

The plasma membrane NADH oxidase of soybean has vitamin K₁ hydroquinone oxidase activity

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Abstract

Isolated plasma membrane vesicles and the plasma membrane NADH oxidase partially purified from soybean plasma membrane vesicles exhibited a cyanide-insensitive vitamin K₁ hydroquinone oxidase activity with isolated plasma membrane vesicles. Reduced vitamin K₁ (phyloquinol) was oxidized at a rate of about 10 nmol/min/mg protein as determined by reduced vitamin K₁ reduction or oxygen consumption. The K_m for reduced K₁ was 350 μ M. With the partially purified enzyme, reduced vitamin K₁ was oxidized at a rate of about 600 nmol/min/mg protein and the K_m was 400 μ M. When assayed in the presence of 1 mM KCN, activities of both plasma membrane vesicles and of the purified protein were stimulated (0.1 μ M) or inhibited (0.1 mM) by the synthetic auxin growth factor 2,4-dichlorophenoxyacetic acid. The findings suggest the potential participation of the plasma membrane NADH oxidase as a terminal oxidase of plasma membrane electron transport from cytosolic NAD(P)H via reduced vitamin K₁ to acceptors (molecular oxygen or protein disulfides) at the cell surface. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

Plasma membranes of both plant and animal cells contain quinones capable of participating in electron transport reactions [1,2]. At the plasma membrane, reduced quinones may function primarily as an anti-oxidant by inhibiting lipid peroxidation or protein

carbonylation [3,4]. Alternatively, they may participate in the plasma membrane electron transport chain and growth control [5].

Recently, Morré and coworkers have described a plasma membrane NADH oxidase of HeLa and other transformed cells where activity is inhibited by several quinone site inhibitors including the vanilloid capsaicin [6] and the antitumor sulfonylurea *N*-(4-methylphenylsulfonyl)-*N'*-(4-chlorophenyl)urea (LY181984) [7]. The drug-responsive activity from cancer cells resembles very closely the plant growth regulator-stimulated NADH oxidase reported previously from plasma membrane vesicles isolated from hypocotyl segments of dark-grown soybean seedlings [8,9]. NADH oxidase preparations, partially purified from the cell surface of HeLa cells, also were active

Abbreviations: K₁H₂, phyloquinol, reduced phyloquinone = reduced vitamin K₁; 2,4-D, 2,4-dichlorophenoxyacetic acid; phyloquinone (vitamin K₁), 2-methyl-3-phytyl-4-naphthoquinone (3-phytylmenadione); NOX, plasma membrane-located NADH (hydroquinone) oxidase with protein disulfide-thiol interchange activity

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in the oxidation of reduced quinones [10]. The oxidation of reduced quinones exhibited the same drug responsiveness as NADH oxidation.

Despite progress in identification of ubiquinols as the natural substrates for the mammalian cell surface NADH oxidase, similar information is still lacking for plants. The experiments reported in the present paper sought to determine if the cell surface NADH oxidase of a plant, soybean, would utilize reduced phyloquinone (K_1H_2) as a candidate substrate to represent a potential natural electron donor. Plant plasma membranes lack significant amounts of ubiquinones [2,11] such that ubiquinones would not be expected to serve as significant sources of reducing equivalents for plasma membrane electron transport in plants. Rather than ubiquinone, plant plasma membranes contain phyloquinone (vitamin K_1) [2].

The observations that K_1H_2 can serve as an electron donor for the plasma membrane NADH oxidase of soybean have implications in the protection of plasma membranes against reactive oxygen species. They also lend support to early suggestions [12] that the plasma membrane NADH oxidase under certain conditions may function as a terminal oxidase in the plasma membrane electron transport chain transferring electrons from reduced coenzyme Q to molecular oxygen or to protein disulfides as terminal electron acceptors [13,14]. Additionally, the identification of a natural substrate source of reducing equivalents for the plasma membrane NADH oxidase will facilitate further examination of the structural and functional properties of the enzyme.

2. Materials and methods

2.1. Materials

Phylloquinone (vitamin K_1 = 2-methyl-3-phytyl-1,4-naphthoquinone; 3-phytylmenadione) was obtained from Sigma, St. Louis, MO, and dissolved in ethanol. Vitamin K_1 amounts were estimated from the absorbance at 248 nm in ethanol and calculated from an extinction coefficient of $18.9 \text{ mM}^{-1} \text{ cm}^{-1}$ [15].

The method for preparation of reduced phyloquinone (50 mM stock solution in ethanol) involved

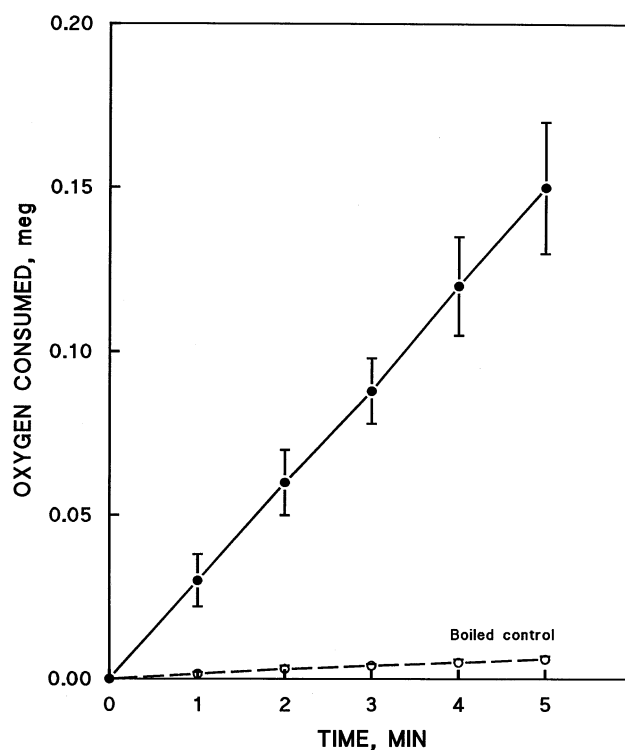


Fig. 1. Linearity of oxygen consumption of isolated plasma membrane vesicles over 5 min. Boiled vesicles (boiled control) or preparations in the absence of K_1H_2 gave only a small background rate. Values are from determinations using five different plasma membrane preparations, \pm standard deviations.

addition of 0.4 of the initial volume of ethanol and 0.4 of the initial volume of 0.25% NaBH_4 , both under nitrogen gas. After 5 min, the excess NaBH_4 was degraded by the addition of 0.2 of the initial volume of 0.1 N HCl. The colorless solution was kept at ice-cold temperatures and protected from light.

2.2. Plant material and isolation of plasma membranes

Soybean seeds (*Glycine max* L. Merr., cv. Williams) were soaked for 4 h in deionized water and grown in the dark (20–22°C) in moist vermiculite contained in foil-covered $18 \times 23 \times 10$ cm plastic boxes normally without supplemental additions of water. After 4–6 days, 2-cm hypocotyl segments, cut 5 mm below the cotyledon, were harvested under diminished light ($0.15 \mu\text{E s}^{-1} \text{ m}^{-2}$) and placed in cold water. Hypocotyl segments (25 g) were homogenized in 100 ml of a medium containing 25 mM

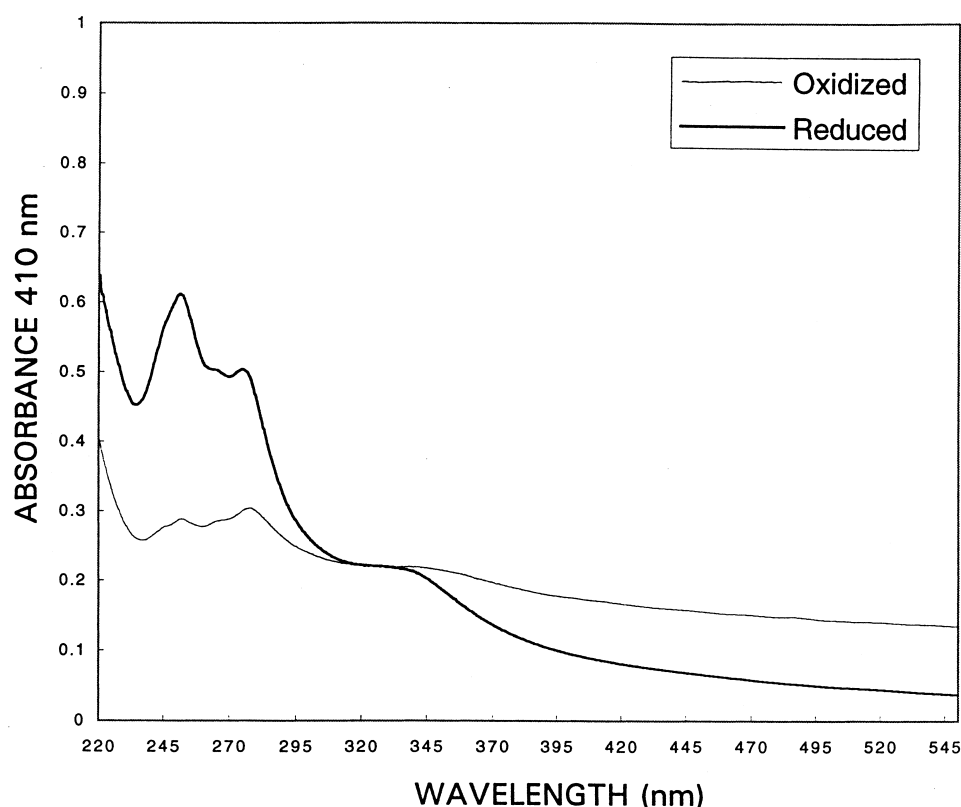


Fig. 2. Absorption spectra of 0.5 μmol vitamin K_1 in 2.5 ml 50 mM Tris-MES, pH 7. The thin line represents the oxidized form. The thick line represents the reduced form after treatment with sodium borohydride. The increase in absorbance at 410 nm was used as a measure of K_1H_2 oxidation.

Tris-MES (pH 7.4), 300 mM sucrose, 10 mM KCl and 1 mM MgCl_2 using a blender. The homogenates were filtered through one layer of Miracloth (Chicopee Mills, NY) and centrifuged for 10 min at $6000\times g$ (HB-rotor). The supernatant was recentrifuged at $60\,000\times g$ (Beckman 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 [16,17] that yielded predominantly right side-out and sealed vesicles [18,19]. Resuspended $60\,000\times g$ pellets were mixed with 6.4% (w/w) polyethylene glycol 3350 (Fisher), 6.4% (w/w) dextran T500 (Pharmacia), 0.25 M sucrose and 5 mM potassium phosphate (pH 6.8). After mixing the tubes by 40 inversions in the cold, the phases were separated by centrifugation at $750\times g$ for 5 min. The lower phase was re-partitioned once with fresh upper phase. The upper phases were diluted approximately 4-fold with buffer and collected by centrifugation at $100\,000\times g$ for 30

min. The membranes were stored at -70°C prior to assay. The yield was 4 mg of plasma membrane protein. The purity based on morphometric analysis after specific staining with phosphotungstic acid at low pH [20] and assay of marker enzymes was $>95\%$.

2.3. NADH oxidase purification

Isolated soybean plasma membrane vesicles were thawed and resuspended in 50 mM Tris-MES, pH 7.0 and 1% Triton X-100 and the mixture was shaken overnight at 4°C . The solubilized preparation was cleared by centrifugation at $108\,000\times g$ for 30 min after which the supernatant was applied at room temperature to a concanavalin A-Sepharose affinity column, equilibrated with 50 mM Tris-MES buffer, pH 7.0. Once loaded, flow was stopped for 30 min to facilitate binding. The column was then washed four times with 1.0 ml of Tris-MES, pH 7.0, then twice with 1.0 ml of 0.1 M α -methylmannoside (in Tris-

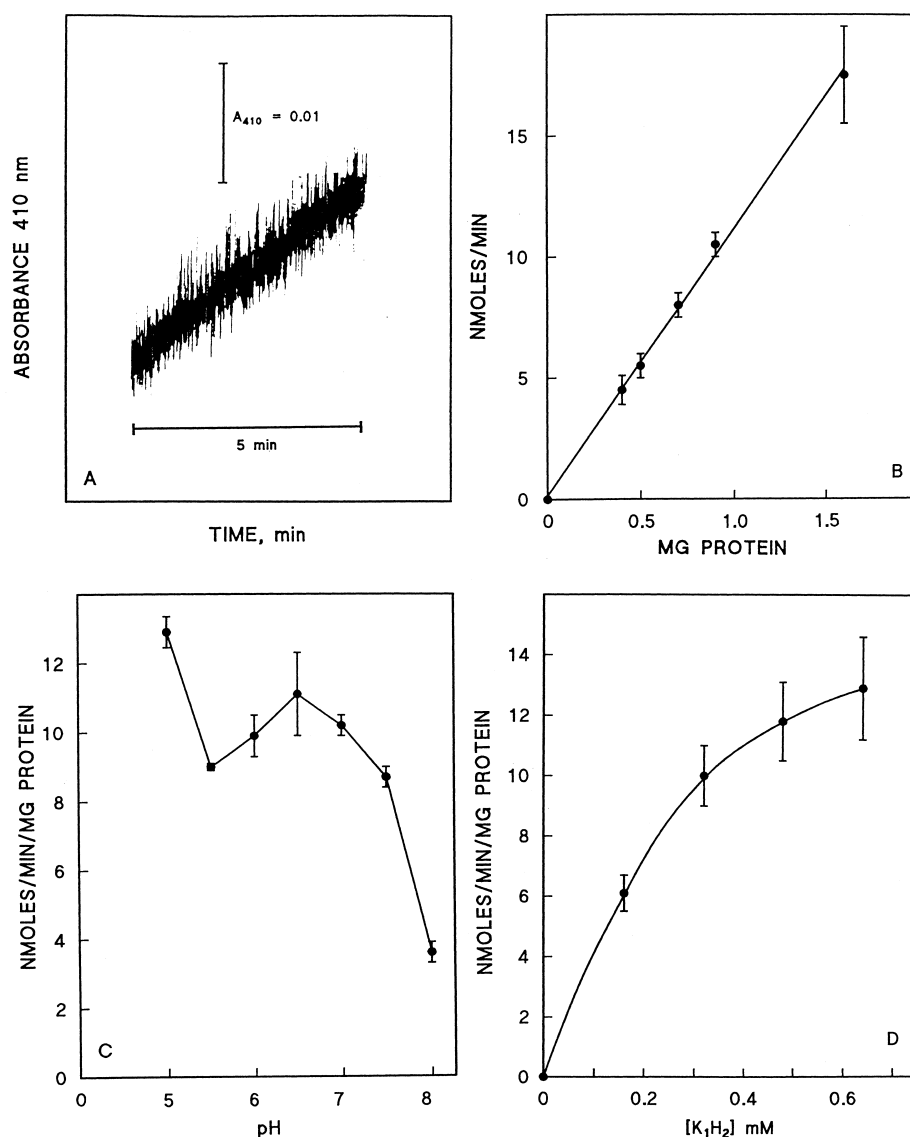


Fig. 3. Oxidation of K_1H_2 by plasma membrane vesicles isolated from 1-cm tissue sections excised from the zone of cell elongation of hypocotyls of dark-grown soybean seedlings. The disappearance of K_1H_2 was determined from the increase in absorbance at 410 nm. (A) Increase in absorbance at 410 nm as a function of time in the presence of 0.8 mg of plasma membrane protein. (B) Rate of K_1H_2 oxidation in proportion to amount of added protein. (C) Response to pH. Blank values for spontaneous decomposition at pH 8 were subtracted. (D) Saturation kinetics with increasing amounts of K_1H_2 in the assay. Unless specified otherwise, the concentration of reduced phyloquinone was 0.32 mM, the pH was 7 and the protein amount was 0.4–0.7 mg. Assays were at 37°C for two intervals of 5 min each. Rates were based on the slope of the second 5-min assay. Values are from four (B) or five (C,D) determinations, each with a different plasma membrane preparation, \pm standard deviations.

MES, pH 7.0), and once more with Tris-MES buffer, pH 7.0. The third, fourth, and fifth fractions were combined and concentrated, and used as the source of protein. The activity was 50–70-fold purified relative to the starting plasma membrane.

2.4. Protein

Protein content was determined by the bicinchoninic acid procedure [21]. Standards were prepared with bovine serum albumin.

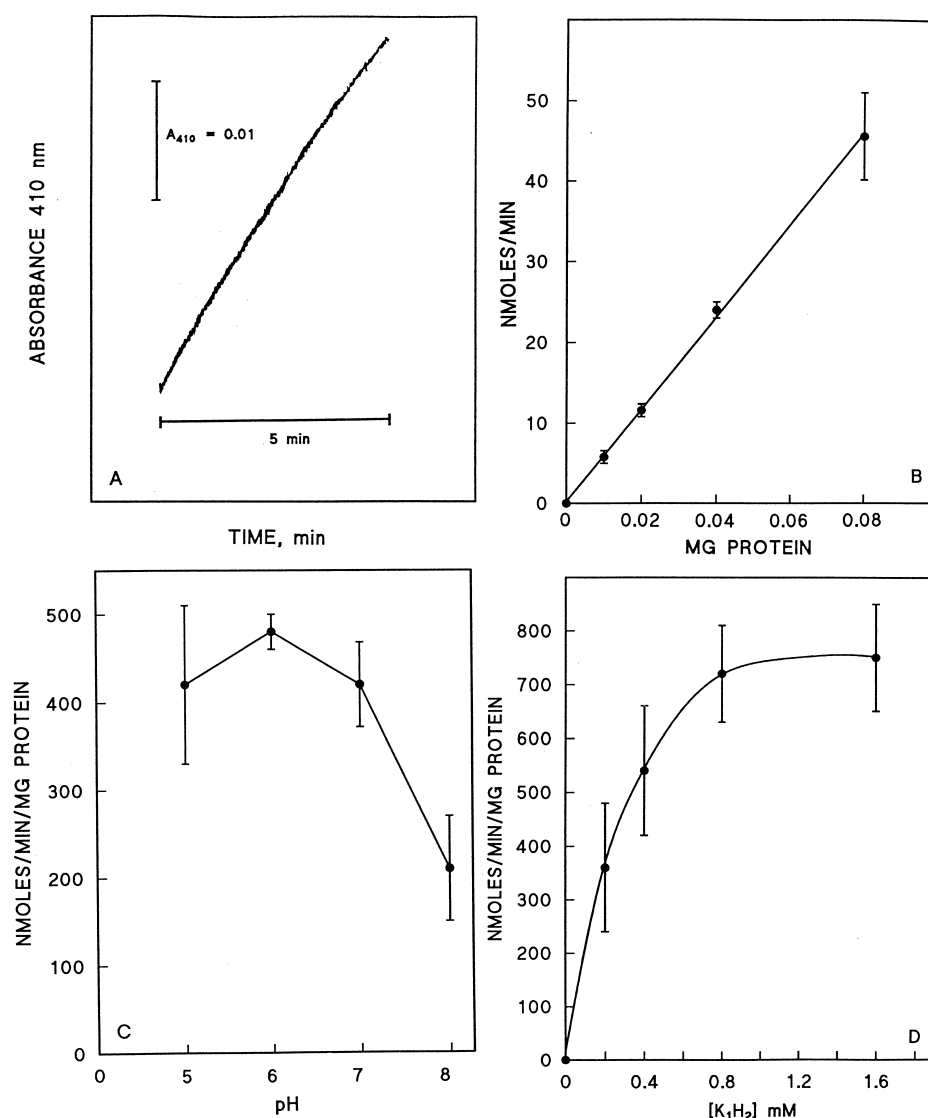


Fig. 4. Oxidation of K_1H_2 by an enzymatic preparation enriched in NADH oxidase activity solubilized from isolated plasma membrane vesicles from soybean hypocotyls prepared as described in Section 2.3. The disappearance of K_1H_2 was determined from the increase in absorbance at 410 nm. (A) Increase in absorbance at 410 nm as a function of time. (B) Rate of K_1H_2 in proportion to amount of added protein. (C) Response to pH. Blank values for spontaneous decomposition at pH 8 were subtracted. (D) Saturation kinetics with increasing amounts of K_1H_2 in the assay. Unless specified otherwise, the concentration of phyloquinone was 0.32 mM, the pH was 7 and the protein amount was 35 μ g per assay. Assays were at 37°C for two intervals of 5 min each. Rates were based on the slope of the second 5-min assay. Values are from three determinations, each with a different plasma membrane preparation, \pm standard deviations.

2.5. Enzyme assay for reduced phyloquinone oxidase

For oxidation of reduced phyloquinone, the reaction mixture contained indicated amounts of soybean plasma membranes or partially purified NADH oxidase in 2.5 ml of 50 mM Tris-MES buffer, pH 6.5. The reaction was started by the addition of 40 μ l of

the reduced 20 mM phyloquinone. The reduced phyloquinone oxidase activity was measured spectrophotometrically at a wavelength of 410 nm at 27°C as described by Sun et al. [22] for reduced coenzyme Q using a Hitachi U3210 spectrophotometer for two consecutive 5-min periods. The decrease in absorbance during the second 5-min period was

used to calculate the specific activity. A blank rate was subtracted in which the assay was carried out in the absence of added proteins. The extinction coefficient used for K_1H_2 oxidation was $0.74 \text{ mM}^{-1} \text{ cm}^{-1}$.

3. Results

Both oxygen consumption in the presence of K_1H_2 and oxidation of K_1H_2 were measured. The former used an oxygen electrode and was linear with time and dependent upon both K_1H_2 and a source of undenatured enzyme (Fig. 1). Both boiled controls and controls lacking K_1H_2 generated only negligible rate of oxygen consumption. For the spectrophotometric method, increase in absorbance at 410 nm as K_1H_2 was oxidized was measured (Fig. 2). This method was used previously to monitor reduced coenzyme Q oxidation [5,10,22] and permits the spec-

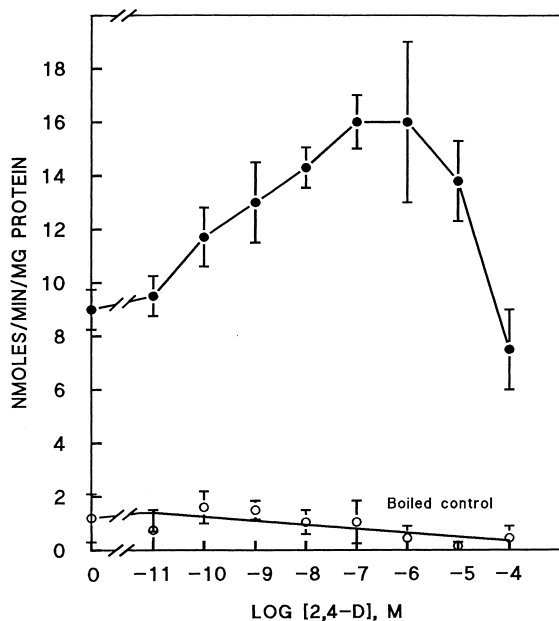


Fig. 5. Response of the oxidation of K_1H_2 by plasma membrane vesicles isolated from 1-cm tissue sections excised from the zone of cell elongation of hypocotyls of dark-grown soybean seedlings to the synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D) (solid symbols). The assay conditions were as described in Fig. 3 and in the presence of 1 mM potassium cyanide. A boiled enzyme blank was included in each experiment as a control (open symbols). Values are average rates from five determinations, each from a different plasma membrane preparation, \pm standard deviations.

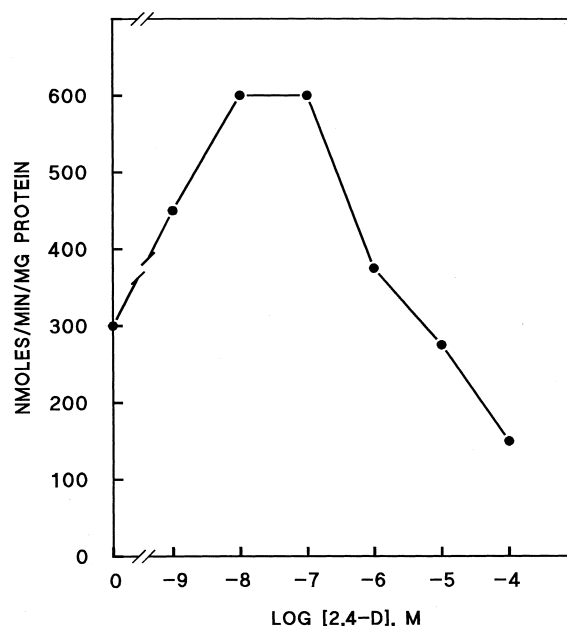


Fig. 6. Response of the oxidation of K_1H_2 by an enzymatic preparation enriched in NADH oxidase activity solubilized from isolated plasma membrane vesicles to the synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D). The assay conditions are as described in Fig. 4. Values are from a single experiment.

trophotometric analyses of reduction of mM amounts of K_1H_2 in turbid solutions containing mg quantities of isolated plasma membrane vesicles.

With the plant preparations, the phytoquinol oxidase activity was measured both with isolated plasma membrane vesicles with solubilized, or partially-purified and membrane-free preparations containing the NADH oxidase activity with similar results. The specific activity with plasma membrane vesicles was low (ca. 10 nmol/min/mg protein), but of the same order of magnitude as for the oxidation of NADH [8,9,14]. Oxygen consumption measured in parallel using an oxygen electrode was 6.2 ± 0.6 ($n=3$) nmol/min/mg protein (Fig. 1).

For isolated plasma membranes, the activity was proportional to time (Fig. 3A) and protein concentration (Fig. 3B). Maximum activity was given at pH 5 and in the range pH 6–7 (Fig. 3C). Reduced vitamin K_1 was unstable at pH 8, but sufficient reduced K_1 was retained in freshly prepared solutions to permit estimation of enzymatic activity. The activity exhibited saturation kinetics with respect to substrate (Fig. 3D). The K_m for reduced vitamin K_1 was $350 \pm 50 \mu\text{M}$ with a V_{\max} of 20 ± 1 nmol/min/mg pro-

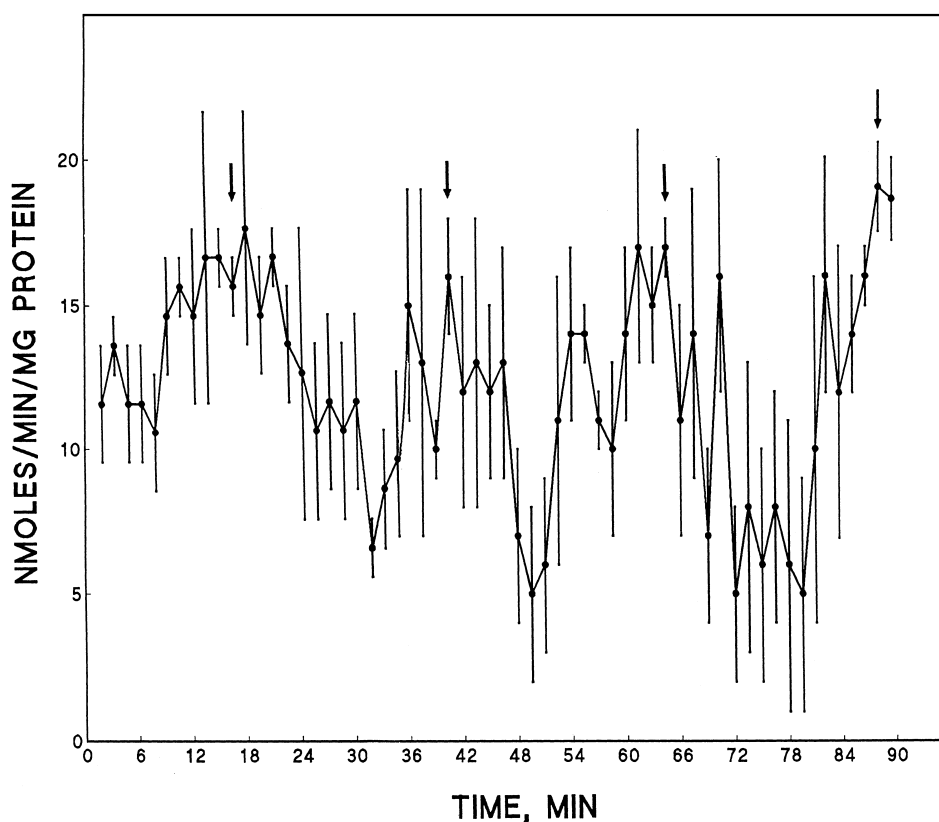


Fig. 7. Periodic variation in the rate of oxidation of K_1H_2 (0.32 mM) as a function of time over 90 min showing 4 maxima (arrows at 16.5, 40.5, 64.5 and 88.5 min) for plasma membrane vesicles isolated from 1-cm tissue sections excised from the zone of cell elongation of hypocotyls of dark-grown soybean seedlings. Rates were determined over 1 min every 1.5 min. Conditions were as given for Fig. 3. Two determinations were carried out simultaneously using two side-by-side Hitachi U3210 spectrophotometers. A second set of two determinations with the same plasma membrane preparation was then begun with the periodicity in phase with the first set of determinations. Results are means of the four sets of data \pm standard deviations to show the reproducibility of the pattern of the periodicity.

tein (Table 1). Absorbance changes at 410 in the absence of K_1H_2 were negligible. Oxidation of K_1H_2 by the soybean preparations was unaffected by either superoxide dismutase or catalase. The rate of spontaneous K_1H_2 oxidation or with a boiled enzyme blank was 0.1 ± 0.07 ($n=6$) nmol/min at a concentration of $32 \mu M$ K_1H_2 . Assay of the spectrophotometric change at 410 nm under anaerobic

conditions revealed no unknown enzymatic destruction of K_1H_2 .

Oxidation of K_1H_2 also was measured with the partially purified preparations with NADH oxidase activity. As with the plasma membrane vesicles, the oxidation of K_1H_2 was proportional to time (Fig. 4A) and protein concentration (Fig. 4B). Activity was similar at pH 5, 6 or 7 (Fig. 4C). The rate was

Table 1

Kinetic constants of the oxidation of reduced phyloquinone by isolated plasma membrane vesicles and the NADH oxidase-enriched fraction released from soybean plasma membranes

| Oxidase source | Apparent K_m (mM) | V_{max} (nmol/min/mg protein) |
|---|------------------------|------------------------------------|
| Isolated plasma membrane vesicles | 0.35 ± 0.05 | 20 ± 1 |
| NADH oxidase-enriched fraction solubilized and partially purified from plasma membrane vesicles | 0.4 ± 0.08 | 1250 ± 250 |

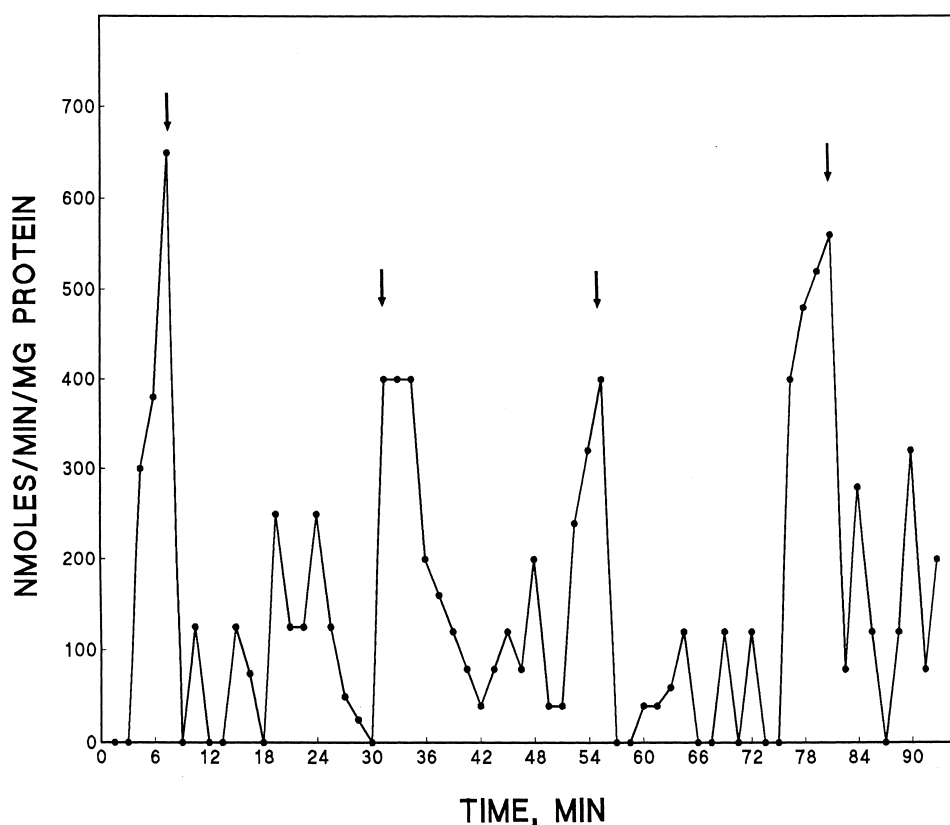


Fig. 8. Periodic variation in the rate of oxidation of K_1H_2 (0.32 mM) as a function of time over 93 min showing four maxima (arrows at 7.5, 31.5, 55.5 and 79.5 min) for an enzymatic preparation enriched in NADH oxidase activity solubilized from isolated plasma membrane vesicles. The average period length was about 24 min. The assay conditions were as described in Fig. 4. Values are from a single experiment.

proportional to concentration of reduced vitamin K_1 (Fig. 4D) with an apparent K_m of $400 \pm 80 \mu M$ (Table 1) and a V_{max} of 1250 ± 250 nmol/min/mg protein.

The oxidation of K_1H_2 both by plasma membranes and the solubilized preparations were resistant to KCN (1 mM), one of the distinguishing characteristics of the plasma membrane NADH oxidase. A second characteristic of the soybean plasma membrane NADH oxidase was its response to growth factors of the auxin type, such as 2,4-dichlorophenoxyacetic acid (2,4-D).

When tested either with the plasma membrane preparations (Fig. 5) or with the solubilized and partially purified NADH oxidase preparations (Fig. 6), a typical bimodal response was obtained. Activity was stimulated about 1.7–2-fold by 2,4-D to a near optimum of $0.1 \mu M$. Higher 2,4-D concentrations were less stimulatory and 0.1 mM inhibited.

A property of the plasma membrane-associated NADH oxidase is that its activity oscillates (see Section 4). The activity for the oxidation of NADH [23] and reduced ubiquinone [10] fluctuates with a period of about 24 min. The K_1H_2 oxidase activity also was periodic both for isolated plasma membrane vesicles (Fig. 7) and for the solubilized and partially purified preparations (Fig. 8). The activity cycled through four periods in about 96 min with an average period of about 24 min. This periodicity provides additional evidence that the hydroquinone oxidase and NADH oxidase activities are catalyzed by the same protein.

4. Discussion

In 1986, our laboratory described from plants an NADH oxidase activity that was growth factor stimulated [8]. This activity was purified from plants

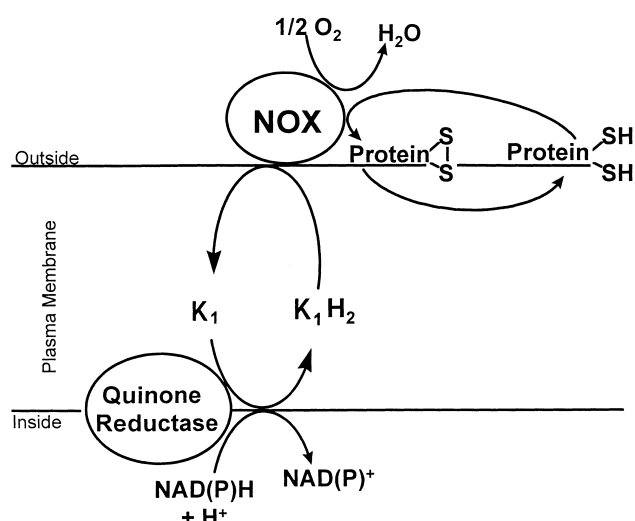


Fig. 9. Diagram illustrating the spatial relationships across the plasma membrane of the inside NAD(P)H: quinone reductase, the membrane pool of K_1/K_1H_2 and the external NADH oxidase (NOX) protein for plants. As illustrated, the NOX protein could function as a terminal oxidase of plasma membrane electron transport donating electrons from cytosolic NADH either to molecular oxygen in a two-electron transfer or to reduce protein disulfides. Modified from Kishi et al. [10].

[9], shown to be present in mammalian plasma membranes where it was also hormone and growth factor stimulated [24] and subsequently was associated with the external cell surface [25,26]. Since it is unlikely that external concentrations of NADH would ever reach 1 mM, an alternative function was sought in protein disulfide thiol interchange [14,27,28]. Activity was shown by measurement of restoration of activity to scrambled (inactive) RNase [27,28] and from an overall stimulation of the reduction of protein disulfides in the presence of NADH (NADH:protein disulfide reductase) and other reducing agents [13]. Both molecular oxygen [14] and protein disulfides [13,14] apparently serve as electron acceptors for electron transfer from NADH. However, evidence for a physiological electron donor other than thiols was unavailable. In a parallel report, we show that hydroquinone ($Q_{10}H_2$ and Q_oH_2) can serve as electron donor for the activity from the surface of HeLa cells [10]. In this report, we show that the reduced form of the corresponding plant quinol, K_1H_2 , can serve as an electron donor for oxygen or protein disulfide reduction catalyzed by the plasma membrane located NADH (hydroquinone) oxidase with

protein disulfide-thiol interchange activity (NOX) as well.

That plasma membranes contain quinones in mammalian cells including coenzyme Q [1] and phyloquinones in plants [2] is well established. These quinones could become reduced at the inner surface of the plasma membrane through the activity of an NAD(P)H coenzyme Q reductase or diaphorase at the plasma membrane [29,30]. The quinol would be free to migrate through the lipid interior of the membrane where it would become available for oxidation by the NOX protein (Fig. 9). To our knowledge, this is the first formulation of a plasma membrane electron transport chain for either animal [10] or for plants (this report) based on identification of known proteins and constituents in the plasma membrane and involving physiological electron donors and acceptors.

Crane et al. [31] had previously described electron transport chains in the plasma membrane based on reduction of ferricyanide [32] which is not a physiological electron acceptor or to iron bound to diferric transferrin. The NOX protein is distinct from the NADH-ferricyanide reductase [33] and shows no activity with ferricyanide or diferric transferrin as an electron acceptor. Both ferricyanide and diferric transferrin exhibit growth factor activity and do stimulate the NOX activity of rat liver plasma membrane as growth factors [34,35], but not as electron acceptors.

The specific activity of K_1H_2 oxidation, while low, ca. 10 nmol/min/mg protein, is of the same order of magnitude as measured for the transmembrane flow of electrons across HL 60 [36] and HeLa cells. Additionally both the oxidation of hydroquinones and the oxidation of NADH [14,33] are resistant to KCN and are stimulated by 2,4-D when assayed in the presence of KCN in plants. Both the activities, hydroquinone and NADH oxidation, from HeLa cells [10] are inhibited competitively by two inhibitors of the HeLa plasma membrane NADH oxidase, capsaicin [6] and the antitumor sulfonylurea, LY181984 [7]. These findings suggest that these compounds act at the same site and that the two activities reside on the same protein. An ability of the NOX protein to oxidize hydroquinones certainly does not prove a role in transmembrane electron transport, but does raise the possibility. The NOX protein might also function

in the scavenging of active oxygen forms at the plasma membrane or in oxygen sensing [37].

We have suggested previously that the principal function of the NOX protein is not in transplasma membrane electron transport, but as a protein disulfide-thiol interchange protein active in a growth related process of the breakage and reformation of disulfide bonds [14]. Transfer of electrons from NADH or NADPH via hydroquinone in the membrane might have important implications in keeping plasma membrane proteins reduced, in sensing of the redox state of the cytoplasm in relation to growth control, or as a possible mechanism of linking sensing systems at the internal cell surface to redox changes at the external cell surface as have been postulated to occur in aging and senescence [38–40].

A recently described property of the NADH oxidase is its periodicity [23,41,42]. The activity oscillates to a maximum with a regular period of about 24 min. The basis for and the significance of the periodicity is unknown, but a potential time keeping function is indicated. That the oxidation of ubiquinol [10], the oxidation of K_1H_2 (this report) and the oxidation of NADH [23] show a similar 24-min periodicity support our suggestion that both activities may be attributed to the same protein or protein complex.

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