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Solubilization and Separation of a Plant Plasma Membrane NADPH-O_2^- Synthase from Other NAD(P)H Oxidoreductases[1]

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Solubilization and ion-exchange chromatography of plasma membrane proteins obtained from bean (*Phaseolus vulgaris* L.) seedlings resulted in a single NAD(P)H-O_2^--synthase protein peak. This enzyme showed a high preference toward NADPH as a substrate (reaction rate, 27.4 nmol O_2^- produced min^{-1} mg^{-1} protein), whereas NADH reactions ranged from 0 to maximally 15% of the NADPH reactions. The protein functions as an oxidase and it was clearly resolved from NAD(P)H dehydrogenases identified with commonly used strong oxidants (ferricyanide, cytochrome *c*, DCIP, and oxaloacetate). The involvement of peroxidases in O_2^- production is excluded on the basis of potassium-cyanide insensitivity and NADPH specificity. The NADPH oxidase is only moderately stimulated by flavins (1.5-fold with 25 μM flavine adenine dinucleotide and 2.5-fold with 25 μM flavin mononucleotide) and inhibited by 100 μM *p*-chloromercuribenzenesulfonic acid, 200 μM diphenyleneiodonium, 10 mM quinacrine, 40 mM pyridine, and 20 mM imidazole. The presence of flavins was demonstrated in the O_2^--synthase fraction, but no *b*-type cytochromes were detected. The effect of these inhibitors and the detection of flavins and cytochromes in the plant O_2^- synthase make it possible to compare this enzyme with the NADPH O_2^- synthase of animal neutrophil cells.

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 apoplast. 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 $p22^{phox}$ and $gp91^{phox}$), 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_{558} cofactors toward O_2 in the phagocytic lumen. Regulation and activation of this oxidase involves the association with a small GTP-binding protein ($p21^{rac}$) and at least two cytosolic phosphoproteins ($p47^{phox}$ and $p67^{phox}$; Segal and Abo, 1993; Segal, 1995). Activation of the constitutively ex-

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Abbreviations: AOS, active oxygen species; DCIP, dichlorophenolindophenol; DPI, diphenyleneiodonium; E'_0, 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 $p22^{phox}$ and to the cytosolic-activating factors $p47^{phox}$ and $p67^{phox}$ 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 O_2^- production in plant cells and isolated plasma membranes have been shown to be sensitive to known 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 O_2^- 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 O_2^- 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 O_2^--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 O_2^- 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 $MgCl_2$, 50 mM KCl, and 0.025% [w/v] Triton X-100 in a protein:detergent ratio of 4:1 [w/w]) and repelleted at 100,000*g* 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 $MgCl_2$, and 25% [v/v] glycerol).

The effectiveness of the washing treatment was controlled by measuring cytosolic Glc-6-P dehydrogenase (EC 1.1.1.49, Valenti et al., 1984), plasma membrane H^+-ATPase (Asard et al., 1989), and NAD(P)H-O_2^- 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 100,000*g* 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 100,000*g* 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% (w/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 M KCl, and 2-mL fractions were collected.

NADPH-dependent O_2^- 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 O_2^-. This reduction is detected spectrophotometrically (Aminco DW2000 spectrophotometer, SLM Instruments, Urbana, IL) at 530 nm. Monoformazan concentrations (and therefore O_2^- 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 O_2^- 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 (NAD[P]H) 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 Cyts (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-oxidoreductase reactions with artificial oxidants (Van Gestelen et al., 1996) were fully retained after the hypotonic lysis. Also, the majority of NAD(P)H-O_2^--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-O_2^- enzyme.

Solubilization and Chromatography of Plasma Membrane Proteins

To obtain information on the nature of the NAD(P)H-oxidoreductase enzyme(s) participating in the production of AOS in plants, we solubilized plasma membrane proteins of bean hooks and screened for O_2^- synthesis after separation with anion-exchange FPLC. The presence of NAD(P)H-dependent O_2^- generation was tested in all protein fractions using the SOD-sensitive reduction of NBT. SOD was added during the linear reaction to ascertain the O_2^- contribution to the NBT reduction. The elution profile of one representative experiment is shown in Figure 1. NADPH-dependent O_2^- production was detected in one single peak eluting from the column between 41 and 48 min (280–400 mM salt). No additional plasma membrane proteins showing NADH or NADPH-dependent O_2^- generation could be demonstrated. Without added nucleotides

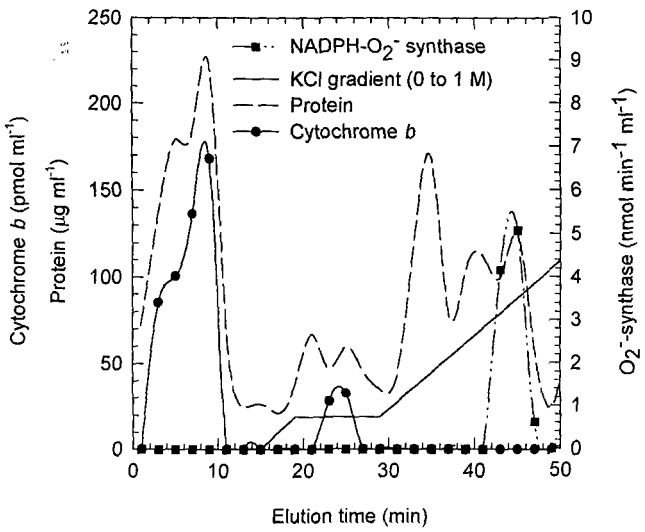

Figure 1. Elution chromatogram of plasma membrane proteins (discontinuous line, BCA protein test), solubilized with octylglucoside and separated by anion-exchange FPLC. The NADPH-O_2^- activity (■), measured by the SOD-sensitive reduction of NBT, is compared with the presence of dithionite-reducible *b*-type Cyts (●).

or without protein no NBT reduction and therefore no O_2^- 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 O_2^- synthase activity described in this paper. It was consistently found that these oxidoreductase activities were clearly resolved from the FPLC protein fraction with O_2^--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 (NADP$^+$ reduction), plasma membrane-bound H^+-ATPase (phosphate liberation), and NAD(P)H-O_2^- synthase were determined*

Plasma Membrane Treatment	Glu-6-P Dehydrogenase	H^+-ATPase	O_2^-Synthase	
			NADPH	NADH
	nmol min^{-1} mg^{-1} protein			
Unwashed	1.0 ± 0.7 (*n* = 2)	990.0 ± 200.0 (*n* = 3)	8.2 ± 1.4 (*n* = 9)	7.3 ± 2.2 (*n* = 5)
Hypotonic wash	0 (*n* = 2)	1150.0 ± 140.0 (*n* = 3)	6.9 ± 1.8 (*n* = 6)	4.9 ± 0.8 (*n* = 2)
Isotonic wash	1.1 ± 0.8 (*n* = 2)	920.0 ± 120.0 (*n* = 3)	8.1 ± 2.1 (*n* = 2)	NT[a]

[a] NT, Not tested.

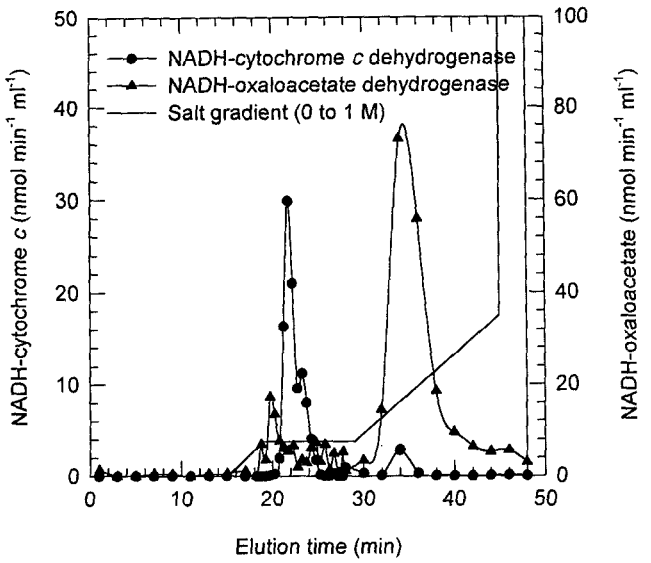

Figure 2. Elution chromatogram of plasma membrane proteins solubilized by octylglucoside and detection of NADH-dependent oxidoreductase activities using Cyt *c* and oxaloacetate as electron acceptors.

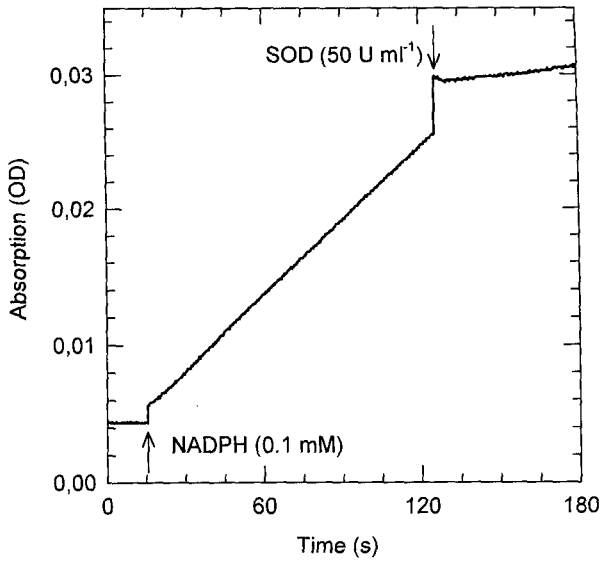

Figure 3. Detection of the NAD(P)H-dependent O_2^- production in the partially purified plant plasma membrane oxidase fraction by reduction of NBT followed at 530 nm. Plasma membranes were solubilized with octylglucoside and separated by anion-exchange chromatography. NADPH (0.1 mM) and SOD (50 units [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).

75 mM salt. The second dehydrogenase eluted in the following salt gradient between 32 and 38 min, peaking at 34 min and 125 mM salt. Both oxidoreductases showed a high preference for NADH as the reductant with the tested electron acceptors (Table II), which indicates that the O_2^--synthase activity is clearly different from other NAD(P)H-acceptor reductases demonstrated previously.

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_2O_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 the 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 Cyts. 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 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 SES are presented from different plasma membrane preparations.

Acceptor	Dehydrogenase at 21.75 min		Dehydrogenase at 34 min	
	NADH	NADPH (n = 1)	NADH	NADPH (n = 1)
	nmol min^{-1} mL^{-1}			
Ferricyanide	78.0 ± 50.0 (n = 2)	0	40.08 ± 0.08 (n = 2)	0
Cyt *c*	43.0 ± 6 (n = 5)	1.0	5 (n = 1)	0.7
DCIP	16.0 ± 7.0 (n = 2)	0	0.4 ± 0.4 (n = 2)	9.0
Duroquinon	21 (n = 1)	0	0 (n = 1)	0
Oxaloacetate	2.8 ± 2.8 (n = 2)	0	106.0 ± 24.0 (n = 3)	0

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- to 2.5-fold (Table III).

Since the neutrophil oxidase contains both flavins and Cyts, the presence of Cyts was tested spectrophotometrically after nonspecific reduction by sodium dithionite (Cross et al., 1981; Asard et al., 1989). The majority of *b*-type Cyts 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 Cyts has a calculated detection limit of about 10 pmol mL^{-1} (using an molar extinction coefficient [E_{430}] of 112 mM^{-1} cm^{-1} and 0.001 *A* units as a minimal reliable measurement). Since the flavin concentration in the sample was between 15 and 20 pmol mL^{-1}, and since the flavin and Cyt occur in a 1:1 ratio in the animal O_2^- synthase, this raised the possibility that the Cyt concentration was below the detection limit. To overcome this problem the O_2^--synthase-containing fraction was concentrated on a 10-kD exclusion filter (Filtron Microsep 10K, Schleicher & Schuell). Measuring the total O_2^--synthase activity and flavin content after concentration demonstrated that the fraction was concentrated 4-fold and that this treatment resulted in virtually no loss of the enzyme (data not shown). The concentrated fraction, however, still showed no detectable Cyt *b*.

Finally, a limited number of potential inhibitors of plasma membrane redox enzymes were tested. KCN, a powerful inhibitor of plant peroxidase enzymes, at a relatively high concentration (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.

Table III. *Characterization of the NADPH-O_2^--synthase activity obtained from the Mono-Q anion-exchange column*

Effects are expressed as percentages of control values without effectors (100% = 27.4 ± 2.7 nmol min^{-1} mg^{-1} protein). Means and SES are calculated from two to three independent plasma membrane preparations.

Effector	Concentration	Percentage Activity
	μM	%
FMN	5	170 ± 40
	25	250 ± 110
FAD	5	127 ± 9
	25	153 ± 13
DPI	10	89 ± 4
	30	75 ± 6
	100	27 ± 15
	200	7 ± 4
Quinacrine	500	76 ± 2
	1,500	48 ± 8
	5,000	47 ± 9
pCMBS	10	30 ± 10
	60	2 ± 2
	100	0
Imidazole	1,000	81 ± 1
	3,000	58 ± 9
	10,000	39 ± 9
	20,000	47 ± 11
Pyridine	2,000	62 ± 12
	6,000	63 ± 19
	20,000	37 ± 9
	40,000	19 ± 9

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-

bution of lipoxygenase activity is doubtful because no activating fatty acids (Chamulitrat et al., 1991) are expected in these solubilized and separated protein fractions.

Screening with alternative electron acceptors (ferricyanide, Cyt *c*, DCIP, and oxaloacetate) for plant plasma membrane redox reactions revealed the presence of two other NAD(P)H-dependent oxidoreductase proteins, which preferentially 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 cofactors (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, unpublished 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 chromophore will undoubtedly help to resolve this problem. However, we also observed that a large excess of exogenously 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 preparations, *b*-type Cyts were not detectable in this fraction, even when it was not concentrated 4-fold. When a detection 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 fraction 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 apparently 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) resulted 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 pyridine) 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 proteins from purified plasma membrane vesicles may be biased by the presence of soluble proteins that became trapped inside the vesicle lumen during tissue homogenization. Despite the presence of cytoplasmic enzymes capable of generating 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-O_2^-, 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 O_2^- 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-O_2^- 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 O_2^- 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

Asard H, Horemans N, Mertens J, Caubergs RJ (1994) The plant plasma membrane *b*-type cytochrome: an overview. Belg J Bot **127:** 171–183

Asard H, Venken M, Caubergs RJ, Reijnders W, Oltmann FL, De Greef JA (1989) *b*-Type cytochromes in higher plant plasma membranes. Plant Physiol **90:** 1077–1083

Askerlund P, Larsson C, Widell S, Møller IM (1987) NAD(P)H oxidase and peroxidase activities in purified plasma membranes from cauliflower inflorescences. Physiol Plant **71:** 9–19

Auh CK, Murphy TM (1995) Plasma membrane redox enzyme is involved in the synthesis of O_2^- and H_2O_2 by *Phytophthora* elicitor-stimulated rose cells. Plant Physiol **107:** 1241–1247

Baker CJ, Orlandi EW (1995) Active oxygen in plant pathogenesis. Annu Rev Phytopathol **33:** 299–321

Bérczi A, Asard H (1995) NAD(P)H-utilizing oxidoreductases of the plasma membrane: an overview of presently purified proteins. Protoplasma **184:** 140–144

Bielski BHJ, Shiue GG, Bajuk S (1980) Reduction of nitro blue tetrazolium by CO_2^- and O_2^- radicals. J Phys Chem **84:** 830–833

Bradley DJ, Kjellbom P, Lamb CJ (1992) Elicitor- and wound-induced oxidative cross linking of a proline rich plant cell wall protein: a novel, rapid defense response. Cell **70:** 21–30

Brisson LF, Tenhaken R, Lamb C (1994) Function of oxidative cross-linking of cell wall structural proteins in plant disease resistance. Plant Cell **6:** 1703–1712

Chamulitrat W, Hughes MF, Eling TE, Mason RP (1991) Superoxide and peroxyl radical generation from the reduction of polyunsaturated fatty-acid hydroperoxides by soybean lipoxygenase. Arch Biochem **290:** 153–159

Chen Z, Silva H, Klessig DF (1993) Active oxygen species in the induction of plant systemic acquired resistance by salicylic acid. Science **262:** 1883–1886

Cross AR, Garcia RC, Segal AW (1982) The association of FAD with cytochrome b_{-245} of human neutrophils. Biochem J **208:** 765–768

Cross AR, Jones OTG (1986) The effect of the inhibitor diphenylene iodonium on the superoxide-generating system of neutrophils: specific labelling of a component polypeptide of the oxidase. Biochem J **237:** 111–116

Cross AR, Jones OTG (1991) Enzymic mechanisms of superoxide production. Biochim Biophys Acta **1057:** 281–298

Cross AR, Jones OTG, Harper AM, Segal AW (1981) Oxidation-reduction properties of the cytochrome *b* found in the plasma-membrane fraction of human neutrophils. Biochem J **194:** 599–606

Dempsey DA, Klessig DF (1994) Salicylic acid, active oxygen species and systemic acquired resistance in plants. Trends Cell Biol **4:** 334–338

Dixon RA, Harrison MJ, Lamb CJ (1994) Early events in the activation of plant defense responses. Annu Rev Phytopathol **32:** 479–501

Dixon RA, Lamb CJ (1990) Molecular communication in interactions between plants and microbial pathogens. Annu Rev Plant Physiol Plant Mol Biol **41:** 339–367

Doke N, Miura Y, Sanchez LM, Kawakita K (1994) Involvement of superoxide in signal transduction: responses to attack by pathogens, physical and chemical shocks and UV irradiation. *In* CH Foyer, PM Mullineaux, eds, Causes of Photooxidative Stress and Amelioration of Defense Systems in Plants, Vol 1. CRC Press, Boca Raton, FL, pp 177–197

Dwyer SC, Legendre L, Low PS, Leto TL (1996) Plant and human neutrophil oxidative burst complexes contain immunologically related proteins. Biochim Biophys Acta **1289:** 231–237

Elstner EF, Heupel A (1976) Formation of hydrogen peroxide by isolated cell walls from horseradish (*Armoracia lapathifolia* Gilib.). Planta **130:** 175–180

Glass GA, De Lisle DM, De Togni P, Gabig TG, Magee BH, Markert M, Babior BM (1986) The respiratory burst oxidase of human neutrophils. Further studies of the purified enzyme. J Biol Chem **261:** 13247–13251

Jones OTG, Jones SA, Wood JD (1995) Expression of components of the superoxide generating NADPH oxidase by human leucocytes and other cells. Protoplasma **184:** 79–85

Legendre L, Heinstein PF, Low PS (1992) Evidence for participation of GTP-binding proteins in elicitation of the rapid oxidative burst in cultured soybean cells. J Biol Chem **267:** 20140–20147

Legendre L, Yueh YG, Grain R, Haddock N, Heinstein PF, Low PS (1993) Phospholipase C activation during elicitation of the oxidative burst in cultured plant cells. J Biol Chem **268:** 24559–24563

Levine A, Tenhaken R, Dixon R, Lamb C (1994) H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. Cell **79:** 583–593

Low PS, Merida JR (1996) The oxidative burst in plant defense: function and signal transduction. Physiol Plant **96:** 533–542

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

Mehdy MC, Sharma YK, Sathasivan K, Bays NW (1996) The role of activated oxygen species in plant disease resistance. Physiol Plant **98:** 365–374

Murphy TM, Auh CK (1996) The superoxide synthases of plasma membrane preparations from cultured rose cells. Plant Physiol **110:** 621–629

Nürnberger T, Nennstiel D, Jabs T, Sacks WR, Hahkbrock K, Scheel D (1994) High affinity binding of a fungal oligopeptide elicitor to parsley plasma membranes triggers multiple defense responses. Cell **78:** 449–460

Rossi F (1986) The O_2^--forming NADPH oxidase of the phagocytes: nature, mechanisms of activation and function. Biochim Biophys Acta **853:** 65–81

Ryals J, Lawton KA, Delaney TP, Friedrich L, Kessmann H, Neuenschwander U, Uknes S, Vernooij B, Weymann K (1995) Signal transduction in systemic acquired resistance. Proc Natl Acad Sci USA **92:** 4202–4205

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

Segal AW, Abo AW (1993) The biochemical basis of the NADPH oxidase of phagocytes. Trends Biochem Sci **18:** 43–47

Serrano A, Córdoba F, Gonzáles-Reyes JA, Navas P, Villalba JM (1994) Purification and characterization of two distinct NAD(P)H dehydrogenases from onion (*Allium cepa* L.) root plasma membrane. Plant Physiol **106:** 87–96

Sutherland MW (1991) The generation of oxygen radicals during host plant responses to infection. Physiol Mol Plant Pathol **39:** 79–83

Tenhaken R, Levine A, Brisson LF, Dixon RA, Lamb C (1995) Function of the oxidative burst in hypersensitive disease resistance. Proc Natl Acad Sci USA **92:** 4158–4163

Valenti V, Stanghellini MA, Pupillo P (1984) Glucose 6-phosphate dehydrogenase isoenzymes of maize leaves. Plant Physiol **75:** 521–526

van der Werf A, Raaimakers D, Poot P, Lambers H (1991) Evidence for a significant contribution by peroxidase-mediated O_2 uptake to root respiration of *Brachipodium pinnatum*. Planta **183:** 347–352

Van Gestelen P, Asard H, Caubergs RJ (1996) Partial purification of a plasma membrane flavoprotein and NAD(P)H-oxidoreductase activity. Physiol Plant **98:** 389–398

Vera-Estrella R, Higgins VJ, Blumwald E (1994) Plant defense response to fungal pathogens. II. G-protein-mediated changes in host plasma membrane redox reactions. Plant Physiol **106:** 97–102

Widell S, Lundborg T, Larsson C (1982) Plasma membranes from oats prepared by partition in an aqueous polymer two-phase system. Plant Physiol **70:** 1429–1435

Wojtaszek P, Trethowan J, Bolwell GP (1995) Specificity in the immobilisation of cell wall proteins in response to different elicitor molecules in suspension-cultured cells of French bean (*Phaseolus vulgaris* L.). Plant Mol Biol **28:** 1075–1087

Wood PM (1987) The two redox potentials for oxygen reduction to superoxide. Trends Biochem Sci **12:** 250–251