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Iron Uptake by Symbiosomes from Soybean Root Nodules[1]

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To identify possible iron sources for bacteroids in planta, soybean (*Glycine max* L. Merr.) symbiosomes (consisting of the bacteroid-containing peribacteroid space enclosed by the peribacteroid membrane [PBM]) and bacteroids were assayed for the ability to transport iron supplied as various ferric [Fe(III)]-chelates. Iron presented as a number of Fe(III)-chelates was transported at much higher rates across the PBM than across the bacteroid membranes, suggesting the presence of an iron storage pool in the peribacteroid space. Pulse-chase experiments confirmed the presence of such an iron storage pool. Because the PBM is derived from the plant plasma membrane, we reasoned that it may possess a ferric-chelate reductase activity similar to that present in plant plasma membrane. We detected ferric-chelate reductase activity associated with the PBM and suggest that reduction of Fe(III) to ferrous [Fe(II)] plays a role in the movement of iron into soybean symbiosomes.

Rhizobia are totally dependent on their plant hosts for nutrients when living within the nodule. Bacteroids are surrounded by a PBM of plant origin (reviewed by Mellor, 1989), which may influence the transfer of all substances that pass between the plant and the bacteroids. The PBM possesses some of the properties of the plant PM from which it is derived but also has a set of PBM-specific proteins, some of which are presumed to be involved in the nitrogen fixation process (reviewed by Verma, 1992). Because the PBM is the interface between the plant host and the bacteroid, its role in controlling the flux of metabolites between the two compartments is likely to be important in the regulation of nitrogen fixation.

Studies of the PBM have shown that this membrane has selective permeability to metabolites. Symbiosomes (consisting of the bacteroid-containing PBS enclosed by the PBM; Roth et al., 1988) prepared from soybean and French bean have dicarboxylate-transporting ability (Udvardi et al., 1988; Herrada et al., 1989), and dicarboxylates are thought to be the major carbon and energy source available for bacteroids. The form(s) of nitrogen transported to bacteroids is not currently known (Whitehead et al., 1995); however, the rescue of various amino acid auxotrophs in planta suggests that amino acids can reach bacteroids (Truchet et al., 1980; Kerppola and Kahn, 1988). Recently, a channel capable of transporting ammonium out of the symbiosome to the plant was discovered on the soybean PBM (Tyerman et al., 1995), confirming that ammonium is the form of nitrogen exported to the plant. The PBM has an ATPase that pumps protons into the symbiosome space, thus energizing the PBM and causing the PBS to have an acidic pH (Blumwald et al., 1985; Udvardi and Day, 1989).

Although some information is available regarding the types of carbon and nitrogen sources available to bacteroids, little is known about the availability and transport of mineral nutrients within the nodule. We are interested in the movement of iron within nodules because there is an increased iron demand for synthesis of iron-containing proteins necessary for nitrogen fixation. For example, experiments in which a soybean split-root system was used showed that induction of iron-deficiency responses [proton release and Fe(III) reduction] is much stronger in the roots of plants that have actively fixing nodules than in the roots of uninoculated plants (Terry et al., 1991). *Lupinus angustifolius* has a higher requirement for iron when nodulated than when uninoculated (Tang et al., 1990a), and iron is directly involved in nodule initiation but not nodule development (Tang et al., 1990b). In contrast, work with peanuts inoculated with *Bradyrhizobium* sp. has shown that iron-deficient plants initiated nodules but had decreased nodule development, lowered amounts of leghemoglobin in nodules, fewer bacteroids present in nodules, and lower nitrogen fixation activity (O'Hara et al., 1988). These studies demonstrate that iron plays a key role in nitrogen-fixing symbioses, although the stage of nodule development most dependent on iron supply varies among legume species.

Bacteroids must be supplied with iron throughout rhizobial/legume symbioses, but it is not known whether they are supplied with iron passively by the plant or whether the bacteroids must actively work to acquire iron. There are two general strategies by which microsymbionts could acquire iron within host plants. One strategy would be the direct utilization of host iron compounds, such as Fe(III)-citrate. Alternatively, microorganisms could synthesize reductants or chelators to dissociate iron from host iron complexes, such as Fe(III)-organic acid complexes

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Abbreviations: BM, outer and inner bacteroid membranes; BPDS, 4,7-di(4-phenyl-sulfonate)-1,10 phenanthroline; PBM, peribacteroid membrane; PBS, peribacteroid space; PM, plasma membrane.

(Tiffin, 1966) and ferritin (reviewed by Andrews et al., 1992), and thus make iron available for their own use. Siderophores, small-molecular-weight, high-affinity, Fe(III) chelators synthesized and secreted by microorganisms and some plants under conditions of iron limitation (Guerinot, 1994), may function to supply the bacteria with iron both in the soil and in planta. In all Gram-negative bacteria studied to date, internalization of iron from Fe(III)-siderophore and chelate complexes involves several outer and inner-membrane receptor proteins and is absolutely dependent on the presence of a functional TonB protein necessary for energetically coupling inner and outer membranes (reviewed by Guerinot, 1994). Several bradyrhizobial strains, including *Bradyrhizobium japonicum* USDA110, have been shown to release organic acids that have the ability to chelate Fe(III) (Guerinot et al., 1990; Carson et al., 1992; Lesueur et al., 1993).

In this study we investigated the acquisition of iron by *B. japonicum* bacteroids by measuring ^{55}Fe uptake by isolated soybean symbiosomes. Experiments were designed to determine what source(s) of iron is available for utilization by symbiosomes and bacteroids in planta by supplying isolated suspensions with various sources of Fe(III)-chelates and then assaying for transport of iron. We also assayed PBM vesicles for the presence of PM-type Fe(III)-chelate reductase activity to determine whether an Fe(III) reduction step is a necessary prerequisite for the movement of iron into symbiosomes.

MATERIALS AND METHODS

Plant Materials, Bacterial Strains, and Chemicals

Soybean (*Glycine max* L. Merr. cv Stevens) seeds were inoculated with *Bradyrhizobium japonicum* USDA110 and grown in pots in a naturally illuminated greenhouse as described by Day et al. (1989). Plants were harvested 3 to 5 weeks after sowing. [U-^{14}C]Suc (20 GBq/mmol), $^{3}H_2O$ (37 GBq/mL), and $^{55}FeCl_3$ (37 MBq/mg Fe) were obtained from Amersham. Silicon oil (AR-200; density 1.04 g/mL) was purchased from Wacker Chemie (Munich, Germany). Percoll was purchased from Pharmacia. All other chemicals were purchased from Sigma or Boehringer Mannheim.

Preparation of Symbiosomes, Bacteroids, and PBM Vesicles

Root nodules were harvested from soybean plants previously inoculated with *B. japonicum* USDA110. Symbiosomes were isolated from the nodules using Percoll gradient centrifugation, as described by Day et al. (1989), with the modification that all buffers were adjusted to pH 6.8. After centrifugation for 15 min at 4000*g* in a Sorvall HB-4 rotor (DuPont), symbiosomes from 5-week-old plants were recovered as a band at the 60/80% interface on a Percoll step gradient; symbiosomes from 3-week-old plants were recovered at the 55/70% interface. The integrity of symbiosome preparations was routinely checked by light microscopy. Washes of symbiosomes and preparation of bacteroids were as described by Day et al. (1989). Briefly, bacteroids were prepared by rupturing intact symbiosomes in wash buffer (350 mM mannitol, 25 mM Mes, 3 mM $MgSO_4$, pH 6.8) by vortexing for 4 min. PBM vesicles were prepared according to the method of Udvardi and Day (1989).

Transport Studies

Metabolite uptake was measured using a silicon oil filtration technique (Palmieri and Klingenberg, 1979). Briefly, cells/compartments to be analyzed were incubated in the presence of radiolabeled substrate for a defined amount of time and then loaded into tubes on top of an AR-200 undiluted silicon oil layer. Tubes were spun briefly to pellet cells/compartments through the oil away from the incubation medium, and the radioactive content of the pelleted material was determined. Unless stated otherwise, transport assays were performed on nodules harvested from 5-week-old plants. Symbiosomes and bacteroids were suspended in cold wash buffer. Fe(III)-chelating compounds were supplied as filter-sterilized stocks at pH 6.8 (adjustments to pH were made with a solution of 5 M NaOH:5 M KOH [3:1, v/v] according to the method of Langman et al., 1972). Chelating compounds were mixed with 10 μM $FeCl_3$ with $^{55}FeCl_3$ present in trace amounts (specific activity of 0.8 μCi/mL for time courses and 8 μCi/mL for 1-min assays with various iron-chelate concentrations) in the following molar ratios: anthranilic acid, 30:1; malic acid, 30:1; pyruvic acid, 30:1; and salicylic acid, 20:1. The number of molecules determined to chelate one molecule of Fe(III) was increased by 10-fold to ensure that all Fe(III) was chelated. Citric acid was used at a 200:1 molar ratio (Messenger and Ratledge, 1982). Chelating agents were added to $FeCl_3$ 30 min prior to initiation of transport assays; the reactions were periodically mixed. Uptake reactions were performed at room temperature (25°C) at pH 6.8 and terminated after 1 min unless otherwise stated. To terminate reactions, bacteroids were centrifuged for 10 s and symbiosomes were centrifuged for 5 s; the shorter time was used for symbiosomes to prevent pelleting of any contaminating free bacteroids.

In this paper ^{55}Fe is described as being transported across membranes after being presented as various ^{55}Fe(III)-chelates; experiments did not address the movement of the chelating compounds themselves. Experiments performed here do not rule out the possibility that chelating compounds are transported along with the iron. Uptake of $^{3}H_2O$ and [^{14}C]Suc was used to estimate total and external water volumes of bacteroids and symbiosomes, respectively (Udvardi et al., 1991). When inhibitors and effectors were used, they were added to the reaction mixture (wash buffer plus symbiosomes or bacteroids) 10 min prior to the addition of the radiolabeled substrate (at a final concentration of 8 μM). Arsenate and KCl were added to final concentrations of 5 mM; *N,N'*-dicyclohexylcarbodiimide, KCN, and NADH were added to final concentrations of 1 mM; valinomycin was added to a final concentration of 5 μM (supplied with 5 mM [final] KCl), and carbonyl cyanide *p*-(trifluoromethoxy)-phenylhydrazone was added to a final concentration of 2 μM. Statistics were calculated using Student's *t* test.

Pulse-Chase Experiments

Pulse-chase experiments to monitor iron partitioning within symbiosomes were performed as follows: radiolabeled ^{55}Fe(III)-citrate was added to a final concentration of 8 μM to symbiosomes in wash buffer in a Corex tube (Corning, Corning, NY) and incubated for 10 min; the mixture was gently swirled periodically with a soft-bristled paintbrush. Cells were centrifuged at 478*g* for 10 min, followed by a brief 15-s centrifugation at 2987*g*. The supernatant was removed and the pellet was resuspended with a paintbrush in an equal volume of fresh wash buffer supplemented with nonradioactive Fe(III)-citrate to a final concentration of 8 μM. Cells were incubated for various times in a shaking water bath at a slow speed at 25°C. Incubations were terminated in two ways: (a) for determination of iron transported into symbiosomes, 150 μL of symbiosomes in reaction medium [wash buffer plus Fe(III)-citrate] were removed from the reaction mixture and pelleted immediately through silicon oil for 5 s; (b) for determination of iron transported into bacteroids, 150 μL of symbiosomes in reaction medium were removed from the reaction mixture and transferred to microfuge tubes and vortexed for 4 min to disrupt the PBM. The resulting mixture was centrifuged over silicon oil for 30 s to pellet bacteroids. Because the protein content of the PBM and PBS is insignificant compared with that of bacteroids (Ouyang et al., 1991), protein determination of each fraction allowed for direct comparison of radioactive iron accumulation per milligram of protein in symbiosomes versus bacteroids.

Fe(III)-Chelate Reduction Assay

Fe(III)-chelate reductase activity was assayed spectrophotometrically according to the method of Holden et al. (1991), using 50 μg of PBM vesicle protein in each assay. Control assays were conducted in which either NADH or PBM was omitted: Fe(III)-chelate reduction was dependent on the presence of both NADH and PBM (data not shown). Assays were conducted for 10 min at room temperature. NADH-ferricyanide reductase assays using $[Fe(CN)_6]^{3-}$ as an artificial electron acceptor (Holden et al., 1991) were performed to measure overall PBM redox activity. To determine whether PBM vesicles were contaminated with PM, we measured the activity of an enzyme, NAD(P)H Cyt *c* reductase, which is known to be associated with the plant PM (Møller and Lin, 1986).

Protein Estimation

Protein concentrations were estimated by the modified Lowry method (Schacterle and Pollack, 1973) with BSA, fraction V, as a standard.

RESULTS

Movement of Iron across Symbiotic Membranes

Iron is transported in soybean xylem in the form of Fe(III)-citrate (Tiffin, 1970). This and the fact that citrate is present in significant amounts in the cytosol of soybean nodule cells (Streeter, 1987; Moreau et al., 1995) prompted us to investigate Fe(III)-citrate as an iron source for symbiosomes and bacteroids in the nodule. Cultured cells of *B. japonicum* USDA110 had previously been demonstrated to use Fe(III)-citrate as an iron source in growth assays in which iron-starved cells were rescued by the addition of Fe(III)-citrate (Guerinot et al., 1990) and in transport assays in which iron-starved but not iron-sufficient cells transported ^{55}Fe(III)-citrate (Plessner et al., 1993). Transport assays with purified symbiosomes and bacteroids isolated from 5-week-old plants were performed using ^{55}Fe(III)-citrate as an iron source at a final concentration of 10 μM. Symbiosomes were able to accumulate ^{55}Fe when presented with ^{55}Fe(III)-citrate and did so at significantly higher rates ($P < 0.05$) than did free bacteroids (Fig. 1). Uptake of ^{55}Fe from ^{55}Fe(III)-citrate was linear over the first 3 min for symbiosomes, and over the first 4 min for bacteroids, under the conditions used in these experiments.

Concentrations of ^{55}Fe(III)-citrate were varied and uptake experiments performed with reactions terminated after 1 min. Symbiosome ^{55}Fe accumulation from ^{55}Fe(III)-citrate followed standard Michaelis-Menten saturation kinetics until the concentration of Fe(III)-citrate reached 10 μM (data not shown). At concentrations greater than 10 μM Fe(III)-citrate, the rate of uptake began to decrease sharply, suggesting that the mechanism of ^{55}Fe transport is extremely sensitive to iron concentrations greater than 10 μM or to citrate concentrations greater than 2.0 mM within a short time. This result, observed consistently in a number of separate experiments, was not due to changes in pH, since more Fe(III)-citrate was added because the pH of reaction mixtures was monitored throughout the experiments and remained constant at pH 6.8. Symbiosomes respired normally in response to added glutamate over the concentration range of 1 to 100 μM Fe(III)-citrate, as monitored with an oxygen electrode (data not shown). Because

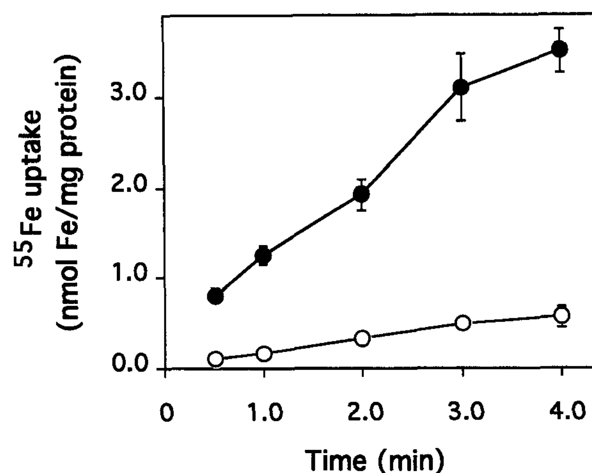

Figure 1. ^{55}Fe uptake by isolated symbiosomes (●) and bacteroids (○) using the silicon oil centrifugation technique. ^{55}Fe(III)-citrate was used at a final concentration of 10 μM, and reactions were terminated at specified times. Values shown represent the means ± SE of four independent experiments; each data point is the average of quadruplicate determinations.

glutamate cannot permeate the PBM but is transported readily into bacteroids where it stimulates respiration, this indicates that the inhibition of ^{55}Fe uptake was not due to PBM rupture (Herrada et al., 1993).

Ten minutes prior to the addition of $^{55}Fe(III)$-citrate, NADH and various inhibitors that are known to be freely membrane permeable were added to symbiosome suspensions, and standard 1-min uptake assays were conducted. NADH supplementation increased ^{55}Fe uptake from $^{55}Fe(III)$-citrate approximately 2-fold, suggesting that the mechanism responsible for ^{55}Fe transport across the PBM may use NADH as an electron donor (Fig. 2). To address whether bacteroids act as a sink to drive the movement of iron into symbiosomes, KCN, a bacterial respiratory chain inhibitor, and the protonophore carbonyl cyanide *p*-(trifluoromethoxy)-phenylhydrazone, were added to incubations. Neither compound inhibited ^{55}Fe transport across the PBM (data not shown), indicating that BM membrane energization is not necessary for uptake of ^{55}Fe into symbiosomes. Valinomycin, a potassium ionophore, and *N,N'*-dicyclohexylcarbodiimide, a proton channel blocker, likewise did not inhibit ^{55}Fe transport across the PBM (data not shown), lending further support to the suggestion that bacteroid respiration does not drive the movement of iron.

Bacteroid ^{55}Fe accumulation was also monitored, but uptake levels from $^{55}Fe(III)$-citrate in the concentration range of 1 to 100 μM Fe(III)-citrate were so low that kinetic values could not be determined (data not shown). Iron demand may be greater in younger bacteroids, and so uptake studies with Fe(III)-citrate as an iron source were performed with bacteroids from 3-week-old plants. Uptake of ^{55}Fe from $^{55}Fe(III)$-citrate was not appreciably different in bacteroids from nodules from 3-week- versus 5-week-old plants (data not shown).

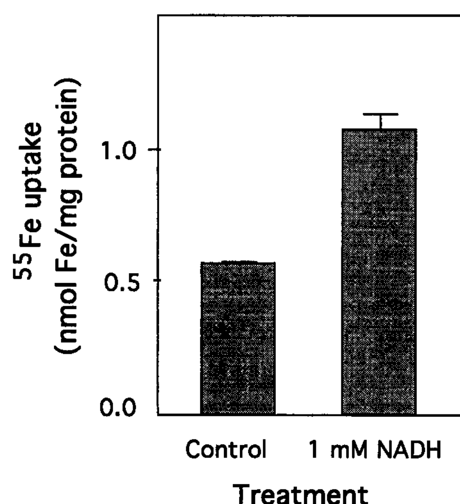

Figure 2. ^{55}Fe uptake by isolated symbiosomes presented with $^{55}Fe(III)$-citrate using the silicon oil centrifugation technique. NADH was added to symbiosome suspensions at a final concentration of 1 mM 10 min prior to the addition of $^{55}Fe(III)$-citrate to a final concentration of 8 μM in standard 1-min uptake assays. Values shown represent the means ± SE of three independent experiments; each data point is the average of quadruplicate determinations.

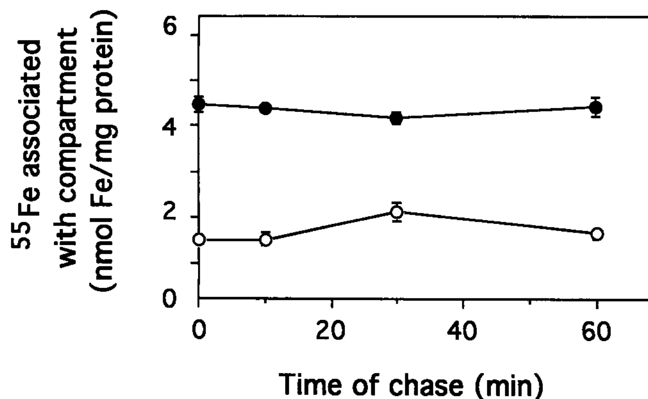

Figure 3. Pulse-chase of ^{55}Fe into symbiosomes to monitor iron partitioning over time. $^{55}Fe(III)$-citrate (8 μM final concentration) was pulsed into a suspension of symbiosomes in reaction medium for 10 min and then chased with cold Fe(III)-citrate for various times. Symbiosomes (●) were pelleted directly after the chase period, and bacteroids (○) were prepared by rupturing symbiosomes after the chase period and pelleting bacteroids for 30 s. Values are the means ± SE of four trials; each data point is the average of quadruplicate determinations. Ten- to 60-min time point values for each type of compartment are not statistically different from one another.

Investigation of Iron Partitioning within Symbiosomes through Pulse-Chase Experiments

The above-described experiments indicated that iron was transported more rapidly across the PBM than the BM. This suggested that iron may accumulate in the PBS before entering the bacteroids, at least within the time frame of these experiments. A pulse-chase experiment was designed to address this hypothesis. Purified symbiosomes were incubated with $^{55}Fe(III)$-citrate, washed, and then resuspended in medium containing unlabeled Fe(III)-citrate. Aliquots of symbiosomes were removed at various times and subjected to silicon oil centrifugation, either as intact symbiosomes or as bacteroids. When symbiosomes are ruptured to release bacteroids, PBS contents are also released, but this material is not dense enough to pellet through silicon oil.

The results of pulse-chase experiments showed that the majority of the radioactive iron that was taken up into symbiosomes was not accumulated in the bacteroids (Fig. 3). This suggests that iron is stored in the PBS immediately after it crosses the PBM. The ^{55}Fe concentration in symbiosomes remained stable after the pulse, indicating that ^{55}Fe did not leak out of the symbiosomes into the reaction medium during the course of the experiment (up to 60 min). The rate of uptake of ^{55}Fe from Fe(III)-citrate into symbiosomes slowed dramatically over a 10-min uptake period (compare Fig. 1 with Fig. 3), indicating that uptake was at or near saturation at the beginning of the pulse-chase experiments.

Transport of Iron from Various Fe(III)-Chelates across the Bacteroid Membrane

Because bacteroids transported ^{55}Fe from $^{55}Fe(III)$-citrate at low rates relative to symbiosomes, other metabolites

with the potential to chelate iron and known to be present in soybean tissues were assayed as potential iron sources for bacteroids. Malic acid, pyruvic acid, and salicylic acid were chosen as test Fe(III) chelators. Malic acid is abundant in nodules (Streeter, 1987) and has some ability to chelate iron (Guerinot, 1994). Pyruvic acid and other α-keto acids have been demonstrated to serve as iron transport agents in staphylococci, micrococci, and *Pasteurella* spp., despite the fact that α-keto acids do not form very stable complexes with Fe(III) compared with other siderophore compounds (Reissbrodt et al., 1994; Heuck et al., 1995). Salicylic acid, which is used as a signal molecule in plant defense, is an intermediate in the synthetic pathway of the siderophore pyochelin and has been identified as a siderophore in a number of pseudomonad species (Meyer, 1992; Visca et al., 1993). Anthranilic acid, which has been identified as a siderophore in *Rhizobium leguminosarum* (Rioux et al., 1986a, 1986b), was also included as a test compound based on a report in the literature that a lesion in the *Rhizobium meliloti* gene for anthranilate synthase leads to an ineffective symbiosis with alfalfa, whereas mutations in genes later in the Trp biosynthetic pathway do not (Barsomian et al., 1992).

Transport assays using purified bacteroids and 10 μM final concentration of the different ^{55}Fe-chelates showed that bacteroids were able to accumulate ^{55}Fe from Fe(III)-anthranilate, Fe(III)-malate, Fe(III)-pyruvate, and Fe(III)-salicylate at rates similar to those from Fe(III)-citrate (Table I). Symbiosomes were also assayed for their ability to transport each of the Fe(III)-chelate compounds, and ^{55}Fe from each compound was taken up into symbiosomes at rates similar to those from Fe(III)-citrate (Table I). These results demonstrate that uptake of ^{55}Fe from no one Fe(III)-chelator is clearly superior to others and that the level of transport of ^{55}Fe from all Fe-chelates assayed was significantly ($P < 0.05$) higher across the PBM than across the BM. It is important to note that there was a rapid association of ^{55}Fe with both bacteroids and symbiosomes over the first 30 s for all Fe(III)-chelates tested except Fe(III)-citrate; this rapid association was then followed by a period of slower, linear uptake. The rates presented in Table I describe this second linear phase of uptake. Others have also seen an initial association of Fe(III) complexes with the cell surface (as discussed by Guerinot et al., 1990).

Reductive versus Nonreductive Transport of Iron from Fe(III)-Citrate across the PBM

Reduction of Fe(III) to Fe(II) is a necessary step prior to the transport of iron into the cells of many organisms. Soybeans and other dicotyledonous plants respond to iron deprivation by inducing a root PM-bound Fe(III) reductase, which reduces Fe(III) to Fe(II) prior to its transport into the plant root (reviewed by Guerinot and Yi, 1994). The PM from leaf mesophyll cells of *Vigna unguiculata* also possesses a Fe(III)-chelate reductase activity that can reduce a variety of Fe(III) chelates, suggesting that reduction of Fe(III) is necessary prior to iron uptake into leaf cells (Brüggemann et al., 1993). Because the PBM is derived from the soybean PM (Verma et al., 1978), we reasoned that Fe(III) presented to the PBM by Fe(III)-chelates may be reduced as a prerequisite to transport into the symbiosome. We assayed purified soybean PBM for the presence of Fe(III)-chelate reductase activity by exposing the PBM to Fe(III)-citrate and the Fe(II) chromophore BPDS. BPDS has a high affinity for Fe(II) and changes color when bound by the Fe(II) ion. If a Fe(III)-chelate reductase is present in association with the PBM, any Fe(III) from Fe(III)-citrate that is reduced will be captured by BPDS and can be quantified colorimetrically (Holden et al., 1991). BPDS assays demonstrated that soybean PBM possesses an NADH-dependent Fe(III)-citrate reductase activity (Table II). It is thought that there are actually two PM reductases in plants that can reduce external electron acceptors such as ferricyanide and Fe(III)-chelates and that one of these reductases is involved in the iron-deficiency response (Moog and Brügemann, 1995). Because the ability to reduce the nonphysiological oxidant ferricyanide does appear to be a ubiquitous feature of plant PM, preparations of soybean PBM were also assayed for ferricyanide reductase activity; the PBM vesicles assayed had high levels of ferricyanide reductase activity (Table II). Although the absolute levels of Fe(III)-chelate reductase activity varied between experiments, the relative ratios between the Fe(III)-citrate reduc-

Table I. *^{55}Fe uptake from various Fe(III)-chelating compounds by isolated symbiosomes and bacteroids*

Symbiosomes and bacteroids were exposed to ^{55}Fe(III)-chelates (final concentration 10 μM), and reactions were terminated at 0.5-, 1-, 2-, 3-, and 4-min time points. Each experiment represents assays performed with symbiosomes or bacteroids prepared from nodules from separate pots of plants, and each data point is the average of quadruplicate determinations.

Fe(III)-Chelate	Rate of ^{55}Fe Uptake			
	Symbiosomes		Bacteroids	
	Experiment 1	Experiment 2	Experiment 3	Experiment 4
	nmol mg^{-1} min^{-1}			
Fe(III)-citrate	0.73	0.85	0.16	0.19
Fe(III)-anthranilate	0.52	1.03	0.17	0.24
Fe(III)-malate	0.79	0.81	0.15	0.19
Fe(III)-pyruvate	0.66	0.71	0.15	0.20
Fe(III)-salicylate	0.81	0.85	0.18	0.27

Table II. *Reductase activities associated with soybean PBM vesicles*

Each experiment represents assays performed with PBMs prepared from nodules from separate pots of plants. Fifty micrograms of PBM vesicle protein were used for each assay. Data for Fe(III)-citrate reductase activities are means (±SE) of four independent assays from the same preparation of purified PBMs.

Experiment	Reduction Rate		
	Fe(III)-citrate	$[Fe(CN)_6]^{3-}$	Cyt *c*
	nmol mg^{-1} min^{-1}		
1	11.1 (0.5)	200	ND[a]
2	23.1 (0.5)	434	ND

[a] ND, Not detected.

tase activity and the ferricyanide reductase activity were consistent, with ferricyanide reductase activity approximately 20 times that of Fe(III)-citrate reductase activity. To determine whether PBM vesicles were contaminated with PM, we measured the activity of an enzyme, NAD(P)H Cyt *c* reductase, which is known to be associated with the plant PM (Møller and Lin, 1986); no NAD(P)H Cyt *c* reductase activity was detected (Table II).

Isolated PBM showed Fe(III) reductase activity similar to those detected in *V. unguiculata* leaf mesophyll PM (Brüggemann et al., 1993) and barley chloroplasts (Jacobs and Jacobs, 1995). These rates of Fe(III) reduction were of the same order of magnitude as those of Fe uptake across the PBM. This suggests that the reduction of Fe(III) to Fe(II) plays a role in the movement of iron into soybean symbiosomes.

DISCUSSION

Because the PBM is derived from the PM, it might be expected to have similar mechanisms for transporting iron. Soybean PM has a Fe(III)-chelate reductase activity that is induced under conditions of iron deprivation (Guerinot and Yi, 1994). The reduction of Fe(III) to Fe(II) is a strict prerequisite to iron uptake in dicotyledonous plants (Chaney et al., 1972), yeast (Lesuisse et al., 1987), and some mammalian cells (Jordan and Kaplan, 1994); indeed, this reduction step has been suggested to be obligatory in all organisms. We have demonstrated that soybean PBM has Fe(III)-chelate reductase activity, and our data are consistent with a model in which the reduction of Fe(III)-chelates presented to symbiosomes is a prerequisite to the internalization of iron into the PBS.

Fe(III)-citrate is likely to be a readily available iron source, and ^{55}Fe presented to symbiosomes as Fe(III)-citrate is internalized into the PBS. Other physiological Fe(III)-chelating compounds, however, cannot be ruled out, because ^{55}Fe presented to the PBM by a number of Fe(III)-chelates in this study was competent for uptake into the symbiosome. This result is not surprising if the Fe(III)-chelates are being reduced by a PBM reductase, because Fe(III)-chelate reductases are able to reduce a variety of Fe(III)-chelates, including nonbiological ones such as Fe(III)-EDTA (Moog and Brüggemann, 1995). The use of radiolabeled, iron-chelating compounds could allow the determination of whether any of the assayed chelators are internalized into the symbiosomes along with ^{55}Fe, if the chelator itself is not internalized in the absence of iron.

Pulse-chase experiments established that a significant portion of the ^{55}Fe that was transported across the PBM was not immediately transported into bacteroids and appeared to remain in the PBS. Thus, we hypothesize that the PBS serves as an iron storage pool within symbiosomes, which might be available for use by bacteroids. This is consistent with early autoradiographic observations by Dilworth and Kidby (1968), who showed that iron was associated with the PBS and with the bacteroid cell wall. The hypothesized PBS iron storage pool may be the previously described nonheme iron fraction in the PBS, which represents approximately 25% of the total soybean nodule iron (Wittenberg and Wittenberg, 1990; Wittenberg et al., 1996). Wittenberg et al. (1996) stated that this iron fraction is bound by low-molecular-weight siderophore compounds and might be expected to be involved in iron storage in the symbiosome. Because the Fe(III)-siderophore fractions isolated by Wittenberg et al. (1996) differed in size and optical spectra when isolated from nodules of plants inoculated with three different strains of bradyrhizobia, the PBS siderophore compounds are hypothesized to be of bacterial origin. The PBS iron storage pool inferred from our studies might be made up of siderophore compound(s) synthesized by bacteroids and released into the PBS. Cultured *B. japonicum* cells have not been demonstrated to produce classical siderophores for iron acquisition, although one study demonstrated that a single strain released citrate under conditions of iron limitation (Guerinot et al., 1990), and another study showed that some strains, including USDA110, excreted citrate and malate regardless of iron status (Carson et al., 1992).

Symbiosomes have been compared with vacuoles and have been found to contain a number of lysosomal activities normally found in vacuoles. The acidic PBS contains α-mannosidase isoenzyme II, at least three acid hydrolases, a protease, and a protein protease inhibitor (reviewed by Mellor, 1989), and it has been suggested that symbiosomes may functionally replace vacuoles in nodule cells (Mellor, 1989). It has been demonstrated that the vacuole is the major iron storage compartment in yeast cells (Raguzzi et al., 1988). Because the solubility of iron decreases by a factor of 1000 for each pH unit increase above 4.0 (Yi et al., 1994), an acidic compartment would be well suited for a role in iron storage. This information fits well with data from pulse-chase experiments presented here, in which the majority of iron transported across the PBM is accumulated in the PBS, and supports the idea of the PBS as an iron storage pool within the symbiosome.

Bacteroids did not transport iron at high levels when presented with any of the Fe(III)-chelates assayed. This may reflect the fact that the form of iron-chelate that is used to supply bacteroids in planta is not one that was tested here. Alternatively, symbiosomes may begin to serve a role in iron storage during the early stages of nodule biogenesis, and mature bacteroids may take up only low levels of

iron from this storage pool for cellular maintenance. Mechanisms for the high-affinity uptake of iron in bacteria and plants are usually only activated when organisms find themselves under conditions of iron limitation. Therefore, a low-affinity bacteroid iron acquisition system may be involved in iron movement into mature bacteroids.

Moreau et al. (1995) monitored uptake of ^{55}Fe from Fe(III)-citrate into symbiosomes and bacteroids and reported both lower levels of ^{55}Fe uptake into symbiosomes and higher levels of ^{55}Fe uptake into bacteroids than we report here. These differences may be due to the use of two different assay methods (silicon oil centrifugation versus filtration), the differing ages of the plants from which nodules were isolated, or differences in the soybean cultivars or the iron nutrition of the plants. Moreau et al. (1995) also reported that soybean PBM lacks Fe(III)-citrate reductase activity. However, in contrast to our studies in which we used BPDS at pH 6.0 in the presence of detergent, they used Ferrozine to assay Fe(III)-citrate reductase activity at pH 7.4 and they did not include a detergent in their assays. BPDS consistently gives higher Fe(III)-citrate reductase values than does FerroZine (HACH, Ames, IA) (Cowart et al., 1993). pH is a key determinant in Fe(III)-chelate reduction reactions, and multiple researchers have shown significant decreases in Fe(III)-chelate reductase activities when reaction pH is increased from 6.0 to 7.0 (reviewed by Cowart et al., 1993). Finally, the Fe(III)-chelate reductase in plant PM is hypothesized to be a transmembrane enzyme or enzyme complex, and activity has been shown to be latent in assays performed in the absence of detergent (Holden et al., 1991). Taken together, the above factors probably account for our ability to detect Fe(III)-chelate reductase activity.

The movement of citrate as well as that of iron was followed by Moreau et al. (1995), who showed that [^{14}C]-citrate was transported poorly across the PBM unless $FeCl_3$ was supplied simultaneously. This work indicates that Fe(III)-citrate moves into the symbiosome as a complex, which is in contrast to our data implicating a PBM Fe(III)-chelate reductase that would lead to the movement of uncomplexed Fe(II) across the PBM. These two experimental findings need not be contradictory; rather, there may be multiple mechanisms by which iron can enter the symbiosome.

In summary, our results suggest that Fe(III)-chelates are reduced by a membrane-bound PBM reductase. The Fe(II) would then presumably be transported via a Fe(II) transport system into the PBS, where it would be stored for future use by the bacteroids. *B. japonicum* mutants impaired in iron uptake should allow us to address what forms of iron are taken up by bacteroids to supply nitrogenase and other iron-containing proteins with this essential metal.

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