Uptake of iron by symbiosomes and bacteroids from soybean nodules

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Abstract Symbiosomes and bacteroids isolated from soybean nodules are able to take up the iron–citrate complex. The kinetics are characterized by initial high rates of iron internalization, and ATPase inhibitors significantly lower the uptake. This is consistent with an energy-dependent process on both membranes, although the involvement of a simultaneous facilitated diffusion can not be completely ruled out. Citrate alone is poorly absorbed by symbiosomes; this uptake is greatly enhanced by addition of iron. Iron–citrate was found both in the nodule cytosol and in the bacteroids. These results provide the first experimental evidence for the existence, at least in young nodules, of an important iron trafficking system from the plant host cell to the microsymbiont, through the peribacteroid membrane.

Key words: Iron uptake; Peribacteroid membrane; Citrate; Nitrogen fixation; Root nodule

1. Introduction

Plants of the family Leguminosae are, together with rhizobial microsymbionts, able to form root nodules capable of reducing atmospheric dinitrogen to ammonia [1]. Iron metabolism is of particular importance in nodules since this metal is a constituent of key proteins such as nitrogenase and leghemoglobin (Lb). Because of this, heme and heme proteins are of special interest because the cytochrome content changes both qualitatively and quantitatively during bacterial differentiation into symbiotic bacteroids [2]. In addition, there is an important need for heme in the synthesis of leghemoglobin in vivo, which makes up to 20% of the plant protein mass in the nodule cytosol [2]. Based on the failure to detect heme biosynthetic enzyme activities in the plant cytosol of root nodules, it was first proposed that the heme for Lb is exclusively synthesized by the rhizobial microsymbiont [3,4]. More recent investigations have shown that the levels of several plant enzymes in the heme biosynthesis pathway are increased in nodules, compared to roots, indicating that the plant may also contribute to heme formation [5–8]. Although the symbiont that carries out Lb heme synthesis remains uncertain [9], the fact remains that, to synthesize their cytochromes and other redox components organized as a branched system [2], bacteroids need to be supplied with iron in functioning nodules.

Whereas free-living bacteria have to cope with other species in the rhizosphere and to evolve specific and high affinity mechanisms to compete for iron, all the nutritional requirements of bacteroids, including iron, are supplied by the host plant. The situation of the bacteroids in the plant cell is particular: they are always surrounded by a membrane of plasmic origin [1]; the so-called peribacteroid membrane (PBM) isolates the microbial symbiont from the cytoplasm of the host cell to form the symbiosome, delimiting a peribacteroid space (PBS). Thus, the symbiosome constitutes the entire functional entity suitable for exchange studies between the two partners. There are several lines of evidence that PBM exhibits a restricted permeability. Till now, only dicarboxylate transport activities [10,11] and glucose permeability [11] have been observed.

In this work, we characterize for the first time the uptake of iron by purified symbiosomes from soybean nodules. The role of citrate in this uptake is highlighted and the involvement of these processes in functioning nodules is discussed.

2. Materials and methods

2.1. Materials

Soybeans (*Glycine max* Merr cv. Labrador) were grown in a glasshouse (temperature range 20–27°C) and supplied with a nitrogen-free solution as previously described [12]. Plants were inoculated with *Bradyrhizobium japonicum* strain USDA 110. Nodules were harvested when they were 4 weeks old.

All chemicals were purchased from Sigma except sodium arsenate from Merck, [^{59}Fe] (chlorid form, specific activity 13.7 mCi/mg; Amersham), [1,5-^{14}C]citric acid (specific activity 561 μCi/mg; Amersham), [U-^{14}C]succinic acid (150 mCi/mmol; CEA) and L-[U-^{14}C]glutamic acid (250 mCi/mmol; CEA).

2.2. Preparation of symbiosome and bacteroid fractions

The preparation of symbiosome and bacteroid fractions in mannitol medium was as described elsewhere [13]. A bacteroid fraction was obtained by vortexing symbiosomes for 5 min to rupture PBMs.

2.3. Uptake studies

2.3.1. Iron uptake. Symbiosomes were suspended in mannitol medium at an optical density of 0.8 units at 600 nm. A 9 ml aliquot of this suspension was incubated in aerobic conditions, at 30°C in a shaking water bath, for 10 min before addition of 1 ml of a radiolabelled mixture. This mixture, prepared 30 min before the experiments to ensure formation of the iron complexes, contained 0.2 μCi of $^{59}FeCl_3$, 1 μM $FeCl_3$ and 0.1 mM citrate in mannitol medium. Samples (1 ml) were withdrawn in duplicate at timed intervals and rapidly filtered through a Millipore HA filter (0.45 μm porosity). The filters were washed five times with 1 ml of uptake medium supplemented with 10 mM nitrilotriacetic acid (NTA) and the radioactivity counted (CG 4000 Intertechnique). NTA is used to solubilize iron retained on the filter and reduce non-specific binding of ^{59}Fe to bacterial cell surfaces. The effect of inhibitors were tested with preincubation at 30°C for 15 min with symbiosome or bacteroid suspensions.

2.3.2. Citrate uptake. 50 nCi of [1,5-^{14}C]citric acid in uptake medium which contained 100 μM citrate and 1 mM $FeCl_3$ were added to a suspension of symbiosomes (0.8 units at 600 nm). Iron was present at a 10-fold excess to ensure all the citrate available for the uptake experiments was in the form of ferric dicitrate complexes. Samples (100 μl) were taken at timed intervals and filtered through Whatman GF/F glass microfibre filters. Filters were washed five times with 1 ml of mannitol medium and radioactivity counted in 4 ml of Aqualumat using a Beckman scintillation counter (LS 6000SC). The same incubations were performed with succinic and glutamic acid as controls.

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2.4. Siderophore detection and protein determination

Nodules (60 g f.wt.) were crushed on ice in a Chelex-treated 100 mM phosphate buffer, pH 7, containing insoluble polyvinylpyrrolidone (10%, w/v). After a first centrifugation at $350 \times g$ to eliminate cell debris, bacteroids were sedimented at $8000 \times g$. The supernatant was clarified by centrifugation at $50{,}000 \times g$ and considered as the nodule cytosolic fraction. It was then filtered through Amicon membranes and the resulting filtrate < 3 kDa was concentrated under vacuum and then fractionated on silica gel plates using 50% ethanol as the solvent. Spots were visualized by spraying chromatograms with chrome azurol-S (CAS) solution [14]. Ferric citrate was used as a reference standard. Citrate concentration was determined enzymatically by the citrate lyase assay [15] using the kit developed by Boehringer (Mannheim). Protein was assayed by the method of Bradford with BSA as the standard [16].

2.5. Reductase assays

The ferric citrate reductase activity was determined by a procedure described by Dailey and Lascelles [17], based on the spectrophotometric measurement at 562 nm of the ferrozine–iron(II) complex formed during the assay. For a final volume of 1 ml in a spectrophotometric cuvette, the standard assay contained 0.8 mM ferrozine, 0.2 mM ferric citrate, 0.15 mM NADH, 0.05 mM FMN in 25 mM Tris-HCl, pH 7.4, and peribacteroid membrane fractions containing 0.2–0.8 mg protein added at zero time. The assays were performed over a period of 30 min and under aerobic conditions as ferric citrate reductase is not inhibited by oxygen [18].

3. Results

All experiments described here were performed with symbiosomes and bacteroids isolated from young nodules (3–4 weeks) for the following reasons: (i) in these nodules the iron demand by bacteroids is probably maximal for their de novo syntheses; (ii) the yield of intact symbiosomes after purification is greater than with older nodules. Under these conditions, the uptake of ferric citrate (measured by ^{59}Fe internalization) by purified symbiosomes and free bacteroids showed very similar kinetics (Fig. 1). The uptake level of the symbiosomes was always slightly higher than that of the bacteroids. This clearly demonstrates that the iron uptake capacity of the bacteroids in vivo is not restricted by the presence of the PBM. To assess the symbiosome integrity, control experiments were always performed with [^{14}C]glutamate, which is known to be unable to cross the PBM (Fig. 1). Other iron chelating compounds, e.g. the siderophores (pyoverdine and desferrioxamine) were used under the same experimental conditions: no iron uptake was observed with these ligands. The uptake levels by free bacteroids were of the same magnitude range as that described previously for free bacteria [19]. The kinetics of iron uptake were characterized by initial high rates during the first 2 min, followed by a slower step. This has also been observed in transport studies with free *Bradyrhizobium japonicum* [19] and *Azotobacter vinelandii* [20]. In a study with *Pseudomonas aeruginosa*, Cox [21] found that iron accumulation via ferric citrate was composed of at least two steps; an initial association of the ferric complex with the cell surface, followed by a slower rate of energy-dependent transport. To distinguish between iron complexes possibly bound to the outside of the symbiosomes or bacteroids from those that had been internalized, we tried washing symbiosomes and cells with NTA. We were unable to completely eliminate the initial high values which we observed in the transport assays. However, when experiments were performed at 0°C, no ferric citrate uptake was observed, strongly suggesting that these high values correspond to an internalization of the ^{59}Fe radioactivity into the cells.

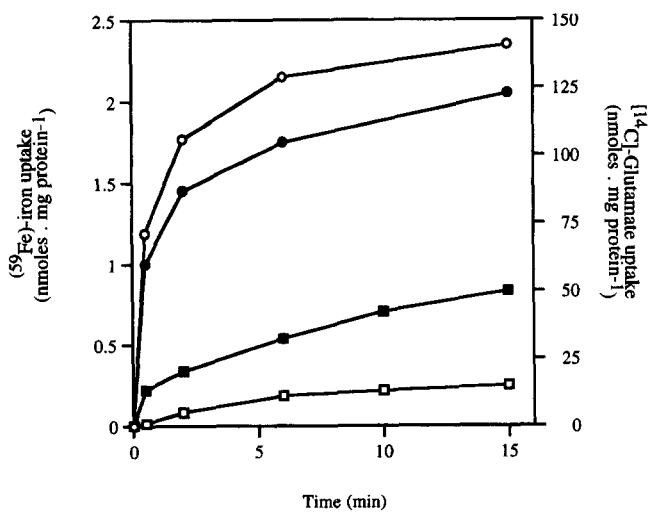

Fig. 1. Citrate-mediated [^{59}Fe]iron incorporation in symbiosomes (○) or bacteroids (●) and [^{14}C]glutamate uptake by symbiosomes (□) or bacteroids (■). Each uptake plot is the average value of duplicate assays repeated with three independent preparations.

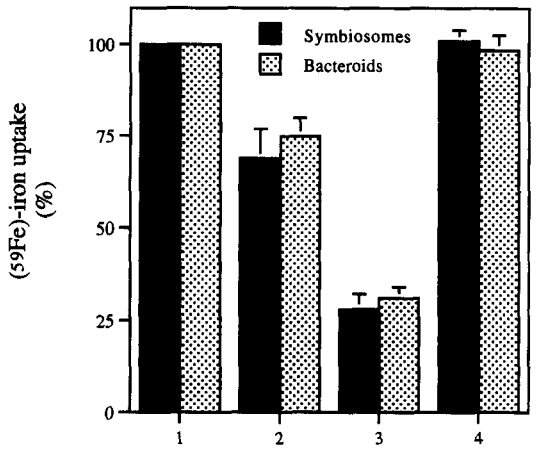

Fig. 2. Effect of inhibitors on [^{59}Fe]iron uptake by intact symbiosomes or bacteroids. (1) Control; (2) 500 μM arsenate; (3) 250 μM vanadate; (4) 1 mM NEM. The 100% uptake value corresponds to 2.5 nmol ^{59}Fe absorbed · min^{-1} · mg protein^{-1}. The data points are the means of results of six experiments.

In the presence of increasing concentrations of ferric citrate, uptake plots gave rise to saturation curves for both symbiosomes and bacteroids, although some variability was observed from one preparation to another. Under these conditions, bacteroids exhibited a slightly higher affinity for iron ($K_m = 7 \pm 4\,\mu M$) than did symbiosomes ($K_m = 14 \pm 9\,\mu M$). Values of V_{max} were, respectively, 2.9 ± 1.8 and 1.6 ± 0.8 nmol $^{59}Fe \cdot min^{-1} \cdot mg$ protein^{-1} for symbiosomes and bacteroids.

The effect of inhibitors on this process is shown in Fig. 2. Similar results were obtained with symbiosomes and bacteroids with all tested inhibitors. Arsenate, which lowers the ATP pool, and vanadate, which inhibits the plasma-type ATPases, gave rise to inhibitions ranging from 30% to 75%, whereas *N*-ethyl maleimide (NEM), a specific reagent of thiol groups, was without effect.

The [^{14}C]citrate internalization was also measured (Fig. 3).

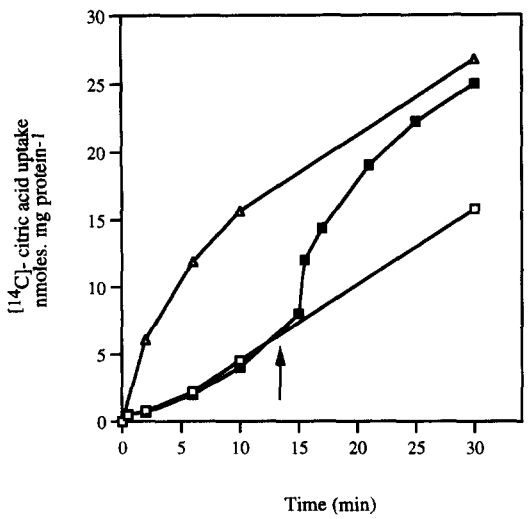

Fig. 3. Influence of iron upon citrate incorporation by symbiosomes. [^{14}C]Citric acid (100 μM) uptake (□) without iron; (△) with 1 mM $FeCl_3$; (■) with 1 mM $FeCl_3$ added 15 min after the zero time of incubation. The arrow indicates iron addition. Each uptake plot is the average value of duplicate assays repeated with three independent preparations.

Citrate alone was poorly absorbed by the symbiosomes compared to dicarboxylates, as found previously [11]. This uptake was greatly stimulated by the presence of iron in the incubations. When iron was preincubated with citrate before the zero time of the kinetics experiment, an uptake curve presenting a high initial rate was observed. After 6 min of incubation, the uptake level was six-fold higher than that obtained without iron in the incubation. On the other hand, when iron was added once the kinetics experiment had already been started, a stimulation of the citrate uptake was observed, almost immediately after the addition of iron. Here again a high initial rate was observed. In control experiments, the addition of iron had no effect on [^{14}C]succinate uptake by symbiosomes under the same experimental conditions, ruling out any stimulatory effect of iron on citrate absorption via a permeabilisation of the PBM due to side effects, i.e. lipid peroxidation [22].

Incubations of symbiosomes with labelled iron and increasing amounts of iron-free citrate allowed a 80% quenching of the iron uptake when the Fe:citrate ratio reached 1:1000; a total inhibition of this uptake occurred with a 1:10,000 ratio. The percentage of inhibition was calculated from a 100% uptake value corresponding to a 1:10 ratio.

When the nodule cytosolic fraction was checked for the presence of siderophores using the CAS test, only one band was detected under our experimental conditions. This band co-migrated with iron–citrate with an R_f of 0.44, close to the R_f previously described (0.42) for this complex in the same solvent [19]. In the bacteroid fraction, the same band was present; however, in this case a minor band with a R_f of 0.11 was also present. On the other hand, the concentration of citrate in the cytosolic fraction, determined enzymatically, was 100 μg/g fresh weight of nodule.

No ferric citrate reductase activity has been detected in PBMs from soybean root nodules. On the other hand, the huge amounts of mannitol in our extracts precluded any accurate measurement of the ferric citrate reductase activity in the PBS.

4. Discussion

When one considers the iron import necessary for the synthesis of rhizobial iron and heme proteins (e.g. nitrogenase and cytochromes) and the additional demand for iron that may be required for the synthesis of Lb, it is clear that a substantial iron flux into bacteroids is essential for symbiotic nitrogen fixation [23]. The only way for the bacteroid in the symbiosome to be supplied with iron is to take it up from the host cell cytosol. To our knowledge, this is the first report characterizing the transport of iron through the PBM; this process was only very briefly mentioned in [24] as unpublished results. The results presented here clearly show that, in spite of its restricted permeability, the PBM allows a sufficient flux of iron to the bacteroid to allow an optimal synthesis of, at least, all the iron-containing molecules necessary for the microsymbiont's metabolism. This uptake activity appears to occur via a citrate–iron complex transport, as ferric citrate was detected in the host cell cytosol and in the bacteroids while no other siderophore could be detected (except for a minor band in the bacteroids). The concentration of citrate in the nodule cytosolic fraction found in this study (100 μg/g fresh weight of nodule) is in good agreement with that previously described (142 μg/g fresh weight of nodule) [25]. This process can be related to the possibility, for soybeans, of transporting iron as ferric citrate [26] and to the presence of high concentrations of iron chelates in the 300–600 Da fraction of soybean nodule cytosol [27]. All these results are consistent with iron citrate uptake by symbiosomes and bacteroids occurring in vivo. We failed to detect citrate in the PBS; this suggests that this molecule is not stored in this space but immediately absorbed by the microsymbiont.

The similar sensibilities of symbiosomes and bacteroids to inhibitors suggests the importance of the motive capacities of the microsymbiont in both cases. The absence of effect of NEM addition excludes the involvement of thiol groups in this iron transport. The effect of vanadate suggests that a plasma-type ATPase is involved in this process. Moreover, it must be noted that this molecule has been shown to inhibit the PBM ATPase of lupin root nodules [28]. The action of arsenate, although less pronounced, confirms the involvement of ATPases in this transport process. These results, together with the inhibition of uptake at 0°C, are consistent with an energy-dependent process on both membranes. However, the possible involvement of a simultaneous facilitated diffusion process could not be completely ruled out as the low molecular mass of the ferric iron dicitrate complex of 443 Da may allow its diffusion through the PBM, as observed in *E. coli* for the cloned iron uptake system of *Serratia marcescens* [29]. The simultaneous existence of these two processes could explain the variability in the K_m determination [30].

The absence of ferric citrate reductase activity in PBMs is consistent with previous studies reporting ferric citrate reductases as soluble enzymes, located in the cytoplasm [31,32]. Moreover, a cytoplasmic location of this enzymatic activity implies that the ferric citrate complex crosses the cell membranes before being dissociated and this is in agreement with the observed uptake of ^{14}C-labelled citrate.

The *Bradyrhizobium* strain used in this study has been shown to be non-siderophore producing [19]; however, it was described, as a free-living bacteria, to be able to utilize ferric citrate as an iron source [19]. Moreover, the USDA 110 strain

was able to grow in a modified YEM medium where the carbon source was citrate (10 g/l) instead of mannitol (unpublished results). Thus, these bacteria may incorporate the iron–citrate complex by the same pathway that they use to incorporate citrate itself as a carbon source or via a specific receptor for the iron part of the complex. In this way, a TonB-like receptor may be implicated, as the finding of TonB-like genes in a variety of organisms suggests that the general scheme for iron transport, involving TonB, ExbB and ExbD, will probably be the same in all Gram-negative bacteria [33]. The problem appears to be different concerning the PBM: citrate alone is poorly transported through this membrane, however, the inhibition of iron citrate uptake by excess citrate suggests that both are recognized by the same site on the PBM, but with largely different affinities. Indeed high concentrations of free citrate are necessary to produce an inhibitory effect.

The existence of similar iron uptake capacities in both free-living and symbiotic bacteria is related in vivo to an important iron demand by the microsymbiont. This significant iron uptake capacity of bacteroids isolated from young nodules supports the hypothesis of the microsymbiont contribution to heme synthesis for leghemoglobin. In that way, heme could be excreted in the PBS and then cross the PBM, as the initial rate of efflux of heme from biological membranes is fast [34].

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References

[1] Bergersen, F.J. (1982) in: Root Nodules of Legumes: Structure and Function, pp. 23–50, Research Studies Press, Chichester.
[2] Appleby, C.A. (1984) Annu. Rev. Plant Physiol. 35, 443–478.
[3] Cutting, J.A. and Schulman, H.M. (1969) Biochim. Biophys. Acta 192, 486–493.
[4] Nadler, K.D. and Avissar, Y.J. (1977) Plant Physiol. 60, 433–436.
[5] Dimitrijevic, L., Puppo, A., Trinchant, J.C. and Rigaud, J. (1989) J. Plant Physiol. 134, 642–644.
[6] Jacobs, N.J., Borotz, S.E. and Guerinot, M.L. (1989) J. Bact. 171, 573–576.
[7] Sangwan, I. and O'Brian, M.R. (1992) Plant Physiol. 98, 1074–1079.
[8] Madsen, O., Sandal, L., Sandal, N.N. and Marcker, K.A. (1993) Plant Mol. Biol. 23, 35–43.
[9] Kaczor, C.M., Smith, M.W., Sangwan, I. and O'Brian, M.R. (1994) Plant Physiol. 104, 1411–1417.
[10] Udvardi, M.K., Price, G.D., Gresshoff, P.M. and Day, D.A. (1988) FEBS Lett. 231, 36–40.
[11] Herrada, G., Puppo, A. and Rigaud, J. (1989) J. Gen. Microbiol. 135, 3165–3171.
[12] Puppo, A., Dimitrijevic, L. and Rigaud, J. (1982) Planta 156, 374–379.
[13] Herrada, G., Puppo, A. and Rigaud, J. (1992) Biochem. Biophys. Res. Commun. 184, 1324–1330.
[14] Schwyn, B. and Neilands, J.B. (1987) Anal. Biochem. 160, 47–56.
[15] Moellering, H. and Gruber, W. (1966) Anal. Biochem. 17, 369–376.
[16] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[17] Dailey, H.A. and Lascelles, J. (1977) J. Bacteriol. 129, 815–820.
[18] Hallé, F. and Meyer, J.M. (1989) Biol. Metals 2, 18–24.
[19] Guerinot, M.L., Meidl, E.J. and Plessner, O. (1990) J. Bacteriol. 172, 3298–3303.
[20] Knosp, O., von Tigerstrom, M. and Page, W.J. (1984) J. Bacteriol. 159, 341–347.
[21] Cox, C.D. (1980) J. Bacteriol. 142, 581–587.
[22] Herrada, G., Puppo, A., Moreau, S., Day, D.A. and Rigaud, J. (1993) FEBS Lett. 326, 33–38.
[23] Nadler, K.D., Johnston, A.W.B., Chen, J.W. and John, T.R. (1990) J. Bacteriol. 172, 670–677.
[24] Guerinot, M.L. (1991) Plant Soil 130, 199–209.
[25] Streeter, J.G. (1987) Plant Physiol. 85, 768–773.
[26] Tiffin, L.O. (1970) Plant Physiol. 45, 280–283.
[27] Becana, M. and Klucas, R.V. (1992) Proc. Natl. Acad. Sci. USA 89, 8958–8962.
[28] Domigan, N.M., Farnden, K.J.F., Robertson, J.G. and Monk, B.C. (1988) Arch. Biochem. Biophys. 264, 564–573.
[29] Angerer, A., Klupp, B. and Braun, V. (1992) J. Bacteriol. 174, 1378–1387.
[30] Ter Kuile, B.H. and Cook, M. (1994) Biochim. Biophys. Acta 1193, 235–239.
[31] Moody, M.D. and Dailey, H.A. (1985) J. Bacteriol. 163, 1120–1125.
[32] Cox, C.D. (1980) J. Bacteriol. 141, 199–204.
[33] Guerinot, M.L. (1994) Annu. Rev. Microbiol. 48, 743–772.
[34] Liem, H., Noy, N. and Muller-Eberhard, U. (1994) Biochim. Biophys. Acta 1194, 264–270.