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In vitro Indole-3-Acetic Acid Uptake in Symbiosomes from Soybean (*Glycine max* L.) Root Nodules

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Abstract

Symbiosomes isolated from *Bradyrhizobium japonicum*-soybean (*Glycine max* L.) root nodules were incubated *in vitro* with [^{3}H] indole-3-acetic acid (IAA). This resulted in a time-dependent increase in radioactivity in the symbiosomes indicating IAA uptake. A plot of 1/V versus 1/[IAA] indicated that a nonsaturable uptake system was present in the symbiosome membrane with an additional, saturable uptake mechanism, which operates at low IAA concentrations. The transporter exhibited an apparent K_m of 140 nM and a V_{max} of 4.1 pmol • min^{-1} • mg^{-1} protein. The auxin-transport inhibitor naphthylphthalamic acid (NPA) inhibited IAA accumulation in the symbiosomes at low IAA concentrations. This indicates that the saturable uptake mechanism may be ascribed to the presence in the symbiosome membrane of an auxin-efflux carrier system, which directs transport of IAA from the plant cytosol towards the bacteroids.

Keywords: Auxin transport, indole-3-acetic acid, soybean nodules, symbiosomes, symbiosome membrane

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1. Introduction

The soil bacterium *Bradyrhizobium japonicum* elicits the formation of root nodules on soybean (*Glycine max* L.). Within the nodule microenvironment *Bradyrhizobium* bacteria are transformed to bacteroids which fix atmospheric nitrogen into ammonia. The formation of root nodules and maintenance of an effective symbiosis is expected to be based on a continuous and carefully balanced exchange of molecular signals and metabolites between the symbionts.

The phytohormone indole-3-acetic acid (IAA) was proposed to play a role in nodule development half a century ago (Thimann, 1936). Phytohormone ratios are believed to be important in nodule development because it has been found that IAA transport inhibitors such as naphthylphthalamic acid (NPA) can induce nodule-like structures on alfalfa roots (Hirsch et al., 1989). Flavonoids produced by legume roots can function as endogenous IAA transport inhibitors (Jacobs and Rubery, 1988), thereby possibly affecting the endogenous hormone balance and perhaps nodule development. Rhizobia are capable of producing IAA, and flavonoids have been shown to stimulate IAA production by rhizobia (Prinsen et al., 1991). Bacterial production of IAA may, however, not be essential for nodule development as purified bacterial lipooligosaccharides have been demonstrated to be the sole bacterial compound needed to initiate formation of meristematic foci and empty nodules on alfalfa (Truchet et al., 1991).

Established root nodules contain higher levels of auxin than the corresponding root tissue and the most abundant auxin in the nodules is IAA (Thimann, 1936). Soybean nodules infected by wild-type bradyrhizobia contain approximately 200 nM free IAA and about 3 μM peptidyl IAA (Hunter, 1987). It has not been established which of the symbiotic partners is the source of the nodule IAA, and the physiological role of IAA in the functioning nodule still needs to be assigned. *Bradyrhizobium japonicum* mutants that accumulate high levels of IAA in culture induce nodules containing higher levels of IAA than nodules induced by the parental strain (Hunter, 1987, 1989). This indicates that the bacteroids may contribute to the IAA level in the nodule, but it has not yet been established to what extent IAA may be transferred between the symbionts. Thus, it cannot be ruled out that IAA of bacterial origin in soybean nodules resides within the symbiosomes. Symbioses induced by the IAA-overproducing mutants have reduced symbiotic performance (Hunter, 1987), indicating that a carefully balanced level of IAA is required to maintain symbiotic nitrogen fixation.

In addition to the ability to synthesize IAA, *Bradyrhizobium japonicum* strains possess enzymes able to catabolize IAA (Egebo et al., 1991), thus the

bacteroids may potentially constitute an overall sink, as well as a source, of IAA in nodules.

Bacteroids in infected nodule cells are maintained extracellular in that they are enclosed by the plant-derived, tissue specific, symbiosome membrane. Any exchange of compounds between the symbionts must pass through this symbiosome membrane. The membrane has many features in common with the plasma membrane (see Brewin, 1990). Plant plasma membranes transport IAA by transmembrane diffusion and via two specific and saturable IAA transporters, the auxin-H^+ symport and the auxin-anion efflux carrier (Hertel et al., 1983; Sabater and Sabater, 1986; Heyn et al., 1987; Lomax and Hicks, 1992). The symbiosome membrane has been demonstrated to contain an auxin-binding protein, which was suggested to be part of an auxin-efflux carrier system (Jacobi et al., 1993). The plasma membrane auxin-efflux carrier is directed to transport IAA out of the cell, but the mode of action of the proposed symbiosome membrane auxin-efflux carrier system was not determined.

The aims of the present work were to investigate the capability of the symbiosome membrane to transport IAA, and to determine by *in vitro* experiments the extent of operation and the direction of IAA transport mediated by the symbiosome membrane auxin-efflux carrier system.

2. Materials and Methods

Plant material

Soybeans (*Glycine max* L. cv "Evans") were grown in a growth cabinet under conditions as previously described (Rosendahl and Jakobsen, 1987). Inoculation was performed at sowing with 5 ml per seed of a 3-day-old yeast broth suspension culture of *Bradyrhizobium japonicum* strain 110 (Hahn and Hennecke, 1984). Six weeks after seedling emergence, 10 g of nodules were harvested on ice, and all subsequent handling of the biological material was performed at 4°C using chilled buffers.

Isolation of symbiosomes

Symbiosomes were isolated by Percoll density-gradient centrifugation as previously described for pea-*Rhizobium* symbioses (Rosendahl et al., 1992) except for the use of a Percoll concentration of 55% in the continuous Percoll gradients. The 55% Percoll gradients were required to isolate soybean symbiosomes, which have a higher density than pea symbiosomes. The present procedure for isolation of symbiosomes reveals pure symbiosomes with no cross

contamination from other organelles or membrane fragments as evident from marker enzyme activities (Christiansen et al., in press). The integrity of the symbiosomes was monitored by microscopy with a Zeiss light microscope equipped with differential interference contrast.

Protein was determined according to Bradford (1976) on ultra sonicated organelles using bovine serum albumin as a standard.

Transport studies

Indole-3-[5-^{3}H]-acetic acid ([^{3}H]IAA) 962 GBq • mmol^{-1} was purchased from Amersham, UK. ^3H_2O was obtained from Institute for Atomic Energy, Oslo, Norway. NPA was kindly donated by Professor R. Rajagopal, The Royal Veterinary and Agricultural University, Copenhagen, Denmark. AR-200 silicone oil was obtained from Wacker-Chemie, Mänchen Werk Burghausen, Germany. Scintillation cocktail was purchased from Packard Instr., Groningen, The Netherlands. All other chemicals were purchased from Sigma.

Uptake of [^{3}H]IAA into symbiosomes was measured using the silicone oil filtration technique (Palmieri and Klingenberg, 1979). The reaction mixture (300 µl) contained purified symbiosomes (600 µg protein) in wash buffer (25 mM MES (pH 7.0) containing 350 mM mannitol, 3 mM $MgSO_4$, and 0.5 mM $CaCl_2$), [^{3}H]IAA at a specific radioactivity of 123 MBq • µmol^{-1}, and, where indicated, the auxin transport inhibitor NPA at 200 µM or the protonophore, carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone (FCCP) at 10 µM which constitutes the upper concentration limit for dissolving FCCP in a stock solution. The reaction mixture was layered in 500 µl microfuge tubes on top of a silicone oil layer (70 µl) which was layered on 30 µl 1.6 M $HClO_4$. The reactions were conducted in triplicate at 20°C and were initiated by applying [^{3}H]IAA to the reaction mixture. At the appropriate times, the reaction was terminated by centrifuging in a Sigma 2 K 15 laboratory centrifuge in a swinging-bucket rotor at 4000 • g for 5 s. The short centrifugation time allows symbiosomes to pass the silicone oil layer, whereas most of any contaminating free bacteroids in the reaction mixture are prevented from pelleting. The liquid volume of the pelleted symbiosomes was determined in a parallel experiment by incubating symbiosomes with 11 kBq of ^3H_2O. After centrifugation, a U-shaped metal wire was introduced into the tubes to the bottom portion of the silicone oil layer, and the tubes were quickly immersed in liquid N_2. The aqueous phase on top of the silicone oil layer was removed by pulling the metal wire. The residual silicone oil in the test tube was removed keeping the acid layer frozen. When thawed the radioactivity in the acid layer was measured on a liquid scintillation analyzer (Packard 1900 TR).

3. Results

The silicone oil filtration technique facilitates the rapid removal of symbiosomes from the incubation medium. All metabolic activity is instantly stopped when the symbiosomes reach the acidic layer. The short centrifugation time (5 s) allows only symbiosomes to pass the silicone oil layer whereas any contaminating free bacteroids remain in the incubation medium as the mass of individual bacteroids is lower than that of symbiosomes (Udvardi et al., 1988). The separation of symbiosomes from free bacteroids by the silicone oil centrifugation was controlled by substituting the acid layer with wash buffer including 4% dextran and examination by light microscopy of the lower wash buffer-dextran layer and the incubation layer. Large soybean multi-bacteroid symbiosomes can be distinguished from single-bacteroid symbiosomes and free bacteroids by differential interference contrast light microscopy (Fig. 1). The volume of the symbiosomes pelleted in the acidic layer was estimated from results of experiments with ^3H_2O. Symbiosome liquid volumes were approximately 5 µl when 600 µg protein was applied to the incubation mixture. The amount of ^3H_2O in the pellet did not change between 1 min to 30 min incubation, supporting the validity of the estimates of volume.

The incubation of symbiosomes in [^{3}H]IAA resulted in a time-dependent increase in radioactivity in the symbiosome pellet (Fig. 2). The increase in the presumed IAA concentration in symbiosomes as a function of time is an index of uptake, thus the results appear to demonstrate that IAA is transported across the symbiosome membrane. At 15 min, a steady level was reached at 5 pmol IAA in the symbiosome pellet. This corresponds to an internal IAA concentration of 1 µM in the pelleted symbiosomes as calculated from the estimated symbiosome volume.

Uptake of IAA as a function of substrate concentration over a range from 5 nM to 10 µM was studied. A plot of V versus IAA concentration suggested that a nonsaturable uptake system was present in the symbiosome membrane (Fig. 3). When the data were plotted in a double-reciprocal manner, the resulting curve was consistent with a biphasic uptake system (Fig. 4). The values at the high substrate concentrations (> 1 µM) are assumed to represent a nonsaturable uptake system, whereas the values at the low substrate concentrations (< 200 nM) probably represent a saturable uptake mechanism. The latter values were used to calculate an apparent K_m of 140 nM and a V_{max} of 4.1 pmol • min^{-1} • mg^{-1} protein for the saturable uptake system.

Controls with unlabeled IAA were performed to test specificity of the accumulation of radioactivity in the symbiosome pellet. When the IAA concentration was varied by varying the amount of unlabeled IAA and keeping the amount of [^{3}H]IAA constant, the overall radioactivity in the symbiosome

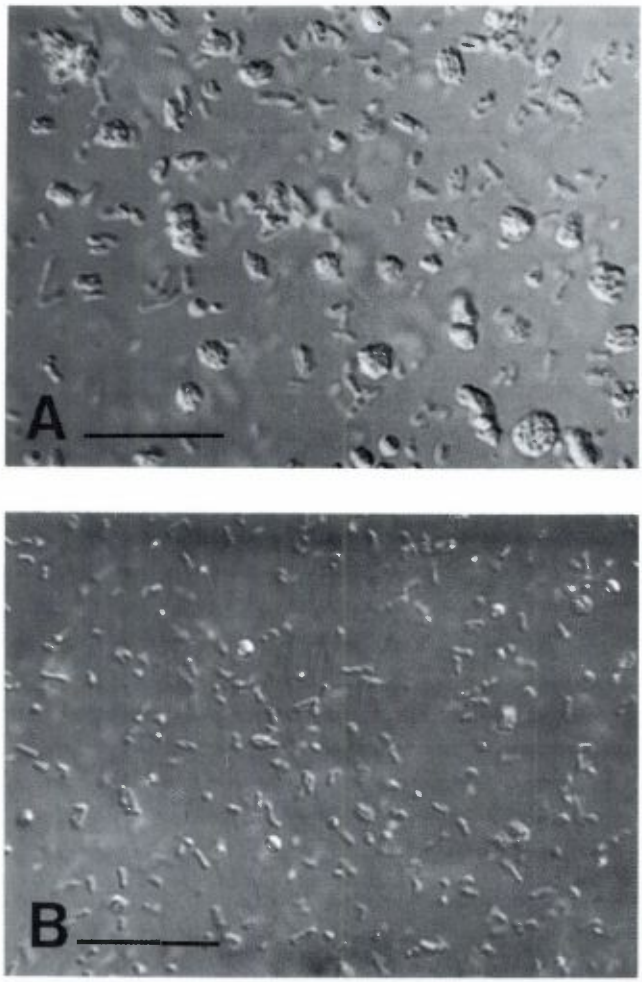

Figure 1. Differential interference contrast light micrographs of (A) symbiosomes and (B) free bacteroids from soybean root nodules. The free bacteroids arise from symbiosomes in which the symbiosome membrane was broken by osmotic shock. Bar represents 10 μm.

pellet declined at increasing concentrations of carrier IAA. Furthermore, when symbiosomes were pre-incubated for 15 min with excess of unlabeled IAA (5 mM), the accumulation of radioactivity in the symbiosomes was inhibited in a competitive manner, i.e. the inhibitory effect of pre-incubation decreased at increasing [^{3}H]IAA concentrations (data not shown).

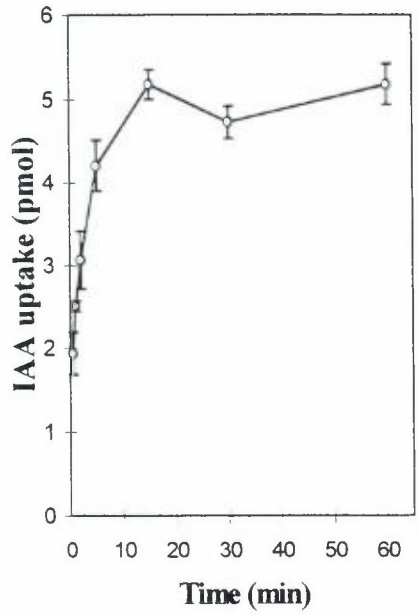

Figure 2. Time dependent accumulation of [^{3}H]IAA in symbiosomes from soybean-*Bradyrhizobium* nodules. Assay mixture contained 100 nM IAA. Each value represents the mean ± SEM (n = 3).

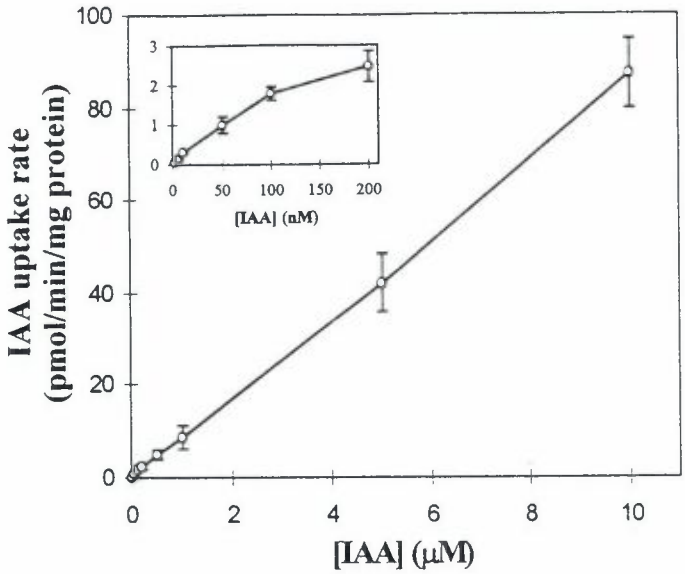

Figure 3. Effect of IAA concentration on the rate of [^{3}H]IAA uptake by symbiosomes from soybean-*Bradyrhizobium* nodules. Insert illustrates the uptake of [^{3}H]IAA at low substrate concentrations. Incubation time was 5 min. Each value represents the mean ± SEM (n = 3).

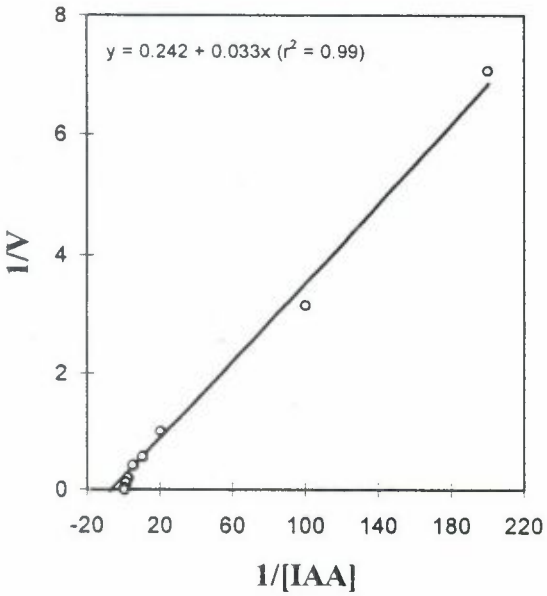

Figure 4. Double-reciprocal plot for the effect of IAA concentration on uptake rate (data from Fig. 3). The values for the five lowest IAA concentrations were used to estimate K_m and V_{max}.

The inclusion of the auxin transport inhibitor NPA at a concentration of 200 µM resulted in a decrease in IAA accumulation in the symbiosomes at the low IAA concentrations (Fig. 5). At IAA concentrations above 500 nM, there was no significant effect of NPA on IAA accumulation (data not shown). The effect of NPA on IAA accumulation indicates the presence of a specific auxin carrier in the symbiosome membrane. The protonophore FCCP applied at a concentration of 10 µM did not affect accumulation of IAA in symbiosomes (Fig. 5). Thus, there is no indication for presence of an auxin-specific symport in the symbiosome membrane.

4. Discussion

The data suggest the presence of a biphasic IAA uptake system in the symbiosome membrane. A nonsaturable uptake system operates at IAA concentrations above 500 nM indicating passive uptake. At IAA concentrations below 200 nM, a saturable carrier-mediated uptake system may operate (Figs. 3 and 4).

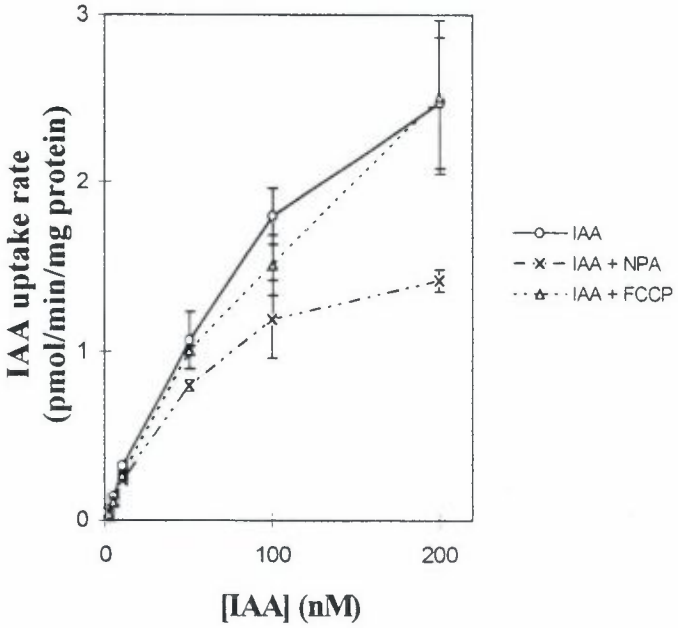

Figure 5. Effect of NPA (200 μM) and of FCCP (10 μM) on the uptake of IAA in symbiosomes from soybean-*Bradyrhizobium* nodules.

The specific auxin-transport inhibitor NPA decreases IAA accumulation in symbiosomes (Fig. 5) confirming the presence of an auxin-efflux carrier system in the symbiosome membrane (Jacobi et al., 1993). NPA acts on IAA accumulation only at IAA concentrations below 500 nM, we thus postulate the presence of a biphasic uptake system with the saturable auxin carrier operating only at low IAA concentrations. The inhibitory effect of NPA on IAA accumulation in the symbiosomes indicates, that the auxin carrier in the symbiosome membrane directs the transport of IAA towards the bacteroids. This direction of carrier-mediated IAA transport in the symbiosome membrane is equivalent to that of the plasma membrane as the IAA carrier in both membranes mediates an efflux of IAA from the plant cytosol (Hertel et al., 1983; Sabater and Sabater, 1986; Heyn et al., 1987; Lomax and Hicks, 1992). The plasma membrane auxin-efflux carrier works against a pH gradient. The symbiosome membrane contains a proton-pumping ATPase (Udvardi and Day, 1989), which is likely to mediate a pH gradient across the symbiosome membrane. An auxin-efflux carrier in the symbiosome membrane would thus operate against a pH gradient similar to the mode of operation of the auxin-efflux carrier in the plasma membrane when the carrier directs auxin into the

symbiosomes. The physiological role of a symbiosome membrane IAA carrier is unknown, but it may be involved in the control of the overall auxin concentration in infected nodule cells.

The content of free IAA in soybean root nodules infected by wild-type bradyrhizobia was reported to be 0.04 μg • g fresh weight of nodules^{-1} (Hunter, 1987). Based on an even distribution of IAA in the tissue and a volume of 1 ml per g nodule this content is estimated to correspond to a concentration of free IAA in soybean nodules of approximately 200 nM. The estimated K_m of 140 nM for the IAA-carrier system in soybean symbiosome membranes (Fig. 4), and the inhibitory effect of NPA on the uptake of IAA in symbiosomes in the IAA concentration range of 5 to 500 nM (Fig. 5) indicate that the carrier system may mediate transfer of IAA from the plant cytosol to the interior of the symbiosomes *in vivo*.

Some *Bradyrhizobium japonicum* strains including the one used in the present study are able to catabolize IAA in the free-living state (Egebo et al., 1991), but the extent of IAA catabolism in the bacteroid state of bradyrhizobia has not yet been determined. The present results, which demonstrate *in vitro* uptake of IAA in symbiosomes (Figs. 2 and 3) indicate that IAA catabolism in bacteroids may occur.

IAA-overproducing *Bradyrhizobium japonicum* strains induce nodules with reduced symbiotic nitrogen fixation (Hunter, 1987). The physiological background for this reduction in nitrogen fixation has not been identified, but it indicates that symbiotic nitrogen fixation in established nodules requires certain levels of IAA in the tissue. The IAA transport mechanisms identified in the symbiosome membrane may be involved in the control of the auxin concentration in infected nodule cells, although at IAA concentrations above 500 nM, transport of IAA across the symbiosome membrane occurs in an unsaturable manner apparently independent of the auxin-efflux carrier.

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