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Protoporphyrinogen Oxidation, a Step in Heme Synthesis in Soybean Root Nodules and Free-Living Rhizobia

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Extracts of the crude bacteroid fraction of symbiotically grown *Bradyrhizobium japonicum* were much more active in oxidizing protoporphyrinogen to protoporphyrin than were extracts of cells grown under free-living conditions, especially when assayed in atmospheres containing only traces of oxygen. This correlates with the higher heme content of the microaerophilic nodules. Furthermore, the high level of oxidative activity in the crude bacteroid fraction was associated with an uncharacterized membrane fraction, probably of plant origin, that was separable from the bacteroids by Percoll gradient centrifugation.

During symbiotic association within the root nodules of leguminous plants, rhizobia synthesize large amounts of heme for the prosthetic group of leghemoglobin. Although the symbiotic bacterium is thought to play an exclusive role in this heme synthesis (6, 10, 24), a role for plant enzymes within the nodule to assist in this process has not been eliminated (11, 12). One of the late steps of heme synthesis as well as chlorophyll synthesis, the oxidation of protoporphyrinogen to protoporphyrin (Fig. 1), has not been characterized for rhizobia, although a mutant strain deficient in this activity forms Fix^- nodules which are deficient in leghemoglobin (25). Protoporphyrinogen oxidation has been demonstrated for a variety of mammalian, bacterial, and plant cells (4, 5, 7–9, 14–21, 23, 27, 29). The mechanism is not known, but in mammalian and yeast mitochondria the oxidation seems to require molecular oxygen (27), while in facultative and anaerobic bacteria alternate electron acceptors can replace oxygen (17, 18, 23). The question of a role for oxygen is especially important for rhizobia, since leghemoglobin synthesis occurs under microaerophilic conditions within the root nodule (1).

Preparation and assay of extracts. Free-living cells of *Bradyrhizobium japonicum* 61A152 (The Nitragin Co., Inc., Milwaukee, Wis.) were grown at 30°C in YEM broth (1% mannitol, 0.2% yeast extract, 0.05% K_2HPO_4, 0.02% $MgSO_4 \cdot 7H_2O$, 0.01% NaCl) for about 72 h to late log phase. Cells were centrifuged (4,000 × *g*, 4°C), washed with buffer A (0.1 M HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid] [pH 7.4], 0.25 M pentaerythritol, 1 mM $MgCl_2$), and suspended in buffer B (buffer A with 10 mM dithiothreitol instead of $MgCl_2$). Symbiotically grown cells of strain 61A152, termed bacteroids, were prepared from nodules harvested from the roots of 6-week-old soybeans (*Glycine max* var. harsoy) as described previously (11). Crude bacteroids, prepared as described previously (22), were resuspended in buffer B. All cells were broken by sonication (22) and centrifuged at 12,000 × *g* (10 min) and then at 17,000 × *g* (10 min); the residue was discarded each time. When indicated, extracts were dialyzed overnight against 0.01 M HEPES (pH 7.5) at 4°C (Spectrapor tubing, 12,000 to 14,000 molecular weight cutoff). Cells, nodules, and fractions were stored at −70°C.

Percoll gradient-purified fractions were prepared from crude bacteroids harvested from about 10 g of nodules as described above except that the grinding buffer was 0.15 M KCl–50 mM KH_2PO_4(pH 7.6) (buffer C) (28). After centrifugation, the crude bacteroids were resuspended in 2 to 3 ml of buffer C and purified in a Percoll gradient (28). The purified fractions were suspended in buffer A without $MgCl_2$. Percoll-purified fractions were analyzed by transmission electron microscopy of specimens negatively stained with phosphotungstic acid (13). The purified bacteroid fraction showed large numbers of intact cells with the typical appearance of bacteroids from soybean nodules (28) (data not shown). Almost no bacteroids were observed in the fraction designated plant membranes.

Protoporphyrinogen oxidation was assayed as described previously (14, 15), except that the reaction mixture (14) contained 0.7 mM glutathione in a test tube (12 by 75 mm) with a total volume of 250 μl. We used extracts heated at 95°C (10 min) and dispersed by brief sonication to determine the rate of nonenzymatic oxidation. Incubation occurred either in air or under an atmosphere of prepurified nitrogen gas (containing O_2 at approximately 8 ppm (8 μl/liter) as a contaminant); the nitrogen atmosphere was established by quickly evacuating a desiccator and replacing the atmosphere with nitrogen gas twice. Proteins (2) and ferrochelatase, with deuteroporphyrin as a substrate (7), were measured as described previously.

Activity of free-living and symbiotic cell extracts. Protoporphyrinogen was oxidized at a much more rapid rate by dialyzed extracts from crude bacteroids than by dialyzed extracts from free-living cells under aerobic assay conditions (Fig. 2). The rate was 4- to 10-fold-higher than that of the heated control, suggesting the enzymatic nature of the oxidation (Fig. 2). Table 1 compares the rate of oxidation under air and under an atmosphere of nitrogen gas containing only traces of oxygen. The dialyzed crude bacteroid extract functioned almost as well in the nitrogen atmosphere, whereas the dialyzed extract of free-living cells was markedly suppressed.

When extracts were assayed before dialysis, there was a marked decrease in aerobic activity of both the crude

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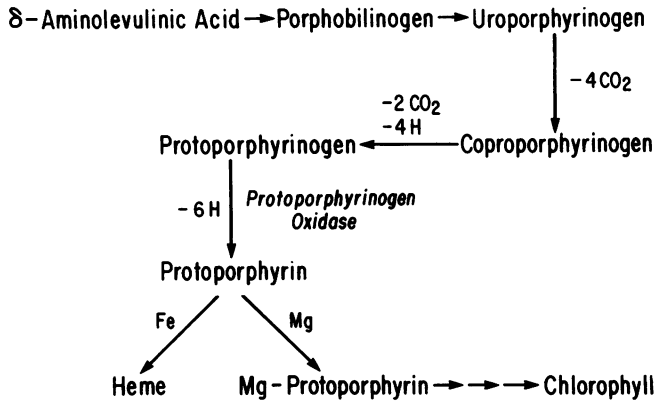

FIG. 1. Pathway for heme and chlorophyll biosynthesis.

bacteroid and free-living extracts, although the former were still more active. This was also apparent during assay under a nitrogen atmosphere, when the undialyzed extract of free-living cells exhibited no protoporphyrinogen-oxidizing activity, while the undialyzed crude bacteroid extract showed a decreased but still measurable rate (0.15 nmol of protoporphyrin formed per h per mg of protein). This was about half the rate of activity of the undialyzed crude bacteroid extract in the aerobic assay. The inhibitory factor removed by dialysis is probably dithiothreitol, since extracts prepared from cells in the absence of dithiothreitol exhibited activities similar to those shown for dialyzed extracts (data not shown). Protoporphyrinogen oxidation by extracts of other cells has been previously shown to be inhibited by reducing agents such as dithiothreitol (15, 16, 20). These

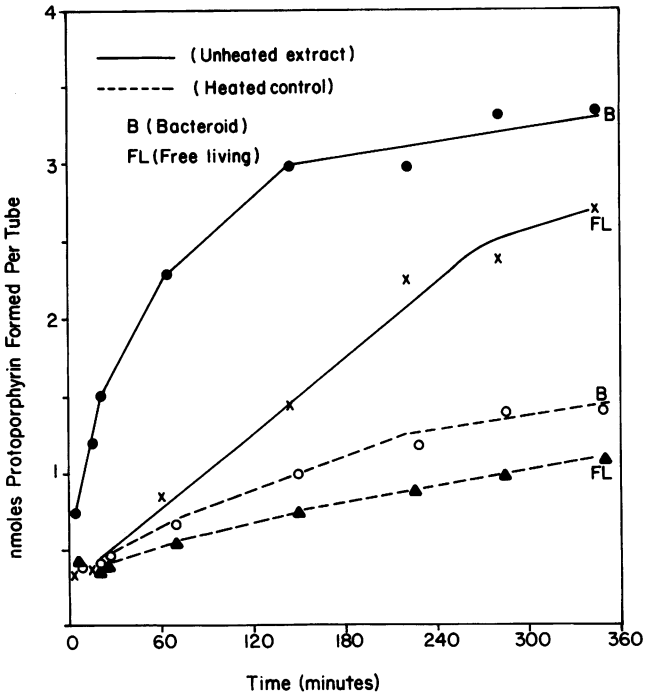

FIG. 2. Protoporphyrinogen oxidation by dialyzed extracts prepared from crude bacteroids or from free-living cells. The assay tubes, incubated aerobically, contained 0.52 and 0.82 mg of protein for the crude bacteroid and free-living extracts, respectively.

TABLE 1. Protoporphyrinogen oxidation by dialyzed extracts of crude bacteroids and free-living cells

Extract assayed	Assay atmosphere	nmol of protoporphyrin formed/h per mg of protein[a]
Crude bacteroids	Air	5.8
	N_2	5.2
Free-living cells	Air	0.19
	N_2	0.03

[a] All values are corrected for heated controls incubated under the same conditions as unheated extract and are means of duplicate tubes which varied less than 5%. The extract of crude bacteroids (0.5 mg of protein per tube) was incubated for approximately 29 min. The extract of free-living cells (1.5 mg of protein per tube) was incubated for approximately 308 min. At least four additional assays were conducted for each extract, with equivalent results.

observations illustrate that, in contrast to free-living cells, the crude bacteroid extract can oxidize protoporphyrinogen in an atmosphere containing only traces of oxygen even if a reducing agent is present.

Activity in gradient-purified fractions from the nodule. When the crude bacteroids were purified, most of the protoporphyrinogen-oxidizing activity was found in a fraction designated plant membranes (28) (Table 2). The activity of this fraction was considerably higher than that observed for crude bacteroids. This may be due to the presence of reductants or inhibitory factors in the bacteroids themselves. The purified bacteroids contained very little or no protoporphyrinogen-oxidizing activity (Table 2). We also sedimented free-living cells in Percoll, and their activity was comparable to that of the dialyzed free-living extract (compare Tables 1 and 2). When the assay was conducted under a nitrogen atmosphere, the purified plant membrane fractions were as active as when assayed aerobically (Table 2). In contrast, the extracts of free-living cells exhibited a 78% decrease and the extract of purified bacteroids exhibited a 95% decrease under a nitrogen atmosphere (Table 2).

Although deficient in protoporphyrinogen-oxidizing activity, purified bacteroids exhibited other bacteroid-associated enzymes (26, 28). In one of our preparations, ferrochelatase, the next enzyme in the heme synthesis pathway, was present at 11.6 nmol of heme formed per h per mg of protein, and β-hydroxybutyrate dehydrogenase was present at 175 nmol of NADH formed per min per mg of protein (28). Similar

TABLE 2. Protoporphyrinogen oxidation by Percoll-purified fractions

Extract assayed[a]	Assay atmosphere	nmol of protoporphyrin formed/h per mg of protein[b]
Plant membranes	Air	90.8
	N_2	89.7
Free-living cells	Air	0.24
	N_2	0.03
Bacteroids	Air	0.11[c]
	N_2	0.006

[a] Extracts were prepared without added dithiothreitol to maximize activity. Plant membranes were used directly after sonication without centrifugation.

[b] Plant membranes (0.06 mg of protein per tube) were incubated for approximately 26 min, and extracts from free-living cells (0.45 mg) and bacteroids (0.84 mg of protein per tube) were incubated for approximately 305 min. The values are means of duplicate tubes which varied by less than 5% and are corrected as described for Table 1.

[c] This was the most active of a total of four different preparations of purified bacteroids examined in the aerobic assay. Two others showed no activity, and one showed half the activity of this preparation.

levels of ferrochelatase enzyme were detected in extracts of free-living cells. We also tested the effect of supplements such as ATP, $MgSO_4$, NADP, and methionine, which are required for the anaerobic oxidation of coproporphyrinogen to protoporphyrinogen in bacteroids of *B. japonicum* (22). When these supplements were added to extracts of purified bacteroids at concentrations described previously (22) and under the conditions shown in Table 2, we found no effect on protoporphyrinogen oxidation by purified bacteroid extracts when tested either in air or under nitrogen (data not shown).

In conclusion, our findings have implications for the regulation of rhizobial heme synthesis. Nodule-derived fractions were much more active in protoporphyrinogen oxidation than were free-living cells, especially under conditions of lowered oxygen tension. This correlates with the in vivo ability of the symbiotic root nodule system to synthesize much larger amounts of heme for leghemoglobin under the microaerophilic conditions within the nodule than free-living cells synthesize during aerobic growth.

Although high levels of protoporphyrinogen-oxidizing activity are associated with the plant membrane fraction, the composition of this fraction is not known. It does not contain free bacteroids; it probably consists of plant-derived membrane structures such as mitochondria and plastids which sediment with the crude bacteroids during the initial differential centrifugation of the nodule homogenate. The peribacteroid membrane which surrounds the bacteroids in the plant cell is probably also present. This membrane is plant derived but may be decorated with bacteroid-derived proteins (3). The significance of this compartmentalization of protoporphyrinogen-oxidizing activity for leghemoglobin synthesis is unclear. One possibility is that some of these plant-derived membranes, containing high levels of protoporphyrinogen oxidase, may act synergistically with the bacteroid, which contains other heme synthesis enzymes. This could increase the efficiency of the late stages of heme synthesis in the microaerophilic environment within the nodule, since the protoporphyrinogen-oxidizing activity of the plant-derived membranes can function very well at low oxygen tensions. Perhaps it would be advantageous for this oxygen-requiring step to be physically separated from the bacteroids themselves, since they contain several oxygen-sensitive enzymes such as nitrogenase and ferrocheletase.

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