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Oxidation of NADH by intact segments of soybean hypocotyls and stimulation by 2,4-D

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Abstract

Intact sections of soybean cut from regions of cell elongation of hypocotyls of etiolated soybean seedlings oxidized externally supplied NADH (NADH is an impermeant substrate). The oxidation of NADH by 1-cm intact sections was stimulated by the plant growth factor 2,4-dichlorophenoxyacetic acid (2,4-D). The optimum concentration of 2,4-D for stimulation was about 1 μM. Stimulations also were given by the naturally occurring 2,4-D analog, indole-3-acetic acid (IAA), but not by the growth inactive 2,4-D analog 2,3-dichlorophenoxyacetic acid (2,3-D). The findings confirm studies comparing inside-out and right side-out vesicles that show the 2,4-D-stimulated NADH oxidase to be located at the external cell surface. Since plant cells are unlikely to encounter NADH at their external cell surface, functions of the oxidase in reactions other than oxidation of NADH are discussed.

Keywords: Cell enlargement; NADH oxidase; Auxin; 2,4-Dichlorophenoxyacetic acid; Plasma membrane; Protein disulfide-thiol interchange; (*Glycine max*)

1. Introduction

Our laboratory has described an NADH oxidase activity of plasma membrane vesicles prepared from segments cut from the region of cell elongation of etiolated hypocotyls of soybean (*Glycine max*) [1–3]. The activity is stimulated by auxin growth factors such as 2,4-dichlorophenoxyacetic acid (2,4-D), α-naphthyleneacetic acid (α-NAA), and indole-3-acetic acid (IAA). Growth is in proportion to the logarithm of the auxin concentration and is unresponsive to growth inactive auxin analogs such as 2,3-dichlorophenoxyacetic acid (2,3-D) and β-naphthyleneacetic acid (β-NAA) [3].

The NADH oxidase activity is associated with both right side-out and inside-out vesicles [4]. Interestingly, the growth factor-stimulated component of the activity appeared to be associated not only with inside-out vesicles but also with the external plasma membrane surface as shown by analyses of vesicles having a right side-out orientation [4]. These observations raised the possibility that a growth factor-stimulated oxidation of NADH could be elicited by addition of NADH to intact segments of plant tissues.

In this report, we describe a simple method to demonstrate measurement of NADH oxidation by intact tissues. Sections cut from hypocotyls of dark-

Abbreviations: IAA, indole-3-acetic acid; 2,4-D, 2,4-dichlorophenoxyacetic acid; 2,3-D, 2,3-dichlorophenoxyacetic acid

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grown soybean seedlings were used. The activity was responsive to the growth factor, 2,4-D. The observation of the phenomenon of a growth factor-responsive NADH oxidase located at the outer surface of the plant plasma membrane implies that some electron donor endogenous to the membrane such as a reduced quinones or protein thiols may be the physiological donors for the activity rather than NADH.

2. Materials and methods

2.1. Plant material

Seeds of soybean (*Glycine max* L. Merr., cv. Williams) were soaked 4–6 h in deionized water, planted in moist vermiculite, and grown 4–5 days in darkness and 25°C in foil-covered 18×10-cm plastic boxes. Hypocotyl segments of different lengths (1/8, 1/4, 1/2 and 1 cm) were excised below the apical hook of four-day old seedlings and floated in deionized water to wash off the contents of broken cells at the cut surface. The sections were maintained at room temperature before and during all experiments.

2.2. NADH oxidase activity

The assay for NADH oxidase activity consisted of 3 ml of 50 mM Tris-MES (pH 6.8) and 1 mM potassium cyanide, the latter to inhibit mitochondrial NADH oxidases as well as cellular and surface peroxidases. The above were added to 30 1-cm, 35 1/2-cm, 45 1/4-cm, or 70 1/8-cm sections which had been placed in the bottom of 10×10×48-mm polystyrene cuvettes (Sarstedt, Newton, NC, USA). The assay was initiated by the addition of 150 μM NADH. The assay could not be stirred due to possible mechanical disturbance of the sections as well as interference with the spectrophotometric assay. Therefore, the assay components were mixed once each minute by pipetting with a plastic pipette. The reaction was monitored by the decrease in the A_{340} with A_{430} as a reference. An SLM DW-2000 (SLM-Aminco) spectrophotometer was used in the dual-wavelength mode of operation. The change of absorbance was recorded manually after mixing, once each minute, for 20 min.

After the first 20 min, 2,4-D, 2,3-D or IAA were added as indicated for individual experiments and the assay was continued for an additional 20 min. Unless indicated otherwise, results were averages from triplicate assays. A millimolar extinction coefficient of 6.22 cm^{-1} was used in calculations of specific activities.

3. Results

Intact 1-cm sections cut from etiolated hypocotyls of soybean oxidize NADH based on the decrease in absorbance at 340 nm (Fig. 1). The rate in the absence of 2,4-D calculated from the disappearance of NADH (decrease in absorbance at 340 nm) was 6.25 nmol/min/30 1-cm sections (Fig. 1). Following addition of 2,4-D, the rate of NADH disappearance approximately doubled to about 12 nmol/min/30 sections (Fig. 1). The blank rate in the presence of NADH but in the absence of tissue (<0.05 nmol/

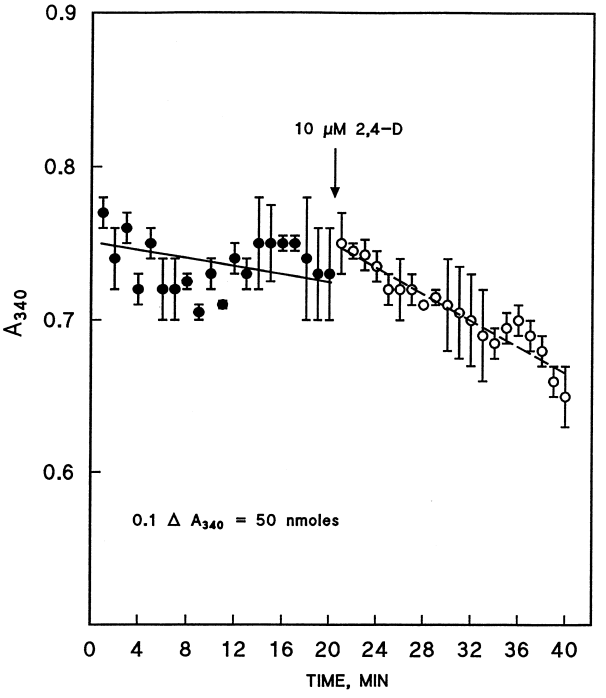

Fig. 1. Time course of NADH oxidation by 1-cm sections of etiolated sections of soybean hypocotyls. Results are averages of four different sets of 30 sections each done on the same day from the same lot of soybeans. The reaction was initiated by the addition of NADH.

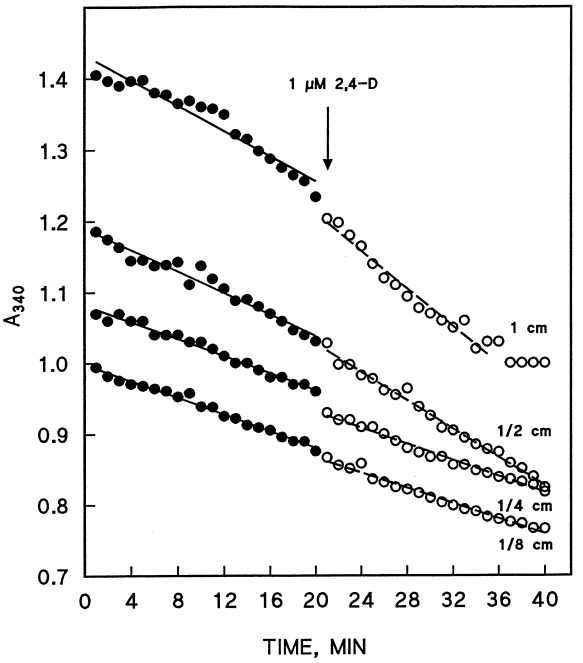

Fig. 2. Time course of oxidation of NADH by intact sections of soybean hypocotyls as a function of section length. Approximately 20 1-cm sections, 35 1/2-cm sections, 40 1/4-cm sections or 70 1/8-cm sections were incubated as described for Fig. 1 for 20 min to establish a basal rate. After 20 min 2,4-D was added to a final concentration of 1 μM and the incubations were continued for an additional 20 min.

min) was subtracted. In the presence of tissue but in the absence of NADH, the absorbance increased slightly. No rates were observed with tissue segments boiled for 10 min prior to assay.

A contribution from an internal oxidase either exposed on cut surfaces or coming from broken cells was unable to account for the 2,4-D-stimulated response of the intact sections. When progressively shorter segments were included in the assay, the proportion of the total activity responding to 2,4-D was reduced rather than augmented (Fig. 2). When rates were normalized for number of sections to a linear section length of 1 cm (i.e. 8 1/8-cm sections, 4 1/4-cm sections, 2 1/2-cm sections or 1 1-cm section) (Fig. 3), rates in the absence of 2,4-D were greatest with tissue cut into 1/8-cm sections. However, the component of activity responding to 2,4-D remained constant or declined as section length was decreased. The rate in the absence of 2,4-D was least with 1-cm sections. The response to 2,4-D was 0.25 nmol/20 min/1-cm section equivalent for 1/4-cm sections, 0.3 nmol/20 min/1-cm section equivalent for 1/2-cm sections and 0.35 nmol/20 min/1-cm section equivalent with the 1-cm sections (Fig. 2).

The response of NADH oxidation to the logarithm of 2,4-D concentration revealed an optimum at 1 μM with the 1-cm sections (Fig. 4). Similar results were found with 1/4- and 1/2-cm sections (not shown). The weak auxin, 2,3-D, at 1 μM elicited an auxin-stimulated rate of about 5 nmol/20 min/25 1-cm sections compared to the rate of 50 nmol/20 min/25 1-cm sections for 1 μM 2,4-D (Table 1). With indole-3-acetic acid the optimum also was approximately 1 μM (Fig. 5).

An apparent periodicity that was both reproducible and significant statistically was observed for the NADH oxidation rate (Fig. 1). It appeared as though the rate of NADH oxidation accelerated for a period of about 12 min and then declined for about 12 min followed by another period of acceleration. The apparent periodicity was seen both in the absence and presence of auxin and to a lesser extent

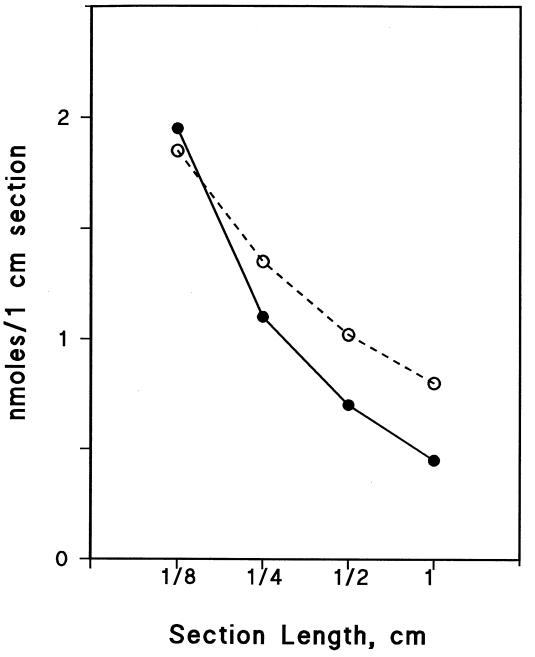

Fig. 3. Results from an experiment similar to that of Fig. 2 where data were normalized for the number of sections and were expressed as nmol/1-cm section (i.e. per 8 1/8-cm sections, 4 1/4-cm sections, 2 1/2-cm sections and 1 1-cm section) as a function of section length. Solid line and symbols, no 2,4-D. Broken line and open symbols, 1 μM 2,4-D.

in data of Fig. 2 especially with the sections 1/2 and 1 cm in length. No such oscillations were observed with NADH in the absence of tissue or with tissue in the absence of NADH.

4. Discussion

The possibility of an external NADH oxidase in plant cells was first raised by Lin for plant roots [5]. That a plasma membrane NADH oxidase activity also might be external was raised by studies of the antitumor sulfonylurea-inhibited NADH oxidase of a cervical carcinoma (HeLa) derived human cell line [6]. Using plasma membrane vesicles of known absolute orientation, the drug-inhibited NADH oxidase was observed only with vesicles of right side-out orientation [6]. Similar results were obtained as well with oriented vesicles from soybean hypocotyls [4]. 2,4-D, presumably, can readily permeate isolated plasma membrane vesicles but NADH cannot. As with the drug-responsive activity of HeLa cells [6], both right side-out and inside-out vesicles exhibited NADH activity but only with the right side-out vesicles was the oxidation of NADH stimulated by 2,4-D [4]. Intact HeLa cells were subsequently shown to exhibit the drug-responsive oxidation of NADH [7] as would be expected of an external drug-inhibited NADH oxidase. That both the drug and NADH sites were external was shown subsequently by inhibition of NADH oxidation by means of impermeant conjugates of an antitumor sulfonylurea [8].

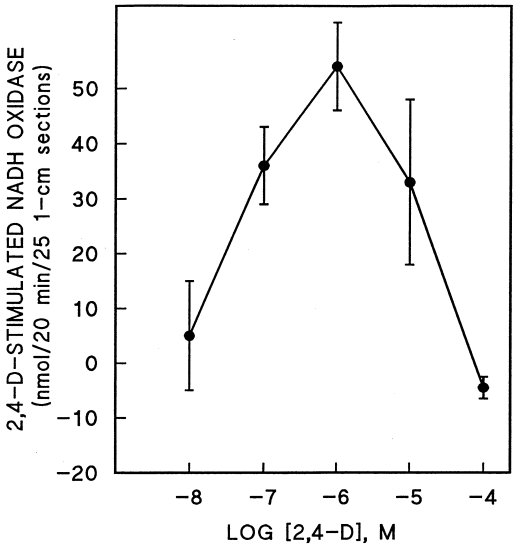

Fig. 4. Dose-response of NADH oxidation by 25 1-cm hypocotyl segments as a function of the logarithm of concentration of the synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D). Results are averages from 3 experiments ± standard deviations.

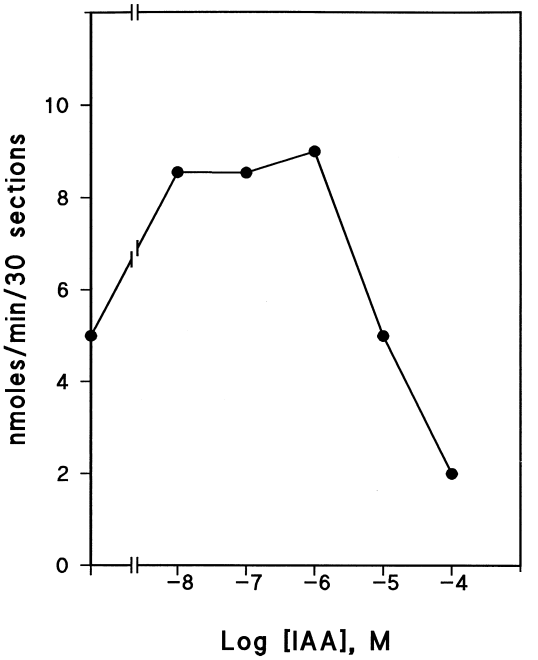

Fig. 5. Dose-response of NADH oxidation by 1-cm hypocotyl segments as a function of the logarithm of auxin concentration for the natural auxin, indole-3-acetic acid (IAA). Results are from rates averaged over 20 min after IAA addition as described for 2,4-D in Fig. 4.

The physiological electron dontor for the 2,4-D-stimulated NADH oxidase is assumed to be some constituent endogenous to the plasma membrane such as reduced quinones or protein thiols rather than NADH since an extracellular source of NADH in plants would be highly unlikely. Protein disulfides have been indicated as the acceptor of pro-

Table 1
Auxin specificity of the stimulation of NADH oxidation by intact segments of etiolated soybean hypocotyls

Auxin	Rate (nmol/20 min/25 sections)
None	50
2,4-D (1 μM)	100
2,3-D (1 μM)	55
IAA (0.1 μM)	73

tons and electrons from NADH in addition to oxygen [9] for the 2,4-D-stimulated activity. This conclusion is based on the reported findings of a 2,4-D-stimulated increase in protein thiols and a corresponding decrease in membrane disulfides in the presence of NADH [9]. The protein appears also to have the ability to function in protein disulfide-thiol interchange [10]. A model has been presented whereby such an activity could function in physical membrane displacements possibly important to plant cell enlargement [11].

In support of the above hypothesis as well as in support of an external form of the NADH oxidase are results with the impermeant thiol reagent PCMS [9]. This compound inhibits specifically 2,4-D-stimulated cell elongation with 1-cm sections of hypocotyl sections as well as inhibition of oxidation of NADH by isolated plasma membrane vesicles.

The basis for the apparent oscillations in A_{340} as a function of time is not known. The oscillations appear to be significant and not due to instrument variation. Studies are in progress to elucidate the source of these variations. They may reflect a natural periodicity in the activity of the cell surface NADH oxidase.

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