Plant and Soil 100, 225–236 (1987). Ms. 100-30

Total and CO-reactive heme content of actinorhizal nodules and the roots of some non-nodulated plants

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Key words Actinorhizae Alnus Casuarina Frankia Hemoglobin Myrica Nitrogen fixation Root nodules

Summary The concentration of total and CO-reactive heme was measured in actinorhizal nodules from six different genera. This gave the upper limit to hemoglobin concentration in these nodules. Quantitative extraction of CO-reactive heme was achieved under anaerobic conditions in a buffer equilibrated with CO and containing Triton X-100. The concentration of CO-reactive heme in nodules of Casuarina and Myrica was approximately half of that found in legume nodules, whereas in Comptonia, Alnus and Ceanothus the concentrations of heme were about 10 times lower than in legume nodules. There was no detectable CO-reactive heme in Datisca nodules, but low concentrations were detected in roots of all non-nodulating plants examined, including *Zea mays*. Difference spectra of CO treated minus dithionite-reduced extracts displayed similar wavelengths of maximal and minimal light absorption for all extracts, and were consistent with those of a hemoglobin. The concentration of CO-reactive heme was not correlated to the degree to which CO inhibited nitrogenase activity nor was it affected by reducing the oxygen concentration in the rooting zone. However, there was a positive correlation between heme concentration and suberization or lignification of the walls of infected host cells. These observations demonstrate that, unlike legume nodules, high concentrations of heme or hemoglobin are not needed for active nitrogen fixation in most actinorhizal nodules. Nonetheless, a significant amount of CO-reactive heme is found in the nodules of Alnus, Comptonia, and Ceanothus, and in the roots of *Zea mays*. The identity and function of this heme is unknown.

Introduction

Until recently, hemoglobins were not thought to occur in higher plants, except in the root nodules of legumes. However, hemoglobins have now been purified from the root nodules of two different nonlegumes, and there is spectroscopic evidence for hemoglobin in the nodules of additional nonlegumes[3,12,26,29]. Moreover, using cloned leghemoglobin cDNA as a probe, there is evidence for cross-hybridizing sequences in genomic DNA from a wide variety of plants, including plants that do not form root nodules[14,20,21,23,24]. All of the above suggests that the gene for hemoglobin is widespread in the plant kingdom. Hemoglobins have also been found in bacteria[1,30].

In the present work we have measured the concentration of total heme and CO-reactive heme in selected actinorhizal root nodules and in roots

of non-nodulating plants as a first step in investigating the function of hemoglobin in nonlegumes. Nodule properties that might be related to hemoglobin function were also studied. With a single exception, we found that soluble, CO-reactive heme was a substantial fraction of total heme in all nodules and roots investigated. However, high concentrations of heme were found in only two of the six genera of actinorhizal plants studied.

Materials and methods

Plant growth

All nodulated plants were grown on vermiculite and were watered twice weekly with a one-fourth strength -N Hoagland's solution and with distilled water on other days. Between the time of seed germination and nodule development, the -N solution was supplemented with 1 m*M* urea. The potted plants were kept in a growth chamber at a constant temperature of 23°C, relative humidity of 70%, light intensity of 300 to 400 μmol $m^{-2} s^{-2}$ (400–700 nm), and photoperiod of 17 h. Casuarina was inoculated with Frankia strain HFPCcI3[32], Alnus with strain HFPArI3[5],Myrica with strain LLR161101, Datisca and Ceanothus with crushed nodules of *Ceanothus americanus*, and Comptonia was inoculated with crushed nodules of Comptonia that were collected in the town of Orono. The crushed nodules of Ceanothus were from plants whose original inoculum was soil and nodules from a Ceanothus site in Massachusetts. Plants were used in experiments at two to three months after inoculation. Roots of *Zea mays* were harvested eight days after planting the seeds in a flat of vermiculite.

Acetylene reduction assays

Nitrogenase activity was measured using the acetylene reduction assay[8,13]. Intact potted plants (7.5 cm diameter pots) were placed in 1000-ml tall form beakers that were sealed with a plastic lid and modeling clay. Acetylene was added to the jars to give a mixture of 10% acetylene and 90% air. The jars were incubated at 23°C in the dark, and gas samples were taken at 15, 30, and 45 min after acetylene addition. The concentration of acetylene and ethylene was measured using a gas chromatograph equipped with a flame ionization detector. The rate of acetylene reduction was constant as a function of time.

The effect of CO on nitrogenase activity was measured using the acetylene reduction assay as described above. After a 60-min acetylene reduction assay in the absence of CO, CO was injected into the incubation jars, and the assay was continued for an additional 60 min. In control jars to which no CO was added, the acetylene reduction rate was usually constant during the 2-h assay. Inhibition by CO was calculated from the acetylene reduction rate for individual plants before and after the addition of CO. If there was any change in acetylene reduction rate by the control jars during the assay, this was taken into account in the calculations.

Extraction of total and CO-reactive heme

CO-reactive heme was extracted from root nodules using a modification of the method of Appleby[3]. The extraction buffer (0.1 *M* potassium phosphate, 1 m*M* EDTA, 1% Triton X-100, pH 7.4) was equilibrated with CO before use and 5 ml was added to between 50 and 1000 mg of nodules placed in a 15 ml glass centrifuge tube (Corex No. 8441). Then 10 mg of sodium dithionite was added and the tube was sealed onto a Biospec homogenizer (No. 1281-0, 10,000 rpm; 1/2 inch stator) using a rubber stopper. The headspace was flushed with argon or nitrogen gas for 60 s, then the homogenizer was run at room temperature for 40 s. The tube was then removed from the homogenizer, stoppered, flushed with argon or nitrogen, and centrifuged at 4500 g for 30 min at 20°C. The supernatant was analyzed for heme content.

Roots were extracted in the same way, except that 0.2 g of insoluble polyvinylpolypyrrolidone

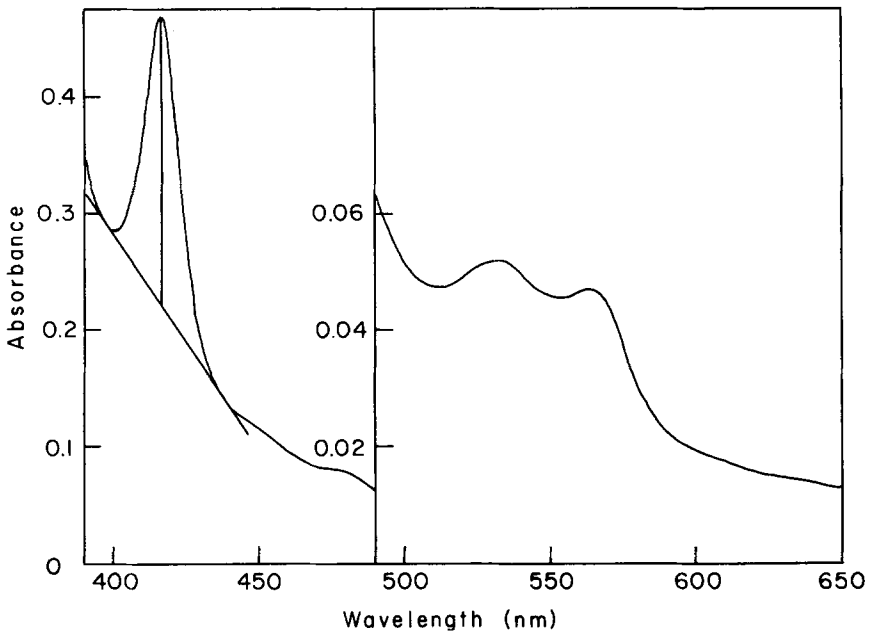

Fig. 1. Absorption spectrum of a CO-equilibrated extract from the nodules of *Myrica gale*. The vertical line at 416 nm is the value of A from which the concentration of CO-reactive heme was calculated.

(Sigma No. P-6755) was added to each tube after homogenization. This reduced background absorbance in the 420 nm region that interfered with quantification of the CO-reactive heme.

Total heme was extracted in the same way as CO-reactive heme, except that 2 ml of pyridine was added to the 5 ml of homogenate immediately after the homogenization was completed. This method was derived from that of Davenport[10].

Spectroscopic methods

Spectra were recorded using a Bausch and Lomb Spectronic 2000 spectrophotometer of 2 nm slit width, set at a 100 nm min^{-1} scan speed. Wavelength and absorbance accuracy were checked with neutral density and holmium oxide filters (Thomas Scientific) and agreed with the factory settings. Samples of about 1.0 ml were measured in semi-micro cuvettes.

For difference spectra, CO was omitted from the extraction buffer, and extracts were added to both sample and reference cells. The sample cell was then gently bubbled with CO.

The concentration of CO-reactive heme was calculated by drawing a "baseline" between the minima at 400 and 435–445 nm on either side of the Soret absorption peak as indicated in Fig. 1. The ΔA was then measured from the baseline to the absorption peak at 416 to 420 nm. To calculate the concentration of CO-reactive heme we assumed a $\Delta E(mM)$ value of 180. This value (C.A. Appleby, personal communication) was determined from pure Parasponia carboxyhemoglobin and is representative of similar hemoglobins from a variety of sources.

Total heme concentration was calculated in a manner similar to that used for CO-reactive heme. The "baseline" was drawn from the minima at about 538 and 573 nm on either side of the alpha peak of pyridine proto hemochrome (see Fig. 2). To calculate total heme concentration, we derived a ΔE (mM) value of 28.7 from an enlargement of Fig. 1 of Paul *et al.*[19].

Growth of plants at 5 kPa O_2

After initiation of root nodules and growth on vermiculite, seedlings (0.5 to 1.6 g fresh weight)

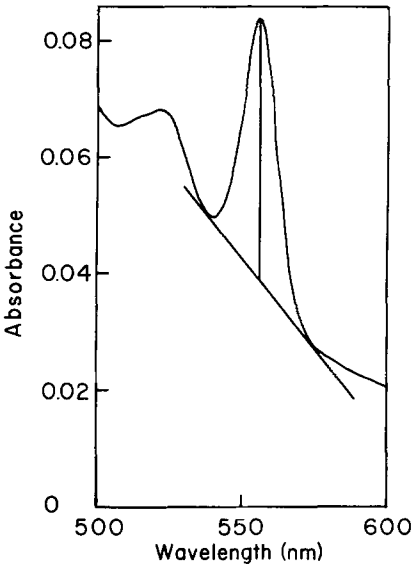

Fig. 2. Absorption spectrum of a pyridine-treated extract from nodules of *Myrica gale*. The vertical line at 556 nm is the value of A from which the concentration of total heme was calculated.

were transferred to water cultures (1/4 strength -N Hoagland's solution). Plastic containers (20 cm diameter, 20 cm high) with snap-on lids were used, with all six plants of a given treatment being placed in the same container. The plants were sealed into split rubber stoppers by wrapping the stems with thin strips of closed cell foam. The stoppers were inserted in holes in the lid, and the cultures were continuously bubbled with either air or a mixture of 95% N_2, 5% O_2, and 0.04% CO_2. A fritted disk was used to disperse the gas and the gas exiting from the cultures bubbled with 5% O_2 was collected for analysis by gas chromatography[31]. Both entering and exiting gas was 5.0% O_2, while entering gas was 0.041% CO_2, and exiting gas was 0.074% CO_2. The gas flow rate was 30 ml min^{-1} or more. The exterior of the containers was covered with aluminum foil to prevent the growth of algae. The water level was kept high, to minimize gas leakage at the seal between the rubber stoppers and plant stems. Seedlings of *Myrica gale* were grown in the water cultures for 14 days, while *Alnus rubra* seedlings were grown for 20 days in the cultures.

Tests for suberin and lignin

Autofluorescence was observed by the method of Berg[4], except that 350–500 nm excitation and 550 nm barrier filters were used. Phloroglucinol staining was as described by Berg, while chromic acid digestion was only 1 day instead of the 4 days used by Berg.

Results and discussion

Extraction of CO-reactive heme

In our initial experiments[26], soluble polyvinylpyrrolidone (PVP) was used instead of Triton X-100 in the extraction buffer. We discontinued use of PVP because it resulted in extracts that absorbed light more strongly at the shorter wavelengths and thus interfered with the assay for CO-reactive heme. However, both methods yielded about the same

Table 1. Total heme, CO-reactive heme, and acetylene reduction rates in actinorhizal nodules, nodules of Lupinus, and roots of *Zea mays*. (Means ± SE, n = 3 to 6)

Species	Total heme	CO-reactive heme	Acetylene reduction rate
	nmol g^{-1} (fresh wt)		μmol h^{-1} g^{-1} (fresh weight)
Lupinus albus (legume)	197 ± 11	174 ± 0.4	–
Casuarina cunninghamiana	95.8 ± 23	80.1 ± 4.4	66.8 ± 2.2
Myrica gale	127 ± 11	103 ± 7	43.5 ± 4.3
Comptonia peregrina	31.5 ± 2.2	17.4 ± 0.9	16.4 ± 0.4
Alnus rubra	23.7 ± 1.2	15.4 ± 0.6	43.1 ± 1.5
Ceanothus americanus	15.6 ± 2.7	11.6 ± 1.5	49.0 ± 3.2
C. americanus – roots only	4.3 ± 0.4	1.4 ± 0.2	–
Datisca glomerata	13.0 ± 0.6	0.0	48.4 ± 2.1
Zea mays – roots	4.1 ± 0.3	1.7 ± 0.1	–

amount of CO-reactive heme: when 5% soluble PVP (Sigma PVP-10, 10,000 mol. wt) was used the yield of CO-reactive heme from nodules of *Myrica gale* was 86 ± 8 nmol g^{-1}, while with 1% Triton X-100 the yield was 98 ± 10 nmol g^{-1} (mean ± SE, n = 3).

CO-reactive heme could also be extracted when Tween 80 or CHAPS (Sigma No. C 3023) were substituted for the Triton X-100. No CO-reactive heme could be extracted in the absence of soluble PVP or the detergents mentioned. The insoluble form of PVP was completely ineffective when used without soluble PVP or detergent.

These results suggest that the CO-reactive heme in actinorhizal nodules is either in an insoluble form when present in the nodule, or has a strong tendency to become insoluble during nodule homogenization. This confirms the results of Davenport[10], who could find only insoluble hemoglobin in actinorhizal nodules.

Concentration of total and CO-reactive heme

The Soret absorption band for the CO-bound heme in our extracts had a peak at 416 to 420 nm. This is consistent with the properties of a hemoglobin and is evidence against certain of the other hemoproteins that are CO reactive[1,17]. Cytochrome P-450, whose CO complex has a peak at 450 nm is ruled out. Likewise, cytochrome a_3 is probably not involved, since it is not readily solubilized and its CO peak is at 430 nm. However, there are other CO-reactive hemoproteins that might be present in our extracts. Horseradish peroxidase has an absorption peak at 423 nm, while cytochrome o has a peak at about 416–418 nm[9]. Thus the absorption maximum at 416 to 420 nm in our extracts is consistent with the presence of a hemoglobin, but does not rule out certain other CO-reactive hemoproteins.

The concentrations of total and CO-reactive heme that we found in nodules of Lupinus and Casuarina (Table 1) were comparable to the results of other investigators[3,10,11,12]. This further confirms that actinorhizal nodules such as Casuarina may have hemoglobin contents that approach those found in legume nodules. However, *Myrica gale* was the only other actinorhizal plant where we found this to be true. In Comptonia, Alnus, and Ceanothus, the concentrations of CO-reactive heme were 5 to 8 times lower than for Casuarina. In Datisca we found no CO-reactive heme at all. Except for Comptonia, these low heme concentrations were accompanied by high nitrogenase (acetylene reduction) activities. Thus there was no indication that the low heme contents were due to a defective symbiosis or other abnormal condition.

In work reported previously[28], we found much lower concentrations of CO-reactive heme, even though the concentrations of total heme were about the same as reported in Table 1. The reason is that these earlier extractions were done in the absence of CO. Following the method of Appleby[3], we modified our procedure and now extract in the presence of CO. We find that the absorption bands of the CO-containing extracts are quite stable when the extract is aerated, whereas the absorption bands of CO-free extracts are rapidly lost.

Because we found evidence for hemoglobins in unrelated genera of actinorhizal nodules, it seemed possible that hemoglobins might be found outside of root nodules. We first examined roots of Ceanothus from which all nodules were removed and found a low but measurable concentration of CO-reactive hemoprotein (Table 1). We found similar results in the roots of all non-nodulating plants examined: *Ulmus americana, Acer saccharinum, Helianthus annuus, Triticum aestivum*, and *Zea mays*. *Zea mays* is the only non-nodulating plant included in Table 1, since total heme was determined only for this species. As can be seen, CO-reactive heme is responsible for a substantial fraction of total heme in *Zea mays*.

Difference spectra

Depending on the material being examined, substances other than CO-reactive hemoproteins absorbed light in the 400 to 650 nm wavelength range, and distorted the absorption bands. Thus difference spectra were used to compare CO-reactive hemoproteins from various plant sources. In the case of *Ceanothus americanus* (Fig. 3), the difference spectra clearly showed the presence of a CO-reactive hemoprotein, whereas in extracts and in spectra of nodule slices[26] the absorption bands are partially obscured by other substances.

The absorption maxima and minima for the difference spectra of plus CO minus dithionite reduced extracts were similar for all nodules and

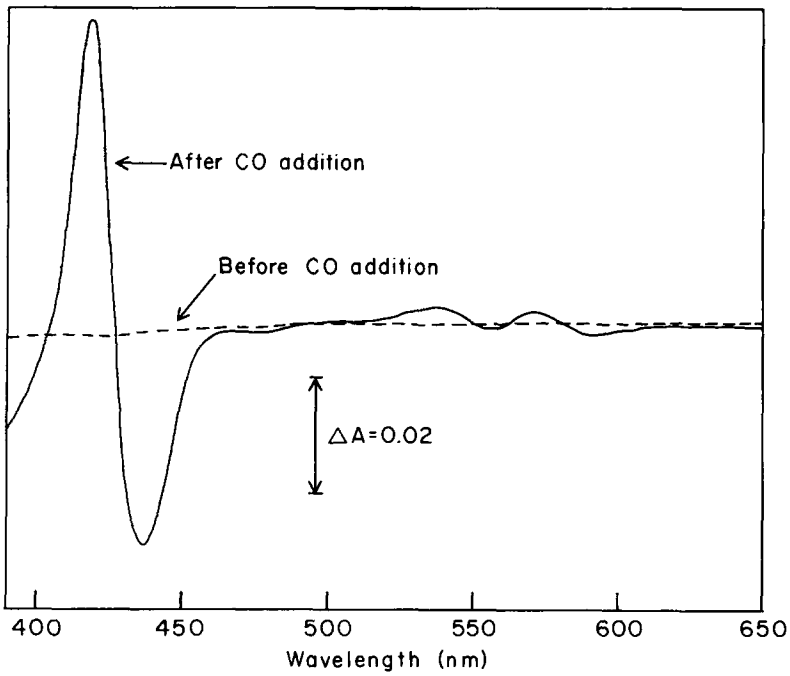

Fig. 3. Difference spectrum of a CO-equilibrated extract from Ceanothus minus a dithionite reduced extract.

roots (Table 2). The wavelengths are consistent with those of a hemoglobin[1,17], but as discussed above, they could be due at least in part to some other CO-reactive hemoprotein. Whatever the identity of the CO-reactive heme, it has three similarities in all nodules and roots examined: 1. It is soluble in 1% Triton X-100 or 5% soluble PVP. 2. It makes up a substantial fraction of total heme. 3. The plus and minus CO difference spectra are consistent with those of a hemoglobin.

The question of whether the CO-reactive heme is partly or entirely a hemoglobin is best pursued using the purified protein. This has been done for the root nodules of *Casuarina glauca,* and the protein extracted has all the properties of a hemoglobin, including ligand binding, mole-

Table 2. Difference spectra of hemoprotein extracts (CO minus dithionite reduced). The wavelengths of maximum difference are given (nm)

Species	+	−	+	−	+
Casuarina cunninghamiana	420	437	537	557	572
Myrica gale	416	433	532	553	568
Alnus rubra	419	436	536	555	571
Ceanothus americanus	419	437	538	557	571
C. americanus — roots only	418	437	–	–	–
Zea mays – roots	419	438	534	555	570

Table 3. Effect of pO_2 on nodule weight and heme content (mean ± SE, n = 3 to 6)

	pO_2	Total heme	CO-reactive heme	Nodule fresh wt
	kPa	nmol g^{-1} (fresh wt)		mg/plant
Myrica gale	5	99.2 ± 3.5	60.7 ± 4.6	88 ± 7
	20	108 ± 6	70.7 ± 5.2	192 ± 24
Alnus rubra	5	22.0 ± 0.8	8.6 ± 0.4	94 ± 18
	20	23.7 ± 0.9	14.1 ± 0.6	260 ± 42

cular weight, and amino acid sequence[12,15]. Other evidence comes from the absorption spectra of slices of nodules of *Myrica gale, Comptonia peregrina,* and *Alnus rubra*[26]. These display the absorption bands of oxyhemoglobin when taken in an atmosphere of O_2, while the bands disappear when N_2 is substituted for O_2. In spite of this evedence, the identity of the heme found in the present study must be considered an open question until further studies are completed.

Possible functions of hemoglobin in actinorhizal nodules

Much higher concentrations of presumed hemoglobin were found in the nodules of Myrica and Casuarina than in other actinorhizal nodules. Since both of these genera often grow in wet soils, where the oxygen concentration may be reduced[25,26], we hypothesized that hemoglobin might be serving to facilitate oxygen transport at low oxygen concentrations. Such a function would be similar to the function of leghemoglobin in transporting oxygen at the low oxygen concentrations that prevail within the diffusion barrier of legume nodules[2,27]. An increase in hemoglobin concentration caused by reduced oxygen concentration in the root environment would support this hypothesis. We examined Alnus as well as Myrica, since it also grows in relatively wet soils.

Our results provide no support for the measured heme being involved in an adaptation to reduced partial pressures of O_2 (pO_2). As found by others[6,16,25], reduced pO_2 in the root zone caused reduced plant growth (results not shown) and reduced nodule weight (Table 3). In spite of this oxygen stress, there was little effect on the concentration of total and CO-reactive heme.

The nodules of Casuarina are unique among actinorhizal plants in that the endophyte, Frankia, forms no vesticles in the nodule[4,18]. Studies of Frankia strain HPCcI3, isolated from Casuarina nodules, show that vesicles are necessary for nitrogen fixation when the cultures are grown at atmospheric pO_2 (20 kPa), but are absent when the cultures fix nitrogen at 0.1 to 0.3 kPa pO_2[18]. These results suggest that the pO_2 within Casuarina nodules may be low compared to other actinorhizal nodules. A possible cause is the suberization or lignification of the walls of

Table 4. Evidence for the suberization of the wall of infected cells in selected actinorhizal plants

Species	Test result for lignin-suberin		
	Chromic acid	Phloroglucinol	Autofluorescence
Casuarina cunninghamiana	++	+	++
Myrica gale	+	+	+
Comptonia peregrina	+	+	+
Alnus rubra	−	−	−
Ceanothus americanus	−	−	−
Datisca glomerata	−	−	−

infected host cells in Casuarina, which could restrict oxygen diffusion into these cells[4]. If so, the hemoglobin in Casuarina nodules might function to facilitate oxygen transport in a zone of low pO_2, as does leghemoglobin in legume nodules.

We decided to investigate the relationship between heme content, suberization, and lignification in the six actinorhizal genera where we measured heme concentration. By comparing Tables 1 and 4 one can see that there is a correlation between the presence of suberization and lignification and elevated heme concentration, except in the case of *Comptonia peregrina*. Comptonia is closely related to Myrica and we found identical results in the two species when testing for suberin and lignin. However the hemoglobin concentration was much lower in Comptonia, and was similar to that in Alnus, which lacks suberin and lignin. It is possible that these particular nodules of Comptonia were not fully effective, as suggested by their relatively low acetylene reduction rates (Table 1). Overall the data support a relationship between elevated heme content and the presence of suberized and lignified walls, but more information is needed to elucidate the nature of this relationship.

Since hemoglobins have a high affinity for CO, it seemed possible that nodules with little or no hemoglobin might be less sensitive to inhibition by CO than those with high concentrations of hemoglobin. If so, this might be used to study the function of the hemoglobin. As can be seen by comparing Tables 1 and 5, there was no correlation between hemoglobin content and the inhibition of acetylene reduction by CO. The

Table 5. Inhibition of acetylene reduction in actinorhizal and legume nodules by 0.33 kPa of CO (mean ± SE, n = 3 to 6)

Species	Inhibition(%)
Alnus rubra	43 ± 4
Casuarina cunninghamiana	54 ± 1
Datisca glomerata	60 ± 7
Myrica gale	62 ± 4
Ceanothus americanus	95 ± 2
Lupinus albus	90 ± 2
Pisum sativum	82 ± 3

inhibition observed could have been due to the effect of CO on a cytochrome oxidase, hemoglobin, or nitrogenase itself[17,22]. Using ^{15}N, Bond found comparable results for inhibition by CO in nodules of Alnus, Myrica, and Pisum[7].

Conclusions

Our results establish that there are high concentrations of total and CO-reactive heme in the nodules of *Myrica gale* and *Casuarina cunninghamiana*. However, the concentrations of heme in other actinorhizal genera were about 5 to 8 times lower, with no significant differences in acetylene reduction rates by nodules with high and low heme contents. Moreover, Datisca nodules had typical acetylene reduction rates, but no detectable heme that was extractable and CO-reactive. We thus conclude that high heme contents are not essential for nitrogen fixation in actinorhizal nodules.

The CO-reactive heme in *Casuarina cunninghamiana* is probably a hemoglobin, since large amounts of a hemoglobin have been purified from the nodules of *Casuarina glauca*[12]. It is now of great interest to purify and characterize the CO-reactive heme extracted from actinorhizal plants such as *Alnus rubra* and non-nodulating plants such as *Zea mays*. If this proves to be a hemoglobin, it would suggest that hemoglobins have some relatively general function in plants.

Our initial investigations into the function of the CO-reactive heme in actinorhizal nodules have been inconclusive. There is a possible relationship with suberization of the cell wall of the infected host cells, but this needs more study. No correlation was observed between heme content and either root zone pO_2 or the sensitivity of nitrogenase activity to CO inhibition.

The simplest hypothesis is that the CO-reactive heme measured in Table 1 is a hemoglobin and functions in facilitating oxygen transport in tissues where the pO_2 is low. This is the most likely function of the lethemoglobin present in legume nodules[2]. Variations in the concentration of hemoglobin in nodules and roots might be due to variations in the intensity of respiration and the resistance to oxygen diffusion to the sites of respiration.

Acknowledgements We thank Linda Krywy for expert technical assistance, Mary Lechevalier for providing Frankia strain LLR 161101, John Torrey for providing some of the seed and Frankia strains used, Larry Winship for seeds of Datisca, Christa Schwintzer for helpful comments on the manuscript, and Cyril Appleby, Tony Fleming, Jonathan Wittenberg, Beth Mullin, Douglas Johnson and Ian Sussex for helpful discussions and data in advance of publication. This work was supported by US National Science Foundation Grant No. DMB-8315415.

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