The N-1-Naphthylphthalamic Acid-Binding Protein Is an Integral Membrane Protein

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N-1-Naphthylphthalamic acid (NPA)-binding protein is a plasma-lemma (PM) protein involved in the control of cellular auxin efflux. We re-evaluated the spatial relationship of this protein with the PM of zucchini (Cucurbita pepo L.) hypocotyls. First, Triton X-114 partitioning indicated that the NPA-binding protein was more hydrophobic than most PM proteins. Second, the NPA-binding activity was found to be resistant to proteolytic digestion in membranes. Maximum concentrations of binding sites for NPA were virtually identical in untreated and protease K-treated PMs: 19.2 and 20.6 pmol [3H]NPA bound/mg protein, respectively. The insensitivity of the NPA-binding protein was not due to its presence inside tightly sealed vesicles or due to lack of protease activity in the conditions tested. This protein could be made sensitive to proteolytic degradation upon solubilization by 3-(3-cholamidopropyl)dimethylammonio-1-propanesulfonate in the presence of sodium molybdate. Proteinase K treatment decreased the concentration of binding sites to 0.84 pmol [3H]NPA bound/mg protein from 9.2 for untreated, solubilized PM. Third, this activity could not be solubilized by chaotropic agents or sodium carbonate treatment of intact PM. This study indicates that the NPA-binding protein may be an integral membrane protein and contradicts previously reported findings that suggested that this protein was peripheral to the PM.

NPA is a synthetic phytotropin that severely stunts the growth of plants (Hoffman and Smith, 1949). Early physiological characterization of the NPA mode of action revealed that it reduced auxin transport in stems and roots (Morgan, 1964) and that the stunted growth and the inhibition of the gravitropic response were a consequence of NPA-induced auxin accumulation in the cells (Morgan, 1964). Although the physiology of plants treated with NPA has been intensely studied and the compound itself has been made into a commercial herbicide (Naptalam, Uniroyal, Middlebury, CT), surprisingly little is known about the biochemical aspects of its mode of action (for a review, see Lomax et al., 1995).

It is now well established that NPA specifically inhibits the efflux of auxin from the cytoplasm of the cell into the periplasm (Lomax et al., 1995). The availability of radiolabeled NPA allowed the characterization of a single, saturable NPA-binding activity located on the PM (Lembi et al., 1971; Muday et al., 1993).

A further understanding of the identity of the NPA-binding activity and the mechanism by which it regulates auxin efflux was attempted by several groups. One approach has been to search for the natural counterpart of NPA, and several candidates such as quercentin (Jacobs and Rubery, 1988), hydrangeic acid, or lunularic acid (Katekar et al., 1993) have been proposed. Another approach has been to investigate the NPA-binding transduction pathway. These studies have uncovered a potential role of Tyr kinase for the NPA-binding protein (Bernasconi, 1996), which possibly phosphorylates a small soluble protein (Morris et al., 1991). This phosphorylated protein would in turn regulate the activity of the auxin carrier.

Independently, it has been proposed that the NPA-binding protein is peripheral to the PM and interacts with actin filaments (Cox and Muday, 1994). The significance of such an association with respect to auxin efflux regulation is not yet clear. A peripheral location of the NPA-binding protein is surprising in several ways. First, the NPA-binding activity has been difficult to solubilize (Brunn et al., 1994), a property that is usually not associated with peripheral proteins. Second, and more important, several analogs of NPA have been prepared and a correlation has been found between the hydrophobicity of a compound and its ability to displace bound NPA (Katekar and Geissler, 1977; Brunn et al., 1994). This finding could not be explained if the binding protein were peripheral to the membrane, since the compound would partition in the membrane, becoming inaccessible to the binding protein. It is therefore important, at this point, to further refine the subcellular localization of the NPA-binding activity. With that intent, we undertook a study of the sensitivity of the NPA-binding protein to proteolysis in both intact PM and in solubilized fractions obtained using a novel solubilization protocol.

MATERIALS AND METHODS

Chemicals

NPA was from Chem Services (West Chester, PA). [2,3,4,5-3H]NPA (58 Ci/mmol) was from American Radiolabeled Chemicals (St. Louis, MO). Proteinase K was from Life Technologies. Sodium molybdate was from Mallinckrodt (Paris, KY). Dextran T500 was from Pharmacia-LKB. Ready Protein scintillation cocktail was from Beckman Instruments. The Micro BCA protein assay kit was from Pierce. Triton X-114 was obtained from Boehringer Mannheim and precondensed as described by Bordier (1981) before use. All other chemicals were from Sigma.

Abbreviations: Rmax, maximum concentration of binding sites; Chaps, 3-(3-cholamidopropyl)dimethylammonio-1-propanesulfonate; NPA, N-1-naphthylphthalamic acid; PM, plasmalemma.

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Plant Material and PM Preparation

Zucchini (Cucurbita pepo L.) seedlings were grown for 5 d at 28°C in the dark. PM was prepared from the upper 5 cm of the hypocotyls using the two-phase partitioning method described by Widell and Larsson (1981). The PM fraction obtained from 70 g of tissue was resuspended in 1 mL of PM buffer (250 mM Suc, 20 mM sodium citrate, 1 mM magnesium chloride, pH 5.3) to yield approximately 5 mg protein/mL (Pierce Micro BCA protein assay). Membranes were aliquoted, frozen in liquid nitrogen, and stored at −80°C.

Protein Solubilization

Published solubilization methods were adapted for the material used (Jones et al., 1987; Haga et al., 1990). PM fractions were adjusted to 2.25 µg protein/µL and incubated for 2 h at 4°C in PM buffer containing 0.6% Chaps, 10 mM sodium molybdate, 1 mM DTT, and 10% glycerol. Unsolubilized material used (Jones et al., 1987; Haga et al., 1990). PM fractions 4°C in PM buffer containing 0.6% Chaps, 10 mM sodium molybdate, 1 mM DTT, and 10% glycerol. Unsolubilized material was pelleted by a 45-min centrifugation at 100,000g. The supernatant, containing the solubilized fraction, was frozen in liquid nitrogen and stored at −80°C.

NPA-Binding Assays

NPA-binding assays were conducted using 10 to 30 µg PM protein/sample. Samples were incubated for 2 h at 4°C with 10 nM [3H]NPA in a volume of 200 µL of PM buffer. Nonspecific binding was determined in the presence of 10 µM unlabeled NPA. Bound [3H]NPA was separated from free [3H]NPA by filtering over GF/B filters (Whatman). Nonspecific binding was determined in the presence of 100 nM unlabeled NPA. Bound [3H]NPA was separated from free [3H]NPA by filtering over GF/B filters (Whatman) presoaked in 0.1% polyethyleneimine (Brusn et al., 1983). The filters were washed with 5 mL of cold PM buffer and counted in 4 mL of Ready Protein cocktail. Saturation assays were conducted with concentrations of [3H]NPA ranging from 0 to 50 nM. Bmax and Kd values were determined from the saturation curves by the program LIGAND (Munson and Rodbard, 1980). NPA binding in the solubilized material was conducted in a similar fashion but in the presence of 0.2% Chaps and 1 mM sodium molybdate in the PM buffer. In the cases in which a saturation curve was not determined, the experiments were performed at least three times and the average value of specific binding from a typical experiment was reported.

Triton X-114 Phase Partitioning

PM fractions (0.5-1 mg) were submitted to Triton X-114 partitioning (Pryde, 1986). The published method (Pryde and Phillips, 1986) was followed exactly with one exception: the aqueous phase obtained after the Suc cushion separation was used directly without dialysis. As a consequence, a small amount of contamination by the detergent phase may be present. After determination of the protein content by the Micro BCA protein assay, NPA-binding assays were performed as described above using 10 µg protein/assay.

Protease Treatments

Unless noted otherwise, proteinase K treatments were performed in PM buffer for 16 h at 4°C. The ratio of proteinase K to PM protein was 1:2, with the final proteinase K concentration being 1 µg/µL. Alternatively, pronase E and trypsin were also used under similar conditions. After protease treatment, the fractions were diluted in PM buffer and assayed for NPA binding as above and analyzed by SDS-PAGE.

SDS-PAGE and Western Blot Analysis

SDS-PAGE and western blot analysis were performed according to published methods (Ausubel et al., 1995). Monoclonal anti-actin antibodies (Cox and Muday, 1994) were used at a 1:400 dilution according to the manufacturer’s instructions (ICN Pharmacologics). Detection was performed using the ECL detection kit (Amersham Life Sciences) and Fuji medical x-ray film RX (Fisher Scientific).

RESULTS AND DISCUSSION

NPA-Binding Is Associated with the Purified PM Fraction

The two-phase partitioning protocol was used for the preparation of the PM fraction. An average of 6% of the total microsomal protein partitioned as PM, with the remaining protein partitioning as a mixture of subcellular membranes. Cyt c oxidase activity, a mitochondria marker (Hodges and Mills, 1988), was used as a marker. The almost complete depletion of this activity in the PM fraction (more than 90%) was an indication of the small extent of contamination by other membranes. These results were comparable to those obtained with other plant tissues (Hodges and Mills, 1988). It is well established that the NPA-binding activity co-purifies with PM markers. Indeed, after the initial study by Lembi et al. (1971), NPA-binding activity has often been used as a marker of PM fractions (Chanson et al., 1984; Hodges and Mills, 1988). In our material, the specific activity of the NPA-binding protein was enriched 3-fold in the PM fraction when compared to the total membrane fraction (Table I). On average, the Bmax value for the NPA-binding protein increased from 6 to 8 pmol/mg protein in the total microsomal fraction to 19 to 24 pmol/mg protein in the PM fraction. A representative saturation curve for the NPA-binding activity in PM is shown in Figure 1A. These results are well in accordance with several previous reports (Muday et al., 1993; Lomax et al., 1995).

Table I. Bmax and Kd values of the NPA-binding activity

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Treatment</th>
<th>Bmax (pmol/mg protein)</th>
<th>Kd (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total membranes</td>
<td>None</td>
<td>6.7</td>
<td>6.8</td>
</tr>
<tr>
<td>PM</td>
<td>Untreated</td>
<td>19.2</td>
<td>7.09</td>
</tr>
<tr>
<td></td>
<td>Proteinase K</td>
<td>20.6</td>
<td>8.23</td>
</tr>
<tr>
<td>Solubilized PM</td>
<td>Untreated</td>
<td>9.20</td>
<td>6.93</td>
</tr>
<tr>
<td></td>
<td>Proteinase K</td>
<td>0.84</td>
<td>6.32</td>
</tr>
</tbody>
</table>
N-1-Naphthylphthalamic Acid-Binding Protein Is Integral to the Plasmalemma

A

Figure 1. NPA-binding activity in membrane-bound (A) and solubilized (B) PM fractions. The saturation curves were obtained as in Table I. The binding experiment was conducted with PM fractions incubated overnight at 4°C in the absence (○) or presence (□) of 1 μg/μL proteinase K.

B

NPA-Binding Activity Partitions in the Highly Hydrophobic Phase during Triton X-114 Partitioning

To give a first indication of the hydrophobicity of the NPA-binding protein, PM fractions were subjected to Triton X-114 phase partitioning. Although this partitioning is not a solubilization protocol per se (Pryde, 1986), it allows an approximate determination of the hydrophobicity of a protein and has been successfully used in animal (Pryde and Phillips, 1986) and plant systems. A recent example is the resolution of the membrane localization of the fusicocin receptor in Commelina communis tissues (Oecking et al., 1994). In our system, this protocol yielded three separated phases of increasing hydrophobicity: an aqueous phase, a detergent-rich phase, and a lipid-rich phase. The result of a representative experiment in which 500 pg of PM protein were used is given in Figure 2. About 50% of the initial protein was recovered in the three phases, with the majority of the protein found in the detergent-rich phase. The several pellet washes and Suc gradient interphases were responsible for the protein loss. The NPA-binding activity partitioned preferentially in the lipid-rich phase, indicating that the NPA-binding protein was more hydrophobic than the majority of the PM protein (Pryde, 1986). Although some activity was found in the detergent-rich phase, none was detected in the aqueous phase. The partitioning protocol somewhat denatured the NPA-binding protein; therefore, the specific NPA-binding activity, even in the fraction enriched in this activity, was only about 30% of the binding in the starting material.

NPA-Binding Activity Is Insensitive to Proteolysis

Since the NPA-binding protein partitioned with the highly hydrophobic phase, it could be postulated that this protein is intrinsic to the PM and inaccessible to proteases. Incubation of PM fractions with proteases would therefore not affect NPA binding. Our investigations indicated that the NPA-binding activity is essentially insensitive to an overnight treatment in the presence of a large amount of proteinase K (1 μg proteinase K/2 μg PM protein). The results reported in Figure 1A and Table I showed no change in B_max or in K_d between proteinase K-treated and untreated fractions. As a consequence, this treatment did not decrease the number of binding sites, and it did not alter the affinity for NPA of the binding sites. These findings contradicted the results of Sussman and Gardner (1980), showing that this activity was sensitive to trypsin digestion. This contradiction may be due to three differences between our study and the published report. First, we benefitted from the development by Widell and Larson (1981) of the two-phase PM preparation system, allowing us to work with highly purified PM fractions as opposed to crude microsomal preparations. Second, we could replace the column chromatography method for NPA-binding assays used by these authors with the filtration on GF/B filters described by Bruns et al. (1983). This method allowed measurements of NPA binding, with nonspecific binding contributing 5% or less of the total binding. It also avoided the limitation due to the rapid off-rate of NPA from the binding protein (77 and 90% of the radioactivity released from the protein after 5 and 10 min, respectively; Patel et al., 1995). Third, it appears that the binding activity...
observed by Sussman and Gardner (1980) was very unstable, since, even in the absence of a protease, a 20-min incubation at room temperature of their sample reduced the NPA binding from 68,467 dpm/4 g fresh weight equivalent (Sussman and Gardner, 1980, table II) to 584 dpm/1.1 g fresh weight equivalent (Sussman and Gardner, 1980, table IV). Hence, their analysis of trypsin degradation was conducted on a membrane fraction having lost more than 97% of its NPA-binding activity. Our results also contradicted the findings of Cox and Muday (1994) that indicated that the NPA binding was peripheral to the PM and located on the cytoplasmic side of the membrane. Hence, we investigated alternative explanations that would reconcile our findings with their hypothesis.

**NPA-Binding Protein Was Not Protected because of Tightly Sealed Vesicles**

PM preparation by the two-phase partitioning method has been shown to yield sealed vesicles with the periplasmic side of the membrane on the inside of the vesicle. One possibility was that proteinase K was not reaching the NPA-binding protein because it could not cross the tightly sealed membrane vesicle. This was ruled out by two experiments. First, PM vesicles were subjected to three rounds of freezing and thawing. Second, PM vesicles were subjected to three 10-s bursts of sonication. These two methods decreased the integrity of the membrane vesicles (Hertel et al., 1983). After these treatments, the fractions were incubated with proteinase K and assayed for NPA binding. No increase in sensitivity toward the protease was observed after sonication or freeze/thawing (data not shown, but similar to the $B_{\max}$ and $K_d$ values reported for PM in Table I).

**In the Conditions Tested, Proteinase K Was Active**

Another possible explanation for the insensitivity of the NPA-binding protein to digestion by proteinase K was simply the lack of activity of the protease in the conditions tested. Proteinase K is stable between pH 4 and 12.5 and at temperatures up to 65°C. Our experimental design used this protease at pH 5.3 and 4°C. The choice of these conditions was dictated by the need to preserve the NPA-binding activity. We demonstrated that proteinase K was sufficiently active in these conditions in three ways. Figure 3 shows an SDS-PAGE analysis of the fractions subjected to an overnight incubation in the absence or presence of proteinase K. The majority of the proteins were degraded by the protease, resulting in an accumulation of silver-stain-reactive material of small molecular mass. The second demonstration of proteinase K effectiveness utilized actin, a cytoplasmic protein associated with the membrane, as a marker for peripheral proteins. By immunoblotting, actin was detected in total microsomal preparations, and a small fraction, estimated at 5% of the total immunoreactivity, was found in the PM preparation (Fig. 4). This result was in accordance with the findings reported by Sonesson and Widell (1993). Upon proteinase K treatment, no actin was detected in the sample, indicating that the protease degradatively degraded this protein. The third demonstration of proteinase K effectiveness was obtained by conducting the protease digestion at 37°C for 1 h instead of the overnight incubation in the cold. Although this treatment resulted in about 20% inactivation of the binding activity in the absence of the protease, no further decrease in activity was detected in the presence of proteinase K (data not shown).

**Pronase E and Trypsin Could Substitute for Proteinase K**

To further demonstrate that the NPA-binding protein was insensitive to proteolytic digestion, the same experiments were repeated with pronase E and trypsin instead of proteinase K. The results are reported in Figure 3. Likewise, incubation for 1 h at 37°C did not decrease NPA-binding activity in PM (data not shown). Our trypsin treatment is shown. Pronase E was directly compared to the one
both membrane-bound and solubilized fractions (Table I). The variation of the $B_{\text{max}}$ was probably due to the differential solubilization of certain proteins versus others, affecting the number of sites per unit of protein. The role played by molybdate in this protocol was not fully understood, but the same effect on solubilization yields was observed only with tungstate, albeit to a lesser extent. It can be argued that this ion stabilizes the activity by inhibiting possible phosphatases or other protein-modifying activities (Manchester et al., 1987). This protocol differed from the solubilization methods reported by three groups (Sussman and Gardner, 1980; Jacobs and Gilbert, 1983; Cox and Muday, 1994). The common feature of these published reports is the use of Triton X-100 in concentrations ranging from 0.1 to 2.5%. Sussman and Gardner (1980) described a 32% (their table I) or 56% (their table II) solubilization of the activity from the total microsomal fraction in the presence of 1% Triton X-100. No solubilization yield was given in the other two publications, but it appeared to be much lower than the one obtained by Sussman and Gardner (1980).

In our experimental system, no solubilized NPA-binding activity was recovered irrespective of the amount of Triton X-100 used. The apparent discrepancy may come from the differences in the analytical method used for assessing solubilization. In our case, solubilized material was defined as one that could not be pelleted during a 45-min centrifugation at 100,000g (Hjelmeland and Chrambach, 1984). A different criterion was used in the Sussman and Gardner and Cox and Muday articles, in which solubilized material was defined as the supernatant of a 30-min centrifugation at 40,000g. The 100,000g criterion was applied by Jacobs and Gilbert (1983) in their analysis of solubilized NPA-binding protein from pea. In this latter case, the solubilized activity was low and could not be compared directly with our findings (466 dpm/assay in table I or 752 dpm/assay in table II; Jacobs and Gilbert, 1983). The same low activity was recovered by Thein and Michalke (1988) in their solubilization protocol involving 1% Chaps. This result was consistent with our preliminary studies using 0.4% Chaps without sodium molybdate, yielding about 2.3% solubilized activity. Another solubilization method, reported by Cox and Muday (1994), utilized 0.4 M potassium iodide or 1 M sodium carbonate with solubilization yields of 80%, leading these authors to propose a peripheral location for the NPA-binding protein. Attempts to replicate these results with our material were unsuccessful. In the case of the carbonate treatment, we found that addition of 1 M sodium carbonate to the reaction mixture increased the pH to 11 and irreversibly denatured the NPA-binding protein. As a consequence, the activity was not recovered in either the pellet or the supernatant. Finally, the tight interaction of the NPA-binding protein with actin was proposed by Cox and Muday (1994) as a possible reason for the low yield of solubilization of the activity by detergents. In our studies, using nonsolubilized PM, digestion of actin by proteinase K (demonstrated by western blot analysis; see Fig. 4) did not release any NPA-binding activity. This activity was quasitatively recovered in the pellet after centrifugation at
Solubilized NPA-Binding Protein Was Susceptible to Protease Treatment

The solubilized fractions containing the NPA-binding activity were subjected to the protease treatments. As reported in Figure 1B and Table I, an overnight incubation at 4°C with proteinase K completely abolished NPA-binding activity in these fractions. Similar results were obtained when the proteinase K was replaced by trypsin and pronase E or when the proteinase K digestion was performed for 1 h at 37°C (data not shown). The extent of the digestion was demonstrated by SDS-PAGE analysis (Fig. 3B) and immunoblotting (Fig. 4, B and C). Since only 25% of the activity was solubilized in this protocol, we also investigated the remaining activity that pelleted after treatment with Chaps and molybdate. The nonsolubilized protein was also readily digested by proteinase K and no activity was observed (data not shown).

Taken together, these results suggest that the NPA-binding protein may be hydrophobic and integral to the PM. This will be of importance for evaluating potential candidates for this protein isolated by molecular cloning methods. Although the present data directly contradict Cox and Muday’s (1994) work that found the NPA-binding protein to be peripherally associated with the PM, it does not rule out a possible interaction between a domain of this protein exposed to the cytoplasm and actin filaments. The findings in the present report are in accordance with the correlation between the hydrophobicity of a compound and its effectiveness in displacing NPA (Katekar and Geissler, 1977). They also raise the interesting possibility that, since the NPA-binding protein is mostly hydrophobic and NPA or NPA-displacing ligands are hydrophobic, the NPA-binding site may reside inside the membrane and be accessible to the ligand only after its partitioning in the membrane.

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