Mutant Strains of *Rhodopseudomonas spheroides*
Lacking δ-Aminolevulate Synthase: Growth, Heme, and Bacteriochlorophyll Synthesis

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Two mutant strains of *Rhodopseudomonas spheroides* were described which lacked δ-aminolevulate synthase activity. They required δ-aminolevulate for growth; they did not respond to protoporphyrin or magnesium protoporphyrin, and only poorly to hemin. Synthesis of cytochromes and heme by mutant H-4 was dependent upon δ-aminolevulate; this strain did not form bacteriochlorophyll either with or without δ-aminolevulate and, consequently, grew only under aerobic conditions. Mutant H-5 formed bacteriochlorophyll in response to δ-aminolevulate and grew both anaerobically in the light and aerobically in the dark; the amount of δ-aminolevulate needed for optimal anaerobic growth was higher than that required aerobically. Synthesis of bacteriochlorophyll and heme by suspensions of mutant H-5 incubated anaerobically in the light was dependent upon δ-aminolevulate; bacteriochlorophyll production was completely inhibited by high aeration and by puromycin. The mutants differed in their ability to take up radioactive δ-aminolevulate from the external environment; mutant H-5 was less active than mutant H-4 or the wild type. It was suggested that *R. spheroides* made only one form of δ-aminolevulate synthase, which provided δ-aminolevulate for bacteriochlorophyll and heme synthesis.

δ-Aminolevulinic acid (ALA) is a common intermediate in the biosynthesis of vitamin B12, hemes, and chlorophyll, and is formed from glycine and succinyl CoA by the action of ALA synthase. The cobalt, iron, and magnesium tetrapyroles are all formed by *Rhodopseudomonas spheroides* and related photosynthetic bacteria, and the regulation of the common part of the biosynthetic pathway raises particular problems because of the great disparity in the concentration of the respective end products (16). Also, bacteriochlorophyll synthesis can be drastically curtailed under certain conditions without preventing synthesis of vitamin B12 and heme and without overproduction of common intermediates. In a previous paper, evidence was presented in support of feedback control by heme of ALA synthase in the regulation of the common part of the biosynthetic pathway leading to the iron and magnesium tetrapyroles (18). However, the synthase cannot be the sole locus of regulation of the branched biosynthetic path.

The isolation of mutant strains of *R. spheroides* which lack ALA synthase activity has provided an opportunity to study heme and bacteriochlorophyll synthesis without the complications arising from participation of the synthase. This paper reports studies of the growth characteristics of the mutants and their ability to form bacteriochlorophyll and heme under various conditions. Their ability to take up ALA from the external environment has also been studied.

**MATERIALS AND METHODS**

**Organisms.** The wild-type strain of *R. spheroides* and its maintenance have been previously described (14). Mutants H-4 and H-5 were derived from it by treatment with the mutagen, N-methyl-N-nitroso-N'-nitroguanidine. The procedure followed that used previously (15), except that the medium for development of potential mutants contained 0.1 μM ALA. Colonies arising on the supplemented medium were tested individually for ability to grow without ALA, since replica plating with this organism was not satisfactory; isolates which responded to ALA were replated on the supplemented medium.

**Growth of organisms and preparation of cell suspensions.** The basal medium used for growth was the malate-glutamate one, supplemented with 0.1% yeast extract (14). For the preparation of suspensions, organisms were grown under low aeration in the yeast extract-malate-glutamate medium with 0.1 μM ALA.
Cells were harvested at the beginning of the stationary phase and resuspended to an optical density of 2 (1-cm light path) at 680 nm in mixture GS (18). For measurement of heme synthesis, \(^{55}\)FeCl\(_3\) (0.5 \(\mu\)c per ml) was added. The various conditions of incubation were defined previously (18).

**Measurement of growth.** Growth was measured either with a Klett colorimeter using filter number 660 or with a Zeiss spectrophotometer at 680 nm. A Klett reading of 100 was equivalent to 0.4 mg (dry weight) of cells per ml; an absorbancy at 680 nm of 1 (1-cm light path) was equivalent to 0.5 mg (dry weight) per ml.

**Analytical methods.** Bacteriochlorophyll, heme, and protein were determined as previously described (18). The cytochrome content was determined by measurement of the reduced-minus-oxidized spectrum of extracts prepared by sonic treatment (6). The activities of ALA synthase and ALA dehydratase were determined in cell extracts, as described by Burnham and Lascelles (4).

**Uptake of \(^{14}C\)-ALA.** Cells were grown under low aeration in the yeast extract-malate-glutamate medium with 0.1 mM ALA, in the case of the mutants. The harvested organisms were washed in 0.04 M potassium phosphate buffer (pH 7.0) and resuspended to the desired density in a salts mixture which contained the inorganic components of the malate-glutamate growth medium (pH 7.0). After temperature equilibration for 5 min, the reaction was begun by addition of ALA-\(^{14}C\), usually to a final concentration of 0.01 mM (0.04 \(\mu\)c per ml). Incubation was at 30°C without shaking. Samples (1.0 ml) were removed at intervals, filtered on membrane filters (Millipore Corp., Bedford, Mass.; pore size 0.65 \(\mu\)m), and washed three times with 5-ml portions of 0.04 M potassium phosphate buffer (pH 7.0). After drying, the radioactivity on the filters was determined by liquid scintillation counting, using a toluene-based scintillation fluid and the Beckman Scintillation System.

**Materials.** Hemin, protoporphyrin, and its dimethyl ester were from Calbiochem, Los Angeles, Calif. Magnesium protoporphyrin was prepared by the method of Baum, Burnham, and Plane (2) with protoporphyrin dimethyl ester as the starting material; the magnesium dimethyl ester derivative was converted to potassium salt by the method of Granick (12). Magnesium protoporphyrin monomethyl ester was isolated by the procedure of Cooper (8) from the culture fluids of a mutant strain of *R. spheroides*, kindly provided by T. Hatch. Stock solutions of hemin were in 0.01 N NaOH in 50% ethyl alcohol; protoporphyrin and the magnesium derivatives were in 0.01 N KOH in 2% Tween-80. The concentration of the stock solutions of tetrapyrroles was 1 to 2 mM, and they were prepared freshly before use; they were added without sterilization to media after autoclaving. \(^{5}\)Aminolevulinic acid-\(^{14}C\) was from Calbiochem, and \(^{55}\)FeCl\(_3\) (43 mc/mg of iron) was from New England Nuclear Corp., Boston, Mass.

**RESULTS**

**Growth and characteristics of mutants.** Strains H-4 and H-5 grew aerobically at a maximum rate with 0.1 mM ALA (Fig. 1); the lag was prolonged with 1 mM ALA, but the final rate was similar to that attained with 0.1 mM ALA. The mutants differed in their response to low concentrations of ALA. Strain H-4 grew with 0.01 mM ALA, although at a reduced rate, whereas strain H-5 did not grow at this concentration (Fig. 1).

Hemin (0.01 mM) promoted only very slow linear growth of the mutants. With strain H-5, the further addition of 0.01 mM ALA, which alone gave no response, considerably stimulated growth (Fig. 2). Neither strain responded to protoporphyrin, magnesium protoporphyrin or its monomethyl ester (each tested at 0.01 mM), or to cyano-cobalamin (0.07 \(\mu\)M), nor did these compounds promote growth of mutant H-5 in the presence of 0.01 mM ALA. Strain H-4 did not grow anaerobically in the light with concentrations of ALA up to 1 mM; this behavior was consistent with its failure to make bacteriochlorophyll. In contrast, mutant H-5 grew under these conditions, but for maximal growth it needed a higher concentration of ALA than under aerobic conditions (Fig. 3).

**Tetrapyrrole content of cells.** The cytochrome content was determined in the mutants after aerobic growth with various amounts of ALA. In cells of both strains grown with 1 mM ALA, the level was comparable with that in the wild type cultured under the same conditions but without ALA (Table 1). Reduced minus oxidized spectra of both mutants showed predominantly *b* and *c* type cytochromes in equal proportions. This pattern was constant in cells grown with high and low concentrations of ALA and was similar to

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Fig. 1. Aerobic growth of mutants H-4 and H-5 in response to ALA. Cultures were shaken at 200 rev/min in naphtho flasks (300-ml volume) containing 50 ml of yeast extract-malate-glutamate medium, and supplemented with ALA at the concentrations (mm) shown on the curves. Each flask was inoculated with 0.01 ml (about 2 X 10\(^7\) cells) from a fully grown aerobic culture in the same medium with 0.1 mM ALA. Growth was observed with a Klett colorimeter using filter number 660.
that found in aerobically grown wild-type cells (20).

Synthesis of bacteriochlorophyll by mutant H-5 under anaerobic light conditions was markedly increased by raising the concentration of ALA from 0.1 to 1 mM (Table 1).

Since mutant H-4 did not grow anaerobically in the light, the bacteriochlorophyll content was determined in cells grown under low aeration. Only traces were detectable in cells grown with concentrations of ALA ranging from 0.1 to 1 mM.

Enzymatic activities of mutants H-4 and H-5. ALA synthase activity was not detected in extracts of either mutant grown under the low aeration conditions which gave maximal activity in the wild type. The level of ALA dehydratase in both mutants was similar to that found in the wild type when grown under comparable conditions. The specific activity of extracts prepared from cells grown under low aeration with 0.1 mM ALA was within the range of 55 to 65 nmoles of porphobilinogen per mg of protein per hr; the enzyme activity did not vary in cells grown with suboptimal and optimal concentrations of ALA.

Properties of a revertant strain of mutant H-4. The failure of mutant H-4 to form bacteriochlorophyll might have resulted from a second mutation in the magnesium branch of the biosynthetic pathway. The isolation of a spontaneous revertant of H-4 gave an ALA mutant which grew aerobically with low or high ALA levels. The revertant was designated H-5. The optimal growth conditions for mutant H-5 were found to be 0.1% Tween-80 and 0.1% hemin in the yeast extract-malate-glutamate medium with 0.1 mM ALA. The cells were harvested and resuspended to a density (at 680 nm) of 0.26 in fresh medium, with addition of ALA to give the concentrations (mM) shown on the curves, and incubated anaerobically in the light. Growth was observed by measuring the absorbance at 680 nm.

**TABLE 1. Concentration of cytochrome and bacteriochlorophyll in mutant and wild-type cells**

<table>
<thead>
<tr>
<th>ALA concn in medium (mM)</th>
<th>Total cytochrome</th>
<th>Bacteriochlorophyll</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H-4</td>
<td>H-5</td>
</tr>
<tr>
<td>0.01</td>
<td>0.02</td>
<td>No growth</td>
</tr>
<tr>
<td>0.1</td>
<td>0.48</td>
<td>0.56</td>
</tr>
<tr>
<td>0.4</td>
<td>1.08</td>
<td>0.69</td>
</tr>
</tbody>
</table>

*Values are expressed as nanomoles per milligram of protein. Cytochromes were determined in cells grown aerobically, as described in Fig. 1, in yeast extract-malate-glutamate medium with ALA as shown; the cells were harvested in the early stationary phase of growth. Bacteriochlorophyll was determined in mutant H-5 harvested from the cultures shown in Fig. 3 after 10.5 hr. The values for the wild type were determined in cells grown under the same conditions as the mutants and harvested at a density (680 nm) of 2.0. Values for the wild type without ALA were 1.25 and 17 nmoles/mg of protein for cytochrome and bacteriochlorophyll, respectively.*
ant with wild-type characteristics made this possibility unlikely.

The revertant of mutant H-4 was isolated from a large colony which developed on unsupplemented yeast extract-malate-glutamate-agar. This organism behaved like the wild type in all respects examined. It exhibited the same growth rate under both aerobic and anaerobic light conditions and did not require ALA.

Also, the revertant formed bacteriochloroprophyl and heme in quantities similar to the wild type when grown under appropriate conditions. Suspensions of the revertant formed bacteriochloroprophyl and heme at the same rate as did the wild type when incubated anaerobically in the light in mixture GS under the conditions described previously (18).

The activity of ALA synthase in extracts of the revertant was similar to that found in the wild type; specific activities of 127 and 122 nmoles of ALA per mg of protein per hr were observed in extracts of mutant and wild type, respectively, grown anaerobically in the light in unsupplemented malate-glutamate medium.

**Tetrapyrrole synthesis by suspensions of mutant H-5.** Formation of tetrapyrroles was studied with suspensions of H-5 harvested after growth under low aeration with 0.1 mM ALA.

Bacteriochlorophyll and heme were formed when the cells were incubated anaerobically in the light in mixture GS supplemented with 0.1 to 1 mM ALA (Fig. 4). The maximal rate of bacteriochlorophyll synthesis was achieved with 0.4 mM ALA, but a higher concentration was needed for maximal production of heme. Only small amounts of coproporphyrin or other tetrapyrroles were found in the supernatant fluids after incubation of H-5 anaerobically in the light. After incubation for 5 hr in mixture GS with 1 mM ALA, the porphyrin concentration in the medium was approximately 0.002 mM. The wild type, under the same conditions, amassed considerably greater amounts of porphyrins (18).

Protoporphyrin, magnesium protoporphyrin and its monomethyl ester, in concentrations up to 0.02 mM, were completely ineffective in replacing or sparing the requirement for ALA for bacteriochlorophyll or heme formation. Nor was there any response to a mixture of hemin and magnesium protoporphyrin each at a concentration of 0.01 mM.

Bacteriochlorophyll and heme synthesis by H-5 was affected by aeration and by inhibitors in a similar manner to the wild type. High aeration prevented bacteriochlorophyll production, but heme synthesis continued, although at a reduced rate (Fig. 5). Puromycin completely stopped bacteriochlorophyll formation, whereas 8-azaguanine and threonine were severely inhibitory (Table 2). Heme synthesis was also retarded by these compounds; threonine was particularly effective, as observed previously in the wild type (Table 2).

**Tetrapyrrole synthesis by suspensions of mutant H-4.** The capabilities of H-4 were examined under aerobic conditions, since the mutant made only traces of bacteriochlorophyll and consequently
could not obtain energy under anaerobic-light conditions.

Heme synthesis by suspensions of mutant H-4 was dependent upon the concentration of ALA (Table 3). The mutant also accumulated porphyrins and magnesium derivatives in the medium. Spectroscopic examination indicated the presence of coproporphyrin, protoporphyrin, and magnesium protoporphyrin or its methyl ester, or both. This mixture of tetrapyrroles was similar in quantity and composition to that formed by the wild type when incubated with ALA under the same conditions (15).

Only traces of bacteriochlorophyll were found in cells of mutant H-4 incubated with up to 1 mM ALA. Neither magnesium protoporphyrin nor its monomethyl ester (each at 0.01 mM) promoted bacteriochlorophyll formation. Protoporphyrin (0.02 mM) was also ineffective, and it neither replaced nor spared the requirement for ALA in heme synthesis.

Uptake of ALA by wild type and mutant strains. The difference between the two mutants in their growth response to low concentrations of ALA (see Fig. 1) might arise from differences in their ability to transport the compound from the external environment. Therefore, uptake of ALA by wild-type and mutant cells was examined.

The wild type took up 14C-ALA when exposed to the compound in the presence of a salts mixture. Uptake was not linear but continued at a decreasing rate for about 30 min (Fig. 6). The radioactivity in the cells then declined, due possibly to conversion of ALA to condensation products such as porphobilinogen. Similar kinetics of ALA uptake were observed with mutant H-4 incubated under the same conditions (Fig. 6). In contrast, mutant H-5 fixed ALA at a considerably lower rate (Fig. 6). This strain also showed a greater response to increasing concentrations of ALA than did either the wild type or mutant H-4. Within the range of 0.001 to 0.1 mM ALA, the initial rate of uptake by mutant H-5 increased by 30-fold, whereas the other strains showed an 8- to 10-fold increase (Table 4).

The requirements for uptake of ALA were examined with the wild-type organism. It was temperature-dependent in that uptake did not occur at 0 C, but the rate did not vary significantly within the range of 25 to 34 C. Energy was apparently not required, since ALA uptake oc-

Table 2. Effect of inhibitors on bacteriochlorophyll and heme synthesis by suspensions of mutant H-5a

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Amt synthesized in 4 hr (nmoles/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bacteriochlorophyll</td>
</tr>
<tr>
<td>Nil</td>
<td>7.1</td>
</tr>
<tr>
<td>Phenylalanine (40 μg/ml)</td>
<td>0.7</td>
</tr>
<tr>
<td>8-Azaguanine (1 mM)</td>
<td>0.9</td>
</tr>
<tr>
<td>DL-Threonine (5 mM)</td>
<td>0.01</td>
</tr>
</tbody>
</table>

* Incubation conditions were as described in Fig. 5 in the presence of 1 mM ALA. The inhibitors were added after incubation for 2 hr. The concentrations of bacteriochlorophyll and heme at this time were 6.5 and 1.6 nmoles/ml, respectively, and the values shown are for subsequent synthesis in 4 hr.

Table 3. Heme synthesis by suspensions of mutant H-4 incubated under low aerationb

<table>
<thead>
<tr>
<th>ALA (mM)</th>
<th>Heme formed (nmoles/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nil</td>
<td>0.025</td>
</tr>
<tr>
<td>0.1</td>
<td>0.15</td>
</tr>
<tr>
<td>0.3</td>
<td>0.41</td>
</tr>
<tr>
<td>1.0</td>
<td>0.71</td>
</tr>
</tbody>
</table>

* Cells were suspended in mixture GS with 14Fe and with addition of ALA as shown. Incubation was for 6 hr under low aeration.

![Figure 6](http://jb.asm.org/) Uptake of 14C-ALA by wild type and mutant strains. Cells were grown, washed, and suspended in the salts mixture as described in Materials and Methods. The cell density at 680 nm was 0.5 (0.25 mg dry weight per ml). The concentration of 14C-ALA was 0.01 mM, 31,000 counts/min per nmole. Incubation was at 30 C; samples (1 ml) were removed at intervals and the radioactivity was determined.
TABLE 4. Effect of ALA concentration on uptake by mutants and wild type

<table>
<thead>
<tr>
<th>ALA</th>
<th>Wild type</th>
<th>H-4</th>
<th>H-5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 min</td>
<td>10 min</td>
<td>5 min</td>
</tr>
<tr>
<td>mM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.001</td>
<td>0.15</td>
<td>0.20</td>
<td>0.16</td>
</tr>
<tr>
<td>0.01</td>
<td>0.62</td>
<td>1.14</td>
<td>0.66</td>
</tr>
<tr>
<td>0.10</td>
<td>1.25</td>
<td>1.90</td>
<td>1.50</td>
</tr>
</tbody>
</table>

* The procedure was as in Fig. 6, except that the concentration of ALA was varied. Results are expressed as nanomoles of ALA fixed by 1 ml of suspension, containing 0.25 mg (dry weight) of cells.

DISCUSSION

It has been suggested that *R. spheroides* produces two forms of ALA synthase, regulated specifically by the iron and magnesium branches of the biosynthetic pathway (16, 17). If this were so, loss of the "magnesium enzyme" by mutation should not affect aerobic growth, since the "iron enzyme" might be expected to provide sufficient ALA for the synthesis of heme. The presence of the magnesium enzyme alone might also be expected to compensate for lack of the other form. The notion of isoenzymes is made unlikely by the isolation of mutant H-5. This strain lacks synthase activity and requires added ALA for both heme and bacteriochlorophyll synthesis; consequently, growth under aerobic and anaerobic-light conditions is completely dependent upon added ALA. The behavior of mutant H-4 is also incompatible with the concept of two enzymes. This strain lacks ALA synthase activity and requires ALA for heme synthesis. However, mutant H-4 does not make bacteriochlorophyll with or without ALA and, consequently, grows only under aerobic conditions.

The inability of mutant H-4 to form bacteriochlorophyll from ALA may be due to the toxicity of this compound or its metabolic products at the concentration needed to support synthesis of the pigment. Aerobic growth of mutant H-4 is delayed considerably by high concentrations of ALA (Fig. 1). In the wild type, inhibition of growth and of bacteriochlorophyll synthesis by added ALA has been observed by many workers, but no explanation has so far been provided (15). The relative incapacity of strain H-5 to take up ALA from the medium presumably protects this mutant from inhibition by ALA.

The possibility that the strains are double mutants precludes any firm conclusions based on their response to ALA. The isolation of a spontaneous revertant strain of H-4 with wild-type characteristics suggests that a single mutation is involved in this mutant. In contrast, mutant H-5 is likely to be the result of a double mutation, one of which has interfered with its ability to take up ALA from the medium. Spontaneous revertants of this strain have now been isolated which can grow both aerobically and anaerobically without ALA, but under both conditions addition of ALA stimulates their growth. These revertants still exhibit diminished uptake of ALA, but more detailed investigation of their properties has not yet been undertaken.

The supply of ALA has been suggested as a major factor in the normal regulation of chlorophyll synthesis in bacteria and plants (9, 16). This would be influenced by the availability of the primary substrates, glycine and succinyl CoA, as well as by the activity of ALA synthase. The observations with mutant H-5 show that control at this stage cannot alone account for the regulation of bacteriochlorophyll synthesis in *R. spheroides*. The insertion of magnesium is likely to be another important locus, as suggested previously (18). The sensitivity of this step to oxygen is evident in suspensions of mutant H-5, forming tetrapyrrroles from ALA. Thus, production of bacteriochlorophyll and other magnesium derivatives was immediately arrested upon aeration, whereas heme synthesis continued. Such sensitivity of the first enzyme leading specifically to bacteriochlorophyll synthesis might play a key role in the overall regulation of pigment formation by the oxygen pressure and by light intensity (7).

Bacteriochlorophyll production by mutant H-5 in response to ALA shows the same dependence upon protein synthesis as that observed in the wild type with glycine and succinate as substrates. Therefore, the obligatory link between pigment and protein synthesis cannot be attributed solely to a continuous need to form new ALA synthase to counterbalance its rapid destruction (3, 9, 16). The observations with mutant H-5 accord with the possibility that steps of the magnesium branch of the biosynthetic pathway are integrated with the development of proteins of the membrane structure in which bacteriochlorophyll is located.

A growth response to ALA has been observed with mutant strains of *Escherichia coli* (21), *Bacillus subtilis* (1), and *Spirillum itersonii* (19).
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Hemin fully replaces ALA for growth of the latter two organisms, in contrast to the behavior of the mutant strains of *R. spheroides*. In mutants H-4 and H-5, the poor response to hemin could reflect lack of permeation. The same explanation might be invoked for the inactivity of protoporphyrin and its magnesium derivatives; alternatively, these compounds may participate in biosynthesis in bound forms which do not exchange freely with the exogenous materials.

Vitamin B₁₂ is also derived from ALA, and the wild-type strain is rich in this factor (5). Assay of the mutants grown with optimal concentrations of ALA showed them to contain levels similar to the wild type (B. Rittenberg, personal communication). All attempts to demonstrate a growth response or sparing action to vitamin B₁₂, added as cyanocobalamin, were negative. Possibly, *R. spheroides* cannot convert this form of vitamin B₁₂ to the coenzyme form.

**ACKNOWLEDGMENTS**

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**LITERATURE CITED**


