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Photoaffinity Labeling of Soluble Auxin-binding Proteins*

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Heather Macdonald‡, Alan M. Jones§, and Patrick J. King‡¶

From the ‡Friedrich Miescher-Institut, Postfach 2543, 4002 Basel, Switzerland and the §Department of Biology, University of North Carolina, Chapel Hill, North Carolina 27599

The photoaffinity labeling agent azido-IAA (5-N_3-[7-^{3}H]indole-3-acetic acid), a biologically active analogue of the endogenous auxin indole-3-acetic acid, was used to search for auxin-binding proteins in the soluble fraction of *Hyoscyamus muticus* cells. Azido-IAA became covalently attached to three polypeptides with a high specific activity. The labeling was specific for IAA and not due to random tagging. Two polypeptides with molecular masses of 31 and 24 kDa in the 0–30% ammonium sulfate fraction were labeled after UV photolysis at 0 °C but not at −196 °C, and appeared to have a high affinity indole-binding site(s) for which active, non-indole auxins were not good ligands. A third polypeptide with a molecular mass of 25 kDa present in the 50–60% ammonium sulfate fraction labeled exclusively at −196 °C and had a significant affinity for active auxins but not for inactive indoles. The azido-IAA labeling pattern, pI, competition results, and immunoprecipitation all indicate that the 31- and 24-kDa polypeptides are related to the basic form of endo-1,3-β-glucanase (EC 3.2.1.39). Azido-IAA labeling polypeptides equivalent to the 31- and 24-kDa species were apparently also present in the cell wall. The low pH optimum for binding of azido-IAA to the 25-kDa polypeptide suggests the location of the active protein in a compartment such as the vacuole or a transport vesicle rather than in the cytosol.

Auxin (indole-3-acetic acid) is thought to be a signaling molecule important for the regulation of plant growth and development. The investigation of auxin's mode of action has been strongly influenced by the models of peptide and steroid hormones and their receptors in animal cells, such that it has become implicit that a membrane-bound auxin receptor initiates a signal transduction cascade in plant cells or that auxin forms a complex with a soluble receptor to regulate transcription in the nucleus. These two divergent models have led to searches for an auxin receptor both in the membrane fraction and in the cytosol.

Support for the concept of a plasma membrane receptor is to be found in the changes in plasma membrane activity (enhanced electrogenic proton pumping and cell wall acidification) (Cleland, 1987) that accompany auxin stimulation of growth. Extensive studies of membrane-associated binding sites in maize coleoptiles have resulted in the isolation of a 43-kDa auxin-binding protein (ABP)[1] with 22-kDa subunits (Löbler and Klämbt, 1985; Shimomura *et al.*, 1986; Venis, 1987) for which cDNA clones have recently been obtained (Hesse *et al.*, 1989; Tillmann *et al.*, 1989; Inohara *et al.*, 1989). Further polypeptides, possibly related to this ABP, have been detected immunologically (Hesse *et al.*, 1989; Napier *et al.*, 1988) and by photoaffinity labeling (Jones and Venis, 1989). The maize protein appears to be involved in auxin-stimulated H^+-ATPase activity at the plasma membrane (Barbier-Brygoo *et al.*, 1989). Photoaffinity labeling of plasma membrane vesicles from zucchini hypocotyls and tomato seedlings with 5-azido-[7-^{3}H]IAA further revealed a polypeptide doublet (40–42 kDa) that is thought to form part of a pH-dependent auxin uptake symport (Hicks *et al.*, 1989a, 1989b).

The concept of a plasma membrane receptor, however, must be set against the evidence for a specific auxin-uptake system and the fact that auxins are small hydrophobic molecules that are likely to be highly membrane-penetrant in their uncharged form (Rubery, 1987). These facts, together with evidence for auxin-regulated gene expression (Hagen, 1987), have encouraged the search for soluble receptors (Ihl, 1976; Jacobsen, 1982; Libbenga *et al.*, 1987; Kikuchi *et al.*, 1989). A soluble ABP from tobacco callus with a native molecular weight of 150–200 kDa appeared to show modest auxin-dependent stimulation of transcription in isolated tobacco callus nuclei (Libbenga *et al.*, 1987). Two further ABPs from mung bean hypocotyls (Sakai and Hangata, 1983; Sakai, 1985) were claimed to bring about auxin-dependent qualitative changes in transcription of isolated nuclei (Kikuchi *et al.*, 1989).

The investigations of soluble auxin "receptors" described above involved conventional binding assays to detect auxin-binding sites among the fractionated proteins, mainly using synthetic auxin analogues. We have now used a photoaffinity labeling agent (5-N_3-[7-^{3}H]IAA), an analogue of the endogenous auxin IAA, to covalently tag soluble proteins with which auxin interacts. The proteins were partially purified by ammonium sulfate fractionation and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

EXPERIMENTAL PROCEDURES

Cell Cultures—The origin of the suspension culture cells of *Hyoscyamus muticus* and the culture conditions were as described by Gebhardt *et al.* (1983), except that auxin was omitted from the medium.

Soluble Protein Extraction—Cells 10–14 days after subculture were collected by filtration on nylon net (45-μm pore size) in a Buchner funnel and washed with several volumes of distilled water. Free water was removed by brief suction, and the samples were frozen in liquid

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¶ To whom correspondence should be addressed: Tel.: 61-697-55-83; Fax: 61-697-39-76.

[1] The abbreviations used are: ABP, auxin-binding protein; IAA, indole-3-acetic acid; azido-IAA, 5-azido-[7-^{3}H]IAA; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; BSA, bovine serum albumin; HPLC, high performance liquid chromatography; MOPS, 3-(*N*-morpholino)-propanesulfonic acid; NAA, naphthaleneacetic acid.

nitrogen. Samples were stored for 1–21 days at −20 °C. Frozen tissue was then thawed at 4 °C and homogenized using a Polytron homogenizer for 30 s at low speed and 30 s at full speed, with a ratio of 20 ml of buffer to 10 g of tissue. The homogenization buffer contained 200 mM boric acid, 2 mM disodium tetraborate, 250 mM KCl, and 20 mM Tris-HCl, pH 6.8. Polyclar AT (25 mg/ml) was added to the buffer at least 1 h before use, and sodium diethyldithiocarbamate (1.8 mg/ml) and mercaptoethanol (0.3%, v/v) were added immediately before use. The homogenate was squeezed through nylon cloth and the filtrate was centrifuged at 175,000 × *g* for 2 h at 4 °C. The supernatant was filtered through a Whatman No. 1 filter prior to ammonium sulfate precipitation.

Ammonium Sulfate Precipitation—Ammonium sulfate (Merck) was ground to a fine powder and added gradually. Protein mixtures were stirred for at least 30 min after addition of the ammonium sulfate, and the precipitate was collected by centrifugation at 10,000 × *g* for 15 min at 4 °C. The ammonium sulfate pellets were resuspended slowly on ice with a minimum volume of 20 mM Tris-HCl, pH 7.4, containing 25 mM NaCl and desalted by dialysis against the same buffer (or 25 mM sodium citrate, pH 6.0) for at least 4 h (four changes of buffer to a total volume of 2 liters). The protein samples were stored at −20 °C.

Photoaffinity Labeling—The labeling buffer contained 100 mM sodium citrate, 250 mM sucrose, and 0.5 mM $MgSO_4$. The pH was normally adjusted to 5.5 after addition of the $MgSO_4$, but in later experiments it was adjusted to 5.0 or 4.5. Protein samples (typically 100 μg of a complex mixture) were made up to 40 μl with labeling buffer in disposable acrylic, UV-transparent cuvettes (Fisher) and kept on ice (0 °C). Competing substances or scavengers were added at this point as appropriate. A stock solution of labeling buffer containing 0.7 μM 5-N_3-[7-^{3}H]IAA (azido-IAA) was prepared under a red safelight and 40 μl were added to each cuvette to give a final azido-IAA concentration of 0.35 μM. Samples were then mixed and kept on ice for 20 min in the dark. Where indicated, the samples to be irradiated at −196 °C were then frozen in liquid nitrogen for 30 s before irradiation. Two UV lamps set 15 cm apart were used for irradiation: a long wavelength UV lamp (Black Ray, XX-15) and a short wave transilluminator (both from UV Products). Cuvettes were held between the two UV sources for the appropriate times. Competing substances were prepared as stock solutions using dimethylsulfoxide or ethanol as solvents, if necessary.

SDS-PAGE, Staining, and Fluorography—Electrophoresis was performed on 12% discontinuous gels (Laemmli, 1970) using Dalton Mark VII (Sigma) molecular weight markers. Purified β-1,3-glucanase (basic form) (Felix and Meins, 1985) was a gift from Dennis Keefe, Friedrich Miescher-Institut, Basel. Proteins were detected by staining with Coomassie Brilliant Blue (0.2% (w/v) in 40% methanol, 10% acetic acid). Gels were photographed on a light box using a yellow filter. For fluorography, destained gels were soaked in water for 30 min and then shaken for 20 min in 20 volumes of sodium salicylate solution (16%, w/v) containing 5% methanol and 0.5% glycerol. The treated gels were dried onto filter paper and exposed to Kodak X-Omat AR film at −80 °C.

Quantification of Radioactivity in SDS Gels—Gel slices were dissolved in 1–2 ml of freshly prepared 2% (w/v) periodic acid in Eppendorf tubes at 55 °C overnight or until completely dissolved. The solution was then transferred to scintillation vials with 18 ml of Insta-Gel scintillation fluid and counted.

Temperature Sensitivity—Protein samples were heated in a block heater for the times indicated and cooled on ice before labeling.

HPLC Analysis of Azido-IAA—Photoaffinity labeling mixtures were prepared as described above. 40-μl samples were taken into 1 ml of ice-cold methanol immediately after mixing and after 30 min. The methanolic extracts were centrifuged to remove proteins, and the supernatant was evaporated to ~50 μl and taken up in 300 μl of distilled water. The samples were chromatographed on a 25 × 0.46-cm Nucleosil C18 HPLC column with a 30-min linear gradient of 10–60% methanol in 20 mM acetic acid/triethylamine, pH 3.5, 0.8 ml/min. Peaks were detected using an on-line radioactivity detector.

Cell Wall Preparation—Water-washed suspension culture cells were homogenized in 10 mM MOPS, pH 7.0, or in water using a Polytron homogenizer for 2 × 30 s at full speed. Cell walls were pelleted at 5000 × *g* for 5 min and washed five times with water. Proteins were extracted from the washed walls by incubating overnight on ice in 5 M NaCl. The supernatant after centrifugation at 5000 × *g* for 5 min was taken and dialyzed against several changes of 20 mM Tris-HCl, pH 7.4.

Immunoprecipitation—Antibodies to the basic form of tobacco β-1,3-glucanase (Keefe *et al.*, 1990) and chitinase, and preimmune serum were a gift from Ursula Hinz, Friedrich Miescher-Institut, Basel. Approximately 15 mg of protein A-Sepharose CL-4B (Pharmacia LKB Biotechnology Inc.) were swollen in phosphate-buffered saline (PBS) for 1 h at room temperature. Approximately 100 μg of IgG fraction (or an equivalent amount of preimmune serum) was added and allowed to adsorb for at least 1 h at room temperature. After washing with PBS, the Sepharose beads were resuspended in an adsorption buffer consisting of PBS containing 0.5% (w/v) BSA, 0.2% (w/v) sodium deoxycholate, and 1% (v/v) Triton X-100. The labeled protein sample was then added, the volume was made up to 500 μl with adsorption buffer, and the mixture was incubated overnight at 4 °C with gentle shaking. After the beads were allowed to settle, the supernatant was discarded and the beads were washed three times with PBS. SDS sample buffer was added and the samples were boiled for 4 min prior to SDS-PAGE.

Isoelectric Focusing—Servalyte Precotes, pH 3–10 (Serva AG, Heidelberg), were used according to the manufacturer's instructions and were prefocused for 1 h prior to sample application. The samples were ~20 μl of photoaffinity labeling mix without sample buffer. Protein standards were run to allow estimates of pI.

Western Blotting—Proteins were transferred from Laemmli gels to reinforced nitrocellulose membranes (Schleicher and Schuell) using a Bio-Rad Transblot apparatus. Transfer was for at least 3 h at 200 mA in 25 mM Tris, 192 mM glycine, pH 8.3, 20% methanol, with cooling. Remaining protein-binding sites were blocked by overnight incubation in TBS buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl) containing 5% dried milk powder. The antibodies used were 1) polyclonal IgGs raised to partially purified 22-kDa ABP from maize (8) and 2) alkaline phosphatase-linked goat anti-rabbit IgG. Antibodies were incubated at 1/1000 dilution with the membrane in TBS + 0.2% BSA for 3 and 1 h, respectively, with a TBS + 0.1% Tween 20 wash between each incubation. Alkaline phosphatase activity was detected using nitro blue tetrazolium (0.7 mg liter^{-1}) and 5-bromo-4-chloro-3-indolyl phosphate (0.15 mg liter^{-1}) in 100 mM Tris, 100 mM NaCl, 5 mM $MgCl_2$, pH 9.0. Partially purified maize ABP was included on blots as a positive control.

RESULTS

Labeling of Soluble Proteins—Soluble proteins of wild-type *H. muticus* cell cultures were fractionated by ammonium sulfate precipitation. The fractions were desalted by dialysis and incubated at 0 °C in the presence of the photoaffinity label 5-N_3-[7-^{3}H]IAA. After photolysis, the polypeptides of each fraction were separated by SDS-PAGE (Figs. 1*A* and 2*A*) and radiolabeled polypeptides were detected by fluorog-

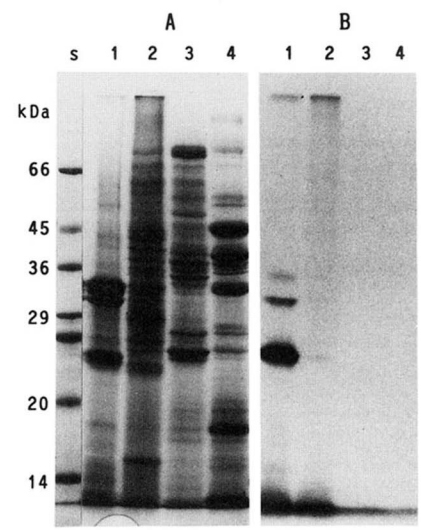

FIG. 1. **Photoaffinity labeling of soluble proteins of *H. muticus* at 0 °C.** *A*, Coomassie-stained SDS-PAGE gel with equivalent amounts per lane of proteins precipitated at different ammonium sulfate concentrations (% saturation) before labeling. *Lane 1*, 0–40%; *lane 2*, 40–50%; *lane 3*, 50–60%; *lane 4*, 60–80%. *Lane s* contains molecular weight markers. *B*, fluorograph of gel shown in *A*.

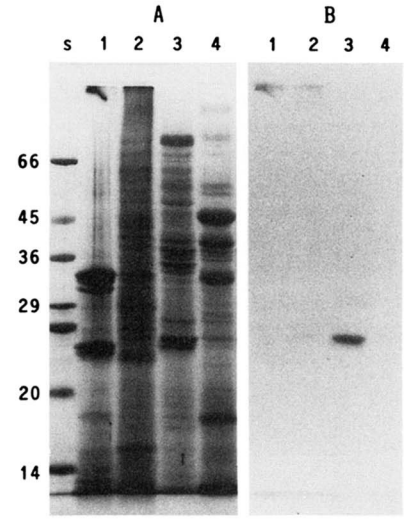

FIG. 2. **Photoaffinity labeling of soluble proteins of *H. muticus* at −196 °C.** *A*, Coomassie-stained SDS-PAGE gel with equivalent amounts per lane of proteins precipitated at different ammonium sulfate concentrations (% saturation) before labeling. *Lane 1*, 0–40%; *lane 2*, 40–50%; *lane 3*, 50–60%; *lane 4*, 60–80%. *Lane s* contains molecular weight markers. *B*, fluorograph of gel shown in *A*.

raphy (Figs. 1*B* and 2*B*). In general, the results in Figs. 1 and 2 show that only very few polypeptides were labeled and that labeling was not proportional to protein abundance.

The number and identity of the labeled polypeptides depended upon the photolysis conditions. In samples cooled on ice (0 °C) during labeling and photolyzed without further treatment, two polypeptides with molecular masses of 31 and 24 kDa repeatedly incorporated the photoaffinity label to a high degree (Fig. 1). The 24-kDa polypeptide was normally less abundant but more strongly labeled than the 31-kDa species. Both polypeptides were precipitated by 40% ammonium sulfate, and the 24-kDa peptide was also precipitated at 30%. In contrast, when samples were frozen in liquid nitrogen (−196 °C) immediately prior to photolysis, the 31- and 24-kDa polypeptides were not significantly labeled, but a polypeptide of 25 kDa molecular mass in the 50–60% ammonium sulfate fraction was specifically labeled (Fig. 2). Labeling at −196 °C was carried out to maximize selectivity of labeling; free azido-IAA molecules will be immobilized in the solvent matrix at this temperature and should not label proteins by nonspecific collisions occurring after photolysis of the azide. In both cases, the polypeptides described are those that labeled consistently. In some experiments, other bands appeared in the background that behaved differently in competition experiments and are considered to represent nonspecific labeling (see below).

Control Experiments with Azido-IAA—The stability of azido-IAA during incubation with protein solutions at 0 °C in the dark was investigated to confirm that the labeled proteins had bound azido-IAA and not an azido-IAA metabolite. HPLC analysis of methanol extracts of labeling reactions revealed no significant degradation of the labeled compound during 30 min of incubation with either the 0–30% or 50–60% ammonium sulfate fractions. More than 95% of the radioactivity was associated with a single peak with the same retention time as azido-IAA both initially and after 30 min (data not shown). Some degradation was observed with cell wall extracts (see below), but at least 70% of the label remained in the azido-IAA peak after 30 min.

The labeling reaction at 0 °C saturated within 30 s of photolysis and, at −196 °C, saturation occurred after about 1 min (Fig. 3). Further experiments showed that ultraviolet (UV) irradiation was required for labeling to occur and that preirradiated azido-IAA added immediately to protein mixtures did not result in labeling (data not shown). These experiments suggest that the reactive nitrene generated during photolysis is very short lived compared to the duration of the binding experiments and that the labeling patterns observed are specific for IAA and are not due to random tagging. 1,4-Dithio-*dl*-threitol and glutathione, which are effective scavengers of nonbound, photoactivated azide only slightly reduced labeling of the 31- and 24-kDa polypeptides at 1 mM and did not affect labeling of the 25-kDa polypeptide, again indicating that the photolabeling is specific and occurs at or near an IAA-binding site. In contrast, the scavenger *p*-aminobenzoic acid, even at low concentrations for a scavenger function (200 μM), reduced labeling in all polypeptides and in the background, presumably by competition (data not shown).

Competition for Labeling—Specificity of labeling is also indicated by the reduced incorporation of azido-IAA into all three polypeptides caused by introduction of IAA into the labeling mixture (Fig. 4). To obtain an estimate of the apparent displacement constant (K_i) for IAA of the three polypeptides, labeling reactions were prepared with a series of concentrations of competing IAA. The polypeptides were separated by SDS-PAGE, and bound radioactivity was determined in the band after excision from the gels. The K_i was estimated from the concentration of IAA required for half-maximal displacement of the label (Fig. 5). The displacement curves for the 31- and 24-kDa polypeptides are almost identical (K_i = 3 μM). In contrast, the 25-kDa polypeptide labeled at −196 °C has an apparent K_i of ~100 μM.

Studies of the competition for labeling by other auxin analogues and related indoles revealed differences in properties between the polypeptides labeled at 0 °C and that labeled after freezing at −196 °C (Fig. 4). The major difference is that non-indole auxin analogues, 1-naphthaleneacetic acid (1-NAA) (Fig. 4, *lane 3*), 2-naphthaleneacetic acid (2-NAA) (Fig. 4, *lane 5*), and 2,4-dichlorophenoxyacetic acid (Fig. 4, *lane 4*), did not reduce azido-IAA incorporation into the 31- and 24-kDa polypeptides (Fig. 4*A*). In contrast, both 1-NAA and 2-NAA, and to a lesser extent 2,4-dichlorophenoxyacetic acid, competed with IAA for binding to the 25-kDa polypeptide (Fig. 4*B*). The competition by 2-NAA was particularly strong. Conversely, in the case of indolic compounds, particularly L-tryptophan (Fig. 4, *lane 8*) and D-tryptophan (Fig. 4, *lane 9*), competition for binding to the 31- and 24-kDa polypeptides

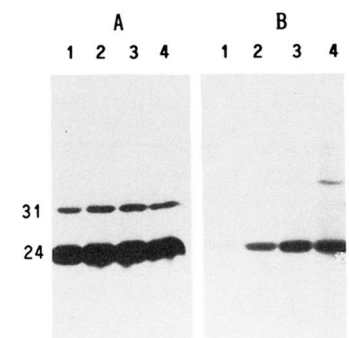

FIG. 3. **The effect of increasing photolysis time on photoaffinity labeling.** Shown are fluorographs of SDS-PAGE gels with equivalent amounts of protein per lane. *A*, 0–30% ammonium sulfate fraction photolyzed at 0 °C. The labeled polypeptides have estimated masses of 31- and 24-kDa. *B*, 50–60% ammonium sulfate fraction photolyzed at −196 °C. The major labeled polypeptide has an estimated mass of 25 kDa. Photolysis time in seconds: *lane 1*, 10; *lane 2*, 30; *lane 3*, 60; and *lane 4*, 300.

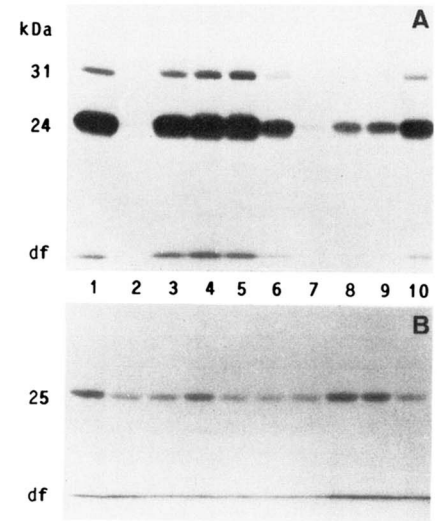

FIG. 4. **The effect on photoaffinity labeling of indoles and non-indole IAA analogues present in the reaction mixture.** Shown are fluorographs of SDS-PAGE gels with equivalent amounts of protein per lane. *A*, 0–30% ammonium sulfate fraction photolyzed at 0 °C. *B*, 50–60% ammonium sulfate fraction photolyzed at −196 °C. The competing substances (each at 200 μM) were added prior to the addition of azido-IAA. *Lane 1*, control, with dimethyl sulfoxide as solvent, added at the same concentration used in all other treatments; *lane 2*, IAA; *lane 3*, 1-NAA; *lane 4*, 2,4-dichlorophenoxyacetic acid; *lane 5*, 2-NAA; *lane 6*, quercetin; *lane 7*, indole; *lane 8*, L-tryptophan; *lane 9*, D-tryptophan; *lane 10*, indole-3-carboxylic acid. The estimated molecular weights of the major labeled polypeptides are shown. *df*, dye front.

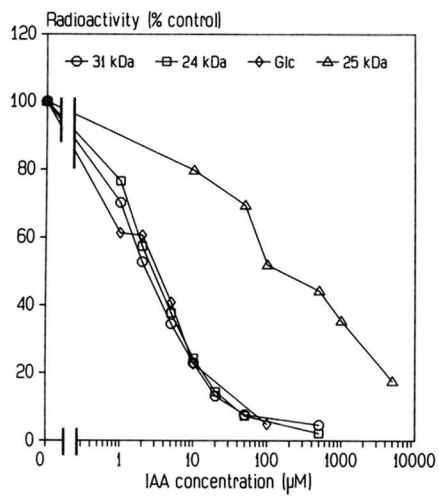

FIG. 5. **The effect of the addition of IAA at increasing concentrations on the incorporation of azido-IAA into soluble proteins of *H. muticus*.** The competing IAA was added to the reaction mixtures (in triplicate for each concentration) prior to the addition of azido-IAA. After separation by SDS-PAGE, bound radioactivity was measured by excision of bands at the molecular weights indicated, digestion, and scintillation counting. The 31- and 24-kDa bands were present in the 0–30% ammonium sulfate fraction photolyzed at 0 °C. The 25-kDa band was present in the 50–60% ammonium sulfate fraction photolyzed at −196 °C. Authentic tobacco β-1,3-glucanase (*Glc*) was photolyzed at 0 °C. The control reaction mixtures contained no competing IAA.

was greater than for the 25-kDa polypeptide.

The random labeling of other polypeptides that occurred in some experiments (the "background") also differed in sensitivity to the presence of competing IAA, according to the labeling conditions. Background labeling after photolysis at 0 °C was reduced by IAA (and all other competing compounds), whereas after photolysis at −196 °C, only the 25-kDa polypeptide showed competitive labeling. This effect can be seen on labeling of the "dye-front" in Fig. 4. An exception is the effect of the anti-auxins and auxin transport inhibitors tested at 200 μM 2-NAA (*lane 5*), quercitin (*lane 6*), 1-naphthylphthalamic acid (data not shown), and 2,3,5-triiodobenzoic acid (data not shown), which dramatically reduced all labeling after photolysis at −196 °C (Fig. 4*B*) but had a negligible effect at 0 °C (Fig. 4*A*). Measurement of the absorption spectra for the scavengers and competing compounds showed that competition is for binding and is not due to UV absorbance (data not shown).

pH Dependence of Labeling—Labeling with azido-IAA was pH-dependent, and the pH optimum differed between the polypeptides labeled at the two temperatures (Fig. 6). Labeling of the 31- and 24-kDa polypeptides was similar over a wide pH range (3.0–7.0) (Fig. 6*A*), being slightly reduced at the extremes of the range. At lower pH, a new polypeptide appeared that was also labeled and that was probably a degradation product. The 25-kDa polypeptide had a sharp labeling optimum at pH 4–4.5, and labeling was inhibited at pH 3.0 and pH 6.5–7.0 (Fig. 6*B*).

Labeling of Cell Wall Proteins—The extraction buffer used for preparing soluble protein extracts contained a relatively high salt concentration, and extraction with low salt buffer substantially reduced the yield of these proteins. It is, therefore, possible that the 31- and 24-kDa proteins are cell wall proteins found as contaminants in soluble extracts. To test this possibility, proteins extracted from washed cell walls using 5 M NaCl were labeled at 0 and −196 °C (Fig. 7). At 0 °C (*lanes 1–3*), two polypeptides were strongly labeled in cell wall extracts (*lane 3*) that have the same molecular weights (31 and 24 kDa), the same pH dependence for labeling, and the same pI as the two polypeptides labeled in the 0–30% ammonium sulfate cut of the soluble proteins (*lane 1*). In addition, the labeling was competed with IAA and tryptophan but not with NAA (data not shown). It is, therefore, very likely that the same 31- and 24-kDa polypeptides were

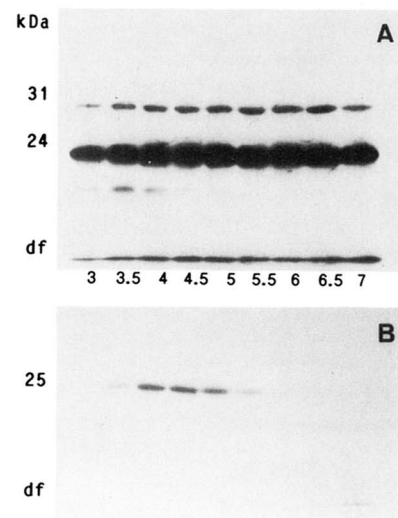

FIG. 6. **The effect of changes in pH of the reaction mixture on photoaffinity labeling of soluble proteins of *H. muticus*.** Shown are fluorographs of SDS-PAGE gels with equivalent amounts of protein per lane. *A*, 0–30% ammonium sulfate fraction photolyzed at 0 °C. *B*, 50–60% ammonium sulfate fraction photolyzed at −196 °C. The pH of the reaction mixtures are shown in the center. The experiment was carried out by adjusting the pH of the standard labeling buffer over its maximum possible pH range. The estimated molecular weights of the major labeled polypeptides are indicated, *df*, dye front.

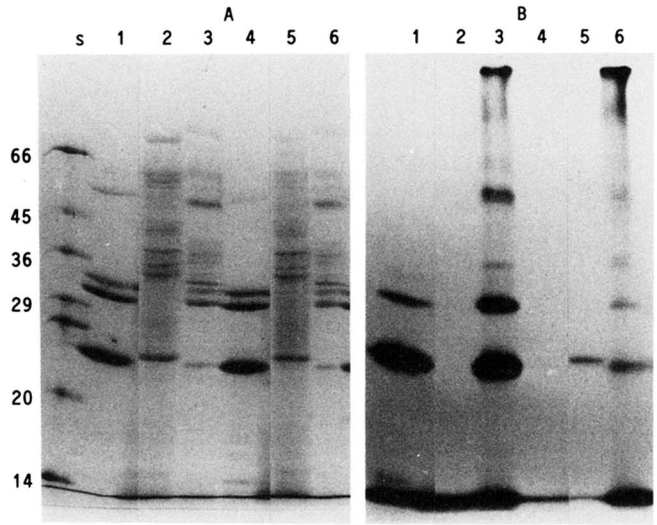

FIG. 7. **Photoaffinity labeling of cell wall and soluble proteins of *H. muticus*.** *A*, Coomassie-stained SDS-PAGE gel of equivalent amounts of protein per lane. *Lanes 1* and *4*, 0–30% ammonium sulfate fraction of soluble proteins after photolysis at 0 and −196 °C, respectively; *lanes 2* and *5*, 50–60% ammonium sulfate fraction of soluble proteins after photolysis at 0 and −196 °C, respectively; *lanes 3* and *6*, cell wall proteins after photolysis at 0 and −196 °C, respectively. *Lane s* contains molecular weight markers. *B*, fluorograph of gel shown in *A*.

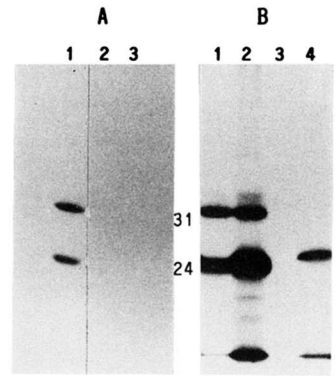

FIG. 8. **The precipitation of azido-IAA-labeled soluble proteins of *H. muticus* by antibodies to basic β-1,3-glucanase.** *A*, fluorograph of an SDS-PAGE gel of proteins in the 0–30% ammonium sulfate fraction precipitated by incubation with antibodies to β-1,3-glucanase (*lane 1*), chitinase (*lane 2*), and preimmune serum (*lane 3*) after photolysis at 0 °C. The estimated molecular masses of the labeled polypeptides in *lane 1* are 31 and 24 kDa. *B*, fluorograph of an SDS-PAGE gel of proteins precipitated by incubation with antibodies to β-1,3-glucanase. *Lane 1*, immunoprecipitation of 0–30% ammonium sulfate fraction after photolysis at 0 °C; *lane 2*, labeled proteins from the same fraction not treated with the antibodies; *lane 3*, immunoprecipitation of 50–60% ammonium sulfate fraction after photolysis at −196 °C; *lane 4*, labeled proteins from the same fraction not treated with the antibodies. The estimated mass of the labeled polypeptide is 25 kDa.

labeled in the cell wall extract and in the soluble protein extract. Several other polypeptides in the cell wall extract were labeled (Fig. 7), but it is not known whether the binding is specific. At −196 °C (*lanes 4–6*), the 31- and 24-kDa polypeptides were labeled in cell wall extracts (*lane 6*) but less than at 0 °C. The 25-kDa polypeptide that labeled at −196 °C in the 50–60% ammonium sulfate cut of the soluble proteins (*lane 5*) was not labeled in the cell wall fraction. Furthermore, the yield of this protein was not reduced by the use of low salt extraction buffer.

Identity of the 31- and 24-kDa Polypeptides—The abundance of the 31-kDa polypeptide, its low ammonium sulfate solubility, and the fact that the amount of the protein decreased in cultures treated with auxin (data not shown) led to the hypothesis that this protein was the basic form of β-1,3-glucanase (Felix and Meins, 1985). The hypothesis was tested by immunoprecipitation experiments and by photoaffinity labeling of authentic β-1,3-glucanase. The 31-kDa polypeptide in *H. muticus* labeled at 0 °C was precipitated almost quantitatively by incubation with tobacco anti-glucanase IgG (Fig. 8). The 24-kDa polypeptide was also precipitated by the glucanase antibody but with lower efficiency. No labeled polypeptide was precipitated by anti-chitinase IgG or by preimmune serum used as controls (Fig. 8*A*). The 25-kDa polypeptide seen after freezing at −196 °C was not precipitated by the glucanase antibody (Fig. 8*B*). Purified glucanase protein in the presence of an excess of BSA and cytochrome *c* was efficiently labeled by azido-IAA at 0 °C (Fig. 9*A*). BSA was only slightly labeled under these conditions, and cytochrome *c* was not labeled. Competition experiments with purified glucanase (Fig. 9*B*) gave results very similar to those obtained with the 31- and 24-kDa polypeptides (Fig. 4*A*), *i.e.* reduction of azido-IAA incorporation by IAA and indoles but not by non-indole auxins.

Labeled samples of the 0–30% ammonium sulfate fraction run on analytical isoelectric focusing gels gave two major radioactive bands near the basic end of the gel (pI ≈ 9.5). The basic form of tobacco β-1,3-glucanase labeled with azido-IAA

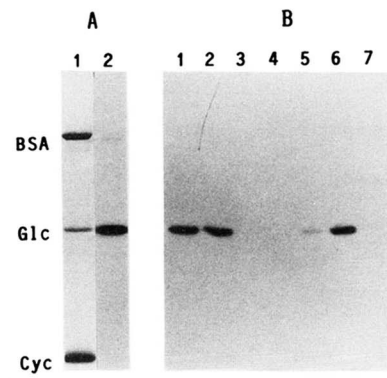

FIG. 9. **Photoaffinity labeling of purified β-1,3-glucanase with azido-IAA.** *A*, *lane 1*, Coomassie-stained SDS-PAGE gel showing separation of a mixture of BSA (10 μg), cytochrome *c* (*Cyc*, 10 μg), and β-1,3-glucanase (*Glc*, 5 μg) after labeling with azido-IAA and photolysis at 0 °C; *lane 2*, fluorograph of the gel described in *lane 1*. *B*, fluorograph of an SDS-PAGE gel of β-1,3-glucanase showing the effect of indoles and non-indole IAA analogues on the photoaffinity labeling of the protein. Photolysis was carried out at 0 °C. Substances were added to the reaction mixture at 50 μM prior to the addition of azido-IAA. *Lane 1*, control, no addition; *lane 2*, solvent control, 0.05% (v/v) ethanol; *lane 3*, IAA; *lane 4*, indole-3-butyric acid; *lane 5*, L-tryptophan; *lane 6*, NAA; *lane 7*, indole.

focused at approximately the same pI on these gels (data not shown). Thus the 31- and 24-kDa polypeptides behave similarly in azido-IAA labeling, are both basic, have the same competition spectrum, and apparently share all of these features and common antigenic determinants with β-1,3-glucanase. They differ, however, in temperature sensitivity. Preincubation of proteins for 15 min at 60 °C substantially reduced the binding activity of the 24-kDa but not the 31-kDa polypeptide (data not shown).

Maize ABP Antibody Studies—No specific reactions were detected on Western blots between antibodies to the maize ABP and either the 0–30 or 50–60% ammonium sulfate fractions of soluble proteins (data not shown).

DISCUSSION

The auxin analogue azido-IAA was used to search in the soluble protein fraction of *H. muticus* cells for polypeptides that bind IAA. After incubation of azido-IAA and protein fractions in the dark to allow binding, and exposure to UV irradiation to generate a reactive nitrene, three polypeptides were identified to which azido-IAA was covalently attached. We have shown that no catabolism of the azido-IAA occurred during the labeling period, and as it has been demonstrated previously that azido-IAA has auxin activity (Melhado *et al.*, 1984), the labeled polypeptides may be of significance for auxin action. In addition to putative auxin receptors, azido-IAA labeling may also identify auxin-sequestering proteins or auxin-metabolizing enzymes.

Many chemical artifacts are possible with photoaffinity labeling (Potter and Haley, 1983) and careful study and characterization of the photolysis conditions are necessary. Clearly, the use of one set of conditions, as has been the case in many published photolabeling experiments, is insufficient to conclude that photolabeling is specific. We have shown by several control experiments that the labeling of *H. muticus* proteins described here is not due to random tagging but is specific for IAA. However, strikingly different labeling patterns were obtained by simply altering the photolysis temperature from 0 to −196 °C. The reason for this difference is not clear, but there are several possibilities. First, binding is typically an equilibrium reaction, whereas the photolytic reaction is not, and lower temperatures decrease the off rates of the photogenerated nitrene (equilibrium) but have little effect on the nitrene half-life (nonequilibrium). Therefore, for equilibrium binding where the residence time of the nitrene is similar to its half-life, lower temperatures should promote, and may be required for, photolabeling. This may explain why the 25-kDa polypeptide was only seen after photolysis at −196 °C. Second, the orientation of the reactive nitrene at the binding site may be different for the two classes of polypeptides. The 31- and 24-kDa polypeptides were labeled strongly after photolysis at 0 °C but negligibly at −196 °C. Since low temperatures would decrease the gross molecular movements of the polypeptide and its side chains, it is possible in cases where the initial binding of the azido-IAA to the polypeptide is via the carboxyl side chain that the nitrene in position 5 would be adjacent to an attachment site for a substantially shorter time at −196 °C as compared with 0 °C. This may be the case for the 31- and 24-kDa polypeptides. In contrast, the indole ring may be inserted into a binding site on the 25-kDa polypeptide, trapping the reactive nitrene. Third, temperature effects on tertiary structure, solvation, and environment pH could also affect photolabeling.

The specificity and the biological significance of binding was also examined through the competition between azido-IAA and related molecules for the binding sites. The introduction of IAA into the reaction mixture reduced incorporation of azido-IAA into all three polypeptides, indicating that photolabeling occurred at or near the IAA-binding site. The labeled polypeptides could be distinguished, however, on the basis of the competition for binding with indoles and non-indole auxin analogues. The two polypeptides labeled at 0 °C appeared to have a high affinity indole-binding site(s) (K_i for IAA = 3 μM), for which non-indole, but active, auxins were not good ligands, whereas the 25-kDa polypeptide labeling at −196 °C had a significant affinity for active auxins but not inactive indoles. The surprisingly high displacement constant for IAA at this latter site (K_i = 100 μM) should not be taken to indicate low affinity for IAA since the values are derived from nonequilibrium reactions. The optimal conditions for attachment of the photolabel are probably not optimal for binding *in vivo*. In this respect, it is interesting that Hicks *et al.* (1989a) found that IAA at 1 mM was required to compete with the azido-IAA labeling of a putative auxin-binding protein in zucchini membranes at −196 °C. A binding protein with an affinity for several active auxin analogues but not for indoles in general (such as the 25-kDa polypeptide) would appear more likely to have a role in auxin action in the cell than a protein that binds indoles but not non-indole auxin analogues (like the 31- and 24-kDa polypeptides). However, this assumes that non-indole auxins have their effects by substituting for the IAA molecule at a binding site involved in auxin action. The possibility remains that the auxin analogues are effective indirectly by regulating IAA availability in the cell. Thus the 25-kDa polypeptide might represent the site at which IAA and its analogues interact, and the 31- and 24-kDa polypeptides, the site to which IAA itself binds to produce auxin effects.

A further difference between the 0 and −196 °C photolysis conditions was the effect of competing agents on background labeling. Whereas at the higher temperature the background was also reduced by addition of unlabeled IAA, at −196 °C only the 25-kDa polypeptide labeling was reduced, again suggesting that the 25-kDa polypeptide labeled more specifically than the 31- and 24-kDa polypeptides. The anti-auxins and auxin transport inhibitors tested (2-NAA, 1-naphthylphthalamic acid, and 2,3,5-triiodobenzoic acid) were very efficient competitors for all labeling, including the background, at −196 °C. Quercetin, a widely distributed plant flavonoid shown to inhibit polar auxin transport (Jacobs and Rubery, 1988), was also a very effective azido-IAA binding competitor. The intervention of these compounds and the narrow pH range for photolabeling suggests that recognition of the 25-kDa polypeptide by azido-IAA is biologically significant.

Further investigation of the importance of the labeled proteins for IAA metabolism or IAA action can best be carried out with suitable antibodies and other probes derived from protein sequence analysis. In the meantime, four major conclusions can be drawn from the experiments described above. First, none of the polypeptides in the soluble fraction of *H. muticus* that are labeled by azido-IAA are immunologically related to the plasma membrane/endoplasmic reticulum lumen-associated, 22-kDa subunit, auxin-binding protein of maize coleoptiles (Hesse *et al.*, 1989; Tillmann *et al.*, 1989; Inohara *et al.*, 1989; Jones and Venis, 1989).

Second, based upon the ammonium sulfate precipitation data, the azido-IAA labeling pattern, pI, the competition experiments, and immunoprecipitation, the 31-kDa polypeptide is related to the basic form of β-1,3-glucanase. These features are shared also by the 24-kDa polypeptide labeled at 0 °C, which is possibly a degradation product of the same enzyme. β-1,3-Glucanase is a very abundant protein, and binding of auxins to common proteins, *e.g.* peroxidase (Lobarzewski and Dawidowicz, 1983) and BSA (Murphy, 1979), has previously been demonstrated. Such binding has been considered to be the result of nonspecific, hydrophobic interactions rather than an indication of biologically relevant sites. However, the specific activity of auxin binding to β-1,3-glucanase in the experiments above is many times more than that for BSA. Furthermore, the levels of such glucanases in plants are clearly influenced by auxin treatment (Felix and Meins, 1986; Wong and Maclachlan, 1980), and the enzymes may be involved in the cell wall-loosening process stimulated by auxin (Huber and Nevins, 1981; Tanimoto and Masuda, 1968). Thus, it is tempting to contemplate a biological role for the azido-IAA binding to β-1,3-glucanase.

Third, polypeptides equivalent to the 31- and 24-kDa species found in the soluble fraction were apparently present in the cell wall, and the possibility exists that the recorded soluble activity represents proteins removed from the wall through the use of high ionic strength extraction buffers. Although the presence of auxin-binding proteins in the cell wall would be compatible with the "wall-loosening" model of auxin-induced cell elongation (see Cleland, 1987), the present results are somewhat ambivalent because the azido-IAA-labeled polypeptides in both cases have the physical properties of the *basic* form of β-1,3-glucanase, which (in tobacco) is located primarily in the vacuole (Keefe *et al.*, 1990). Furthermore, this form of the enzyme adheres strongly to chromatography supports, dialysis membranes, etc. Thus the apparent cell wall activity may be due to contamination with vacuolar enzyme binding to cell walls during homogenization.

Fourth, the 25-kDa polypeptide was not found among the proteins stripped from the cell wall and appears to be an authentic soluble protein. Its pH optimum of 4–4.5 for binding suggests this protein is active in a compartment such as the vacuole or a transport vesicle rather than in the cytosol. In several properties, such as pH optimum, binding specificity, displacement constant, and location, IAA binding to the 25-kDa polypeptide resembles the auxin binding in maize designated Site I (Dohrmann, 1978).

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REFERENCES

Barbier-Brygoo, H., Ephritikhine, G., Klämbt, D., Ghislain, M., and Guern, J. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 891–895

Cleland, R. E. (1987) in *Plant Hormones and Their Role in Plant Growth and Development* (Davies, P. J., ed) pp. 132–148, Martinus Nijhoff, Dordrecht

Dohrmann, U., Hertel, R., and Kowalik, H. (1978) *Planta (Berl.)* **140,** 97–106

Felix, G., and Meins, F. (1985) *Planta (Berl.)* **164,** 423–428

Felix, G., and Meins, F. (1986) *Planta (Berl.)* **167,** 206–211

Gebhardt, Ch., Shimamoto, K., Lázár, G., Schnebli, V., and King, P. J. (1983) *Planta (Berl.)* **159,** 18–24

Hagen, G. (1987) in *Plant Hormones and Their Role in Plant Growth and Development* (Davies, P. J., ed) pp. 149–163, Martinus Nijhoff, Dordrecht

Hesse, T., Feldwisch, J., Balshüsemann, D., Bauw, G., Puype, M., Vandekerkhove, J., Löbler, M., Klämbt, D., Schell, J., and Palme, K. (1989) *EMBO J.* **8,** 2453–2461

Hicks, G. R., Rayle, D. L., Jones, A. M., and Lomax, T. L. (1989a) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 4948–4952

Hicks, G. R., Rayle, D. L., and Lomax, T. L. (1989b) *Science* **245,** 52–54

Huber, D. J., and Nevins, D. J. (1981) *Planta (Berl.)* **151,** 206–214

Ihl, M. (1976) *Planta (Berl.)* **131,** 223–228

Inohara, N., Shimomura, S., Fukui, T., and Futai, M. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 3564–3568

Jacobs, M., and Rubery, P. H. (1988) *Science* **241,** 346–349

Jacobsen, H.-J. (1982) *Physiol. Plant.* **56,** 161–167

Jones, A. M., and Venis, M. A. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 6153–6156

Keefe, D., Hinz, U., and Meins, F. (1990) *Planta (Berl.)* **182,** 43–51

Kikuchi, M., Imaseki, H., and Sakai, S. (1989) *Plant Cell Physiol.* **30,** 765–773

Laemmli, U. K. (1970) *Nature* **227,** 680–685

Libbenga, K. R., van Telgen, H. J., Mennes, A. M., van der Linde, P. C. G., and van der Zaal, E. J. (1987) in *Molecular Biology of Plant Growth Control* (Fox, J. E., and Jacobs, M., eds) pp. 229–243, Alan R. Liss, Inc., New York

Lobarzewski, J., and Dawidowicz, A. (1983) *Phytochemistry (Oxf.)* **22,** 2427–2430

Löbler, M., and Klämbt, D. (1985) *J. Biol. Chem.* **260,** 9848–9853

Melhado, L. L., Jones, A. M., Ho, T.-H. D., and Leonard, N. J. (1984) *Plant Physiol.* **74,** 289–294

Murphy, G. J. P. (1979) *Plant Sci. Lett.* **15,** 183–191

Napier, R. M., Venis, M. A., Bolton, M. A., Richardson, L. I., and Butcher, G. W. (1988) *Planta (Berl.)* **176,** 519–526

Potter, R. L., and Haley, B. E. (1983) *Methods Enzymol.* **91,** 613–633

Rubery, P. H. (1987) in *Plant Hormones and Their Role in Plant Growth and Development* (Davies, P. J., ed) pp. 341–362, Martinus Nijhoff, Dordrecht

Sakai, S. (1985) *Plant Cell Physiol.* **26,** 185–192

Sakai, S., and Hangata, T. (1983) *Plant Cell Physiol.* **24,** 685–693

Shimomura, S., Sotobayashi, T., Futai, M., and Fukui, T. (1986) *J. Biochem.* **99,** 1513–1524

Tanimoto, E., and Masuda, Y. (1968) *Physiol. Plant.* **21,** 820–826

Tillmann, U., Viola, G., Kayser, B., Siemeister, G., Hesse, T., Plame, K., Löbler, M., and Klämbt, D. (1989) *EMBO J.* **8,** 2463–2467

Venis, M. A. (1987) in *Molecular Biology of Plant Growth Control* (Fox, J. E., and Jacobs, M., eds) pp. 219–228, Alan R. Liss, Inc., New York

Wong, Y.-S., and Maclachlan, G. A. (1980) *Plant Physiol.* **65,** 222–228