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Different properties of two types of auxin-binding sites in membranes from maize coleoptiles

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Abstract. Two types of auxin-binding sites (sites I and II) in membranes from maize (*Zea mays* L.) coleoptiles were characterized. Site I was a protein with a relative molecular mass of 21000, and the distribution of site I protein on sucrose density gradient fractionation coincided with that of NADH-cytochrome-*c* reductase (EC 1.6.99.3), a marker enzyme of the endoplasmic reticulum. Immunoprecipitation and immunoblotting studies showed that the content of site I protein in maize coleoptiles was approx. 2 $\mu g \cdot (g\ FW)^{-1}$. Site II occurred in higher-density fractions and also differed immunologically from site I. Site I was present at the early developmental stage of the coleoptile and increased only twice during coleoptile growth between day 2 and 4. Site II activity was low at the early stage and increased more substantially between day 3 and 4, a period of rapid growth of the coleoptile. Both sites decreased concurrently after day 4, followed by a reduction in the growth rate of the coleoptile. Coleoptiles with the outer epidermis removed showed a lower site I activity than intact coleoptiles, indicating that site I was concentrated in the outer epidermis. Site II, in contrast, remained constant after removal of the outer epidermis. The results indicate that site I is not a precursor of site II and that the two sites are involved in different cellular functions.

Key words: Auxin binding – Coleoptile – *Zea* (auxin binding)

Abbreviations: FW = fresh weight; M_r = relative molecular mass; 1-NAA = 1-naphthaleneacetic acid; 2-NAA = 2-naphthaleneacetic acid; SDS-PAGE = sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Introduction

Specific binding sites for auxin have been demonstrated in both soluble and membrane fractions of various plant tissues (Hertel et al. 1972; Ray et al. 1977b; Jacobs and Hertel 1978; Vreugdenhil et al. 1979; Sakai and Hanagata 1983; van der Linde et al. 1984). The different locations of these binding sites may reflect the fact that auxins appear to affect not only gene expression in nuclei (Guilfoyle 1986; Key et al. 1986; Sakai et al. 1986; Theologis 1986) but also various functions of isolated cellular membranes (Buckhout et al. 1981; Morré et al. 1984a, b, 1986; Gabathuler and Cleland 1985). Furthermore, multiple types of auxin-binding sites have been found in both soluble and membrane fractions (Batt et al. 1976; Batt and Venis 1976; Dohrmann et al. 1978; Jacobs and Hertel 1978; Sakai 1985; Shimomura et al. 1986).

Dohrmann et al. (1978) reported the presence of three types of auxin-binding sites in different membrane fractions of maize coleoptiles: site I in the endoplasmic reticulum, site II in the tonoplast, and site III in the plasma membranes. Site III has been shown to be the auxin-transport system in closed membrane vesicles (Hertel et al. 1983; Hertel 1983; Lomax et al. 1985; Benning 1986; Sabater and Rubery 1987; Heyn et al. 1987), but the roles of sites I and II are unknown.

We recently purified an auxin-binding protein from the membrane fraction of maize shoots (Shimomura et al. 1986). The purified preparation was composed of two molecular species with different molecular masses (M_r), 21000 and 20000. Both species possessed auxin-binding activity. We prepared antibody against the purified protein and

examined by immunological methods whether the protein corresponded to site I or II. In this paper we show that sites I and II differ in their structure, development and tissue distribution. These results indicate that these two sites participate in different cellular functions.

Material and methods

Chemicals. 1-Naphthalene-[1-^{14}C]acetic acid ([^{14}C]1-NAA), 2.1 GBq·mmol^{-1}, was synthesized as described in Shimomura et al. (1986). 1-Naphthaleneacetic acid (1-NAA) was obtained from Tokyo Kasei Kogyo, Tokyo, Japan. 2-Naphthaleneacetic acid (2-NAA) was from Nakarai Chemicals, Kyoto, Japan. Goat anti-rabbit immunoglobulin G conjugated to peroxidase was purchased from Jackson Immunoresearch Laboratories, Avondale, Penn., USA. All other chemicals were obtained at reagent grade from various commercial sources.

Plant material. Maize seeds (*Zea mays* L., cv. Golden Cross Bantam; Fujita Seed Co., Osaka, Japan) were soaked in water for 3–5 h and allowed to germinate on moist vermiculite at 26–27° C for the indicated periods in a darkroom (1.7·0.8·1.7 m^3) with 2 h dim red light/day, provided by four 13-W red-colored fluorescent tubes (Matsushita Electric Industrial Co., Osaka, Japan) at a distance of 50–100 cm above the seedling trays.

Preparation of antiserum. The auxin-binding protein was purified from whole shoots of 5-d-old maize seedlings as described in Shimomura et al. (1986). A sample of 100 μg of the purified protein was injected subcutaneously into an albino rabbit with complete Freund's adjuvant (Wako Pure Chemical, Osaka). A booster injection was given six weeks later, and antiserum was obtained one more week later. The immunoglobulin G fraction was obtained by precipitation with ammonium sulfate followed by ion-exchange chromatography on diethylaminoethyl (DEAE)-cellulose.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 12.5% polyacrylamide gels as described by Laemmli (1970). Proteins separated by SDS-PAGE were located either by staining with Coomassie Brilliant Blue R-250 or by the immunological procedure after blotting onto a nitrocellulose sheet, as described by Towbin et al. (1979). The blot was soaked in saline (0.15 M NaCl, 10 mM potassium phosphate, pH 7.4) containing 5% bovine serum albumin and 0.1% NaN_3 for 1 h at room temperature. The sheet was transferred to a solution containing anti(purified auxin-binding protein) antiserum diluted 200-fold into saline containing 5% albumin and 0.1% NaN_3, and incubated at 4° C overnight. After washing the sheet with 0.05% Nonidet P-40 (Sigma Chemical Co., St Louis, Mo., USA) in saline, it was incubated for 2 h at room temperature with anti-rabbit immunoglobulin G conjugated to peroxidase diluted 1000-fold into 5% albumin in saline. The color reaction was performed in saline containing 0.1 mg·ml^{-1} 4-chloro-1-naphthol and 0.01% H_2O_2.

Sucrose-density-gradient centrifugation. Coleoptiles were chopped up with a razor blade in homogenization medium (0.25 M sucrose, 50 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris)-citric-acid buffer, pH 8.0, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM $MgCl_2$; Ray et al. 1977a), and then ground in a mortar and squeezed through nylon cloth (pore size 56 μm). The filtrate was centrifuged for 10 min at 5000·*g* to remove cell debris. Sucrose-density-gradient fractionation was performed according to Dohrmann et al. (1978). The gradient was composed of a cushion of 2 ml of 45% (w/w) sucrose, 20 ml of a linear gradient of 15–45% sucrose, and 2 ml of 15% sucrose; 17 ml of the 5000·*g* supernatant was applied. All sucrose solutions contained 10 mM Tris-acetic-acid buffer, pH 7.0, 1 mM EDTA, 1 mM KCl and 0.1 mM $MgCl_2$. The gradient was centrifuged at 25000 rpm (113000·*g*) for 2 h at 4° C in a Beckman SW 27 rotor (Beckman Instruments, Palo Alto, Cal., USA) and fractions of 1.5 ml were collected.

Auxin-binding assay. Standard assays of the auxin-binding sites in the membrane fraction were performed by the centrifugation procedure described by Ray et al. (1977a). The 5000·*g* supernatant of a homogenate was centrifuged for 30 min at 170000·*g*, and the pellet was washed once with a solution containing 0.25 M sucrose, 10 mM sodium-citrate buffer, pH 6.0, and 0.5 mM $MgCl_2$. The final pellet was resuspended using a paintbrush in assay medium (0.25 M sucrose, 10 mM sodium-citrate buffer, pH 5.5, 5 mM $MgCl_2$), and promptly used for auxin-binding assays to minimize acid inactivation of the auxin-binding sites (Shimomura et al. 1986). Resuspended membranes were mixed with 0.1 μM [^{14}C]1-NAA ± 0.1 mM 1-NAA in a final volume of 1 ml. Specific binding denotes the difference between the radioactivities of duplicate pellets with and without unlabeled 1-NAA.

For assay of the auxin-binding activites of sites I and II by the method of Dohrmann et al. (1978), membrane preparations were introduced into three tubes, each containing 0.1 μM [^{14}C]1-NAA. The second and third tubes also contained 10 μM 2-NAA and 10 μM 2-NAA + 0.1 mM 1-NAA, respectively. Site I activity (with high affinity toward 2-NAA) was calculated from the difference between the radioactivities of the first and second tubes, and site II activity (with low affinity toward 2-NAA) was calculated from the difference between the radioactivities of the second and third tubes.

Binding assays of the solubilized auxin-binding protein were performed by the gel-filtration method described in Shimomura et al. (1986).

The binding activities are represented as average values ± SD in respective duplicate samples.

Assay of marker enzymes. Acid-phosphatase (EC 3.1.3.2) activity of the membrane fractions on sucrose-density gradients was assayed by the method of Dohrmann et al. (1978). Each fraction (50 μl) was diluted twice with 10 mM Tris-acetic-acid buffer, pH 7.0, and 30 μl of the diluted sample were assayed for determination of total activity. The residual diluted sample was centrifuged at 436000·*g* for 20 min to remove vesicles, and 30 μl of the supernatant were assayed for determination of non-membrane-associated activity. The membrane-associated activity was calculated as the difference between these two values. The sample (30 μl) was mixed with 50 μl of a substrate solution (1.5 mg·ml^{-1} *p*-nitrophenyl phosphate, 60 mM sodium acetate, pH 5.0, 1.2% Triton X-100) and incubated for 20 min at 35° C. The reaction was terminated by adding 80 μl of 1 M $HClO_4$ and 0.8 ml of 0.2 M Na_2CO_3. Absorbance at 400 nm of the sample was measured.

NADH-cytochrome-*c* reductase (EC 1.6.99.3) was assayed by the method of Lord et al. (1973). The assay medium (1 ml) contained 20 μM cytochrome *c*, 0.1 mM NADH, 50 mM potassium-phosphate buffer, pH 7.2, 2.5 mM KCN, 1 μM antimycin A. The reaction was followed by recording the absorbance at 550 nm.

Other procedures. Protein concentration was determined with a Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, Cal., USA) with γ-globulin as standard. The trichloroacetic-acid-insoluble fractions were used for protein assays. The DNA content was assayed by the diphenylamine method of Schneider (1957).

Results

Characterization of the anti(purified auxin-binding protein) antibody. Specificity of the anti(auxin-binding protein) antibody was examined by analysis of the immunoprecipitated proteins on SDS-PAGE (Fig. 1). Since the efficiency of immunoprecipitation was dependent on the ratio of antibody to auxin-binding protein, we had preliminarily examined conditions giving a maximum yield of immunoprecipitated proteins. Under these conditions, both molecular species of M_r 21000 and 20000 of the purified auxin-binding protein (lane 1) were completely precipitated with the antibody by centrifugation at 15000·*g* (lane 2).

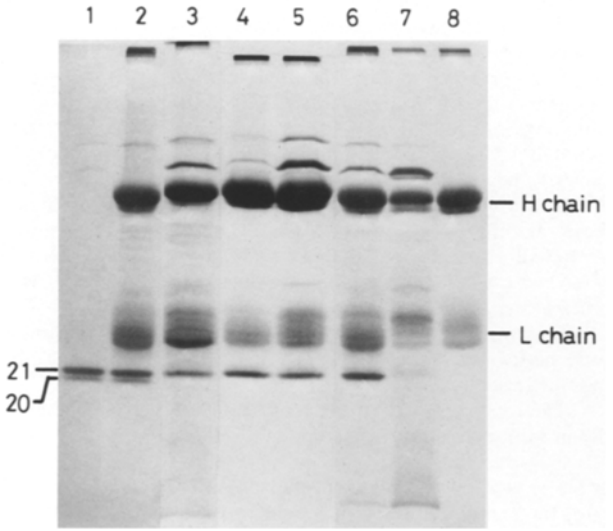

Fig. 1. Electrophoretic profiles of immunoprecipitated proteins from maize membrane proteins. The membrane fraction (5000–170000·*g*) from each organ of maize seedlings was washed with 20 volumes of cold acetone, and the resulting powder was homogenized with extraction medium (10 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$) and centrifuged at 20000 *g* for 1 h. The supernatant (corresponding to 0.7–1.5 g FW of tissue·ml^{-1}) or the purified auxin-binding protein (3.8 μg·ml^{-1}) was incubated with the anti(purified auxin-binding protein) antibody (0.6–0.7 mg·ml^{-1}) at 0° C for 1 h. The immunoprecipitated proteins were collected by centrifugation at 15000·*g* for 30 min, washed once with the extraction medium and analysed by SDS-PAGE. *Lane 1*, 8 μg purified auxin-binding protein; *2*, immunoprecipitated proteins from 8 μg purified protein; *3*, immunoprecipitated proteins from 28 coleoptiles (2.2 g FW) on day 4; *4*, from 29 primary leaves (2.3 g FW) on day 5; *5*, from 7.3 whole shoots (2.3 g FW) on day 5; *6*, from 44 mesocotyls (3.9 g FW) on day 4; *7*, from 37 roots (3.8 g FW) on day 4; *8*, 20 μg of the antibody

When the antibody was incubated with the membrane proteins solubilized from the acetone-washed membranes prepared from whole shoots of 5-d-old maize seedlings, three proteins of M_r 21000, 60000 and 80000 in addition to the H- and L-chains of the antibody were immunoprecipitated (lane 5). Similar protein patterns on SDS-PAGE were also obtained when the membrane proteins were prepared by extraction with detergent, 1% Triton X-100, and immunoprecipitated in the presence of 0.7% Triton X-100 (data not shown). The M_r-21000 protein was immunologically stained by the immunoblotting method as shown later (Figs. 4, 6), while the M_r-60000 and -80000 proteins were not detected by this method (Figs. 4, 6). These difference might be either the result of the association of the M_r-60000 and -80000 proteins with the M_r-21000 protein under the nondenaturing conditions, or the result of the loss of the antigenic activities of the M_r-60000 and -80000 proteins by denaturation with SDS.

The M_r-21000 protein was distributed in all organs of maize seedling tested, i.e. coleoptile (Fig. 1, lane 3), primary leaf (lane 4) and mesocotyl (lane 6), and also in the primary root as a faint band (lane 7). Specific auxin binding by membranes had already been demonstrated in the former three organs (Ray et al. 1977b; Walton and Ray 1981). We found that the membranes from the primary roots of 3-d-old maize seedlings possessed 9% of the 1-NAA binding activity of the membranes from the coleoptiles based on the fresh weight (FW) of the tissues; in the standard assay as described in *Material and methods*, the membranes from 0.88 g FW of roots (corresponding to eight roots) bound specifically 294±26 cpm of [^{14}C]1-NAA in the presence of 16800 cpm of total radioisotope, and the membranes from 0.4 g FW of coleoptiles (eight coleoptiles) bound 1406±16 cpm in the presence of 15600 cpm.

The M_r-20000 species present in the purified preparation (Fig. 1, lane 1) was not detected in this experimental series, but in some experiments (Fig. 4) it could be detected by this antibody.

Figure 2 shows the effect of the antibody on the auxin-binding activity. The residual activity of the solubilized auxin-binding protein was measured by both equilibrium gel filtration and equilibrium dialysis. By the gel-filtration method, the activity of the supernatant after immunoprecipitation decreased linearly with increase in the concentration of the antibody and was completely lost at 1 mg·ml^{-1} antibody (Fig. 2). When the activity

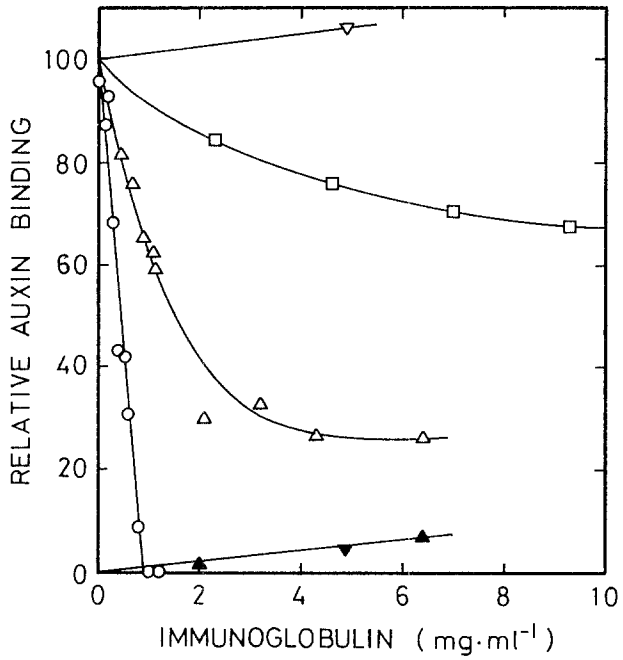

Fig. 2. Effect of the anti(auxin-binding protein) antibody on the auxin-binding activity of the solubilized or the membrane-associated auxin-binding sites from maize shoots. ○ Residual activity, measured by the gel-filtration method, of the supernatant after immunoprecipitation: the purified auxin-binding protein (6 μg·ml^{-1}) was incubated at 4° C for 2.5 h with its antibody in 10 mM Tris-HCl, pH 7.2, 5 mM $MgCl_2$ followed by centrifugation at 15000·*g* for 10 min. △▽▲▼ Auxin binding by the solubilized binding sites assayed by the equilibrium-dialysis method. To one half compartment of an equilibrium-dialysis cell was added: 4 μg·ml^{-1} binding protein + its antibody (△) or + immunoglobulin G prepared from the preimmune serum (▽), or antibody alone from immune serum (▲) or preimmune serum (▼). To the remaining half compartment was added 0.2 μM [^{14}C]1-NAA in equilibrium medium (0.15 M NaCl, 20 mM sodium citrate, pH 6.0, 5 mM $MgCl_2$); equilibrium was performed at 4° C for 5 h; the auxin-binding activity was obtained by subtraction of the radioactivities of two compartments. □ Auxin binding of the membranes from 1 g FW of whole shoots on day 5 in the presence of the anti(binding protein) antibody in 1 ml of the equilibrium medium containing 0.1 μM [^{14}C]1-NAA ± 0.1 mM 1-NAA; after incubation at 0° C for 2 h, the radioactivity in the pellet (at 127000·*g* for 20 min) was measured

of the immunocomplex was measured by the equilibrium-dialysis method, 60% of the activity was detected at this concentration of the antibody (Fig. 2). Further addition of the antibody resulted in more reduction of the activity, but some 20% of the activity remained at 6.4 mg·ml^{-1} antibody.

The immunoglobulin prepared from the preimmune serum, in contrast to the immune serum, showed no inhibitory effect on the auxin binding of the solubilized auxin-binding protein (Fig. 2). The small increase in auxin binding observed in the presence of the preimmune antibody could be attributed to auxin binding by the antibody (Fig. 2).

The effect of the anti(auxin-binding protein) antibody on the auxin binding of the membrane fraction was also examined (Fig. 2). In contrast to the solubilized binding sites, the antibody showed less inhibitory effect on the binding sites in the membranes, about 70% of the binding activity of the membranes remained at 9.3 mg·ml^{-1} antibody, while the same extent of inhibitory effect was achieved at about 0.8 mg·ml^{-1} antibody in the solubilized sites. This difference in the inhibitory effect should not be the result of the presence of larger amounts of the antigen in the membrane sample than in the solubilized one because the membranes from 1 g FW of shoots were estimated to contain about 2 μg of the auxin-binding protein (Fig. 1, lane 5) and, thus, the concentration of the protein in the immunoreaction was approx. 2 μg·ml^{-1}, while the concentration of the protein in the equilibrium dialysis was 4 μg·ml^{-1}. This difference in the inhibitory effect might be a consequence of steric hindrance by the membrane or a conformational change during solubilization of the binding sites, or else there may be auxin-binding sites immunologically different from the purified auxin-binding protein in the membranes.

Subcellular localization of the auxin-binding protein. Two types of auxin-binding sites (sites I and II) in the membranes were distinguishable by differences in their affinity for 2-NAA as well as in their positions upon isopycnic density-gradient fractionation (Fig. 3A, B). Site I activity was found in about 25% sucrose and its distribution coincided with that of NADH-cytochrome-*c* reductase, a maker enzyme of the endoplasmic reticulum. Membranes possessing site II activity were detected at higher sucrose concentrations, and their peak position was dependent on age of the coleoptile: about 32% sucrose on day 3 (Fig. 3A) and about 30% sucrose on day 5 (Fig. 3B). Dohrmann et al. (1978) showed that site II activity co-migrates under centrifugal force with acid phosphatase, a putative tonoplast marker. However, we observed that the peak density of the vesicles containing acid phosphatase was dependent on the age of the coleoptile (Fig. 3A, B), and that the distribution of site II activity coincided with that of acid phosphatase on day 5 (Fig. 3B) but differed from it on day 3 (Fig. 3A).

Figure 4 shows the immunoblots of the membrane proteins of the coleoptile which had been fractioned by density-gradient centrifugation. Ten nanograms of purified auxin-binding protein in the

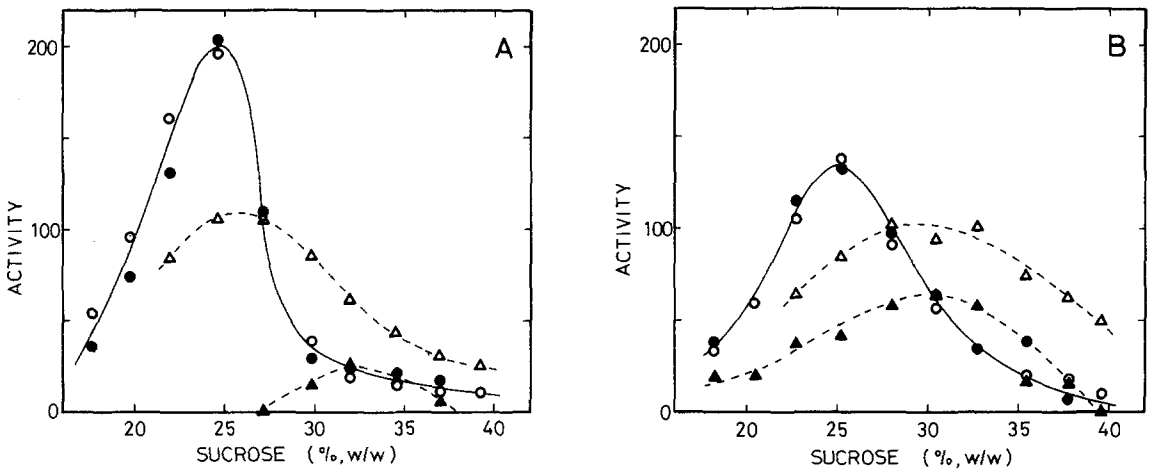

Fig. 3A, B. Sucrose-density-gradient fractionation of two types of auxin-binding sites in the membranes of maize coleoptiles on day 3 (**A**) and on day 5 (**B**). The supernatant (5000·*g*) of a homogenate from 178 coleoptiles (6.3 g FW) on day 3 or from 104 coleoptiles (12 g FW) on day 5 was fractionated on two linear gradients. The corresponding fractions (each 1.5 ml) from two parallel gradients were pooled and analysed for auxin-binding and enzyme activity. The figures represent the activity values corresponding to a single coleoptile. ● Site I activity (fmol of [^{14}C]1-NAA bound): in the binding assay, 0.9 ml of each fraction was mixed with 0.9 ml of 20 mM sodium citrate, pH 5.5, 10 mM $MgCl_2$, 0.2 μM [^{14}C]1-NAA (28800 cpm) ± 20 μM 2-NAA; at the peaks of the binding profiles, specific bindings of 1-NAA were 1736 cpm (**A**) and 654 cpm (**B**). ▲ Site II activity (fmol of [^{14}C]1-NAA bound): each fraction was mixed with an equal volume of 0.2 μM [^{14}C]1-NAA, 20 μM 2-NAA ± 0.2 mM 1-NAA; at the peaks, the specific bindings of 1-NAA were 216 cpm (**A**) and 289 cpm (**B**). ○ NADH-cytochrome-*c* reductase (nmol·min^{-1} × 4): 5 μl of each fraction were assayed; at the peaks, the activities were 0.308 (**A**) and 0.127 A_{550}·min^{-1} (**B**). △ Acid phosphatase (nmol·min^{-1} × 10): 15 μl of each fraction were assayed; at the peaks, the activities were 0.789 (**A**) and 0.443 A_{400}·(20 min)$^{-1}$ (**B**)

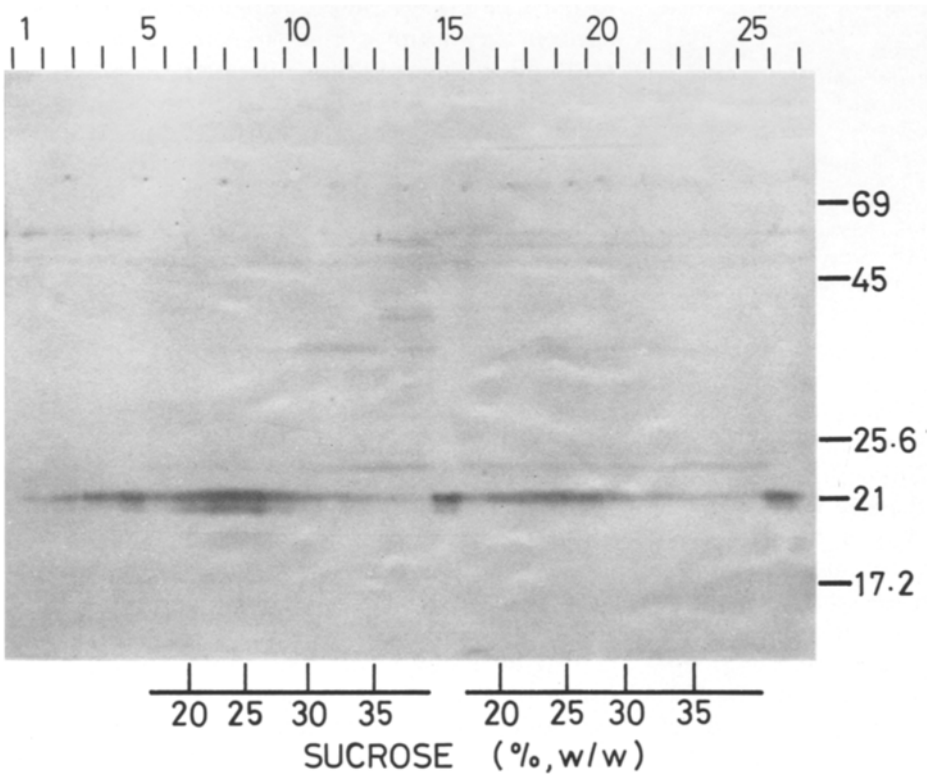

Fig. 4. Immunoblot analysis of auxin-binding sites in membrane fractions from maize coleoptiles. The supernatant (5000·*g*) of homogenates of 28 coleoptiles on day 3 or on day 5 was fractionated on a sucrose-density gradient, 0.2 ml of each fraction (1.5 ml) was precipitated by trichloroacetic acid, and the proteins were solubilized and subjected to SDS-PAGE followed by immunoblotting as described in *Material and methods*. *Lanes 1–4*, 10, 20, 40, and 80 ng of purified auxin-binding protein; *lanes 5–14*, fractions at 18.8, 20.4, 23.0, 25.3, 27.8, 30.1, 32.5, 34.7, 36.9, and 39.3% (w/w) sucrose from coleoptiles on day 3; *lanes 16–25*, fractions at 18.3, 20.8, 22.5, 24.8, 27.7, 30.0, 32.0, 34.1, 36.3, and 38.6% (w/w) sucrose from coleoptiles on day 5; *lanes 15* and *26*, 80 ng of purified binding protein

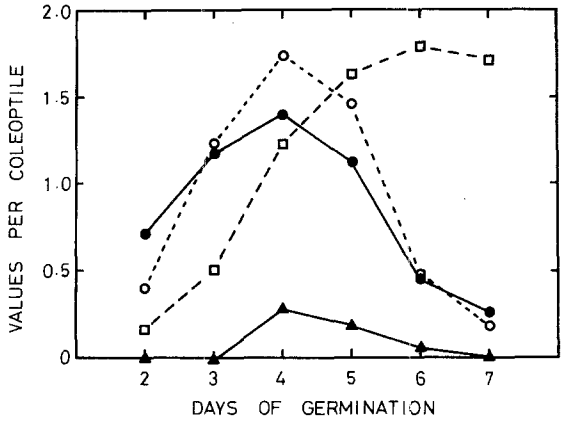

Fig. 5. Developmental patterns of two types of auxin-binding sites during growth of maize coleoptile. The membrane fraction (5000–170000·*g*) prepared from 100 coleoptiles on each day was used for auxin-binding and enzyme-activity determinations. In the binding assay, the membranes from eight coleoptiles were mixed with 1 ml of [^{14}C]1-NAA (16300 cpm) solution as described in *Material and methods*. The observed auxin bindings on day 4 were 1747 ± 67 cpm at site 1 and 345 ± 42 cpm at site II. The figures represent the values corresponding to a single coleoptile. ● Site I (pmol); ▲ site II (pmol); ○ NADH-cytochrome-*c* reductase (μmol·min^{-1} × 10); □ fresh weight (mg × 0.01)

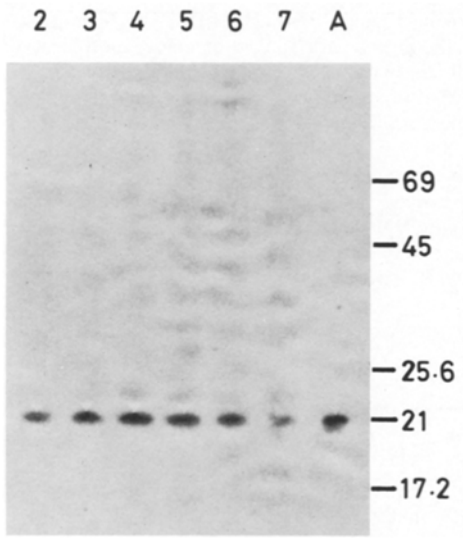

Fig. 6. Immunoblot of the membrane proteins from maize coleoptiles. *Lanes 2–7*, membrane fractions (5000–170000·*g*) from coleoptiles on, in this sequence, days 2–7. Samples applied on each lane corresponded to half a coleoptile. *Lane A*, 80 ng of purified auxin-binding protein of M_r-21000 and 20000. M_r markers used were bovine serum albumin (69000), ovalbumin (45000), chymotrypsinogen A (25600) and myoglobin (17200)

blot as a marker for color reaction of peroxidase conjugated to the second antibody was a sufficient amount for detection of the protein by the antiserum (Fig. 4, lane 1). The color reaction was proportional to the amount of the protein at least up to 80 ng (lanes 1–4). In coleoptiles of day 3 (lanes 5–14), two proteins of M_r 21000 and 20000, coinciding in positions with the two species of the purified auxin-binding protein, were stained immunologically. The color intensities of these two proteins were maximal in the 25%-sucrose fraction (lane 8), and the fractions above 30% sucrose (lanes 10–14) showed only faint bands. The distribution of these two proteins on the sucrose-density gradient coincided with that of site I activity and differed from that of site II activity (Fig. 3A). In coleoptiles of day 5 (Fig. 4, lanes 16–25), the M_r-21000 protein was stained in about 25% sucrose (lane 19) but the M_r-20000 protein was not detected. As on day 3, the distribution of the M_r-21000 protein on day 5 coincided with that of site I but differed from that of site II (Fig. 3B).

Development of sites I and II during coleoptile growth. Based on fresh-weight increase, the maize coleoptile grew, under the conditions used, through day 6 and then stopped growing (Fig. 5). The DNA content in the coleoptile was constant during growth (about 7 μg per coleoptile), indicating that the growth was mainly based on cell enlargement and not accompanied by an increase in cell number. Site I activity was observed on day 2, increased twice until day 4 and decreased thereafter, corresponding to the period of the decrease in the growth rate of the coleoptile (Fig. 5). Site II activity was low between day 2 and 3, increased between day 3 and 4, the period of rapid growth of the coleoptile, and thereafter decreased.

Figure 6 shows the result of immunoblots of membrane proteins from coleoptiles on days 2–7. The M_r-21000 protein was visible and its intensity was dependent on the age of the coleoptile; it increased until day 4 and thereafter decreased. This developmental pattern coincides with that of site I (Fig. 5). The color intensity of the protein band from half a coleoptile (Fig. 6, lanes 2–7) was comparable to that of 80 ng of purified auxin-binding protein (Fig. 6, lane A). This concentration was also comparable to that obtained from the immunoprecipitation experiments; there were roughly 0.2 μg of the protein per coleoptile on day 4 (Fig. 1, lane 3).

Distribution of sites I and II in the maize coleoptile. The auxin-binding activities of intact maize coleoptiles and coleoptiles with the outer epidermis removed are compared in Table 1. In three separate experiments, the specific activity (based on membrane protein) of total auxin-binding sites (site I plus II) in coleoptiles with the outer epidermis re-

Table 1. Comparison of auxin-binding activities between intact coleoptiles and coleoptiles with the outer epidermis removed (by abrasion with emery cloth). The membrane fractions 5000–170000·*g*) were used for analyses. In the auxin-binding assays, 0.7–0.9 mg of membrane proteins was mixed with 1 ml of [^{14}C]1-NAA (16000 cpm) solution as described in *Material and methods*. The values are means ± SD

Coleoptile	Specific activity of auxin binding (cpm·(mg protein)$^{-1}$)			Membrane protein (mg·(coleoptile)$^{-1}$)
	Total	Site I	Site II	
Expt. 1 (coleoptiles on day 4)				
Intact	1728 ± 42			86
Abraded	1567 ± 29			56
Expt. 2 (coleoptiles on day 5)				
Intact	1771 ± 45	1486 ± 45	286 ± 26	58
Abraded	1167 ± 24	886 ± 22	282 ± 24	44
Expt. 3 (coleoptiles on day 5, grown in the dark)				
Intact	1231 ± 21	950 ± 27	281 ± 27	114
Abraded	908 ± 91	580 ± 27	328 ± 91	90

moved was lower than that in the intact coleoptiles. In experiments 2 and 3 (Table 1), this low activity could be attributed to that of site I but not to site II.

Discussion

Our results demonstrate that the localization of the two molecular species, M_r 21000 and 20000, of the purified auxin-binding protein from maize shoots (Shimomura et al. 1986) corresponds to site I in the endoplasmic reticulum. Since the minor M_r-20000 protein was not always detected, it might be a modification of the M_r-21000 protein artificially formed after homogenization of the tissues.

The content of the M_r-21000 protein in maize shoots on day 5 was immunochemically estimated to be about 2 μg·(g FW)$^{-1}$ which corresponds to 100 pmol of auxin-binding sites (or 50 pmol, assuming that one binding site is composed of two subunits; Shimomura et al. 1986). Murphy (1980) has estimated the number of the auxin-binding sites in maize shoots to be at least 100 pmol·(g FW)$^{-1}$. Therefore, the M_r-21000 protein would be the major auxin-binding protein in site I. The M_r-21000 protein was present in primary leaf, coleoptile and mesocotyl of the maize seedling in comparable amounts, based on organ fresh weight. A small amount of the protein was also detected in the primary root of maize seedling. The content of the M_r-21000 protein was of the order of 0.01% of total cellular proteins of maize coleoptiles, a value one order higher than those of animal hormone or neurotransmitter receptors located in the plasma membrane such as insulin receptor (Siegel et al. 1981), β_2-adrenergic receptor (Benovic et al. 1984) and muscarinic acetylcholine receptor (Haga and Haga 1985). However, the content of a thyroid-hormone-binding protein located in the endoplasmic reticulum of human cells has been reported to be approx. 0.03% of total cellular proteins (Cheng et al. 1986), i.e. of the same order as that of the auxin-binding protein located in the endoplasmic reticulum of the maize coleoptile.

Another type of auxin-binding site (site II) was detected in the maize coleoptile in about 30% sucrose on the sucrose-density gradient, whereas site I was at about 25% sucrose (Fig. 3A, B). In the immunoblotting experiments (Fig. 4), the antibody against the purified auxin-binding protein could not detect any proteins except for the M_r-21000 protein in the corresponding fractions in which site II activity was observed (Fig. 3A, B). If site II were an identical protein to site I, the color intensity of the M_r-21000 protein in 30% sucrose on the immunoblots of the 5-d-old coleoptiles (Fig. 4, lane 21) should have been comparable to that in 25% sucrose (lane 19) and stronger than that in 20% sucrose (lane 17) since the site II activity had been underestimated under the assay conditions (the presence of 10 μM 2-NAA and the lower affinity of site II for 1-NAA than that of site I; Dohrmann et al. 1978).

Different properties between sites I and II have been reported: the two sites exhibit differences in their binding affinities for a series of auxin-relating compounds (Batt et al. 1976; Batt and Venis 1976; Dohrmann et al. 1978); a supernatant factor (benzoxazolinones; Venis and Watson 1978) inhibits auxin binding at site I but not at site II (Dohrmann et al. 1978); dithioerythritol inactivates site I but not site II (Dohrmann et al. 1978). In addition, there are differences in their development during coleoptile growth (Fig. 5) and in their localization in the coleoptile tissues (Table 1). Site I was present at the early developmental stage and its level increased during coleoptile growth twice, between day 2 and 4. Site II activity was, in contrast, low at the early stage and increased at the rapid-growth stage of the coleoptile between day 3 and 4 (Fig. 5). In contrast to their different development during the early growth of the coleoptile, both auxin-binding sites concurrently decreased after day 4, soon followed by a reduction in the growth rate of the coleoptile (Fig. 5). A reduction of auxin-binding

sites in the membrane fraction has been reported by Walton and Ray (1981) in the mesocotyl of red-light-irradiated maize seedling, and this irradiation also resulted in the reduction in auxin-mediated growth of the mesocotyl.

The specific activity of auxin binding to site I in coleoptiles with the outer epidermis removed was lower than that in intact coleoptiles, while the specific activity of site II remained constant after removing the outer epidermis (Table 1). Löbler and Klämbt (1985) have found, using indirect immunofluorescence-labeling, that site I protein was located in the outer epidermis of the maize coleoptile. Our results may be consistent with this observation. Assuming that the outer epidermis constitutes 11% of the total coleoptile volume (Löbler and Klämbt 1985) and that the membrane proteins are uniformly distributed in the coleoptile tissues, approx. 47% (Table 1, experiment 2) or 46% (experiment 3) of site I in the coleoptile was localized in the outer epidermis. The specific activity of site I in the outer epidermis was 7.2-fold (experiment 2) or 6.8-fold (experiment 3) higher than that in the residual coleoptile sections.

Our results indicate that site I is not merely a precursor of site II, as previously speculated (Dohrmann et al. 1978; Jacobs and Hertel 1978) but rather that the two sites are involved in different cellular functions. Site I activity has been demonstrated to be a result of complex formation between auxin and a binding protein but it remains unclear whether site II activity is to be attributed to auxin-binding protein or to auxin uptake by membrane vesicles like the site III activity in the plasma membrane (Hertel et al. 1983).

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