Bacteriochlorophyll and Heme Synthesis in *Rhodopseudomonas spheroides*: Possible Role of Heme in Regulation of the Branched Biosynthetic Pathway

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Synthesis of heme, measured by incorporation of iron-59, and of bacteriochlorophyll was studied with wild-type and mutant strains of *Rhodopseudomonas spheroides*. The wild type formed heme from glycine and succinate at one-fortieth the rate of bacteriochlorophyll under anaerobic-light conditions. Added δ-aminolevulinate stimulated heme synthesis 10-fold without increasing bacteriochlorophyll production. Heme synthesis from glycine and succinate was increased when the magnesium branch of the biosynthetic pathway was curtailed by mutation or by p-fluorophenylalanine or 8-azaguanine. Synthesis of bacteriochlorophyll by the wild type from glycine and succinate stopped immediately after addition of puromycin, but heme production continued for a period. Porphyrins and other precursors did not appear upon addition of puromycin alone, but simultaneous addition of o-phenanthroline resulted in the accumulation of coproporphyrin. Production of this porphyrin by a mutant strain with impaired ability to form heme was unaffected by puromycin. Heme synthesis from glycine and succinate or from δ-aminolevulinate was decreased by limitation of methionine; it is suggested that coproporphyrin accumulation from glycine and succinate under conditions of methionine deficiency results from relief of feedback inhibition of δ-aminolevulinate synthase by heme. The development of δ-aminolevulinate synthase activity in response to low aeration is prevented by addition of δ-aminolevulinate. This repressive action of the latter is abolished when its conversion to heme is impeded by mutation or by methionine deficiency. It is suggested that heme, the quantitatively minor end product of the branched biosynthetic pathway, may regulate the flow of common intermediates when utilization of protoporphyrin by the magnesium branch is diminished. This regulation may be exerted by feedback inhibition of δ-aminolevulinate synthase and also by repression of enzyme formation.

Chlorophyll and heme (a general term for iron porphyrins) share a common biosynthetic pathway which diverges at the stage of protoporphyrin into the magnesium and iron branches (Fig. 1). The former predominates in photosynthetic organisms, since the chlorophyll concentration is 10 to 100 times more than that of heme (16). In *Rhodopseudomonas spheroides* and related bacteria, marked variations in bacteriochlorophyll synthesis occur in response to environmental changes. Cells contain maximum amounts of the pigment when grown either anaerobically in the light or in the dark under low aeration (5). Under conditions of high aeration, synthesis is repressed to a level of less than 1% that found under favorable conditions for pigment formation. Production of heme is also influenced by the environment, but the fluctuations are far less dramatic. The cytochrome content of *R. spheroides* shows only a two- to fourfold variation in response to environmental changes which cause a 50- to 100-fold variation in bacteriochlorophyll concentration (23).

These observations imply control mechanisms which ensure the diversion of a major proportion of the common intermediate protoporphyrin into the magnesium branch under appropriate conditions. Also, this branch must be under some in-
dependent control, since chlorophyll synthesis can be terminated without preventing heme synthesis. However, closure of the magnesium branch does have repercussions on the common part of the biosynthetic sequence, since intermediates do not accumulate. Such a lack of intermediates suggests that termination of bacteriochlorophyll synthesis in some way interferes with the operation of δ-aminolevulinate (ALA) synthase, the first enzyme of the biosynthetic pathway.

ALA synthase has been partially purified from R. spheroides; it is inhibited by low concentration of iron protoporphyrin in a manner consistent with a role for heme in the control of the enzyme by feedback inhibition (2). The excess production of coproporphyrin by R. spheroides under conditions of iron deficiency could represent the failure of such a heme-mediated control (13).

Regulation of the synthase by heme is apparently paradoxical in an organism in which the main quantitative function of the synthase is directed towards bacteriochlorophyll synthesis, and one aim of the present work was to determine the physiological significance of the in vitro sensitivity of the enzyme to heme. Bacteriochlorophyll and heme synthesis have been studied with suspensions of cells using succinate and glycine as primary substrates and also with ALA present to bypass the synthase. The observations support a control system outlined in Fig. 1. The magnesium and iron branches of the biosynthetic pathway compete for a common pool of protoporphyrin; a decrease in the utilization of protoporphyrin by the magnesium branch favors its diversion to heme, which in turn regulates ALA synthase by feedback inhibition. Evidence is also presented for repression of the enzyme by heme.

MATERIALS AND METHODS

Organisms. The wild-type strain of R. spheroides and the mutants derived from it, together with details of the maintenance of the cultures, have been previously described (14, 17). Table 1 summarizes the characteristics of the mutants, which are described more fully elsewhere (17).

Growth of organisms and preparation of cell suspensions. Cells were grown in the malate-glutamate medium (14), supplemented with 0.5 mM L-methionine in the case of the auxotrophs 2-33 and 6-10. In the studies of tetrapyrrrole synthesis, cultures were grown under low aeration in Erlemeyer flasks filled to 80% of their nominal capacity and shaken on a gyratory shaker at 200 rev/min; such conditions promote synthesis of the bacteriochlorophyll in the wild type. In the experiments on the development of ALA synthase activity, the cultures were grown with high aeration in 500-ml volumes in 2-liter Fernbach flasks, shaken on a reciprocal shaker; such conditions provided cells which were low in bacteriochlorophyll and in ALA synthase activity (14).

The harvested cells were suspended to an optical density of 2 (1-cm light path) at 680 nm (equivalent to about 1 mg, dry weight, per ml) in mixture GS, which consisted of the malate-glutamate growth medium supplemented with 10 mM glycine and 10 mM sodium succinate. Mixture GS contained 0.01 mM ferric citrate; for measurement of heme synthesis, 59FeCl3 (about 0.5 μCi per ml) was added.

Suspensions were incubated anaerobically in the light or in the dark under low or high aeration. Incubation in the light was in 11-mm tubes in a water bath, illuminated by strip lights giving an intensity of 150 ft-c to the cells; the tubes were flushed with nitrogen and capped with Parafilm. For incubation under low aeration, 15-ml volumes of suspension were incubated in 25-ml flasks on a gyratory shaker at 200 rev/min. All incubations were at 30°C.

Estimations. Bacteriochlorophyll was determined (3, 5) by extraction of the cells with methanol, by using the extinction coefficient of 60 mm (1 cm). Coproporphyrin was estimated in the supernatant fluids after dilution in 1.4 N HCl (13); the cells produced coproporphyrinogen but this was converted to the porphyrin by acid treatment.

Heme synthesis was measured by incorporation of 59Fe, based on a method described by Jones (10). After incubation of the suspensions with the isotope, 3-ml samples were acidified with 0.2 ml of 1 N HCl and extracted with 3.0 ml of cyclohexanone. After standing for at least 2 hr in the cold, the tubes were centrifuged and 1-ml samples of the solvent layer were removed for the determination of radioactivity. 59Fe was measured with a scintillation detector (model 810B; Baird Atomic, Cambridge, Mass.). This method was checked with the more specific procedure of Labbe and Nishida (11), which involves crystallization of heme after addition of carrier hemoglobin in the form of red blood cells. The results agreed to within 10%. The extraction procedure is assumed to measure free heme and non-covalently bonded heme (25).

Protein was estimated by the Lowry procedure, with crystalline bovine serum albumin as standard (20).

Assay of ALA synthase. The method described by Burnham and Lascelles (2) was used to assay ALA synthase in extracts prepared by sonic oscillation. One unit of enzyme is that which produces 1 nmole of ALA in 1 hr under the standard conditions.

Materials. 59FeCl3 was from the New England Nuclear Corp., Boston, Mass.; stock solutions were in 0.1 N HCl. Puromycin, p-fluorophenylalanine, 8-azaguanine, and ALA were from the Sigma Chemical Co., St. Louis, Mo.

RESULTS

Synthesis of bacteriochlorophyll and heme by the wild-type strain and the effect of ALA. Suspensions of the wild-type strain in mixture GS (containing glycine and succinate as primary substrates) formed bacteriochlorophyll at about 40 times the rate of heme under anaerobic-light conditions of incubation (Fig. 2a).
Glycine

\[ \text{Glycine} \xrightarrow{\text{SuccinylCoA}} \text{ALA synthesis} \xrightarrow{} \text{ALA} \xrightarrow{\text{PBG}} \text{Coproporphyrinogen} \xrightarrow{} \text{Protoporphyrin} \]

Heme → Hemoproteins

Fe

\[ \text{Mg} \xrightarrow{\text{Mg-protoporphyrin}} \text{Methyl} \xrightarrow{\text{Mg-protoporphyrin monomethyl ester}} \text{Bacteriochlorophyll} \]

**Fig. 1. Outline of biosynthetic pathway of heme and bacteriochlorophyll. The dotted lines indicate areas for control. ALA, L-aminolevulinate; PBG, porphobilinogen; CoA, coenzyme A.**

**TABLE 1. Properties of mutant strains of R. spheroides**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Nutritional requirement</th>
<th>Bacteriochlorophyll synthesis</th>
<th>Compounds* accumulated</th>
<th>Presumed block in tetrapyrrole synthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-10</td>
<td>Methionine</td>
<td>+</td>
<td>Nil</td>
<td>Protoporphyrinogen → protoporphyrin</td>
</tr>
<tr>
<td>2-33</td>
<td>Methionine</td>
<td>-</td>
<td>Coproporphyrin</td>
<td>PBG → uroporphyrinogen</td>
</tr>
<tr>
<td>6-6</td>
<td>Nil</td>
<td>-</td>
<td>Porphobilinogen</td>
<td>Lack ability to form Mg derivatives; apparently normal in other respects</td>
</tr>
<tr>
<td>L-57 and 8-13</td>
<td>Nil</td>
<td>-</td>
<td>Nil</td>
<td></td>
</tr>
</tbody>
</table>

* Compounds accumulated by cultures grown in malate-glutamate medium, supplemented with 0.5 mM L-methionine in the case of auxotrophs, under conditions of low aeration which permit bacteriochlorophyll synthesis by the wild type.

Heme production was apparently limited by ALA synthase, since addition of ALA to bypass this step increased heme production by about 10-fold (Fig. 2b). In contrast, bacteriochlorophyll formation was not stimulated by ALA and was actually inhibited as incubation proceeded.

**Heme synthesis by mutant strains.** Figure 1 predicts that heme synthesis is favored when competition by the magnesium pathway for a limited supply of protoporphyrin is diminished. Mutant strains which could not form magnesium derivatives were therefore examined for their capacity to synthesize heme. They were compared with the wild-type organism under conditions of low aeration which permitted synthesis of bacteriochlorophyll by the latter (Table 2). In mixture GS, heme production by mutants 8-13 and L-57 was two to three times that of the wild-type strain. With ALA present, these mutants and the wild type formed similar amounts of heme (Table 2).

Mutant strains 2-33 and 6-6 which have partial blocks in the biosynthetic path prior to protoporphyrin were also examined (Table 2). Strain 2-33 formed less heme than the wild type in mixture GS and was only slightly stimulated by ALA. Strain 6-6 was more active than the wild type in mixture GS but also showed little or no response to ALA. The behavior of these mutants in the presence of ALA is consistent with their presumed enzymatic defects. The difference in their ability to form heme from glycine and succinate suggests that the lesion in 2-33 creates a more severe shortage of protoporphyrin than in strain 6-6.

**Effect of inhibitors of protein synthesis on bac-**
TABLE 2. Bacteriochlorophyll and heme synthesis by mutant and wild-type R. spheroides

<table>
<thead>
<tr>
<th>Strain</th>
<th>Tetrapyrrole formed (nmoles/ml)</th>
<th>Heme</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bacteriochlorophyll (without ALA)</td>
<td>Without ALA</td>
</tr>
<tr>
<td>Wild type</td>
<td>8</td>
<td>0.32</td>
</tr>
<tr>
<td>8-13</td>
<td>ND</td>
<td>0.80</td>
</tr>
<tr>
<td>L-57</td>
<td>ND</td>
<td>0.72</td>
</tr>
<tr>
<td>6-6</td>
<td>ND</td>
<td>0.79</td>
</tr>
<tr>
<td>2-33</td>
<td>ND</td>
<td>0.20</td>
</tr>
</tbody>
</table>

*a The cells were suspended in mixture GS with 55Fe supplemented with 0.5 mM L-methionine in the case of strain 2-33, and with ALA as shown. Incubation was with low aeration in the dark for 5 hr.

*b Not detectable.

FIG. 2. Synthesis of bacteriochlorophyll and heme by wild-type cells. The cells were incubated anaerobically in the light in mixture GS with 55Fe (a). ALA (1 mM, final concentration) was added to a portion of the suspension after 3 hr (b). Bacteriochlorophyll (●) and heme (○) were determined on samples removed at intervals.

Bacteriochlorophyll and heme formation. There have been numerous observations that inhibition of protein and of nucleic acid synthesis prevents the formation of bacteriochlorophyll, indicating an obligatory link between pigment production and macromolecular synthesis (16).

The relative susceptibility of bacteriochlorophyll and heme formation to such inhibition was examined with suspensions of the wild type incubated anaerobically in the light in mixture GS. Addition of puromycin immediately stopped bacteriochlorophyll synthesis; however, heme production continued for a period at the same rate as the control and then inhibition became evident (Fig. 3). p-Fluorophenylalanine and 8-azaguanine, at concentrations which inhibited bacteriochlorophyll synthesis significantly, stimulated heme synthesis (Table 3). Under the conditions of these experiments, puromycin completely inhibited protein synthesis, measured by incorporation of 14C-phenylalanine, whereas the analogues reduced the rate only to approximately one-half that of the control.

The inhibitors were also tested in the presence of ALA (Table 3). Puromycin reduced heme production to one-third of the control value; the analogues had no effect.

Accumulation of coproporphyrin in the presence of puromycin. Intermediates on the biosynthetic pathway did not accumulate when bacteriochlorophyll production in mixture GS was terminated by addition of puromycin. This suggested that inhibition of protein synthesis had secondary consequences on the activity of ALA synthase. Inactivation of the synthase might result from inhibition by heme, whose synthesis continued for a time after addition of puromycin. Experiments in which heme synthesis by the wild type was blocked with the iron chelator, o-phenanthroline, supported this. When o-phenanthroline was added simultaneously with puromycin, accumulation of coproporphyrin occurred, indicating restoration of
The effects of puromycin was provided by observations with mutant 2-33, which is defective in heme synthesis (Table 2). The accumulation of coproporphyrin by this strain in mixture GS was not prevented by puromycin (Table 4).

**Effect of methionine deficiency on heme and coproporphyrin production.** Deprivation of methionine prevents bacteriochlorophyll synthesis in *R. spheroides*, since the methylation of magnesium protoporphyrin involves S-adenosylmethionine as methyl donor (8). In the wild-type strain, limitation of methionine can be achieved by addition of threonine, which inhibits its formation at the stage of homoserine dehydrogenase (7). Accumulation of coproporphyrin is another consequence of methionine deficiency, possibly because of feedback control of ALA synthase (6, 19). Our observations with the methionine auxotroph 6-10 and with the wild-type strain suggest that methionine deficiency may alleviate such a control by impeding the synthesis of heme.

In mixture GS, methionine deprivation decreased heme synthesis by both mutant and wild-type strains (Table 5). This was observed with the mutant by comparing heme synthesis with and without addition of methionine, and in the wild-type strain by the addition of threonine. Depres-

**TABLE 4. Effect of puromycin and o-phenanthroline on coproporphyrin production by wild type and mutant 2-33**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Additions</th>
<th>Tetrapyrrole formed (nmoles/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Bacteriochlorophyll (without ALA)</td>
</tr>
<tr>
<td>Wild type</td>
<td>Nil</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>Puromycin (40 μg/ml)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>dl-p-Fluorophenylalanine</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>(4 mM)</td>
<td>0</td>
</tr>
<tr>
<td>2-33</td>
<td>Nil</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td>Puromycin (40 μg/ml)</td>
<td>35</td>
</tr>
</tbody>
</table>

* The wild-type cells were incubated anaerobically in the light in mixture GS for 3 hr; puromycin and o-phenanthroline were then added to give the final concentrations shown. The concentration of bacteriochlorophyll and of heme at the time of the additions was 10 and 0.24 nmoles/ml, respectively; the values shown are for subsequent synthesis in 6 hr.

ALA synthase activity (Table 4). Heme synthesis was completely inhibited by o-phenanthroline in these experiments.

Further support for this interpretation of the
sion in heme synthesis under conditions of methionine starvation was particularly evident when ALA was added to the incubation systems (Table 5).

Both mutant and wild-type strains accumulated coproporphyrin when incubated in mixture GS under conditions of methionine deficiency (Table 5). Addition of ALA did not increase such porphyrin production, indicating that the synthase was not rate-limiting under these circumstances. In contrast, addition of ALA to the controls resulted in the accumulation of a mixture of porphyrins containing predominantly coproporphyrin (Table 5).

Effect of ALA on development of synthase activity. ALA synthase in R. spheroides is repressed by growth under high aeration, and derepression occurs upon transfer to low aeration (13). The development of enzyme activity in response to low oxygen pressure is prevented by the addition of ALA, suggesting that this compound or a metabolic derivative represses enzyme formation (14). Since the present work has shown that the organism forms excess heme from exogenous ALA, the repression of the synthase might be due to heme rather than to ALA itself. This possibility was explored in mutant organisms with varying capacities to form heme from ALA and also in the wild-type strain, with threonine used to limit heme synthesis.

The cells were grown under high aeration to repress ALA synthase; they were then suspended in mixture GS with Tween 80 and incubated under low aeration. Both wild-type and mutant strains showed a considerable increase in enzyme activity under these conditions (Table 6). Addition of ALA to the suspensions prevented development of enzyme activity in the wild type and in those mutants (L-57, 8-13) which formed excess heme from ALA (see Table 2). In contrast, ALA did not affect enzyme development in strains 6-6 and 2-33; this is consistent with their relative inability to form large amounts of heme from this intermediate. It is notable that the activity of ALA synthase developed in strain 2-33 is two to three times higher than that found in the other strains (Table 6).

DL-Threonine (5 mM) abolished the effect of ALA upon development of enzyme activity in the wild type; heme synthesis from ALA was also considerably reduced by this concentration of threonine (Table 7).

DISCUSSION

Our observations suggest that in R. spheroides the intracellular concentration of heme may in-
fluence both the activity of ALA synthase and also its formation. Regulation by the quantitatively minor end product of the branched biosynthetic pathway may be important in preventing wastage of common intermediates when production of bacteriochlorophyll, the major product, is curtailed.

The mechanism outlined in Fig. 1 centers upon control of ALA synthase by heme. In addition to the inhibition by heme of the enzyme in vitro, the evidence to support our proposals can be summarized as follows. (i) ALA synthase is apparently rate-limiting for heme synthesis, since addition of ALA to cells incubating in mixture GS considerably increased heme production. (ii) Heme formation in mixture GS rises when the operation of the magnesium pathway is curtailed by mutation or by 8-azaguanine or p-fluorophenylalanine. This could indicate diversion of protoporphyrin from the magnesium to the iron branch. (iii) Accumulation of coproporphyrin in mixture GS presumably represents enhanced ALA synthase activity, and the phenomenon is correlated with impeded heme synthesis. Such overproduction occurs when heme formation is limited by an enzymatic defect (mutant 2-33) or by methionine deficiency. It also occurs under conditions of iron deficiency (13). (iv) Precursors do not accumulate when bacteriochlorophyll formation in mixture GS is terminated with puromycin, but heme synthesis continues for a period. ALA synthase activity may be checked with heme, since addition of o-phenanthroline together with puromycin causes coproporphyrin to accumulate. Also, puromycin did not affect coproporphyrin production by mutant 2-33.

The ultimate decline in heme synthesis, which occurs in mixture GS after addition of puromycin, could be due to inhibition of the synthase upon the attainment of a critical level of heme. However, this explanation is not entirely satisfactory, since puromycin also curtails heme synthesis in the presence of ALA. Possibly, the biosynthetic enzymes are rapidly degraded when protein synthesis is inhibited. Destruction of enzymes, if occurring, does not impede heme synthesis in the presence of p-fluorophenylalanine or 8-azaguanine, which did not completely inhibit protein synthesis. These analogues stimulated heme synthesis in mixture GS, but severely depressed bacteriochlorophyll formation. We interpret the rise in heme to be the result of an increase in the pool of protoporphyrin consequent to the curtailment of the magnesium branch.

The proposed control mechanism is presented merely as a working hypothesis which is consistent with our experimental observations but which requires more rigorous testing. Assuming that free heme is the inhibitor of ALA synthase, a particular deficiency of the present work is that the method for estimation of heme does not discriminate between free and protein-bound forms. The intracellular concentration of free heme is governed by its rate of formation and its rate of utilization for hemoprotein synthesis. Critical examination of our proposal requires kinetic experiments to determine changes under various conditions in the pool levels of free heme and precursors in relation to bacteriochlorophyll synthesis. For instance, it is important to know the kinetics of formation of free heme immediately after addition of puromycin and of its utilization for hemoprotein formation upon restoration of protein synthesis. Knowledge of the fluctuation of pool levels of protoporphyrin is also necessary.

A role for heme as co-repressor of the formation of ALA synthase is supported by the positive correlation between the development of enzyme activity and impaired ability to convert ALA to heme. Also, the exceptionally high level of synthase found in mutant 2-33 suggests lack of the natural co-repressor; this strain showed only feeble ability to form heme. Alternatively, the observations with ALA could be interpreted as inhibition of enzyme activity by heme rather than as repression. However, previous experiments with mixed extracts from "ALA-repressed" and untreated cells gave no evidence of an inhibitor (15). We did not obtain direct evidence of repression by heme; addition of heme to the cell suspensions did not affect development of ALA synthase activity. This compound probably did not penetrate the cells, since mutant strains requiring ALA for growth show little response to hemin
The lack of effect of hemin upon enzyme development contradicts previous reports (9, 15). In the present experiments, Tween 80 was included in the incubation mixture to maintain the hemin in solution, whereas it was not used in the previous work. The earlier observations may be attributed to inhibition of the enzyme by precipitation of hemin, which was carried over with the cell-free extract into the assay system.

The insertion of magnesium into the protoporphyrin nucleus must be a key point in the regulation of the branched pathway. It is presumably this enzyme which determines the preferential diversion of protoporphyrin towards bacteriochlorophyll synthesis when the supply of the common intermediate is limited. To achieve this, the magnesium enzyme in comparison with the iron one (ferrochelatase) may have a greater affinity for protoporphyrin. Information about these enzymes is lacking; ferrochelatase is readily demonstrable in particulate preparations from R. spheroides, but in vitro formation of magnesium protoporphyrin has not been observed (22, 23).

Another critical point in the biosynthetic pathway is the conversion of coproporphyrinogen to protoporphyrin, since coproporphyrin rather than protoporphyrin is the predominant porphyrin which accumulates when control of ALA synthase fails or when ALA is added. Attempts to detect this step in extracts of R. spheroides have failed so far (23). In animal tissues, the enzyme system requires oxygen as electron acceptor (1, 24).

Recently, conversion of coproporphyrinogen to protoporphyrin has been shown in cell-free extracts of Chromatium, but only in the presence of oxygen (12). Under physiological conditions, bacteria (such as Chromatium) which form tetrapyroles in the complete absence of oxygen must use other mechanisms.

The reason for the apparent participation of methionine in heme synthesis is obscure. Its involvement is apparently in the area between coproporphyrinogen and heme, since coproporphyrin accumulates under conditions of methionine deficiency. One possibility is that methionine might serve as a methyl donor for the synthesis of phosphatidylcholine, in turn needed for ferrochelatase activity. This enzyme in extracts of R. spheroides is stimulated by a lipid extract of the organism which is rich in this phosphatide (21).

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LITERATURE CITED


