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Effects of Iron Deficiency on Heme Biosynthesis in *Rhizobium japonicum*

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The effects of iron deficiency on heme biosynthesis in *Rhizobium japonicum* were examined. Iron-deficient cells had a decreased maximum cell yield and a decreased cytochrome content and excreted protoporphyrin into the growth medium. The activities of the first two enzymes of heme biosynthesis, δ-aminolevulinic acid synthase (EC 2.3.1.37) and δ-aminolevulinic acid dehydrase (EC 4.2.1.24), were diminished in iron-deficient cells, but were returned to normal levels upon addition of iron to the cultures. The addition of iron salts, iron chelators, hemin, or protoporphyrin to cell-free extracts did not affect the activity of these enzymes. The addition of levulinic acid to iron-deficient cultures blocked protoporphyrin excretion and also resulted in high δ-aminolevulinic acid synthase and δ-aminolevulinic acid dehydrase activities. These results suggest the possibility that rhizobial heme synthesis in the legume root nodule may be affected by the release of iron from the host plant to the bacteroids.

Leghemoglobins, the hemoproteins required for nitrogen fixation in the legume root nodule, are unique products of the *Rhizobium*-legume symbiosis. The heme is produced by the *Rhizobium* bacteroids (8, 13, 18), whereas the apoprotein is produced by the host plant (9, 11, 25). During the latter stages of nodule formation, leghemoglobins are produced in such large amounts that they comprise >20% of the total soluble protein of the nodule, equivalent to 100 to 200 nmol of heme per g (wet weight) (18). By comparison, laboratory-cultured rhizobia contain approximately 10 nmol of heme per g (wet weight). It is thus evident that a large increase in rhizobial heme synthesis occurs during nodulation. However, the mechanisms involved in the regulation of heme biosynthesis in *Rhizobium* have not been characterized to any great extent.

The regulation of rhizobial heme synthesis might occur via the transfer of a regulatory compound from the host plant to the bacteroids. Several reports have suggested that iron may play a regulatory role in heme biosynthesis in certain organisms. The first enzyme unique to the heme biosynthetic pathway, δ-aminolevulinic acid synthase (ALAS), has been reported to have increased activity in the presence of iron in animals (3, 26), plants (16), and microorganisms (23). In addition, the second enzyme of the pathway, δ-aminolevulinic acid dehydrase (ALAD), has been shown to have decreased activity in *Neurospora* grown under iron-limiting conditions (5). There have also been several reports suggesting that iron is required at the step where coproporphyrinogen is converted to protoporphyrin (PROTO) in certain organisms (14, 22). In this investigation, we have grown *Rhizobium japonicum* cells under iron-limiting conditions to examine the possibility that iron released by the host plant may play a regulatory role in heme biosynthesis in *R. japonicum*.

MATERIALS AND METHODS

Media and growth conditions. The chemicals used for media preparation were chosen from readily available sources for having low amounts of iron contamination. In addition, all culture flasks were soaked overnight in 6 N HCl to remove contaminating iron.

Low-iron (LI) medium contained (in grams per liter of distilled, deionized water): mannitol, 2; $Na_2HPO_4 \cdot 7H_2O$, 1.3; $NaH_2PO_4 \cdot H_2O$, 0.1; $MgSO_4 \cdot 7H_2O$, 0.05; NaCl, 0.5; $(NH_4)_2SO_4$, 1; $Ca(NO_3)_2 \cdot 4H_2O$, 0.02; biotin (Sigma Chemical Co.), 0.0025; and Casamino Acids (Difco Laboratories), 1. High-iron (HI) medium was obtained by adding 4 mg of ferric citrate (Baker Chemical Co.; 16% iron by assay) per liter of LI medium. Yeast extract-mannitol medium has been described previously (1).

The concentration of iron in LI medium was determined with a Varian model AA6 atomic absorption spectrophotometer with carbon rod attachment CRA-90. LI medium contained 14 ng of iron per ml, corresponding to a concentration of 0.25 μmol of iron per liter. HI medium contained 11.7 μmol of iron per liter.

R. japonicum 3I1b-110 was kindly supplied by D. C. Weber, U.S. Department of Agriculture, Beltsville, Md. The cultures were maintained on yeast extract-mannitol slants and were routinely tested for nodulation ability on soybeans (*Glycine max* var. Hark) as

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previously described (18). Broth cultures (500 ml) for experimental assay were grown in 1-liter Erlenmeyer flasks at 30°C on an orbital shaker (120 rpm). Cells were routinely harvested at early stationary phase. Viable cell counts were made by serial dilution of culture samples into sterile 0.7% NaCl which were then spread out on yeast extract-mannitol plates.

Enzyme assays. Cell-free extracts were prepared by sonication as previously described (1), and results are presented as the average value of two or more experiments, with the range of variation indicated.

(i) ALAS. δ-Aminolevulinic acid production in cell-free extracts was measured by a modification of the method of Mauzerall and Granick (17). The reaction mixture contained (in millimoles per liter): HEPES (*N*-α-hydroxyethylpiperazine-*N'*-2-ethane-sulfonic acid) buffer (pH 8.0), 100; $MgCl_2$, 16.5; Na_2-succinate, 100; glycine, 100; ATP, 7; coenzyme A, 0.05; pyridoxal phosphate, 0.3; 2-mercaptoethanol, 0.5; and the cell-free extract to give a final protein concentration of between 0.5 and 1.0 mg/ml. After incubation for 2 h at 30°C, the reaction was stopped by placing the tubes on ice and adding trichloroacetic acid to a final concentration of 5%. After the precipitated protein was removed by centrifugation, 1 ml of the supernatant solution was combined with 0.25 ml of 0.65 M Na_3PO_4 and 0.1 ml of ethylacetoacetate and placed in a boiling-water bath for 15 min. After cooling, 1.35 ml of modified Ehrlich reagent (24) was added to each tube, and the resulting color complex was analyzed after 15 min in a spectrophotometer (Gilson model 240) at a wavelength of 553 nm. The molar extinction coefficient of the δ-aminolevulinic acid pyrrole-Ehrlich complex at 553 nm was determined to be 6.2×10^4. A unit of ALAS activity is defined as 1 nmol of δ-aminolevulinic acid formed per h.

(ii) ALAD. Porphobilinogen production in cell-free extracts was measured colorimetrically, using modified Ehrlich reagent as previously described (18) except that HEPES buffer (pH 7.5) was used in place of Tris-hydrochloride buffer. The molar extinction coefficient of the porphobilinogen-Ehrlich complex at 555 nm was determined to be 5.0×10^4. A unit of ALAD activity is defined as 1 nmol of porphobilinogen formed per h.

(iii) Succinic thiokinase. Succinic thiokinase activity was estimated by the detection of succinic hydroxamate generated from succinyl-coenzyme A as previously described (1). A unit of succinic thiokinase activity is defined as 1 μmol of succinyl-coenzyme A formed per h.

Analytical methods. (i) Cytochromes. Difference spectra (sodium dithionite–reduced minus ferricyanide–oxidized) were used to detect cytochromes in cell-free extracts obtained by sonicating cells in 0.1 M sodium phosphate buffer (pH 7.0). Extracts were clarified by centrifugation at 30,000 × *g* for 15 min, and the protein concentration was adjusted to 3.0 mg/ml. Difference spectra were recorded with a Cary 15 recording spectrophotometer.

(ii) Porphyrins. Porphyrins excreted by *R. japonicum* cells were insoluble in the culture medium and adhered either to the sides of the culture flask or to the cells. No porphyrins were detected in the supernatant fluid after pelleting the cells. Porphyrin excretion was followed fluorometrically with a Turner model 111 fluorometer (excitation filter, λ = 405 nm; secondary filter, λ = >570 nm). Samples (5 ml) were periodically removed from cultures, mixed with 1 ml of concentrated HCl, and placed in the dark at 4°C for fluorometric determination after centrifugation at 30,000 × *g* for 15 min.

Rinsing the sides of the culture flask with 5 ml of 1 N HCl followed by centrifugation at 30,000 × *g* for 20 min provided a sample suitable for spectrophotometric analysis of the excreted porphyrin in acidic solution. Absorption spectra were obtained with a Cary 15 recording spectrophotometer.

The methyl ester of the excreted porphyrin was obtained by rinsing the sides of the culture flask with a methanol-sulfuric acid solution (20:1, vol/vol). After this solution was allowed to remain in the dark overnight, the porphyrin methyl ester was purified by the method of Doss and Ulshofer (12). The absorption spectrum of the porphyrin methyl ester at neutral pH could then be obtained. Additional analysis of the excreted porphyrin along with porphyrin methyl ester standards (obtained from Sigma Chemical Co.) was accomplished by thin-layer chromatography in the three different systems described below.

(iii) Protein. The concentration of protein in extracts was measured by the method of Bradford (2), using bovine gamma -globulin as a standard.

RESULTS

Effects of iron deficiency on growth rate. The initial growth rates of cells in low- and high-iron media were identical, but after five generations the growth rate of cells in LI medium rapidly decreased (Fig. 1), leading to a short stationary

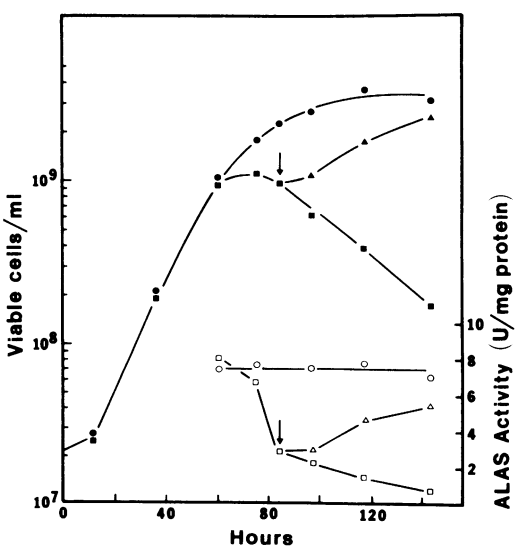

FIG. 1. ALAS activity and growth of *R. japonicum* in HI and LI media. ALAS activity was measured as described in the text. Closed symbols refer to cell counts; open symbols refer to ALAS activity. Symbols: (●, ○) cells grown in HI medium; (■, □) cells grown in LI medium; (▲, △) cells grown in LI medium to which ferric citrate (4 mg/liter) had been added at the point indicated by the arrow.

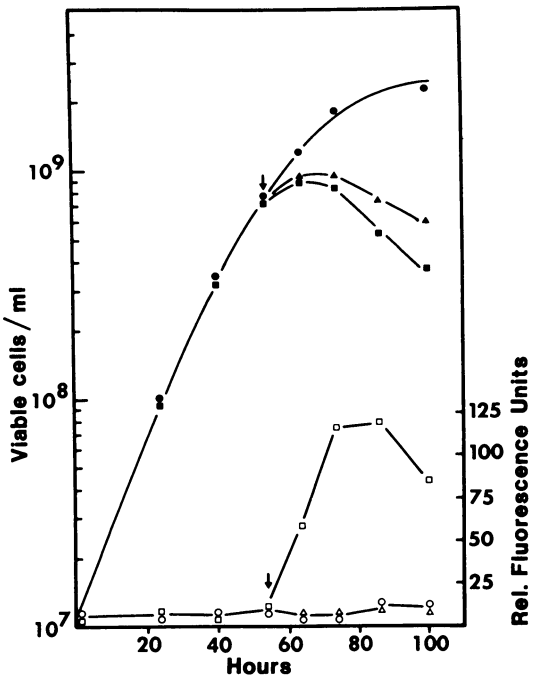

FIG. 2. Porphyrin fluorescence of *R. japonicum* cultures grown in HI and LI media. Cultures grown in HI and LI media were periodically measured for growth and fluorescence due to porphyrin accumulation. Closed symbols represent cell counts; open symbols represent relative fluorescence units. Symbols: (●, ○) cells grown in HI medium; (■, □) cells grown in LI medium; (▲, △) cells grown in LI medium to which 10 mM LA had been added at the point indicated by the arrow. Twenty-five relative fluorescence units are equivalent to 10 pmol of PROTO per ml.

phase which was followed by a decline in cell viability. The addition of iron to stationary-phase cultures in LI medium resulted in a resumption of cell growth. The reason for the decline in cell viability is unclear; perhaps the iron released from lysed cells is unavailable for new cell growth.

Effect of iron deficiency on cytochrome content. The most prominent cytochrome observed in cell-free extracts of *R. japonicum* was a type *c* cytochrome with an α peak at 552 nm. The level of this cytochrome in extracts from cells grown in HI medium was found to be three times the level in extracts from iron-deficient cells.

Effect of iron deficiency on porphyrin excretion. Porphyrin excretion was observed with *R. japonicum* cells grown in LI medium but not with cells grown in HI medium (Fig. 2). There was no fluorescence due to porphyrins above background levels in cultures grown in LI medium until the cells entered an iron-limited stationary phase, at which time a large increase in fluorescence could be observed.

The excreted porphyrin was identified as PROTO based on the absorption spectrum of the excreted porphyrin in 1 N HCl (absorbance maxima at 408, 556, and 600 nm) and of the porphyrin methyl ester in chloroform at neutral pH (absorbance maxima at 408, 508, 541, 577, and 632 nm). In addition, the methyl ester of this porphyrin co-chromatographed with authentic protoporphyrin IX dimethyl ester in three thin-layer chromatography systems (Table 1).

Effects of iron deficiency on heme biosynthesis. The activity of ALAS, the first and rate-limiting enzyme of the heme biosynthetic pathway, was shown to decrease rapidly as cells grown in LI medium entered the iron-limited stage of growth (Fig. 1). This decrease in ALAS activity preceded the decrease in cell viability. The addition of iron to such cultures led to a restoration of ALAS activity as well as a resumption of growth. ALAS activity of cells grown in HI medium remained high throughout the growth cycle. It is clear from simple replacement experiments that iron is the essential component for the high ALAS activity of HI medium-grown cells; cells grown with high concentrations of other iron salts such as $FeCl_3$ have high ALAS activity (10.1 ± 1.3 U/mg of protein), whereas cells grown with equimolar levels of sodium citrate or salts of other transition metals (e.g., $CoCl_2$, $CuSO_4$, and $ZnSO_4$) have low ALAS activity (2.9 ± 1.7 U/mg of protein). Similar results were observed with cells grown in a complex medium (yeast extract-mannitol). When cell-free extracts of iron-replete cells were mixed with those from iron-deficient cells, the ALAS activity was the average of the two activities (data not shown).

TABLE 1. Chromatographic properties of the methyl ester of the porphyrin excreted by *R. japonicum* grown in LI medium

System[a]	R_f value			
	URO I + III standard[b]	COPRO III standard[b]	PROTO IX standard	Excreted porphyrin
I	0.13	0.34	0.49	0.49
II	0.18	0.40	0.48	0.48
III	0.93	0.68	0.38	0.38

[a] I, Benzene-ethyl acetate-ethanol (190:20:7.5, vol/vol/vol) on Brinkmann Silica Gel G thin-layer chromatography plates (12); II, petroleum ether-paraffin oil-chloroform (1:1:10, vol/vol/vol) on Eastman chromagram sheets (6); III, water–acetonitrile–*p*-dioxane (2:7:1, vol/vol/vol) on Whatman KC_{18} reverse-phase thin-layer chromatography plates (solvent contained 0.5 M NaCl to stabilize the hydrocarbon chains).

[b] URO, Uroporphyrin; COPRO, coproporphyrin.

The activity of ALAD, the second enzyme of the heme biosynthetic pathway, was decreased by approximately the same degree as ALAS when the cells were subjected to iron limitation (Table 2). On the other hand, the activity of succinic thiokinase, the enzyme generating succinyl-coenzyme A (a substrate for ALAS), was not decreased in iron-limited cells. In fact, there was a slight increase in the activity of this enzyme.

We could not demonstrate activation of ALAS or ALAD by addition of $FeCl_3$ or $FeSO_4$ (up to 1 mM) to cell-free extracts of iron-deficient cells. Conversely, the addition of desferal (a ferric iron chelator) and α,α'-dipyridyl (a ferrous iron chelator) at high concentrations (1 mM) did not decrease the high ALAS or ALAD activities in extracts from iron-replete cells (data not shown). Since iron-deficient cells have little iron available for conversion of PROTO to heme, a possible explanation for the decreased ALAS and ALAD activities might involve enzyme activation by heme. However, the addition of hemin (200 μM) to extracts from iron-deficient cells did not increase the activity of ALAS or ALAD (data not shown). Similarly, the addition of hemin (200 μM) was without effect on the high ALAS and ALAD activities of extracts from iron-replete cells, suggesting that hemin does not feedback-inhibit these enzymes.

Since PROTO is accumulated by iron-deficient cells, we also examined the possibility that PROTO inhibits ALAS activity. However, addition of PROTO (200 μM) did not diminish the high ALAS activity in cell-free extracts of iron-replete cells (data not shown). We could not determine the effect of PROTO on ALAD activity since this porphyrin interfered with the colorimetric determination of porphobilinogen.

Levulinic acid (LA), a competitive inhibitor of ALAD (19), blocks PROTO excretion by cells growing in LI medium (Fig. 2). The addition of LA (10 mM) to cells growing in LI medium resulted in ALAS activity about twice as high as that of cells grown in LI medium in the absence of LA (2.6 ± 0.2 and 5.1 ± 0.4 U/mg of protein, respectively, without and with LA). ALAD was similarly affected (1.5 ± 0.2 and 2.9 ± 0.3 U/mg of protein). The LA was added shortly before visible porphyrin excretion normally occurred, and enzyme activities were measured 18 h after this addition. The addition of LA directly to the reaction mixture did not affect ALAS activity.

TABLE 2. Activities of enzymes of heme biosynthesis in cell-free extracts of *R. japonicum* grown in HI and LI media

Growth medium	U/mg of protein		
	STK activity[a]	ALAS activity	ALAD activity
LI		4.1 ± 0.9	2.1 ± 0.2
HI		8.9 ± 1.6	6.9 ± 1.0
LI	35.2 ± 7.8	2.8 ± 0.1	
HI	28.6 ± 7.8	9.8 ± 1.3	

[a] STK, Succinic thiokinase.

DISCUSSION

R. japonicum cells growing in LI medium underwent several changes that are characteristic of iron-limited growth. There was a decreased maximum cell yield, a decrease in the cytochrome content of the cells, and excretion of PROTO.

Porphyrin excretion by microorganisms growing under iron-deficient conditions is a common phenomenon (15). The excreted porphyrin is often coproporphyrin, which has led many investigators to suggest that iron is required for the conversion of coproporphyrinogen to PROTO (14, 22). However, it is apparent from our results that iron is not required for coproporphyrinogen oxidation in *R. japonicum*.

The data presented here suggest that the iron supply of the cell affects heme biosynthesis in *R. japonicum*. It appears that high ALAS and ALAD activities are maintained only when sufficient levels of iron are available to the cell. It does not appear that decreased ALAS and ALAD activities are a result of cell death, since (i) the activities begin to decline before cell death is first observed and (ii) the activity of succinic thiokinase is not diminished in iron-deficient cells.

Brown (3) reported that iron was required for maximal rates of δ-aminolevulinic acid formation in chick erythrocyte preparations and suggested that this was due to increased catalytic efficiency of pyridoxal phosphate. In contrast, we found that iron chelators did not affect ALAS or ALAD activity in cell-free extracts of *R. japonicum*, suggesting that iron does not play a direct role in the activity of these enzymes. This finding is supported by the fact that the addition of iron to extracts from iron-deficient cells did not lead to enhanced ALAS or ALAD activities, although it is possible that the iron needs to be metabolized to some other form before having an enhancing effect.

We were not able to demonstrate the presence of activators or inhibitors of ALAS in *R. japonicum* by the use of mixing experiments with cell-free extracts from iron-deficient and iron-replete cells. Similarly, Tait (23) reported that growth of *Micrococcus* in different media resulted in varying ALAS activities, but mixing experiments did not indicate the presence of activators or inhibitors. On the other hand, studies with *Rhodo-*

pseudomonas have indicated that thiol-containing compounds are able to activate ALAS in this organism (20, 21).

We observed no inhibition by hemin or PROTO of ALAS activity in cell-free extracts. This is somewhat surprising when one considers that feedback inhibition by these compounds is a rather common phenomenon. For example, ALAS activity in *Rhodopseudomonas spheroides* is 50% inhibited by a concentration of hemin between 1 and 10 μM or by PROTO at 10 times this concentration (4). The same degree of inhibition of ALAS in *Spirillum itersonii* occurs with 10 μM heme (7). In addition, it should be noted that there has been a report that heme biosynthesis in *R. japonicum* is 50% inhibited by 100 μM heme at a step subsequent to ALAS (10). Experiments which examined the possibility of feedback inhibition of ALAD by PROTO were inconclusive due to the interference by PROTO with the colorimetric reaction involving porphobilinogen and Ehrlich reagent.

The addition of LA to iron-deficient cultures prevented PROTO excretion and also resulted in increased ALAS and ALAD activities. This suggests the possibility that PROTO (or heme) may be able to repress the formation of these enzymes. However, the decreased rate of heme biosynthesis in LA-containing cultures results in a decreased utilization of cellular free iron, thus increasing the levels of available cellular iron. Therefore, the cultures containing LA may not be truly iron deficient.

The results of this study raise the possibility that rhizobial heme synthesis in the legume root nodule may be affected to some degree by the release of iron from the host plant to the bacteroids. It is not clear whether iron (or some metabolite reflecting the iron supply of the cell) acts as a direct modulator of heme synthesis or whether the decreased ALAS and ALAD activities are a result of altered general metabolism in iron-deficient cells. However, from the results of this study it does not appear that iron, PROTO, or heme directly affects the activities of these enzymes.

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