An Auxin Binding Protein is Localized in the Symbiosome Membrane of Soybean Nodules

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Binding of tritiated indole-3-acetic acid ([^{3}H]IAA) to symbiosome membranes of soybean nodules occurred in a protein-dependent manner and was competitively inhibited by unlabeled indole-3-acetic acid (IAA), 1-naphthaleneacetic acid (1-NAA) and dithiothreitol (DTT), but not by tryptophan and benzoic acid. The symbiosome membranes bound IAA with a K_D of 1×10^{-6} M. Photoaffinity labeling identified an auxin-binding protein (ABP) in the symbiosome membrane with an apparent molecular mass of 23 kDa. This 23 kDa protein was labeled either with 5-azido-[7-^{3}H]indole-3-acetic acid ([^{3}H]N_3IAA) or with 5′-azido-[3,6-$^3H_2$]-1-naphthylphthalamic acid ([$^3H_2$]N_3NPA). Labeling of the 23 kDa protein with [^{3}H]N_3IAA was competitively inhibited by unlabeled IAA and 1-NAA. NPA and quercetin, inhibitors of polar auxin transport, as well as rutin, a glycosylated derivative of quercetin, competed with IAA for binding. Conversely, [$^3H_2$]N_3NPA labeling was inhibited by unlabeled IAA and NPA. The 23 kDa symbiosome membrane protein was partially solubilized with Triton X-100 and nearly completely using Triton X-114. The observation that auxin transport inhibitors compete with IAA for binding suggests that the symbiosome membrane ABP could be part of an auxin efflux carrier system required to control the auxin concentration in infected soybean nodule cells.

Introduction

Soil bacteria such as *Rhizobium, Bradyrhizobium,* and *Azorhizobium* can form a beneficial relationship with various members of the plant family *Leguminosae*. As a result, root nodules are formed in which atmospheric nitrogen can be fixed into organic nitrogen. Hence an understanding of the factors at the molecular level regulating the formation of root nodules by these bacteria will ultimately contribute to the understanding of plant development in general.

Although the factors that trigger nodule development are largely unknown, a change in phytohormone ratios may be important in the regulation of this process. It has been found that auxin transport inhibitors such as naphthylphthalamic acid (NPA) or triiodobenzoic acid (TIBA) can induce nodule-like structures on alfalfa roots [1]. Moreover, root nodules of various legumes contain higher levels of auxin than do the roots [2] and the most abundant auxin in root nodules is indole-3-acetic acid (IAA) [3]. IAA has been shown to be produced in plant apical meristems and then transported to the root nodules [4]. Rhizobia themselves are also capable of producing auxins as well as other phytohormones [5]. The isolation of mutants of *Bradyrhizobium japonicum* that accumulate IAA in culture and induce nodules containing higher IAA levels, compared to nodules elicited by the parent strain, indicates that nodule IAA can be at least in part of bacterial origin [6, 7]. Associations induced by these mutant strains show reduced symbiotic fitness indicating that auxins could play an important role in the communication between microsymbiont and host plant in mature root nodules.

Auxin perception and the ensuing responses are thought to be mediated by auxin receptors. Several auxin binding sites have been identified in the en-

Abbreviations: ABP, auxin binding protein; BSA, bovine serum albumin; DTT, DL-dithiothreitol; EDTA, ethylenedinitrilotetraacetic acid; [^{3}H]N_3IAA, 5-azido-[7-^{3}H]IAA; [$^3H_2$]N_3NPA, 5′-azido-[3,6-$^3H_2$]NPA; IAA, indole-3-acetic acid; NPA, 1-naphthylphthalamic acid; PMSF, phenylmethylsulfonylfluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TIBA, 2,3,5-triiodobenzoic acid; Tris, tris-(hydroxymethyl)aminomethane.

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doplasmic reticulum, the tonoplast and the plasma membrane [8] and proteins corresponding to these sites have been proposed to play a role in auxin perception or transport [9–11]. Using photoaffinity labeling with synthetic light-sensitive auxin analoges or auxin transport inhibitors, several proteins were identified in membrane fractions of zucchini, tomato and maize [11–15]. One of the proteins that was identified by photoaffinity labeling in maize plasma membranes was shown to be immunological related to another, now well studied, ER-located auxin binding protein [15]. The finding that this protein was also labeled with $[^3H_2]N_3NPA$ suggested that it is a component of the auxin efflux carrier which is thought to play an important role in controlling the physiological concentration of auxins [11].

In this report, we demonstrate that symbiosome membranes isolated from soybean nodules bind specifically IAA and using synthetic auxin-derived photoaffinity probes we have identified a 23 kDa protein which shares similarities in auxin and phytotropin binding with pm23 from maize, a protein suggested to be a component of the auxin efflux carrier [11, 15].

Materials and Methods

Plant material and preparation of membrane vesicles

Glycine max L. Merr. *cv.* Preston (Ottawa research station, Canada) was infected with *Bradyrhizobium japonicum* 110spc4 and grown under controlled conditions as previously described [16]. Nodules were harvested 24 to 30 days post-infection and used immediately for the isolation of the symbiosome membrane. Nodule cell fractionation and isolation of the symbiosome membrane were performed essentially as described in Mellor and Werner [17] with some modifications [18]. The resulting symbiosome membrane vesicles were frozen in liquid nitrogen and stored at −80 °C or used directly for photoaffinity labeling or for equilibrium binding studies.

[^{3}H]IAA binding assays

[^{3}H]IAA binding was assayed by equilibrium dialysis according to Reinhard and Jacobsen [19]. One half of the dialysis chamber was filled with 250 µl membrane vesicles (50 µg protein) in buffer A (10 mM Na-citrate, pH 5.5, 5 mM $MgCl_2$) and the other half with 250 µl buffer A. For competition studies, various amounts of unlabeled competitor were added to both half cells of the dialysis chamber. Ten microliters of [^{3}H]IAA solution were added to each cell of the dialysis chamber, resulting in a final concentration of 55 nM IAA. Assays were performed in triplicate for each competitor concentration. After 8 h of incubation at 20 °C, 200 µl aliquots were transfered from each half cell into 8 ml of scintillation cocktail and counted in a liquid scintillation counter (Beckman Instruments). The difference in radioactivity between the half-cells of the dialysis chamber, which reflects binding of labeled IAA to membrane vesicles, was determined. Results of saturable IAA binding to symbiosome membranes are plotted according to Scatchard [20].

Gel electrophoresis

Polypeptides were separated on 12.5% (w/v) SDS-PAA gels [21] using the SDS loading buffer of Day *et al.* [22]. Polyacrylamide gels were stained with silver nitrate [23] or 0.25% (w/v) Coomassie Brilliant Blue R-250 in 45% (v/v) methanol and 10% (v/v) glacial acetic acid.

Photoaffinity labeling

Photoaffinity labeling using $[^3H]N_3IAA$ as ligand was performed according to Campos *et al.* [14], and with $[^3H_2]N_3NPA$ as described by Zettl *et al.* [11]. After labeling, the membranes were pelleted at 100,000 × *g* (11 min, 2 °C). The pelleted symbiosome membrane vesicles were then dissolved in 20 µl SDS-loading buffer and the proteins separated using 12.5% (w/v) SDS-PAA gels. Gels were fluorographed [24], dried and used to expose Kodak XAR-5 X-ray film for at least two weeks.

To study solubilization of photoaffinity labeled symbiosome membrane proteins, pelleted membranes were resuspended in 100 µl 10 mM Tris-Mes (pH 6.5), 250 mM sucrose containing either 1% (w/v) Triton X-100 or 1% (w/v) Triton X-114. After incubation on ice for 30 min, followed by centrifugation at 100,000 × *g* (11 min, 2 °C), the pellets were resuspended in 20 µl SDS-loading buffer and the supernatant was diluted 1 : 1 with SDS-

loading buffer. Samples were then analyzed by SDS-PAGE and fluorography as described above.

Protein determination

Protein concentrations were determined by the method of Lowry [25] using BSA as standard.

Results and Discussion

Characterization of [^{3}H]IAA binding to the symbiosome membrane

Binding of [^{3}H]IAA to symbiosome membranes attained equilibrium after dialysis for 8 h at 20 °C. Binding occurred in a protein concentration dependent manner (Fig. 1). Addition of 1% (w/v) Triton X-100 resulted in a 2.5-fold increase in [^{3}H]IAA binding. A low level of unspecific binding was observed with heat-treated membranes. The observation that Triton X-100 enhances rather than decreases equilibrium binding of [^{3}H]IAA to symbiosome membranes excludes the possibility that "apparent" IAA binding is the result of IAA accumulation in sealed membrane vesicles.

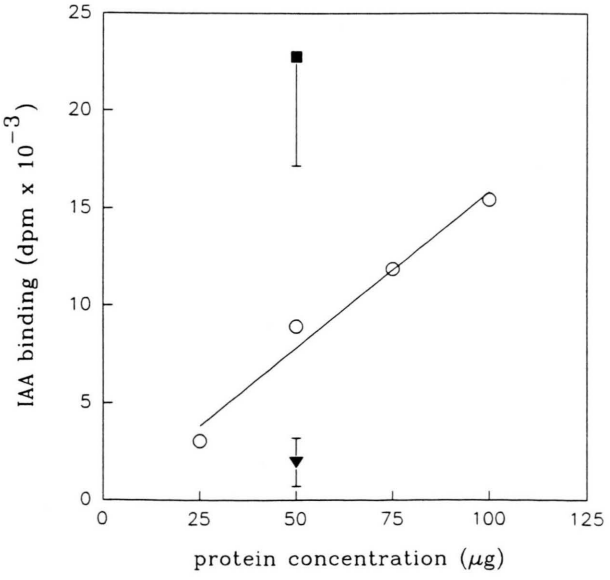

Fig. 1. Correlation between equilibrium binding of [^{3}H]IAA to the symbiosome membrane and protein concentration of the membrane preparation: (○) non-denaturated membrane, (▼) 50 µg heat-treated membrane protein, (■) 50 µg membrane protein in the presence of 1% Triton X-100.

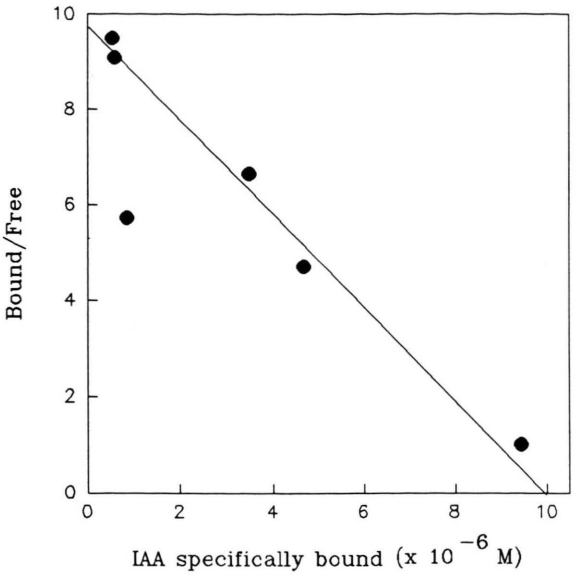

Fig. 2. Scatchard plot for specifically bound IAA. Binding of [^{3}H]IAA to symbiosome membranes (50 µg protein/assay) was studied by equilibrium dialysis. Unlabeled competitor was added ranging from 5×10^{-4} M to 1×10^{-8} M.

To determine the dissociation constant for IAA, symbiosome membranes were incubated with [^{3}H]IAA and unlabeled IAA as competitor in the concentration range from 5×10^{-4} M to 10^{-8} M. The results shown in Fig. 2 are plotted according to Scatchard [20] and indicate a dissociation constant for IAA of 1×10^{-6} M. This value is in agreement with K_D-values determined for binding of [^{3}H]IAA to maize plasma membranes [26] and to the major auxin binding protein (ERabp1) found in the endoplasmic reticulum of maize coleoptiles [9].

1-NAA competes with [^{3}H]IAA for binding to symbiosome membranes

To demonstrate the specificity of IAA binding, symbiosome membranes were incubated with [^{3}H]IAA at 20 °C in the presence of an excess of unlabeled IAA, 1-NAA (a synthetic auxin analoge) or compounds having no auxin activity (Fig. 3). [^{3}H]IAA binding was inhibited by unlabeled IAA and to a lesser extent by 1-NAA. Hydrophobic compounds having no auxin activity,

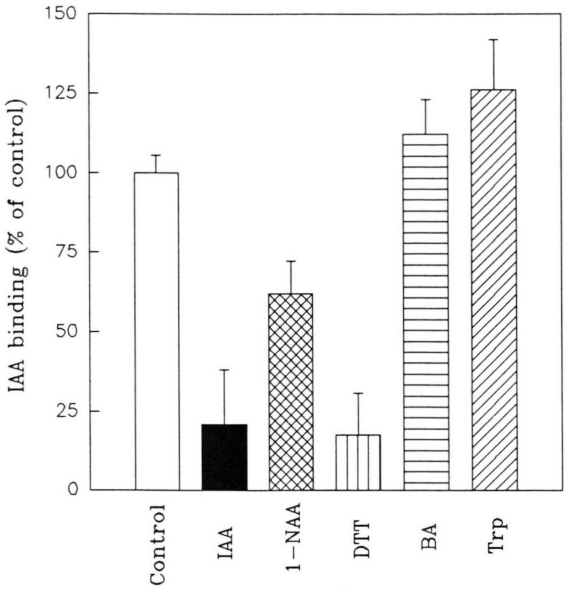

Fig. 3. Effects of unlabeled competitors (5×10^{-5} M) and of DTT (10^{-3} M) on [^{3}H]IAA equilibrium binding to the symbiosome membrane (50 µg protein/assay).

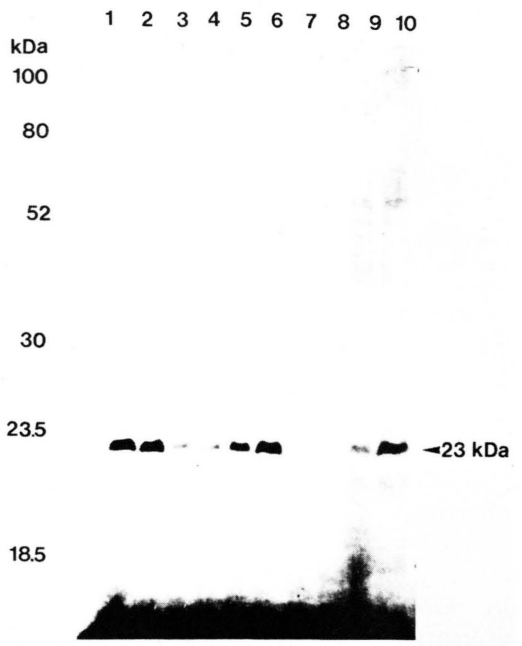

Fig. 4. Symbiosome membrane proteins (100 µg) were photoaffinity labeled with [^{3}H]N_3IAA and analyzed by SDS-PAGE and fluorography. The membrane vesicles were labeled without competitor at pH 5.5 (lane 1), pH 6.5 (lane 2) and pH 7.5 (lane 3) or in presence of 1 mM unlabeled competitor at pH 5.5: IAA (lane 4), 1-NAA (lane 5), L-tryptophan (lane 6), quercetin (lane 7), rutin (lane 8), NPA (lane 9) or benzoic acid (lane 10).

such as tryptophan (Trp) and benzoic acid (BA), were not able to displace IAA from the symbiosome membrane. This suggests that only functionally active auxins are able to bind to the symbiosome membrane. Interestingly, addition of 10^{-3} M DTT nearly completely inhibited auxin binding to symbiosome membranes. The data demonstrate the specificity of auxin binding to symbiosome membranes and are consistent with auxin binding data reported by Dohrmann *et al.* [8] for maize plasma membranes. A similar reduction in auxin binding by dithioerithritol (DTE) was previously found in maize microsomal fractions suggesting the involvement of disulfide bridges in stabilizing the putative auxin receptor [8, 27].

Photoaffinity labeling of symbiosome membrane proteins with [^{3}H]N_3IAA

In order to confirm and extend the equilibrium dialysis binding data and to identify the auxin binding proteins, photoaffinity labeling experiments, were performed [14]. Following photoaffinity labeling of symbiosome membranes with [^{3}H]N_3IAA, a single labeled protein with an apparent molecular mass of 23 kDa was identified (Fig. 4). Maximum labeling occurred from pH 5.5 to 6.5, whereas labeling at pH 7.5 resulted in a weak signal (Fig. 4, lanes 1–3). An ABP of similar size and labeling characteristics was recently reported following photoaffinity labeling of maize coleoptile membranes [15].

Auxins and phytotropins compete with [^{3}H]N_3IAA for labeling

To study the specificity of photoaffinity labeling of the 23 kDa protein, symbiosome membranes were preincubated at 4 °C with [^{3}H]N_3IAA in combination with various unlabeled compounds thought to bind either specifically or non-specifically to auxin binding proteins (Fig. 4, lanes 4–10). Labeling of the 23 kDa protein with [^{3}H]N_3IAA was strongly inhibited by unlabeled IAA and less strongly by a physiologically active auxin analoge 1-naphthaleneacetic acid (1-NAA).

1-naphthylphthalamic acid (NPA), quercetin and its glycoside derivative rutin, well known inhibitors of polar auxin transport [28], also competed with $[^3H]N_3IAA$ for binding. Only negligible competition was observed with benzoic acid and tryptophan, compounds which are structurally related to IAA but have no auxin activity. A similar pattern of competition of photoaffinity labeling has recently been observed for the 23 kDa *Zea mays* plasma membrane ABP [15].

Solubilization of the symbiosome membrane ABP

In order to extract the photoaffinity labeled 23 kDa protein from symbiosome membranes, membranes were either incubated with Triton X-100 or Triton X-114. Extracted proteins were separated by SDS-PAGE (Fig. 5). While Triton X-100 solubilized only approximately 60% of the 23 kDa protein from the symbiosome membranes, we observed a more effective extraction using Triton X-114 (compare lanes 1, 2, and 3, 4 in Fig. 5). We conclude from these data that the symbiosome membrane ABP is probably peripherally associated with the symbiosome membrane. Similar solubilization characteristics have been observed for the maize plasma membrane ABP, pm 23 [11, 15].

Photoaffinity labeling of symbiosome membranes with $[^3H_2]N_3NPA$

Unlabeled NPA inhibited labeling of the 23 kDa auxin binding protein by $[^3H]N_3IAA$ and therefore it was interesting to attempt photoaffinity labeling of the symbiosome membrane with $[^3H_2]N_3NPA$ (Fig. 6). It could be demonstrated that a sym-

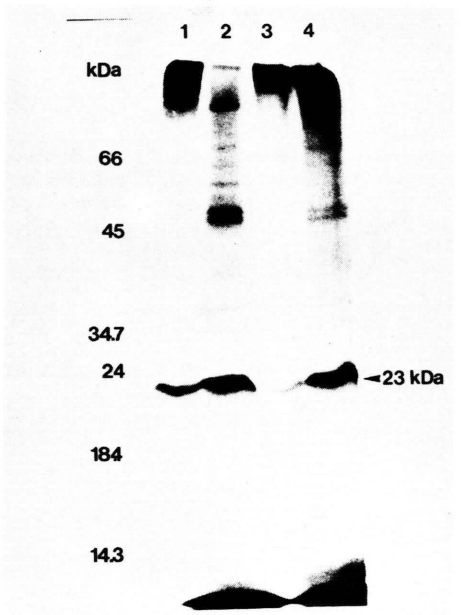

Fig. 5. Symbiosome membrane vesicles were photoaffinity labeled with $[^3H]N_3IAA$ and then extracted with 1% Triton X-100 (lane 1: insoluble membrane pellet, lane 2: Triton X-100 soluble proteins) or with Triton X-114 (lane 3: insoluble membrane pellet, lane 4: Triton X-114 soluble proteins). Proteins were analyzed by SDS-PAGE and fluorography.

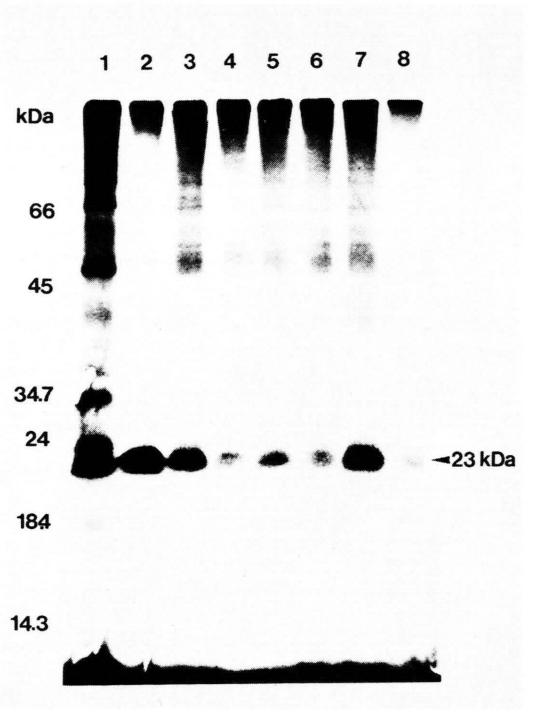

Fig. 6. Photoaffinity labeling of symbiosome membrane vesicles by $[^3H_2]N_3NPA$ and $[^3H]N_3IAA$. Labeling was performed with $[^3H]N_3IAA$ at 4 °C (lane 1) and at −196 °C (lane 2) or with $[^3H_2]N_3NPA$ at 4 °C (lane 7) and at −196 °C (lane 8). Inhibition of $[^3H_2]N_3NPA$ labeling was studied at 4 °C in the presence of 1 mM unlabeled competitor: benzoic acid (lane 3), IAA (lane 4), TIBA (lane 5) and NPA (lane 6). Photoaffinity labeled membrane proteins were analyzed by SDS-PAGE and fluorography.

biosome membrane protein of identical molecular weight was photoaffinity labeled by both [^{3}H]N_3IAA and [$^3H_2$]N_3NPA (Fig. 6, lanes 2, 7). Labeling with [^{3}H]N_3IAA was optimal at −196 °C. An increase in the temperature resulted in high background labeling (Fig. 6, lanes 1, 2). In contrast, photoaffinity labeling using [$^3H_2$]N_3NPA was optimal at 4 °C (Fig. 6, lanes 7, 8).

NPA, TIBA and IAA compete with [$^3H_2$]N_3NPA for binding

Photoaffinity labeling of the 23 kDa symbiosome membrane ABP with [$^3H_2$]N_3NPA was decreased in the presence of unlabeled NPA, IAA and TIBA. Like NPA, TIBA is an inhibitor of polar auxin transport. [$^3H_2$]N_3NPA labeling was only weakly competed by benzoic acid, whereas NPA, IAA and TIBA were strong competitors (Fig. 6, lanes 3–7). These data indicate that the symbiosome membrane ABP, like the 23 kDa maize plasma membrane ABP [11], interacts with both auxins and phytotropins. These labeling and competition patterns suggest that the symbiosome membrane ABP, like the maize plasma membrane ABP, is probably part of an auxin efflux carrier system. However, despite the striking similarities observed in photoaffinity labeling and equilibrium binding data reported here, the symbiosome membrane protein seems to be immunologically distinct from the plasma membrane protein of *Zea mays, i.e.* polyclonal antibodies raised against the maize plasma membrane ABP failed to recognize the symbiosome membrane ABP [unpublished information]. Further investigations are required to finally establish the function of the symbiosome ABP.

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