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Further Characterization on the Transport Property of Plasmalemma NADH Oxidation System in Isolated Corn Root Protoplasts[1]

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ABSTRACT

Recent experiments show that exogenous NADH increases the O_2 consumption and uptake of inorganic ions into isolated corn (*Zea mays* L. Pioneer Hybrid 3320) root protoplasts (Lin 1982, Proc Natl Acad Sci USA 79: 3773–3776). A mild treatment of protoplasts with trypsin released most of the NADH oxidation system from the plasmalemma (Lin 1982 Plant Physiol 70: 326–328). Further studies on this system showed that exogenous NADH (1.5 millimolar) tripled the proton efflux from the protoplasts thus generating a greater electrochemical proton gradient across the plasmalemma. Trypsin also released ubiquinone (11.95 nanomoles per milligrams protein) but not flavin or cytochrome from the system. Kinetic analyses showed that 1.5 millimolar NADH quadrupled V_{max} of the mechanism I (saturable) component of K^+ uptake, while K_m was not affected. Diethylstibestrol and vanadate inhibited basal (ATPase-mediated) K^+ influx and H^+ efflux, while NADH-stimulated K^+ uptake was not or only slightly inhibited. *p*-Chloromercuribenzenesulfonic acid, *N,N'*-dicyclohexylcarbodiimide, ethidium bromide, and oligomycin inhibited both ATPase- and NADH-mediated H^+ and K^+ fluxes. A combination of 10 millimolar fusicoccin and 1.5 millimolar NADH gave an 11-fold increase of K^+ influx and a more than 3-fold increase of H^+ efflux. It is concluded that a plasmalemma ATPase is not involved in the NADH-mediated ion transport mechanism. NADH oxidase is a -SH containing enzyme (protein) and the proton channel is an important element in this transport system. Fusicoccin synergistically stimulates the effect of NADH on K^+ uptake.

Plasmalemma redox systems have been suggested in various eukaryotic cells (8, 9, 22, 23, 26). Evidence for a direct linkage of this redox system to ion transport via the generation of an electrochemical proton gradient in plant cells is emerging (20, 21). Recently, a terminal oxidase which can accept electrons from exogenous NADH has been isolated from the plasmalemma of corn root protoplasts (20, 21). Exogenous NADH was also found to increase the O_2 consumption and the uptake of inorganic ions in corn root segments (21). In plant cells, it is believed that an electrochemical proton gradient can be established by a plasmalemma ATPase and the hydrolysis of ATP (28). Is the plasmalemma ATPase system also involved in the generation of a H^+ gradient by the NADH redox system? Attempts were made in the present study to address this question and to further characterize the transport property of this system.

Inhibitors of the plasmalemma ATPase, such as DCCD,[2] DES, PCMBS, and vanadate, are well characterized (2, 7, 12, 16, 17, 30). In the experiments reported here, it is shown that in corn root protoplasts, DES and vanadate have little effect on K^+ influx driven by NADH oxidation although they partially inhibit net H^+ efflux. On the other hand, DCCD, ethidium bromide, oligomycin, and PCMBS strongly inhibit both H^+ efflux and K^+ influx linked to NADH oxidation. Kinetic studies show that exogenous NADH is primarily effective in the low (mechanism I) K^+ range. The isolated NADH oxidase appears to function with a quinone cofactor; flavoprotein and Cyt *c* are not required although they may be present in the membrane-linked redox reaction.

MATERIALS AND METHODS

Protoplast Isolation. Root protoplasts were isolated from 3-d-old etiolated corn (*Zea mays* L. Pioneer Hybrid 3320) seedlings as described (19) and suspended for all experiments in a basic medium of 0.6 M mannitol, 0.2 mM $CaCl_2$, and 1 mM Hepes buffer at pH 6.0.

K^+ and H^+ Fluxes Measurement. K^+ influx, with the exception of the kinetic study, was measured by adding 1 mM KCl with carrier-free $^{86}Rb^+$ to the basic medium. After 20 min of incubation at 30°C in the presence of radiolabeled ion, protoplasts were isolated by rapid centrifugation through silicone oil (18). Proton flux was measured at 30°C with a thermostatic reaction vial containing 0.1 ml of protoplast suspension (about 10^6 protoplasts) and 0.9 ml of 0.6 M mannitol, 1 mM KCl, 0.2 mM $CaCl_2$, and 1 mM Hepes buffer at pH 6.0. A pH electrode was used to monitor continuously the pH change of the medium and 0.01 N HCl or NaOH was used for titration. Apparent (net) H^+ efflux was calculated as previously reported (18).

Determination of Cytochromes, Flavin, and Quinones. Previously reported methods were used to extract and measure the Cyt (5), flavin (13), and quinones (11, 15) in the trypsin-released plasmalemma NADH redox system of corn root protoplasts (20).

In brief, Cyt were extracted by adding 0.2 ml of 0.1 M $Al_2(SO_4)_3$ into 2 ml of trypsin-treated protoplast supernatant (20) and adjusted with 3% $Al_2(SO_4)_3$ or 5 N NaOH to pH 4.5. The mixture was kept in an ice bucket and stirred for 2 h. It was then filtered through a No. 1 prewetted Whitman filter paper. The filtrate was adjusted to pH 8.2 to 8.4 with concentrated NH_4OH

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[2] Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; DES: diethylstilbestrol, FC, fusicoccin; PCMBS, *p*-chloromercuribenzenesulfonic acid; NEM, *N*-ethylmaleimide.

and stirred with 50 mg of Celite Filter Aid (Johns Manville Co.) and passed through an Amberlite IRC-50 (Aldrich Chemical) column (1 × 4.5 cm). The column was then washed with 20 ml of 20 mM Na_2HPO_4 buffer (pH 8.0). The colored or total (if no color could be observed) resin was removed and resuspended with 2 volumes of 20 mM Na_2HPO_4 buffer. The supernatant was then removed and the resin was washed three times. Finally, resin was poured back into a column, and 15 to 20 ml of 0.5 M NaCl in Na_2HPO_4 buffer was slowly added to elute the Cyt from the resin. The eluant was then concentrated with a Rota-Vac concentrator down to 1 ml. An absorption spectrum (400–600 nm) was taken with a Beckman DU-8 spectrophotometer.

Flavin was determined by comparing the absorption spectra (250–650 nm) of the trypsin-treated protoplast supernatant before and after the removal of flavin with saturated $(NH_4)_2SO_4$ and 0.1 M Na pyrophosphate (13).

Anhydrous *n*-pentane was used to extract quinones from the trypsin-treated protoplast supernatant (20). Five ml of *n*-pentane (dried with anhydrous Na_2SO_4) was added to the supernatant and vortexed occasionally for 5 min at 4°C. The mixture was centrifuged at 4000 *g* for 3 min. After the *n*-pentane extract was removed, the pellet was reextracted three times with 5 ml each of *n*-pentane. The combined extracts were concentrated down to 1 ml and an absorption spectrum (200–400) was taken for the quinone measurement.

FC was a generous gift from Dr. E. Marrè, and DES, DCCD, ethidium bromide, oligomycin, mersalyl, and PCMBS were purchased from Sigma. All other chemicals were ACS reagent grade.

RESULTS

Inhibitor Studies. The net H^+ efflux from corn root protoplasts was tripled by 1.5 mM NADH (Table I). In the presence of DES or vanadate, H^+ efflux due to NADH was inhibited 60% to 70%. Inhibition was also obtained with the water-soluble sulfhydryl reagents, PCMBS and mersalyl, membrane-permeable sulfhydryl reagent NEM, and the proton channel inhibitors, DCCD (7, 17, 30), oligomycin (25), and ethidium bromide (3). Since only net H^+ efflux can be measured, there is uncertainty in the interpretation of this result, but it seems probable that the redox mechanism responsible for driving H^+ efflux possesses a H^+ transport pathway exceptionally sensitive to mercurials and mitochondrial ATPase H^+-channel inhibitors. Fusicoccin is believed to act by stimulating the activity of the H^+-ATPase (24). As shown in Table I, the extra net H^+ efflux elicited by NADH (Column III) was essentially unaltered by addition of FC. These data suggest that the electrogenic H^+ efflux driven by NADH oxidation must possess a 'proton channel' for H^+ transport with properties similar to those proposed for the H^+-transporting ATPase.

Table I. *Effect of Chemicals on NADH Stimulated Apparent (NET) H^+ Efflux in Isolated Corn Root Protoplasts*

Period of determination was 20 min. NADH concentration was 1.5 mM. Units for I, II, and III were μmol per 10^6 protoplasts per h.

	I (−NADH)		II (+NADH)		III (II − I)	
		%		%		%
Control	0.88	100	2.61	100	1.73	100
DES, 50 μM	0.29	33	1.00	38	0.71	41
Vanadate, 100 μM	0.39	44	0.93	36	0.54	31
PCMBS, 1 mM	0.35	39	0.35	13	0	0
Mersalyl, 50 μM	1.03	117	1.88	72	0.85	49
NEM, 25 μM	0.41	46	1.04	40	0.63	36
DCCD, 50 μM	0.37	42	0.34	13	0.03	2
Oligomycin, 5 μg/ml	0.25	29	0.10	4	−0.15	
Ethidium Bromide, 50 μM	0.44	50	0.01	0	−0.43	
FC, 10 μM	1.41	160	3.02	116	1.61	93

Table II. *Effect of Chemicals on NADH-Stimulated K^+ ($^{86}Rb^+$) Influx in Isolated Corn Root Protoplasts*

Uptake periods were 20 min. NADH concentration was 1.5 mM. Units for I, II, and III were nmol per 10^6 protoplasts per h.

	I (−NADH)		II (+NADH)		III (II − I)	
		%		%		%
Control	25.70	100	62.28	100	36.58	100
DES, 50 μM	6.08	24	31.55	51	25.47	70
Vanadate, 100 μM	14.62	57	51.20	82	36.58	100
PCMBS, 1 mM	15.74	61	25.05	40	9.31	25
Mersalyl, 50 μM	24.67	96	19.93	32	−4.74	
NEM, 25 μM	2.29	9	8.32	13	6.03	16
DCCD, 50 μM	14.75	57	13.28	21	−1.47	
Oligomycin, 5 μg/ml	6.03	23	10.16	16	4.13	11
Ethidium bromide, 50 μM	8.27	32	10.74	17	2.47	7
FC, 10 μM	108.34	422	283.96	456	175.62	480

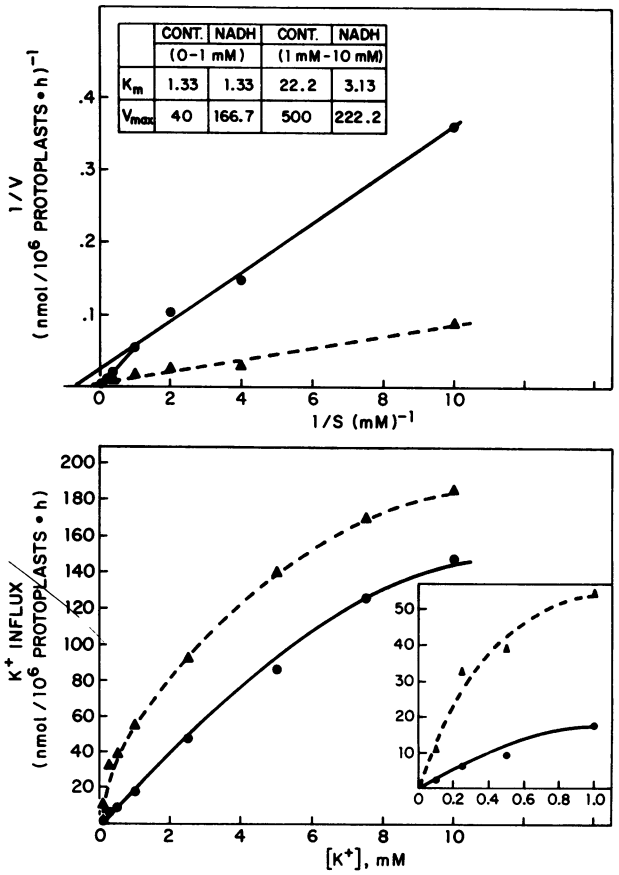

FIG. 1. Effect of exogenous NADH on the concentration-dependence of K^+ ($^{86}Rb^+$) influx in isolated corn root protoplasts. Protoplasts were incubated in radiolabeled ion ($^{86}Rb^+$) uptake solution (see "Materials and Methods" for the composition) for 20 min at 30°C with (▲) and without (●) 1.5 mM NADH. Upper panel, double reciprocal plot of the concentration (kinetic) curves were used to calculate the V_{max} and K_m values.

The possibility that the inhibitors and FC were affecting NADH oxidation was investigated. Within 20 min there was very little or no effect of DCCD, oligomycin, ethidium, PCMBS, mersalyl, NEM, or FC on the basal respiration or extra O_2 consumption due to NADH.

Table II gives comparable data for K^+ ($^{86}Rb^+$) influx. DES gave only 30% inhibition of NADH-driven K^+ influx, and van-

Table III. *Effect of Chemicals on NADH Oxidation in Isolated NADH-Oxidase*

NADH oxidation was calculated from the decrease of A_{340} ($\epsilon = 6.22 \times 10^6$ cm^2) in 10 min. NADH (0.1 mM) and about 50 μg protein of concentrated trypsin-treated protoplast supernatant (20) was used in each assay.

	NADH Oxidation	
	μmol NADH/μg protein·h	%
Control	0.123	100
Quinacrine, 0.5 mM	0.023	19
DCCD, 50 μM	0.120	97
Oligomycin, 5 μg/ml	0.124	101
Ethidium bromide, 50 μM	0.116	94
PCMBS, 1 mM	0.110	89
Mersalyl, 50 μM	0.122	99

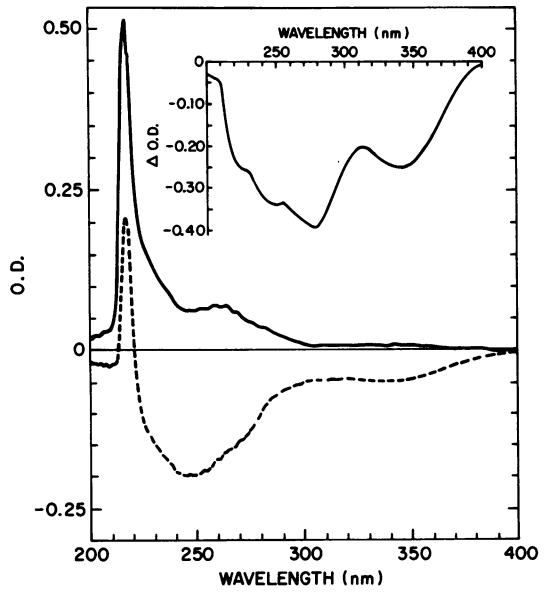

FIG. 2. Absorption spectra of reduced (——) and oxidized (- - -) quinone extracted from trypsin-released plasmalemma NADH redox system in *n*-pentane solution. NADH (1.5 mM) was used to reduce quinone extract and H_2O_2 (1.5 mM) was used to oxidize it. Inset, difference spectrum (reduced *versus* oxidized) of quinone extract.

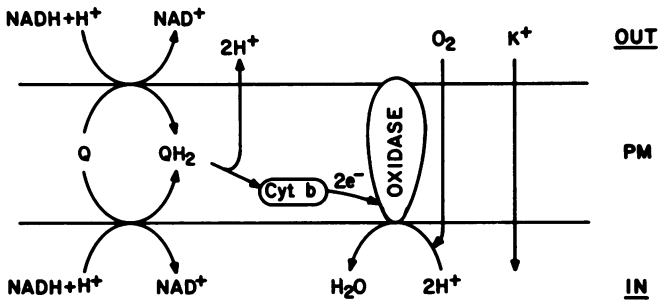

FIG. 3. Model of a plasmalemma $NADH/O_2$ redox system involved in creating an electrogenic potential.

adate none (Column III). Again, however, PCMBS, mersalyl, NEM, DCCD, oligomycin, and ethidium bromide were much more effective than with endogenous K^+ transport. The fact that endogenous K^+ transport was insensitive to mersalyl (column I), whereas NADH-driven K^+ transport was completely inhibited by mersalyl, further substantiates the differentiation of these two K^+ transport systems.

Unlike the result with net H^+ efflux (Table I), FC gave a 400+% increase in K^+ influx with both endogenous and NADH-driven systems. Superficially, this makes it appear that K^+ transport is the same in both systems, and that the action of FC is not confined to H^+-ATPase activity. It is well established that activation of H^+-pumping by FC is dependent on K^+ or other readily transported cation (24).

Figure 1 shows the kinetics of K^+ influx in endogenous and NADH-driven transport. NADH has its primary effect at low K^+ concentrations, the mechanism I range of Epstein (10). It is in this range that corn root cells appear to actively accumulate K^+ against an electrochemical gradient (6). At K^+ concentrations greater than 2 mM, the rate of increase in K^+ influx is essentially parallel for endogenous and NADH-driven transport, with only slight evidence for saturation kinetics. In the mechanism I range, NADH has no effect on the apparent K_m for transport although there is a 4-fold increase in V_{max}. With high K^+ concentrations the NADH system maintains a lower K_m and V_{max} than the endogenous system, but the significance of these calculations is suspect (14).

NADH Oxidase. The trypsin-released NADH oxidase, which appears as a 42,000-D polypeptide on SDS-PAGE (20) was studied for sensitivity to inhibitors and the presence of electron-transport cofactors. It was previously reported (21) that quinacrine, suggested to be a specific inhibitor of a transmembrane oxidoreductase (9), specifically inhibited the NADH-driven influx of K^+ and Pi in protoplasts. As shown in Table III, quinacrine also inhibited NADH oxidation by the isolated oxidase. However, DCCD, oligomycin, ethidium bromide, PCMBS, and mersalyl had no effect. The action of quinacrine is thus directly on the oxidase while the proton channel and SH inhibitors are only effective when the oxidase is associated with the plasmalemma in a conformation producing H^+-pumping, hyperpolarization, and salt uptake.

No evidence could be found for the presence of Cyt or flavoproteins with the isolated oxidase. These may be intrinsic proteins which remain within the membrane. However, using 12.2 mM cm^{-1} as the extinction coefficient for Δ275 to 290 nM absorption (11, 15), about 12 nmol quinone/mg protein was found. The difference spectrum of oxidized and reduced states indicates the quinone to be ubiquinone (Fig. 2, inset).

Plasmalemma vesicles from *Bacillus subtilis* which oxidizes NADH at the outer surface appear to pass electrons directly to menaquinone −7 (4). Direct addition of menadione, a quinone analog, stimulated NADH oxidation by the isolated corn root NADH oxidase by more than 50% (data not shown). It appears that the isolated oxidase may transfer electrons to ubiquinone which is then oxidized by O_2. At some point quinacrine interferes with electron transfer.

We are continuing to investigate the possibility that the oxidase has only ubiquinone as a cofactor. As reported previously (20) removal of the oxidase from the outer surface of the protoplast does not impair the basic K^+ transport capacity of the membrane, but does sharply reduce the ability of exogenous NADH to stimulate transport. The enzymic mechanism for transport seems to be intrinsic, and the presence of the oxidase simply couples another source of energy for driving it.

DISCUSSION

The evidence that a H^+-ATPase is responsible for energizing salt uptake is extensive (28) and unquestioned here. However, at least for corn root protoplasts, there is now evidence that a redox reaction driven by an NADH oxidase which is localized in the plasmalemma can also drive H^+ efflux and K^+ influx and hyperpolarize the protoplast membrane potential. The present findings with inhibitors indicate that although the energy-linkage of the endogenous and NADH systems are different, there may be

common elements with respect to H^+ and K^+ transport. NADH oxidation appears to operate through an avenue for driving H^+ efflux which is sensitive to proton channel inhibitors (Table I) and an avenue for K^+ influx which may be responsive to FC (Table II). These results were obtained at low K^+ concentrations where the K_m for K^+ influx is unaltered by NADH. Obviously, more must be learned about H^+ and K^+ channels before these findings can be interpreted, but it seems possible that these channels may be similar for both energy-linked systems.

It should be pointed out that there is an imbalance between the H^+ efflux and the K^+ influx in the system reported here (Table I *versus* Table II). However, the responses of these fluxes to the addition of NADH and other chemicals tested do correlate reasonably well. The results do appear to make out a 1:1 of H^+ to K^+ exchange mechanism. They do also suggest that there must be a large movement of other ion(s) to balance the H^+ efflux which may or may not be directly related to the addition of chemicals.

A question arises as to whether the endogenous system incorporates some transport driven by exogenous NADH. Quinacrine, which seems to specifically inhibit the NADH oxidase (Table III), is also a strong inhibitor of K^+ influx in corn root segments (21). NADH-driven ion uptake in roots and protoplasts is commonly inhibited by the uncoupler (p-trifluoromethoxy)carbonyl cyanide phenylhydrazone (21). The question cannot be answered with any more assurance than previously (1, 27, 29), but there is now more reason to ask it.

The present findings and those reported previously (20, 21) clearly demonstrate at least for corn root protoplasts, that the oxidation of exogenous NADH on the outer surface of the plasmalemma can result in an increase of H^+ efflux and inorganic ion influx and a hyperpolarization of the membrane potential. A model outlined in Figure 3 is proposed that a quinone pool in the plasmalemma can be reduced by NADH either from the apoplastic or cytoplasmic side of the membrane. When quinone is reoxidized, it passes e^- to a plasmalemma Cyt *b* and NADH-oxidase, and the H^+ is then released to the outside. The external O_2 accepts electrons and consumes the internal H^+ at the oxidase site. A completion of this NADH/O_2 electron transport chain results in a net H^+ efflux thus generating an electro-proton gradient for ion transport.

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