

Cytoplasmic Orientation of the Naphthylphthalamic Acid-Binding Protein in Zucchini Plasma Membrane Vesicles¹

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Polar transport of the plant hormone auxin is blocked by substances such as *N*-1-naphthylphthalamic acid (NPA), which inhibit auxin efflux and block polar auxin transport. To understand how auxin transport is regulated in vivo, it is necessary to discern whether auxin transport inhibitors act at the intra- or extracellular side of the plasma membrane. Populations of predominantly inside-in plasma membrane vesicles were subjected to treatments that reverse the orientation. These treatments, which included osmotic shock, cycles of freezing and thawing, and incubation with 0.05% Brij-58, all increased NPA-binding activity and the accessibility of the binding protein to protease digestion. Marker activities for inside-out vesicles also increased, indicating that these treatments act by altering the membrane orientation. Finally, binding data were analyzed by multiple analyses and indicated that neither the affinity nor abundance of binding sites changed. Kinetic analyses indicated that the change in NPA-binding activity by Brij-58 treatment was due to an increase in the initial rates of both association and dissociation of this ligand. These experiments indicated that the NPA-binding site is on the cytoplasmic face of the plasma membrane in zucchini (*Cucurbita pepo* L. cv Burpee Fordhook).

The auxins are a class of phytohormones that control plant growth, development, and response to the environment through regulation of elongation, cell division, differentiation, and tropic responses to light and gravity. Auxins, of which IAA is the predominant naturally occurring type, are delivered to plant cells by a unique polar transport mechanism (for review, see Goldsmith, 1977; Lomax et al., 1995). Polar transport is critical for the growth and development of plants and their ability to respond to environmental stimuli. Auxins are transported from their source in the shoot apex at the growing tip toward the base of stems. Polar auxin transport depends on the proton gradient across the plasma membrane of plant cells and has been described as chemiosmotic (Rubery and Shelldrake, 1974). The ionization states of IAA in the extracellular space and in the cytoplasm affect the membrane permeability of this phytohormone and lead to an accumulation

of the anionic form of the molecule within the cytoplasm. Polar transport is believed to be mediated by an uptake $2\text{H}^+/\text{IAA}^-$ symport carrier (Lomax et al., 1985; Sabater and Rubery, 1987) and an IAA^- efflux carrier (Rubery and Shelldrake, 1974). Basal localization of this IAA^- efflux carrier has been proposed to drive the polarity of IAA transport (Rubery and Shelldrake, 1974; Jacobs and Gilbert, 1983).

Several synthetic auxin transport inhibitors, or phytochemicals, have been identified, but NPA is the best characterized (Rubery, 1990). These phytochemicals specifically block auxin transport at the site of the efflux carrier (Rubery, 1990). Natural inhibitors, or flavonoids, have also been shown to block transport at the site of the efflux carrier (Jacobs and Rubery, 1988). Radiolabeled auxin transport inhibitors can be used to study the auxin efflux carrier. [³H]NPA has been shown to bind to a protein, since the binding is heat- and protease-sensitive (Sussman and Gardner, 1980). Plasma membrane vesicles prepared by aqueous two-phase partitioning have been shown to exhibit high-affinity binding ($K_d = 15$ nM) by [³H]NPA to a single site using Hill plots, Scatchard plots, and Lineweaver-Burk plots, as well as the computer program Ligand, to analyze the data (Muday et al., 1993). Auxin transport inhibitors also perturb plant growth and development. Applications of these compounds inhibit the ability of plants to respond to gravity and also inhibit growth and lateral root development (Muday and Haworth, 1994; Lomax et al., 1995). Understanding how these molecules regulate auxin movement requires characterization of the protein to which these molecules bind.

Little is known about the biochemical characteristics of the efflux carrier. Two lines of experimentation suggest that the efflux carrier is composed of at least two separate polypeptides, with a distinct regulatory polypeptide binding NPA. First, pretreatment of zucchini (*Cucurbita pepo* L., cv Burpee Fordhook) hypocotyl segments with cycloheximide, an inhibitor of protein translation, was shown to inhibit NPA-regulated IAA efflux without affecting the saturable binding of NPA (Morris et al., 1991). These re-

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Abbreviations: B_{max} , maximum concentration of binding sites; BTP, bis-Tris propane; GFB, glass microfiber filters, type B; k_{on} , association rate constant; k_{off} , dissociation rate constant; NBB, NPA-binding buffer; NPA, naphthylphthalamic acid; [³H]NPA, [2,3,4,5-(³H)]NPA.

sults suggest that the NPA binding and auxin efflux sites are on separate proteins with different stabilities following cycloheximide treatment. Wilkinson and Morris (1994) prepared microsomal membranes from etiolated zucchini hypocotyls pretreated with monensin, a monovalent cationophore that permeates cells and disrupts late protein-processing events in the Golgi apparatus. Treatment with monensin inhibited IAA efflux, the regulation of efflux by NPA, and polar auxin transport, but did not affect saturable binding of NPA. These results suggest that the sites for auxin efflux and NPA binding are located on separate polypeptides (Wilkinson and Morris, 1994). Second, treatment of plasma membrane vesicles with KI or Na₂CO₃ released the NPA-binding protein into the supernatant after ultracentrifugation, indicating that NPA binds to a peripheral protein (Cox and Muday, 1994). Furthermore, the NPA-binding protein is still active in membrane-free, detergent-insoluble pellets, indicating that it does not require a lipophilic environment for activity (Cox and Muday, 1994).

Recent evidence suggests that the NPA-binding or regulatory subunit of the efflux carrier is associated with the cytoskeleton. Indication of a possible interaction between the NPA-binding protein and the cytoskeleton was shown by Cox and Muday (1994). Treatment of zucchini plasma membrane vesicles with Triton X-100 resulted in a detergent-insoluble pellet enriched in cytoskeletal components, which contained 50 to 60% of the starting [³H]NPA-binding activity. [³H]NPA-binding activity was found in the supernatant upon depolymerization of the cytoskeleton and then recovered in the pellet upon repolymerization of the cytoskeleton. Treatment of detergent-insoluble pellets with cytochalasin B released actin monomers and [³H]NPA-binding activity into the supernatant (Cox and Muday, 1994). Further experiments using phalloidin, cytochalasin D, and taxol to stabilize F-actin, G-actin, and microtubules, respectively, have shown a more direct interaction between the NPA-binding protein and the actin cytoskeleton (Butler, 1995).

Monoclonal antibodies against the NPA-binding protein provided evidence for a basal localization of this protein in pea stem cells (Jacobs and Gilbert, 1983). The association of the NPA-binding protein with the actin cytoskeleton suggests a mechanism by which the mobility of this protein may be restricted to localize it on one plane of the plasma membrane. The association of proteins with the cytoskeleton has been well documented and has been shown to play a significant role in the localization of proteins in the cell (Bloch and Resneck, 1986; Yoshihara and Hall, 1993).

If the NPA-binding protein interacts with the actin cytoskeleton, then it must be located on the inside face of the plasma membrane. Discerning the orientation of the NPA-binding protein is an important step in understanding the nature of the protein and its regulation of the efflux carrier. It was originally thought that the NPA-binding protein faced the extracellular space, since the optimal pH for binding of [³H]NPA (pH 5.3) is closer to the extracellular pH. However, experimental evidence contradicts this hypothesis. Michalke (1982) isolated two corn microsomal

membrane vesicle preparations based on differential sedimentation with two PEG concentrations. One preparation appeared to be inside-in and this preparation bound only the protonated and uncharged NPA (membrane-permeable form) and was unable to bind the NPA anion. An increase in [³H]NPA binding when microsomal membranes are subjected to freezing and thawing, which may flip the vesicles from inside-in to inside-out, has been reported (Hertel et al., 1983). These experiments suggest that the NPA-binding protein may face inside the membrane, a conclusion that can be strengthened by further analyses.

To demonstrate the membrane-sidedness of the NPA-binding protein, the best approach would be to assay highly pure populations of tightly sealed plasma membrane vesicles of both inside-in and inside-out orientations. Plasma membrane vesicles separated by aqueous two-phase partitioning produce populations of membrane vesicles that are reported to be oriented uniformly, with greater than 80% of the vesicles having the cytoplasmic side facing inside (Palmgren et al., 1990). Recently, the addition of 0.05% Brij-58 to inside-in spinach leaf plasma membrane vesicles was shown to produce a high percentage of tightly sealed inside-out membrane vesicles (Johansson et al., 1995). We present data that show an increase in protease-sensitive [³H]NPA binding in preparations of zucchini plasma membrane vesicles when the membranes are converted to inside-out populations by exposure to cycles of freezing and thawing, osmotic shock, or Brij-58.

MATERIALS AND METHODS

Chemicals and Radiochemicals

[³H]NPA (58.0 Ci/mmol) was obtained from American Radiolabeled Chemicals (St. Louis, MO). NPA was purchased from Chemical Services (West Chester, PA). PEP, NADH, lactate dehydrogenase (50% glycerol), and pyruvate kinase (50% glycerol) were purchased from Boehringer Mannheim. Brij-58 was obtained from Pierce. Trypsin (from bovine pancreas with 10,000 N α -benzoyl-L-Arg ethylester units mg⁻¹ activity), ATP disodium salt, and valinomycin (resuspended in ethanol) were purchased from Sigma. Dextran T500 was purchased from Pharmacia Biotech (Piscataway, NJ). Scintiverse scintillation fluid, sodium citrate, DTT, and Whatman GFB filters were obtained from Fisher Scientific. Multiscreen HA plates were purchased from Millipore (Burlington, MA). All other chemicals were purchased from Sigma and were of the highest grade possible.

Plant Material and Plasma Membrane Preparation

Zucchini (*Cucurbita pepo* L. cv Burpee Fordhook) seedlings were grown in the dark for 5 d in vermiculite at 30°C. The top 4 cm of 5-d-old hypocotyls were harvested for plasma membrane preparation. Plasma membrane vesicles were prepared using two different procedures, since the experiments in this paper were performed in two different laboratories and several years apart. Plasma membrane vesicles prepared with procedure A were used in the

Brij-58 treatments, and those prepared with procedure B were used in freeze-thaw and osmotic shock studies.

For procedure A, hypocotyls (100 g) were harvested and homogenized in an equal volume per weight of buffer A (25 mM Tris, pH 7.2, 0.25 M Suc, 3.0 mM EDTA, 0.25 mM PMSF, 1 mM DTT). The homogenate was then filtered through one layer each of Miracloth (Calbiochem) and nylon cloth followed by centrifugation at 6,700g at 4°C for 10 min to remove cellular debris. The supernatant was centrifuged at 100,000g for 33 min at 4°C. The resulting microsomal membrane pellets were resuspended in buffer B (5.0 mM K₂HPO₄, pH 7.8, and 0.25 M Suc) at a ratio of 1 mL/25 g tissue. The membranes were then subjected to aqueous two-phase partitioning using the method of Widell and Larsson (1981), with 6.3% (w/w) dextran and 6.3% (w/w) PEG, as described by Muday et al. (1993). The tubes were centrifuged at 1,000g for 10 min. The purified plasma membrane top phase was removed and re-extracted using a fresh bottom phase. This final top phase, containing purified plasma membrane vesicles, was then diluted 3:1 with buffer B and centrifuged at 100,000g for 33 min at 4°C. The membranes were resuspended in buffer C (50 mM Tris, pH 7.2, 0.05 mM CaCl₂, 5 mM MgCl₂, and 50 mM KCl) at a ratio of 0.6 mL/100 g starting material and were used fresh in experiments with Brij-58. Previous analysis indicated that these membranes were more than 85% pure (Muday et al., 1993). For H⁺-pumping and ATPase activity assays, plasma membrane vesicles were resuspended in buffer C buffered with 50 mM Mops pH 7.2 instead of Tris, since Tris acts as an uncoupler (Palmgren, 1990). Protein concentration was determined by the bicinchoninic acid assay procedure (Smith et al., 1985).

For plasma membrane procedure B, hypocotyls were homogenized in buffer D (50 mM Mops pH, 7.2, 0.25 M Suc, 5.0 mM EDTA, 0.2% BSA [Sigma A3294], 0.2% casein enzymatic hydrolysate, 0.6% polyvinylpolypyrrolidone, 0.5 mM PMSF, 5 mM DTT). The homogenate was then filtered through one layer of nylon cloth followed by centrifugation at 6,700g at 4°C for 10 min to remove cellular debris. The supernatant was then centrifuged at 100,000g for 30 min at 4°C. The pellets were resuspended in 1 mL of buffer E (5.0 mM K₂HPO₄, pH 7.8, and 0.25 M Suc, 0.1 mM EDTA, and 0.1 mM DTT). The membranes were then subjected to aqueous two-phase partitioning as described above but using buffer E (250 mM Suc, 5 mM K₂HPO₄, pH 7.8, 20 mM NaCl, 1 mM DTT). The purified plasma membrane top phase was removed and saved, and the bottom phase was re-extracted using a fresh top phase. The two top phases were combined and diluted 3:1 with buffer E and centrifuged at 100,000g for 30 min at 4°C. The membranes were resuspended at a protein concentration of 5 mg/mL in buffer E and then either used fresh or frozen in liquid nitrogen and stored at -80°C to be used in freeze-thaw and osmotic shock experiments.

[³H]NPA-Binding Assay

Assays were performed in 200 μL of NBB (20 mM sodium citrate, pH 5.3, 1.0 mM MgCl₂, 0.25 M Suc). Plasma membrane vesicles with a final concentration of 0.2 mg/mL

were incubated with 5.0 nM [³H]NPA or in a range from 1 to 68 nM as described by Muday et al. (1993). Background was assessed by incubating with 10 μM cold NPA. For competition assays, 5 nM [³H]NPA and unlabeled NPA in the range of 10 pM to 1 μM were used. Two procedures were followed for incubating and filtering.

With method A, samples were incubated for 45 min (or for the indicated time) with slight shaking at 4°C. After incubation, 190 μL of each sample was poured through a filter (Whatman GFB circle that had been pretreated with 0.3% polyethylenimine for 1 h). The filter was then washed with 5 mL of NBB, soaked in 2.5 mL of scintillation fluid overnight, and counted in a liquid scintillation counter (Tm Analytic, Elk Grove Park, IL) for 2 min. Binding is reported in picomoles of [³H]NPA bound per milligram of protein in the assay. The values represent an average of three different replicates. The average and SE for a sample mean difference were calculated using the method of Gravetter and Wallnau (1992).

With method B, after a 20-min incubation with slight shaking at 4°C, plates (Multiscreen HA) were filtered and washed with 200 μL of NBB. The plastic backings were removed and the plate was incubated for 15 min at 50°C, after which the filters were punched out with a cork borer and soaked in 0.5 mL H₂O for 30 min. Finally, 4 mL of scintillation fluid was added and the samples were counted for 2 min. The values represent an average of four different replicates. Binding activity is reported in picomoles of [³H]NPA per milligram of protein in the assay.

Formation of Inside-Out Vesicles from Inside-In Vesicles

Purified inside-in plasma membrane vesicles prepared by method A, with a final concentration of 2 mg/mL, were incubated in buffer C with and without 0.05% Brij-58 for 20 min at 4°C on ice. The tubes were gently mixed prior to incubation. Inside-in plasma membrane vesicles prepared using procedure B at 2 mg/mL were subjected to freezing in liquid nitrogen for 30 s per freeze-thaw cycle. For osmotic shock studies, the membranes were diluted into NBB with reduced Suc concentration to yield the indicated concentrations.

Trypsin Digestion and Marker Enzyme Assays

Trypsin digestions were performed under two separate conditions. Inside-out vesicles isolated by cycles of freezing and thawing and with a final protein concentration of 1.25 mg/mL were incubated at room temperature for 3 min in buffer B with a final trypsin concentration of 1 μg/mL. The reaction was stopped after 3 min by the addition of 3 μg/mL trypsin inhibitor. Following a 20-min incubation with Brij-58, trypsin (final concentration, 10 μg/mL) was added to the microfuge tube for 30 min at room temperature (23°C). The tubes were gently agitated several times during incubation. To verify that trypsin was active under the conditions used for the Brij-58 experiments, protease activity was measured by examining the A₂₄₇ change using the artificial substrate *N*-α-tosyl-L-Arg methyl ester (Worth-

ington, Freehold, NJ) according to the manufacturer's recommendations (data not shown).

H⁺-pumping activity was measured using the method of Palmgren (1990) and Palmgren and Sommarin (1989). The reaction buffer contained 10 mM Mops, pH 7, with BTP; 2 mM ATP, pH 7, with BTP; 140 mM KCl; 1 mM EDTA; 1 mM DTT (made fresh); 1 mg/mL BSA, pH 7, with BTP; 20 μ M acridine orange; 2.5 μ g/mL valinomycin; and 50 μ g/mL plasma membrane vesicles. Samples were mixed after the addition of the plasma membrane vesicles and allowed to incubate at room temperature for 5 min. The reaction was initiated by addition of 4 mM MgCl₂ and the rate of A₄₉₅ decrease of the probe for pH change, acridine orange, was recorded. The blank contained everything except plasma membrane vesicles and MgCl₂.

ATPase activity was measured using the method of Palmgren (1990) and Palmgren and Sommarin (1989). The reaction buffer contained 10 mM Mops, pH 7, with BTP; 2 mM ATP, pH 7, with BTP; 140 mM KCl; 1 mM EDTA; 1 mM DTT (made fresh); 1 mg/mL BSA, pH 7, with BTP; 50 μ g/mL plasma membrane vesicles; 0.25 mM NADH (made fresh); 1 mM PEP; 50 μ g/mL pyruvate kinase; and 25 μ g/mL lactate dehydrogenase. Reagents were mixed and allowed to incubate for 5 min at room temperature, and then 4 mM MgCl₂ was added to initiate the hydrolysis of ATP, which is enzymatically linked to the oxidation of NADH. The rate of NADH oxidation was measured as the A₃₄₀ decrease. The blank contained everything except plasma membrane vesicles and MgCl₂.

RESULTS

Osmotic Shock of Zucchini Plasma Membrane Vesicles

The sidedness of the NPA-binding protein was initially investigated using inside-in plasma membrane vesicles

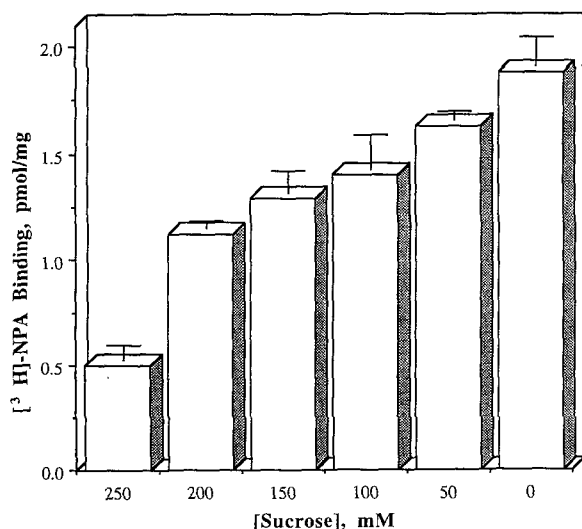


Figure 1. Osmotic shock of zucchini plasma membrane vesicles. Vesicles were resuspended in 20 mM sodium citrate, pH 5.3, and the indicated concentrations of Suc. The vesicles were then assayed for binding of 5 nM [³H]NPA after a 10-min incubation in the indicated buffer.

Table I. Effect of freezing and thawing of plasma membranes on NPA-binding activity and marker enzyme activity

Assay	Unfrozen	Frozen	Increase
NPA-binding activity (pmol mg ⁻¹ protein) ^a	3.0	4.1	1.4-fold
NPA-binding susceptibility to trypsin (%) ^b	4.8	22.0	4.5-fold
H ⁺ Pumping (change in A ₄₉₅ mg min ⁻¹ × 10 ³)	4.8	19.4	4.0-fold

^a NPA-binding activity was measured using 5 nM [³H]NPA. ^b The susceptibility of NPA-binding activity was calculated by subtracting binding activity after trypsin digestion from the total activity and dividing by the total activity.

subjected to osmotic shock. Suspensions consisting of primarily inside-in zucchini plasma membrane vesicles were resuspended in buffer with reduced Suc concentration. Osmotic shock occurred, causing the plasma membrane vesicles to burst and reseal with a random orientation, thereby exposing inward-facing binding sites. Figure 1 shows that as Suc was incrementally decreased from 250 to 0 mM the amount of [³H]NPA-binding activity steadily increased. Proton pumping in these vesicles decreased with increasing osmotic shock, indicating that the vesicles became leaky (data not shown).

Freezing and Thawing of Plasma Membrane Vesicles

The location of the NPA-binding site was further investigated using inside-in plasma membrane vesicles that had been frozen and then thawed to reverse the orientation. Palmgren et al. (1990) were able to use this same method to convert inside-in plasma membrane vesicles from sugar beet leaves to inside-out vesicles. We subjected zucchini plasma membrane vesicles to zero or one cycle of freezing and thawing and assayed for NPA-binding activity using 5 nM [³H]NPA after 20 min of incubation. A 1.4-fold increase in binding activity for frozen and thawed plasma membrane vesicles relative to the unfrozen sample was determined (Table I).

The increase in NPA-binding caused by freezing and thawing could have been due to the orientation of the membrane vesicles being changed to increase the accessibility of this site, to the increase in the permeability of the membrane, or to the change in activity of the protein. If either of the first two hypotheses are correct, then frozen and thawed samples should lose NPA-binding activity after trypsin treatment, and the controls should be unaffected. Protease digestion of NPA-binding activity in the controls caused a loss of 0.14 pmol/mg NPA-binding activity, which corresponds to only 4.8% of the total activity, as shown in Table I. In vesicles subjected to one cycle of freezing and thawing, the amount of NPA-binding activity digested increased to 0.9 pmol/mg. This increase corresponds to 22% of the total activity and represents a 4.5-fold increase in the percentage of NPA-binding activity that is sensitive to protease digestion. The increase in protease sensitivity of NPA binding more closely approached the effectiveness of these treatments

on marker enzyme levels than on total NPA-binding activity. The smaller effect of freezing and thawing on NPA-binding activity may reflect the ability of NPA to bind to sites facing the inside of the vesicle, since this molecule is membrane-permeant. In contrast, the marker enzymes and protease digestion experiments more accurately measured events that occur only at the external face of vesicles. Therefore, it appears that inside-in zucchini plasma membrane vesicles were converted to inside-out vesicles when frozen and then thawed, thereby exposing the NPA-binding protein to trypsin and providing easier access to the protein by [³H]NPA. Our interpretation is that 22% of the NPA-binding sites were altered in orientation by this treatment and that most of these sites were then protease-sensitive.

Palmgren et al. (1990) have shown that a greater number of inside-out plasma membrane vesicles can be obtained by increasing the number of cycles of freezing and thawing. The amount of NPA-binding activity as a function of the number of cycles of freezing and thawing is shown in Figure 2. These data suggest that, as the number of freezing and thawing cycles increases, the specific activity of [³H]NPA binding (in picomoles per milligram of zucchini plasma membrane vesicles) also increases.

Marker Enzyme Analysis of Frozen and Thawed Plasma Membrane Vesicles

To demonstrate that freezing and thawing changes the accessibility of the NPA-binding proteins through reorientation of the membrane rather than by altering membrane permeability, the ability of frozen and thawed plasma membrane vesicles to pump H⁺ across the membrane was examined. Plasma membrane vesicles need to be tightly sealed and facing inside-out for the rate of ATP-dependent

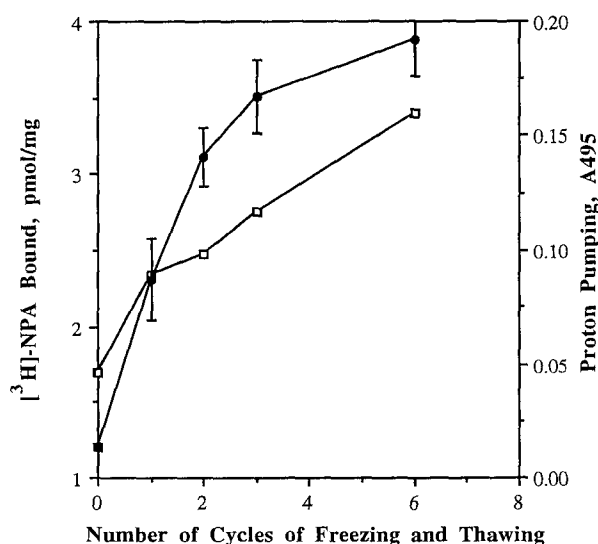


Figure 2. Effect of the number of cycles of freezing and thawing on H⁺ pumping and NPA-binding activity. Zucchini plasma membrane vesicles were frozen and thawed for the indicated number of times and then assayed for binding of 5 nM [³H]NPA for 20 min or for H⁺ pumping. □, NPA binding; ●, proton pumping.

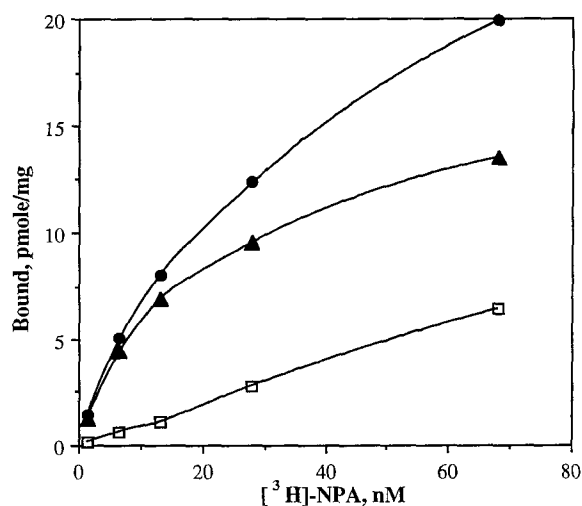


Figure 3. Saturation curve of [³H]NPA binding to zucchini plasma membranes using GFB filters (Whatman). Total, specific, and nonspecific binding were determined and plotted as a function of the [³H]NPA concentration after a 45-min incubation at 4°C. Background was determined by inclusion of 10 μM radioinert NPA in the assay. ●, Total binding; □, nonspecific binding; ▲, specific binding.

H⁺ pumping to be measured (Sze, 1985) by the method of Palmgren et al. (1990). Therefore, measurement of proton pumping was a control to verify that the effect of freezing and thawing of vesicles was to alter the orientation of the vesicle without increasing the permeability of the membrane to molecules such as [³H]NPA and trypsin. A 4-fold increase in H⁺ pumping was observed for plasma membrane vesicles frozen and thawed for one cycle (Table I). A plot of the decrease in absorbance of the pH probe acridine orange as a function of the number of freezing and thawing cycles is shown in Figure 2. As the number of cycles of freezing and thawing increased, so did the rate at which protons were pumped across the membrane. These results suggest that the number of inside-in plasma membrane vesicles converted to inside-out plasma membrane vesicles increases with each additional cycle of freezing and thawing. Based on these data, it appears that tightly sealed inside-out zucchini plasma membrane vesicles were formed when inside-in vesicles were frozen and thawed, thereby increasing [³H]NPA-binding activity by providing greater accessibility to the binding protein.

Measurement of [³H]NPA Binding to GFB Filters

A saturation curve for the binding of 1 to 68 nM [³H]NPA to zucchini plasma membrane vesicles is presented in Figure 3. The total, specific, and nonspecific binding are plotted as a function of the [³H]NPA concentration after a 45-min incubation using GFB filters (Whatman). The background for the saturation curve is equal to or less than 30% of the total binding at NPA concentrations up to 68 nM. At the 5 nM concentration of [³H]NPA used in most GFB filter assays reported in this paper, the background is equal to or less than 5% of the total binding.

Brij-58 and Trypsin Treatment of Plasma Membrane Vesicles

The detergent Brij-58 was shown to convert inside-in plasma membrane vesicles made from spinach leaves to inside-out vesicles, with a maximum effect at 0.05% (Johansson et al., 1995). Zucchini plasma membrane vesicles were incubated with 0.025, 0.05, 0.1, 0.5, and 1% Brij-58. Since the greatest increase in binding activity was found with the 0.05% concentration (data not shown), this concentration was used to treat the vesicles reported in this paper. This low concentration of Brij-58 did not result in any solubilization of NPA-binding activity or release into a supernatant sample after ultracentrifugation for 30 min at 100,000g following Brij treatment (data not shown).

Zucchini plasma membrane vesicles were treated with and without 0.05% Brij. The [³H]NPA-binding activity from a representative experiment is shown in Figure 4. When the incubation with [³H]NPA was for 45 min, there was a 1.4-fold increase in binding with 0.05% Brij-treated plasma membrane vesicles compared with vesicles incubated without Brij-58. A summary of multiple experiments is presented in Table II. The average increase in NPA-binding activity by 0.05% Brij-58 treatment in 37 experiments was 1.5-fold. The average amount of NPA-binding activity digested by trypsin increased from 0.15 pmol/mg in plasma membrane vesicles not treated with Brij-58 to 1.0 pmol/mg in treated vesicles in an average of 14 separate experiments. To normalize the amount of NPA-binding activity digested by trypsin by the total amount of activity, the percentage of activity susceptible to digestion is reported in Table II. When the average amount of NPA-binding activity digested by trypsin was compared from 14 separate experiments, Brij-58 treatment increased this protease-sensitive binding 5-fold, as shown in Table II. Since vesicles treated with 0.05% Brij-58 were still tightly sealed, only the binding activity that was exposed was digested. As described above

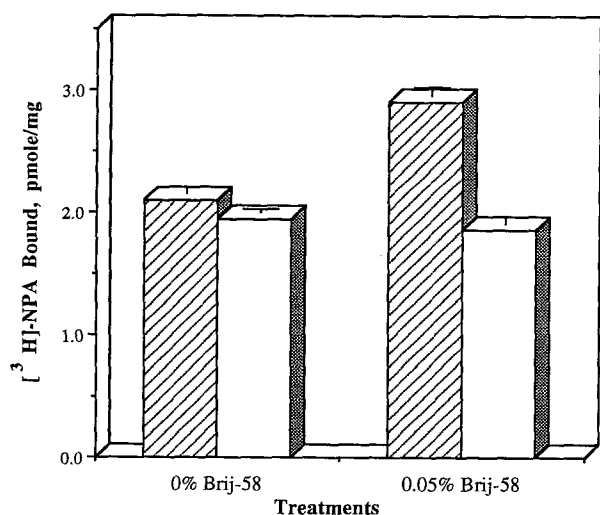


Figure 4. Trypsin sensitivity of [³H]NPA binding to zucchini plasma membrane vesicles incubated with and without 0.05% Brij-58. Samples were subjected to protease treatment before [³H]NPA-binding activity was measured. ▨, Control; □, 10 µg/mL trypsin.

Table II. Effect of Brij-58 treatment on NPA-binding activity and marker enzymes

Assay	Brij-58 Concentration		Increase
	0%	0.05%	
NPA-binding activity (pmol mg ⁻¹ protein) ^a	2.8 ± 0.8	3.9 ± 1.1	1.5-fold
NPA-binding susceptibility to trypsin (%) ^b	7.5 ± 5.5	37.0 ± 9.5	5.0-fold
ATPase activity (µmol ADP mg ⁻¹ protein min ⁻¹) ^c	7.5 ± 1.3	16.9 ± 4.4	2.3-fold
H ⁺ Pumping (change in A ₄₉₅ mg ⁻¹ protein min ⁻¹) ^d	1.3 ± 0.5	4.7 ± 1.9	3.6-fold

^a The data and increase are the average of 37 separate experiments. ^b The susceptibility of NPA-binding activity was calculated by subtracting binding activity after trypsin digestion from the total activity and dividing by the total activity. The data represent the averages of 14 separate experiments. ^c The data and increase are the averages of three separate experiments. ^d The data and increase are the averages of four separate experiments.

for trypsin treatments of frozen vesicles, the trypsin effect more closely approached the effect of the marker enzymes. Treatment of vesicles with 3% Brij-58, which is sufficient to solubilize proteins from the lipid bilayer, resulted in a more dramatic reduction in NPA-binding activity after trypsin treatment, with greater than 50% of the NPA-binding activity destroyed (data not shown). These data are consistent with the hypothesis that 0.05% Brij-58 enhances NPA binding by converting inside-in plasma membrane vesicles to inside-out vesicles, thereby exposing the NPA-binding protein to trypsin and enabling it to bind more easily to the protein.

Marker Enzyme Analysis of Brij-58-Treated Samples

To verify whether Brij-58 increased the binding of [³H]NPA by changing the orientation of plasma membrane vesicles, the ability of Brij-58-treated plasma membrane vesicles to pump protons across the membrane was investigated. For the rate of the ATP-dependent H⁺ pumping to be assessed, plasma membrane vesicles had to be tightly sealed and oriented inside-out (Palmgren et al., 1991). As shown in Table II, there was a 3.5-fold increase in pumping when vesicles were treated with Brij-58. These values represent the decrease in absorbance of the pH probe acridine orange. This value is similar to the 3-fold increase in H⁺ pumping for spinach plasma membrane vesicles treated with Brij-58 shown by Johansson et al. (1995).

The activity of cytoplasmic ATPase was also measured to verify the action of Brij-58. For ATPase activity to be measured by the procedure reported by Palmgren et al. (1991), the plasma membrane vesicles had to be oriented inside-out, exposing the ATPase protein. The assay measures the decrease of A₃₄₀ of the hydrolysis of NADH, which is enzymatically linked to the hydrolysis of ATP. As shown in Table II, there was a 2.3-fold increase in ATPase activity when plasma membrane vesicles were treated with Brij-58. A 2-fold increase in ATPase activity was reported by Johansson et al. (1995) for spinach plasma membrane vesicles treated with Brij-58. These

data from the two marker enzymes provide further evidence that tightly sealed inside-out plasma membrane vesicles are formed from inside-in vesicles upon treatment with Brij-58.

Determination of Equilibrium

Figure 5 shows the plot of zucchini plasma membrane vesicles treated with and without 0.05% Brij-58 and incubated with [3 H]NPA from 1 to 450 min. Samples with and without Brij-58 treatment ultimately reached the same equilibrium. Plasma membrane vesicles treated with 0.05% Brij-58 reached equilibrium after 2 h, whereas those not treated reached equilibrium after 3 h. A plot of the ratio of [3 H]NPA bound to Brij-58-treated plasma membrane vesicles to [3 H]NPA bound to plasma membrane vesicles not treated with Brij-58 is shown in the inset of Figure 5. The inset shows that the difference in binding activity of the two samples equalized over time. These data are consistent with an increase in [3 H]NPA binding in plasma membrane vesicles incubated with Brij-58 due to greater accessibility of the NPA-binding protein. This improved accessibility was caused by the reorientation of some of the plasma membrane vesicles from an inside-in to an inside-out orientation, in combination with binding to inside-in vesicles due to the diffusion of the lipophilic protonated NPA. To demonstrate that this efflux was not due to an altered state of the protein, the binding constants K_d and B_{max} , which describe the protein activity, were measured.

Saturation of [3 H]NPA Binding

Since [3 H]NPA appeared to have greater accessibility to the binding site in plasma membrane vesicles treated with

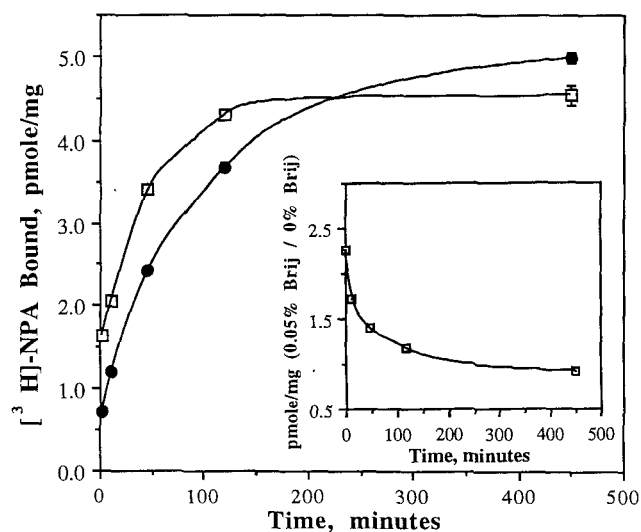


Figure 5. [3 H]NPA-binding activity measured as a function of incubation time in zucchini plasma membrane vesicles treated with and without 0.05% Brij-58. Samples were incubated with 5 nM [3 H]NPA. Inset represents the ratio of [3 H]NPA bound to plasma membrane vesicles treated with Brij-58 to [3 H]NPA bound to vesicles treated without Brij-58 at each time assayed. ●, No Brij-58; □, 0.05% Brij-58.

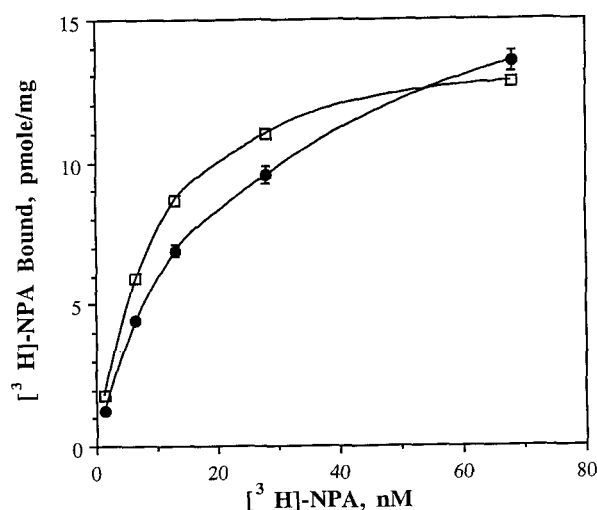


Figure 6. Saturation curve of [3 H]NPA binding to zucchini plasma membrane vesicles treated with and without 0.05% Brij-58. Plasma membrane vesicles were incubated with 1 to 68 nM [3 H]NPA for 45 min at 4°C and then assayed. ●, No Brij-58; □, 0.05% Brij-58.

Brij-58, the next step was to verify that the number of binding sites did not change. A saturation curve for the binding of 1 to 68 nM [3 H]NPA after a 45-min incubation to zucchini plasma membrane vesicles treated with and without Brij-58 is shown in Figure 6. Both samples saturate at similar levels of binding, with plasma membrane vesicles not treated with Brij-58 requiring higher concentrations of [3 H]NPA to saturate the binding site.

Determining K_d and B_{max}

A Scatchard plot is shown in Figure 7. The K_d and B_{max} values for plasma membrane vesicles treated with and without Brij-58 were calculated and are presented in Table

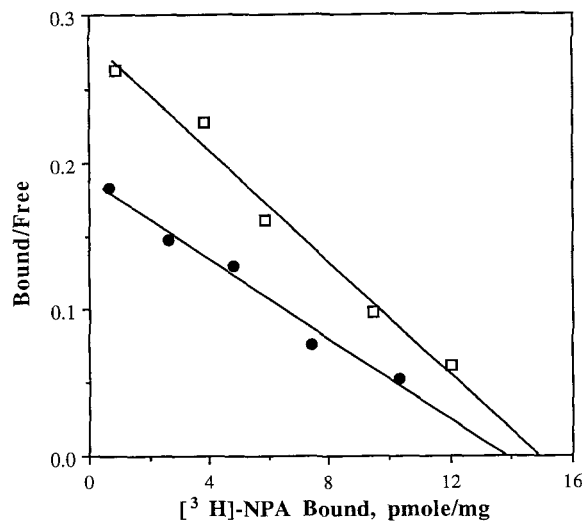


Figure 7. Scatchard plot of plasma membrane vesicles treated with and without 0.05% Brij-58. Plasma membrane vesicles were incubated with varying concentrations of [3 H]NPA for 45 min at 4°C. ●, No Brij-58; □, 0.05% Brij-58.

III. For the control and Brij-58-treated plasma membrane vesicles, K_d was determined to be 11 and 15 nM, respectively, and B_{max} was determined to be 13 and 15 pmol/mg, respectively. These values were calculated using the method presented by Burt (1986). K_d and B_{max} were also determined using a Lineweaver-Burk plot and are presented in Table III. These values coincide with the previously published K_d and B_{max} values for zucchini plasma membrane vesicles by Muday et al. (1993). Thus, treatment with Brij-58 does not appear to change the protein's affinity or the number of binding sites available.

The binding of [3 H]NPA to control and Brij-58-treated samples was analyzed by the computer program Ligand (Munson and Rodbard, 1980). This program can determine whether the data are best explained by a one- or two-site model. Competition assays on plasma membrane vesicles treated with and without Brij-58 were also analyzed using the Ligand program (data not shown). For both samples, the data were described by a single-site fit; a two-site fit was not possible. K_d and B_{max} were also determined using the Ligand program and are presented in Table III. These data further indicate that the binding affinity and number of binding sites do not change; only the accessibility of the binding site to the NPA molecule changes.

A plot of the kinetics of association of [3 H]NPA binding to plasma membrane vesicles treated with and without Brij-58 is shown in Figure 8. By plotting the first half of the association as a function of time, the K_{on} can be determined using the slope for the K_{on}' value in the following equation: $k_{on} = k'_{on}/[(^3\text{H]NPA})B_{max}]$ (Bennett and Yamamura, 1985). For plasma membrane vesicles treated with and without Brij-58, the k_{on} was found to equal 0.0022 and 0.0020 $\text{nm}^{-1} \text{min}^{-1}$. These values agree with the k_{on} values for zucchini plasma membrane vesicles reported by Muday et al. (1993).

A plot of the kinetics of dissociation of [3 H]NPA binding to plasma membrane vesicles treated with and without Brij-58 is shown in Figure 9A. The plot consists of the log of [3 H]NPA bound in picomoles per milligram as a function of time after the addition of 10 μM unlabeled NPA. [3 H]NPA binding is fully reversible, with a K_{off} value of 0.090 and 0.085 min^{-1} for Brij-58-treated and non-treated plasma membrane vesicles, respectively. k_{off} was calcu-

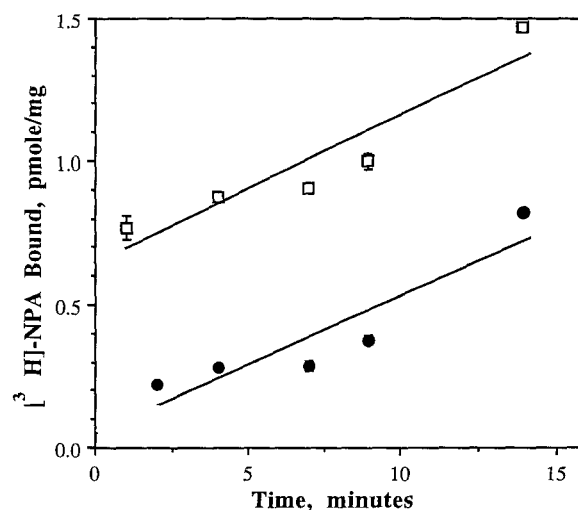


Figure 8. Kinetics of association of 5 nM [3 H]NPA in plasma membrane vesicles incubated with and without 0.05% Brij-58. ●, No Brij-58; □, 0.05% Brij-58.

lated using the constant -2.303 times the slope of the plot (Hrdina, 1986). These values agree with the k_{off} values for zucchini plasma membrane vesicles reported by Muday et al. (1993). Dividing the k_{off} by the K_{on} provides an alternate method for computing K_d values. K_d was found to be 45 and 43 nM for Brij-58- and non-Brij-58-treated plasma membrane vesicles, respectively, using this method (Table III). It is not clear why these values are 3-fold higher than those obtained by Scatchard or Lineweaver Burk plots, but similar values were obtained previously by Muday et al. (1993). These data suggest that Brij-58 does not increase the binding of [3 H]NPA by altering either the affinity or the number of binding sites, but rather by increasing the availability of these sites.

Dissociation of [3 H]NPA Binding

Although the K_{off} values were very similar between control and Brij-58-treated samples (Fig. 9A), there was an apparent difference in dissociation in samples immediately after the addition of unlabeled NPA. To magnify this difference, [3 H]NPA binding was allowed to reach equilibrium, and the dissociation of binding was measured after the addition of a much lower concentration of unlabeled NPA. In this analysis, 20 nM radioinert NPA was used (10 μM was used in the previous analysis). The results from this analysis are in Figure 9B. The differences in rates of dissociation of samples analyzed for less than 5 min are clear, although the rates of dissociation for both samples from points later than 5 min are similar. These results indicate that Brij-58 treatment produces a subset of NPA-binding sites that more rapidly bind and release NPA, although the affinity or the total abundance of NPA-binding sites does not change. Furthermore, the remaining sites that are presumably located within inside-in plasma membrane vesicles bind and release NPA at very similar rates with or without Brij-58 treatment.

Table III. [3 H]NPA-binding constants determined by several graphical methods

Graphical Method	Binding Constant	Treatment of Zucchini Plasma Membranes	
		Control (no Brij-58)	0.05% Brij-58
Scatchard ^a	K_d	15 nM	11 nM
	B_{max}	14 pmol/mg	15 pmol/mg
Lineweaver-Burk ^b	K_d	18 nM	16 nM
	B_{max}	14 pmol/mg	17 pmol/mg
Ligand program	K_d	9.3 nM	7.7 nM
	B_{max}	13.1 pmol/mg	13.3 pmol/mg
Kinetic analyses ^c	K_d	43 nM	45 nM

^a K_d is defined as $-1/\text{slope}$ and B_{max} is equal to the x intercept (Burt, 1986). ^b K_d is equal to $-1/x$ intercept and B_{max} is equal to $1/y$ intercept. ^c K_d was calculated by dividing k_{off} by the k_{on} .

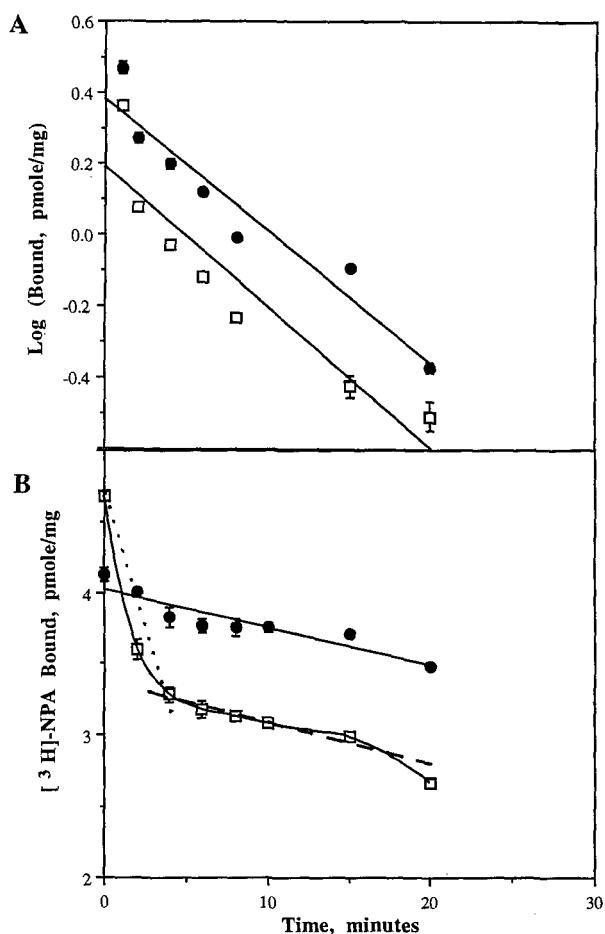


Figure 9. Kinetics of dissociation of plasma membrane vesicles incubated with and without 0.05% Brij-58. A, Plasma membrane vesicles were incubated with 5 nM [3 H]NPA for 2 h and then 10 μ M NPA was added. The log of the bound [3 H]NPA after the addition of the unlabeled NPA is plotted as a function of time after the addition; B, samples were incubated with 5 nM [3 H]NPA for 2 h and then 20 nM NPA was added. ●, No Brij-58; □, 0.05% Brij-58.

DISCUSSION

The experiments presented in this paper were designed to determine the orientation of the NPA-binding protein in relation to the cytoplasm in zucchini plasma membrane vesicles. Standard procedures were used to obtain plasma membrane vesicles of predominantly (greater than 80%) inside-in orientation and then to partially reverse the orientation. The complexity of this analysis lies in the fact that to follow the NPA-binding protein, it is necessary to measure the binding of a molecule that is lipophilic in the protonated form. As a result, there is binding of this molecule to sites on the inside of vesicles, but the rate of binding is increased when the orientation of vesicles is reversed and the site is facing out from the vesicles. It was necessary to analyze the binding carefully to separate binding at the two sides of the membrane.

The first approach to alter the vesicle orientation was osmotic shock, which involves the resuspension of membranes in buffer with reduced Suc to osmotically rupture vesicles.

Two other approaches were used to convert inside-in plasma membrane vesicles to an inside-out orientation: freezing and thawing inside-in plasma membrane vesicles (Palmgren et al., 1990) and treating them with low concentrations of Brij-58 (Johansson et al., 1995). Treatment of plant plasma membrane vesicles with low concentrations of Brij-58, a polyoxyethylene acyl ether, has been shown to convert vesicle orientation from inside-in to inside-out. This treatment results in tightly sealed vesicles, as demonstrated by electron microscopy and by the measurement of transport protein activity requiring tightly sealed vesicles. In a series of polyoxyethylene acyl ethers tested, this ability to alter membrane orientation rather than solubilize proteins is specific for molecules with a large head group (Johansson et al., 1995). The effectiveness of this treatment in these experiments was assessed by measuring the change in NPA-binding activity, the sensitivity of NPA binding of each sample to protease digestion, and the level of marker enzymes that also have specificity in orientation.

When inside-in plasma membrane vesicles are resuspended in buffer with reduced Suc concentration, the vesicles rupture, providing easier access to inside-facing binding sites. An increase in [3 H]NPA-binding activity is observed for osmotically shocked inside-in plasma membrane vesicles. As the Suc concentration in the resuspension buffer decreases, the number of ruptured plasma membrane vesicles increases, resulting in more NPA-binding activity. The ruptured inside-in plasma membrane vesicles are unable to maintain the necessary pH gradient, resulting in lower H^+ pumping. The level of H^+ pumping continues to decrease as the inside-in plasma membrane vesicles are subjected to higher levels of osmotic shock. It appears that the increase in NPA-binding activity is due to an increase in the accessibility of the binding sites.

These initial studies revealed a corresponding increase in binding activity as the accessibility of internal binding sites for NPA were increased. These data are consistent with an internal NPA-binding site on inside-in plasma membrane vesicles. More direct means of studying internal binding sites involve the conversion of plasma membrane vesicles from inside-in to inside-out with either freezing and thawing procedures or 0.05% Brij-58. Conversion from inside-in to inside-out plasma membrane vesicles by both procedures yielded similar increases in NPA-binding activity and susceptibility of NPA-binding activity to protease digestion. The effectiveness of the treatments on NPA-binding activity are consistent ($n = 37$ separate Brij-58 experiments), but the increase in activity is smaller than optimal, since the membrane-permeant NPA ligand also can cross the membrane to bind internally facing sites. The effectiveness of these treatments on increasing trypsin-sensitive NPA-binding activity is more dramatic, with 4 to 5-fold increases. This greater effect is due to the lack of permeability of the membrane to trypsin.

To verify that these treatments are not simply acting to increase membrane permeability to NPA and protease, H^+ pumping was measured as a marker enzyme. H^+ pumping is a particularly useful marker assay, since it measures membrane orientation and requires that the vesicles have sealed membranes (Sze, 1985). An increase in another

membrane orientation marker, ATPase activity, was also seen for Brij-58-treated plasma membrane vesicles. As the orientation of plasma membrane vesicles is changed from inside-in to inside-out, it appears that the position of the NPA-binding protein also shifts to one that is more accessible to the NPA molecule and to the protease trypsin. The decrease in NPA-binding activity upon digestion with trypsin in samples treated with Brij-58 or treated by freezing and thawing reduces binding activity but does not destroy it completely, since the membrane vesicles are intact and only a subset of NPA-binding sites become exposed. One surprising observation from this analysis is that, although trypsin digestion dramatically reduces binding in Brij-58-treated or frozen/thawed vesicles, the levels of binding after digestion with both trypsin and proteinase K (data not shown) are reduced only to the control values. The expectation would be that digestion would reduce binding in these samples to values lower than the controls. Our best explanation for this difference is that there is some accumulation of NPA within the vesicles, which obscures the true number of binding sites on the inside of the membrane. It is possible to get greater digestion of binding activity with trypsin. If high concentrations of Brij-58 (3%) are used to release proteins from the lipid bilayer, a majority of the NPA-binding activity is lost after protease digestion (data not shown).

If the only effect of these treatments is to alter membrane orientation, then both the number and affinity of NPA-binding sites should not change, since NPA is a membrane-permeant molecule. The only change in binding should be in the accessibility of NPA binding to sites that are now available for binding without NPA crossing the membrane. The constant number and affinity of NPA-binding sites was demonstrated using several approaches. First, when binding of NPA is measured as a function of the duration of incubation, both control and Brij-58-treated samples and unfrozen and frozen samples reach the same maximum binding. Likewise, when NPA binding is measured with multiple [^3H]NPA concentrations for control and Brij-58-treated samples, the same maximum binding is reached. When these data are analyzed by Scatchard or Lineweaver-Burk plots, the K_d and B_{max} can be calculated. As shown in Table III, the K_d and B_{max} values obtained with these two graphical approaches are similar in both control and Brij-58-treated samples. Calculation of K_d using the kinetics of association and dissociation also yields similar values. Finally, analysis of [^3H]NPA binding using the computer program Ligand also indicates that the K_d and B_{max} are unchanged. Together, these results indicate that the number of binding sites and affinity of NPA are unchanged.

Although the affinity and number of NPA-binding sites remain unchanged by Brij-58 treatment, NPA-binding activity increases, particularly at points early in the experiment or at low [^3H]NPA concentrations. A change in the rate of association and dissociation may account for this increase in binding activity. The K_{on} , as described above, is calculated after the first 20 min of incubation. Except for the 1st min of association, almost equal K_{on} rates are recorded for both control and Brij-58-treated plasma mem-

brane vesicles. However, there is a large increase in the association of [^3H]NPA for Brij-58-treated plasma membrane vesicles during the 1st min of incubation. This increase in binding activity levels off after another 1 min and the slopes for the association of [^3H]NPA for both the control and Brij-58-treated plasma membrane vesicles are essentially parallel after this time. A similar effect can be seen in the dissociation of [^3H]NPA for the NPA-binding site. There appears to be a slightly faster off rate in samples treated with Brij-58. Upon incubation with radioinert NPA, more [^3H]NPA is displaced in Brij-58-treated plasma membrane vesicles than in the control sample. Once again, this faster dissociation occurs during the 1st min of incubation. After the initial increase in displacement, the remaining points parallel the control sample. Based on the trypsin data, it appears that only about one-third of the inside-in plasma membrane vesicles are converted to an inside-out orientation. This may account for why the overall slopes parallel one another for the control and Brij-58-treated vesicles, whereas the initial association and dissociation of [^3H]NPA appears to be greater for samples containing more inside-out plasma membrane vesicles. Although the affinity remains unchanged, it is apparent that the samples containing more inside-out plasma membrane vesicles bind and release NPA faster during the 1st min of incubation. Thus, the accessibility of the binding protein appears to increase after Brij-58 treatment, allowing NPA to associate and dissociate with the protein more easily. This difference can be more clearly seen in Figure 9B, in which the dissociation of [^3H]NPA by a lower concentration of radioinert NPA is analyzed.

The leading argument against an inward-facing binding site for NPA relies on the fact that the optimal pH for binding of NPA at a pH of 5.3 is close to the extracellular pH. It may be that the binding of a ligand to this protein does not occur at the optimum pH in vivo. Also, this pH optimum could coincide with the pH of the cell at the location of binding, or, it may result from optimal protein or ligand conformation or be due to increased uptake of the ligand at this pH. Since other auxin transport inhibitors such as the flavonoid quercetin do not exhibit similar pH profiles for optimal binding (Michalke, 1982; Rubery, 1990), the former possibility is probably not correct. We tested the latter possibility, that the low pH is optimal due to an increased uptake of the protonated NPA, by determining the pH optima of samples treated with and without Brij-58. Both preparations had similar pH optima for NPA binding (data not shown), suggesting that the pH effect is not due simply to the ligand's permeability. Consequently, the best hypothesis for the pH optimum is that the NPA ligand and/or the protein reach the optimal conformation for binding at a lower pH.

The cytoplasmic orientation of the NPA-binding protein may complicate studies. One important question is whether this protein is integral or peripheral to the membrane. Although chaotropic agents or high salt concentrations may release cytoplasmically localized peripheral membrane proteins in purified inside-in plasma membrane vesicles, the protein will remain trapped in the vesicle. In a

previous experiment, Cox and Muday (1994) found that NPA-binding activity could be released by such treatments only when membranes were also osmotically shocked by resuspension of samples in Suc-free medium. As Figure 1 demonstrates, osmotic shock does permeabilize or alter orientation of plasma membrane vesicles so that NPA-binding sites are more accessible. The results in the current study do not directly address whether the NPA-binding protein is integral or peripheral to the membrane, but the ability of proteases to digest this protein in intact inside-out vesicles clearly shows that the NPA-binding site is not internal to the membrane.

An important question is whether the cytoplasmic orientation of the NPA-binding site will allow efficient regulation of auxin transport in vivo. Naturally occurring auxin transport inhibitors are presumed to bind to the same site as synthetic inhibitors, such as NPA, and to modulate auxin transport in plants. The best candidates for natural ligands are flavonoids (Jacobs and Rubery, 1988; Rubery and Jacobs, 1990). These compounds can compete for NPA binding in vitro and exhibit other characteristics of auxin transport inhibitors (Jacobs and Rubery, 1988; Rubery and Jacobs, 1990). In their initial characterizations of these ligands, Rubery and Jacobs (1990) interpreted their physiological analysis to suggest that auxin transport inhibitors were acting at cytoplasmic sites. Also, addition of a sulfate group to quercetin, the most active flavonoid, reduced its ability to block IAA efflux, presumably by decreasing its membrane permeability (Faulkner and Rubery, 1992). The location of flavonoid synthesis is well characterized and has been determined to be cytoplasmic (Hrazdina and Jensen, 1992). This suggests that flavonoids can have ready access to the NPA-binding site of the auxin efflux carrier to modulate the activity of this protein to alter auxin distribution and plant growth and development.

The intracellular location of the auxin transport inhibitor-binding site would allow interaction with and regulation by cytoplasmic structures such as the cytoskeleton. Recent evidence has suggested an interaction between the NPA-binding protein and the actin cytoskeleton in zucchini (Cox and Muday, 1994; Butler, 1995). A cytoplasmic orientation of the NPA-binding protein allows association with the cytoskeleton, which may serve as a means to regulate the activity or localization of the NPA-binding protein.

In conclusion, the experimental results described in this paper are consistent with an NPA-binding site that faces the cytoplasm. The specificity of several treatments that convert predominantly inside-in plasma membrane vesicles to inside-out vesicles without altering membrane integrity were demonstrated with marker enzyme analysis. These treatments increase NPA-binding activity and more dramatically increase the amount of NPA-binding activity that is sensitive to protease digestion. The increase in NPA-binding activity is due to an increased rate of association and dissociation of the NPA ligand, but does not reflect a change in the total number of NPA-binding sites or the affinity of the protein for this ligand. These results are consistent with an NPA-binding protein oriented toward the cytoplasm.

NOTE ADDED IN PROOF

A recent study has used similar approaches to examine the nature of the association of the NPA-binding protein with the zucchini plasma membrane (Bernasconi et al., 1996); specifically to determine whether the NPA-binding protein was integral or peripheral to the plasma membrane. Our results are similar to theirs in that NPA-binding activity was protected from digestion by proteases in plasma membrane vesicles and was sensitive to digestion in solubilized vesicles. Our results differ in the effectiveness of proteases in digesting NPA-binding activity in vesicles subjected to cycles of freezing and thawing. They report that protease sensitivity did not increase in response to freezing and thawing vesicles or sonication, but they did not demonstrate the effectiveness of these treatments on membrane orientation through assay of marker enzymes. Although their results support their conclusion that the NPA-binding protein is integral to the membrane, our data suggest that there is an alternative interpretation. One possible explanation is that localization of the NPA-binding protein to the cytoplasmic face of zucchini plasma membrane vesicles can also protect binding sites from protease digestion.

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