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Reevaluation of the Role of Auxin Binding Site II

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Abstract: Binding of 1-naphthylacetic acid (1-NAA) was assayed in microsomal membranes from *Zea mays* coleoptiles and from hypocotyls of *Cucurbita pepo*. Auxin binding site II was differentiated from site I binding by using phenylacetic acid (PAA) to saturate site I binding capacity. The amount of type-II binding sites, per gram original fresh weight, was 34 pmol with *Zea* and 6.4 pmol with *Cucurbita*. When maize membranes were separated by dextran gradient centrifugation, auxin binding site II migrated coincident with tonoplast marker enzymes. The physiologically active auxin 4-chloroindoleacetic acid (4-Cl-IAA) competed very poorly with 1-NAA binding to both site I and site II. This result suggests that sites I and II are not involved in the regulation of growth. When comparing isolated outer epidermis with intact coleoptile of *Zea*, similar amounts and ratios of site I and site II binding activities were observed.

The plant hormone auxin participates in the regulation of many developmental processes; it is transported in a polar (basipetal) manner from cell to cell. No receptors have been identified which could explain the pleiotropic effects auxin exerts. Auxin may act via a single receptor. Alternatively, individual auxin binding proteins may mediate different responses or different aspects of a single response. Soluble and membrane-associated auxin binding proteins which may be involved in auxin-regulated developmental processes have been described (reviewed in Napier and Venis 1990). Certain soluble auxin binding proteins may mediate the transcriptional activation of auxin-responsive genes (McClure et al. 1989), some of which are thought to participate in the growth response (Guilfoyle 1986).

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In membrane fractions from maize, three auxin binding sites have been identified (Dohrmann et al. 1978). Auxin binding site I and site II differ from site III in that they have high affinity for 1-NAA. The plasma membrane-localized auxin binding site III was suggested to be an uptake carrier possibly involved in auxin transport (Lomax et al. 1985).

Auxin binding site I (auxin binding protein, ABP1) has been localized at the endoplasmic reticulum (ER) by density gradient centrifugation (Ray 1977), and possesses the ER retention signal Lys-Asp-Glu-Leu at the carboxy terminal end of its predicted amino acid sequence (Hesse et al. 1989; Inohara et al. 1989). However, electron microscopic localization studies revealed that ABP1 is secreted, though inefficiently, to the plasma membrane and the cell wall (Jones and Herman 1993). It has been proposed that this extracellular form of ABP1 is involved in auxin signal perception (Barbier-Brygoo et al. 1989; Rück et al. 1993). In contrast to eukaryotic membrane receptors, ABP1 does not contain any transmembrane spanning domains. There are also discrepancies between the growth promoting activity of different auxin analogs and their ability to bind to site I (Ray et al. 1977b).

Auxin binding site II was first described by Batt and Venis (1976); its physiological role remains unclear, although the amounts of site II seem to be highest during the rapid-growth stage of the coleoptile (Shimomura et al. 1988). Site I and site II appear to be different proteins because antibodies raised against site I did not cross-react with site II (Shimomura et al. 1988), and site I but not site II was inactivated by a supernatant factor (benzooxazolinone, Venis and Watson 1978) and reducing agents (Ray et al. 1977a; Dohrmann et al. 1978). In addition, site II differs from site I in its binding specificity for different auxin analogs, having a much lower affinity to the antiauxin 2-NAA and the weak auxin PAA than does site I (Dohrmann et al. 1978). Site II but not site I auxin binding activity was re-

ported to be present in zucchini hypocotyls (Jacobs and Hertel 1978). In the study presented here, the potential contribution of auxin binding sites I and II to physiological processes such as growth is examined.

Materials and Methods

Plant Material

Zea mays L. (hybrid Inracorn cat. 5A, Samen Hamprecht, Freiburg, FRG) was grown in the dark for 4 days as described by Ray et al. (1977a). Seeds of zucchini squash (*Cucurbita pepo* L., zucchini "Cocozelle von Tripolis"; Wagner, Heidelberg, FRG) were planted in moist vermiculite and grown in the dark at 25°C for 5 days.

Radiochemicals and Chemicals

HPLC-purified [4-^{3}H]1-NAA (418 GBq/mmol) was provided by Ludwig Wälder, Friedrich Miescher Institut, Basel, Switzerland. [^{3}H]1-N-naphthylphthalamic acid (NPA, 2.03 TBq/mmol) was purchased from CEA (Gif-sur-Yvette, France).

Benzoic acid (BA), 1-NAA, and IAA were purchased from Merck (Darmstadt, FRG). PAA was purchased from Roth (Karlsruhe, FRG). 4-Cl-IAA was synthesized by K. C. Engvild (see Böttger et al. 1978). NPA was synthesized by Thomson et al. (1973). All auxins, analogs, and NPA were stored as ethanolic stock solutions and used such that the final ethanol concentration was never higher than 1%. This concentration did not influence ligand binding (data not shown).

Preparation of Membrane Vesicles

Whole maize coleoptiles with their primary leaves removed and hypocotyl segments from *Cucurbita* were harvested and placed on ice. The tissue was homogenized in a herb grinder (Moulinex) for 2 × 2 s in ice-cold extraction buffer (50 mM Tris, pH 7.9, 1 mM EDTA, 0.1 mM $MgCl_2$, 250 mM sucrose). The homogenate was squeezed through a fine nylon mesh prior to centrifugation at 3000 *g* for 15 min at 4°C. The pellet was discarded and the supernatant centrifuged at 100,000 *g* for 20 min at 4°C to yield a microsomal membrane pellet.

Epidermal peels from maize coleoptiles were prepared by creating small incisions on either side of the vascular bundles. The tissue between the vascular bundles was gently bent, and the epidermis was peeled along the axis. Adhering cortical tissue was removed and the epidermal peels were immediately frozen in a glass homogenizer filled with liquid nitrogen. The epidermal tissue was homogenized in liquid nitrogen and subsequently on ice in extraction buffer containing 2.5% (w/v) PVP. The homogenate was centrifuged as described above to yield a microsomal membrane preparation.

Fractionation of Microsomal Membranes

Microsomal vesicles from 15 g of coleoptile tissue were resuspended on ice in 2 ml of gradient medium (50 mM Tris, pH 7.6, 250 mM sucrose, 1 mM EDTA) and layered on top of a 26-ml linear gradient of 1%–12% (w/v) dextran T-500 (Pharmacia, Freiburg, FRG) in the gradient medium. Beneath the gradient was a 3-ml cushion of 45% (w/v) sucrose. The membranes were centrifuged at 80,000 *g* for 2 h at 4°C in a SW28 rotor (Beckman, Palo Alto, CA) followed by collection of 2-ml fractions from the top of the gradient. The density of the fractions was determined as sucrose equivalents by refractometry, and binding and marker enzyme activities were assayed. ER was shifted to higher densities, by preparing microsomes in extraction buffer and gradient medium containing 3 mM $MgSO_4$.

Binding Tests

Reversible binding of 1-NAA and NPA to microsomal membranes was determined by a sedimentation assay according to Dohrmann et al. (1978) with modifications. All steps were carried out at 4°C. 1-NAA binding was determined by mixing microsomes (0.5 ml) with an equal volume of test buffer (10 mM Na-citrate, pH 5.5, 5 mM $MgCl_2$) containing radiolabelled 1-NAA (concentrations indicated in Figure legends), 3 μM carbonylcyanide-3-trifluoromethoxyphenylhydrazone, 1 μM nigericin, 1 μM valinomycin. The ionophores were added to dissipate any pH gradients. Binding to site II was differentiated from site I by the presence of 50 μM PAA. *Nonspecific binding of 1-NAA* was defined as the amount of radiolabel associated with membranes in the presence of 0.1 mM unlabelled 1-NAA.

NPA binding was assayed by the incubation of microsomes in test buffer containing 0.3 nM [^{3}H]NPA in the presence or absence of 10 μM unlabelled NPA. The NPA binding assays were incubated for 30 min at 4°C.

After the incubation period, membranes were centrifuged for 5 min at 400,000 *g* at 4°C. The supernatant was decanted and pellets were rinsed, drained, and incubated in 0.5 ml methanol for 2 h. Aliquots of the supernatant and the methanol solutions were transferred to scintillation vials and counted in Bray's solution in a Beckman scintillation counter.

Marker Enzyme Activities

ATPase and latent IDPase activities were determined according to Caubergs et al. (1986). Vanadate-sensitive ATPase and nitrate-sensitive ATPase were determined by the inhibition of 50 μM Na-orthovanadate and 0.1 M KNO_3, respectively, of hydrolysis of 1 mM Na-ATP in the presence of 15 mM Tris-MES, pH 7, 1 mM $MgSO_4$, 0.1 mM ammonium molybdate, 1 μM oligomycin, and 0.05% (w/w) Triton X-100. Activity was defined as the difference in ATP hydrolysis in the presence and absence of inhibitor. Latent IDPase activity was measured by the hydrolysis of 2.5 mM Na-IDP in the presence of 15 mM Tris-MES, pH 7, 1 mM $MgSO_4$, 0.1 M KCl. *Activity* was defined as the difference in hydrolysis in the presence or absence of 0.05% (w/w) Triton X-100. Inorganic phosphate was determined according to Dulley (1975).

Pyrophosphatase activity was determined according to Hager et al. (1980) with modifications. Uptake of 30 μM neutral red (Sigma, Munich, FRG) into membrane vesicles was assayed in 20 mM HEPES-MES, pH 7.8, 50 mM KNO_3, 300 mM sucrose, 1 mM $MgCl_2$ with 2 mM pyrophosphate as substrate. Activity was determined by the increase in absorbance at 560 nm after 2.5 min. Absorbance changes were converted into pH changes by calibrating the pH-dependent absorbance change with a standard test solution of neutral red as specified above.

NADH-dependent cytochrome-c oxidoreductase (CCR) and cytochrome-c oxidase (CCO) were assayed as described by Lord et al. (1973) and Appelmans et al. (1955), respectively. Protein was determined by the method of Spector (1978) using BSA as standard.

Protein Gel Electrophoresis

Soluble and membrane proteins were precipitated with 10% (w/v) TCA, washed with 80% (v/v) acetone, resuspended in sample buffer containing 2 mM PMSF, and separated by SDS-PAGE according to Læmmli (1970). Polyacrylamide gels (10%) were stained with Coomassie blue. Phosphorylase (97 kD), bovine serum albumin (66 kD), ovalbumin (45 kD), carboanhydrase (31 kD), trypsin inhibitor (22 kD), and lysozyme (14 kD) served as molecular weight standards.

Results and Discussion

*NAA-Binding Site II Is Found in Both Monocots (*Zea*) and Dicots (*Cucurbita*)*

Binding of labelled 1-NAA was tested in microsomal membranes prepared from maize coleoptiles and, on the other hand, from hypocotyls of *Cucurbita*. Site II was differentiated from site I by using 50 μM PAA to saturate site-I binding capacity (see Dohrmann et al. 1978). The data (Fig. 1) confirm earlier findings of saturable 1-NAA binding of type II in *Zea mays* (Dohrmann et al. 1978, Shimomura et al. 1988) as well as in the dicot zucchini (see Jacobs and Hertel 1978).

Scatchard analysis (Scatchard 1949) of site II 1-NAA binding in material from maize coleoptiles yielded a straight line indicating the presence of a single binding site with the K_D-value of 1.4 μM (Fig. 1A). The number of binding sites were determined to be 34 pmol/g fresh weight in agreement with Dohrmann et al. (1978).

The saturation kinetics of 1-NAA binding was also analyzed for *Cucurbita*. A dissociation constant of 1.2 μM was determined assuming a single class of binding sites. This value agrees well with the K_D of 1.4 μM for auxin binding site II in maize. Thus affinity is the same in both plants while the amounts of site II (per original g fresh weight) differed somewhat. *Cucurbita* had only 6.4 pmol which correspond to approximately 20% of the amount in maize. On the other hand, concerning site-I binding, in maize membranes approximately 40 pmol/g fresh weight were determined (Dohrmann et al. 1978; own data, not shown), whereas in microsomes from *Cucurbita* very little if any (<0.5 pmol/g fresh weight) binding sites of type I could be found (data not shown; see also Jacobs and Hertel 1978).

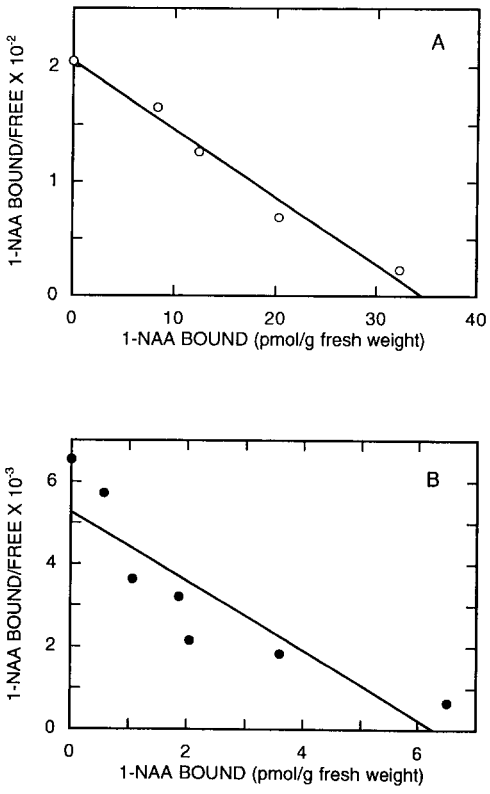

Fig. 1. Scatchard plot analysis of 1-NAA binding to microsomes from (**A**) maize coleoptiles and (**B**) hypocotyls of *Cucurbita*. Crude membranes were incubated with [^{3}H]1-NAA (2 nM) and increasing concentrations of unlabelled 1-NAA in the presence of 50 μM PAA.

Subcellular Localization of Auxin Binding Site II

Site I and site II proteins are associated with membrane material. From published results of Ray (1977) and Dohrmann et al. (1978), and from data in Fig. 1 it can be calculated that specific binding activity per protein increases in pelletable material when sites I or II are purified after centrifugation lasting several hours. When microsomal particles were recentrifuged and washed in extraction medium, no significant loss of binding activity could be observed (data not shown). This is evidence for a stable and tight membrane association of the NAA binding sites; a loose membrane attachment of a soluble binding protein is ruled out.

In order to test whether 1-NAA binding in the presence of 50 μM PAA—at site II—could be separated from site I, maize microsomes were fractionated in linear dextran gradients (Fig. 2). Sucrose gradients have already been reported in the past (e.g., Dohrmann et al. 1978); dextran gradients, however, allow to separate membranes according to different characters. Specific binding of 1-NAA coincided with activities of the marker enzymes ni-

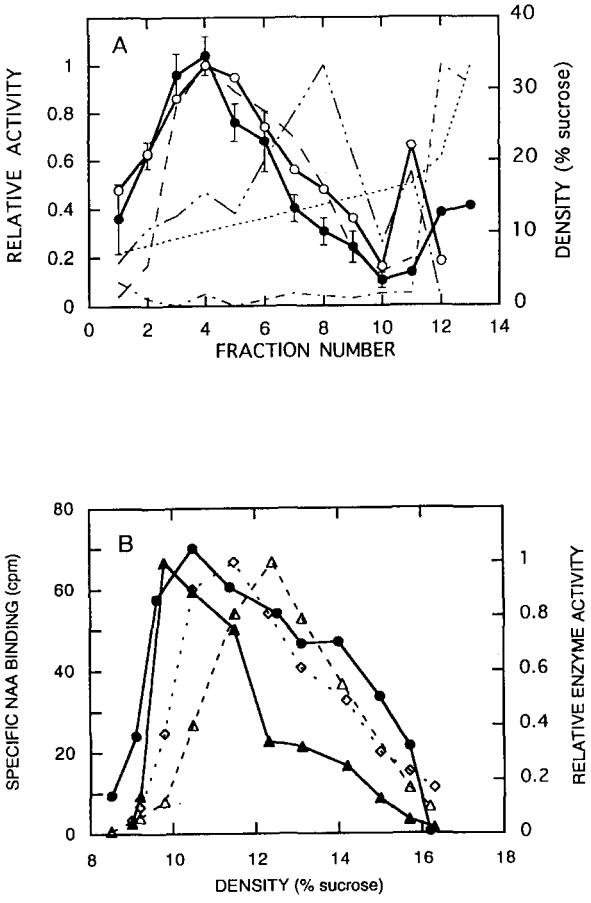

Fig. 2. Fractionation of microsomes from maize coleoptiles by dextran gradient centrifugation in the absence (**A**) and presence (**B**) of $MgSO_4$. (**A**) Fractions were tested for specific [^{3}H]1-NAA binding (3 nM) in the presence of PAA. Data points represent means of four experiments ± SD. Maximal specific binding was 125 cpm (●). Relative density (· · · · ·) is expressed as percent sucrose equivalents. Maximal marker enzyme activities were as follows: nitrate-sensitive ATPase (○) 0.6 μmol/h/ml; vanadate-sensitive ATPase (- · · · -) 0.9 μmol/h/ml; CCR (- - -) 1.4 μmol/min/ml; CCO (- · - · -) 1.9 μmol/min/ml. (**B**) Specific [^{3}H]1-NAA binding (5 nM) in the presence of PAA (●). Maximal enzyme activities were as follows: IDPase (◇) 45 μmol/h/ml; CCR (△) 0.5 μmol/min/ml. Maximal intravesicular acidification by pyrophosphatase (▲) was 0.3 pH-units.

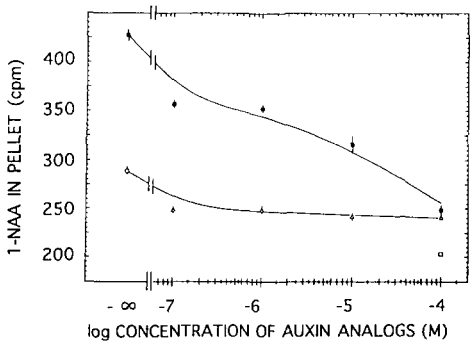

Fig. 3. Displacement of [^{3}H]1-NAA (1 nM) by unlabelled auxin analogs. Crude microsomes from maize coleoptiles were incubated in [^{3}H]1-NAA in the presence (△) and absence (●) of 50 μM PAA with increasing concentrations of 4-Cl-IAA. 50 μM PAA (○) was assumed to saturate auxin binding site I. Site I and site II were saturated with 0.1 mM 1-NAA (□). Means of three replicates ± SE are shown.

trate-sensitive ATPase (tonoplast) and CCR (ER). Moreover, 1-NAA binding was separated from the mitochondrial marker enzyme CCO and the plasma membrane marker vanadate-sensitive ATPase.

Retention of ribosome binding to the ER by Mg^{2+} permitted separation of ER membranes from the tonoplast (Fig. 2B). Specific 1-NAA binding in the presence of PAA was coincident with another tonoplast marker, pyrophosphatase. In this gradient system 1-NAA binding was separated from the marker enzymes CCR and latent IDPase (Golgi apparatus).

Thus, on maize membranes, separated by dextran gradient centrifugation, auxin binding site II migrated coincident with the tonoplast marker enzymes, nitrate-sensitive ATPase and pyrophosphatase, and was not found along with the other membranes listed. Similarly, site II binding to *Cucurbita* has been found in corresponding zones of sucrose gradients (Jacobs and Hertel 1978).

Such findings could be interpreted as evidence that the majority of site II binding occurs at the tonoplast (Dohrmann et al. 1978). However, significant additional 1-NAA binding occurred at densities where high activities of CCR were observed, indicating that not all of the binding to site I was competed away by the presence of 50 μM PAA (see Fig. 2B). Furthermore, Shimomura et al. (1988) found a shift of site II activity away from the soluble vacuolar marker enzyme acid phosphatase in sucrose gradients when membranes were used from 3-day-old instead of 5-day-old maize coleoptiles. Aducci and Hertel (unpublished) had failed to find 1-NAA binding to site II after vacuole isolation from maize mesocotyls and zucchini hypocotyls. Therefore, the subcellular localization of site II requires further investigation. Centrifugation without prepelleting and other gradient materials should be used.

Competition of 1-NAA Binding by 4-Cl-IAA

4-Chloroindoleacetic acid is a naturally occurring hormone (Marumo et al. 1968; Hofinger and Böttger 1979) and a physiologically very active auxin (see Fischer et al. 1992; Hertel 1993). It was used in

competition assays with [^{3}H]1-NAA (Fig. 3). 1-NAA binding in the absence of PAA reflects the cumulative binding to site I and site II. This form of binding was displaced by high concentrations of 4-Cl-IAA. There was even less competition of 4-Cl-IAA with 1-NAA binding in the presence of 50 μM PAA which may be explained by residual NAA binding to site I. At 0.1 mM 4-Cl-IAA, the same amount of [^{3}H]1-NAA was displaced in the presence or absence of PAA. In comparison, more [^{3}H]1-NAA was displaced by 0.1 mM unlabelled 1-NAA which was bound to sites I and II. This suggests that 4-Cl-IAA hardly bound to site II. The estimated ligand concentration necessary to displace 50% of the [^{3}H]1-NAA (IC_{50}) that was bound to site I was estimated to be less than 10 μM in the case of 4-Cl-IAA. This value is approximately two orders of magnitude higher than the IC_{50} value for 1-NAA (Ray et al. 1977a; Löbler and Klämbt 1985a).

The inability of 4-Cl-IAA to bind to site II and its weak interaction with site I contrasts its strong growth promotion in monocots and dicots (Katekar and Geissler 1983; Fischer et al. 1992). Hertel (1993) compared the effects of 1-NAA and 4-Cl-IAA on maize coleoptile elongation with the binding of these auxins to sites I and II using the same plant material and stock solutions. Growth promotion of 4-Cl-IAA was 10 times higher than that of 1-NAA whereas binding affinities were 100-times lower amounting to a 1000-fold discrepancy (Hertel 1993). These data do not support the concept that site II is involved in the regulation of growth and seriously question the proposed receptor model for site I/ABP1.

1-NAA Binding in the Outer Epidermis of the Coleoptile

Outer epidermis was manually separated from the rest of maize coleoptiles. The epidermal peels were analyzed by SDS-PAGE. Soluble and microsomal proteins were separated and stained with Coomassie (Fig. 4). Most of the proteins bands were common to both types of tissue. Differences in the abundance of a few bands were, however, apparent in both soluble and membrane protein fractions. These differences indicate a separation of the tissues.

The epidermal membrane preparation was further characterized by analyzing marker enzyme activities (Table 1). Similar nitrate-sensitive ATPase activities were found in the outer epidermis and the whole coleoptile; vanadate-sensitive ATPase activities were higher in the outer epidermis than in the whole coleoptile (Table 1) in agreement with immunohistochemical data (Villalba et al. 1991).

NPA (an inhibitor of auxin efflux) specifically bound to microsomes of the outer epidermis although at somewhat lower levels than in the whole coleoptile (Table 1). This data suggests that auxin is transported primarily in the inner tissues of the co-

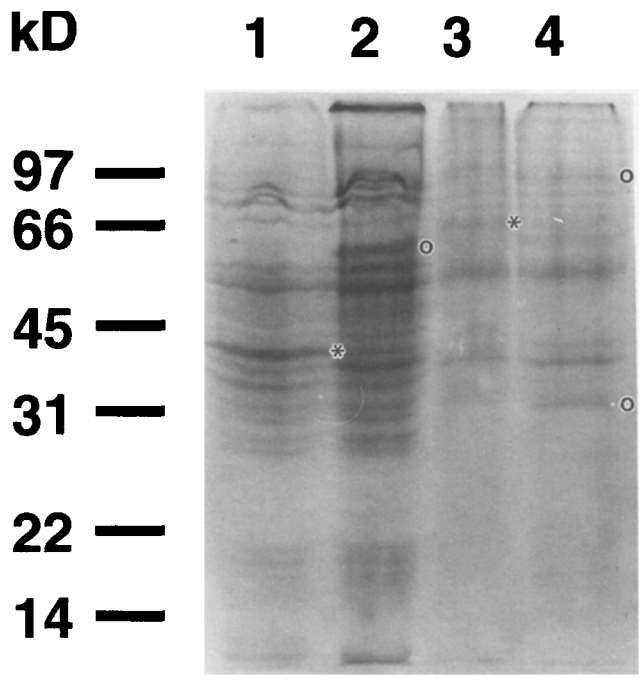

Fig. 4. SDS-PAGE analysis of soluble (lanes 1 and 2) and microsomal membrane proteins (lanes 3 and 4) from the outer epidermis (lanes 1 and 3) and the whole coleoptile (lanes 2 and 4) of maize. Protein (40 μg) was separated and stained with Coomassie blue. *Asterisks* and *open circles* indicate proteins that were more abundant in the epidermis and whole coleoptile, respectively. Molecular masses of marker proteins are indicated.

Table 1. Binding data and marker enzyme activities in crude microsomal fractions from the outer epidermis and the whole coleoptile of maize.

Activity	Epidermis	Coleoptile	[a]Ratio
Auxin binding site I	0.19	0.12	1.6
Auxin binding site II	0.07	0.05	1.4
NPA binding	0.94	1.84	0.5
CCR (μmol/min)	0.5	1.2	0.4
CCO (μmol/min)	0.2	0.2	1.0
VO_4-ATPase (μmol/h)	0.9	0.5	1.8
NO_3-ATPase (μmol/h)	0.3	0.4	0.8

Note: Saturable [^{3}H]1-NAA binding was measured in the absence (site I) or in the presence of 50 μM PAA (site II). 1-NAA and NPA binding are expressed as a ratio of cpm specifically bound to cpm free. All of the data is expressed per mg protein.
[a] Ratio, activity in epidermis to activity in coleoptile.

leoptile, consistent with previous results (Hertel and Leopold 1963; Jacobs and Gilbert 1983). On the other hand, the finding contradicts the interpretation of azido-IAA labelling data by Jones (1990) that auxin was transported primarily in the epidermis.

Similar quantities of 1-NAA binding per protein were observed using crude membranes from outer epidermis and whole coleoptiles (Table 1). There may be slightly more binding activity in the outer epidermis especially in the case of site I (ratio 1.6). These results agree with previous data using abraded coleoptiles (Shimomura et al. 1988). The authors observed similar site-II binding activities in intact and abraded coleoptiles; site-I binding activities were somewhat higher in intact than abraded coleoptiles. In contrast, the results reported here disagree with a tissue-specific distribution of site I or ABP1 (Löbler and Llämbt 1985b).

Conclusions

Auxin binding site II was differentiated from site I by performing the binding assay in the presence of 50 μM PAA, concentrations that eliminate most of the 1-NAA binding to site I. Under these conditions a single class of binding sites was detected. The sites were localized at membranes that comigrate with tonoplast markers under many but not all conditions. The nature of the microsomes that carry NAA-binding site II, remains to be elucidated. The auxin binding sites I and II were found in the isolated outer epidermis of the coleoptile, but they occur as well in the inner tissues of the organ.

The weak competition of 4-Cl-IAA with 1-NAA binding to site I and especially site II did not correlate with the biological activity of this hormone. These data make a contribution of site II to growth unlikely and cast doubt on the proposed receptor function of site I. Instead, "auxin" binding site II may play a role in some other important, general metabolic or physiological process where substances similar to NAA/IAA are involved because the sites have a high affinity for 1-NAA and are present in monocots as well as dicots.

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