Evidence for a Plasmalemma Redox System in Sugarcane

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ABSTRACT

A plasmalemma-bound NADH-dependent redox system has been identified in protoplasts isolated from cell suspensions of sugarcane. This system oxidized NADH as well as NADPH, increased O2 consumption 3-fold, and increased the pH of the external medium while the cytoplasmic pH was decreased. In the presence of NADH, ferricyanide was rapidly reduced and the external medium was acidified. The uptake rates of K+, 3-O-methylglucose, leucine, and arginine were all decreased in the presence of NADH.

A redox system localized within the plasmalemma of higher plants is an appealing concept for which there is at present still little direct evidence. Nevertheless, convincing data have been obtained from corn root (4) and oat root preparations (8). Additional indirect evidence supporting the functioning of a redox chain in the plasmalemma of plants has been reported (1, 2). The possible significance of oxidation-reduction in the plasmalemma was considered in a review by Löw and Crane (6) who suggested that redox reactions may control glycolytic functions, peroxidation of unsaturated lipids, and other cell functions via sulfhydryl sites. A trans-membrane electron transfer has also been proposed for iron reduction in bean roots (12). Of particular interest would be a redox chain able to function as an adjunct or alternative to ATPase in energization of the plasmalemma for nutrient transport. Evidence for such a redox system is gaining wider credence (5).

The addition of NADH to intact corn roots or protoplasts greatly increases O2 consumption (4) and NADH oxidation can be coupled to the reduction of added ferricyanide (2). Moreover, Lin (5) has demonstrated that K+ influx, particularly at low concentrations, is stimulated by the addition of NADH to corn root protoplasts, is accompanied by proton efflux, and causes an increase in the membrane potential.

Our interest in a possible relationship between a redox function and sugar transport across the plasmalemma originated with an observation that membrane preparations from sugarcane cells showed NADH bound to the plasmalemma and a promotion of its oxidation to NAD in the presence of glucose (9). In the present investigation we wanted to determine whether the observations made by Lin (4, 5) in corn root protoplasts could be extended to another species, such as sugarcane. Our results revealed some similarities in the behavior of these two species, but also some important differences that cast doubt on the significance of an NADH-linked redox chain to nutrient transport across the plasmalemma in sugarcane.

MATERIALS AND METHODS

Sugarcane cell suspensions from a subclone of Saccharum sp. hybrid H50-7209 were grown in White's inorganic salt mixture supplemented with yeast extract, arginine, sucrose, vitamins, and 2,4-D (7). Protoplasts were isolated from 9-d-old cultures as previously described (11). For all experiments, protoplasts were resuspended in White's basal salt mixture containing 0.5 mM mannitol, pH 5.5 (medium A).

O2 consumption was measured at 25°C, using an O2 electrode (Yellow Springs Instruments). NADH oxidation and ferricyanide reduction were measured as the decrease in A at 340 and 420 nm, respectively. The pH of the medium was measured with a pH electrode. Membrane potential was measured as the accumulation of [14C]tetraphenylphosphonium ion. The lyophilic cation (5 μM) was added to protoplast suspension 20 min before addition of NADH. It had previously been determined (3) that equilibration was reached within 10 to 15 min. Samples were withdrawn at intervals after NADH addition and the protoplasts separated from medium by centrifugation through silicone oil (14). Radioactivity in the supernatant and protoplast pellet was determined and the membrane potential calculated according to the Nernst equation. Internal pH was measured as the distribution of the weak acid [14C]-5,5-dimethyl-2,4-oxazolidinedione in a manner similar to the membrane potential measurements. Internal pH was calculated according to the formula as described by Waddell and Butler (13). Protoplast volume was determined as the [3H]H2O accessible but [14C]dextran excluding space (10). Cytoplasmic volume had been previously determined (10) to be 32% of the protoplast volume.

For uptake measurements, protoplasts were incubated in medium A at 25°C. Radioactive substrate was added at zero time and samples were withdrawn after 1, 3, 5, and 7 min of incubation. Protoplasts were separated from the medium by centrifugation through silicone oil. Radioactivity was determined by scintillation spectrometry. [14C]NADH was prepared from [14C]NAD by reduction with sodium thiosulfate. NAD and NADH uptake was measured after incubations for 10 s and 15 min. Protoplasts were separated from the medium by silicone oil centrifugation.

Concentration of cations (Na+, K+, and Mg2+) in the protoplast suspension medium was determined by inductively coupled argon plasma spectroscopy (Jarrell-Ash Division, Fisher Chemicals).

Radiochemicals were purchased from New England Nuclear: NAD (adenosine [U-14C], 554 mCi/mmol; rubidium-86, 75 mCi/mmol; 3-O-methylglucose (glucose [U-14C], 300 mCi/mmol; leucine [U-14C], 503 mCi/mmol; arginine [U-14C], 287 mCi/mmol; tetraphenylphosphonium [phenyl-14C], 19.2 mCi/mmol; 5,5-dimethyl-2,4-oxazolidinedione [2-14C], 47 mCi/mmol. All organic chemicals were purchased from Sigma Chemical Co.

RESULTS

O2 Consumption. Prior work by Lin (4) has shown that the addition of NADH to corn roots or to protoplasts prepared from

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roots stimulated \( \text{O}_2 \) consumption. \( \text{O}_2 \) consumption by protoplasts from sugarcane cells grown in suspension cultures was stimulated almost 3-fold in the presence of NADH (Fig. 1). NADPH elicited the same response. Cyanide reversed the rate of \( \text{O}_2 \) consumption to a value lower than that of the control (Fig. 1), suggesting that flavoprotein and/or Cyt are involved in the increase. Succinate did not stimulate \( \text{O}_2 \) consumption by the protoplast suspension (results not shown); therefore, the observed increase is not from mitochondrial contamination by broken protoplasts. There was no stimulation of \( \text{O}_2 \) consumption by catechol or IAA; therefore, neither phenol oxidase nor IAA oxidase was involved.

**NADH Oxidation and Ferricyanide Reduction.** Sugarcane protoplasts oxidized exogenous NADH at a rate of \( 7 \ \mu\text{mol} \cdot 10^6 \text{ protoplasts}^{-1} \cdot \text{h}^{-1} \) (Fig. 2). The oxidative enzyme was not specific for NADH since NADPH was oxidized at the same rate. NADH added to a suspension of \( 10^6 \) protoplasts at 1.5 mm concentration was almost completely removed from the medium within 10 min after addition. Uptake of NADH into the cells could not account for its disappearance from the medium since the rate of NADH uptake was 18 nmol \( \cdot 10^6 \) protoplasts \( ^{-1} \cdot \text{h}^{-1} \) and the NADH uptake rate was 7.8 nmol \( \cdot 10^6 \) protoplasts \( ^{-1} \cdot \text{h}^{-1} \). These rates are less by a factor of 1,000 than the NADH oxidation rate. Hence, NADH oxidation occurs at or near the outer surface of the protoplasts. No NADH oxidation was detected in the supernatant when protoplasts were removed by low speed centrifugation (40g for 5 min). Cyt c was not oxidized by a suspension of intact protoplasts, further evidence that the oxidation is not caused by broken or intact mitochondria or by plastids. There was no reduction of NAD to NADH, therefore, no basis for direct cycling between these two substances.

**Effect of NADH Addition on Protonmotive Potential.** NADH added to protoplast suspensions caused an immediate and rapid alkalization of the medium (Fig. 4). Addition of an electron acceptor, ferricyanide, to the protoplast suspension simultaneously with or following the addition of NADH caused an acidification of the incubation medium (Fig. 4). Ferricyanide was rapidly reduced in the presence of NADH, and once the acceptor was used up there was no further acidification of the medium. With second and third additions of ferricyanide, medium acidification resumed at the initial rate.

In the absence of ferricyanide, and concurrent with medium alkalization caused by NADH addition, acidification of the cytoplasm from a normal value of pH 7.2 to 6.8 occurred within 10 min (Fig. 5). The decrease of cytoplasmic pH probably was not reflected by a transient depolarization of the membrane potential (Fig. 6) and, in fact, there was slight hyperpolarization. The shift in medium pH probably caused the slight change of membrane potential (3) which, together with the collapse of the pH gradient by the addition of NADH, decreased the protonmotive potential.
from −170 mV to approximately −88 mV.

Concentration of several major cations (Na⁺, K⁺, Mg²⁺) was not increased in the medium when NADH was added to the protoplast suspension, showing that influx of H⁺ was not accompanied by efflux of any of the major cations in the cells. The proton influx rate was 10.6 μmol·10⁶ protoplasts⁻¹·15 min⁻¹ and a cation efflux rate of the same magnitude would be required to balance the charge. The analytical procedure used in these experiments would be sensitive enough to detect such a change.

**Effect of NADH Oxidation on Nutrient Uptake.** Uptake rates of K⁺, 3-O-methylglucose (a glucose analog), leucine, and arginine were inhibited in the presence of NADH (Table I). The inhibition is greater than would be expected from a pH change of the external medium and may be a function of acidification of the cytoplasm. These data show conclusively that, in sugarcane protoplasts, external NADH oxidation does not stimulate nutrient uptake.

**DISCUSSION**

Our results demonstrate the existence of an NADH/NADPH oxidation system on the external surface of sugarcane protoplasts isolated from suspension cultures. In contrast to the results from maize roots (2) and yeast cells (6) where internal NADH was oxidized by reduction of external ferricyanide, ferricyanide reduction in sugarcane protoplasts required exogenous NADH. Our data confirm results reported for maize roots (2) and yeast cells (6), i.e. that ferricyanide reduction is accompanied by proton efflux. However, NADH oxidation in the absence of an electron acceptor caused alkalinization of the external medium and acidification of the cytoplasm. These results suggest that the presence of a nonmembrane-bound, impermeable artificial acceptor, such as ferricyanide, prevents proton transfer across the membrane and facilitates their release into the medium. On the other hand, in the absence of an artificial acceptor, an as yet unidentified component of a trans-membrane electron chain accepts and transfers the proton to the inside of the cell, rather than to the outside as proposed by Lin (5). Conceivably, the NADH-dependent ferricyanide reduction system may be an independent redox system on the membrane with as yet unknown functions. Addition of NADH to a protoplast suspension may cause the plasmalemma redox system of sugarcane to function in a reverse direction so that protons are pumped into the cytoplasm rather than to the cell exterior. Perhaps such a system provides an inwardly directed 'proton pump' as a safety valve in case of massive alkalinization of the cytoplasm induced by some external factor.

The components of the plasmalemma-bound redox system in sugarcane protoplasts are not known, but it must be assumed that a membrane-bound quinone is the acceptor and NADH or...
NADPH the most likely internal electron donor. Lin (5) has reported that in corn protoplasts a quinone pool in the plasmalemma can be reduced by NADH from either side of the cell membrane. When the quinone is reoxidized, electrons are transferred to Cyt b and NADH oxidase.

Acidification of the cell interior should be accompanied by a transient polarization of the membrane potential to a less negative value. Our data showed no NADH-elicited depolarization. Tetraphenylphosphonium ion may bind nonspecifically to membranes and therefore may not be a reliable probe for measurement of membrane potential when absolute values are desired, but it permits the monitoring of changes in membrane potential. Since there was no increase in the concentration of cations in the external medium, it is conceivable that cations were sequestered in the vacuoles. Alternatively, anions such as Cl\(^-\) or SO\(_4\)\(^{2-}\) could have either entered the cell along with protons or been mobilized from the vacuoles. These changes would reestablish the charge balance in the cytoplasm and retain the normal membrane potential on the plasmalemma. However, the effect of Cl\(^-\) or SO\(_4\)\(^{2-}\) movement out of vacuoles would disturb the charge balance and presumably cause the membrane potential on the tonoplast to be polarized to a more positive value.

Evidence for existence of the NADH redox system is most pronounced in protoplasts. NADH oxidation cannot be detected in intact cells and the NADH-ferricyanide reduction rate was approximately two orders of magnitude lower in intact cells than in protoplasts. The cell wall probably acts as a diffusion barrier preventing NADH (whatever its source) from reaching the oxidation site on the plasmalemma. In addition, it cannot be ruled out that the plasmalemma redox system is not an artifact of cell wall removal, i.e. a wound response. It is unlikely that the redox system is the result of leaky or broken protoplasts, since (a) removal of protoplasts by low speed centrifugation showed no evidence for redox activity in the supernatant solution, and (b) NADH oxidation rates were similar in intact protoplasts and in a suspension in which >50% of the protoplasts were broken.

The decreased uptake rates for K\(^+\), 3-O-methylglucose, and amino acids is a rational consequence of the observed decrease in protonotive potential caused by NADH oxidation. Evidence presented here shows that in sugarcane protoplasts an exogenous NADH oxidation system does not provide the energy for nutrient transport.

LITERATURE CITED

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