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5′-Azido-[3,6-$^3H_2$]-1-naphthylphthalamic acid, a photoactivatable probe for naphthylphthalamic acid receptor proteins from higher plants: Identification of a 23-kDa protein from maize coleoptile plasma membranes

(auxin efflux carrier/auxin transport/indole-3-acetic acid/*Zea mays*)

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ABSTRACT **1-Naphthylphthalamic acid (NPA) is a specific inhibitor of polar auxin transport that blocks carrier-mediated auxin efflux from plant cells. To allow identification of the NPA receptor thought to be part of the auxin efflux carrier, we have synthesized a tritiated, photolabile NPA analogue, 5′-azido-[3,6-$^3H_2$]NPA ([$^3H_2$]N_3NPA). This analogue was used to identify NPA-binding proteins in fractions highly enriched for plasma membrane vesicles isolated from maize coleoptiles (*Zea mays* L.). Competition studies showed that binding of [$^3H_2$]N_3NPA to maize plasma membrane vesicles was blocked by nonradioactive NPA but not by benzoic acid. After incubation of plasma membrane vesicles with [$^3H_2$]N_3NPA and exposure to UV light, we observed specific photoaffinity labeling of a protein with an apparent molecular mass of 23 kDa. Pretreatment of the plasma membrane vesicles with indole-3-acetic acid or with the auxin-transport inhibitors NPA and 2,3,5-triiodobenzoic acid strongly reduced specific labeling of this protein. This 23-kDa protein was also labeled by addition of 5-azido-[7-^{3}H]indole-3-acetic acid to plasma membranes prior to exposure to UV light. The 23-kDa protein was solubilized from plasma membranes by 1% Triton X-100. The possibility that this 23-kDa polypeptide is part of the auxin efflux carrier system is discussed.**

The group of phytohormones known as auxins [indole-3-acetic acid (IAA) and related natural and synthetic analogues] influence fundamental processes in plant growth, differentiation, and development (for review see ref. 1). Auxins are thought to be synthesized in apical shoot meristems or young leaves and transported to subapical target tissues. It is generally assumed that polar auxin transport by carrier proteins plays an important role in controlling auxin concentrations in various parts of the plant, but little is known about the molecular elements required for auxin transport through the various tissues of a plant (for review see ref. 2). Basipetal polar auxin transport in shoots requires energy and occurs with a velocity of 1–2 cm·hr^{-1} (3, 4). Binding and inhibitor studies using active auxins or auxin-transport inhibitors, as well as electrophysiological evidence, point to the presence of auxin uptake and efflux carriers localized on the plasma membrane of responsive plant cells (5–8). Transport of auxin through shoot tissues apparently involves a saturable, specific nH$^+$/IAA$^-$ influx carrier (3, 6, 9–13). In addition, passive uptake of undissociated IAA across the plasma membrane also occurs. Binding studies have indicated that an nH$^+$/IAA$^-$ influx carrier must be evenly distributed on the plasma membrane of maize (*Zea mays*) coleoptile or zucchini (*Cucurbita pepo*) hypocotyl cells. The auxin efflux carrier was found to be inhibited by phytotropins such as 1-naphthylphthalamic acid (NPA) or 2,3,5-triiodobenzoic acid (TIBA) (14, 15). NPA, for example, inhibits polar auxin transport, affects root growth, and abolishes gravitropic responses (16–20). These *in vivo* responses to NPA correlate with an *in vitro* inhibition of auxin efflux from plasma membrane vesicles isolated from zucchini hypocotyls or maize coleoptiles, resulting in a net accumulation of IAA in these membrane vesicles (10, 21).

Analysis of high-specificity binding of [^{3}H]NPA to maize microsomes or plasma membranes led to the identification of NPA binding sites, called NPA receptors (7, 22). NPA receptor proteins could be partially solubilized from plasma membranes by detergents such as Triton X-100 (22). Both membrane-bound and solubilized receptors had similar affinities for NPA, with K_d values in the range of 10^{-7} M. However, solubilization with Triton X-100 resulted in an increase of affinity for both 1-naphthylacetic acid and 2-naphthylacetic acid. Further analysis of high- and low-specificity NPA-binding activities revealed that this binding site is possibly one conformation of an auxin-binding protein involved in polar auxin transport (22). It was proposed that the NPA-binding protein could be either an auxin efflux carrier or a regulatory element of the auxin efflux carrier. It is therefore likely that NPA-binding proteins play an important role in auxin transport and perception (15, 23, 24). Identification of molecular elements of presumed auxin efflux carriers and molecular analysis of its components should contribute to our understanding of auxin action.

Although NPA-binding activities were detected in membrane vesicles by the use of highly specific binding of radioactively labeled NPA, such assays were not sensitive enough to allow successful isolation and molecular characterization of NPA-binding proteins. Photoaffinity labeling has been successfully applied to detect several auxin-binding proteins (25–27). Furthermore, recent data indicate that 5′-azido-NPA (N_3NPA) competes with [^{3}H]NPA for similar binding sites on zucchini membrane vesicles (28). We therefore synthesized a tritiated and light-sensitive NPA analogue, [3,6-$^3H_2$]N_3NPA, to specifically label membrane proteins after exposure to light. A protein of 23 kDa in plasma membranes from maize coleoptiles can be selectively photoaffinity labeled with both [$^3H_2$]N_3NPA and 5-azido-[7-^{3}H]IAA ([^{3}H]N_3IAA).

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Abbreviations: IAA, indole-3-acetic acid; [^{3}H]N_3IAA, 5-azido-[7-^{3}H]IAA; NPA, 1-naphthylphthalamic acid; [$^3H_2$]N_3NPA, 5′-azido-[3,6-$^3H_2$]NPA; TIBA, 2,3,5-triiodobenzoic acid.

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MATERIALS AND METHODS

Chemicals. Phthalic anhydride was obtained from Aldrich and used without further purification. 1,5-Diaminonaphthalene (Aldrich) was recrystallized from toluene and further purified by passage through an AlO_3 column.

Phthalic anhydride was brominated to 3,6-dibromophthalic anhydride according to Allen *et al.* (29). 3,6-Dibromo-5'-amino-NPA (3,6-dibromo-NH_2NPA) was synthesized from 3,6-dibromophthalic anhydride and 1,5-diaminonaphthalene at room temperature under argon. The reaction mix was stirred in acetonitrile overnight. 3,6-Dibromo-NH_2NPA precipitated and was collected by filtration. The precipitate was washed with acetonitrile and the crude product was dissolved in acetonitrile/ethanol (2:1, vol/vol) by addition of concentrated NH_4OH. After acidification with 2 M HCl the final product was precipitated, collected, and characterized by ^1H NMR and high-resolution mass spectroscopy (HR-MS). ^1H NMR [free acid in $(CD_3)_2SO$]: δ 10.5 (s, 1H, COOH); 7.98 (d, 1H, arom.); 7.72 (AB, 2H, arom.); 7.62 (d, 1H, arom.); 7.44 (d, 1H, arom.); 7.38 (t, 1H, arom.); 7.22 (t, 1H, arom.); 6.71 (d, 1H, arom.). HR-MS: m/z 445.9285 ($C_{18}H_{12}N_2O_2Br_2$, $M^+ \cdot -H_2O$, calcd. 445.92657). ^1H NMR spectra [in $(CD_3)_2SO$ with trimethylsilane as internal standard] were obtained with a Bruker (Karlsruhe, F.R.G.) Cryospec WM 250 or a Bruker WM 400. Mass spectra and high-resolution mass spectra were obtained with a Finnigan MAT 90 GC–MS system.

Synthesis of [$^3H_2$]N_3NPA. The synthesis is summarized in Fig. 1. 3,6-Dibromo-NH_2NPA was tritiated by catalyzed halogen displacement using tritium gas with 10% Pd on charcoal as catalyst. Fifteen millicuries (1 mCi = 37 MBq) of the resulting material, [3,6-$^3H_2$]NH_2NPA, was suspended in 200 μl of 80% acetic acid in a microcentrifuge tube. $NaNO_2$ (900 μg in 20 μl of water) was added and the mixture was incubated on ice for 1 hr (30). After centrifugation for 3 min at 10,000 × g, the supernatant was transferred to a fresh microcentrifuge tube. The insoluble pellet was discarded.

NaN_3 (780 μg) was added in 10 μl of ice-cold water. The reaction continued for 2 hr on ice with occasional gentle mixing. All further steps were performed in the dark or under dim red safety light. The solution was dried under vacuum in a Speed Vac centrifuge (Savant). The pellet was dissolved in 100% methanol and applied to a TLC plate (Merck 5583) with 44.5% water/40% acetonitrile/15% methanol/0.5% acetic acid) as the mobile phase. After chromatography a Hyperfilm-^3H film (Amersham, RPN.535) was exposed to the dry TLC plate for 2 hr at room temperature. The film was developed and aligned with the TLC plate, allowing identification of a band with a R_f of 0.82. This band was marked and scratched out. The material was extracted three times with methanol and concentrated in a Speed Vac centrifuge. Concentration was measured by comparing UV spectra obtained from spectroscopically characterized unlabeled N_3NPA and the labeled material. By using an extinction coefficient of 13,300 liter·mol^{-1}·cm^{-1} (28), the concentration was adjusted to 1 mM. The solution was stored at −20°C. The specific activity was determined by liquid scintillation counting (2.3 Ci/mmol).

Unlabeled NH_2NPA and N_3NPA were synthesized according to Voet *et al.* (28). NPA was prepared from 1-aminonaphthalene (Aldrich) and phthalic anhydride. After reaction for 2 hr in hot acetonitrile the precipitated product was washed with acetonitrile.

[^3H]N_3IAA, 21 Ci/mmol, was synthesized as described (31).

Plant Material and Preparation of Membrane Vesicles. Maize caryopses [*Zea mays* (L.) cv. Mutin, from Kleinwanzlebener Saatzucht, Einbeck, F.R.G.] were soaked in water and grown on moist cotton–wool without light for 3 days at 28°C. Coleoptiles including primary leaves were harvested and homogenized in 2.5 volumes of ice-cold buffer A [100 mM Tris/citric acid, pH 8.0/250 mM sucrose/1 mM EDTA/0.1 mM $MgCl_2$/10 mM ascorbic acid, containing aprotinin, (3.5 μg/ml) and leupeptin (0.1 μg/ml)] with a Waring Blendor and a Polytron homogenizer at maximum speed. After filtration through two layers of nylon (135-μm mesh), the particulate material retained in the net was reextracted with buffer A (1:1, vol/vol). The combined homogenates were centrifuged at 10,000 × g for 15 min at 4°C. The supernatant was centrifuged at 142,000 × g for 35 min at 4°C. The microsomal pellet was suspended in buffer B (5 mM potassium phosphate, pH 7.8/250 mM sucrose) and adjusted to 9 g per 100 g of starting material.

Plasma membrane vesicles were enriched from the microsomal fraction by partitioning between dextran T 500 and poly(ethylene glycol) 3350 (32). The resulting plasma membrane vesicles were homogenized with a glass homogenizer in buffer C (10 mM Tris/Mes, pH 6.5/250 mM sucrose) and frozen in liquid nitrogen. Vesicles were either stored at −80°C or used directly for photoaffinity labeling assays or binding studies. Concentration of membrane protein was

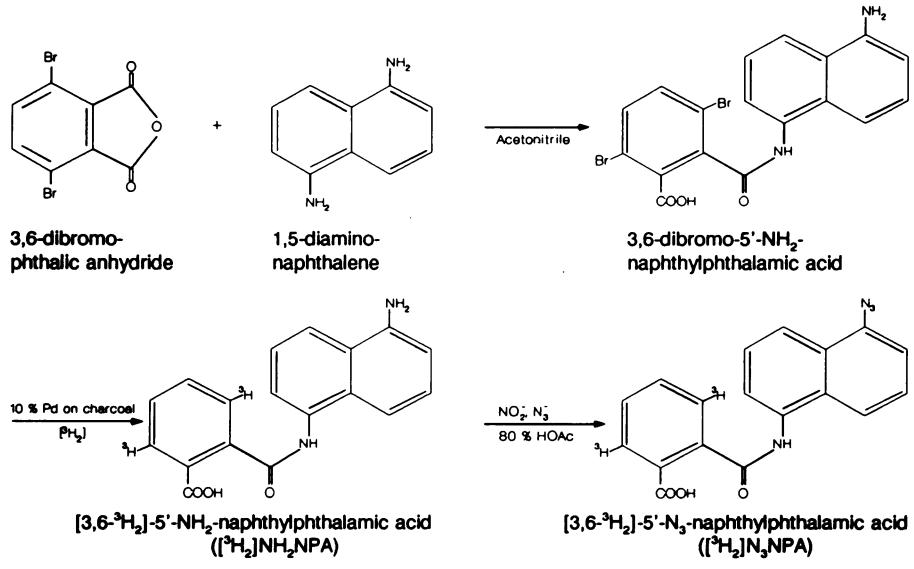

FIG. 1. Synthesis of [$^3H_2$]N_3NPA.

determined by Bradford assay (33) using bovine serum albumin as a standard.

Photoaffinity Labeling. All manipulations were performed on ice under red safety light. Aliquots of resuspended plasma membrane vesicles containing 100 μg of protein were diluted to 80 μl with buffer C. The pH was adjusted to 4.5 or 5.5 with 50 mM citric acid or to pH 7.5 with 20 mM Tris. The competitors were used from 10 mM stock solutions in 20 mM Tris. To each assay mixture, 10 μl of 20 μM $[^3H_2]N_3NPA$ in buffer C was added, resulting in a final concentration of 2 μM. The final concentration of the competitors was 1 mM. After addition of $[^3H_2]N_3NPA$, samples were mixed and incubated on ice for 10 min. For illumination, suspensions were transferred to lids of microcentrifuge tubes and placed on an aluminum block cooled on ice. The samples were illuminated with a high-pressure mercury lamp (Philips HPK 125W/L) for 25 min. After illumination the membranes were pelleted at 100,000 × *g* (11 min, 2°C). After resuspension in 100 μl of buffer C containing 1% (wt/vol) Triton X-100 and incubation on ice for 30 min, suspensions were centrifuged at 100,000 × *g* (11 min, 2°C). The pelleted plasma membrane vesicles were resuspended in 20 μl of SDS loading buffer. Solubilized proteins were precipitated from the supernatant with 12% (wt/vol) trichloroacetic acid for 2 hr on ice. After centrifugation for 45 min in a microcentrifuge, the pellets were washed with 1 ml of ice-cold acetone and dissolved in 20 μl of SDS loading buffer. The plasma membrane proteins were separated by SDS/12.5% PAGE. Gels were fluorographed (34) prior to drying. Exposure of gels to Kodak XAR-5 x-ray films was for 5–40 days. Photoaffinity labeling using $[^3H]N_3IAA$ as ligand was performed according to Campos *et al.* (31). After illumination, samples were further analyzed as described above.

$[^3H_2]N_3NPA$ Binding Assays. $[^3H_2]N_3NPA$ binding was assayed by equilibrium dialysis according to Reinhard and Jacobsen (35). All manipulations were performed under red safety light on ice. One half of the dialysis chamber was filled with 255 μl of plasma membrane vesicles in buffer C (50 μg of protein), and the other half with 255 μl of buffer C with or without competitor. Five microliters of $[^3H_2]N_3NPA$ solution was added to each cell of the dialysis chamber, resulting in a final concentration of 25 nM and a final volume of 260 μl. Identical reaction mixtures (50 μg of protein, 25 nM $[^3H_2]N_3NPA$) were used in each assay. Dialysis against buffer C instead of plasma membrane vesicles resulted in a background activity of 31 cpm (2% of control). For each concentration the assays were performed in triplicate. After 12 hr of dialysis at 4°C, aliquots (100 μl) were removed from each half of the cell. Radioactivity was analyzed in 5 ml of Rotizint cocktail (Roth, Karlsruhe, F.R.G.) in a liquid scintillation counter (Beckman). The difference in radioactivity in the half-cells of the dialysis chamber, which reflects the binding efficiency, was determined.

RESULTS

Synthesis and Characterization of $[^3H_2]N_3NPA$. The synthesis of $[^3H_2]N_3NPA$ is outlined in Fig. 1. 3,6-Dibromo-NH_2NPA was prepared as described in *Materials and Methods* and converted by catalytic tritium exchange to $[3,6\text{-}^3H_2]NH_2NPA$ (specific activity, 2.3 Ci/mmol). The final product, $[^3H_2]N_3NPA$, was obtained in a one-step conversion reaction with an overall yield of ≈20%. After final purification by TLC, the absorbance spectrum of $[^3H_2]N_3NPA$ was determined. In addition, unlabeled N_3NPA was synthesized according to Voet *et al.* (28). Comparison of R_f values from TLC and of absorbance spectra demonstrated identical physical properties for both compounds. After exposure to UV light, kinetic analysis of spectra revealed an identical decay of absorbance maxima at 310 nm for both compounds (data not shown).

Table 1. Binding of $[^3H_2]N_3NPA$ to maize coleoptile plasma membrane vesicles

Competitor	Conc.	Binding	
		cpm	% control
None	—	616	100
NPA	10 μM	532	86
	10 nM	393	64
	10 μM	223	36
Benzoic acid	100 μM	613	99

Binding was assayed by equilibrium dialysis in the presence or absence of competitors.

Characteristics of $[^3H_2]N_3NPA$ Binding to Plasma Membrane Vesicles. To assess specific binding of $[^3H_2]N_3NPA$ to membranes, we performed binding assays with highly enriched plasma membrane vesicles isolated from maize coleoptiles. Equilibrium binding of $[^3H_2]N_3NPA$ to the vesicles was attained by incubation for 12 hr at 4°C in the absence of UV light (Table 1). Unlabeled NPA was used as a competitor. Increasing concentrations of NPA resulted in successful competition of $[^3H_2]N_3NPA$ binding, whereas benzoic acid was not able to compete. Specific binding of $[^3H_2]N_3NPA$ was detected only with highly enriched plasma membrane vesicles, but not with crude microsomal membranes or heat-treated plasma membrane vesicles.

Photoaffinity Labeling of Plasma Membrane Proteins. Photoaffinity labeling was performed after incubation of plasma membrane vesicles with $[^3H_2]N_3NPA$ for 10 min at 4°C. Since solubilization of NPA-binding activities by Triton X-100 had

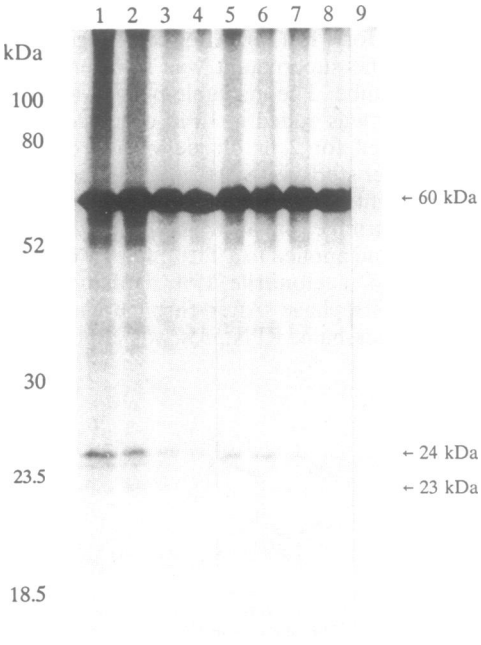

FIG. 2. Maize plasma membrane proteins photoaffinity labeled with $[^3H_2]N_3NPA$ and analyzed by SDS/PAGE and fluorography. Plasma membrane vesicles containing 100 μg of protein were incubated for 10 min at pH 4.5 (lane 1), pH 5.5 (lane 2), pH 6.5 (lane 3), or pH 7.5 (lane 4) or in the presence of competitor (1 mM) at pH 5.5 [IAA (lane 5), NPA (lane 6), benzoic acid (lane 7), or TIBA (lane 8)]. After incubation of membrane vesicles with 2 μM $[^3H_2]N_3NPA$, photoaffinity labeling was performed as described in *Materials and Methods*. Prior to SDS/PAGE, membrane vesicles were extracted for 30 min in buffer C containing 1% Triton X-100. After centrifugation the insoluble pellet (lanes 1–8) and the supernatant (lane 9, pH 5.5, without competitor) were analyzed by SDS/PAGE and fluorography. Sizes of molecular mass marker proteins are indicated at left.

been reported (22), we extracted the vesicles after photoaffinity labeling with buffer C containing 1% Triton X-100. After centrifugation the insoluble pellet and the supernatant containing the solubilized proteins were analyzed by SDS/PAGE and fluorography. Earlier experiments had shown an effect of pH on NPA-binding activity. We therefore tested the influence of pH on the efficiency of photoaffinity labeling of plasma membrane vesicles with $[^3H_2]N_3NPA$. A major polypeptide of 60 kDa (pm60) and several minor polypeptides were found to be associated with $[^3H_2]N_3NPA$ (Fig. 2). Different pH values (see legend to Fig. 2) did not influence the efficiency of pm60 labeling (lanes 1–4), nor was the photoaffinity labeling of pm60 affected by NPA, IAA, benzoic acid, or TIBA (Fig. 2, lanes 5–8). We conclude that labeling of this polypeptide is nonspecific. In addition, we found that pm60 could not be solubilized by Triton X-100 (lane 9).

After exposure of films for >7 days, fluorography revealed two additional proteins of 23 kDa (pm23) and 24 kDa (pm24) (Fig. 2, arrows; see also Figs. 3–5).

pm23 and pm24 could be partially extracted from the plasma membrane vesicles by Triton X-100 (Fig. 3). Photoaffinity labeling of pm24 was influenced by pH. Photoaffinity labeling was strong at pH 4.5, whereas labeling at pH 6.5 or 7.5 reduced the signal intensity. Addition of NPA or TIBA (Fig. 4, lanes 3 and 4) or IAA (Fig. 5, lane 2) prior to illumination did not reduce photoaffinity labeling of pm24 by $[^3H_2]N_3NPA$.

Photoaffinity Labeling of pm23 Is pH-Dependent. Labeling of pm23 was influenced by pH during preincubation of plasma membrane vesicles with $[^3H_2]N_3NPA$. Optimal labeling occurred at pH 5.5, the labeling was reduced at pH 4.5 and 6.5, and only weak labeling occurred at pH 7.5 (Fig. 3, compare lanes 1–4). After incubation in buffer C containing 1% Triton X-100, >50% of the pm23 molecules were solubilized (Fig. 3, compare lanes 5–8).

NPA and TIBA Compete for $[^3H_2]N_3NPA$ Labeling of pm23. When competitors for IAA transport were added to the photoaffinity labeling assay prior to illumination, both NPA and TIBA prevented specific labeling of pm23 by $[^3H_2]N_3NPA$ (Fig. 4, lanes 3 and 4). Preincubation with benzoic acid, an inactive competitor, did not result in reduction of labeling (Fig. 4, lane 2).

pm23 Is Labeled with $[^3H]N_3IAA$. To demonstrate that pm23 can specifically bind IAA we performed photoaffinity labeling experiments using both $[^3H_2]N_3NPA$ and $[^3H]N_3IAA$ as ligands (31, 36). Plasma membranes were first labeled with $[^3H_2]N_3NPA$ in either the absence (Fig. 5, lanes 1 and 3) or the presence (lanes 2 and 4) of IAA. Photoaffinity labeling of pm23

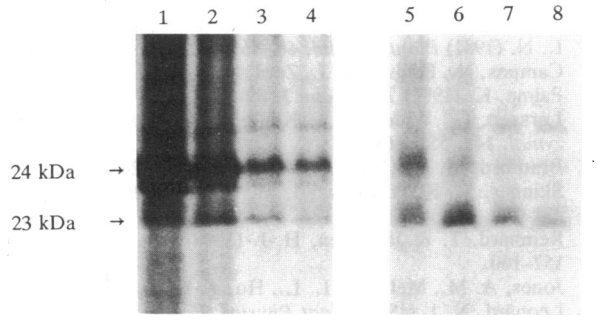

FIG. 3. Photoaffinity labeling of maize plasma membrane proteins is influenced by pH. Plasma membrane vesicles (100 μg of protein) were incubated for 10 min at pH 4.5 (lanes 1 and 5), pH 5.5 (lanes 2 and 6), pH 6.5 (lanes 3 and 7), or pH 7.5 (lanes 4 and 8). After incubation of membrane vesicles with 2 μM $[^3H_2]N_3NPA$, photoaffinity labeling was performed. After extraction of vesicles by Triton X-100 and centrifugation, the insoluble pellet (lanes 1–4) and the supernatant (lanes 5–8) were analyzed by SDS/PAGE and fluorography.

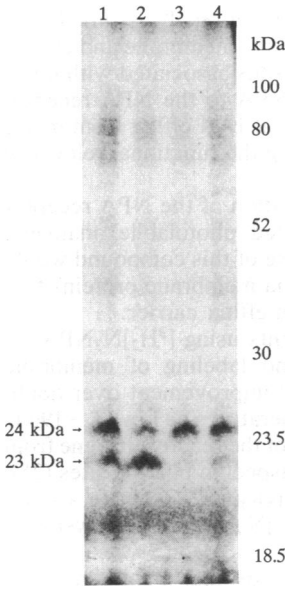

FIG. 4. Competition of photoaffinity labeling by $[^3H_2]N_3NPA$ of Triton X-100-soluble plasma membrane proteins. Plasma membrane vesicles (100 μg of protein) were incubated at pH 5.5 in the absence (lane 1) or presence of 1 mM benzoic acid (lane 2), 1 mM NPA (lane 3), and 1 mM TIBA (lane 4). Labeling with 2 μM $[^3H_2]N_3NPA$ was performed at pH 5.5. After photoaffinity labeling, proteins were extracted with buffer C containing 1% Triton X-100. Prior to gel electrophoresis, solubilized proteins were precipitated with 12% trichloroacetic acid.

but not of pm24 was significantly reduced by addition of IAA in both the Triton X-100-insoluble membrane pellet (lane 2) and Triton X-100-soluble proteins (lane 4). Using $[^3H]N_3IAA$ for photoaffinity labeling, we observed that only pm23 was labeled in the Triton X-100 membrane pellet (lane 5) as well as in Triton X-100-soluble proteins (lane 6). Although background labeling of membrane proteins was comparable for the two photoaffinity probes, it is interesting that only pm23 labeling was blocked by both NPA and IAA. In addition, only pm23 was labeled by $[^3H]N_3IAA$. Thus, pm23, but not pm24, must contain binding sites for both NPA and IAA.

DISCUSSION

Since Went's discovery of auxin (37), basipetal polarity of transport of auxins and auxin-like compounds in stem, petioles, and coleoptiles has been well documented (2). According to the chemiosmotic hypothesis, movement of auxin is not a simple diffusion but is an energy-dependent active transport process (6, 15). Kinetic measurements of uptake of radioactive IAA in coleoptile segments or membrane vesicles suggested the presence of a saturable, inwardly directed carrier-mediated cotransport of IAA anions and H^+ ions, as well as transmembrane efflux of IAA anions on a separate carrier.

Synthetic compounds such as NPA, TIBA, or other phytotropins have been found to inhibit auxin transport by binding to the auxin efflux carrier (38). Binding studies

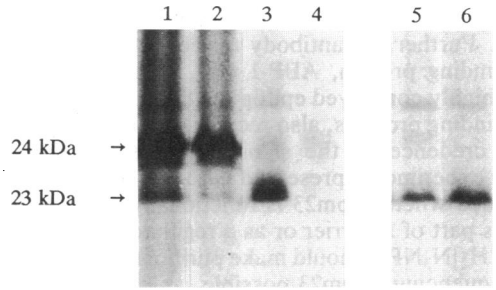

FIG. 5. Photoaffinity labeling of plasma membrane vesicles by $[^3H_2]N_3NPA$ and $[^3H]N_3IAA$. Vesicles (100 μg of protein) were incubated in the presence of either $[^3H_2]N_3NPA$ (lanes 1–4) or $[^3H]N_3IAA$ (lanes 5 and 6). Competition with 1 mM IAA is shown in lanes 2 and 4. After photoaffinity labeling, plasma membranes were extracted with buffer C containing 1% Triton X-100. Both membrane pellets (lanes 1, 2, and 5) and Triton X-100-soluble proteins (lanes 3, 4, and 6) were analyzed by SDS/PAGE and fluorography.

revealed that NPA binding sites, operationally assumed to be receptors, are localized on the plasma membrane and clearly separable from the auxin binding sites associated with intracellular membranes. Binding of NPA to the NPA receptor protein rapidly leads to inhibition of IAA efflux from maize coleoptile sections, thus suggesting the functional relevance of this protein.

To allow identification and isolation of the NPA receptor protein, we synthesized a tritiated photolabile analogue, [$^3H_2$]N_3NPA. We expected that use of this compound would result in specific labeling of plasma membrane proteins that could be components of the auxin efflux carrier.

Photoaffinity labeling experiments using [$^3H_2$]N_3NPA resulted in a very low background labeling of membrane proteins at 4°C. This is a marked improvement over application of [^{3}H]N_3IAA, where temperatures as low as −196°C had to be used to reduce diffusion of the reactive nitrene from the binding site to apparently nonspecific binding sites (25). This difference in chemical reactivity may indicate a more specific and stable binding of [$^3H_2$]N_3NPA to its physiologically relevant binding sites.

We observed no labeling in microsomal membrane fractions. We therefore used only highly enriched plasma membrane vesicles obtained by aqueous two-phase partitioning for photoaffinity labeling. Photoaffinity labeling of these vesicle preparations at 4°C resulted in crosslinking of three polypeptides with apparent molecular sizes of 23, 24, and 60 kDa. It was shown earlier that binding of [^{3}H]NPA to membrane fractions from various plants had a pH optimum between pH 5 and 6 (22, 39). We therefore analyzed the influence of both pH and competitors on photoaffinity labeling of membrane proteins. We found optimal labeling at pH 5.5–6.5, with a general increase of background labeling at more acidic pH. These results are in good agreement with data obtained for binding of [^{3}H]NPA to maize coleoptile membranes (20). After incubation of the plasma membrane vesicles with [$^3H_2$]N_3NPA in the presence of competitors such as NPA, TIBA, and IAA prior to illumination, only pm23 labeling was significantly reduced. Benzoic acid was not able to compete with [$^3H_2$]N_3NPA either in binding assays or in photoaffinity labeling. Sussman and Gardner (22) solubilized ≈50% of [^{3}H]NPA-binding activity from maize coleoptile membranes by using 1% Triton X-100. We observed that >50% of both pm23 and pm24 were solubilized with 1% Triton X-100, whereas the pm60 polypeptide remained completely in the plasma membrane vesicles. Because no competition for photoaffinity labeling of pm60 and pm24 by unlabeled NPA or TIBA was observed, we assume that these two proteins are not related to the NPA receptor protein. In contrast, our competition analysis and the observed labeling of pm23 with [^{3}H]N_3IAA fit the characteristics reported for the NPA receptor protein (for review, see ref. 15). Further, an antibody raised against another maize auxin-binding protein, ABP-1 (40), which probably recognizes a highly conserved epitope within a variety of different auxin-binding proteins, also recognizes pm23 (41). This adds further credence to the significance of the photoaffinity labeling experiments presented here. Although it cannot yet be decided whether pm23 is functionally involved in auxin efflux as part of a carrier or as a regulatory component, the use of [$^3H_2$]N_3NPA should make purification and subsequent microsequencing of pm23 possible.

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