Solubilization and Separation of a Plant Plasma Membrane NADPH-O$_2^-$ Synthase from Other NAD(P)H Oxidoreductases

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Plants respond to pathogenic infection by the induction of a variety of physiological responses to survive the induced stress. Several strategies, including cell wall strengthening, production of microbicidal components and hydrolytic enzymes, and the induction of controlled cell death are used in this defense response (Dixon and Lamb, 1990; Dixon et al., 1994). These mechanisms are induced after specific recognition of the invading pathogen. The recognition process is based on the binding of ligands (elicitors), which are released after the pathogen intrusion, to plasma membrane receptor proteins (Nürnberger et al., 1994).

It has been convincingly shown that plant cells respond to this infection with the rapid production of AOS, either in compatible or incompatible interactions (Sutherland, 1991; Baker and Orlandi, 1995; Low and Merida, 1996; Mehdy et al., 1996). So-called incompatible species also induce a second, sustained burst of AOS a few hours after the initial transient response. There is little doubt that the AOS play a central role in the regulation of defense strategies of the plant. These reactive O$_2^-$ molecules may serve in early plasma membrane events such as microbial degradation and cell wall cross-linking (Bradley et al., 1992; Brisson et al., 1994; Wojtaszek et al., 1995). Moreover, they may also function as intracellular signal molecules, triggering other defense conditions such as the hypersensitive reaction (Tenhaken et al., 1995) and systemic acquired resistance (Chen et al., 1993; Dempsey and Klessig, 1994; Ryals et al., 1995). The combination of these reactions is essential for the plant cell to survive the invading pathogen stressor.

The production of the extracellular AOS in plants may be mediated by the activity of enzymes located in the plasma membrane or in the apoplasm. Different classes of enzymes are thought to contribute to this action, including peroxidases, lipoxygenases, and NAD(P)H-dependent oxidases (Baker and Orlandi, 1995). The contribution of an NAD(P)H oxidase is most commonly accepted as the responsible enzyme in AOS production (Doke et al., 1994; Baker and Orlandi, 1995; Low and Merida, 1996; Mehdy et al., 1996). Plasma membrane oxidase is thought to use cytosolic NADPH to reduce O$_2^-$ at the apoplastic membrane face. Based on the potential similarities to an animal cell oxidative burst oxidase, the hypothesis of a plasma membrane NADPH oxidase generating O$_2^-$ radicals in plant cells has recently received much attention.

The respiratory burst oxidase of animal cells is a plasma membrane protein producing O$_2^-$ molecules as part of the inflammatory immune response destroying invading microorganisms (Segal, 1995). The protein consists of an integral plasma membrane protein (a heterodimer of subunits p22phox and gp91phox), bearing a flavin and a Cyt b cofactor. The oxidase uses cytosolic NADPH to reduce molecular O$_2$ to O$_2^-$ inside the phagocytic vacuoles. In this reaction electrons from NADPH are sequentially transferred through the FAD-flavin and Cyt b$_{58}$ cofactors toward O$_2$ in the phagocytic lumen. Regulation and activation of this oxidase involves the association with a small GTP-binding protein (p21ras) and at least two cytosolic phosphoproteins (p47phox and p67phox; Segal and Abo, 1993; Segal, 1995). Activation of the constitutively ex

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Abbreviations: AOS, active oxygen species; DCIP, dichlorophenolindophenol; DPI, diphenylenedioneium; E$_{50}$, standard redox potential; FPLC, fast-protein liquid chromatography; NBT, nitroblue tetrazolium; pCMBS, p-chloromercuribenzoic acid; SOD, superoxide dismutase.
pressed flavo-Cyt subunit occurs upon treatment of cells with ligands, such as immune-inducing factors, arachidonic acid, and phorbol esters (Rossi, 1986).

The possible similarity of the plant oxidative burst oxidase with the enzyme present in animal cells is based on several lines of evidence. In plant cells the presence of proteins homologous to the small subunit p22phox and to the cytosolic-activating factors p47phox and p67phox has been suggested because of the cross-reactivity with antibodies raised against the neutrophil proteins (Levine et al., 1994; Tenhaken et al., 1995; Dwyer et al., 1996). Moreover, the O2− production in plant cells and isolated plasma membranes have been shown to be sensitive inhibitors of the animal oxidase, such as DPI, quinacrine, imidazole, and pyridine (Auh and Murphy, 1995; Murphy and Auh, 1996). Certain ligands inducing the activity of the neutrophil oxidase, such as phorbol esters and the G-protein activator mastoparan, are also able to induce O2− production by plant cells (Legendre et al., 1992; Vera-Estrella et al., 1994). Finally, the signal transduction pathway leading to AOS production involves similar intermediates, including G-proteins, phospholipase C activity, and Ca and protein phosphorylation in both the animal and plant kingdoms (Legendre et al., 1992, 1993; Levine et al., 1994; Nürnberger et al., 1994; Vera-Estrella et al., 1994).

Although strongly suggestive, it should be stressed that these elements provide only indirect evidence of similarity between the plant and animal O2− synthase. The existence of similar regulatory components does not by itself provide information on the nature of the NAD(P)H oxidase. In addition, the NAD(P)H-dependent O2−-producing enzyme in plant plasma membranes has not been demonstrated to contain a flavin or Cyt.

We present results on the solubilization and separation of an NADPH-dependent O2− synthase from other NAD(P)H oxidoreductases of plant plasma membranes. Attempts have been made to detect flavin and Cyt cofactors in the protein fraction and the similarity to the animal respiratory burst oxidase is discussed.

**MATERIALS AND METHODS**

Hypocotyl hooks of 5-d-old etiolated beans (*Phaseolus vulgaris* L. cv Limburgse Vroege) were harvested and collected on ice. Highly purified plasma membranes were isolated by aqueous two-phase partitioning, as described previously (Widell et al., 1982; Asard et al., 1989). To avoid contamination by proteins potentially trapped inside the tightly sealed plasma membrane vesicles, the fraction was subjected to a washing procedure (Van Gestelen et al., 1996). Plasma membrane vesicles were diluted in a hypotonic medium (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.1 mM MgCl2, 50 mM KCl, and 0.025% w/v Triton X-100) in a protein:detergent ratio of 4:1 (w/w) and repelleted at 100,000g for 45 min at 4°C. The pellet was resuspended in solubilization buffer (50 mM Tris-HCl, pH 8.0, 250 mM Suc, 0.1 mM MgCl2, and 25% v/v glycerol).

The effectiveness of the washing treatment was controlled by measuring cytosolic Gsc-6-P dehydrogenase (EC 1.1.1.49, Valenti et al., 1984), plasma membrane H+ -ATPase (Asard et al., 1989), and NAD(P)H-O2− synthase (see below), as described previously. For comparison, an unwashed plasma membrane preparation suspended in isotonic medium (hypotonic medium supplemented with 250 mM Suc and without KCl or Triton X-100) was diluted either in hypotonic or in isotonic medium. After washing, the membranes were recovered at 100,000g for 45 min at 4°C and resuspended in isotonic medium. In all enzyme tests 0.02% w/v Brij-58 (polyoxyethylene[20]cetyl ether, Janssen Chimica, Geel, Belgium) as a detergent was added to make the plasma membrane vesicles leaky to measure maximal activities. Protein content of the plasma membranes was measured according to the method of Markwell et al. (1978) with BSA as standard.

Solubilization of the plasma membrane proteins was achieved by the addition of 1.4% w/v octylglucoside (1-O-n-octyl-β-D-glucopyranoside, Sigma) at a protein to detergent ratio of 1:10 (w/w) and stirring on ice for 60 min. The solubilized proteins were recovered in the supernatant after centrifugation at 100,000g for 60 min at 4°C. The plasma membrane proteins were subjected to anion-exchange chromatography on an FPLC system (Mono-Q HR 5/5 column, Pharmacia). The column was equilibrated with column buffer containing 20 mM Tris-HCl, pH 8.0, 250 mM Suc, 10% (v/v) glycerol, 0.5% (w/v) Triton X-100, and 0.2% (v/v) Berol 185 (nonionic detergent, Berol Chemicals, Stenungsund, Sweden). The solubilized proteins were diluted 2.5-fold in 1.25% (v/v) Triton X-100 to get below the critical micellar concentration of the detergent octylglucoside used for solubilization and to change to equimolar concentrations of the column buffer. Proteins were eluted from the column at a flow rate of 1.0 mL min−1 with a discontinuous salt gradient in column buffer supplemented with 1 mM KCl, and 2-mL fractions were collected.

NADPH-dependent O2− generation was measured using the tetrazolium dye NBT as an electron acceptor (Bielski et al., 1980). NBT is rapidly converted to monoformazan by two molecules of O2−. This reduction is detected spectrophotometrically (Aminco DW2000 spectrophotometer, SLM Instruments, Urbana, IL) at 530 nm. Monoformazan concentrations (and therefore O2− concentrations) were calculated using an extinction coefficient of 12.8 mm−1 cm−1. The NBT reduction rates are strictly linear with time up to 10 to 15 min and are linearly dependent on the protein concentration (10–50 μg) in the sample. The selective reduction of NBT by O2− was calculated from the difference in the NBT reduction rate in the presence and absence of SOD (50–100 units mL−1, EC 1.15.1.1, Fluka). The reaction mixture consisted of a Tris buffer (50 mM Tris-HCl, pH 7.4, 250 mM Suc) with additions as specified in the text. No NBT reduction with reduced nucleotides (NADPH) was observed in the absence of protein fractions.

Quantitative determination of flavins, NAD(P)H-dehydrogenase activities (Van Gestelen et al., 1996), and b-type Cys (Asard et al., 1989) was performed as previously described. Protein concentrations in the FPLC fractions were estimated by the bicinchoninic acid protein assay (Sigma) using BSA as a standard.
RESULTS

Washing of Plasma Membrane Preparations

Plasma membrane vesicles obtained with aqueous two-phase partitioning are likely to contain entrapped or loosely bound soluble proteins. To ascertain that only plasma membrane-associated enzymes were studied, these purified vesicles were subjected to a washing procedure in both a hypotonic and an isotonic buffer (Table I). The hypotonic treatment effectively removed the cytosolic marker Glc-6-P dehydrogenase. Similar observations have been made in our laboratory with determinations of soluble SOD after hypotonic treatment of vesicles obtained from the same tissue in slightly different buffer conditions (R.J. Caubergs, unpublished results).

Plasma membrane H+-ATPase activity (Table I), flavins, and NAD(P)H-oxidoredutase reactions with artificial oxidants (Van Gestelen et al., 1996) were fully retained after the hypotonic lysis. Also, the majority of NAD(P)H-O2- synthase activities were recovered in the hypotonic washed plasma membranes (Table I). The isotonic controls showed that no degradation of the measured enzymes was induced by the extra dilution and centrifugation step. Therefore, hypotonic treatment of plasma membrane vesicles in the presence of salt and low-detergent concentrations is necessary to remove cytosolic proteins that may be enclosed during tissue homogenization. This washing step has been included in the subsequent characterization of the NADPH-O2- enzyme.

Solubilization and Chromatography of Plasma Membrane Proteins

To obtain information on the nature of the NAD(P)H-oxidoredutase enzyme(s) participating in the production of AOS in plants, we solubilized plasma membrane proteins of bean hooks and screened for O2- synthesis after separation with anion-exchange FPLC. The presence of NAD(P)H-dependent O2- synthase described in this paper. It was consistently found that these oxidoreductase activities were clearly resolved from the FPLC protein fraction with O2- synthase activity (Fig. 2). Screening with the artificial oxidants revealed two distinct proteins. The first eluted in a sharp peak at 21.75 min during the isocratic elution with or without protein no NBT reduction and therefore no O2- synthesis was present. The rate of enzymatic activity in this peak fraction was estimated to be 27.4 ± 2.7 nmol min-1 mg-1 protein (average of 10 experiments) in the presence of 0.1 mM NADPH, 0.1 mM NBT, and 50 units mL-1 SOD. Comparing this specific activity with the one measured in hypotonic, washed plasma membrane vesicles (Table I) showed a 4-fold enrichment after solubilization and anion-exchange FPLC.

Since several plasma membrane NAD(P)H-dependent redox enzymes have already been described using a variety of artificial electron acceptors (Serrano et al., 1994; Bérczi and Asard, 1995), we tested the oxidoreductase activity using ferricyanide, Cyt c, DCIP, and oxaloacetate as oxidants. NAD(P)H-dehydrogenases have already been purified using these oxidants as substrates and it was interesting to test whether these enzymes coincide with the O2- synthase activity described in this paper. It was consistently found that these oxidoreductase activities were clearly resolved from the FPLC protein fraction with O2- synthase activity (Fig. 2). Screening with the artificial oxidants revealed two distinct proteins. The first eluted in a sharp peak at 21.75 min during the isocratic elution with

Table I. Activities of cytosolic Glc-6-P dehydrogenase (NADPH reduction), plasma membrane-bound H+-ATPase (phosphate liberation), and NAD(P)H-O2- synthase were determined

<table>
<thead>
<tr>
<th>Plasma Membrane Treatment</th>
<th>Glu-6-P Dehydrogenase (nmol min-1 mg-1 protein)</th>
<th>H+-ATPase (nmol min-1 mg-1 protein)</th>
<th>O2- Synthase (nmol min-1 mg-1 protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unwashed</td>
<td>1.0 ± 0.7 (n = 2)</td>
<td>990.0 ± 200.0 (n = 3)</td>
<td>8.2 ± 1.4 (n = 9)</td>
</tr>
<tr>
<td>Hypotonic wash</td>
<td>0 (n = 2)</td>
<td>1150.0 ± 140.0 (n = 3)</td>
<td>6.9 ± 1.8 (n = 6)</td>
</tr>
<tr>
<td>Isotonic wash</td>
<td>1.1 ± 0.8 (n = 2)</td>
<td>920.0 ± 120.0 (n = 3)</td>
<td>8.1 ± 2.1 (n = 2)</td>
</tr>
</tbody>
</table>

NT: Not tested.
Figure 2. Elution chromatogram of plasma membrane proteins solubilized by octylglucoside and detection of NADH-dependent oxidoreductase activities using Cyt c and oxaloacetate as electron acceptors.

Figure 3. Detection of the NAD(P)H-dependent O$_2^-$ production in the partially purified plant plasma membrane oxidase fraction by reduction of NBT followed at 530 nm. Plasma membranes were solubilized with octylglucoside and separated by anion-exchange chromatography. NADPH (0.1 mM) and SOD (50 units [U] mL$^{-1}$) were added at the times indicated (arrows) to a reaction mixture consisting of 800 µL of Tris buffer (20 mM Tris-HCl, pH 7.4, 0.1 mM NBT, and 250 mM Suc) and 200 µL of the oxidase-containing fraction (42 µg of protein).

Characteristics of the O$_2^-$ Synthase

The O$_2^-$ synthase-containing protein fractions from the anion-exchange column were collected and used for further characterization. Because of the relative difficulty of obtaining sufficient amounts of protein material, some of these determinations were performed only twice.

O$_2^-$ production was quantified using the SOD-sensitive NBT reduction rate. Inhibition by SOD (50–100 units mL$^{-1}$) was always nearly complete (Fig. 3), indicating that no other O$_2$ radicals or direct electron transfer to NBT was involved. In addition, we found that H$_2$O$_2$ (up to 10 mM) was not able to reduce NBT. The O$_2^-$ synthase activity was initiated almost exclusively by the addition of NADPH.

When NADPH was replaced by NADH as the reductant, the activity ranged from 0 to a maximum of 15% of the NADPH-dependent activity in different preparations. This NADH-dependent reaction was not enriched compared with the hypotonic, washed plasma membrane vesicles (Table I). This indicates that the enzyme affinity for NADH is lowered by changes in the enzyme due to solubilization and separation or, alternatively, that another, more NADH-specific enzyme has been lost upon solubilization.

Since plasma membrane O$_2^-$ generation in plants is often attributed to a flavoprotein similar to the neutrophil flavo-Cyt oxidase, the NADPH-oxidase-containing protein fraction was tested for the presence of flavins and b-type Cyt s. Flavins were extracted from the O$_2^-$ synthase fractions by heat treatment to release noncovalently bound flavins from the proteins. Previous work on total plasma membrane fractions suggested that only noncovalently

<table>
<thead>
<tr>
<th>Table II. Substrate specificity of the two NAD(P)H-acceptor reductases that were resolved from the O$_2^-$ synthase fraction during the anion-exchange chromatography. Means and s$^*$s are presented from different plasma membrane preparations.</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Acceptor</td>
<td>Dehydrogenase at 21.75 min</td>
</tr>
<tr>
<td></td>
<td>NADH</td>
</tr>
<tr>
<td>Ferricyanide</td>
<td>78.0 ± 50.0 (n = 2)</td>
</tr>
<tr>
<td>Cyt c</td>
<td>43.0 ± 6 (n = 5)</td>
</tr>
<tr>
<td>DCIP</td>
<td>16.0 ± 7.0 (n = 2)</td>
</tr>
<tr>
<td>Duroquinon</td>
<td>21 (n = 1)</td>
</tr>
<tr>
<td>Oxaloacetate</td>
<td>2.8 ± 2.8 (n = 2)</td>
</tr>
</tbody>
</table>

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bound flavins were present in this membrane (Van Gestelen et al., 1996).

Flavin determination with HPLC and fluorescence detection revealed the existence of an FMN-type flavin in the protein fraction containing the NADPH-dependent O$_2^-$ production. The amount of FMN was calculated to be 280 ± 30 pmol mg$^{-1}$ protein. In the previous section, we suggested that the O$_2^-$-synthase-containing FPLC fraction contained an FMN-type flavin. However, it is conceivable that a large part of noncovalently bound flavins may have been lost from the protein, resulting in largely underestimated enzyme activity. To test the possibility that flavins stimulate the O$_2^-$ production, exogenous flavins were added. Supplementation of the fraction with a large excess of FAD or FMN (25 nmol of flavin to 10–50 µg of total protein in the test) stimulated the O$_2^-$ production only 1.5–2.5-fold (Table III).

Since the neutrophil oxidase contains both flavins and Cys, the presence of Cys was tested spectrophotometrically after nonspecific reduction by sodium dithionite (Cross et al., 1981; Asard et al., 1989). The majority of b-type Cys was detected in the nonbound protein fractions (about 113 ± 9 pmol mL$^{-1}$, Fig. 1). In addition, one other protein fraction distinct from the O$_2^-$ synthase was shown to possess Cyt b proteins (approximately 31.6 ± 1.3 pmol mL$^{-1}$, Fig. 1). Surprisingly, no Cyt was detected in the O$_2^-$ synthase-containing fraction.

The method used to measure Cyt b has a calculated detection limit of about 10 pmol mL$^{-1}$ (using an molar extinction coefficient of 112 mM$^{-1}$ cm$^{-1}$ at 260 nm). This result by itself indicates that the isolated protein is not a peroxidase, since O$_2^-$ production was highly specific for NADPH (Elstner and Heupel, 1976; van der Werf et al., 1991). The lack of inhibition by KCN (0.05–1 mM) was completely ineffective as an inhibitor (data not shown). Other products were able to inactivate the NADPH-O$_2^-$ activity with varying efficiencies (Table III). The tested inhibitors have been reported to be effective inactivators of the animal neutrophil oxidase (Cross and Jones, 1986, 1991) and have been shown to be active on plant plasma membrane NAD(P)H-O$_2^-$ production, as well (Auh and Murphy, 1995; Murphy and Auh, 1996). The thiol reagent pCMBS showed strong inhibitions at micromolar concentrations (50% inhibition at approximately 5 µM and complete inhibition with 60 µM).

DPI, which has been suggested to interact with flavin cofactors on enzymes, showed an almost complete enzyme inactivation at 200 µM (50% inhibition of approximately 60 µM). The other tested components were only effective in millimolar concentrations. Quinacrine, imidazole, and pyridine inhibited the O$_2^-$-synthase activity by about 50% at concentrations of 1.5, 10, and 10 mM, respectively. However, in quinacrine and imidazole no higher inhibitions were observed at higher concentrations.

### DISCUSSION

The existence of a plant NADPH-oxidase that produces O$_2^-$ has been suggested from physiological studies and from comparisons with a well-characterized animal neutrophil oxidase. However, the presence of an enzyme identical to the neutrophil oxidase has not yet been demonstrated in plants. Therefore, we studied O$_2^-$-synthase activities after solubilization and separation of NAD(P)H-dependent oxidases of the plant plasma membrane.

Plasma membrane proteins were obtained from etiolated bean hook tissue and were subjected to ion-exchange chromatography. One protein fraction, eluting at approximately 340 mM KCl, showed NADPH-dependent O$_2^-$ production, as demonstrated by the SOD-sensitive reduction of NBT. O$_2^-$ production was highly specific for NADPH compared with NADH (ranging from nonactive to a maximum 15% of the NADPH reaction). This result by itself indicates that the isolated protein is not a peroxidase, since O$_2^-$ production by plant peroxidases was induced by NADH, as well (Elstner and Heupel, 1976; van der Werf et al., 1991). The lack of inhibition by KCN (0.05–1 mM) further supports this conclusion. Furthermore, the contri-

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**Table III. Characterization of the NADPH-O$_2^-$-synthase activity obtained from the Mono-Q anion-exchange column**

<table>
<thead>
<tr>
<th>Effector</th>
<th>Concentration µM</th>
<th>Percentage Activity %</th>
</tr>
</thead>
<tbody>
<tr>
<td>FMN</td>
<td>5</td>
<td>170 ± 40</td>
</tr>
<tr>
<td>FAD</td>
<td>25</td>
<td>250 ± 110</td>
</tr>
<tr>
<td>FAD</td>
<td>5</td>
<td>127 ± 9</td>
</tr>
<tr>
<td>DPI</td>
<td>25</td>
<td>153 ± 13</td>
</tr>
<tr>
<td>Quinacrine</td>
<td>10</td>
<td>89 ± 4</td>
</tr>
<tr>
<td>Quinacrine</td>
<td>30</td>
<td>75 ± 6</td>
</tr>
<tr>
<td>Quinacrine</td>
<td>100</td>
<td>27 ± 15</td>
</tr>
<tr>
<td>Quinacrine</td>
<td>200</td>
<td>7 ± 4</td>
</tr>
<tr>
<td>pCMBS</td>
<td>5,000</td>
<td>47 ± 9</td>
</tr>
<tr>
<td>Imidazole</td>
<td>1,500</td>
<td>48 ± 8</td>
</tr>
<tr>
<td>Imidazole</td>
<td>5,000</td>
<td>76 ± 2</td>
</tr>
<tr>
<td>Pyridine</td>
<td>60</td>
<td>2 ± 2</td>
</tr>
<tr>
<td>Pyridine</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Pyridine</td>
<td>1,000</td>
<td>81 ± 1</td>
</tr>
<tr>
<td>Pyridine</td>
<td>3,000</td>
<td>58 ± 9</td>
</tr>
<tr>
<td>Pyridine</td>
<td>10,000</td>
<td>39 ± 9</td>
</tr>
<tr>
<td>Pyridine</td>
<td>20,000</td>
<td>47 ± 11</td>
</tr>
<tr>
<td>Pyridine</td>
<td>2,000</td>
<td>62 ± 12</td>
</tr>
<tr>
<td>Pyridine</td>
<td>6,000</td>
<td>63 ± 19</td>
</tr>
<tr>
<td>Pyridine</td>
<td>20,000</td>
<td>37 ± 9</td>
</tr>
<tr>
<td>Pyridine</td>
<td>40,000</td>
<td>19 ± 9</td>
</tr>
</tbody>
</table>
bution of lipoxigenase activity is doubtful because no ac-
vivating fatty acids (Chamulitrat et al., 1991) are expected in
these solubilized and separated protein fractions.

Screening with alternative electron acceptors (ferricya-
nide, Cyt c, DCIP, and oxaloacetate) for plant plasma mem-
brane redox reactions revealed the presence of two other
NAD(P)H-dependent oxidoreductase proteins, which pref-
ferentially used NADH as the reductant. These other two
NAD(P)H dehydrogenases showed similarities in substrate
specificity with previously purified enzymes (Serrano et al.,
1994). The resolving of the NADPH-O$_2^-$ synthase from the
NAD(P)H oxidoreductases demonstrates that the NADPH-
dependent O$_2^-$ synthase reported in this paper is distinct
from previously demonstrated NAD(P)H oxidoreductases
from plant plasma membranes.

Because of the general suggestion that the plant O$_2^-$
synthase could be similar to the animal respiratory burst
oxidase, we investigated the presence of both animal co-
factors (flavin and Cyt b) in the bean O$_2^-$-synthase protein
fraction. The plant NADPH-oxidase fraction contained a
heat-extractable, and thus noncovalently bound, FMN-type
flavin (280 ± 30 pmol FMN mg$^{-1}$ protein). The FMN
nature of this chromophore was in contrast to the heat-
extractable FAD chromophore of the animal oxidase (Glass
et al., 1986).

In a previous study (Van Gestelen et al., 1996) the major
flavin in plasma membranes from bean hooks was also
identified as being noncovalently bound FMN. Only minor
amounts of FAD were detected in freshly purified plasma
membranes from the same tissue (P. Van Gestelen, unpub-
lished data). This apparent discrepancy with the animal
oxidase is difficult to explain but clearly points to the
existence of a different enzyme system in the plant cell.
However, it should be kept in mind that the co-elution of
the FMN chromophore and NADPH-oxidase activity by
itself is not conclusive proof for the involvement of the
flavin in the O$_2^-$-synthase activity. The further purification
and separation of the apoprotein from the flavin chro-
mophore will undoubtedly help to resolve this problem.
However, we also observed that a large excess of exog-
enuously added flavins (both FAD and FMN) stimulated the
O$_2^-$ production rate only moderately. This stimulatory
action of flavins is in agreement with earlier published data
on O$_2^-$ production in plasma membrane vesicles of rose
cells (Murphy and Auh, 1996).

Although flavins were present in the O$_2^-$-synthase pre-
parations, b-type Cyts were not detectable in this fraction,
even when it was not concentrated 4-fold. When a detec-
tion limit of about 10 pmol mL$^{-1}$ (at the Soret band) was
used, if any b-type Cyts were present in the sample, their
concentration was less than 2.5 pmol mL$^{-1}$ (or 38 pmol
mg$^{-1}$ protein). This level is significantly lower than the
measured FMN concentration of 18.4 pmol mL$^{-1}$ (or 280
pmol mg$^{-1}$ protein). This means that in our enzyme frac-
tion no equimolar ratio of flavin to Cyt is found as for the
animal NADPH-oxidase (Cross et al., 1982).

It is interesting that in animal neutrophil cells there have
also been reported observations of active NADPH-oxidase
enzyme preparations with only flavins and no heme or
Cyts attached (Glass et al., 1986). This activity has been
suggested to result from a direct electron donation from the
flavin to molecular O$_2$ when the natural Cyt oxidant is lost
during enzyme preparations (Glass et al., 1986). NADPH-
dependent O$_2^-$-synthase activity has also been reported in
a variety of animal cells other than neutrophils, and in
some cases these cells contained no detectable heme or
b-type Cyts (Jones et al., 1995). These observations appar-
ently support the idea that a nonheme, NADPH-dependent
O$_2^-$ synthase may be functional in certain cell types.

Still another line of evidence supports the absence of a
typical neutrophil oxidase b-type Cyt homolog in plant
cells. Redox titration analysis in a variety of plant tissues
has demonstrated that the major plant plasma membrane
b-type Cyt (Cyt b$_{561}$) has an E$'_0$ of +120 to +160 mV (Asard
et al., 1989, 1994). This value is very different from the
reported E$'_0$ value of the neutrophil Cyt b$_{558}$ (−245 mV;
Cross and Jones, 1986). A b-type Cyt with an E$'_0$ of +120
mV would not be able to transfer electrons from NADPH
(E$'_0$ = −320 mV) to molecular O$_2$ (E$'_0$ of O$_2$/O$_2^-$ couple
−160 mV; Wood, 1987). In addition to this major Cyt in
plant plasma membranes, small amounts of a Cyt with an
E$'_0$ of about −20 mV were detected (Asard et al., 1989).
Despite the sensitivity of the titration analysis, however, in
no case was a Cyt with an E$'_0$ below −200 mV resolved.
Although the possibility that a low potential component
might still be overlooked cannot be fully excluded, these
results also suggest that a neutrophil oxidase-like Cyt b is
not found at the plant plasma membrane.

Screening of the fractionated bean O$_2^-$ synthase with
known inhibitors of the animal respiratory oxidase revealed
qualitatively similar results. Inhibitors suggested to affect
the flavin moiety of the oxidase (DPI and quinacrine) re-
sulted in the efficient inhibition of the partially purified O$_2^-$
synthase. However, the inhibitors suggested to interact with
the Cyt b of animal oxidase (PCMBs, imidazole, and pyri-
dine) were also effective in our preparations. This result is
somewhat puzzling with respect to the lack of detectable
amounts of Cyt b. The specificity and the mode of action
of these substances therefore needs further documentation.

These results may point to differences between plant and
animal NADPH oxidase. The amount of inhibitor necessary
to obtain inhibition in plant oxidases was sometimes a
magnitude higher in concentration than those effective in
animal oxidase preparations (Cross and Jones, 1986, 1991)
and those used in rose cells and rose cell plasma membrane
preparations (Auh and Murphy, 1995; Murphy and Auh,
1996). These differences may result from different molar
amounts of the studied oxidase in the test conditions or
otherwise point to differences in sensitivity between the
studied enzyme preparations.

The identification of presumptive plasma membrane pro-
teins from purified plasma membrane vesicles may be bi-
ased by the presence of soluble proteins that became trapped
inside the vesicle lumen during tissue homogenization. De-
spite the presence of cytoplasmic enzymes capable of gen-
erating O$_2^-$, this problem is often overlooked. The plasma
membrane vesicles used in this paper were subjected to
hypotonic lysis in the presence of 50 mM KCl and 0.025%
(w/v) Triton X-100 (protein:detergent ratio of 4:1 [w/w])
and were repelleted before protein solubilization. This
washing treatment effectively removed cytoplasmic Glc-6-P dehydrogenase and SOD, whereas full plasma membrane ATPase activity, NAD(P)H–O₂⁻, and other redox activities (Van Gestelen et al., 1996) were retained. It is therefore suggested that a washing procedure is necessary to ascertain the membrane association of the studied oxidase.

The washing procedure was apparently not used in recent work on plasma membranes of rose cells (Murphy and Auh, 1996). These authors reported a considerable loss in NADPH, and, to a lower extent, of NADH-dependent O₂⁻ production, by diluting and repelleting their plasma membrane vesicles in slightly hypertonic conditions. In these conditions the plasma membrane vesicles are considered to be stable, and sealed vesicles and tightly bound plasma membrane proteins should be recovered in the plasma membrane pellet. The recovery of membrane enzymes after diluting and repelleting membrane preparations has been previously shown for peroxidase and NAD(P)H-oxidase activities in cauliflower inflorescences (Askerlund et al., 1987). Because of the considerable loss in NAD(P)H–O₂⁻ activity found by Murphy and Auh (1996) on rose cell preparations, the plasma membrane nature of at least part of their measured NAD(P)H oxidase activities is questionable.

In conclusion, we have demonstrated the presence of an O₂⁻ synthase in the plant plasma membrane and successfully solubilized and clearly resolved this protein from other NAD(P)H oxidoreductases. The enzyme characteristics point to similarities to the animal respiratory burst oxidase with respect to inhibitor sensitivities. The partially purified enzyme, however, apparently contained FMN instead of FAD and no b-type Cyt could be detected in the protein fraction, which suggests differences from the animal oxidase. Although this enzyme may potentially be involved in plant defense responses, this has not yet to our knowledge been demonstrated. A further purification is currently being undertaken and will help resolve the mode of action of this enzyme and allow a more detailed comparison with the animal oxidase.

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LITERATURE CITED

Markwell MAK, Haas SM, Bieber LL, Tolbert NE (1978) A modification of the Lowry procedure to simplify protein determina-
tion in membrane and lipoprotein samples. Anal Biochem 87: 206–210


Segal AW (1995) The NADPH oxidase of phagocytic cells is an electron pump that alkalinises the phagocytic vacuole. Protoplasma 184: 86–103


