Redox Activity at the Surface of Oat Root Cells

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ABSTRACT

Electron transport activity at the cell surface of intact oat seedlings (Avena sativa L. cv Garry) was examined by measuring the oxidation and/or reduction of agents in the medium bathing the roots. Oxidation of NADH with or without added electron acceptors and reduction of ferri-cyanide by an endogenous electron donor were detected. The activities appear to be due to electron transfer at, or across, the plasma membrane and not due to reagent uptake or leakage of oxidants or reductants. NADH-ferri-cyanide oxidoreductase activity was also detected in plasma membrane-enriched preparations from Avena roots. Based on redox responses to pH, various ions, and to a variety of electron donors and acceptors, the results indicate that more than one electron transport system is present at the plasma membrane.

Redox activity, which provides the energy for many important biological processes, is known to be associated with organelles such as mitochondria and chloroplasts. This activity also appears to be present at the plasma membrane of eukaryotic cells. There are reports of electron transport at the surface of intact ascites tumor cells (7), yeast (10), Neurospora (24), carrot phloem cells (8, 23), tobacco callus (2), corn root segments, and protoplasts (12, 15, 19). Electron transport also has been found in plasma membrane fractions from animal tissues (12), Neurospora (4), corn coleoptiles (17, 25), oat roots and shoots, and cauliflower (32). In support of these results, components usually associated with redox activity have been detected on the plasma membrane of animal cells (5, 20, 22), and there is also evidence in plants for flavins and for a b-type Cyt (17, 32).

Cell surface redox activity might be related to proton excretion in a manner analogous to that which occurs in prokaryotes, and in chloroplasts and mitochondria. Although this process is controlled at least in part by a proton-translocating ATPase (29), it is still possible that some of the proton transport is coupled to energy released by electron flow (8, 10, 11, 19). If proton fluxes across the plasma membrane are at least partially mediated by redox activity, this may have important implications for determining the regulation of solute and ion transport as well as for understanding the action of light and auxin, both of which can accelerate proton excretion (26, 31).

Some phenomena have already been linked to redox activity. For example, in Neurospora sporangiohpheres and in corn coleoptiles, blue light induces the reduction of a b-type Cyt, and this event, occurring in the plasma membrane, has been correlated with phototropic curvature of the intact organ (18). Redox activity also appears necessary for the uptake of iron, which must be converted to its reduced form before entering the cells of certain plants (6). Recent evidence suggests that iron is reduced by a plasma membrane-localized redox system (27).

Earlier reports have measured cell surface redox activity in various ways and on a range of plants. We wanted to confirm these reports with one organ of a single species—the roots of intact oat seedlings. Roots were chosen because they need not be peeled or abraded in order for agents to enter the intercellular space; thus, artifacts due to wounding are less likely. Based on data for pH optima, ion requirements, and various electron donors and acceptors, our results indicate that electron transport complexes exist at the cell surface of intact roots; we also show that oxidoreductase activity is present in plasma membrane-enriched preparations from these roots.

MATERIALS AND METHODS

Plant Culture. Oat 'seeds' (Avena sativa L. cv Garry) were germinated on water-moistened vermiculite for 3.5 to 4 d in total darkness except for a 30-s exposure to white light on the 3rd d. To determine the importance of bacterial contamination, seeds were soaked in 25% (v/v) chloroform solution, washed three times in sterile distilled H2O, and planted in autoclaved vermiculite using sterile procedures. Subsequent conditions were the same as above. The amount of bacteria associated with the roots was determined by homogenizing roots from ten seedlings in 20 ml of 25 mM Tris-Mes (pH 6.7), 2% (w/v) sucrose. The diluted extracts were plated on 4% (w/v) agar, 2.75% (w/v) tryptic soy broth, and 2% (w/v) dextrose (pH 7.3). The sterilization procedure reduced detectable bacterial contamination from about 5 × 10^5 colonies to at most 5 × 10^2 colonies/g fresh weight of roots.

In Vivo Redox Assays. Intact plants were removed from the vermiculite, the roots washed in three changes of distilled H2O, and groups of five or ten seedlings were placed in 20-ml beakers containing 5 ml of distilled H2O. Root fresh weight per seedling was approximately 50 mg. After shaking for 30 min on a rotary shaker at 100 rpm, the water was aspirated and 5 ml fresh distilled H2O added for about 30 min more. The seedlings were removed, the roots gently patted dry between layers of tissue, and immersed in 3.5 ml of incubation medium containing 10 mM Tris-Mes (pH 6.7) and freshly added redox agents with or without other additives.

For a zero-time reading, an aliquot of this solution was immediately decanted into a cuvette and optical density measured in a Beckman 25 dual-beam spectrophotometer. The identical medium which had not been in contact with roots was in the reference cuvette. Following determination of absorbance (this took about 20 s), the contents of the sample cuvette were poured back into the beaker containing the seedlings, and the beaker was then placed on the shaker until the next reading was due (usually after 1 or 2 min). All treatments were replicated and experiments were repeated on at least two different occasions; the data that follow represent the results of typical experiments.

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The wavelengths and extinction coefficients for the redox agents were as follows: NADH and NADPH: 340 nm, 6.23 mm⁻¹ cm⁻¹; K₂Fe(CN)₆: 420 nm, 1.00 mm⁻¹ cm⁻¹; Cyt c: 550 nm, 21.1 mm⁻¹. For some experiments in which reduction of ferricyanide was measured without any added electron donor, a diphenyl o-phenanthroline complex was formed with ferricyanide using 2.1 ml of the incubation medium and 0.9 ml of the reagent mix described by Avron and Shavit (1); absorbance was measured at 535 nm, the extinction coefficient was 20.5 mm⁻¹ cm⁻¹ and, again, the identical solution without roots was used to prepare the blank.

Oxygen uptake was measured polarographically with a Clark-type oxygen electrode. The chamber contained 1 ml of the incubation medium described above or the medium of Lin (18). Five or ten 1-cm subapical root segments were then added either immediately after cutting or after 3 h of washing in the same medium bubbled with air.

Preparation of Plasma Membrane-Enriched Fractions. Four-d-old etiolated oat roots were rinsed and ground in the medium of Hodges and Leonard (14) as modified by Stout and Cleland (30). The filtered homogenate was centrifuged at 1,000g and then at 15,000g, and finally at 80,000g with the 13,000g to 80,000g pellet fractionated on a sucrose step gradient containing 25%, 34%, and 45% (v/v) sucrose in 1 mM Tris-Mes (pH 7.5) and 1 mM MgSO₄. The discontinuous gradient was centrifuged for 2 h at 70,000×g in a swinging bucket rotor. Membranes accumulating at the 34 to 45% interface were placed on a 15% to 60% (v/v) renograin gradient containing 250 mM sucrose, 0.1 mM MgSO₄, 25 mM Tris-Mes (pH 7.5) similar to the methods of Leong and Briggs (17). After centrifuging at 70,000×g for 2 h, 1.4-ml fractions were collected.

In Vitro Enzyme Assays. K⁺-ATPase activity was determined in the presence of 0.5 mM (NH₄)₂MoO₄ and 0.5 mM NaN₃ using the procedures of Stout and Cleland (30) except that 25 mM K₂SO₄ was used as the K⁺ salt. The tubes were spun at 200g to remove any renograin precipitate before reading absorbance.

Cyt c oxidase activity was assayed in 1 ml of solution containing 10 mM Tris-Mes (pH 7.5), 0.1% (v/v) Triton X-100, and 250 μg/ml reduced Cyt c. The reaction was initiated by adding 200 μl of the appropriate membrane fraction and loss of absorption was measured at 550 nm for about 2 min; KCN was then added to a final concentration of 1 mM. Any Cyt c oxidation occurring after addition of KCN was subtracted from that observed before the inhibitor was added.

NADH-ferricyanide oxidoreductase activity was assayed in 1 ml of 10 mM Tris-Mes (pH 7.5), 0.8 mM K₂Fe(CN)₆, and 200 μl of the desired membrane fraction. The reaction was initiated by adding NADH to a final concentration of 0.1 mM. The loss of absorption was measured at 340 nm, using the identical solution without membranes as a blank.

RESULTS

Evidence for redox activity at the cell surface was obtained by monitoring the oxidation of electron donors such as NADH and the reduction of electron acceptors such as ferricyanide in the medium surrounding oat root cells. NADH oxidation (Fig. 1A, lower trace) and ferricyanide reduction (Fig. 1B) is detected, and at neutral and alkaline pH values, the presence of both NADH and ferricyanide (Fig. 1A, upper trace) result in a redox rate greater than the sum of either reagent by itself. Oxidation of NADH in the presence of ferricyanide also occurs in the reaction medium without roots, but the rate of this reaction is 10% of that seen when roots are present. Furthermore, the medium containing redox agents is used as a reference, so only the root-stimulated activity is reported. In many cases, however, we tested separately various factors on the spontaneous chemical oxidation of NADH by ferricyanide; the results are reported below.

When assayed with roots, the oxidation of NADH in the presence of ferricyanide and the reduction of ferricyanide alone show a steadily increasing activity with increasing pH, especially from pH 7.0 to 8.3. The oxidation rate of NADH alone, on the other hand, is relatively insensitive to pH from 5.5 to 7.3 but above that pH, the rate decreases (Fig. 1A, lower trace). A similar result is obtained for NADH-stimulated oxygen uptake by corn root protoplasts (19). The chemical reduction of ferricyanide by NADH is insensitive to pH over the entire range tested.

The rate of NADH-ferricyanide oxidoreductase activity is linear for over 10 min (Fig. 2); the leveling off that occurs after this time is not due to injury, since replacement with fresh medium restores redox activity to its original rate. Further evidence that the redox agents are not deleterious to metabolism is the lack of effect of NADH and ferricyanide on oxygen uptake. Also apparent in Figure 2 is that redox activity extrapolates almost to zero-time and that the stoichiometry is close to 1 mol NADH oxidized/2 mol ferricyanide reduced. The root-induced oxidation of NADH and reduction of ferricyanide (when each are present separately) is also linear for over 10 min (data not shown).

Similar kinetics to those in Figure 2 are observed for the oxidation of NADH in the presence or absence of ferricyanide and for the reduction of ferricyanide alone when roots are grown under sterile conditions (see "Materials and Methods"). Furthermore, bacteria grown from root homogenates show little redox activity when approximately 10⁶ bacteria are incubated in 3.5 ml buffer containing NADH and ferricyanide.

The root-stimulated reduction of ferricyanide with or without NADH has a Q₁₀ from 15°C to 25°C of 1.7 to 2.0 compared to a Q₁₀ of 1.1 to 1.3 for the chemical reduction of ferricyanide by NADH. All root-stimulated redox activity is destroyed after the
roots are boiled for 5 min and thoroughly washed. By varying the concentrations of ferricyanide (five concentrations from 0.16 to 0.8 mM) in the presence of NADH, an apparent $K_m$ of about 0.1 mM is obtained, and apparent $K_m$ values of 1.0 to 1.4 mM and 0.6 to 1.0 mM are obtained for the reduction of ferricyanide alone and for the oxidation of NADH alone, respectively (using five concentrations from 0.05 to 0.13 mM).

Redox activities in the presence of oat roots are stimulated strikingly by concentrations of CaCl$_2$ above 0.1 mM (Fig. 3), but no supraoptimal calcium concentration is seen up to 40 mM. Similar concentration-dependent stimulations by calcium occur during the chemical reduction of ferricyanide by NADH (data not shown).

The ability to increase redox activity is not peculiar to calcium as shown in Table I. MgCl$_2$ and SrCl$_2$ are as active as calcium, while LaCl$_3$ and CeCl$_3$ would be 20 to 30 times more active at equimolar concentrations. The monovalent cations Na$^+$ and K$^+$ must be increased to 100-fold higher concentrations to approach the activity of divalent cations (Table I). The relative effectiveness of the ions for the chemical oxidation of NADH by ferricyanide is similar to that seen in the presence of intact roots; however, rates for the chemical reaction remained at 10% of that when roots are present.

The possibility that leakage of agents into the incubation medium might account for the electron transfer observed was investigated by preincubating roots in buffer, removing the seedlings, and then adding the redox agents to the solution. Ferricyanide-stimulated NADH oxidation is apparent in this medium over a comparable period of time, but the rate is 10% or less of that when roots are present. When oxidation of NADH or reduction of ferricyanide were measured alone, rates were 3% of that with roots present. None of the redox activities in the media from which roots were removed are stimulated by calcium and the $Q_0$ is close to 1. And, finally, attempts to stimulate leakage by removing root segments and varying the number of cut surfaces results in identical redox activity when expressed per mg fresh weight, i.e. 58.2 nmol NADH oxidized in the presence of ferricyanide using 25, 5 mm subapical segments versus 58.4

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**Table 1. Effect of Various Ions on Redox Activity**

<table>
<thead>
<tr>
<th>Salt Added (mM)</th>
<th>Redox Activity</th>
<th>NaCl (100)</th>
<th>KCl (100)</th>
<th>MgCl$_2$ (1.0)</th>
<th>SrCl$_2$ (1.0)</th>
<th>LaCl$_3$ (0.1)</th>
<th>CeCl$_3$ (0.1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADH oxidation</td>
<td>177</td>
<td>201</td>
<td>122</td>
<td>257</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NADH oxidation with ferricyanide</td>
<td>76</td>
<td>83</td>
<td>95</td>
<td>332</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NADH oxidation with ferricyanide</td>
<td>114</td>
<td>131</td>
<td>112</td>
<td>112</td>
<td>255</td>
<td>213</td>
<td></td>
</tr>
</tbody>
</table>

nmol NADH oxidized using 50, 2.5-mm segments.

Electron acceptors other than ferricyanide were investigated in order to further characterize cell surface redox activity (Table II). Besides ferricyanide, considerable oxidation is induced by Cyt c and somewhat less by DHA$^2$. We have not determined if Cyt c-induced oxidation of NADH is due to reduction of its iron moiety or to its being a polycation. DQ does not stimulate NADH oxidation.

Additions of 1 mM KCN (active species HCN) have different effects on NADH oxidation depending on the electron acceptor in the medium (Table II). When NADH is oxidized in the presence of ferricyanide or Cyt c, HCN is not inhibitory, even if the roots are pretreated with the poison for 5 min; the reduction

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$^2$ Abbreviations: DHA, dehydroascorbic acid; DQ, duroquinone; SOD, superoxide dismutase.
Table II. Effects of Various Electron Acceptors on Oxidation of NADH by Intact Roots

<table>
<thead>
<tr>
<th>Oxidant</th>
<th>NADH Oxidation Ratea (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>−HCN</td>
</tr>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>Ferricyanide (0.8 mM)</td>
<td>331</td>
</tr>
<tr>
<td>Cyt c (250 µg/ml)</td>
<td>217</td>
</tr>
<tr>
<td>DHA (0.5 mM)</td>
<td>170</td>
</tr>
<tr>
<td>DQ (40 µM)</td>
<td>98</td>
</tr>
</tbody>
</table>

*a Expressed as a per cent of the control containing only NADH. See Figure 3A for typical rates of NADH oxidase activity.

The data presented, we conclude that redox activity, which is not related primarily to epiphytic bacteria, occurs at the outer surface of oat roots. This conclusion, however, depends on the nonpermeating properties of the redox agents used; evidence that NADH and ferricyanide do not enter plant cells is provided by Lin (19) and Craig and Crane (8), respectively. Furthermore, time-course curves for NADH oxidation and for ferricyanide reduction extrapolate almost to zero-time; this is an unlikely result if the reagents must first permeate the cell in order for the redox reaction to occur and then return to the incubation medium. We are also unable to detect any changes in oxygen uptake after addition of NADH and/or ferricyanide. Finally, a surface-localized reaction is suggested by the marked stimulation of redox activity by lanthanum (Table I), a nonpermeating cation (16). It also seems unlikely that Cyt c and DHA permeate the cell.

The striking stimulation of redox activity by multivalent cations (Table I) can be explained by a neutralization of negative surface charges allowing the negatively charged redox agents to approach the plasma membrane. This conclusion, already proposed by others (10, 11), is based on the parameters of Borst-Pauwels (3) which predict in our case that increasing valence increases redox activity (Table I), that no specificity exists within each valence group (Table I), and that relatively high concentrations of cations are required (Fig. 3).

We did note, however, that the ion concentration curves and the ion specificities for redox activity in the presence of roots is identical to the spontaneous chemical oxidation of NADH by ferricyanide in a solution with charged roots; thus, even though the rate of the chemical reaction is 10% of that of root-stimulated reactions and is insensitive to pH from 5.5 to 8.3, it is possible that the ions also serve to facilitate electron transfer. Furthermore, our experiments do not rule out a regulatory role for catalytic (i.e., µM) amounts of endogenous calcium or any other ion.

We considered the possibility that redox activity detected in the incubation medium is due to substances leaking from the cells. One might expect, in such a case, to find these substances when NADH and/or ferricyanide are added to media which had previously bathed the roots. Activity is detected, but the rate is, at most, 10% of that seen when roots are present, and, unlike the activity in the presence of roots, the Q10 is close to 1 and activity is not stimulated by CaCl2. Furthermore, redox activity is not correlated with number of cut surfaces, a condition which should stimulate leakage.

That the cell-surface electron transport or dehydrogenase ac-
activity occurs at the plasma membrane rather than (or as well as) at the wall is suggested by the observation that similar redox activities can be detected using mesophyll protoplasts (Rubinstein, Stern, and Schiff, in preparation) and by the report showing NADH oxidation by corn root protoplasts (19). What is more, the ferricyanide-stimulated oxidation of NADH in vitro (Table III; Fig. 4) further reinforces the suggestion that dehydrogenase activity resides with the plasma membrane. However, fractions off the renogaffin in gradient contain other membranes besides plasma membrane, so a more complete purification is needed before meaningful comparisons can be made of in vitro and in vivo activities.

We can only speculate about the nature of the endogenous electron donors and acceptors. Lin (19) and Misra et al. (23) have implicated oxalic acid as an electron acceptor, but we have not detected any stimulation of oxygen uptake above respiratory rates during oxidation of up to 1.5 mM NADH. Endogenous pyridine nucleotides may be electron donors for the reduction of exogenous ferricyanide as suggested by indirect experiments of Craig and Crane (8) and by more direct determinations in bean (28) and corn roots (Qiu, Rubinstein, and Stern, unpublished results).

It is important to determine whether more than one electron transport complex is present at the cell surface as postulated by Crane et al. (9). We have identified three different redox activities: I, reduction of exogenous ferricyanide by a putative endogenous electron donor; II, oxidation of exogenous NADH by exogenous ferricyanide; and III, oxidation of NADH by oxygen or some other acceptor. It seems reasonable to assume that activity I (catalyzed by endogenous electron donors) is mediated by a different enzyme complex than activities II and III (detected using an exogenous electron donor). In this regard, it is suggestive that the apparent $K_m$ for activity I (reduction of ferricyanide alone) is significantly different from that of activity II (ferricyanide reduction in the presence of NADH). We have also observed that I is more closely associated with proton excretion than II and III (Rubinstein and Stern, unpublished results).

The similar, but unusual, pH profiles for activities I (Fig. 1A, upper trace) and II (Fig. 1B) does not necessarily indicate a similar electron transport system, but may mean that a component of both complexes is a flavin-containing dehydrogenase; purified dehydrogenases from mitochondria show similar responses to pH (21). Furthermore, flavin-containing compounds are associated with plant plasma membranes (17, 32).

We tentatively suggest that redox activities II and III, which are detected with exogenous electron donors, involve the same electron transport system. In the case of activity II, ferricyanide and DHA may remove electrons at different sites along the system compared to activity III which uses a natural acceptor. If only one of these sites were sensitive to HCN, it would explain why HCN does not inhibit NADH oxidation by ferricyanide or Cyt c, but does inhibit oxidation by DHA. The very different pH curves and rates for activities II and III may signify that other carriers become rate limiting when electrons from NADH oxidation are not removed by ferricyanide. One possible pathway for oxidation of NADH is presented by Gross et al. (13).

Even though uncertainty exists as to the number and nature of the electron transporting systems and to the identity of the natural oxidants and reductants, it seems clear that redox activity occurs at the cell surface of intact oat roots. This activity is not due to the breakdown of redox reagents, or to contaminating bacteria, or to leakage of factors from the cells. Ferricyanide-induced NADH oxidation is also present in plasma membrane-enriched fractions of oat roots. It still must be determined how cell surface redox activity is linked to cellular functions.

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