Isolation of a novel auxin receptor from soluble fractions of rice (*Oryza sativa* L.) shoots

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Received 10 September 1998

Abstract An auxin binding protein (ABP) was isolated from the shoots of rice seedlings and characterized. The ABP was found to be a monomer with a molecular mass of 57 kDa and play a crucial role via auxin binding in regulating H^+ translocation activity of the plasma membrane in a typical biphasic manner. The results of binding equilibrium experiments indicate that the ABP binds indole 3-acetic acid with a high affinity ($K_d = 1.9 \times 10^{-8}$ M), having four primary binding sites for auxin and some secondary sites with low auxin affinities. The ABP appears to have an unambiguous auxin receptor function.

Key words: Auxin binding protein; Binding equilibrium; Dose-response curve; H^+ translocation; Indole 3-acetic acid; *Oryza sativa*

1. Introduction

Auxins are a class of plant hormones that influence various processes of plant growth and development [1]. The main auxin in most plants is indole 3-acetic acid (IAA), which appears to control, besides many other effects, cell division and elongation. By analogy with models of hormone action in animals, it is generally accepted that numerous effects of auxin in plants are mediated by a variety of cellular receptors [2]. Several putative receptor sites have been reported in both the membrane and soluble fractions of some plants [3–9]. Auxin binding protein 1 (ABP1), a homodimeric protein with 22 kDa subunits located in the endoplasmic reticulum and the plasma membrane of maize, has been isolated and extensively characterized [3]. Hicks et al. [4,5] identified two auxin receptor proteins with molecular masses of 40 kDa and 42 kDa, respectively, in membranes of zucchini and tomato by photoaffinity labeling. Jones and Venis [6] found a 43-kDa membrane protein in maize also by photoaffinity labeling and claimed this protein to be identical to those of Hicks et al. [4]. Other authors described cytosolic auxin binding proteins. Sakai and co-workers [7] detected a nuclear protein that stimulates the expression of a 18-kDa translation product. A 65-kDa soluble ABP was identified in soybean hypocotyls by Prasad and Jones [8]. Another ABP with a molecular mass of 44 kDa, designated ABP_{44}, that binds a number of active auxins with reasonably high affinities was purified from soluble fractions of pea apices by Reinhard and Jacobson [9].

We previously reported the existence in rice (*Oryza sativa* L.) plants of, at least, two isoforms of soluble protein factor, which are closely associated with an auxin action on plant membranes in the respective concentration ranges of applied IAA, separated by about 3 orders of magnitudes [10]. In the present study, as an extension of the previous one, the dominant isoform of the soluble factor in rice shoots, formerly designated SPF_{II}, which acts as a mediator for IAA-induced stimulation of P-type ATPase in microsomal membranes in a 10^{-7}–10^{-4} M range of IAA, was purified to homogeneity and characterized. The results indicate that this factor is a monomeric protein with a molecular mass of 57 kDa, binds IAA with a high affinity ($K_d = 1.9 \times 10^{-8}$ M) and plays a crucial role via IAA binding in regulating H^+ translocation activity of the plasma membrane, producing a typical bell-shaped dose-response curve for the auxin effect. The protein will hereafter be referred to as ABP_{57}.

2. Materials and methods

Rice seedlings were hydroponically grown in Hoagland medium in complete darkness for 7 days at 25 ± 2°C. The shoots were harvested, immediately frozen in liquid nitrogen and stored at −80°C until use.

The procedures for ABP_{57} isolation comprised six major steps involving ammonium sulfate fractionation, DEAE-Sepharose CL-6B (anion exchange) chromatography, CM-Sepharose CL-6B (cation exchange) chromatography, Sephacryl S-100 (gel permeation) chromatography, tryptophan-attached Sepharose 4B (affinity) chromatography, and Superdex 75 fast protein liquid chromatography (FPLC).

Crude extracts, prepared by homogenizing about 2.5 kg of the frozen shoots in an extraction buffer (0.25 M sucrose, 2.5 mM dithiothreitol, 3 mM EDTA and 20 mM Tris-MES, adjusted to pH 6.7), filtering through cheesecloth and centrifuging at 12 000 × *g*, were ammonium sulfate fractionated. The precipitates obtained at ammonium sulfate concentrations over the range of 30–45% saturation were suspended in a suspension buffer (0.25 M sucrose, 2.5 mM dithiothreitol, 3 mM $MgSO_4$, 20 mM KCl and 20 mM Tris-MES, adjusted to pH 8.0) and loaded onto a DEAE-Sepharose CL-6B column (5 × 8 cm). The flow-through fractions showing ABP_{57} activity were adjusted to pH 6.0 with dilute HCl and applied to a CM-Sepharose CL-6B column (3.5 × 6 cm). The bound proteins were eluted with a linear gradient of NaCl (0–0.5 M) in 20 mM Tris-MES buffer (pH 6.0). The active fractions were pooled, concentrated using microcentrifuge filters (Nihon Millipore, Yonezawa, Japan) at 3500 × *g* and then layered on a Sephacryl S-100 column (2.5 × 80 cm) equilibrated with an elution buffer (1 mM EDTA, 1 mM leupeptin, 0.1 M KCl and 20 mM Tris-MES, adjusted to pH 7.0). The protein was further purified in an affinity column filled with our preparations of tryptophan-attached Sepharose 4B, which is shortly to be described in detail. The final purification was accomplished in a FPLC system (Pharmacia Biotech, Uppsala, Sweden), using a Superdex 75 column equilibrated with 20 mM Tris-MES buffer (pH 7.0).

Coupling of tryptophan to Sepharose 4B gel was performed according to a procedure for the covalent attachment of primary amines to CNBr-activated resin [11], as follows. Pre-swollen CNBr-activated Sepharose 4B gel (20 ml) was mixed with tryptophan (3 mM) in 0.1 M $NaHCO_3$ buffer (pH 8.3) containing 0.5 M NaCl and gently rotated in an end-over-end manner for 15 h at 4°C. Then the gel was filtered off and washed with several cycles of 0.1 M acetate buffer (pH 4.0). In order to block the remaining active groups the gel was mixed with

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PII: S0014-5793(98)01307-6

4 volumes of 1 M ethanolamine (pH 8.0) and gently rotated for 2 h at room temperature. Excess blocking agent was washed away with 0.1 M acetate buffer (pH 4.0) containing 0.5 M NaCl. The concentration of tryptophan coupled to the gel was estimated to be ca. 3 μmol/ml. The tryptophan-attached Sepharose 4B column (1.5×5 cm) was equilibrated with 20 mM Tris-MES buffer (pH 7.0) and the elution was done with a linear gradient of IAA (0–20 mM) in 20 mM Tris-MES buffer (pH 7.0) containing 1 M KCl at a flow rate of 0.2 ml/min. The active fractions were pooled and dialyzed against 20 mM Tris-MES buffer (pH 7.0) for the ensuing FPLC.

Plasma membrane vesicles were prepared, essentially according to Larson et al. [12] as in Laporte and Rossignol [13], by phase partitioning in an aqueous two-phase system of dextran T-500 (6.6%, w/w) and polyethylene glycol 3350 (6.6%, w/w) in 5 mM phosphate buffer (pH 7.0) containing 3.3 M sucrose and 3 mM KCl. The upper phase was diluted in 8 volumes of 10 mM Tris-MES buffer (pH 5.7) containing 0.33 M sucrose and 1 mM PMSF, and centrifuged at 100 000×*g* for 1 h. The pellets of membrane vesicles were suspended in 10 mM MOPS-BTP buffer (pH 7.0) containing 0.25 M sucrose and stored in liquid nitrogen until use.

Vanadate-sensitive ATP hydrolysis by the plasma membrane was measured as previously described [10]. ABP_{57} activity of protein fractions during the purification procedures was determined by assessing the combined effect of the protein plus IAA (5 μM) on the stimulation of P-type ATPase in the inside-out plasma membrane vesicles that were formed during thawing of liquid nitrogen-frozen membranes.

H^+ translocation activity of the membrane was fluorophotometrically assayed, based on the quenching kinetics of acridine orange fluorescence in the membrane suspensions, as described by Palmgren [14]. The basal mixture consisting of the plasma membrane (20 μg/ml) and acridine orange (2 μM) in an assay buffer (1.2 mM Na-ATP, 50 mM KBr, 0.14 M KNO_3 and 10 mM MOPS-BTP, adjusted to pH 7.0) was incubated in the presence of either ABP_{57}, IAA or ABP_{57} plus IAA and their absence for 20 min at 37°C. H^+ translocation into the inside-out membrane vesicles was initiated by adding 20 μl of 120 mM $MgSO_4$ to 2.0 ml of the reaction mixtures in a cuvette.

[^{14}C]IAA binding to ABP_{57} was tested by equilibrium dialysis in 2×250 μl Teflon dialysis cells (Hoefer Pharmacia Biotech, San Francisco, CA), using dialysis membranes with a molecular weight cutoff of 12 000. ABP_{57} solution (200 μl at 0.5 μg/ml) in 50 mM K-phosphate buffer (pH 7.0) was placed on one side of the membrane and [^{14}C]IAA solution (200 μl at 1.75–175 μCi/ml) in the same buffer on the other side, and rotated for 12 h at 4°C. Then 100-μl aliquots were taken from each half of the cells, pipetted into 2 ml of Optiphase 'Hisafe' (Fisons Chemical, Loughborough, UK) and measured for radioactivity in a Wallac 1409 liquid scintillation counter.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of proteins was performed on acrylamide gradient (10–22%) gels which were stabilized by a 5.5–11% linear sucrose gradient in the discontinuous buffer system of Laemmli [15]. Gel permeation HPLC of proteins was conducted under non-denaturation conditions, using a BioSep SEC-S3000 column equilibrated with 20 mM Tris-MES buffer (pH 7.0).

3. Results

The overall scheme of the protein purification is compiled in Table 1. One unit of ABP_{57} activity was defined as the amount of protein causing a 2-fold increase in the rate of ATP hydrolysis by 1 mg protein of the plasma membrane in the presence of the optimum concentration of IAA (5 μM) at 37°C and at pH 7.0. Of the purification procedures the affinity chromatography using tryptophan residue as a ligand for the protein turned out to be the most effective with respect to the column efficiency for separation, in which a fairly well-resolved protein band coincided well with the activity band.

The isolated ABP_{57}, purified about 2500-fold with a yield of 6%, gave a single band on SDS-PAGE corresponding to a molecular mass of 57 kDa and a single protein peak placed between two marker proteins, bovine serum albumin (66 kDa) and ovalbumin (45 kDa) on gel permeation HPLC, as shown in Fig. 1: these results indicate that ABP_{57} is a monomeric protein.

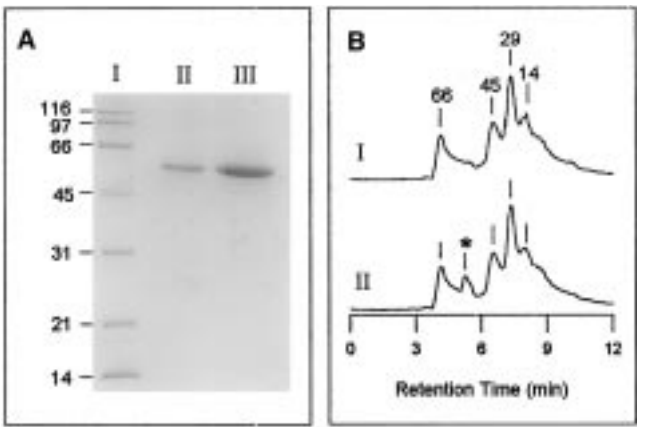

Fig. 1. SDS-PAGE and gel permeation HPLC of ABP_{57}. A: Lane I shows molecular mass markers in kDa, while lanes II and III show the isolated ABP_{57}, loaded at ca. 1.5 μg and 7.5 μg, respectively. B: Trace I is the chromatogram of a mixture of marker proteins under non-denaturation conditions with values in kDa and trace II gives the peak of ABP_{57}, indicated by *, among the same markers.

Biochemical responses of the plasma membrane to ABP_{57}, IAA, and orthovanadate were examined. The H^+-ATPase activity was significantly increased by the inclusion of ABP_{57} (0.1 μg/ml). The addition of IAA (5 μM) to this mixture resulted in a further increase to a much larger extent in H^+ translocation. Such effects of ABP_{57} alone or ABP_{57} plus IAA were completely eliminated by the presence of orthovanadate. The combined effect of ABP_{57} and IAA on the membrane function was found to be irrespective of the order of addition to the membrane samples. The H^+-ATPase, however, did not respond to IAA itself in the absence of the ABP. The relevant data obtained in fluorescence quenching experiments, using acridine orange as an indicator for H^+ accumulation in the inside-out membrane vesicles, are illustrated in Fig. 2.

Changes in the rate of H^+ translocation by the H^+-ATPase responding to IAA were measured and plotted as a function of the concentration of IAA. As shown in Fig. 3, the resulting dose-response curve turned out to be bell-shaped, with the maximum stimulation occurring at ca. 5×10^{-6} M, only in the presence of ABP_{57}.

IAA binding to ABP_{57} was tested over an IAA concentration range of 0.1–10 μM. A Scatchard plot from the binding results is given in Fig. 4. Since the plot showed curvature for a

Table 1
Purification of ABP_{57}

Step[a]	Protein (mg)	Total activity (units)	Specific activity (units/mg)
CEX	942	1072	1.1
ASF	112	406	3.6
AEC	14	361	26
CEC	2.1	183	87
GPC	0.19	124	653
AFC	0.034	81	2382
FPLC	0.022	60	2727

[a]CEX, crude extract; ASF, ammonium sulfate fractionation; AEC, anion exchange chromatography; CEC, cation exchange chromatography; GPC, gel permeation chromatography; AFC, affinity chromatography; FPLC, fast protein liquid chromatography.

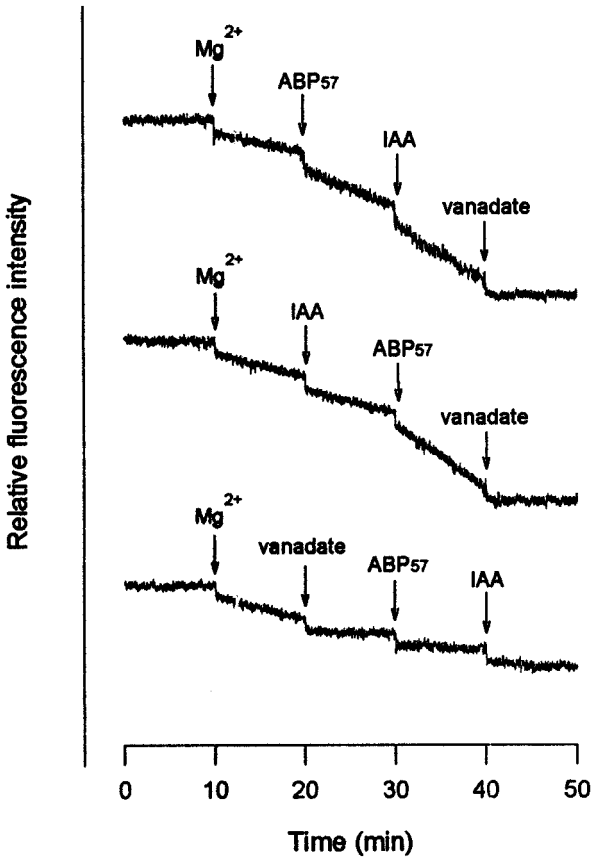

Fig. 2. The effects of ABP_{57}, IAA and orthovanadate on H^+ translocation across the plasma membrane. Time courses of change in fluorescence intensity of acridine orange were traced. The additions of the reaction initiator (1.2 mM Mg^{2+}) and the effectors (0.1 μg/ml ABP_{57}, 5 μM IAA and 2 mM orthovanadate) to the basal mixture of the membrane (20 μg protein/ml) and acridine orange (2 μM) are indicated by arrows.

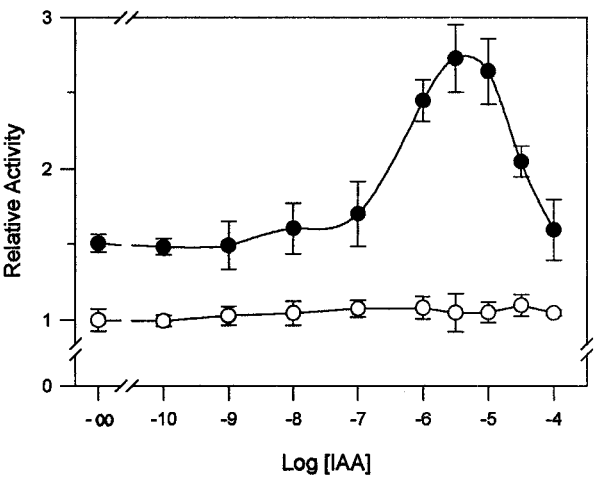

Fig. 3. Dose-response curves for IAA-induced modulation of H^+-ATPase of the plasma membrane in the presence of ABP_{57} (●) and its absence (○). IAA (0–100 μM) was added to the basal mixture either alone or together with ABP_{57} (0.1 μg/ml) and the reaction was initiated by Mg^{2+} (1.2 mM). The H^+-ATPase activity was measured from the fluorescence quenching kinetics as shown in Fig. 2. Data are presented as mean ± S.E.M. ($n = 3$).

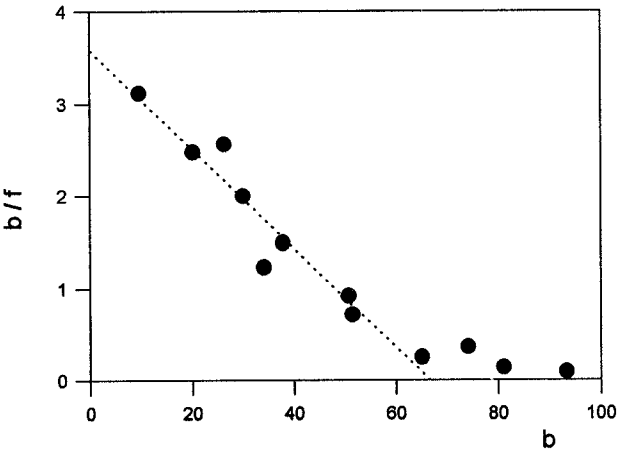

Fig. 4. Scatchard plot of the binding data of [^{14}C]IAA to ABP_{57}. *b*, [^{14}C]IAA bound to ABP_{57} in units of nmol/mg protein; *f*, [^{14}C]IAA unbound in units of nM. Results are averages of duplicate measurements.

larger number of ligands bound (*b*), the data did not appear properly represented by the identical-and-independent-sites model for ligand binding to a macromolecule. Nonetheless, for $b \leq 6.5 \times 10^{-8}$ mol/mg they fitted reasonably well to a straight line. From the slope of the line the dissociation constant (K_d) was determined to be 1.9×10^{-8} M. Although the number of binding sites per protein (*N*) could not be precisely calculated because the Scatchard plot was curved for higher values of *b* and because the amount of ABP_{57} was estimated by the stained band on electrophoretic gels, the intercepts of the extrapolated straight line on the horizontal and vertical axes gave $N = 3.9$, which is close enough to 4.

4. Discussion

Auxin receptors are defined as primary detectors and transducers of auxin signal in plant cells [16]. When a certain protein present in plant materials at a low concentration was found to specifically bind active auxins with high affinities, the protein was regarded as an ABP which might have a receptor function. Therefore, several approaches for the identification and/or isolation of auxin receptor proteins have concerned their auxin binding characteristics, such as photoaffinity labeling, affinity chromatography and anti-idiotypic antibody approach [3–9]. A criterion for ABP is K_d of the protein-auxin complex; that is, when K_d is approximately equal to or smaller than 1 μM, the protein is considered to be an ABP [9].

The results presented herein clearly indicate that ABP_{57} binds the natural auxin IAA with a high affinity ($K_d = 1.9 \times 10^{-8}$ M) and plays a role in regulating an important physiological function of the plasma membrane apparently via auxin binding (Figs. 2 and 3). Furthermore, this ABP appears to bind to the plasma membrane, activating the H^+-ATPase to some extent, independently of IAA (Fig. 2), and yielding a Hill plot that indicates protein-protein binding at a 1:1 ratio (data not shown; virtually identical data were given in the previous communication [10]). Taking these observations all together, it is concluded that ABP_{57} is a novel auxin receptor. To our knowledge this ABP is the first auxin receptor protein ever described that was identified and iso-

lated based on a biochemical functional assay rather than ligand binding assays throughout the purification procedures. Using rather simple techniques, relatively large amounts of the protein (ca. 9 μg from 1 kg of fresh shoots of rice seedlings) can be prepared with purification to homogeneity, which would permit further characterization of the biochemical identity.

It might be irrational to attempt to use tryptophan as a ligand for an ABP in affinity chromatography, since tryptophan is classified as an inactive auxin analogue. The only difference in the structure of tryptophan from an active IAA analogue, indole 3-propionic acid, is seen in the presence of an amino group on α carbon. According to the model of the auxin binding site described for ABP1 [17], there are three regions involved in the protein-auxin interaction: a planar aromatic ring binding platform, a carboxylic acid binding site and a hydrophobic transition region which separates two binding sites. Because amino group is highly hydrophilic, the bridged region, i.e. $-CH_2CH(NH_2)-$, between two putative binding groups of the tryptophan molecule may be incompatible with the hydrophobic transition region of the binding site of ABP, causing a limited binding ability of tryptophan to the protein. We therefore presumed that blocking the amino group and rendering some hydrophobicity to the bridged region by reaction of the amino group with CNBr-activated groups on the polysaccharide gel might enable the tryptophan residue to bind to ABP to a significantly increased extent. Our results with the affinity chromatography experiments appear pertinent to such conjecture. For the present, however, we could not say whether such is also the case with various ABPs other than ABP_{57}, which certainly awaits extensive experimental scrutiny.

An intriguing feature of auxin effects in plants is that auxin regulates some physiological activities in a biphasic manner depending on the applied concentration, as has been demonstrated by bell-shaped dose-response curves for auxin action on the elongation growth of the coleoptiles and apical root segments of several plants [18–21]. A plausible explanation for this may be provided by hypothesizing the existence of two different types of binding sites on an auxin receptor molecule, the primary site(s) with high auxin affinity and the secondary site(s) with low auxin affinity. The binding of auxin to the primary site(s), occurring at lower auxin concentrations, could be associated with stimulatory effects of auxin and a further auxin binding to the secondary sites, taking place at higher auxin concentrations, with disappearance of the stimulation through two-phase conformational changes of the receptor protein. The binding isotherm for ABP_{57} (Fig. 4) seems to conform to this hypothesis. The linear region of the Scatchard plot is thought to correspond to IAA binding to four identical high affinity sites of ABP_{57}. Meanwhile, the occurrence of curvature for $b \geq 6.5 \times 10^{-8}$ mol/mg, approximately equivalent to the number of IAA bound per protein molecule higher than 3.7, is likely related to the binding of IAA to the low affinity sites. It is also noteworthy that the data of the binding equilibrium that fit the Scatchard equation were obtained at IAA concentrations ranging from 1×10^{-7} M to 5×10^{-6} M, where only the stimulation phase of the regulatory effects of IAA on the H^+-ATPase appeared. In this context, our measurements of K_d and N seem to be only for the binding of IAA to the high affinity sites of ABP_{57} that induces the first phase alteration in conformation of the protein to an activated state.

Acknowledgements: The financial support from the Rural Development Administration, Republic of Korea, is acknowledged.

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