Plant Physiol. (1996) 110: 621–629

The Superoxide Synthases of Plasma Membrane Preparations from Cultured Rose Cells

Terence M. Murphy* and Chung-Kyoon Auh[1]

Section of Plant Biology, University of California, Davis, California 95616

Preparations of plasma membranes isolated from cultured rose (*Rosa damascena* Mill. cv Gloire de Guilan) cells synthesized O_2^- when incubated with either NADH or NADPH, as measured by an O_2^--specific assay based on the chemiluminescence of lucigenin. The activities were strongly dependent on the presence of Triton X-100. The K_m for NADH was 159 μM; that for NADPH was 19 μM. Neither NADH- nor NADPH-dependent activity was inhibited by azide, an inhibitor of peroxidase, nor by antimycin A, an inhibitor of mitochondrial electron transport; both activities were inhibited by 30 to 100 nM diphenylene iodonium, an inhibitor of the mammalian NADPH oxidase. The NADH- and NADPH-dependent activities could be distinguished by detergent solubilization and ultracentrifugation: the NADH-dependent activity sedimented more easily, whereas the NADPH-dependent activity remained in suspension. One or both of these enzymes may provide the O_2^- seen when plant cells are exposed to pathogens or pathogen-associated elicitors; however, plasma membranes from rose cells treated with a *Phytophthora* elicitor had the same activity as control cells.

The term "active oxygen" refers to chemically reactive species of oxygen, including singlet oxygen, hydroxyl radical, O_2^-, and H_2O_2. The generation of these species occurs as a byproduct of several natural processes, such as photosynthesis and respiration. Since the products can destroy sensitive functional molecules, plants and other organisms have evolved detoxifying mechanisms (Foyer et al., 1994).

Active oxygen species also play a positive role in normal plant development and in a plant's response to stress. In normal development, H_2O_2 serves as a substrate for the polymerization of lignin, suberin, and possibly other cell-wall components (Hahlbrock and Griesbach, 1979; Olson and Varner, 1993). O_2^- and H_2O_2, produced in response to bacterial or fungal attack, are thought to kill the attacking cells and also adjacent host cells, thus limiting the spread of the pathogen. Active oxygen species have been suggested to play a signaling role in inducing other defenses to pathogens (Levine et al., 1994; Price et al., 1994).

In past publications, our laboratory has reported that suspension-cultured cells of rose (*Rosa damascena*) produce a burst of H_2O_2 after they have been treated with UV-C radiation (Murphy and Huerta, 1990). They also produce the same response when they are challenged with a chemical elicitor, a cell-wall component isolated from a culture of a *Phytophthora* species originally identified as a parasite on rose (Arnott and Murphy, 1991).

The mechanism of synthesis of active oxygen species, and particularly of metabolically required H_2O_2, is a topic of active discussion. In rose cells, the elicitor-induced H_2O_2 is synthesized exclusively from O_2^-, as judged by the ability of *N,N*-diethyldithiocarbamate, an inhibitor of superoxide dismutase, to block the appearance of H_2O_2 in elicitor-stimulated cells (Auh and Murphy, 1995). It has been proposed that apoplastic H_2O_2 synthesized by horseradish roots occurs by dismutation of the O_2^- generated through a complex cycle involving extracellular NADH, phenolic compounds, and peroxidase (Elstner and Heupel, 1976; Gross et al., 1977; Halliwell, 1978). The extracellular NADH is said to be supplied by cell-wall-bound malate dehydrogenase, with the substrate malate provided by a malate-oxalacetate shuttle in the plasma membrane. An alternative source of O_2^- in plant cells has been proposed by Doke (1985): a plasma membrane-associated oxidase that transfers electrons from NAD(P)H in the cytoplasm to oxygen in the apoplast. In rose cells the synthesis of H_2O_2 is stimulated by added NADH and phenolics (or salicylhydroxamic acid), but it is neither stimulated by malate nor inhibited by oxalacetate (Murphy and Huerta, 1990), as it would be if a mechanism similar to that postulated for horseradish roots were active. Furthermore, the synthesis of O_2^- and H_2O_2 by elicitor-stimulated rose cells is inhibited by DPI, an inhibitor of the plasma membrane NADPH oxidase of mammalian neutrophils, consistent with the presence of such an enzyme in the rose cell plasma membranes (Auh and Murphy, 1995). In this paper, we describe NADH- and NADPH-oxidases assayed by the appearance of O_2^- (O_2^- synthases) in rose cell plasma membrane preparations.

MATERIALS AND METHODS

Cells, Plasma Membranes

Suspension-cultured cells of *Rosa damascena* Mill. cv Gloire de Guilan were grown as described by Murphy et al. (1979). Plasma membranes were prepared from 4-d-old cultures by the aqueous two-phase method of Larsson et al. (1983) as previously described (Murphy and Auh, 1992). With this method, we disrupted the cells by sonication in a buffer containing 25 mM Tris, 25 mM Mes, 3 mM EDTA, 2.5

[1] Present address: Department of Genetics, North Carolina State University, Raleigh, NC 27695-7614.

* Corresponding author; e-mail tmmurphy@ucdavis.edu; fax 1-916-752-5410.

Abbreviations: DPI, diphenylene iodonium; O_2^-, superoxide.

mM DTT, and 250 mM Suc, pelleted dense material by centrifugation at 18,800*g* for 15 min, and then collected microsomal membranes by centrifugation at 190,000*g* for 40 min. We resuspended the microsomes and subjected them to three successive partitions in a 12-g mixture containing 6.3% PEG, 6.3% dextran, 4 mM KCl, and 250 mM Suc in 5 mM potassium phosphate buffer, pH 7.6. The membranes from the third upper (PEG) phase were diluted in 1 mM Tris-Mes buffer, pH 6.5, containing 1 mM $MgSO_4$ and 20% Suc, pelleted by centrifugation, and stored at −70°C until used.

An analysis of marker enzymes in the final preparation is given in Table I. K^+-stimulated ATPase (plasma membrane), IDPase (dictyosome), Cyt *c* oxidase (mitochondrion), and Cyt *c* reductase (ER) were assayed as described by Hodges and Leonard (1974), except that the mitochondrial Cyt *c* oxidase was estimated by the difference in activity with and without 1 mM KCN and the Cyt *c* reductase was measured in the presence of 1 μM antimycin A rather than KCN. Peroxidase (soluble) was measured by the oxidation of guaiacol according to the method of Castillo et al. (1984). The specific activity of the ATPase in the plasma membrane preparation was high and within the normal range. The purification procedure removed more than 99.7% of the total Cyt *c* oxidase, Cyt *c* reductase, IDPase, and peroxidase. However, a measurable amount of the oxidase, reductase, and peroxidase remained in the plasma membrane preparation (Table I).

Assay of O_2^- Synthesis

The assay of O_2^- was based on the chemiluminescence of lucigenin (bis-*N*-methylacridinium nitrate, Sigma) as previously described (Auh and Murphy, 1995) but with modifications. A standard assay mixture contained 1.5 to 4 μg of plasma membrane protein, 100 μM NADH or 30 μM NADPH, 0.02% (w/w) Triton X-100, and 0.4 mM lucigenin, plus sufficient buffer (0.1 M Gly brought to pH 9 with NaOH plus 1 mM EDTA) to make a total volume of 1 mL. The components were combined in a 4-mL disposable plastic scintillation vial, and the chemiluminescence was measured by counting the mixture for 1 min in a liquid scintillation counter (Beckman model 9800) operated without the coincidence circuit (single-photon counting mode).

Table I. *Marker enzymes in a representative plasma membrane preparation*

The crude extract and the final plasma membrane fraction prepared from 20 g of washed 4-d-cultured rose cells were assayed for ATPase, KCN-sensitive Cyt *c* oxidase, antimycin A-resistant Cyt *c* reductase, IDPase, guaiacol peroxidase, and NADH- and NADPH-dependent O_2^- synthases. The homogenate (Homog) contained 3.07 mg mL^{-1} protein in 107 mL; the membrane fraction (U3) contained 0.57 mg mL^{-1} protein in 1.2 mL. NT, Not tested. ND, None detected.

Enzyme	Total Activity		Specific Activity	
	Homog	U3	Homog	U3
	nmol min^{-1}		*nmol* min^{-1} mg^{-1}	
ATPase	NT	650	NT	950
Cyt *c* oxidase	2100	6.0	6.5	8.7
Cyt *c* reductase	2400	1.9	7.4	2.8
IDPase	3.2×10^4	ND	98	ND
Guaiacol POD	8400	4	26	5.8
O_2^- synthase				
NADH	5029[a]	53	15[a]	77
NADPH	94[a]	18	0.29[a]	27

[a] O_2^- synthase activity in homogenate was initially rapid but decreased below background within 20 min.

Assay Calibration with Xanthine and Xanthine Oxidase

A sample of xanthine oxidase (grade I or grade III, Sigma) was assayed by the oxidation of xanthine: 10 to 200 μL of a xanthine oxidase dilution (nominally 0.3 unit mL^{-1}) and 0.05 mM xanthine were combined with the Gly-NaOH-EDTA buffer described above, and the rate of increase in A_{290}, representing the oxidation of xanthine to uric acid, was measured in a spectrophotometer over 1 to 3 min. The rate of the reaction (mol O_2^- min^{-1}) was calculated by multiplying the rate of increase of A_{290} (min^{-1}) by the reaction volume (3 mL) and dividing the result by the absorbance constant of uric acid (extinction coefficient at 290 nm = 1.22×10^7 mL mol^{-1} cm^{-1}) and the path length (1 cm) and then multiplying the result by an assumed 2 mol O_2^- produced for each 1 mol xanthine oxidized (Corbisier et al., 1987). The same sample of xanthine oxidase was then further diluted by a factor of 100, and aliquots were combined with 0.05 mM xanthine, 0.4 mM lucigenin, and 1 mM *N,N*-diethyldithiocarbamate in sufficient Gly-NaOH-EDTA buffer to total 1 mL; the enzyme-stimulated chemiluminescence of the mixture was measured. Both the rate of xanthine oxidation and the chemiluminescence increased proportionally with increasing enzyme. By assuming that the proportionality held for comparisons between the assays, we could calculate the reaction rate equivalent to a given level of chemiluminescence. One million counts per minute (10^6 cpm in the chemiluminescence assay) was equivalent to 6.3 ± 1.6 pmol O_2^- produced min^{-1} (mean ± SE, five experiments). This value was used to convert chemiluminescence data to reaction rates for presentation in the tables and figures below.

Solubilization and Differential Centrifugation

Samples of plasma membrane were solubilized by diluting them into buffer (15 mM Tris, pH 7.4, 2 mM $MgSO_4$, 330 mM Suc) containing 0.75 mg mL^{-1} Triton X-100, 2.5 mg mL^{-1} sodium deoxycholate, or 0.75 mg mL^{-1} dodecylmaltoside and incubating them for at least 10 min at 0°C. To sediment unsolubilized membranes, samples were centrifuged for 60 min at 100,000*g*. The supernatants were removed, and the pellets were resuspended in the buffer described above (minus detergent).

RESULTS

Basic Characteristics of the Enzyme

In the presence of 100 μM NADH or 30 μM NADPH and 0.02% Triton X-100, 0.4 mM lucigenin produced a level of chemiluminescence equivalent to 6 to 20 pmol min^{-1}, the level depending on the preparation of dinucleotide; this

chemiluminescence was strongly stimulated by preparations containing plasma membrane. Figure 1 shows the development and persistence of chemiluminescence in typical plasma membrane-containing reaction mixtures. An initial increase peaking at 20 to 40 min was followed by a regular, exponential decay. We assumed that the chemiluminescence closely reflected the rate at which O_2^- was produced, i.e. that the lucigenin reacted with the O_2^- as quickly as it was formed and there was no long-term accumulation of O_2^-. In confirmation of this assumption, we observed that the chemiluminescence of a fixed amount of O_2^-, prepared by incubating DMSO with NaOH (Hyland et al., 1983) and added to lucigenin in buffer, was lost exponentially with a half-life of 0.11 min (data not shown). This observation implies that the assay responded quickly to changes in O_2^- production rate. It also implies that the slow increase and decrease of chemiluminescence shown in Figure 1 represented activation and decay of the enzyme activity. Because initial counts were more variable (affected by room light and other factors), all reaction mixtures were counted six times over a period of at least 75 min, and measurements were based on data taken on the fourth, fifth, and sixth counts. The results from experimental treatments were always compared to those of untreated controls counted at the same times.

The rate of chemiluminescence was proportional to the concentration of lucigenin, up to a concentration of 0.4 to 0.5 mM (Fig. 2A). We used 0.4 mM lucigenin, a near-saturating amount, for all of the experiments reported in this paper. Lucigenin chemiluminescence has been described as specific for O_2^- (Corbisier et al., 1987), and the inhibition by superoxide dismutase of the chemiluminescence produced by rose plasma membrane in the presence of NADH or NADPH (Fig. 3) is consistent with the interpretation that

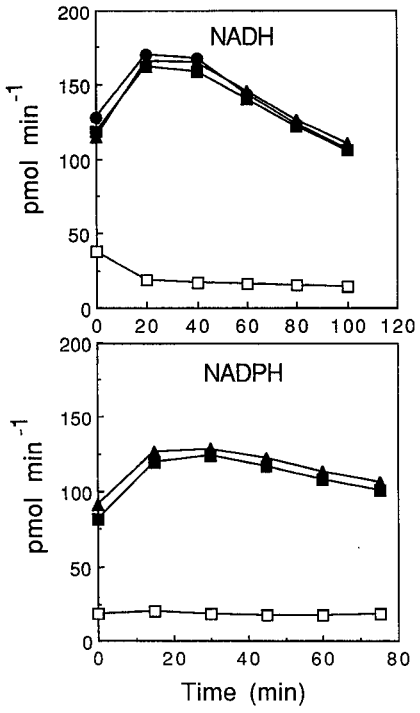

Figure 1. Time course for lucigenin chemiluminescence. Reaction mixtures of 1 mL contained 0.1 M Gly-NaOH buffer, pH 9, 0.02% Triton X-100, 0.4 mM lucigenin, and 100 μM NADH (top) or 30 μM NADPH (bottom), either without (open symbols) or with (filled symbols) plasma membrane (top, 0.84 μg of protein; bottom, 6.2 μg of protein). Different closed symbols represent replicate tubes.

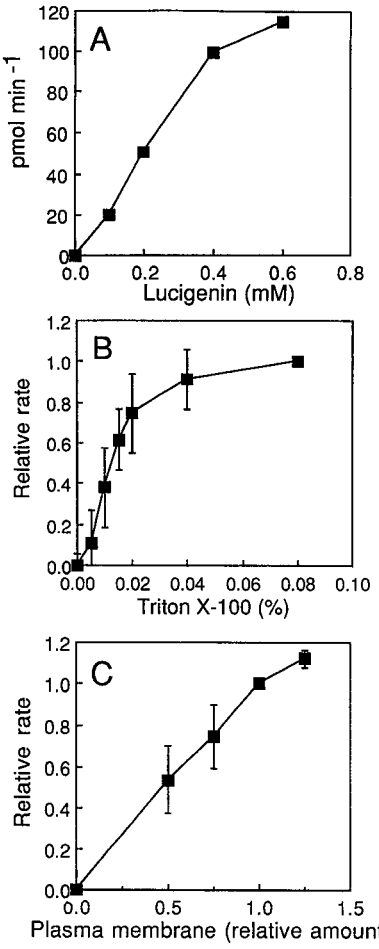

Figure 2. Characteristics of plasma membrane-catalyzed O_2^- synthesis. Except where noted, assays contained 100 μM NADH, 0.02% Triton X-100, and 0.4 mM lucigenin. A, Dependence on lucigenin concentration. Each tube received 0.84 μg of plasma membrane protein, NADH, Triton X-100, as noted above, and the concentration of lucigenin given on the abscissa. B, Dependence on concentration of Triton X-100. Tubes received 0.84, 1.7, 2.2, or 4.1 μg of plasma membrane protein (from different preparations), NADH and lucigenin as noted above, and the concentration of Triton X-100 given on the abscissa. The background luminescence observed with no plasma membrane was subtracted from each value. Within each experiment, values were normalized to the value obtained at 0.08% Triton X-100. C, Dependence on concentration of enzyme. NADH, Triton X-100, and lucigenin as noted above were combined with the amounts of plasma membrane given in B and with measured fractions of those amounts. The background luminescence observed with no plasma membrane was subtracted from each value; the differences were normalized by dividing by the value given by the standard amount ("1" on the abscissa) to correct for the different activities of the enzyme preparations. The data in A come from a single experiment. The data in B and C represent means ± SE (n = 4).

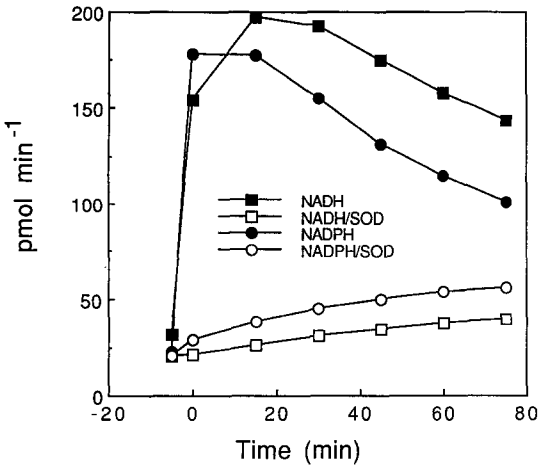

Figure 3. The effect of O_2^- dismutase (SOD) on plasma membrane-stimulated lucigenin chemiluminescence. Mixtures containing buffer, 100 μM NADH or 30 μM NADPH, 0.02% Triton X-100, and 0.4 mM lucigenin, with and without 10^4 units (nominal) of SOD were counted by scintillation spectrometry for 75 min; the value at −5 min shows the last count. At time 0, 0.84 μg of plasma membrane protein was added, and mixtures were counted periodically during the next 75 min. The figure shows the results from a representative experiment; the experiment was performed three times with similar results.

in this system chemiluminescence was at least 90% mediated by O_2^-.

The synthesis of O_2^- by plasma membrane acting with NADH or NADPH was strongly dependent on the inclusion of Triton X-100 in the assay medium (Fig. 2B). The latency (1 − [activity without Triton]/[maximum activity with Triton]) was 1.0 ± 0.06, averaged over four independent plasma membrane preparations. Triton X-100 also had an effect on the chemiluminescence of lucigenin, both alone and in combination with xanthine plus xanthine oxidase. The standard concentration of Triton X-100 (0.02%) produced an increase of 25% in chemiluminescence (data not shown). This effect contributed to the apparent stimulation of plasma membrane-catalyzed chemiluminescence by the detergent.

The rate of chemiluminescence increased proportionally with an increase in plasma membrane preparation (Fig. 2C), confirming the utility of the assay as a measure of O_2^- synthase activity.

The purification procedure resulted in a membrane preparation with O_2^- synthase specific activity that was substantially higher than the activities of Cyt *c* oxidase, Cyt *c* reductase, and peroxidase (Table I). The purification and recovery of the O_2^- synthases were difficult to determine, because the chemiluminescence stimulated by the homogenate decayed more rapidly than that by the plasma membrane and may not have been an accurate measure of the amount of enzyme in the homogenate. The highest specific activity of a plasma membrane preparation was 170 nmol O_2^- min^{-1} mg^{-1} protein, although the value shown in Table I is more typical. Other preparations had specific activities as much as 10-fold lower. Variation in the specific activity of different preparations may have been caused by different levels of contaminants in the preparation, different responses of the membranes to the Triton X-100, different degrees of loss of cofactor (see below), and different durations of storage (some preparations had been stored for as long as 4 years at −70°C). All experiments characterizing the enzymes were performed on at least three independent preparations with similar results.

Substrate and Cofactor Dependence

The dependence of the O_2^- synthetic reaction on substrate concentration was different for NADH and NADPH, although in both cases the kinetics could be modeled by the Michaelis-Menten formula (Fig. 4). With NADH as substrate, the K_m was 159 μM; with NADPH as substrate, the K_m was 19 μM. Although the use of different preparations of plasma membrane, with different specific activities, made it difficult to compare V_{max} for the two substrates, three of the preparations were used with both substrates. The ratios of V_{max} for NADH:NADPH demonstrated by

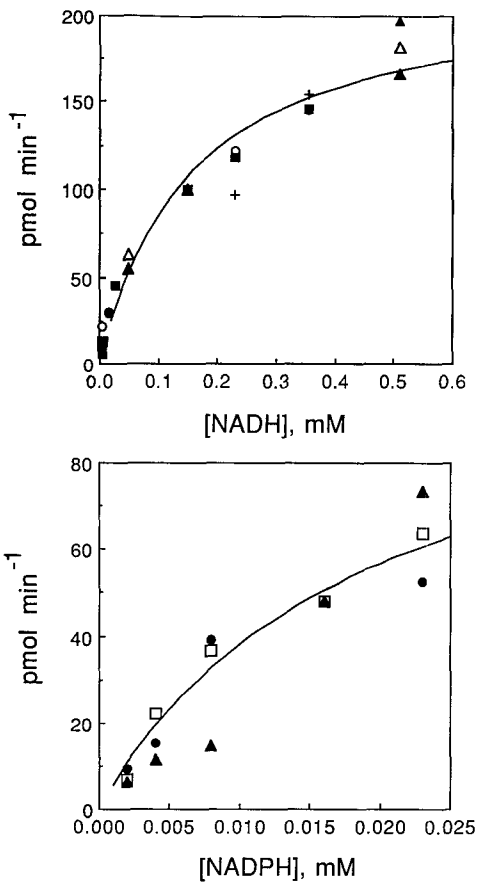

Figure 4. Dependence of O_2^- synthase activity on substrate concentration. All assays contained 0.02% Triton X-100 and 0.4 mM lucigenin. Data were derived from 10 experiments using 4 different enzyme preparations (top, NADH) or from 3 experiments with 3 different enzyme preparations (bottom, NADPH). Data obtained from preparations with different specific activities were normalized to a standard value (averaged from several experiments) at 0.15 mM NADH and 0.016 mM NADPH to obtain more accurate estimates of the K_m. Curves represent best least-squares fits of the data to a Michaelis-Menten equation by a nonlinear regression procedure.

these three preparations were 3.8, 1.3, and 2.5. Thus, the V_{max} for NADH was generally higher than for NADPH, but there was substantial variation in these ratios among the preparations.

The activities of both NADH- and NADPH-dependent enzymes were stimulated by FAD (Fig. 5). For both enzymes, the maximum degree of stimulation over the activity observed without added FAD was approximately 2-fold, reached with 0.3 μM FAD using NADH as substrate and 1 μM FAD using NADPH as substrate. There was a loss of stimulation at 10 μM FAD with NADH, but not NADPH, as substrate. FMN gave no significant increase in NADH-dependent activity, but it did give a significant ($P < 5\%$) stimulation of NADPH-dependent activity. This stimulation reached a maximum of 1.6-fold over the control activity at 6 μM FMN (data not shown).

Inhibitors

The effects of various compounds were investigated to test the possible relationship of the rose cell O_2^- synthases to other electron transport activities (see "Discussion"). Azide is an inhibitor of peroxidases, with 3.3 mM N_3^- giving 50% inhibition of the rose cell peroxidases secreted into the growth medium (data not shown). Antimycin A is an inhibitor of the mitochondrial electron transport chain. Quinacrine, imidazole, and DPI are inhibitors of mammalian O_2^- synthase. Arg is a substrate for mammalian nitric oxide synthase, an enzyme also reported to synthesize O_2^-, and *N*-nitroarginine methyl ester is an inhibitor of O_2^- synthesis by that enzyme (Pou et al., 1992). Auxins (2,4-D, naphthaleneacetic acid) stimulate NADH oxidases in certain plant plasma membranes (Morré and Brightman, 1991).

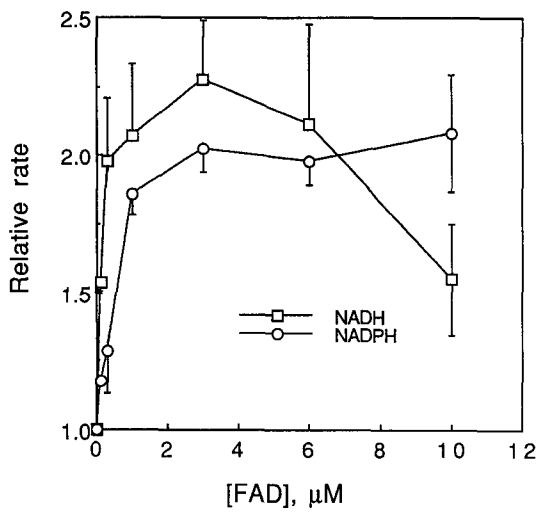

Figure 5. Stimulation of O_2^- synthase activity by FAD. All assays contained 0.02% Triton X-100, 0.4 mM lucigenin, 100 μM NADH or 30 μM NADPH, plasma membrane protein, and the concentration of FAD given on the abscissa. Data represent means ± SE from three experiments using three different enzyme preparations. Background values (without enzyme or FAD) were subtracted, and the differences were related to the value obtained with enzyme but without added FAD. Control activities ranged from 79 to 134 (NADH) and 32 to 36 (NADPH) pmol min^{-1}.

Table II. *Effects of putative inhibitory or activating compounds on O_2^- synthase activity*

All assays contained 0.02% Triton X-100, 0.4 mM lucigenin, either 100 μM NADH or 30 μM NADPH, and 1.5 to 4 μg of plasma membrane yielding an activity in control mixtures of 28 to 160 (NADH) or 12 to 70 (NADPH) pmol min^{-1}. Values are activities in reaction mixtures containing inhibitor relative to control mixtures without inhibitor. A value of 1.0 indicates that there was no effect. Background activities (without enzyme) were subtracted: a negative value indicates that the luminescence with enzyme was less than the luminescence without enzyme. Means and SE were calculated from assays using three independently prepared plasma membrane preparations.

Inhibitor	Substrate	
	NADH	NADPH
NaN_3, 3.3 mM	0.98 ± 0.02	0.91 ± 0.10
Antimycin A, 10 μM	0.97 ± 0.05	1.09 ± 0.03
Arginine, 1 mM	1.37 ± 0.26	1.02 ± 0.06
N-nitroarginine methyl ester, 1 mM	1.07 ± 0.17	0.94 ± 0.02
2,4-D, 10 μM	0.99 ± 0.03	1.10 ± 0.12
Naphthaleneacetic acid, 10 μM	1.03 ± 0.06	1.09 ± 0.14
Quinacrine, 500 μM	0.73 ± 0.20	0.52 ± 0.01[a]
Imidazole, 10 mM	0.82 ± 0.19	1.05 ± 0.22
DPI, 15 μM	−0.02 ± 0.05[a]	−0.02 ± 0.02[a]

[a] Values significantly different from 1.0 ($P < 0.05$ by *t* test).

The O_2^- synthetic reaction showed no sensitivity to azide, antimycin A, Arg, *N*-nitroarginine methyl ester, or auxins and little sensitivity to quinacrine and imidazole when assayed either with NADH or NADPH. However, it was completely inhibited by DPI (Table II). The sensitivity to DPI depended on the substrate provided: with NADH, the reaction was inhibited 50% by 100 nM DPI; with NADPH, the reaction was more sensitive, with 50% inhibition requiring approximately 30 nM DPI (Fig. 6).

Solubilization and Differential Centrifugation

Although the differences in reaction characteristics (K_m and sensitivity to DPI) seen with NADH and NADPH as substrates could be explained by the specificity of the active site of a single enzyme, it is also possible that separate enzymes could be transferring electrons from the two electron donors to O_2. A series of solubilization/sedimentation tests were conducted to determine whether the NADH- and NADPH-dependent O_2^- synthases could be separated from each other and to provide initial information needed for the purification of the enzymes (Fig. 7). The NADH-dependent O_2^- synthase was easily sedimented at 100,000*g* for 1 h in the Suc-Tris medium; it was partially solubilized by 0.75 mg mL^{-1} Triton X-100 and 0.75 mg mL^{-1} dodecylmaltoside but not by 2.5 mg mL^{-1} sodium deoxycholate. The NADPH-dependent O_2^- synthase was not as easily sedimented, even in the absence of detergent, and was more completely solubilized by the detergents. After they were added to reaction medium (a dilution of

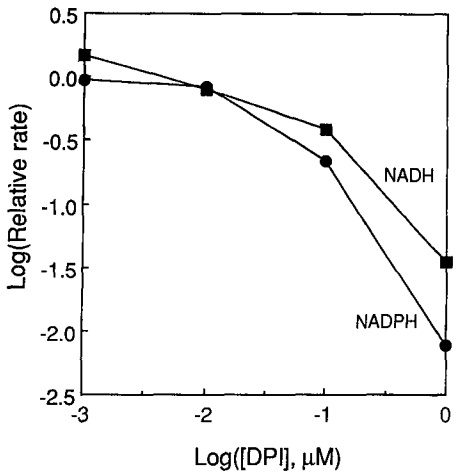

Figure 6. Inhibition of O_2^- synthase activity by DPI. All assays contained 0.02% Triton X-100, 0.4 mM lucigenin, either 100 μM NADH or 30 μM NADPH, and plasma membrane. Values are activities in tubes containing inhibitor relative to control tubes without inhibitor. Background activities without enzyme were subtracted. Control activities for the three experiments ranged from 23 to 158 (NADH) and 23 to 52 (NADPH) pmol min^{-1}. The data points indicate means from three independent experiments. A two-way factorial analysis of variance indicated that the results for NADH and NADPH were significantly different ($P < 0.02$).

50-fold), the activities of NADH- and NADPH-dependent enzymes in the supernatants retained their ability to be stimulated by addition of 0.02% Triton X-100 to the assay medium.

Lack of Activation by Elicitor

The synthesis of O_2^- by rose cells in vivo was stimulated by an elicitor preparation from a *Phytophthora* species (Auh and Murphy, 1995). However, the specific activity in vitro of O_2^- synthase in plasma membranes prepared from elicitor-treated cells was the same as that from control cells, whether NADH or NADPH was used as substrate (Table III).

DISCUSSION

The data presented demonstrate that plasma membrane preparations from suspension-cultured rose cells catalyze the synthesis of O_2^- in the presence of the electron donors NADH and NADPH. Rose cell extracellular peroxidases are sensitive to azide (50% inhibition at 3.3 mM) and not to DPI (Auh and Murphy, 1995; T.M. Murphy and C.-K. Auh, unpublished experiments), whereas the synthesis of O_2^- by our membrane preparations was sensitive to DPI and not to azide (Table II). The activity of guaiacol peroxidase in the plasma membrane preparation was very low (Table I), and there were no added phenolics, compounds required for the formation of O_2^- by peroxidase, in our assay mixtures. We thus suggest that peroxidase cannot be responsible for O_2^- synthesis in this system. This conclusion is consistent with the results of Doke and Miura (1995).

The observation that the O_2^- synthesis of plasma membrane vesicles is strongly stimulated by Triton X-100 is consistent with the idea that the oxidizing sites of the O_2^- synthase(s) are on the cytoplasmic side of the vesicles, unavailable to the substrate NADH or NADPH in the absence of a detergent. However, lipid or detergent may stimulate plasma membrane enzymes by mechanisms in addition to permeabilization of vesicles (for example, see Sandstrom et al., 1987; Bourdil et al., 1990). A direct effect on the O_2^- synthase(s) is not unlikely, since their degree of stimulation by Triton X-100 was greater than that generally measured for ATPase in plasma membranes prepared by the same method.

Evidence for Two O_2^- Synthases

Several observations suggest that the NADH- and NADPH-dependent O_2^- synthase activities represent different enzyme systems. These include the variable ratio of

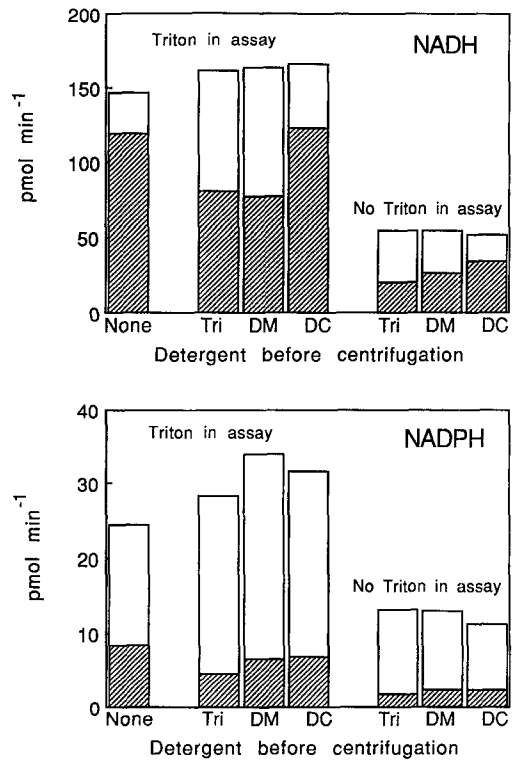

Figure 7. Solubilization and centrifugation of NADH- and NADPH-dependent O_2^- synthase. Aliquots of plasma membrane preparation (8.4 μg of protein) were treated for 10 min at 4°C in 200 μL of 15 mM Tris-Cl buffer, pH 7.4, 2 mM $MgSO_4$, 330 mM Suc, containing 0.75 mg mL^{-1} Triton X-100 (Tri), 0.75 mg mL^{-1} dodecylmaltoside (DM), or 2.5 mg mL^{-1} sodium deoxycholate (DC), or in buffer lacking detergent, and then centrifuged for 1 h at 100,000*g*. Supernatants were removed and the pellets were resuspended overnight in the original volume of buffer. Supernatants and pellets were assayed for O_2^- synthase with NADH (top) or NADPH (bottom) as substrate and in the presence or absence of 0.02% Triton X-100 in the reaction mixture. The upper and lower parts of each bar show the activity in the supernatant and pellet, respectively. Values represent means from three independent experiments.

Table III. *Comparison of O_2^- synthesis by plasma membranes prepared from elicitor-treated and untreated rose cells*

Equal amounts of plasma membrane protein (2.2 μg in one experiment; 3.4 μg in another) from untreated cells and cells treated with elicitor for 30 or 60 min were compared in reaction mixtures containing 0.02% Triton X-100, 0.4 mM lucigenin, and either 100 μM NADH or 30 μM NADPH. Within each experiment, rates for plasma membranes from treated cells were related to those from untreated cells; the activities of membranes from the untreated cells were 76 and 103 (NADH) and 52 and 69 (NADPH) pmol min^{-1}. Data are means ± SE.

Substrate	Control	Time of Treatment	
		30 min	60 min
NADH	1.0	1.11 ± 0.18	0.92 ± 0.03
NADPH	1.0	1.15 ± 0.07	1.04 ± 0.03

NADH- to NADPH-dependent activity in different preparations, the great difference in K_m between the two activities, the difference in concentration of DPI needed to inhibit the two activities by 50%, and the different sedimentation properties of the two activities. The low degree of sedimentation of the NADPH-dependent activity indicates that it is either on regions of the plasma membrane that are smaller or less dense than the regions holding the NADH-dependent activity or it is on a different type of membrane. Although the two-phase isolation procedure is recognized as highly specific for plasma membrane, some contamination of the preparation by other membranes occurs (Table I). The presence of Cyt *c* oxidase suggested that mitochondrial membrane might contribute to the NADH-dependent O_2^- synthase activity. However, the insensitivity of the activity to antimycin A argues against the idea that the full mitochondrial electron transport system is involved.

Relation to O_2^- Synthesis in Vivo

The sensitivity of the lucigenin assay was sufficiently great, and the conditions of the plasma membrane assay and intact-cell assay (Auh and Murphy, 1995) were sufficiently different, that we cannot conclude with certainty that the O_2^- measured in the present plasma membrane experiments came from the same enzyme system that produces O_2^- in intact cells. However, in both cases the O_2^- synthesis was not inhibited by azide or cyanide and was inhibited by low concentrations of DPI (Table II; Auh and Murphy, 1995). The difference in sensitivity to DPI (50% inhibition at 30–100 nM DPI for plasma membranes, at 13 μM for intact cells) can be explained by limitations in the uptake of DPI by cells and the presence of Triton X-100 in the plasma membrane assay. The fact that the sensitivity of the intact-cell system to quinacrine and imidazole (Auh and Murphy, 1995) was greater than that of the plasma membrane system (Table II) suggests that there were components in intact cells that were essential for elicitor-induced O_2^- synthesis and that were lost in the isolation of plasma membranes.

Which of the two substrates is responsible for the synthesis of O_2^- in intact cells? The low K_m of the NADPH-specific system and the supposition that NADH is primarily oxidized in living cytoplasm whereas NADPH is primarily reduced suggest that the NADPH system is the most likely candidate. This suggestion is consistent with an earlier suggestion concerning ferricyanide reduction by *Zea mays* roots (Qui et al., 1985). However, the high V_{max} of the NADH-specific system suggests that its potential contribution should not be ignored.

Doke and his colleagues have extensively investigated O_2^- synthesis by *Phytophthora*-infested potato tuber tissue. Recently, Doke and Miura (1995) reported that NADPH oxidase is associated with plasma membrane isolated from tuber tissue inoculated with an incompatible *Phytophthora* strain or with a cell-free elicitor preparation but not with plasma membrane from uninoculated tubers or from tubers inoculated with a compatible strain (Doke and Miura, 1995). We did not observe such a distinction between plasma membrane preparations from control and elicitor-treated cells (Table III), possibly because of changes in the enzymes during the purification procedure. The fact that the plasma membranes from control rose cells had O_2^- synthesis activity is consistent with our observation (Auh and Murphy, 1995) that control cells produce O_2^- at a level as high as one-fourth that of elicited cells. Doke and Miura (1995) also reported that O_2^- synthesis by isolated potato plasma membrane could be stimulated in vitro by a *Phytophthora* elicitor in the presence of a 100,000*g* supernatant fraction and other components. We have not yet attempted to duplicate these findings.

Relation to Other Aspects of Plasma Membrane Redox Activity

Redox activities of plant plasma membranes have been studied in several other contexts. Electrons, presumably from cytoplasmic donors, can be accepted by extracellular ferricyanide ion (Møller and Lin, 1986). This may be similar to trans-plasma membrane electron transport in plasma membrane O_2^- synthesis. However, there are reasons to believe that the enzyme systems differ in at least some aspects. For instance, the application of a fungal elicitor, which stimulates O_2^- synthesis in rose cells (Auh and Murphy, 1995), inhibits ferricyanide reductase in the same cells (Murphy and Auh, 1992).

Plasma membranes isolated from soybean hypocotyls demonstrate an auxin-stimulated NADH oxidase activity (Morré and Brightman, 1991). The fact that this activity is stimulated under the same conditions in which growth is stimulated and only by those auxin analogs that also stimulate cell elongation suggests that the activity has some role in the growth process. This enzyme is separable from the ferricyanide reductase. The reduced product of this activity has not been determined, but oxygen appears to be the acceptor and O_2^- may be formed. However, it is not clear how O_2^- would participate in the stimulation of growth. In fact there are reasons to believe that its appearance in the apoplast might have the opposite effect through cross-linking cell-wall components (Fry and Miller, 1987).

The auxin-stimulated NADH oxidase shows a low degree of detergent stimulation (Morré and Brightman, 1991), one characteristic that distinguishes it from the O_2^- synthases described in the present paper. Furthermore, the rose cell O_2^- synthases were not stimulated by 10 μM 2,4-D or naphthaleneacetic acid (Table II).

Investigations of blue-light phenomena in plant development have led to the elucidation of a plasma membrane electron transport chain containing a flavin and a Cyt. Irradiation with blue photons leads to changes in absorbance spectra that indicate an initial reduction of the flavin and subsequent reduction of the Cyt. Although measurement of the spectral changes is possible in solutions from which oxygen has not been excluded, the reduction of the Cyt is increased by the inclusion of oxygen-scavenging systems like Glc plus Glc oxidase (Asard et al., 1995). Thus, it is possible that O_2 accepts single electrons from the reduced Cyt and that these studies represent another view of one or both of the O_2^- synthases.

Neutrophils from mammalian blood produce O_2^- as part of their scavenging function, and this ability is centered in the plasma membrane. The composition of the mammalian NADPH oxidase has been determined: there is a flavocytochrome *b* integral to the membrane and three cytoplasmic proteins, including a *rac*-type GTP-binding protein, that are essential for activity (Abo et al., 1992; Segal and Abo, 1993). Like the rose enzyme, the neutrophil enzyme is inhibited by DPI, which binds to the flavocytochrome component (Cross and Jones, 1986).

It has also been reported that the mammalian plasma membrane enzyme nitric oxide synthase can form O_2^- under some conditions (Pou et al., 1992; Culcasi et al., 1994). Nitric oxide synthase is, like the plant O_2^- synthase reported here, inhibited by low concentrations of DPI, suggesting that there is a possible relationship between the two electron transport systems. However, neither Arg nor *N*-nitroarginine methyl ester had any effect on rose cell O_2^- synthesis, and we have no other evidence connecting either rose cell O_2^- synthase with nitric oxide synthase.

The use of the lucigenin assay has allowed us to detect O_2^- synthases that appear to differ from other plant plasma membrane redox systems. The possibility that there are two separate O_2^--generating systems must be considered in further studies of O_2^- synthesis by normal and stressed plant cells.

Received October 5, 1995; accepted November 13, 1995.

LITERATURE CITED

Abo A, Boyhan A, West I, Thrasher AJ, Segal AW (1992) Reconstitution of neutrophil NADPH oxidase activity in the cell-free system by four components: p67-phox, p47-phox, p21rac1, and cytochrome b_{-245}. J Biol Chem **267:** 16767–16770

Arnott T, Murphy TM (1991) A comparison of the effects of a fungal elicitor and ultraviolet radiation on ion transport and hydrogen peroxide synthesis by rose cells. Environ Exp Bot **31:** 209–216

Asard H, Horemans N, Briggs WR, Caubergs RJ (1995) Blue light perception by endogenous redox components of the plant plasma membrane. Photochem Photobiol **61:** 518–522

Auh C-K, 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

Bourdil I, Milat ML, Blein JP (1990) Effect of lysophosphatidylcholine on the NADH-ferricyanide reductase activity associated with the plasma membranes of corn roots. Plant Sci **70:** 143–153

Castillo FJ, Penel C, Greppin H (1984) Peroxidase release induced by ozone in *Sedum album* leaves. Involvement of Ca^{2+}. Plant Physiol **74:** 846–851

Corbisier P, Houbion A, Remacle J (1987) A new technique for highly sensitive detection of superoxide dismutase activity by chemiluminescence. Anal Biochem **164:** 240–247

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

Culcasi M, Lafon-Cazal M, Pietri S, Bockaert J (1994) Glutamate receptors induce a burst of superoxide via activation of nitric oxide synthase in arginine-depleted neurons. J Biol Chem **269:** 12589–12593

Doke N (1985) NADPH-dependent O_2^- generation in membrane fractions isolated from wounded potato tubers inoculated with *Phytophthora infestans*. Physiol Plant Pathol **27:** 311–322

Doke N, Miura Y (1995) In vitro activation of NADPH-dependent O_2^- generating system in a plasma membrane-rich fraction of potato tuber tissues by treatment with an elicitor from *Phytophthora infestans* or with digitonin. Physiol Mol Plant Pathol **46:** 17–28

Elstner EF, Heupel A (1976) Formation of H_2O_2 by isolated cell-walls from horseradish (*Armoracia lapathifolia*). Planta **130:** 175–180

Foyer CH, Descourvières P, Kunert KJ (1994) Protection against oxygen radicals: an important defense mechanism studied in transgenic plants. Plant Cell Environ **17:** 507–523

Fry SC, Miller JG (1987) H_2O_2-dependent cross-linking of feruloyl-pectins *in vivo*. Food Hydrocolloids **1:** 395–397

Gross GG, Janse C, Elstner EF (1977) Involvement of malate, monophenols, and the superoxide radical in hydrogen peroxide formation by isolated cell walls from horseradish (*Armoracia lapathifolia* Gilib.). Planta **136:** 271–276

Hahlbrock K, Griesbach H (1979) Enzymatic controls in the biosynthesis of lignin and flavonoids. Annu Rev Plant Physiol **30:** 105–130

Halliwell B (1978) Lignin synthesis: the generation of hydrogen peroxide and superoxide by horseradish peroxidase and its stimulation by manganese (II) and phenols. Planta **140:** 81–88

Hodges TK, Leonard RL (1974) Purification of a plasma membrane-bound adenosine triphosphatase from plant roots. Methods Enzymol **32:** 392–406

Hyland K, Voisin E, Banoun H, Auclair C (1983) Superoxide dismutase assay using alkaline dimethylsulfoxide as superoxide generating system. Anal Biochem **135:** 280–287

Larsson C (1983) Partition in aqueous polymer two-phase systems: a rapid method for separation of membrane particles according to their surface properties. *In* JL Hall, AL Moore, eds, Isolation of Membranes and Organelles from Plant Cells. Academic Press, London, pp 279–309

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

Møller IM, Lin W (1986) Membrane-bound NAD(P)H dehydrogenases in higher plant cells. Annu Rev Plant Physiol **37:** 309–334

Morré DJ, Brightman AO (1991) NADH oxidase of plasma membranes. J Bioenerg Biomembr **23:** 469–489

Murphy TM, Auh C-K (1992) *Phytophthora* elicitor inhibits ferricyanide reduction by cultured rose cells. Environ Exp Bot **32:** 487–496

Murphy TM, Hamilton CM, Street HE (1979) A strain of *Rosa damascena* cultured cells resistant to UV light. Plant Physiol **64:** 936–941

Murphy TM, Huerta AJ (1990) Hydrogen peroxide formation in cultured rose cells in response to UV-C radiation. Physiol Plant **78:** 247–253

Olson PD, Varner JE (1993) Hydrogen peroxide and lignification. Plant J **4:** 887–892

Pou S, Pou WS, Bredt DS, Snyder SH, Rosen GM (1992) Generation of superoxide by purified brain nitric oxide synthase. J Biol Chem **267:** 24173–24176

Price AH, Taylor A, Ripley SJ, Griffiths A, Trewavas AJ (1994) Oxidative signals in tobacco increase cytosolic calcium. Plant Cell **6:** 1301–1310

Qui Z-S, Rubinstein B, Stern AI (1985) Evidence for electron transport across the plasma membrane of *Zea mays* root cells. Planta **165:** 383–391

Sandstrom RP, DeBoer AH, Lomax TL, Cleland RE (1987) Latency of plasma membrane H^+-ATPase in vesicles isolated by aqueous phase partitioning. Plant Physiol **85:** 693–698

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