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Photoaffinity labeling of indole-3-acetic acid-binding proteins in maize

(auxin receptor/5-azidoindole-3-acetic acid)

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ABSTRACT **The photoaffinity labeling agent 5-azidoindole-3-acetic acid, an analog of the endogenous plant hormone indole-3-acetic acid (an auxin), was used to identify indole-3-acetic acid-binding proteins in maize. Two peptides with subunit molecular masses of 24 and 22 kilodaltons are specifically labeled in a saturable manner. Both peptides are slightly acidic and behave as dimers under nondenaturing conditions. The possibility that one of these peptides is the auxin receptor that mediates cell elongation in maize is discussed.**

One primary function of auxins in plants is to alter the physical and biochemical properties of the restraining cell wall to allow turgor to drive cell expansion (1). The precise mechanism by which cell elongation is evoked by auxin remains unknown, although presumably it involves a complex of auxin and auxin receptor. The identity of the auxin receptor that mediates cell elongation is also unknown. Recently, indirect methods have been used to identify an auxin-binding protein (ABP) (2–5) which has some of the expected characteristics of the receptor that mediates cell elongation (6, 7, 23). We report here the results of using 5-azidoindole-3-acetic acid (5-N_3IAA), a photoaffinity analog of the endogenous auxin indole-3-acetic acid (IAA), for direct labeling of IAA-binding proteins in maize.

Photoaffinity labeling has been successful in identifying and characterizing receptors from animals and plants (8). For example, 2-azido-N^6-[^{14}C]benzyladenine ([^{14}C]N_3BzlAde) was used to identify a cytokinin-binding protein from wheat and recently it has been used to characterize the benzyladenine-binding site of this cytokinin-binding protein (9). These workers found that a histidine residue in or near the binding site becomes specifically labeled by [^{14}C]N_3BzlAde, indicating that this residue may be involved in the binding reaction.

Several photoaffinity labeling azido analogs of IAA have been synthesized and shown to be biologically active (10) and competitive for naphthalene-1-acetic acid (NAA) binding in crude extracts of maize (11). One of these was tritiated and used to label the membrane-bound auxin binding sites in maize, but a high level of nonspecific binding interfered with the identification of proteins that had specifically incorporated the photoaffinity label (12). Modifications of the labeling protocol have enabled us to identify two ABPs in maize that saturably and specifically bind the hormone.

MATERIALS AND METHODS

Materials. *Zea mays* L. (cv. Beaupre) caryopses were sown on wetted Vermiculite and grown in the dark. Coleoptiles were harvested on the 5th day. The sources for all the chemicals used to partially purify ABPs are listed in ref. 4. All other chemicals, except where noted, are from Sigma. IAA was purified further by recrystallization in ethanol/water. The polyclonal antibodies were prepared against a 22-kDa subunit ABP as described by Napier *et al.* (4).

Purification of ABPs. The method used to purify ABPs from maize is described in detail by Napier *et al.* (4) and briefly outlined here. Membrane proteins of the microsomal fraction of etiolated maize shoot tissue were extracted with acetone, then fractionated on an anion-exchange column (DEAE-Bio-Gel, Bio-Rad) followed by size exclusion chromatography (Sephacryl S-200, Pharmacia) and analytical anion-exchange chromatography (Mono Q, Pharmacia). Auxin-binding activity in the fractions was followed throughout the chromatographies by the ammonium sulfate precipitation assay described by Venis (13). The final pooled fractions were desalted on Sephadex G-25, lyophilized, and stored at −20°C. However, up to 9 days of storage at room temperature had no apparent effect on these lyophilized samples. The purification resulted in an approximately 100-fold increase in auxin-binding activity. The 22- and 24-kDa proteins are approximately 10% of the total protein in the samples used in the photolabeling experiments. Since the proteins of this sample are readily soluble in aqueous buffers after their removal from the microsomal fraction by acetone extraction of the lipids, it is assumed that they are not integral membrane proteins.

Photolysis. All manipulations involving unphotolyzed 5-N_3IAA were performed under dim green light. Lyophilized samples enriched for auxin binding were redissolved in buffer containing 10 mM sodium citrate at pH 5.5, 250 mM sucrose, and 0.5 mM magnesium sulfate. An ethanolic stock of 5-N_3IAA was added to the samples so that the final concentration equaled 0.1 μM or less. The mole ratio of ABP to labeling agent is estimated to be approximately 1:1. The final concentration of ethanol never exceeded 2.5%. Aliquots (200 μl) in disposable UV-transparent cuvettes (acrylic, Fisher) were incubated on ice for 30 min in darkness with the indicated amounts of IAA then photolyzed for 15 sec (except for the experiment in Fig. 3, where the photolysis time is indicated). Photolysis was achieved by holding all the cuvettes together between two UV sources mounted in opposition, 15 cm apart. The top and bottom UV sources were a Spectronics XX-15F (254 nm) and a Spectronics XX-15B (300 nm). The total irradiance was approximately 10 mW/cm^2 at the sample level.

SDS/PAGE, Isoelectric Focusing (IEF)/PAGE, Fluorography, Immunoblot Analysis, and Size-Exclusion Chromatography. SDS/PAGE was performed on 12% acrylamide gels as described by Laemmli (14) and IEF/PAGE was performed as described by O'Farrell (15). Samples subjected to immunoblot analysis were transferred from gels to Immobilon (Mil-

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Abbreviations: ABP, auxin-binding protein; 5-N_3IAA, 5-azidoindole-3-acetic acid; IAA, indole-3-acetic acid; NAA, naphthalene-1-acetic acid; IEF, isoelectric focusing.

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lipore, MA) and processed further with the anti-ABP polyclonal antibodies (4) as previously described (16). Goat anti-rabbit immunoglobulin conjugated to alkaline phosphatase was used as the secondary reporter. Samples were subjected to size exclusion chromatography (SEC) on a TSK-2000SW (Toya Soda, Tokyo) high-performance liquid chromatography column. The buffer was 100 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris), pH 7.2/5% (vol/vol) glycerol/1 mM ethylenediaminetetraacetic acid (EDTA) and the flow rate was 0.5 ml/min. Gels used in fluorography were soaked in 1 M sodium salicylate for 30 min, dried down on paper, and exposed to Kodak XAR-5 film at −80°C for 4–14 days. The signals were quantitated by scanning the films and were integrated by cutting out and weighing the peaks. Signals used in the quantitative analyses always fell within the linear response range of the film. A second method of quantitation was to cut out the bands from the dried gels, digest with NCS tissue solubilizer (Amersham), and measure the radioactivity directly by using liquid scintillation counting. The two methods gave comparable results. The specific binding activity of these ABPs is defined here as moles of 5-N_3IAA incorporated per microgram of protein. The amount of protein was estimated by comparing the Coomassie blue staining intensity of the 22- and 24-kDa ABPs to the staining intensity of carbonic anhydrase (molecular mass = 26 kDa) loaded as a dilution series in adjacent lanes. Radioactivity per band for the specific activity parameter was estimated by measuring directly, using liquid scintillation counting. All experiments were repeated at least once.

RESULTS AND DISCUSSION

Membrane proteins of maize were extracted with acetone and enriched for auxin-binding activity by anion-exchange and size-exclusion chromatographies (4). These samples were used in the photoaffinity labeling experiments. Enrichment for auxin-binding activity by partial purification greatly improved affinity labeling in comparison to photolabeling crude extracts of maize shoot tissue (12). Improvement is probably achieved primarily by the removal of lipids and abundant nonspecific binding sites, although we cannot preclude the possibility that the purification scheme favorably alters the conformation of the ABPs. Of the many peptides remaining in the ABP-enriched samples, only peptides with molecular masses of 22 and 24 kDa incorporate the photoaffinity label to a high degree (Fig. 1). The amount of radioactivity incorporated in these bands is at least 10 times greater than of any other band (Fig. 1). The approximate specific binding activity of the ABP is 0.1 mmol of 5-N_3IAA per mol of ABP. Parallel labeling experiments were conducted with nonplant proteins such as bovine serum albumin, carbonic anhydrase, and lysozyme (Fig. 2). The specific binding activity of the 24-kDa peptide was at least 20-fold greater than that of any of these negative controls. A third protein, appearing as a diffuse band at 43 kDa in Fig. 1, also incorporates the labeling agent but to a lesser extent than the major labeled proteins. Because of the low amount of this protein in the samples it is difficult to determine the specific binding activity of the 43-kDa peptide(s) with confidence. By photoaffinity labeling with 5-N_3IAA, Hicks *et al.* (17) have recently identified a plasma membrane-localized ABP of similar molecular mass. Their data indicate that this protein, which may be the same as the 42-kDa diffusely labeled band shown here, is involved in auxin transport.

The presence of exogenous IAA prevents incorporation of 5-N_3IAA into the 22- and 24-kDa peptides, indicating that

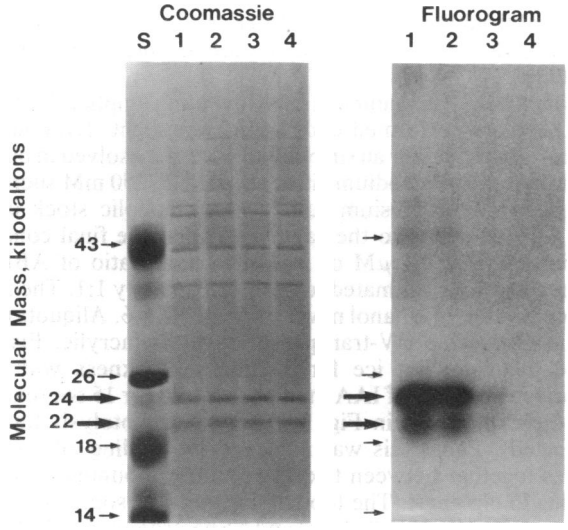

FIG. 1. Specific photoaffinity labeling of ABPs in maize with 5-azido[7-^{3}H]indole-3-acetic acid (5-N_3[^{3}H]IAA). The protein sample (approximately 50 μg/ml) was incubated for 30 min in the dark with 0.2 μM 5-N_3[^{3}H]IAA in 0.25 M sucrose/10 mM sodium citrate, pH 5.5/0.5 mM magnesium sulfate and nonradioactive IAA at the concentrations indicated below. The apparent K_d values for IAA and 5-N_3IAA, estimated from competition assays using [^{14}C]NAA, are 10 and 5 μM, respectively (10). The samples were photolyzed (10 mW/cm^2) for 15 sec and then subjected to SDS/PAGE and fluorography. Each lane contains approximately 5 μg of protein. (*Left*) Coomassie blue-stained gel; (*Right*) corresponding fluorogram. Lane S, standards (prestained low molecular weight group, Bethesda Research Laboratories); lane 1, no IAA; lane 2, 0.16 μM IAA; lane 3, 1.6 μM IAA; lane 4, 16 μM IAA.

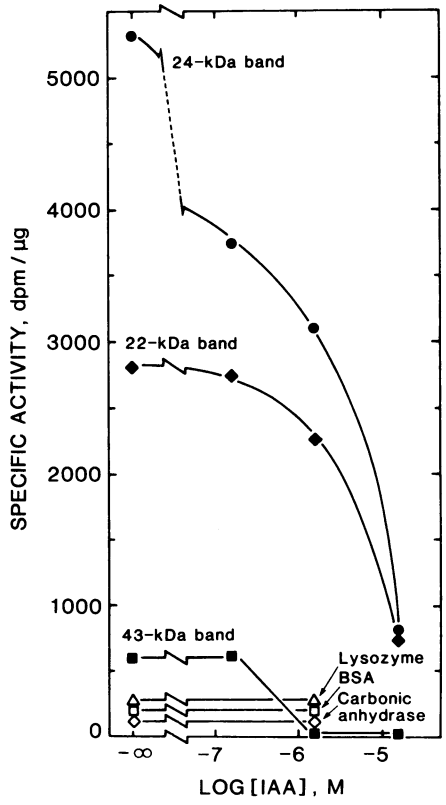

FIG. 2. IAA competition curves of photoaffinity labeling by 5-N_3[^{3}H]IAA of ABPs from maize and other nonplant proteins. The radioactive bands indicated in Fig. 1 were cut out of the gel, digested with tissue solubilizer, and quantitated by liquid scintillation counting. The 43-kDa band is the faintly labeled band seen in Fig. 1. Bovine serum albumin (BSA; fraction V), carbonic anhydrase, and lysozyme were at 0.5 μg/ml. Scanning the fluorograms and integrating the signals gave the same results as measuring radioactivity directly.

photolabeling is specific and occurs at or near the IAA binding sites (Figs. 1 and 2). An apparent K_d for IAA can be calculated from the data in Fig. 2. Both the 22- and 24-kDa ABPs have an apparent K_d for IAA of 5 μM, approximately the same K_d as was previously calculated from equilibrium binding data (11, 18–20). The scavenging agent paraminobenzoic acid at 1 mM had no effect on labeling, again indicating that photolabeling is specific. However, 10 mM dithiothreitol reduced labeling to 9% of the controls without dithiothreitol. This is consistent with previous reports that thioreducing agents inhibit the NAA-binding activity in crude extracts of maize (18). This class of NAA binding is designated site I binding.

The labeling reaction saturates within 30 sec of photolysis. Also, labeling does not occur without photolysis, nor does labeling occur when the maize protein preparation is added to the labeling agent immediately after photolysis (Fig. 3). The latter observation indicates that the photogenerated nitrene intermediate is short-lived relative to the time for experimental manipulations. The observed labeling is, therefore, probably not due to random tagging by an activated nitrene (21).

The majority of the radioactivity in labeled samples migrated in nondenaturing size-exclusion chromatography with an apparent molecular mass between 40 and 50 kDa, indicating that the labeled proteins are either dimeric or have a rigid-rod structure (Fig. 4). Fractions from the size-exclusion column subjected to SDS/PAGE in conjunction with fluorography (Fig. 4*A*) and immunoblot analysis (Fig. 4*B*) reveal that the 24- and 22-kDa ABPs migrate with native molecular masses of 50 and 43 kDa, respectively. The elution volume of the photolabeled ABPs did not change when 10 mM 2-mercaptoethanol was included in the elution and sample buffer. This indicates that, if these proteins are dimers, disulfide bridging is not involved exclusively in maintaining the dimer conformation.

When the labeled proteins were subjected to IEF gel electrophoresis, the radioactivity focused in two peaks at approximately pH 5.0–5.3 (Fig. 5). This was confirmed by autoradiography of two-dimensional SDS gels. Immunoblots of the IEF gels as well as two-dimensional gels of the photolabeled samples were probed with the antibody made against the 22-kDa-subunit ABP. This analysis confirmed that the 22-kDa-subunit ABP was focusing between pH 5.0 and 5.3.

Other techniques have been used to identify an ABP in maize having a subunit molecular mass of approximately 22 kDa. Löbler and Klämbt (2), using immunoaffinity chromatography, purified a 20-kDa maize protein which has binding properties similar to those of binding site I. Shimomura *et al.* (3) used affinity chromatography to purify from maize shoot membranes a 21-kDa ABP which behaves as 43 kDa in nondenaturing size exclusion chromatography. Their data indicated that this protein has site I binding characteristics (3, 5). Finally, Napier *et al.* (4) used a variety of chromatography matrices to purify a 22-kDa subunit ABP from maize shoots. Therefore, it appears that there are several ABPs which have been identified by a variety of experimental approaches. In some cases, the ABPs identified by different groups are possibly the same. For example, the 22-kDa ABP identified here is probably the same ABP identified by Napier *et al.* (4) because both ABPs have approximately the same native and subunit molecular masses and pI. However, our work demonstrates that in a single selected protein population there are two ABPs. It is possible that these are structurally or functionally related.

What role do these ABPs have in cell physiology? There is sufficient evidence to consider a role in auxin-mediated cell elongation. Löbler and Klämbt (6) demonstrated that monospecific polyclonal antibodies directed against the ABP inhibit auxin-induced growth of epidermal cells in maize coleoptile tissue. Recently, the work was extended to include evacuolated tobacco protoplasts (7), where it was shown that

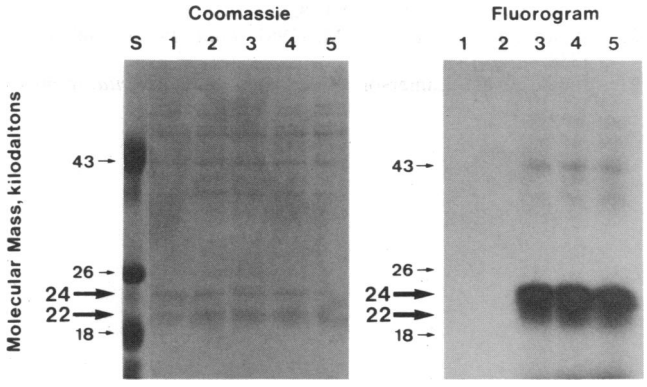

FIG. 3. Saturability of the photolytic reaction. ABP-enriched samples were photolyzed for the indicated times then subjected to SDS/PAGE and fluorography. Each reaction mixture contained 0.2 μM 5-N_3[^{3}H]IAA. Each lane contains approximately 5 μg of total protein. Lane S, low molecular weight standards (see Fig. 1); lane 1, the ABP sample was injected into the photolysis chamber immediately after 15 sec of photolysis of the N_3[^{3}H]IAA without the sample; lane 2, no photolysis; lane 3, 30 sec of photolysis; lane 4, 60 sec of photolysis; lane 5, 300 sec of photolysis.

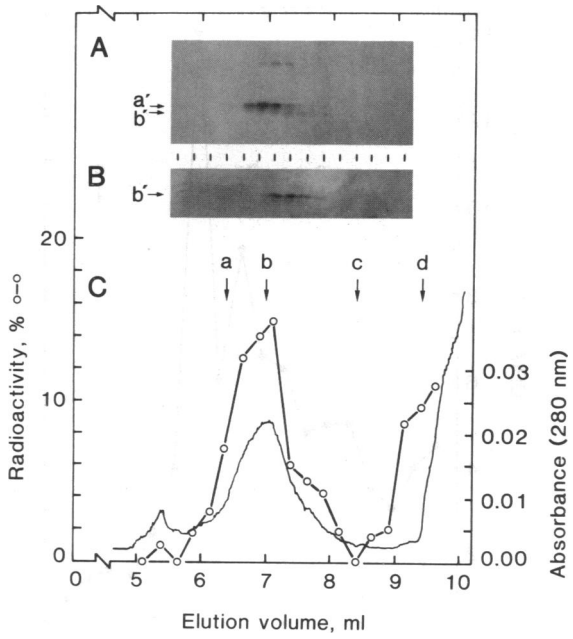

FIG. 4. Size-exclusion chromatography of photolabeled ABPs from maize. Protein samples enriched for auxin binding were photolabeled with 5-N_3[^{3}H]IAA as described for Fig. 1. The samples were subjected to size-exclusion chromatography (TSK-2000SW, Toya Soda) and fractionated. Fractions were assayed by liquid scintillation counting and the radioactivity is expressed as a percent of radioactivity in the included volume (5–9.5 ml) (*C*). Fractions were also subjected to SDS/PAGE, fluorography (*A*), and immunoblot analysis (*B*). The immunoblot in *B* was probed with a polyclonal antibody directed against a 22-kDa ABP (4). The arrows on the fluorogram (*A*) and the immunoblot (*B*) indicate the positions of the 24-kDa peptide (a') and the 22-kDa peptide (b'). The ticks between *A* and *B* represent the position of the corresponding fractions indicated in *C* (o—o). Absorbance at 280 nm is shown as the thin trace. Arrows on the chromatogram indicate the elution volumes of bovine serum albumin, 67 kDa (a); ovalbumin, 46 kDa (b); trypsinogen, 24 kDa (c); and cytochrome *c*, 12 kDa (d). The only peak of incorporated radioactivity elutes at a molecular mass of 43–50 kDa. The radioactivity eluting later (9 ml) is part of the shoulder of unincorporated label migrating in the salt volume.

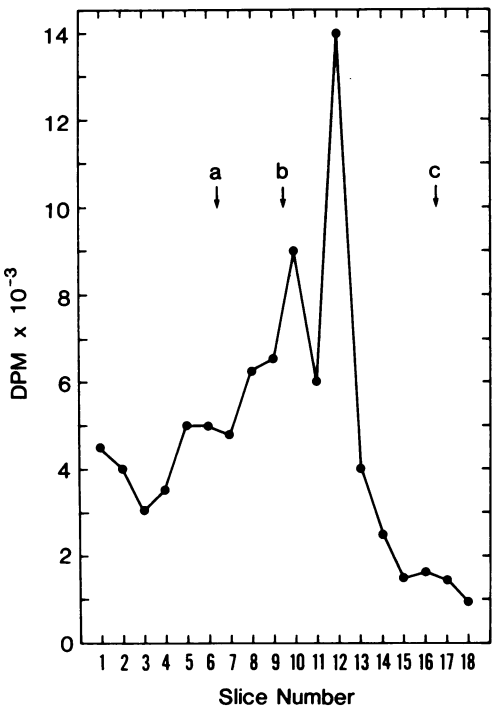

FIG. 5. IEF gel electrophoresis of photoaffinity labeled ABPs from maize. The photolabeled ABP-enriched samples were subjected to IEF gel electrophoresis, the tube gels were sliced, and the sections were digested in NCS (Amersham) solubilizer. The radioactivity was measured by liquid scintillation counting. The pH gradient was determined by using IEF standards (Bio-Rad) in parallel tubes: a, phycocyanin, pI 4.65; b, lactoglobulin, pI 5.1; c, bovine carbonic anhydrase, pI 6.0.

anti-ABP antibodies blocked NAA-induced hyperpolarization of the plasma membrane. In addition, since the ABP and site I binding are the same (2–5, 23), indirect evidence which has indicated that site I binding mediates cell elongation (e.g., see ref. 22) now applies to the ABP.

Our method of detecting ABPs differs from previous methods in two ways. First, we are utilizing a substituted analog of the endogenous hormone, IAA, whereas the other groups have used NAA or diiodobenzoic acid, both synthetic growth substances. Second, and most important, our method of detection is direct; proteins that bind the hormone become tagged. An additional advantage of this technique is that it can be readily used to map and characterize the active binding site(s).

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