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Biochemistry

Bacterial heme synthesis is required for expression of the leghemoglobin holoprotein but not the apoprotein in soybean root nodules

(nitrogen fixation/symbiosis/protoporphyrinogen oxidase/*Bradyrhizobium japonicum* mutant)

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ABSTRACT **In *Bradyrhizobium japonicum*/soybean symbiosis, the leghemoglobin (legume hemoglobin) apoprotein is a plant product, but the origin of the heme prosthetic group is not known. *B. japonicum* strain LO505 is a transposon Tn*5*-induced cytochrome-deficient mutant; it excreted the oxidized heme precursor coproporphyrin III into the growth medium. Mutant strain LO505 was specifically deficient in protoporphyrinogen oxidase (protoporphyrinogen-IX:oxygen oxidoreductase, EC 1.3.3.4) activity, and thus it could not catalyze the penultimate step in heme biosynthesis. Soybean root nodules formed from this mutant did not contain leghemoglobin, but the apoprotein was synthesized nevertheless. Data show that bacterial heme synthesis is required for leghemoglobin expression, but the heme moiety is not essential for apoleghemoglobin synthesis by the plant. Soybean leghemoglobin, therefore, is a product of both the plant and bacterial symbionts.**

Numerous plant species of the family Leguminosae form symbioses with nitrogen-fixing bacteria; the conversion of atmospheric nitrogen (N_2) to fixed nitrogen (NH_3) by the bacteria confers to the plant host the ability to grow in nitrogen-deficient soil. The legume root nodule is the site of nitrogen fixation, and it consists of differentiated plant and bacterial cells. The oxygen-binding protein leghemoglobin (legume hemoglobin) is found in these nodules in very high concentrations; it facilitates the diffusion of oxygen to the rapidly respiring bacteroids (differentiated, N_2-fixing bacteria) and concomitantly buffers the free O_2 concentration at an extremely low tension (for review, see ref. 1). Leghemoglobin, therefore, helps resolve the paradox of the simultaneous requirement of O_2 for respiration to support nitrogen fixation and the exclusion of O_2 to prevent inactivation of the oxygen-labile nitrogenase enzyme. Hemoglobins have also been found in many, but not all, nonlegume symbioses (2), and thus they seem to be nearly ubiquitous in plant symbiotic nitrogen fixation.

It is well established that the soybean leghemoglobin apoprotein is synthesized by the plant (1), but the origin of the heme prosthetic group has been a subject of debate. Some measurements of heme biosynthetic enzyme activities in fractionated soybean nodules (3–5) suggest that leghemoglobin heme is synthesized by the bacterium, whereas other experiments (3) indicate that either or both symbionts can synthesize leghemoglobin heme. Studies of other legume symbioses give conflicting results as well (6–8). The most direct evidence to date does not confirm that the soybean leghemoglobin heme moiety is bacterial in origin. In those experiments (9) it was found that nodules from a *Bradyrhizobium japonicum* δ-aminolevulinic acid (ALA) synthase mutant contain leghemoglobin and have nitrogen-fixing activity, showing that leghemoglobin heme is synthesized despite the lesion in the first step of the bacterial heme biosynthesis pathway. Interestingly, an analogous ALA synthase mutant in *Rhizobium meliloti* yields alfalfa nodules that lack leghemoglobin (7), suggesting important differences between the soybean and alfalfa symbioses. The present report shows that a *B. japonicum* strain mutated in a late enzymatic step of heme biosynthesis affects leghemoglobin expression in soybean root nodules.

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MATERIALS AND METHODS

Chemicals and Reagents. Commercial porphyrins, ALA, and porphobilinogen were obtained from Porphyrin Products (Logan, UT). Gases were purchased from Arundel Sales and Services (Baltimore). Organic solvents and other chemicals were obtained from J. T. Baker Chemical (Phillipsburg, NJ) or Sigma.

Bacteria Growth and Maintenance. Strain LO505 is a transposon Tn*5*-induced cytochrome-deficient mutant of parent strain LO; isolation, growth, and maintenance of both strains were described previously (10). Cells were generally grown in 1- or 2-liter cultures to late logarithmic phase. Bacteroids (cells grown symbiotically) were obtained and isolated from soybean root nodules as previously described (10).

Isolation and Identification of Porphyrins. Soluble porphyrins were isolated from the spent cell growth medium by passing it through a 5-ml column of neutral alumina activated with 3% acetic acid and then eluting the porphrins off the column with 1 M HCl or with CH_3OH/H_2SO_4 (20:1, vol/vol). Methyl esters of the isolated porphyrins were prepared as described (11). Porphyrins were identified by absorption spectra of the acid species in 1 M HCl and the neutral methyl ester species in chloroform and by TLC as described (11, 12). The isomer of the excreted coproporphyrin was determined by using the two-development paper chromatography system of Chu *et al.* (13). In our hands the R_f values of coproporphyrin I and III methyl esters were 0.39 and 0.79, respectively.

Enzyme Assays. Cell-free extracts were prepared as described (10) and used for measuring heme biosynthetic enzyme activities. ALA synthase and ALA dehydratase were assayed as described previously (14). The reaction mixtures were incubated for 2 hr at 30°C using ≈4 mg of crude extract protein per reaction. Oxygen-dependent coproporphyrinogen oxidase activity was measured as the extractable protoporphyrin formed from coproporphyrinogen after photooxidation as described (11); thus the sum of protoporphyrin and protoporphyrinogen formed was quantitated. Approximately 80 mg of crude extract protein in a 4-ml reaction mixture containing 1 mM EDTA, 5 mM dithiothreitol, 50 mM Tris (pH

Abbreviation: ALA, δ-aminolevulinic acid.

8), and 50 μM coproporphyrinogen was shaken in the dark at 180 revolutions per min in a 50-ml Teflon centrifuge tube at 30°C for 3 hr. The reaction was stopped by the addition of 20 ml of ethyl acetate/glacial acetic acid (3:1, vol/vol), which also served as an extraction step. Protoporphyrin was specifically extracted from the acid extract after photooxidation as described (11), and it was quantitated spectrophotometrically using an extinction coefficient (mM) of 275 at 408 nm. Coproporphyrinogen was synthesized from coproporphyrin by chemical reduction with 3% sodium amalgam as described (11). Protoporphyrinogen oxidase (protoporphyrinogen-IX:oxygen reductase, EC 1.3.3.4) activity was assayed by quantitating enzymatically formed protoporphyrin from coproporphyrinogen. The experimental conditions were identical to that of the coproporphyrinogen oxidase assay, except that the porphyrins were extracted into the ethyl acetate/acetic acid and subsequently extracted with 2.5 M HCl in the dark (under red light). The dark extraction excludes the porphyrinogens, and thus only enzymatically formed protoporphyrin was quantitated. The remaining extractions were done under normal light. Nonenzymatic protoporphyrin formation was measured in the cytosolic fraction of cell-free extracts, which does not have protoporphyrinogen oxidase activity; the nonenzymatic rate was subtracted from the total rate. Specific activities of coproporphyrinogen oxidase and protoporphyrinogen oxidase did not vary significantly when the protein concentration was varied from 25 to 100 mg. Whereas protoporphyrinogen oxidase activity did not vary significantly with the growth phase of the cells, coproporphyrinogen oxidase activity was higher in mutant strain LO505 cells harvested in the logarithmic phase of growth compared with those harvested at the stationary phase. Ferrochelatase activity was measured as described (5) by measuring mesoheme formed from mesoporphyrin and Fe^{+2} by 8–10 mg of crude extract protein. Nitrogen fixation in soybean root nodules was measured as ethylene formed from acetylene as described (10).

Quantitation of Heme. Heme was quantitated in nodule cytosol using the pyridine hemochromogen assay described by Bisseling *et al.* (15).

Immunologic Blots. Total nodule cytosol (4 μg per lane) was resolved by $NaDodSO_4$/PAGE (12% acrylamide) and stained for total protein (16) or probed with antileghemoglobin antibodies (17) as described. The antibodies were provided by G. Stacey (Dept. of Microbiology, University of Tennessee, Knoxville, TN).

Protein Assay. Protein concentration was quantitated by the dye-binding method of Bradford (18).

RESULTS

Bradyrhizobium japonicum strain LO505 is a Tn*5*-induced cytochrome mutant of the parent strain LO (10). The general cytochrome deficiency of this mutant was the initial reason for suspecting that it was a heme biosynthesis mutant. In the present study, strain LO505 was found to excrete the oxidized heme precursor coproporphyrin III into the liquid growth media, whereas strain LO did not do so under the same conditions (Fig. 1). Excretion of porphyrins is typical of bacterial heme mutants (ref. 19 and references therein). The absorption peaks at 621, 567.5, 532, 498, and 400 nm of the methyl ester of the isolated porphyrin are essentially identical to those reported in the literature for coproporphyrin (11), and the identification of coproporphyrin was confirmed by TLC of the porphyrin methyl ester. The spectrum of the acid species of the isolated porphyrin also confirmed it as coproporphyrin with absorption peaks at 590, 548, and 401 nm (data not shown). Furthermore, the coproporphyrin was identified as the III isomer by paper chromatography (as described in *Materials and Methods*), which is the physiological isomer in heme biosynthesis. The accumulation of coproporphyrin I would have indicated a lesion in uroporphyrinogen III cosynthase, which results in the production of uroporphyrin I as well (20).

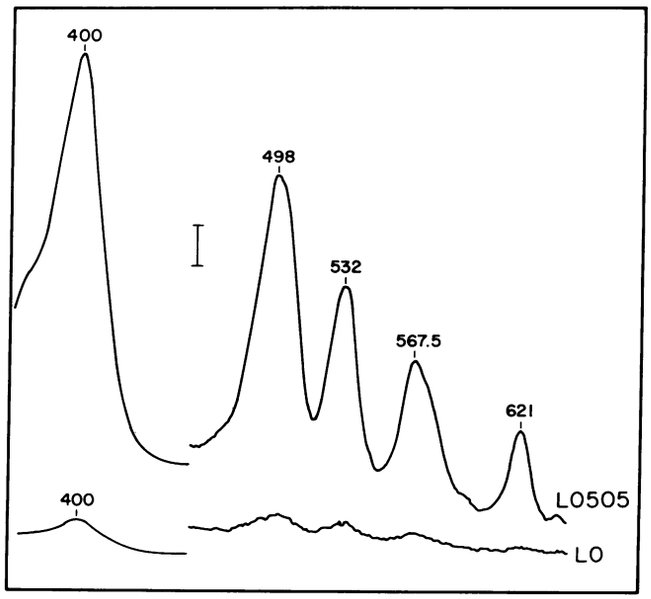

FIG. 1. Absorption spectra of soluble porphyrins excreted by strains LO and LO505. Spectra are the neutral methyl esters of the isolated porphyrins in chloroform. Vertical bar, change in *A* of 0.1 from 360–450 nm and of 0.01 from 450–650 nm.

Coproporphyrin accumulation has been seen in bacteria grown under iron deficiency (21). However, the addition of ferric citrate or $FeCl_3$-$6H_2O$ (4 mg/liter) did not restore the wild-type phenotype to mutant strain LO505, indicating that the mutant was not deficient in iron uptake or assimilation. In addition, Roessler and Nadler (22) found that an iron deficiency in *B. japonicum* cells represses ALA synthase and ALA dehydratase activities, whereas mutant strain LO505 had elevated activities of these two enzymes compared with the parent strain (Table 1).

The production of coproporphyrin III showed that mutant strain LO505 expressed all heme biosynthetic enzymes necessary for coproporphyrinogen III formation, and its accumulation in the medium indicated that the mutation occurred at some step after coproporphyrinogen III synthesis. Activities of the enzymatic steps subsequent to coproporphyrinogen III formation, and of ALA synthase and ALA dehydratase, were measured in extracts of strains LO and LO505 grown in culture (Table 1). Strain LO505 had no detectable protoporphyrinogen oxidase activity, whereas the other enzyme activities of the mutant were at least as great as those of the parent strain. Data show that all enzymes of

Table 1. Heme biosynthesis enzyme activities of strains LO and LO505 grown in culture

Enzyme	Activity	
	LO	LO505
ALA synthase	470	2,310
ALA dehydratase	2,470	3,840
Coproporphyrinogen oxidase	55	176
Protoporphyrinogen oxidase	16	<1
Ferrochelatase	2,140	1,940

Activities were measured in cell-free extracts from cells harvested during the logarithmic phase of growth. Data are expressed as pmol of product formed per hr per mg of protein and are an average of at least three trials, with the exception of coproporphyrinogen oxidase, which is the average of two trials.

the heme biosynthesis pathway are functional in mutant strain LO505, with the exception of protoporphyrinogen oxidase. Protoporphyrinogen oxidase catalyzes the oxidation of protoporphyrinogen to protoporphyrin; this reaction occurred nonenzymatically *in vitro* at 13% of the total rate in three experiments, which, if it can occur also *in situ*, may explain why the mutant expresses a small amount of cytochrome *b*, and can grow, albeit at a very slow rate (10).

A protoporphyrinogen oxidase mutant would possibly accumulate protoporphyrin rather than coproporphyrin resulting from the autooxidation of protoporphyrinogen. Although we found very little protoporphyrin in the growth media of mutant strain LO505, we did see extractable protoporphyrin (*ca.* 50 pmol/mg of protein) in stationary-phase cells of the mutant that was not found in the parent strain. Whether this protoporphyrin was present as the porphyrinogen or the porphyrin in those cells is not known because no precautions were taken to prevent autooxidation during the extraction.

Mutant strain LO505 expressed elevated levels of ALA synthase, ALA dehydratase, and coproporphyrinogen oxidase (Table 1), indicating that protoporphyrin or heme may be a negative regulator of these enzymes in wild-type cells. There is evidence that heme is a regulator of heme biosynthesis enzymes in several systems (23–25), but the enzyme under control differs with the organism.

The effect of the protoporphyrinogen oxidase mutation on symbiosis is shown in Table 2. Small root nodules were formed from mutant strain LO505 and, like the cultured cells, bacteroids isolated from these nodules lacked protoporphyrinogen oxidase activity. Most interestingly, LO505 nodules contained no detectable leghemoglobin in the nodule cytosol, as discerned by the pyridine hemochromogen assay. The latter result shows that bacterial heme synthesis is required for leghemoglobin formation in soybean root nodules.

Because the pyridine hemochromogen assay measured only nodule cytosol heme, it was necessary to establish whether nodules contained the leghemoglobin apoprotein. Although strain LO505 nodules lacked leghemoglobin, the apoprotein could indeed be discerned antigenically (Fig. 2*B*). The presence of the leghemoglobin apoprotein in strain LO505 nodule cytosol was detected from an immunoblot of a $NaDodSO_4$/polyacrylamide gel probed with antileghemoglobin antibodies (Fig. 2*B*). Apoleghemoglobin was also visible on an identical gel stained for total protein as a major, low M_r band (Fig. 2*A*). Results show that the heme biosynthesis mutation in *B. japonicum* strain LO505 did not result in general symbiotic anomalies because soybeans inoculated with this mutant formed nodules and synthesized the leghemoglobin apoprotein. The leghemoglobin heme, therefore, is bacterial in origin, and bacterial heme must be synthesized

Table 2. Symbiotic properties of soybeans nodulated with strains LO and LO505

Property	Strain	
	LO	LO505
Nodules/plant, no.	41 ± 7	30 ± 6
Nodule weight/plant, g	0.67 ± 0.07	0.16 ± 0.01
Nitrogenase activity, μmol of ethylene formed/hr per g of nodules	8.9 ± 0.7	<0.2
Protoporphyrinogen oxidase activity, pmol/hr per mg of protein	18 ± 2	<1
Nodule cytosol heme, nmol/g of nodules	154 ± 7	<5

Data are presented as the average number ± the SD of at least four trials, except for protoporphyrinogen oxidase activity, which is the average of three trials. Properties were quantitated as described in *Materials and Methods*.

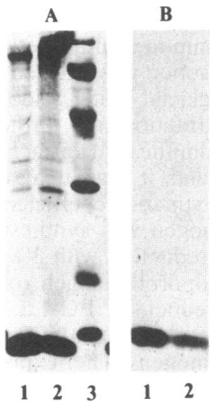

FIG. 2. Immunological detection of leghemoglobin apoprotein in soybean nodule cytosol from strains LO and LO505. (*A*) $NaDodSO_4$/PAGE: the gel was stained for total protein with silver stain. (*B*) Protein from a duplicate gel was electroblotted onto nitrocellulose and probed with antileghemoglobin antibodies. Cross-reactive material was visualized as described (17). Lanes: 1, strain LO; 2, strain LO505; 3, protein standards of 92.5, 67, 45, 30, 21.5, and 14.4 kDa in size.

for the formation of intact leghemoglobin. Furthermore, the data show that the heme moiety is not an essential prerequisite for apoleghemoglobin synthesis by the soybean host, contrary to what is generally assumed (e.g., ref. 1). Less leghemoglobin apoprotein was found in nodules of strain LO505 compared with those of the parent strain, which could be due to a diminished level of synthesis by the plant, or the apoprotein may be less stable than the holoprotein. Soybean nodules containing the leghemoglobin apoprotein, but not the heme, have been observed previously (26), but the nature of the bacterial mutation in that case is not known.

DISCUSSION

Bradyrhizobium japonicum strain LO505 was shown to be a heme biosynthesis mutant, with a lesion in protoporphyrinogen oxidase activity. No other heme enzyme deficiencies were seen; thus strain LO505 is not likely to be mutated in a regulatory gene or in an unrelated gene that incidentally affects heme synthesis.

Data show that bacterial heme synthesis is required for leghemoglobin expression in *B. japonicum*/soybean symbiosis. The lesion in mutant strain LO505 causing a deficiency in protoporphyrinogen oxidase activity resulted in soybean root nodules that lack the heme moiety of leghemoglobin but that do contain the apoprotein. The present findings do not necessarily conflict with those of Guerinot and Chelm (9), who found leghemoglobin in nodules from an ALA synthase mutant of *B. japonicum*. Possibly the plant symbiont can rescue the ALA synthase mutant by providing the bacteria with ALA, but it cannot complement a protoporphyrinogen oxidase mutant, which would require the transport of protoporphyrin across the bacterial and peribacteroid membranes. The presence of nitrogenase activity in the *B. japonicum* ALA synthase mutant (9) strongly indicates that cytochromes are expressed symbiotically in that mutant, and therefore synthesis of bacterial heme must occur. In the present study, cross-feeding of early precursors from the soybean to the bacteria, if it does indeed occur, would be inconsequential because strain LO505 was deficient in a late step of heme biosynthesis.

Studies of heme biosynthesis in the rhizobia raise questions about how the plant and bacterium interact and regulate each other at the molecular level. It has been suggested (e.g., ref. 1) that heme induces leghemoglobin apoprotein synthesis by

the plant. However, our data and those of Noel *et al.* (26) show that apoleghemoglobin can be found in nodules where heme is absent; thus regulation of apoprotein synthesis by *B. japonicum* cannot be exclusively at the level of heme. This does not rule out a regulatory role for a heme precursor, however, because mutant strain LO505 could synthesize porphyrins and other heme intermediates. Differences in soybean and alfalfa leghemoglobin expression resulting from the same ALA synthase gene mutation (7, 9) emphasize that the molecular basis of plant/bacteria interactions and regulation probably differ among the legume symbioses. How other heme biosynthesis mutations affect leghemoglobin expression in legume root nodules will be interesting to observe.

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