrophotometry (1) and comparing its elution pattern in the HPLC with that reported by Fuesler et al. (3). Porphyrin compounds were determined by comparing peak area with standards. Their mM extinction coefficients in diethyl

ether are 158 at 404 nm for protoporphyrin IX (ProtoIX) and 308 at 419 nm for Mg-protoporphyrin IX. Millimolar extinction coefficient of Mg-protoporphyrin monomethyl ester is the same as that of Mg-protoporphyrin IX. Millimolar extinction coefficient of protochlorophyll, that is 289.7 at 432 nm in diethyl ether, substituted for Mg-2, 4-divinyl phaeoporphyrin a_5 monomethyl ester. Bchl was determined by using mM extinction coefficient at 770 nm (2).

Pigment standards and N-methylprotoporphyrin dimethyl ester (NMPD). Mg-protoporphyrin dimethyl ester was prepared by treating protoporphyrin dimethyl ester (Sigma) with Mg-perchlorate (14). The hydrolysis of Mg-protoporphyrin dimethyl ester with KOH produced Mg-protoporphyrin IX and Mg-protoporphyrin monomethyl ester. The porphyrins were separated by the HPLC. Treatment of the Mg-protoporphyrins with HCl produced ProtoIX, protoporphyrin monomethyl ester and protoporphyrin dimethyl ester. The porphyrins were identified by spectrophotometry and comparison of their retention time in the HPLC to authentic standards. ProtoIX was purchased from Sigma. NMPD was synthesized from protoporphyrin dimethyl ester by using the method of Matteis et al. (11) with some modifications. Protoporphyrin dimethyl ester was heated at 95 °C with methyl iodide for 24 h. NMPD was purified by a thin layer chromatography, and identified spectrophotometrically (11). The content was estimated using a mM extinction coefficient of 135 at 419 nm.

RESULTS AND DISCUSSION

Effects of ALA on Erythrobacter longus

As shown in Fig. 1, cellular Bchl level in *E. longus* was not enhanced by the addition of 100 μ M of ALA. No enhancement was at either 1, 10 μ M, or 1 mM. At 1 mM of ALA, a slight inhibition of growth was observed (data not shown). No experiment was done at the concentrations higher than 1 mM, although Shioi et al. (19) have reported that Bchl accumulation in this bacterium was suppressed at 5 mM of ALA.

Tables 1 and 2 show the change in the level of intermediates of tetrapyrrole synthetic pathway at different concentrations of ALA. The cellular level of protoheme increased with ALA, whereas cytochrome *c*, which is synthesized from protoheme, did not increase (Table 1). At 1 mM of ALA, cytochrome *c* level was decreased. Protoporphyrin IX (ProtoIX) and some of the intermediates of magnesium branch were also increased at 100 μ M of ALA (Table 2). Although the data in the tables were based on 16 or 19 h incubation with external ALA, a similar enhancement in the levels of porphyrin compounds was observed also with 1 h incubation. The levels remained relatively constant during 3 h incubation (data not shown). Hence, increase of the intermediates, alone, is insufficient for the enhancement of Bchl synthesis. The accumulation of intermediates in excess may be accompanied with a decrease in their turnover rate. It is also possible that the increase of ProtoIX results in the decrease in the activity of ALA synthesis.

Fig. 1. Effect of 5-aminolevulinic acid (ALA) on bacteriochlorophyll *a* (Bchl) synthesis by *Erythrobacter longus*.

Closed symbols indicate Bchl accumulation in the presence of ALA at 100 μ M. Open symbols are without ALA.

Table 1. Effect of external 5-aminolevulinic acid (ALA) on heme content in *Erythrobacter longus*.

Concentration of ALA (μ M)	Protoheme	Cytochrome <i>c</i>
	nmol/mg dry cell	
1,000	0.52	0.39
100	0.35	0.45
10	0.28	0.45
1	0.19	0.46
0	0.20	0.46

Bacterial strain was incubated for 16 h.

Table 2. Effect of external 5-aminolevulinic acid (ALA) on porphyrin content in *Erythrobacter longus*.

Concentration of ALA (μ M)	ProtoIX	MgProto	MgProtoMe	MgDVP
	pmol/mg dry cell			
100	13.0	2.4	19.1	11.1
10	4.9	6.2	11.4	5.7
1	3.9	4.4	9.5	6.4
0	4.4	4.9	9.4	5.1

Bacterial strain was incubated for 19 h. Magnesium branch proceeds in the order of Mg-protoporphyrin IX (MgProto), Mg-protoporphyrin monomethyl ester (MgProtoMe), and Mg-2,4-divinyl phaeoporphyrin *a*₅ monomethyl ester (MgDVP).

Table 3. Effect of external 5-aminolevulinic acid (ALA) on porphyrin content in *Roseobacter denitrificans*.

ALA (μM)	ProtoIX	MgProto	MgProtoMe	MgDVP	Bchl
	pmol/mg dry cell				nmol/mg
100	61.8	2.8	8.6	8.6	2.5
10	26.1	2.9	6.2	9.7	2.3
1	11.5	2.5	5.2	8.2	2.2
0	11.6	2.2	3.8	—	2.3

Bacterial strain was incubated for 19 h. Abbreviations are the same as in Table 3.

Table 4. Effect of external 5-aminolevulinic acid (ALA) on heme contents in *Roseobacter denitrificans*.

Concentration of ALA (μM)	Protoheme	Cytochrome <i>c</i>
	nmol/mg dry cell	
100	0.10	0.48
10	0.09	0.52
1	0.09	0.56
0	0.10	0.56

Bacterial strain was incubated for 19 h.

Inhibition of ALA synthesis by ProtoIX is reported on *Rhd. sphaeroides* (23).

Increase of heme compounds with external ALA is reported on *Rhodobacter sphaeroides*. With the addition of ALA at 1 mM, heme synthesis was increased tenfold, whereas no increase in Bchl content (10). The increase of heme synthesis is accompanied with the decrease of ALA synthesis, suggesting that ALA synthesis in the bacterium is under a negative feed-back regulation by heme. Although the effect of ALA in *Rhd. sphaeroides* is similar to *E. longus*, chemical identification of the heme compounds has not been done with *Rhd. sphaeroides*. ALA synthetic activity in *E. longus* was not determined.

Effect of ALA on Bchl synthesis of *Roseobacter denitrificans*

Table 3 shows that Bchl level in *Roseobacter denitrificans* was not enhanced by ALA, but ProtoIX was increased. ProtoIX is a substrate of Mg- and Fe-chelatase, but there was no increase of either Mg-porphyrins or heme compounds (Table 4). Hence, increase of ProtoIX, alone, is insufficient for the enhancement of chelation. It is likely that chelations of either iron or magnesium into ProtoIX are rate-limiting steps. This may account for the weak incorporation of external ALA into cell; Shioi et al. (19) reported that external ALA has not been readily incorporated into the cell of *Ro. denitrificans*. Gorchein (4) reported that in *Rhd. sphaeroides* Mg^{2+} insertion into ProtoIX is regulated by oxygen. There is no report on environmental factor acting on ferrochelatase activity. It is also possible that increase of ProtoIX results in the decrease of ALA synthetic activity (23).

Fig. 2. Effect of *N*-methylprotophyrin dimethyl ester (NMPD) on bacteriochlorophyll *a* (Bchl) synthesis.

Solid line indicates growth, dotted line Bchl. Closed symbols are with 500 nM of NMPD, open symbols are without NMPD.

Although Shioi et al. (19) have reported that Bchl accumulation in *Roseobacter* was suppressed by exogenous ALA, no suppression of Bchl accumulation was found in this experiment. This discrepancy should be due to the difference in the concentration of exogenous ALA. The concentrations of ALA was 1 to 100 μ M in this experiment, while Shioi et al. (19) examined the effect of ALA at 0.5 to 10 mM. The suppression of Bchl accumulation is caused by the inhibition of porphobilinogen (PBG) synthase by 4-hydroxy-5-aminovaleic acid (HAVA). HAVA is produced from ALA outside the cell or in periplasms. HAVA does not inhibit PBG synthase at the concentrations lower than 1 mM.

Effect of NMPD

Figure 2 shows the effect of NMPD on Bchl accumulation in the aerobic bacteria. NMPD is an inhibitor of ferrochelatase in iron branch (6). NMPD enhanced Bchl content in *E. longus* but not in *Ro. denitrificans*. The effect of NMPD on *E. longus* differed from that on *Rhd. sphaeroides*: 16 h incubation of *Rhd. sphaeroides* with NMPD resulted in the increase of Mg-protophyrin monomethyl ester but not in Bchl (6).

Although data in this paper is fragmentary and cannot depict the regulatory mechanism of Bchl synthesis in the aerobic bacteria, the difference in the effects of NMPD and ALA suggest that the regulatory mechanism of Bchl synthesis is different between *E. longus* and *Ro. denitrificans*. The difference in the regulation should be discussed in light of evolutionary relationship between these two bacteria; *E. longus* is included in Proteobacteria alpha-4 (5, 20, 22), while *Ro. denitrificans* is

close to *Rhodobacter* in alpha-3 (5, 12, 22, 23).

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