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# NADH oxidase activity present on both the external and internal surfaces of soybean plasma membranes

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**Abstract**

Soybean plasma membranes exhibit NADH oxidase activities accessible to NADH supplied to either the external or internal membrane surfaces. Activity at the external surface was demonstrated using plasma membranes of right side-out orientation prepared by aqueous two-phase partition and using intact soybean cells grown in culture. Activity at the internal membrane surface was demonstrated using vesicles of inside-out orientation obtained by preparative free-flow electrophoresis or by aqueous two-phase partition following freeze–thaw or Brij detergent treatment to invert some of the right side-out vesicles. The NADH oxidase activity of both membrane surfaces appeared to be stimulated by the synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D). Following the freeze–thaw treatment utilized to evert the vesicles, however, the NADH oxidase activity of right side-out vesicles was greatly reduced. A 2,4-D-stimulated activity of these vesicles could be restored by treatment first with reduced glutathione and then with hydrogen peroxide or oxidized glutathione. Upon treatment with 0.1% Triton X-100, both right side-out and inside-out vesicles exhibited approximately the same specific activity and the same 2,4-D-stimulated component of activity equal to the sum of the activities of the right side-out and inside-out vesicles assayed separately. These findings suggest that a significant NADH oxidase activity of the soybean plasma membrane responsive to 2,4-D resides on the external plasma membrane surface. This latter activity is unlikely to function under physiological conditions in the direct oxidation of NADH and alternative functions, such as in protein disulfide–thiol interchange, would be more appropriate. © 1997 Elsevier Science B.V.

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## 1. Introduction

A characteristic of the plant plasma membrane is the ability to oxidize NADH as determined by decrease in absorbance at 340 nm [1]. A portion of this activity is stimulated by both natural and synthetic plant hormones of the auxin type such as 2,4-dichlorophenoxyacetic acid (2,4-D) [2,3]. The stimulation is not given by the inactive, but chemically related

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; DTT, dithiothreitol; GSH, reduced glutathione; GSSG, oxidized glutathione; IAA, indole-3-acetic acid; NTSB, 2-nitro-5-thiosulfobenzoate; PCMB, *p*-chloromercuribenzoate; SHAM, salicylhydroxamic acid

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auxin analog 2,3-dichlorophenoxyacetic acid (2,3-D) or by β-naphthyleneacetic acid (β-NAA) [4] which is a stereoisomer of the active α-naphthyleneacetic acid (α-NAA) [4]. Previous experiments were largely with plasma membrane vesicles prepared by aqueous two-phase partition. Since NADH is regarded as an impermeant substrate, it was likely that soybean plasma membranes exhibited an external activity capable of oxidizing NADH. As plasma membrane vesicles obtained by aqueous two-phase partition are largely right side-out [5,6], it would follow that the 2,4-D-stimulated NADH oxidase might also be located at the external membrane surface.

In this report, different methods of plasma membrane preparation were compared to demonstrate that plasma membrane of soybean exhibit NADH oxidase activities at both the external and internal membrane surfaces. Studies of vesicles of known absolute orientations demonstrated that the activity at the external membrane surface observed with right side-out vesicles was the principal site of the 2,4-D-stimulated activity.

## 2. Materials and methods

### 2.1. Plant material

Soybeans (*Glycine max* L. Merr. cv Wayne) were germinated and grown in moist vermiculite for 5 days in a dark cabinet. On day 5, hypocotyl segments were excised with a razor blade (2-cm sections from the hook downwards) and collected directly into cold distilled water. In experiments of Tables 1 and 2, the sections were infiltrated under vacuum for 5 min with a medium consisting of 0.25 M sucrose, 25 mM Tris-Mes, pH 7.5, 10 mM KCl and 1 mM $MgCl_2$. The harvest yielded between 30 and 50 g hypocotyl segments. The segments were divided into three to five 10-g portions for homogenization using a Waring blender at full speed (three 10 s bursts alternating with 10 s cooling) in 10 ml aliquots of the above isolation medium plus 1 mM PMSF and 0.1% BSA. After the first homogenization, the slurry was strained through a layer of Miracloth and the residue rewashed with 10 ml of fresh isolation medium. The strained suspensions were combined at the end and used for isolating a microsomal fraction by centrifugation. The first centrifugation to remove cell wall material, nuclei and other large particles was in a Sorval centrifuge with a HB-4 rotor at $10{,}000 \times g$ for 10 min. The supernatant was transferred to a Spinco SW-27 rotor and centrifuged at $60\,000 \times g$ for 30 min. The microsomal pellets were suspended in free-flow electrophoresis buffer or in 0.5–1 ml of 5 mM potassium phosphate buffer, pH 6.8, for two-phase partition.

### 2.2. Isolation of right side-out plasma membrane vesicles by aqueous two-phase partition

The isolation of plasma membranes from soybean hypocotyls by partition in a two-phase polymer system also has been described [1]. Basically, the method used here was developed by Kjellbom and Larsson [7] for the isolation of plasma membranes from spinach leaves, but with a modified polymer composition of the two phases. One g of soybean microsomal membrane suspension was added to the two-phase system to give a final composition of 6.4% (w/w)

Table 1
A comparison of NADH oxidation with $0_2$ uptake rates by NADH oxidase from whole soybean cells and plasma membranes from soybean hypocotyls

| Source | nmol · mg protein$^{-1}$ · min$^{-1}$ | | |
|---|---|---|---|
| | NADH oxidized | $O_2$ uptake | Ratio |
| Right side-out plasma membranes (two phase) | 5.5 ± 0.9 | 2.2 ± 0.0 | 1.25 |
| Inside-out plasma membranes (FFE) [a] | 3.0 ± 0.2 | 1.4 ± 0.15 | 1.07 |
| Cultured soybean cells | 1.1 ± 0.1 [b] | 0.75 ± 0.7 [b] | 0.74 |

Results are from a minimum of 3 determinations ± S.D.
[a] Free-flow electrophoresis.
[b] The $O_2$ uptake rates and NADH oxidation rates for whole cells are given on the basis of μmol · g dry wt.$^{-1}$ · min$^{-1}$.

Dextran T500 (Pharmacia) and 6.4% (w/w) Carbowax (polyethylene glycol) 3350 (Fisher Scientific), 0.25 M sucrose and 5 mM potassium phosphate, pH 6.8. The system was mixed by inversion 40 × in 30 ml glass centrifuge tubes and separated into two phases by centrifugation (1000 × *g* for 5 min). The upper phase, enriched in plasma membranes, was partitioned twice against a fresh lower phase and separated by centrifugation as before. The lower phase was repartitioned against a fresh upper phase, followed by a fresh lower phase to collect and wash additional plasma membranes. Finally, all upper phases were combined, diluted with cold distilled water or with 5 mM potassium phosphate buffer, pH 6.8 and centrifuged in a SW-27 rotor at 120 000 × *g* for 45 min to pellet the isolated plasma membranes, which were finally suspended in 0.5–1 ml of 25 mM Tris-Mes containing 0.25 M sucrose or distilled water.

### *2.3. Isolation of inside-out plasma membrane vesicles by preparative free-flow electrophoresis*

Preparative free-flow electrophoresis of the microsomal fraction from soybean hypocotyls was performed as described by Canut et al. [8] to yield an '*E*' fraction with a predominantly cytoplasmic side-out orientation.

### *2.4. Isolation of inside-out plasma membrane vesicles produced by freezing and thawing*

Plasma membrane vesicles, frozen and thawed four times to invert some of the vesicles, were subfractionated by repeating the phase partition step. The lower phase was enriched in inside-out vesicles. The upper phase contained the remaining right side-out vesicles [9]. The vesicles were collected by centrifugation at 120 000 × *g* for 45 min and resuspended in 25 mM Tris-Mes pH 7, containing 0.25 M sucrose.

### *2.5. Isolation of inside-out plasma membrane vesicles produced by Brij 58 ($C_{16}E_{20}$) treatment*

Plasma membrane vesicles, freshly prepared and stored frozen at −70°C were resuspended in the detergent Brij 58 ($C_{16}E_{20}$) to a final concentration of 0.5% (w/w). This treatment instantaneously produces 100% sealed, inside-out vesicles from preparations of 80–90% right side-out vesicles [10]. The inside-out vesicles were collected by centrifugation at 120 000 × *g* for 45 min and resuspended in 25 mM Tris-Mes, pH 7, containing 0.25 M sucrose. The Brij 58 supernatant was retained and assayed for NADH oxidase activity as well.

### *2.6. Measurement of oxygen uptake*

Oxygen uptake by isolated plasma membranes was measured with a Yellow Springs Oxygen Monitor at 24°C in the presence of 1 mM KCN in a total volume of 1.5 ml. Since oxygen uptake was slow, 0.5–1 mg membrane protein was required per assay. The oxygen uptake rate was initiated by adding 150 μM NADH to membranes in 25 mM Tris-Mes, pH 7. Reaction rates were recorded with a Sargent–Welch recorder.

### *2.7. Measurement of NADH oxidation by plasma membrane vesicles*

NADH oxidation was measured spectrophotometrically with a DW-2a SLM 2000 double-beam spectrophotometer in the dual mode, measuring the disappearance of NADH at 340 nm minus 430 nm or using a Hitachi U3210 spectrophotometer measuring only the decrease in absorbance at 340 nm. The assays were performed at 24°C with stirring by a magnetic stirring assembly. Individual reaction mixtures contained 1 mM KCN, 50 mM Tris-Mes, pH 7, and

Table 2
Characteristics of NADH oxidase of whole soybean cells compared to plasma membrane vesicles isolated from soybean hypocotyls

| Membrane type | Parameters of NADH oxidation | |
|---|---|---|
| | $K_m$ | $V_max$ |
| Two-phase plasma membrane | 200 μM NADH | 10.0 nmol NADH · mg protein$^{-1}$ · min$^{-1}$ |
| Plasma membrane isolated by free-flow electrophoresis | 125 μM NADH | 5.7 nmol NADH · mg protein$^{-1}$ · min$^{-1}$ |
| Whole cells | 35 μM NADH | 175.0 nmol NADH · g dry wt$^{-1}$ · min$^{-1}$ |

Table 3
The response of NADH oxidase of isolated plasma membranes and whole cells of soybeans to substances inhibiting or stimulating the activity

| Substance | Concentration (μM) | Right side-out plasma membrane by two-phase partition | | Inside-out plasma membrane by free-flow electrophoresis | | Whole cells | |
|---|---|---|---|---|---|---|---|
| | | Reaction rate [a] | Stimulation (+) or inhibition (−) (%) | Reaction rate [a] | Stimulation (+) or inhibition (−) (%) | Reaction rate [b] | Stimulation (+) or inhibition (−) (%) |
| None | – | 5.5 ± 0.9 | – | 2.9 ± 0.5 | – | 1.13 ± 0.14 | – |
| KCN | 1000 | 5.1 ± 0.4 | −7 | 2.9 ± 0.3 | 0 | 0.29 ± 0.06 | −74 |
| Sodium molybdate | 500 | 4.4 ± 0.2 | −20 | 2.7 ± 0.3 | −7 | 1.10 ± 0.03 | −3 |
| PCMB | 10 | 3.7 ± 0.1 | −33 | 1.8 ± 0.4 | −38 | 0.59 ± 0.14 | −48 |
| SHAM | 1000 | 13.5 ± 0.5 | +146 | 4.7 ± 0.6 | +62 | 4.20 ± 0.72 | +272 |
| 2,4-D | 1 | 9.4 ± 1.3 | +71 | 7.0 ± 0.7 | +141 | – | – |

Spectrophotometric assays were performed as described in Section 2. Membrane fractions and whole cells were incubated with the test substance for 3 min before the addition of NADH.
[a] nmol · mg protein$^{-1}$ · min$^{-1}$.
[b] μmol · g dry wt.$^{-1}$ · min$^{-1}$.

40–50 μg plasma membrane protein. After a 3-min equilibration period, 150 μM NADH was added. Reaction rates at a full scale absorbance of 0.02 or 0.06 were recorded with a linear recorder. The rates of NADH oxidation were calculated using a millimolar extinction coefficient of 6.22 for NADH. Samples stored on ice in a cold cabinet or frozen for more than 48 h required the addition of 1 μM GSH for 10 min followed by 0.03% hydrogen peroxide or 100 μM GSSG to restore the NADH oxidase activity.

### *2.8. Measurement of NADH oxidation by cultured soybean cells*

NADH oxidation by soybean cells grown in tissue culture was measured as follows: cells were harvested by centrifugation in an unrefrigerated table model, international centrifuge at 1500 rpm (setting 4) for 2 min. They were rewashed three times with fresh sucrose/salts solution (0.1 M sucrose with 10 mM each of KCl, NaCl and $CaCl_2$). The washed cells were suspended in 50 ml sucrose/salts solution and kept on a reciprocal shaker. Aliquots of cells were removed for assays as needed. NADH oxidation by whole cells was also assayed spectrophotometrically as the difference between 340 and 430 nm. The reaction mixture contained soybean cells (about 5 μg dry wt. per assay) in sucrose/salts solution and 25 mM Tris-Mes, pH 7. 100 μM NADH was added after 3 min incubation period to start the reaction. The reaction rates for NADH oxidation were calculated using a millimolar extinction coefficient of 6.22.

## 3. Results

### *3.1. NADH oxidase activity observed with both right side-out and inside-out plasma membranes vesicles*

NADH oxidase activity based on the decrease in absorbance at 340 nm was exhibited by plasma membrane vesicles prepared to yield predominately right side-out (aqueous two-phase partition) or inside-out (preparative free-flow electrophoresis) orientation (Table 1). Vesicle orientation was confirmed in parallel for each experimental series either from measurements of ATP latency or from parameters such as electrophoretic mobility [8]. The specific activities

were similar for the two types of preparations although, in these experiments, the vesicles prepared by aqueous two-phase partition exhibited a higher specific activity than those prepared by preparative free-flow electrophoresis. When NADH oxidation was estimated based on oxygen uptake, rates were similar and the ratio of NADH oxidation to $1/2$ $O_2$ consumption was approximately one. NADH is an impermeant substrate and oxidation rates were determined for cultured soybean cells as well.

Kinetic parameters were similar (Table 2) comparing plasma membrane vesicles prepared by aqueous two-phase partition and by free-flow electrophoresis. The $K_m$ for NADH determined for plasma membranes isolated by aqueous two-phase partition of 200 μM was similar to that of 125 μM determined for plasma membranes obtained by preparative free-flow electrophoresis. The $K_m$ for NADH for intact soybean cells was 35 μM. The values for $V_{max}$ for the two different plasma membrane preparations (Table 2) were in the same relative proportions as were the specific activities of Table 1.

Activities of plasma membranes both isolated by aqueous two-phase partition and by preparative free-flow electrophoresis were resistant to inhibition by cyanide or sodium molybdate and were stimulated by salicylhydroxamic acid (SHAM) (Table 3). *p*-Chloromercuribenzoate (PCMB) inhibited both activities (Table 3). The 2,4-D stimulation was reduced by PCMB, but unaffected by SHAM (not shown).

### *3.2. NADH oxidase of right side-out vesicles stimulated by auxin*

The plasma membrane vesicles prepared by aqueous two-phase partition have been previously shown to be predominantly right side-out and the activity of these preparations was stimulated by auxins (Table 3) [2–4,11]. The plasma membrane vesicles isolated by preparative free-flow electrophoresis also were stimulated by auxin (Table 3). The vesicles prepared by aqueous two-phase partition, while predominantly right side-out, may contain some vesicles of a predominantly inside-out orientation and vice versa for the vesicles isolated by preparative free-flow electrophoresis [8,12]. Latency of ATPase activity was used as a measure of an enzymatic activity localized to the inside surface of the plasma membrane (Table 4). The ATPase activity of vesicles prepared by aqueous two-phase partition was 76–79% latent indicating up to 24% inside-out vesicles in these preparations. Similarly with preparative free-flow electrophoresis, the latency of the inside-out vesicles purified by preparative free-flow electrophoresis was between 15 and 30%. This suggested that between 70 and 85% of the vesicles, but not all, were inside-out. Therefore, two additional methods were used to prepare inside-out and right side-out vesicles and the preparations were examined for auxin responsiveness.

### *3.3. Right side-out plasma membrane vesicles everted by repeated freeze–thaw cycles*

Plasma membrane vesicles prepared by aqueous two-phase partition were frozen and thawed to evert some of the vesicles. When fractionated a second time by aqueous two-phase partition, such preparations yielded predominantly right side-out vesicles in the second upper phase and predominantly inside-out vesicles in the second lower phase as determined previously based on latency measurements [5,8]. The specific activity of the NADH oxidase tended to decline with successive freezing and thawing cycles (Fig. 1, Table 5). Whereas, the supernatant from the 6 freeze–thaw cycles contained NADH oxidase activity, this activity represented only about 5% of the total and could not account for the losses accompanying freezing and thawing. The losses in activity upon freezing and thawing varied in extent among different preparations from ca. 20 to $>90\%$. 2,4-D-responsive

Table 4
Latency of ATPase activity of plasma membrane vesicles obtained from soybean hypocotyl microsomes by aqueous two-phase partition and free-flow electrophoresis in series

| Fraction | ATPase latency (%) |
|---|---|
| Aqueous two-phase partition | 76–79 |
| Preparative free-flow electrophoresis, inside-out | 15–30 |
| Preparative free-flow electrophoresis, right side-out | 60–70 |

The ATPase latencies were determined from specific activities measured in the presence and absence of Triton X-100 at a near optimum Triton X-100 to protein ratio of 1:1.8.

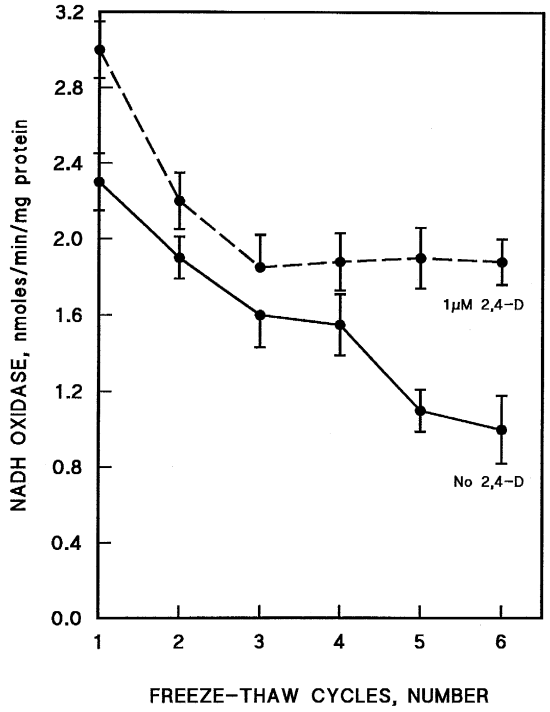


Fig. 1. NADH oxidase activity of plasma membrane vesicles from etiolated soybean hypocotyls in response to 6 cycles of freezing and thawing. With each successive cycle of freezing and thawing, activity was reduced both in the presence and absence of 2,4-D, but the reduction was proportionately greater in the absence of 2,4-D than in its presence. Results are from 3 determinations ± S.D.

activity also was reduced as a result of the freeze–thaw treatments and the reductions were sometimes proportionately greater (Table 5) and sometimes proportionately less (Fig. 1) than for the 2,4-D-unresponsive activities.

Table 5
Loss of 2,4-D-responsive NADH oxidase activity upon freezing and thawing and preparation of right side-out and inside-out plasma membrane vesicles

| Fraction | NADH oxidase activity (nmol · mg protein$^{-1}$ · min$^{-1}$) | |
|---|---|---|
| | No 2,4-D | +1 μM 2,4-D |
| Starting vesicles | 2.3 ± 0.2 | 3.0 ± 0.3 |
| After 6× freeze–thaw | | |
| Vesicle pellet | 1.4 ± 0.4 | 1.6 ± 0.15 |
| Soluble supernatant | 2.4 ± 0.5 | 2.0 ± 0.4 |
| After two-phase partition of pellet | | |
| Upper phase (right side-out) | 2.6 ± 0.15 | 1.8 ± 0.5 |
| Lower phase (inside-out) | 5.0 ± 0.2 | 7.6 ± 0.6 |

Vesicles were frozen and thawed six times. Right side-out and inside-out vesicles were prepared and separated by aqueous two-phase partition as described in Section 2.6.

When separated by a second aqueous two-phase partition, the upper phase containing the right side-out vesicles was usually devoid of auxin-stimulated activity and 1 μM 2,4-D frequently inhibited the activity (Table 5). In contrast, the lower phase fractions enriched in inside-out vesicles contained an NADH oxidase activity of higher specific activity that was stimulated by auxin.

### *3.4. Restoration of activity to right side-out plasma membrane vesicles after the freeze-thaw treatment*

In preparations where activity was reduced after the 6 freeze-thaw cycles, activity could be restored. The preparations were first fully reduced with 1 or

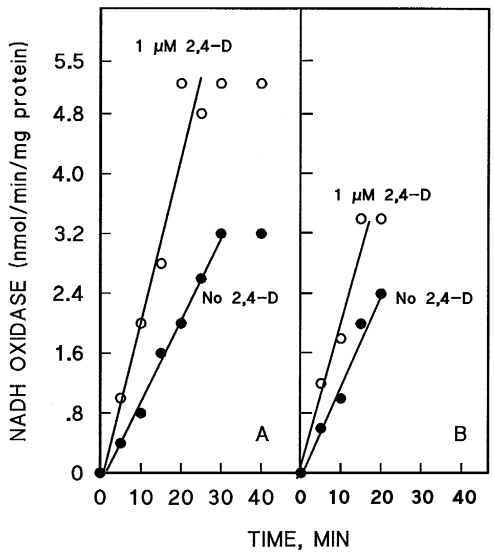


Fig. 2. Restoration of NADH oxidase activity to a plasma membrane preparation where 2,4-D-stimulated activity was lost following freezing and thawing. Restoration of activity was achieved in the presence or absence of 1 μM 2,4-D by first completely reducing the membrane preparation by treatment with 100 μM (A) or 1 μM (B) reduced glutathione (GSH) for 5 min followed by the addition of an oxidizing agent, 0.03% hydrogen peroxide (A) or 100 μM oxidized glutathione (GSSG) (B) in the presence of NADH substrate. Activity was then gradually restored over a period of 20–30 min. The restored activity was 2,4-D-stimulated and maximal at 30 min. Each value represents the specific activity averaged over 5 min which reached a plateau after 20–30 min. Results are from single experiments, but the technique was reproducibly effective in restoring activity to a variety of inactive preparations.

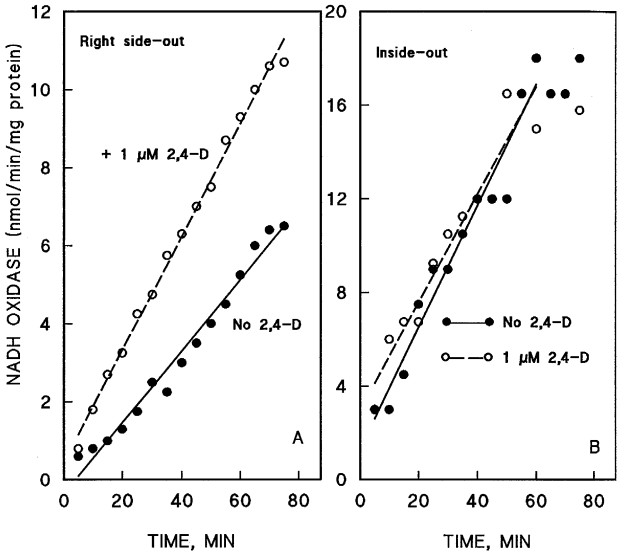


Fig. 3. Restored activity comparing right side-out (upper phase) (A) and inside-out (lower phase) (B) vesicles obtained by two-phase partition following the freeze–thaw treatment according to Larsson et al. [9]. After the series of 6 freeze–thaw cycles, only the inside-out vesicles (B) were initially auxin-responsive. However, when NADH oxidase activity was restored according to the protocol of Fig. 2, a 2,4-D-responsive activity now appeared in the membrane vesicles with a right side-out (cytoplasmic side-in) orientation (A).

100 μM reduced glutathione for 5 min. Subsequent addition of an oxidizing agent, such as 0.03% hydrogen peroxide (Fig. 2A) or 100 μM oxidized glutathione (Fig. 2B), resulted in a restoration of activity. In the experiments of Fig. 2, activity was restored over 20–30 min. The restored activity was 2,4-D stimulated and maximal after about 30 min of incubation. These treatments were less effective or without effect on plasma membrane vesicles freshly prepared with full activity or plasma membrane vesicles which had been frozen with retention of activity.

The restoration technique of Fig. 2 was then applied to vesicles of plasma membranes which had been resolved into right side-out and inside-out fractions by aqueous two-phase partition (Fig. 3). With the right side-out fractions, activity was initially 2,4-D-unresponsive, but a 2,4-D-responsive fraction appeared with time of incubation. With the inside-out fraction of plasma membrane vesicles, the activity was initially 2,4-D-responsive, but, as the activity increased following addition of the oxidant, no further increase in 2,4-D-responsive activity was observed. These results are from single experiments, but the experiments have been repeated several times and the results are summarized in Table 6.

Table 6
Restored activity comparing right side-out (upper phase) and inside-out (lower phase) vesicles obtained by two phase partition following 6 freeze–thaw cycles according to Larsson et al. [6]

| Vesicle orientation | NADH oxidase ($nmol \cdot mg\ protein^{-1} \cdot min^{-1}$) | |
|---|---|---|
| | No addition | +1 μM 2,4-D |
| Right side-out | 10 ± 3 | 14 ± 3 |
| Inside-out | 16 ± 4 | 17 ± 4 |

Results are averages of 4 experiments ± S.D.

To verify that the right side-out vesicles contained the 2,4-D responsive NADH oxidase activity, vesicles were isolated and assayed for activity both before and after treatment with 0.01% Triton X-100 to permeabilize the membranes to NADH under the conditions of activity restoration of Fig. 3 (Table 7). In the absence of Triton X-100, only the right side-out vesicles were responsive to auxin. However, in the presence of 0.01% Triton X-100, both the right side-out and inside-out vesicles exhibited an auxin-responsive oxidation of NADH which was approximately equal to the sum of the specific activities of the right side-out vesicles and the inside-out vesicles assayed in the absence of Triton X-100 (Table 7).

Similar results to those of Table 7 were obtained using 1 mM CHAPS in place of 0.01% Triton X-100.

Table 7
2,4-D response of right side-out and inside-out plasma membrane vesicles prepared by freezing and thawing followed by aqueous two-phase separation and response to 0.01% Triton X-100

| Vesicle orientation | NADH oxidase ($nmol \cdot mg\ protein^{-1} \cdot min^{-1}$) | |
|---|---|---|
| | No addition | +1 μM 2,4-D |
| (A) No Triton X-100 | | |
| Right side-out | 2.9 ± 0.5 | 4.9 ± 0.6 |
| Inside-out | 9.3 ± 1.2 | 9.8 ± 1.5 |
| (B) +0.01% Triton X-100 | | |
| Right side-out | 11.8 ± 0.7 | 15.2 ± 1.9 |
| Inside-out | 12.0 ± 1.0 | 15.7 ± 0.9 |
| (Calculated from A; right side-out + inside-out) | 12.2 | 14.7 |

Assay was as in Fig. 3 except that the incubation with oxidized glutathione was continued for only 30 min. Results are averages from 4 experiments ± S.D.

With the CHAPS, the detergent treatment increased the activity for inside-out vesicles by a factor of 1.25 in the absence of 2,4-D and by a factor of 1.45 in the presence of 2,4-D, compared to a factor of 1.3 in the absence of 2,4-D and a factor of 1.3 in the presence of 2,4-D for 0.01% Triton X-100.

Inside-out vesicles prepared by the Brij method of Johansson et al. [10] yielded specific activities of $12.7 \pm 0.7$ nmol NADH oxidizing $\cdot$ mg protein$^{\times 1} \cdot$ min$^{-1}$ in the absence of 2,4-D and $11.0 \pm 0.8$ nmol NADH oxidizing $\cdot$ mg protein$^{-1} \cdot$ min$^{-1}$ in the presence of 1 μM 2,4-D. These results are averaged from four different experiments. Addition of Triton X-100 (0.02%) to the Brij-isolated vesicles resulted in a decrease in specific activity of about 8% both in the presence and absence of 2,4-D.

## 4. Discussion

The oxidation of NADH by isolated plasma membrane vesicles emerges from these studies as a property of both the external and the internal membrane surfaces. The specific activities of the activities as estimated from measurements using vesicles of different orientations were similar. The existence of NADH oxidation at the external cell surface was verified from measurements with intact soybean cells grown in suspension culture. 2,4-D responsiveness was not determined with the intact soybean cells due to the presence of 2,4-D in the culture medium.

The stimulation by SHAM suggested the presence of a peroxidase capable of NADH oxidation [13,14] associated with the soybean plasma membrane. However, the 2,4-D-responsive component was unaffected by SHAM and, as reported previously [15], appears not to be a peroxidase.

A more definitive test of the distribution of NADH oxidase activity between the inside and outside surfaces of plasma membrane vesicles from soybean would be expected from studies of vesicles of known absolute orientation produced by freezing and thawing. These experiments were initially hampered by an overall reduction in specific activity and loss of 2,4-D responsiveness of frozen and thawed plasma membrane vesicles. The loss of activity was progressive with increasing freeze–thaw cycles up to about 5 cycles and then remained constant at about 1 nmol $\cdot$ mg protein$^{-1} \cdot$ min$^{-1}$ with additional freeze–thaw cycles.

When separated into right side-out and inside-out vesicles by aqueous two-phase partition, the right side-out vesicles were unresponsive to 2,4-D or slightly inhibited, whereas the inside-out vesicles did exhibit a higher specific activity and remained 2,4-D responsive.

In an effort to restore activity and 2,4-D response to the frozen and thawed vesicles, a protocol was devised based on studies with mammalian plasma membrane NADH oxidase activity. The vesicles were first treated with 1 or 100 μM reduced glutathione in the presence of NADH, followed by treatment with either 0.03% hydrogen peroxide or 100 μM oxidized glutathione in the presence or absence of 1 μM 2,4-D. Following the addition of the oxidant, activity increased gradually to a maximum after about 30 min. The restored activity was 2,4-D stimulated.

When applied to right side-out and inside-out vesicles by aqueous two-phase partition, the greatest 2,4-D response was seen with the right side-out vesicles, whereas the initially 2,4-D responsive inside-out vesicles (Table 5) were increased in specific activity, but not in 2,4-D responsiveness, by the treatment with reduced glutathione followed by hydrogen peroxide (Table 6).

Thus, a 2,4-D-responsive component was demonstrated to be associated with the NADH oxidase activity of the external plasma membrane surface. A similar situation was described for HeLa (human cervical carcinoma) cells in culture where an antitumor sulfonylurea-responsive form of NADH oxidase was found to be associated specifically with an external NADH site [16]. As with the study reported here, plasma membrane vesicles from HeLa cells exhibited NADH oxidase activities of nearly equal specific activities with both right side-out and inside-out vesicles, but only with the right side-out vesicles was the oxidation of NADH inhibited by the antitumor sulfonylurea.

With both soybean and HeLa, the association of the hormone or drug-responsive NADH oxidase with the external cell surface makes a function of the activity in the direct oxidation of NADH improbable. It is unlikely that millimolar concentrations of NADH or other appropriate electron donor would ever be achieved at the external cell surface.

That the activity is found at the outer cell surface, raises the possibility of either an electron transport role as part of an electron chain or that the activity might carry out some alternative function. The possibility of an alternative function has been raised with the observation that soybean plasma membranes exhibit an auxin-responsive protein disulfide–thiol interchange activity [17]. This possibility has been reinforced by direct measurements of thiols and disulfides that show an auxin-stimulated increase in thiols of membrane proteins in the presence of NADH or cysteine with a corresponding decrease in membrane disulfides [18]. In the absence of NADH or cysteine, a 2,4-D response of membrane thiols is also seen, but in the opposite direction, i.e., a decrease [18]. Thus, the general reaction catalyzed by the 2,4-D-responsive protein appears to be protein disulfide–thiol interchange [17]. The response to detergent treatment suggests that the protein disulfides reduced are at the outer surface of the plasma membrane.

Indirect evidence for a thiol involvement in auxin-induced growth was provided earlier in that auxin-induced cell enlargement was inhibited by thiol reagents in parallel to inhibition of auxin-stimulated oxidation of NADH [19]. Again, an involvement of thiols at the cell surface is implicated in that auxin-stimulated cell enlargement is inhibited specifically by the impermeant thiol reagent *p*-chloromercuriphenylsulfonic acid [18].

Electron acceptor activities at the external surface of the plasma membrane have been demonstrated previously for NADH-ferricyanide oxidoreductases [20,21]. However, the NADH-ferricyanide activity was distinct from that of the NADH oxidase [11].

While right side-out vesicles appear to contain a 2,4-D-responsive NADH oxidase, NADH oxidation by inside-out vesicles, under certain conditions, also may be auxin responsive. As shown by Table 5, when plasma membrane vesicles of soybean were everted by freezing and thawing cycles, total NADH oxidase activity was lost progressively. When such vesicles were resolved into right side-out and inside-out vesicles by aqueous two-phase partition, the right side-out vesicles no longer showed auxin stimulation, but the predominantly inside-out vesicles were auxin responsive. A similar result was obtained with these plasma membrane preparations of inside-out vesicles prepared by preparative free-flow electrophoresis (Table 3). However, as shown by data of Fig. 3 and Table 6, when activity was restored to such vesicles by treatment first with reduced glutathione followed by treatment with oxidized glutathione or hydrogen peroxide, the 2,4-D stimulated activity appeared in the right side-out vesicles but was lost with the inside-out vesicles (Fig. 3).

Inside-out vesicles, when ruptured by Triton X-100, showed enhanced 2,4-D-stimulated activity, whereas right side-out vesicles did not. Also inside-out vesicles prepared by the Brij method did not show a 2,4-D-stimulated activity. Thus, it was possible that the 2,4-D-stimulated activity of the inside-out vesicles after freezing and thawing both when separated by preparative free-flow electrophoresis or by a second aqueous two-phase partition may be due to a special population of right side-out vesicles present in the preparations or created during assay. Alternatively, the NADH oxidase activities present at the inner and outer surfaces of the plasma membrane may both respond to auxin under certain conditions, or may be the result of different proteins with differing properties in regard to auxin responsiveness. Resolution of the basis for the auxin-responsiveness or lack thereof of the inside-out plasma membrane vesicles from soybean must await additional study.

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