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INDUCTION OF A HIGH AFFINITY BINDING SITE FOR AUXIN IN *AVENA* ROOT MEMBRANE

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Key Word Index—*Avena sativa*; Gramineae; oats; membrane; auxin binding site; roots.

Abstract—A membrane preparation from *Avena sativa* root has been found to contain only one low-affinity IAA-binding site having a K_d value of 8.4×10^{-6} M, while that from coleoptile contains one low-affinity and one high-affinity IAA-binding site, having K_d values of 7×10^{-6} M and 2×10^{-7} M respectively. Our incubation of *Avena* root in the presence of IAA for 1 hr, an IAA-binding site with a high-affinity, ($K_d = 6.8 \times 10^{-7}$ M) has been detected in the membrane. The appearance of this high-affinity site was prevented when induction with IAA was performed in the presence of cycloheximide. Two new protein bands having MW of 2.63×10^4 and 10^4 have been detected in the IAA-induced root using L-[^{35}S]methionine as monitored by subjecting the membrane proteins to slab gel electrophoresis and autoradiography. One of the protein bands (2.63×10^4) coincided with a similar band of protein isolated from the coleoptile membrane. This suggests that one of the early events of auxin action in gene expression is the generation of a membrane high affinity site in the root system.

INTRODUCTION

The mechanism of auxin action is not yet clearly known. It is assumed that in the early event of the process, the auxin molecule must be specifically recognized by the cellular receptors. These receptors complexed with auxin must then signal the cell in some way, leading to the occurrence of physiological responses characteristic of auxin action in the sensitive tissue. Therefore it is important to isolate such receptors or binding sites from auxin-sensitive tissue to understand the mechanism of auxin action[1–4]. Each receptor binds the hormone with a definite affinity and molecular specificity related to the hormonal response, which is the basis of receptor detection. There exists a correlation between the affinity of the auxin with the receptor and its sensitivity to tissue extension.

Although high-affinity auxin binding sites have been reported in coleoptile, hypocotyl or epicotyl membranes of different plants[5–13], it is not detectable in the root membrane[5]. However, the presence of a low-affinity binding site in the root is documented[14]. Since the root membrane does not contain the high-affinity auxin binding site, the question arises as to whether such a binding site could be generated within a short period after incubating the intact roots in presence of IAA. The present study reports the induction of such a site in the root membrane by IAA.

RESULTS

An attempt was made to determine the dissociation constant (K_d) of IAA and membrane (10^3–12×10^3 *g* fraction) binding, using the pelleting assay method of Batt *et al.*[10]. The analysis of Scatchard plots of the results (obtained from Figs. 1 and 2 and compared in Table 1) show only one (low affinity, high capacity) IAA-binding site for control root membrane (Fig. 1a) and two (one low affinity, high capacity and one high affinity, low capacity) IAA-binding sites for the control coleoptile membrane (Fig. 1b).

In order to check whether the auxin binding site is located in the plasma membrane, further purification of the membrane fraction from root and coleoptile was carried out. Membrane marker enzymes were assayed at every step of purification as outlined by Hodges and Leonard[15]. Although no apparent purification of IAA binding activity in the plasma membrane of root and coleoptile was detected by the assay method (Table 2), there was however, a ten-fold and four-fold increase in specific activity of β-glucan synthetase and of K^+-ATPase of root plasma membrane respectively. The cytochrome *c* oxidase activity which is a marker for the mitochondrial fraction has been found to be decreased by eight-fold in the case of the root plasma membrane (Table 3). It is known that *Avena* coleoptiles are more sensitive to auxin compared with roots, so far as the elongation of the tissue after the application of auxin is concerned[16]. Taking this and our previous results into consideration, it is assumed that the highly responsive (towards auxin) tissue has the high affinity site, whereas less responsive or non-responsive tissue does not. Is it possible then to make the root competent so that IAA can generate the high affinity site within a short period of incubation? To approach this problem a different type of experiment was devised, involving the incubation of intact root tissue in IAA solution (5×10^{-5} M) for different time intervals. The result (Table 1) shows that the high affinity site in root membrane is detectable only when

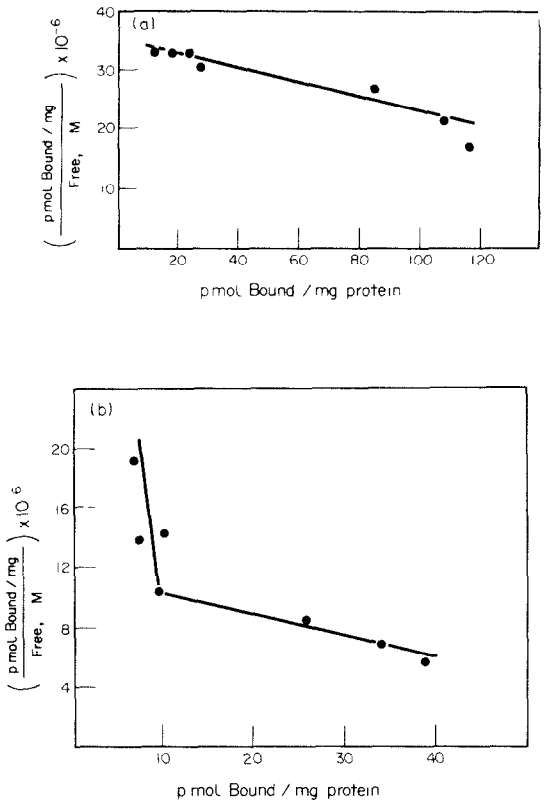

Fig. 1. Scatchard plot of IAA binding with 10^3–12×10^3 g membrane. IAA binding to the membrane was carried out as described in Table (1). (a) Root; (b) coleoptile.

the intact roots are incubated for at least 1 hr in the presence of IAA (Fig. 2).

To confirm whether the induction of the high-affinity site is due to *de novo* protein synthesis, the induction experiment for roots was performed in the presence of 5×10^{-5} M cycloheximide and the K_d for IAA binding with the root membrane was determined. The result shows a total inhibition of the inducible high-affinity site with no effect on the low-affinity site (same as in Fig. 1a). It is also possible to repeat the same type of experiments with purified membrane

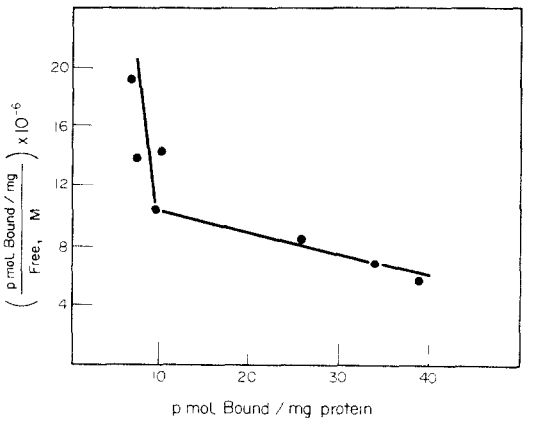

Fig. 2. Scatchard plot of IAA binding with 10^3–12×10^3 g membrane. The membrane was isolated from the root in which a high affinity site has been induced by incubating with 5×10^{-5} M IAA at 32° for 1 hr. Experimental conditions were the same as in Table 1.

and solubilized membrane proteins by Triton X-100 (data not shown). When an IAA induction experiment (1 hr incubation) was performed to monitor protein synthesis in the membrane, L-[^{35}S]methionine was used together with IAA. The membrane proteins were solubilized by SDS and subjected to gel electrophoresis in a vertical slab. After processing and drying the gel, it was exposed to X-ray film. From such an autoradiograph (Fig. 3), it was observed that IAA-treated roots contain two newly-labelled protein bands having MWs of 2.63×10^4 and 10^4 (lane 2 from left), in addition to other proteins synthesized by the control roots (lane 1 from left). An identical protein band having a MW of 2.63×10^4 from the coleoptile membrane was also detectable after staining the same gel (picture not shown). When cycloheximide was used, neither bands 2.63×10^4 nor 10^4 were synthesized by the root (lane 3 from left).

DISCUSSION

The physiological role of the high-affinity binding site for auxin, as described by several workers from different systems [5–13], is not yet well understood. It is assumed that the auxin binding to the receptor may

Table 1. Comparison of dissociation constants for IAA binding in control and IAA induced membranes

Membranes	High affinity site		Low affinity site	
	K_d(M)	n(pmol IAA bound/mg protein)	K_d(M)	n(pmol IAA bound/mg protein)
Control root	Nil	Nil	8.4×10^{-6}	294
IAA induced root	6.8×10^{-7}	15	7.0×10^{-6}	57
Control coleoptile	2.0×10^{-7}	12	7.0×10^{-6}	81

The above values have been obtained from Scatchard plots of the results depicted in Figs. 1 and 2. The different concentrations of IAA used vary from 4.0×10^{-7} M to 7.4×10^{-6} M. [^{14}C]IAA concentration was kept fixed to 4×10^{-7} M and final IAA concentration up to 7.4×10^{-6} M was obtained by adding unlabelled IAA. The non-specific binding corresponding to an IAA concentration of 10^{-4} M was subtracted from each point before plotting.

Table 2. IAA binding activity in 10^3–12×10^3 *g* membrane and purified plasma membrane fractions

Fractions	IAA binding activity (cpm/mg protein)	
	Root	Coleoptile
10^3–12×10^3 *g* pellet	1860	1800
Purified plasma membrane	1890	1830

IAA binding to the membrane was done as described in Table 1.

Table 3. Purity of the membranes

Membrane fractions	Enzyme activity (μmol/min/mg protein)					
	β-Glucan synthetase II ($\times 10^3$)		K^+-stimulated ATP-ase pH 6.0		Cytochrome *c* oxidase	
	Root	Coleoptile	Root	Coleoptile	Root	Coleoptile
10^3–12×10^3 *g*	0.26	0.09	0.05	0.006	0.34	0.38
12×10^3–10^5 *g*	1.04	0.27	0.11	0.013	0.05	0.07
Plasma membrane	2.60	0.55	0.22	0.028	0.04	0.06

The different marker enzymes have been assayed as described in the text. The membrane fractions used were the same as those used for the binding assay in Table 2.

increase either the polar transport of auxin[17] or may activate a membrane bound K^+-dependent ATPase proton pumping system[18] so that the localized pH drop can hydrolyse the cross linkages in the cell wall for extension and elongation of the cell wall during growth. But the evidence is still indirect.

In a continuation of our work on the membrane bound auxin receptors from *Avena* roots[14], it has been observed that the sites for binding auxin with higher affinity or lower K_d values are not detectable (Table 1 and Fig. 1a). This is consistent with the results of Hertel *et al.*[5], in which they did not detect auxin binding sites in maize root and tuber tissue. When the binding sites were solubilized by Triton X–100 from the membrane of both the coleoptile and root and passed through a DEAE–cellulose column, these were resolved into two peaks[19]. However, it was difficult to monitor the profile of proteins in the gel electrophoretogram due to presence of Triton X–100. Using SDS for solubilization, although it is possible to get a definite pattern of protein bands in the gel electrophoretogram, the detection of actual auxin-binding protein in the gel was difficult. From the pattern of labelled proteins (Fig. 3) it is apparent that at least two proteins are synthesized *de novo* in the root by the application of IAA. That these proteins might be the induced IAA-binding sites is indicated by: (1) these protein bands are not detectable in the control root membrane, (2) high-affinity IAA-binding sites are also not present, and (3) when the synthesis of these proteins is induced by IAA in a short period, the detection of IAA-binding sites is possible. If the protein synthesis is suppressed during induction, the IAA-binding sites could not be detected as well. Whether the 2.63×10^4 and 10^4 proteins constitute two subunits of a single high-affinity IAA-binding site cannot be assessed at present. Root, tuber tissues[5] and mesocotyl[13] which respond physiologically to auxin treatment apparently having no detectable auxin-binding activity raised doubts concerning the significance of the high affinity site in the coleoptile. If it is a matter of continuous generation either in the coleoptile or root or other tissues in the presence of a threshold internal concentration of IAA, then it may be possible to identify a correlation between the apparent presence or absence of these sites and the physiological response. It has recently been indicated in the case of artichoke tuber tissue that auxin-binding activity of the crude membrane fractions is detectable only after culturing tuber tissue in presence of 2,4-dichlorophenoxyacetic acid (2,4-D) for a long period[20]. Since no other properties of this auxin-binding site are available, it is difficult to compare the *Avena* root high affinity site with that from tubers.

It appears, therefore, that one of the early events of auxin action in gene expression is the generation of a membrane high-affinity site in the root system. In the case of the coleoptile, since the optimal internal concentration of IAA is maintained, the high-affinity site is synthesized continuously, whereas in the case of root, owing to the sub-optimal level of IAA

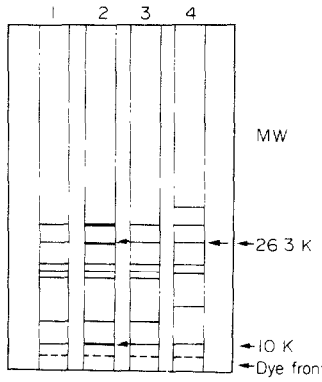

Fig. 3. Diagram of the autoradiograph of SDS-soluble membrane (10^3–12×10^3 *g*). Intact roots were incubated with 5×10^{-5} M IAA alone or 5×10^{-5} M IAA and 5×10^{-5} M cycloheximide for 1 hr in presence of L-[^{35}S]methionine. The membranes were isolated and treated with final sample buffer as described by Laemli[23] and then centrifuged at 12×10^3 *g* for 40 min and the clear supernatant used in electrophoresis in a vertical slab gel containing a 10% separating gel and 3% stacking gel. *Ca* 20 000 cpm was applied in each of lanes 1, 2 and 3 and 10 000 cpm in lane 4 and subjected to electrophoresis. After electrophoresis, the slab was stained and destained as described by Bonner and Lasky[24], dried and then exposed to X-ray film for one month at room temperature. Left to right: lane 1, 10^3–12×10^3 *g* root membrane (control); lane 2, 10^3–12×10^3 *g* root membrane, IAA-induced; lane 3, 10^3–12×10^3 *g* root membrane, IAA induced in presence of cycloheximide; lane 4, 10^3–12×10^3 *g* coleoptile membrane (control).

present, the synthesis of this site is shut off and cannot be detected by the binding assays used. As soon as IAA is given exogenously or by some other process, the synthesis of a high-affinity binding site is induced (within 1 hr) as one of the early effects of IAA detectable in the root membrane.

EXPERIMENTAL

Oat (*Avena sativa*, cv Kent) was purchased from National Seeds Corporation Ltd., New Delhi, India. 3-Indolylacetic acid (IAA), cycloheximide, sodium dodecyl sulfate (SDS), phenylmethylsulfonyl fluoride (PMSF), Triton X-100 and marker proteins were purchased from Sigma. Radioactive 3-indolyl [2-^{14}C]acetic acid (49 Ci/mol) and L-[^{35}S]methionine (1095 Ci/m mol) were obtained from Amersham International, U.K. and INDU medical X-ray film (screen type) was a product of Hindustan Photo Films Mfg Co., Ootacamund, India.

Induction of high-affinity site with IAA alone. Ca 7–12 g intact root or coleoptile were taken, washed thoroughly with H_2O and incubated for different time intervals (15, 30, 60, 120 and 300 min) in 10 mM K-Pi buffer (pH 6) with (experimental) and without (control) 5×10^{-5} M IAA at 32° in the dark with frequent shaking. At the end of the incubation period, the adhering IAA was removed by washing with cold (4°) H_2O and processed for the detection of IAA-binding sites in the membrane as described under binding assay.

Induction with IAA as detected by L-[^{35}S]*methionine incorporation.* The method of induction was the same as described previously, except that the K–Pi buffer used contained *ca* 30–40 μCi L-[^{35}S]methionine with (experimental) or without (control) IAA. The protein pattern of the membrane was then detected as described later.

Membrane isolation and binding assay. 10^3–12×10^3 *g* membrane pellet was obtained according to the method of ref. [21]. The homogenization buffer contained PMSF as a protease inhibitor. The crude homogenate was first centrifuged at 10^3 *g* for 5 min and the supernatant further centrifuged at 12×10^3 *g* for 40 min and the pellet washed as usual. The membrane fraction was further purified by sucrose density gradient centrifugation and characterized by the presence of marker enzymes[15]. The membrane was assayed for IAA-binding activity using the pelleting method of ref. [10]. Protein was determined by the Lowry method[22] using bovine serum albumin as standard.

SDS electrophoresis of membrane and autoradiography. SDS–acrylamide (10%) gel electrophoresis in a vertical slab of L-[^{35}S]methionine-incorporated membrane was done following the method of ref. [23]. The slab gel was dried as described in ref. [24] and autoradiographed using INDU X-ray film. The MW of each protein band was determined by comparing their mobilities with those of the marker proteins.

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