The Hormone-responsive NADH Oxidase of the Plant Plasma Membrane Has Properties of a NADH:Protein Disulfide Reductase*

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Pin-Ju Chueh‡, Dorothy M. Morré‡, Claude Penel§, Tammy DeHahn¶, and D. James Morré††

From the Departments of ‡Foods and Nutrition and ††Medical Chemistry and Molecular Pharmacology, Purdue University, West Lafayette, Indiana 47907 and §University of Geneva, Geneva, Switzerland

Plasma membranes of plant cells are characterized by a plant hormone (auxin)-responsive oxidation of NADH. The latter proceeds under argon. Also, when NADH oxidation is stimulated 50% by auxin addition, oxygen consumption is reduced by 40%. These findings are reconciled by direct assays using 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) (Ellman’s reagent) that show protein disulfides to be electron acceptors for auxin-stimulated NADH oxidation. In the presence of an external reducing agent such as NADH, cysteine, or dithiothreitol, protein disulfides of the membrane are reduced with a concomitant stoichiometric increase in free thiols. In the absence of an external reducing agent, or in the presence of oxidized glutathione, DTNB-reactive thiols of the plasma membrane are decreased in the presence of auxins. Several auxin-reducing combinations were effective, but the same reductants plus chemically related and growth-inactive auxin analogs were not. A cell surface location of the affected thiols demonstrated with detergents and impermeant thiol reagents suggests that the protein may have a different physiological role than oxidation of NADH. For example, it may carry out some other role more closely related to the function of the auxin hormones in cell enlargement such as protein disulfide-thiol interchange.

Brightman et al. (1) described an NADH oxidative activity of the plant plasma membrane that was stimulated by the active auxins 2,4-dichlorophenoxyacetic acid (2,4-D),1 indole-3-acetic acid (IAA), and α-naphthaleneacetic acid (α-NAA). The activity was unaffected by benzoic acid and the structurally related but inactive auxin analogs 2,3-dichlorophenoxyacetic acid (2,3-D) and β-naphthaleneacetic acid (β-NAA) (2). Subsequently, the activity was correlated with plant cell elongation using thiol reagents as inhibitors of both auxin-induced cell elongation and the auxin-stimulated oxidation of NADH (3). Both were inhibited by N-ethylmaleimide, p-chloromercuribenzoate, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), reduced glutathione (GSH), or dithiothreitol (DTT). In the absence of auxin, the oxidation of NADH by plasma membranes of soybean hypocotyls was accompanied by an approximately stoichiometric consumption of oxygen (ratio of NADH reduced to 0.5 O2 consumed of 1) (4). However, when oxygen consumption was measured following stimulation of NADH oxidation by 2,4-D, not only was oxygen consumption no longer stoichiometric, it was less than that measured in the absence of 2,4-D (5). Therefore, alternative electron acceptors in the membrane for the 2,4-D-stimulated activity were sought.

Preliminary indications favored disulfides of membrane proteins (5). Plasma membrane vesicles were subsequently found to catalyze a protein disulfide-thiol interchange activity that was auxin-responsive (6). Both the latter and the auxin-stimulated NADH oxidation were sensitive to inhibition by brefeldin A (7). Based on this and other evidence, it was suggested that the two activities (auxin-stimulated NADH oxidation and auxin-stimulated protein disulfide-thiol interchange) might be catalyzed by the same protein of the plasma membrane. In keeping with that suggestion, experiments were conducted to determine if auxin treatment of isolated plasma membrane vesicles would lead to net changes in the thiol or disulfide content of the isolated plasma membranes vesicles. An auxin (2,4-D or IAA)-induced increase in thiols and decrease in disulfides of the plasma membrane were observed in the presence of NADH, cysteine and DTT but less so with GSH. The changes modulated by 2,4-D in the thiols and disulfides in the presence of NADH occurred in stoichiometric proportions to the reducing equivalents coming from NADH. The results suggest that protein disulfides serve as acceptors for NADH reduction by plasma membrane vesicles. The physiologically relevant function of the activity at the cell surface, however, may be the more general catalysis of disulfide-thiol interchange among membrane proteins that occurs in the absence of NADH (6).

EXPERIMENTAL PROCEDURES

Plant Material—Seeds of soybean (Glycine max L. Merr., cv. Williams 82) were soaked 4–6 h in deionized water, planted in moist vermiculite, and grown 4–5 days in darkness at 20–22 °C in foil-covered 18 cm × 25 cm × 10-cm plastic boxes or enamel trays normally without supplemental additions of water. One- or two-cm long segments, cut 5 mm below the cotyledons, were harvested under low laboratory lighting (0.15 μmol of photons s−1 m−2) and used for elongation measurements (1-cm long segments) or isolation of plasma membranes (2-cm long segments).

Isolation of Plasma Membrane—Hypocotyl segments 2 cm long, cut just below the cotyledon, were harvested and placed in cold water. The segments (40 g) were chopped with razor blades in 40 ml of homogenization buffer (0.3 M sucrose, 50 mM Tris-Mes (pH 7.5), 10 mM KCl, 1 mM MgCl2). The homogenate was filtered through one layer of Miracloth (Chicopee Mills, New York, NY) and centrifuged for 10 min at 6,000 × g (HB-rotor). The supernatant was recentrifuged at 60,000 × g (Man SW 28 rotor) for 30 min, and the pellets were resuspended in 0.25 M sucrose with 5 mM potassium phosphate (pH 6.8). Plasma membrane vesicles were prepared using a 16-g aqueous two-phase partitioning system (8). Resuspended 60,000 × g pellets were mixed with 6.4% (w/v) polyethylene glycol 3350 (Fisher), 6.4% (w/v) dextran T7500 (Pharmacia), 0.25 M sucrose, and 5 mM potassium phosphate (pH 6.8). After mixing the tubes by 40 inversions, the phases were separated by cen-
trifugation at 750 × g for 5 min. The lower phase was repartitioned with a fresh upper phase, and the two upper phases were repartitioned twice with fresh lower phases. The upper phases were diluted approximately 4-fold with buffer, and the membranes were collected by centrifugation at 100,000 × g for 30 min. The membranes were assayed fresh (in Figs. 1 and 3 and Table I) or prepared and stored frozen at −70 °C (all other tables and figures) prior to assay. The yield was 1–2 mg of plasma membrane protein.

**NADH Oxidase Activity**—The assay for the plasma membrane NADH oxidase was in 50 mM Tris-Mes buffer (pH 7.0), 150 μM NADH in the presence of 1 mM potassium cyanide, the latter to inhibit any mitochondrial NADH oxidases contaminating the plasma membranes. Thiols were measured (Figs. 2, 5, and 7–9) values at 100,000 × g for 30 min. The membranes were assayed fresh in a Hitachi model U2000 spectrophotometer. The change of absorbance was recorded as a function of time by a chart recorder. The specific activity of the plasma membrane was calculated using an absorption coefficient of 6.21 nmol/mg protein−1 cm−1.

Assays were initiated by addition of NADH. Following the addition of NADH and for each subsequent addition, the assays were continued for 10 min with the steady state rates between 5 and 10 min being reported.

**Incubation of Plasma Membrane Vesicles with 2,4-D**—Plasma membranes (600 μg) resuspended in 200 μl of homogenization medium were incubated with or without 1 or 10 μM 2,4-D for 0, 10, 20, or 30 min at room temperature (23–25 °C). At the times indicated, aliquots of 50 μl were removed and centrifuged immediately for 3 min at 15,000 × g (Eppendorf model 5414). The supernatants were removed, and the tube completely drained of liquid. Thiols or disulfides were determined as follows.

**Determination of Thiols Using DTNB (or Ellman’s Reagent)**—Plasma membranes (150 μg) resuspended in the incubation medium or in water following centrifugation as described above to recover the plasma membranes were combined with 50 μl of 10 mM DTNB and incubated 20 min at room temperature. The membranes were then diluted with 2.5 ml of 0.1 M sodium phosphate, pH 8.0. Absorbance was determined using a Shimadzu UV-160 (Columbia, MD) double wavelength spectrophotometer at 412 nm with reference at 520 nm. Thiol content was estimated from a cysteine standard curve determined in parallel for each assay. Values for control samples without membranes were equivalent to the reagent blank. The absorbance of the reagent blank (∼ control samples) was subtracted for each set of determinations. Standards were unchanged over a 30-min incubation with or without 2,4-D. Absorbance of preparations heated at 80 °C for 10 min did not change over time in response to NADH, for example, either in the presence or absence of 2,4-D. Thiol content of lower phase membranes depleted in plasma membrane vesicles did not respond to 2,4-D over 20 min of incubation. Results were expressed as nanomoles of thiol/mg of protein based on a cysteine standard. For Figs. 1 and 3, reagent and plasma membrane blanks were subtracted at approximate absolute initial thiol levels exposed to DTNB at the cell surface on a protein basis (5.5 ± 0.5 nmol/mg protein). On a molar basis, the content of membrane surface disulfides approximated that of membrane thiols (i.e. 5.5 ± 0.5 nmol/mg of protein). For the tables and figures where time-dependent changes in thiols were combined with 50 mM Tris-Mes buffer (pH 7.0), 150 μM NADH, 0.1 mM NADH. In contrast, preparation of isolated vesicles of plasma membranes increased in response to 2,4-D (Table III).

**Oxygen Consumption Decreased as NADH Oxidation Is Increased**—Measurements of oxygen consumption using an oxygen electrode (11) also were indicative of some acceptor other than oxygen being responsible for the activity stimulated by auxin. While there was an approximately stoichiometric basal rate of NADH oxidation and oxygen consumption, the stoichiometry was not maintained upon auxin addition. Upon auxin addition, NADH oxidation was increased by approximately 50% whereas oxygen consumption was decreased by 40% (Table II). The activity was resistant as well to 150 μM salicylhydroxamic acid, an inhibitor of the alternate cyanide resistant pathway of NADH oxidation in plant mitochondria (12).

**Direct Determination of Thiols Show Increase with 2,4-D**

**Plus Electron Donor (DTT, NADH, or Cysteine)**—Direct determinations of thiols using DTNB (or Ellman’s reagent) in response to treatment with 1 μM 2,4-D from a series of repeated measurements with isolated plasma membrane vesicles are summarized in Table III. After 10 min of 2,4-D treatment with plasma membranes incubated without thiol reagents, the thiol content was reduced significantly by the 2,4-D treatment (Table III). However, if DTT was present, the thiol content of the plasma membranes increased in response to 2,4-D (Table III).

In the presence of dibuthrotetol, the optimum concentration of 2,4-D to induce the thiol increase was found to be in the range of 1 to 10 μM (Fig. 1). The weak auxin analog 2,5-D was without effect at 1 μM as was 1 μM of a weak acid, benzoic acid, lacking auxin activity (Table III). The relatively high absolute thiol content recorded in these experiments may be attributed at least in part to the use of vesicles that had been frozen and thawed. Similar results were obtained comparing the growth-active auxin analog α-NAA and the growth-inactive auxin analog β-NAA (Table IV). Thiols were decreased by α-NAA in the absence of reductant and increased by α-NAA in the presence of 0.1 mM NADH. In contrast, β-NAA was without effect on membrane thiols in both the presence or absence of NADH.

In the absence of DTT, the decrease in thiols in response to

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**Table I**

| Addition | 2,4-D NADH oxidation (nmol/min/mg protein) |
|----------|------------------------------------------|
| None     | None                                      |
| 1 μM     | 5.6 ± 0.9a                                |
| 10 μM    | 8.5 ± 1.2b                               |
| KCN      | None                                      |
| 1 μM     | 5.1 ± 0.4a                                |
| 1 μM     | 7.4 ± 1.0b                               |
| Nitrogen atmosphere | None                                      |
| 1 μM     | 4.3 ± 0.75a                              |
| Argon atmosphere  | None                                      |
| 1 μM     | 5.1 ± 1.5a                               |
| 1 μM     | 11.5 ± 3.0b                              |

**RESULTS**

**Activity Not Inhibited by Cyanide or an Argon or Nitrogen Atmosphere**—Unlike those NADH oxidase activities where oxygen is the acceptor of electrons, the 2,4-D-stimulated oxidation of NADH by isolated vesicles of plasma membrane prepared from etiolated hypocotyls of soybean was unaffected or even stimulated by an argon or nitrogen atmosphere (Table I). A lack of inhibition by cyanide has served as an important criterion to distinguish the plasma membrane oxidase from that of mitochondria where NADH oxidation is cyanide sensitive. Purging the cuvette of oxygen or use of oxygen-purged solutions in combination with an argon or nitrogen atmosphere reduced but did not eliminate NADH oxidation as with an argon or nitrogen atmosphere alone.

**Oxygen Consumption Decreased as NADH Oxidation Is Increased**—Measurements of oxygen consumption using an oxygen electrode (11) also were indicative of some acceptor other than oxygen being responsible for the activity stimulated by auxin. While there was an approximately stoichiometric basal rate of NADH oxidation and oxygen consumption, the stoichiometry was not maintained upon auxin addition. Upon auxin addition, NADH oxidation was increased by approximately 50% whereas oxygen consumption was decreased by 40% (Table II). The activity was resistant as well to 150 μM salicylhydroxamic acid, an inhibitor of the alternate cyanide resistant pathway of NADH oxidation in plant mitochondria (12).

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**Table II**

| Addition | 2,4-D NADH oxidation (nmol/min/mg protein) |
|----------|------------------------------------------|
| None     | None                                      |
| 1 μM     | 5.6 ± 0.9a                                |
| 10 μM    | 8.5 ± 1.2b                               |
| KCN      | None                                      |
| 1 μM     | 5.1 ± 0.4a                                |
| 1 μM     | 7.4 ± 1.0b                               |
| Nitrogen atmosphere | None                                      |
| 1 μM     | 4.3 ± 0.75a                              |
| Argon atmosphere  | None                                      |
| 1 μM     | 5.1 ± 1.5a                               |
| 1 μM     | 11.5 ± 3.0b                              |
NADH oxidation and oxygen uptake by plasma membrane vesicles prepared from hypocotyls of etiolated soybean seedling

Oxygen uptake was determined using an oxygen electrode (11) by one of us (D. J. M.) in collaboration with the laboratory of Professor James Siedow, Duke University, Durham, NC. The number of determinations is given in parenthesis. Results are ± S.D., n = 3 or ± mean deviations, n = 2. SHAM = salicylhydroxamic acid.

| Addition | NADH disappearance | 0.5 O2 consumption |
|----------|--------------------|--------------------|
| None (3) | 4.2 ± 0.2          | 4.3 ± 0.5          |
| 2,4-D, 1 µM (3) | 6.3 ± 0.2          | 2.7 ± 0.3          |
| SHAM, 150 µM (3) | 5.3 ± 0.2          | 3.5 ± 0.4          |
| SHAM, 150 µM, 1 µM 2,4-D (2) | 7.5 ± 0.2          | 2.65 ± 0.15        |

Estimation using DTNB of the surface thiol content of freshly prepared plasma membranes of soybean hypocotyls (0.15 mg plasma membrane protein) as influenced by a 10-min treatment with 2,4-D, 2,3-D, or benzoic acid, 1 µM final concentration

Values are from four replicates each consisting of averages of four independent determinations ± S.D. among the four replicates. Values not followed by the same letter were significantly different (p < 0.05) as determined by a two-tailed t test.

| Conditions | Treatment (10 min) | Surface thiol content |
|------------|--------------------|-----------------------|
| No DTT     | No auxin 2,4-D     | 11.6 ± 1.9            |
|            | 1 µM 2,4-D         | 5.5 ± 2.8             |
|            | 1 µM 2,3-D         | 12.2 ± 3.0            |
|            | 1 µM Benzoic acid  | 10.0 ± 1.9            |
| +10 µM DTT | No auxin 2,4-D     | 12.6 ± 0.4            |
|            | 1 µM 2,4-D         | 16.3 ± 1.0            |
|            | 1 µM 2,3-D         | 12.6 ± 1.3            |
|            | 1 µM Benzoic acid  | 13.5 ± 2.0            |

THIOLS, NMOLES/MG PROTEIN

FIG. 1. 2,4-D-induced increase in surface thiol content of freshly prepared soybean plasma membranes as a function of 2,4-D concentration in the presence of 10 µM DTI. Thiolos were determined by reaction with DTNB. The results are from a single experiment but the findings were repeated (e.g. Fig. 3) with consistent results.

2,4-D (10 µM) was rapid and sustained for about 20 min (Fig. 2A). In the presence of DTT, the 2,4-D resulted in an increase in thiols that also was sustained for at least 20 min (Fig. 2B). Relative to controls (no 2,4-D), the net increase in thiols in response to 2,4-D was 5.5 nmol/mg of protein over 20 min. Overall, membrane thiols did increase with dithiothreitol treatment including inclusion of dithiothreitol in the incubation medium. However, the differences were small (≤3 nmol/mg of protein) and the 2,4-D response was not affected by DTT preincubation compared with simultaneous DTT plus 2,4-D pretreatment.

Thiols Increased by Treatment with 2,4-D and Electron Donor Located at or Near the External Membrane Surface—The thiols appeared to be those accessible to DTNB at or near the membrane surface (Fig. 3). The increase in thiols in response to 2,4-D plus 10 µM DTT was essentially unchanged as the membranes were incrementally treated by concentrations of 0, 0.1, and 1% Triton X-100. In contrast, total thiols reactive with DTNB were incrementally increased by the treatment with detergent. Triton X-100 was determined to not interfere in the thiol assay over the range of concentrations reported in Fig. 3. The plasma membrane vesicles used in these studies were 50 to 70% right side-out based on measurements of ATP latency.

The membrane impermeant thiol reagent p-chloromercuri-

FIG. 2. Surface thiol content of soybean plasma membranes in response to 10 µM 2,4-D and no 2,4-D in the absence (A) and presence (B) of 10 µM DTI. Assays were with 0.15 mg of membrane protein. Membranes were prepared and stored frozen at −70 °C prior to assay. Results are from three different experiments ± S.D. among experiments. Differences at 10 and 20 min were significantly different for 2,4-D compared with no 2,4-D (p < 0.05).

Plasma Membrane NADH:Protein Disulfide Reductase

Estimation of the surface thiol content of plasma membranes of soybean hypocotyls (0.15 mg plasma membrane protein) as influenced by a 20-min treatment with α-NAA (growth active) or β-NAA (growth inactive) in the presence or absence of 0.1 mM NADH

Membranes were prepared and stored frozen at −70 °C prior to assay. Values are from three replicates ± S.D. among replicates. Values not followed by the same letter were significantly different (p < 0.03) as determined by a two-tailed t test.

| Conditions | Treatment (20 min) | Surface thiol content |
|------------|--------------------|-----------------------|
| No NADH   | No auxin 2,4-D     | 17 ± 4            |
|            | 10 µM α-NAA        | 5 ± 2             |
|            | 10 µM β-NAA        | 20 ± 3            |
| +0.1 mM NADH | No auxin 2,4-D | 8 ± 3             |
|            | 10 µM α-NAA        | 27 ± 6            |
|            | 10 µM β-NAA        | 14 ± 2            |

FIG. 4. These observations support the detergent obser-
vations of Fig. 3, which, when taken together, suggest that the thiol groups increased by 2,4-D and involved in the growth process are located near the external surface of the plasma membrane.

Protein Disulfides Decreased by Treatment with 2,4-D and Electron Donor in Proportion to the Increase in Protein Thiols—

When protein disulfides were estimated by use of NTSB according to the method of Thannhauser et al. (9), the plasma membrane levels of protein disulfides decreased linearly with NADH addition in the presence of 2,4-D (Fig. 5B) in approximate inverse proportion to the thiol content (Fig. 5A). In the presence of NADH, both the auxin-stimulated increase in protein thiols of the plasma membrane vesicles (Fig. 5A) and an auxin-stimulated decrease in protein disulfides of the plasma membrane vesicles (Fig. 5B) were proportional to time of incubation over 30 min.

With NADH present, the net increase in thiols in response to 10 mM 2,4-D in the presence of NADH was 13.4 nmol/mg of protein over 20 min. The response due to 2,4-D in the absence of reducer over 20 min was -13.0 nmol/mg of protein compared with no 2,4-D (Table V). The net change in thiols in response to NADH for the 2,4-D-treated vesicles was therefore 26.4 nmol/mg of protein/20 min or 1.32 nmol/min/mg of protein. The rate of 1.2 nmol/min/mg of protein of Table VI was calculated in a similar manner.

With NADH present, the average rate of disappearance of disulfides of the plasma membrane vesicles in response to 2,4-D was 0.6 nmol/min/mg of protein over 20 min. This rate compared closely to the rate of 2,4-D-stimulated oxidation of NADH measured in parallel (Table VI). By comparison, the rate of thiol appearance stimulated by NADH and 2,4-D (e.g., Fig. 5A) was approximately 1.2 nmol/min/mg of protein over 20 min of incubation (Table VI) above the rate with no addition (e.g., Fig. 2A).

In the presence of 0.1 mM NADH, the 2,4-D-induced increase in protein thiols was proportional to plasma membrane protein.
mixture of 0.1 mM GSH plus 0.1 mM GSSG, neither the thiol nor observed in the absence of GSH (Fig. 9, A)
C GSSG had an opposite effect (Fig. 9, B) compared to no GSH. Similar to that observed for 10 microM 2,4-D (Fig. 9, E) and accompanied by a corresponding net increase in thiols of the isolated plasma membrane vesicles compared with no IAA (Fig. 8B). The magnitude of the response to 10 microM IAA was similar to that observed for 10 microM 2,4-D. The experiments of Fig. 8 with IAA and NADH were conducted in parallel to those of Fig. 2 with 2,4-D and DTT and the results were similar.

Comparison of specific activities of auxin (10 microM 2,4-D)-stimulated NADH oxidation and changes in protein disulfides and thiols in the presence of 0.1 mM NADH at the surface of isolated vesicles of soybean plasma membranes

Results are means of rates averaged over the first 20 min of auxin addition from three experiments ± S.D. Membranes were prepared and stored at -70 °C prior to assay.

| Parameter | No 2,4-D | +10 microM 2,4-D | Δ 2,4-D | n mole/min/mg protein |
|-----------|----------|----------------|---------|----------------------|
| Thiol appearance | 0.4 ± 0.1 | 1.6 ± 0.14 | 1.2 | |
| Disulfide disappearance | 0.25 ± 0.02 | 0.8 ± 0.05 | 0.55 | |
| NADH oxidation | 1.1 ± 0.1 | 1.75 ± 0.2 | 0.65 | |

**TABLE V**

**Summary of changes in thiols and disulfides of the membrane surface after 20 min of incubation in the absence or presence of 10 microM 2,4-D**

| Reductant | Seed lot | 2,4-D-induced changes in plasma membrane thiols or disulfides over 20 min | Membrane storage | Table or figure | Thiols | Disulfides x 2 |
|-----------|----------|-------------------------------------------------|-----------------|----------------|--------|---------------|
| None      | II       | None                                            | Table III<sup>a</sup> | −12.2          | 8      |               |
|           | III      | −70 °C                                          | Fig. 9          | −17.0          | 16     |               |
| GSSG      | IV       | −70 °C                                          | Fig. 8          | −11.3          | 16     |               |
| GSH       | III      | −70 °C                                          | Fig. 9          | −13.0          | 16     |               |
| DTT       | I        | None                                            | Table III<sup>a</sup> | −3            | 28     |               |
|           | II       | None                                            | Fig. 1          | 9.5            |        |               |
| Cysteine  | III      | −70 °C                                          | Fig. 2          | 5.5            |        |               |
| NADH      | III      | −70 °C                                          | Fig. 3          | 5.4            |        |               |
|           |          | −70 °C                                          | Mean (DTT) 7.0 ± 1.9 | 13.3 ± 2.5 | 13.4 ± 2.4 |               |
|           |          | −70 °C                                          | Mean (cysteine and NADH) 13.4 ± 2.4 | 13.4 ± 2.4 | 13.4 ± 2.4 |               |

* Treated for 10 rather than 20 min. Values were doubled for purposes of comparison.

**DISCUSSION**

An NADH oxidative activity of unknown function has been observed in plasma membrane of soybeans as an activity with a 2,4-D-responsive component (1, 2). A component of the basal activity was first thought to represent an NADH-ascorbate free radical oxidoreductase (13). However, the auxin-responsive component of the activity (6 nmol/min/mg of protein out of 30 nmol/min/mg of protein in the presence of monodehydroascor-
bate) was observed in the absence of either added ascorbate or added dehydroascorbate (13). Thus, the auxin-responsive activity represented NADH oxidation with some constituent other than the ascorbate radical as electron acceptor. The activity was subsequently purified by Brightman et al. (1). The constitutive oxidation of NADH, but not the auxin-responsive activity, was observed to be slowed but not eliminated by an argon atmosphere free of oxygen (4). The stoichiometry of NADH reduced to 0.5 O₂ consumed for the constitutive activity was near unity (4). In contrast to the constitutive activity, the 2,4-D-stimulated oxidation of NADH was subsequently shown to be unaffected by an argon atmosphere and not accompanied by a corresponding increase in oxygen consumption (5). Surprisingly, oxygen consumption was actually inhibited by auxin (5) (Table II).

The above observations led to considerations of substrates present in the membrane other than oxygen that might serve as electron and proton acceptors for the auxin-stimulated activity. One possibility suggested by present observations is disulfides of plasma membrane proteins. Morré (14) and Spring et al. (15) had earlier observed that protein thiols increased in response to auxin but experiments with isolated plasma membrane vesicles were lacking. A role of the auxin-stimulated NADH oxidase in disulfide reduction also would be consistent with experiments where the auxin-stimulated activity was shown to be inhibited by thiol reagents (3, 16). Additionally, soybean plasma membranes were found to exhibit an auxin-stimulated restoration of activity to inactive scrambled RNase. Here, activity was restored as interchain disulfides were reformed under conditions of reduction followed by reoxidation under non-denaturing conditions (6).

## Figure 7
Surface thiol content of soybean plasma membranes in response to 10 μM 2,4-D and no 2,4-D in the presence of 0.1 mM NADH (A) or 0.1 mM cysteine (B). Assays were with 0.15 mg of membrane protein. Membranes were prepared and stored at −70 °C prior to assay. Results are from six determinations ± S.D. Values at 20 and 30 min were significantly different for 2,4-D compared with no 2,4-D (p < 0.05).

## Figure 8
Surface thiol content of soybean plasma membranes in response to 10 μM IAA and no IAA in the absence (A) and presence (B) of 0.1 mM NADH. Assays were with 0.15 mg of membrane protein. Membranes were prepared and stored at −70 °C prior to assay. Results are from three different experiments ± S.D. among experiments. Values at 20 and 30 min were significantly different for 2,4-D compared with no 2,4-D (p < 0.05).

## Figure 9
Response of surface thiol (right) and disulfide content (left) of soybean plasma membranes to the presence or absence of 0.1 mM reduced or oxidized glutathione alone or in combination with or without 10 μM 2,4-D. Assays were with 0.15 mg of membrane protein. Membranes were prepared and stored at −70 °C prior to assay. A and D, no addition. B and E, 0.1 mM GSH. C and F, 0.1 mM GSSG. Results are from triplicate determinations in each of three different experiments ± S.D. among experiments. Significant differences for 2,4-D compared with no 2,4-D (p < 0.05) were observed only with the 20-min points of A and C, the 30-min point of F, and the 10- and 20-min points of E.
thiol formation, disulfide disappearance and NADH oxidation were stoichiometric over 20 min of auxin treatment. These relationships support the contention that the membrane acceptor for the auxin-stimulated oxidation of NADH by isolated vesicles of soybean may be disulfides of membrane proteins as indicated initially from inhibitor data (3, 16) and measurements of oxygen consumption (5). The findings do not eliminate the possibility that the electron acceptor for the constitutive basal NADH oxidase activity of soybean plasma membranes may be oxygen or some combination of oxygen and protein disulfides. In this regard, auxin might act as a switch causing the plasma membrane NADH oxidase to favor reduction of protein disulfides over oxygen (5, 16). Evidence for disulfide reduction, as one manifestation of a potentially more general role in protein disulfide-thiol interchange, may provide a mechanism to eventually help explain the physiological function of the auxin-stimulated activity in auxin-regulated plant cell enlargement.

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