Oxygen Radical Generation by Isolated Microsomes from Soybean Seedlings

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ABSTRACT

The generation of active oxygen species by microsomes isolated from soybean seedlings was studied. NADPH-dependent superoxide anion production was 5.0 ± 0.4 nmol min⁻¹ mg⁻¹ of microsomal protein. Hydrogen peroxide generation by microsomes was 1.40 ± 0.05 nmol min⁻¹ mg⁻¹ of protein. Hydroxyl radical production, in the presence of ferric EDTA, evaluated through the generation of formaldehyde from dimethyl sulfide or tert-butyl alcohol was 0.50 ± 0.04 and 0.44 ± 0.03 nmol min⁻¹ mg⁻¹, respectively. NADPH proved to be suitable as cofactor for oxygen radical generation by microsomes from soybean seedlings. Because transition metals are implicated in radical generation by biological systems, the ability of microsomal membranes to reduce iron complexes was studied. Ferric ATP, ferric citrate, ferric ADP, ferric diethylenetriamine pentaacetic acid, and ferric EDTA were efficiently reduced in the presence of either NADPH or NADH as cofactor. The pattern of effectiveness of the different ferric complexes, on superoxide anion, hydrogen peroxide, and hydroxyl radical production, was similar to that found with animal microsomes. The data presented here indicate that microsomal ability to catalyze oxygen radical generation must be considered as an important contribution to cellular radical steady-state concentrations in cells from soybean seedlings.

Hydroperoxide metabolism in plants, particularly during seed germination, has received little attention. However, information about hydroperoxide production and utilization could be relevant to understanding important processes such as lipid peroxidation and redox changes. Previous work from our laboratory has indicated that superoxide anion and H₂O₂ are normal metabolites of plant cells (36, 37). Both oxidants are kept at low steady-state levels by the action of superoxide dismutase, catalase, and GSH and ascorbate peroxidases, presumably located at the cytosol of the soybean embryonic axes (10, 37). Mitochondrial H₂O₂ is generated through the primary production of O₂⁻ and its consequent dismutation (15). Mitochondria seem to be the most important cellular source of H₂O₂ (36), but other sources such as ER and peroxisomes should not be excluded.

In plants, Cyt P-450-dependent mono-oxygenases have been described in several species (2). They are involved in important metabolic pathways leading to lignins (19, 33), phytoalexins (24), pigments (20), steroids (32, 38), alkaloids (26), cutins (42), and hydroxy-fatty acids (3, 40). Plant Cyt P-450s are also implicated in the metabolism of exogenous compounds (14, 44, 45) and in the detoxification of herbicides (29). Most of these oxygenases occur in the microsomal fraction, and all are dependent on molecular O₂ and reducing equivalents provided by NADPH or NADH. Microsomal electron transport leads to the generation of active O₂ species, such as H₂O₂, O₂⁻, and ·OH, in animal tissues (34, 35). Reduction of O₂ to superoxide radical in plants has been demonstrated previously by microsomal membranes from carnation flowers, by a plasma membrane-bound NAD(P)H oxidase upon infection of potato tubers with incompatible races of Phytophthora infestans (27), and by radish plasma-lemma vesicles (43).

In the present study, we provide evidence that microsomal membranes from soybean seedlings produce superoxide anion and H₂O₂ at detectable rates in the presence of either NADPH or NADH as cofactor. Because transition metals are absolutely required to catalyze the generation of active O₂ species, the effect of exogenous iron on the generation of O₂ radicals by microsomes isolated from soybean seedlings was evaluated.

MATERIALS AND METHODS

Plant Material

Soybean seeds (Glycine max var Hood) were grown for 7 d in the dark at 26°C over water-saturated filter paper. The final weight of soybean seedlings was 1.17 ± 0.07 g/seedling.

Microsomal Membrane Preparation

Hypocotyls were homogenized for 30 s in a blender with a buffer containing 0.1 M Tris-HCl (pH 7.0), 0.3 M mannitol, 5 mM EDTA, 0.1% BSA, 0.05% cysteine. The homogenate was filtered through several layers of gauze and then centrifuged at 10,000g for 30 min. The resulting supernatant was centrifuged at 100,000g for 60 min. The pellet (microsomal fraction) was resuspended in buffer containing 0.1 M Tris-HCl (pH 7.0), 20% (v/v) glycerol.

Cyt P-450 Content

Cyt P-450 was estimated according to the procedure of Omura and Sato (31).

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Enzyme Assays

NADH and NADPH Cyt c reductase activities were determined, recording changes in A_550 (E = 21 mM cm⁻¹). The reaction mixture contained 0.3 mM potassium phosphate buffer (pH 7.7), 0.4 mM Cyt c, 100 mM NADPH or NADH, and 0.2 to 0.5 mg of microsomal protein. Superoxide dismutase activity was assayed spectrophotometrically at 550 nm by the method of McCord and Fridovich (28), in a reaction medium containing 50 mM potassium phosphate buffer (pH 7.8), 0.1 mM EDTA, 15 mM Cyt c, 50 mM xantine, and xantine-oxidase. The amount of homogenate inhibiting by 50% the reduction of Cyt c was taken as 1 unit of superoxide dismutase activity. Under the conditions of the assay, this amounts to 0.12 μg of superoxide dismutase/mL. Peroxidase activity was measured spectrophotometrically at 430 nm (E = 2.47 mM cm⁻¹) in a reaction medium consisting of 50 mM potassium phosphate buffer (pH 7.0), 5 mM pyrogallol, and 1 mM H₂O₂ (41). Catalase was assayed spectrophotometrically at 240 nm (E = 40 mM cm⁻¹) in a reaction medium consisting of 50 mM potassium phosphate buffer (pH 7.0) (10).

O₂⁻ Generation

The rate of production of O₂⁻ was determined as the superoxide dismutase-sensitive rate of adrenochrome formation, measured at 485 to 575 nm (E = 2.97 mM cm⁻¹) in a Perkin-Elmer dual-wavelength spectrophotometer. The reaction medium consisted of 40 mM potassium phosphate buffer (pH 7.4), 120 mM KCl, 1 mM EDTA, and 1 mM epinephrine (5).

NADH and NADPH Consumption

Cofactor consumption rates were determined spectrophotometrically at 340 nm (E = 6.2 mM cm⁻¹). The reaction mixture contained 100 mM phosphate buffer (pH 7.4), 0.1 mM NAD(P)H, and about 1 mg of microsomal protein in a final volume of 3 mL.

H₂O₂ Generation

The production of H₂O₂ was determined by the generation of formaldehyde from the oxidation of methanol by the catalase-H₂O₂ compound 1 complex (22). Reactions were carried out in a buffer consisting of 40 mM Tris-HCl (pH 7.4), 120 mM KCl, 100 mM methanol, and about 1 mg of microsomal protein in a final volume of 1 mL in the presence of either catalase or azide. Formaldehyde was determined fluorometrically (excitation = 415 nm, emission = 505 nm).

·OH Generation

The production of ·OH or compounds with the oxidizing power of ·OH was assayed by the generation of formaldehyde from DMSO or tert-butyl alcohol (9). The basic reaction system consisted of 40 mM potassium phosphate buffer (pH 7.4), 120 mM KCl, 0.4 mM NAD(P)H, either 30 mM DMSO or tert-butyl alcohol, and about 1 mg of microsomal protein in a final volume of 1 mL. The production of formaldehyde was determined by the Nash reaction (30).

Rate of Reduction of Iron

The reduction of iron was monitored in a reaction system containing 0.1 mM phosphate buffer (pH 7.4), about 0.1 to 0.35 mg of microsomal protein, either 0.5 mM NADPH or 0.5 mM NADH, and 5 mM α,α′-bipyridyl in a final volume of 3 mL. The formation of the stable ferrous-(bipyridyl) complex was measured at 520 nm (E = 14 μM cm⁻¹) (7).

The ferric complexes were prepared by dissolving ferric ammonium sulfate in 0.1 n HCl and then diluting with the respective chelator to the appropriate stock concentration. Ferric ATP was utilized as a 1:20 complex, whereas the other ferric complexes were all utilized as 1:2 complexes.

RESULTS

Mixed-function oxidase activity in microsomes from hypocotyls of soybean seedlings was characterized. Carbon monoxide was passed through a dithionite-reduced sample of soybean seedlings microsomes, and the detected peak at 450 nm was ascribed to Cyt P-450 content (32 ± 3 pmol/mg of protein). When reduction was carried out with NADPH under aerobic conditions, the difference spectrum of soybean microsomes was similar to that of animal tissues, exhibiting a maximum at 425 nm and representing Cyt b₅ content (40 ± 3 pmol/mg of protein). Table I shows the activities of NADPH- and NADH-Cyt c reductases. Higher activity of NADH-Cyt c reductase than NADPH-dependent reductase is consistent with a higher consumption of NADH by the microsomes as compared with NADPH (Table I).

Figure 1 shows O₂⁻ production by microsomal membranes from soybean seedlings in the presence of NADPH. The rate of O₂⁻ generation was calculated from the superoxide dismutase-sensitive rate of adrenochrome formation. A linear response of the rate of O₂⁻ generation to the microsomal protein addition, in the presence of NADPH, was observed (Fig. 1). Similar patterns were obtained in the presence of NADH. The detected rates of O₂⁻ production were 5.0 ± 0.4 and 5.5 ± 0.4 nmoI-min⁻¹mg⁻¹ of protein in the presence of NADPH and NADH, respectively.

The production of H₂O₂ was assayed by determining the peroxidatic activity of catalase toward methanol (30). The rates of H₂O₂ generation by microsomes were 1.40 ± 0.05 and 0.87 ± 0.05 nmoI-min⁻¹mg⁻¹ of protein in the presence of NADPH and NADH, respectively.

The production of formaldehyde from DMSO or tert-butyl alcohol is a rapid and sensitive technique that has been

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<th>Table I. Mixed Function Oxidase Activities and Cofactor Consumption by Microsomes Isolated from Hypocotyls of Soybean Seedlings</th>
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<td>Values are means ± se.</td>
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<td>Cyt P-450</td>
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utilized to evaluate the generation of 'OH or 'OH-like compounds during animal microsomal electron transfer (34, 35). A time course for the oxidation of DMSO is shown in Figure 2 using either NADPH or NADH as cofactor in the presence of ferric EDTA. Essentially similar results were obtained when tert-butyl alcohol was utilized as the 'OH scavenger. Oxidation of either tert-butyl alcohol or DMSO was not detected in the absence of added iron.

Because transition metals, mainly iron, are implicated in radical generation by biological systems (6, 13), the ability of microsomal membranes to reduce iron complexes was studied. Table II shows that, in the presence of either NADPH or NADH, all the tested iron complexes were reduced. Ferric reduction rates were higher with NADH than with NADPH, with all of the ferric complexes utilized.

The effect of the iron complexes on 'OH generation by soybean seedling microsomes was evaluated. Table III indicates that ferric EDTA and ferric DTPA were very effective in catalyzing the oxidation of DMSO by the microsomes, whereas ferric ADP and ferric citrate were not. The pattern of effectiveness of the different ferric complexes was the same with either NADPH or NADH. Because H₂O₂ appears to serve as the precursor of 'OH through an iron-catalyzed reaction, the effect of iron on H₂O₂ generation was assayed. In the presence of NADPH or NADH, either ferric EDTA or ferric DTPA increased significantly the rate of production of H₂O₂, whereas ferric ADP and ferric citrate were not effective (Table III).

The activities of enzymic antioxidants, such as catalase, peroxidases, and superoxide dismutase, were measured in total homogenate from soybean seedlings (Table IV). Vitamin E content in microsomal membranes, evaluated by HPLC, was 1.2 ± 0.1 pmol/mg of protein, suggesting that both enzymic and nonenzymic antioxidants play an active role in the cellular defense system against O₂ toxicity.

**DISCUSSION**

Previous data from our laboratory indicate that the rate of mitochondrial O₂⁻ production and H₂O₂ steady-state levels in soybean embryonic axes at the onset of germination were 0.8 to 1.3 nmol-min⁻¹ mg⁻¹ of mitochondrial protein (37)
and 0.3 μm (36), respectively. Extramitochondrial \(O_2^-\) production was not considered, because at early stages of germination (2 h of incubation) the microsomal contribution seems less important. As soon as an active metabolism was established in the soybean seedling, production of active \(O_2\) species by microsomal membranes became significant. The enzymic activities responsible for radical generation include Cyt P-450, Cyt c reductase, and Cyt b5. Several studies have identified these activities in different plant tissues (4, 23, 39). In the current report, mixed-function oxidase activities were determined in microsomes isolated from soybean hypocotyls, and \(O_2^-\), \(H_2O_2\), and \('OH\) production were measured in the presence of either NADPH or NADH as cofactor.

Microsomal \(O_2^-\) generation was 5.0 ± 0.4 and 5.5 ± 0.4 nmol-min\(^{-1}\)mg\(^{-1}\) of microsomal protein, in the presence of either NADPH or NADH as cofactor, respectively. These values are within the same range of production by peroxisomal membranes from \(Pisum\) sativum L. leaves (11), reported as 10 nmol-min\(^{-1}\)mg\(^{-1}\) of protein, and of cotton roots extracts (2.2 ± 0.9 nmol-min\(^{-1}\)mg\(^{-1}\) of protein) (8). By comparison with other \(O_2\) radicals, \(O_2^-\) was considered rather unreactive (21), even though it was recently reported that \(O_2^-\) can either be deleterious to [4Fe-4S]-containing dehydratase (17, 18) or react with NO to produce peroxinitrite (1). However, the main danger of \(O_2^-\) seems to lie in its ability to dismutate to \(H_2O_2\) and to form \('OH\) (12).

\(H_2O_2\) generation was detected in the presence of either NADPH or NADH as cofactor. The ratio of NADPH-dependent \(H_2O_2\) to NADH-dependent \(H_2O_2\) was 1.7 for soybean seedling microsomes and 5 for rat liver microsomes (12). This difference could be ascribed to a different relative activity of the enzymes involved in plant, as compared to animal, microsomes.

Isolated microsomes from soybean seedlings in the presence of either NADPH or NADH and iron can catalyze the production of \('OH\)-like species as reflected by the production of formaldehyde from \(DMSO\) and \(\text{tert}-\text{butyl alcohol}\). Iron catalytic activity is required for the production of \('OH\). Ferric EDTA and ferric DTPA are effective catalysts for plant microsomal generation of \('OH\), whereas ferric ATP and ferric citrate are poor catalysts. This pattern of effectiveness could be ascribed to the higher redox potential of iron when compared to EDTA or DTPA (25) or could be dependent on iron availability, which is related to the dissociation constant of the complex.

It would appear that the production of \(H_2O_2\) is a better candidate than reduction of the ferric complexes as the limiting step for \('OH\) generation. For example, with ferric EDTA as the iron catalyst, rates of NADPH-dependent \('OH\) production, \(H_2O_2\) generation, and reduction of iron from the ferric to the ferrous state are about 0.50 ± 0.04, 1.40 ± 0.05, and 3.8 ± 0.1 nmol-min\(^{-1}\)mg\(^{-1}\) of protein, respectively. Regarding the nature of the electron donor, iron reduction is significantly higher in the presence of NADH as compared with NADPH. This increased rate of reduction could reflect the higher activity of NADH-Cyt c reductase as compared with NADPH-Cyt c reductase activity (Table I).

At the cellular level, \(O_2\) radical damage is controlled through enzymic and nonenzymic antioxidants. The activities of catalase, peroxidases, and superoxide dismutase measured in the soybean seedling homogenates were higher, as compared with previous data obtained in embryonic axes allowed to imbibe for 2 h (37). The rearrangement of energy-yielding processes during seed development causes an increase in \(O_2\) uptake, leading to a more active production of \(O_2\) radicals, which would determine the need for an increase in the activity of the enzymic antioxidant system. Regarding the nonenzymic antioxidants, it has been reported that tocopherols and tocotrienols are accumulated in organs and tissues with high sensitivity to oxidation, mainly the seeds (16). It is feasible that during germination and growth vitamin E content could be affected, but our data indicate that microsomal membranes contain detectable amounts of vitamin E. Further study is needed to explore the role of vitamin E in protection during growth.

Research on the microsomal contribution to free radical generation will provide a sound basis for an integrative

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<th>Table IV. Enzymic Antioxidants in Soybean Seedling Homogenates</th>
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<th>Antioxidant</th>
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<tr>
<td>Catalase</td>
<td>14.4 ± 1.3 μmol of H(_2)O(_2)·min(^{-1})mg(^{-1}) of protein</td>
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<tr>
<td>Peroxidases</td>
<td>1.1 ± 0.1 nmol·min(^{-1})mg(^{-1}) of protein</td>
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<tr>
<td>Superoxide dismutase</td>
<td>11.5 ± 1.6 units·mg(^{-1}) of protein</td>
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evaluation of cellular total production rates that will contribute to the understanding of the complex role of these species in plant metabolism. The measurement of rates of production of \( \text{O}_2 \) radicals by microsomes, mitochondria, and peroxisomes will allow the calculation of cellular steady-state concentrations in physiological conditions after stress or in aged soybean. After steady-state concentrations are compared, the involvement of radicals in cellular damage could be evaluated. On the other hand, a detailed study of antioxidant status will contribute to the finding of experimental strategies for improving seed germination and plant growth.

LITERATURE CITED

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