Effector Molecules to Probe Cytochrome c Oxidase Activity in Germinating Phaseolus vulgaris L. Seeds

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Summary

The respiratory effector molecules carbon monoxide (CO), nitrous oxide (N₂O), and deuterium oxide (D₂O) were used to probe cytochrome c oxidase (EC 1.9.3.1) activity in mitochondrial particles and enzyme isolated from germinating bean seeds. Mitochondrial CO effects were dose-dependent and readily reversible, with maximal activity inhibition of 58% and 81% for mitochondria and oxidase, respectively, in the presence of 80% CO. Cytochrome oxidase activity was reversibly inhibited 36% in the presence of 80% N₂O. In the presence of 99.8% D₂O, mitochondrial and enzyme activities were 43% and 52% of control activities, with mitochondrial effects partially reversed in H₂O. Visible spectra of mitochondrial and enzyme preparations showed that CO bound to cytochrome oxidase at heme a₃, while N₂O and D₂O did not directly affect the ligand binding site. Seed germination was not changed in the presence of these molecules, but reductions were observed in seedling respiration and root length corresponding to reductions in cytochrome oxidase activity. As loss of vigor is the first step in seed deterioration, these results would indicate that cytochrome c oxidase activity may be important in the loss of viability.

Key words: Bean seeds; carbon monoxide; nitrous oxide; deuterium oxide; mitochondria; dioxygen utilization.

Abbreviation: CcO = Cytochrome c oxidase.

Introduction

Cytochrome c oxidase (CcO) is the terminal electron acceptor of the mitochondrial respiratory chain in both plants and animals. The catalytic function of this key bioenergetic enzyme is the reduction of dioxygen to water, which is accompanied by proton pumping across the inner mitochondrial membrane (Einarsson and Caughey, 1985), including two heme prosthetic groups, the heme a that accepts electrons from reduced cytochrome c, and the heme a₃ that binds the dioxygen molecule substrate, which is provided four electrons for complete reduction. CcO preparations from sweet potato (Maeshima and Asahi, 1978), pea (Matsuoka et al., 1981), maize (Hawkesford et al., 1989), and wheat germ (Pfeiffer et al., 1990) have been characterized in the literature, but most of the detailed biochemical information about CcO is based on studies of bovine heart enzyme.

During the initial stages of seed germination, the number of mitochondria, their protein and cytochrome contents, enzymatic activities, and amount of DNA have all been shown to increase (Solomos et al., 1972). In electron micrographs of dry seeds, mitochondria often appear to be less abundant and poorly organized, with fewer cristae (Opik, 1972). Studies by Morohashi (1978) describe increases in mitochondrial activity, including dioxygen utilization, that are directly correlated with time of imbibition and/or amount of water uptake. Sato and Asahi (1975) separated mitochondrial membranes from dry seeds into three fractions using sucrose density centrifugation; during imbibition the fractions merged into one with concomitant formation of active and stable mitochondria. Matsuoka and Asahi (1983) described a mechanisms of increased cytochrome c oxidase activity in
pea cotyledons during imbibition and, using immunological techniques, correlated enzyme subunit assembly with hydration; the process did not require protein synthesis. We have previously reported a direct correlation between dioxygen utilization and moisture content during the first 48 h of germination in Phaseolus seeds (Sowa and Roos, 1989).

Carbon monoxide is a well known respiratory effector that inhibits CcO activity by binding to the dioxygen reductase site at the heme a1 iron of CcO (Einarsdottir et al., 1988). This specificity for CO binding has been used to study the structure of the CcO active site in bovine heart enzyme. In plants, no direct effects of CO on CcO activity have been reported. However, CO has been used to block CcO activity in experiments measuring nitrate reduction in plant tissues (Naik and Nicholas, 1986).

Materials and Methods

Seed Germination

Bean seeds, cv. Shamrock, donated by Rogers/NK Seed Company (Nampa, Idaho), were germinated under controlled conditions in sealed desiccators with relative humidity near 100%, and with inlet/outlet ports for gas exchange. Atmospheres of either air (supplied by an aquarium pump), 80% CO2/20% O2, or 80% N2O/20% O2 (mixed using precalibrated flowmeters) were washed in distilled water before being continuously passed over the seeds. Seeds (100 per test) were germinated at room temperature on moist blotting paper before being continuously passed over the seeds. Seeds were also tested in air-saturated D2O. Probes of the gas mixture and tested for dioxygen utilization activity. Recovery in air was determined by exposing the treat seedlings to 30 min air purge before testing in air-saturated water. Recovery in solution was determined by testing gas-exposed seedlings in air-saturated water. Seedlings were also tested in air-saturated D2O. Probe response to N2O was accounted for in all polarographic rate calculations.

Mitochondrial Respiration

Mitochondrial particles were isolated using a modification of an early method (Sowa et al., 1987). Five hundred grams of seed were imbibed for 48 to 60 h between wet paper towels in a 20 to 25°C chamber, and exposed to 30 min of 80% CO2/20% O2 or 80% N2O/20% O2, mixed as described above. Following exposure, the seedlings were quickly placed in distilled water also saturated with the gas mixture and tested for dioxygen utilization activity. Recovery in air was determined by exposing treated seedlings to a 30-min air purge before testing in air-saturated water. Recovery in solution was determined by testing gas-exposed seedlings in air-saturated water. Seedlings were also tested in air-saturated D2O. Probes of the gas mixture and tested for dioxygen utilization activity. Recovery in air was determined by exposing treated seedlings to a 30-min air purge before testing in air-saturated water. Recovery in solution was determined by testing gas-exposed seedlings in air-saturated water. Seedlings were also tested in air-saturated D2O. Probes of the gas mixture and tested for dioxygen utilization activity.

Measurement of CcO Activity

Successful batches of mitochondria from 6 to 7-d etiolated seedlings were isolated on a discontinuous 40 to 60% sucrose gradient (Sakano and Asahi, 1976) and kept frozen at −80°C until use. Pooled batches from 4.5 to 6 kg of fresh tissue were used to prepare submitochondrial fractions (Nakamura and Asahi, 1976), which were solubilized in KCl and deoxycholate, followed by Triton X-100, and subjected twice to DEAE-cellulose chromatography to purify CcO (Nakagawa et al., 1988). The resulting enzyme was concentrat-
Table 1: Bean-seed germination and root length after 72 h in the presence of effector molecules.

<table>
<thead>
<tr>
<th>Germination (%)</th>
<th>Root Length (mm)</th>
</tr>
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<tbody>
<tr>
<td>air</td>
<td>99±1* / 22.2±0.4</td>
</tr>
<tr>
<td>80% CO/20% O₂</td>
<td>99±1 / 10.2±0.3</td>
</tr>
<tr>
<td>80% N₂O/20% O₂</td>
<td>98±1 / 20.7±0.4</td>
</tr>
<tr>
<td>H₂O</td>
<td>82±4 / 14.2±0.5</td>
</tr>
<tr>
<td>D₂O</td>
<td>71±4 / 8.7±0.5</td>
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* Mean ± S.E. Each experiment contained 100 seeds per treatment.

Two experiments were conducted with gaseous effectors and three experiments were conducted in D₂O.

ed in an Amicon Diaflow apparatus. Visible spectra of oxidized and reduced enzyme, and reduced enzyme in the presence of effector molecules were recorded on a Hewlett-Packard 8450A spectrophotometer.

Activity of the CeO preparation, including exposure to the gaseous effector molecules, was measured polarographically in the same manner as the mitochondria. Enzyme response to D₂O was measured by testing activity in D₂O assay buffer.

Results

Seed Germination and Vigor

Seed germination percentage was not changed in the presence of the gaseous effector molecules (Table 1). In the D₂O experiments, germination was lower in control seeds (possibly due to excess water in the germination dishes), but there was no significant difference in germination between the treated and control seeds (Table 1).

Seed vigor, as indicated by mean root length, was significantly lower than controls in the presence of all three effector molecules (Table 1). Seeds germinated in an atmosphere of 80% CO/20% O₂ or in the presence of D₂O had large reductions in root length: 54% and 39%, respectively. Seeds germinated in an atmosphere of 80% N₂O/20% O₂ had only a slight, though significant, reduction in root length after 72 h of germination. D₂O effects were the same in scarified seeds (data not shown).

Seedling Respiration

Germinated seedling uptake of O₂, averaging 35 mmols min⁻¹ kg⁻¹ (fresh mass) in controls, was inhibited by all three molecules (Table 2). Seedling respiration, following 30-min preincubation in 80% CO/20% O₂, was reduced to 85% of the control. Seedlings preincubated in CO showed no significant recovery in solution (90% of control). CO-treated seedlings allowed to recover under a 30-min air purge respired at rates close to control values. Seedlings exposed to CO for 90 min showed the same degree of respiratory inhibition (data not shown).

Seedlings preincubated in 80% N₂O/20% O₂ respired at 78% of control (Table 2). As with the CO, there was no significant recovery in solution (79% of control). Recovery in air approached the control value. Seedling respiration rates did not change after 90 minute preincubation in N₂O (data not shown).

Testing seed respiration in D₂O reduced dioxygen uptake to 80% of control (Table 2). Recovery was not determined.

Binding to Mitochondrial Hemes

Carbon monoxide bound to the bean-seed mitochondrial preparations, as shown by a shift in redox absorbance maxima at 440 nm (Fig. 1a, b) expected for CO binding at heme a₃. Redox difference spectra also illustrate that N₂O did not affect the heme a sites of cytochrome oxidase; absorbance maxima at 444 nm and 605 nm were not changed in the presence of N₂O, and subsequent exposure to CO indicated normal binding at heme a₁ (Fig. 1c, d). Interestingly, the Soret peak of cytochrome b shifted slightly from 429 to 428 nm in the presence of N₂O, and the alpha peak at 566 nm lost intensity. These changes were reversed with CO binding. Spectra of mitochondrial dilutions in H- and D-containing buffers showed no significant differences in absorbance maxima; ligand binding of CO to the heme a₁ site was not affected by D₂O (Fig. 1e, f).

Mitochondrial Respiration

The respiratory activities of mitochondrial particles were partially inhibited in a dose-dependent manner by carbon monoxide (Fig. 2). The maximal inhibition was 42% after 30 min of exposure to 80% CO/20% O₂. Upon exposure to air, recovery of activity was rapid. Assay plots of %O₂ versus time, which were linear under air, became nonlinear when CO was present (data not shown). We previously reported 15% inhibition of CeO activity by 80% N₂O in bean-seed mitochondrial particles, with complete reversibility after an air purge (Sowa et al., 1987).

Dioxygen utilization activity was reduced to 43% of control values by complete exchange of D₂O (Table 3).
Fig. 1: Visible-Soret redox absorption spectra of dilutions of bean-seed mitochondrial particles containing cytochromes a, b, and c diluted to a protein concentration of 3 g L\(^{-1}\) in phosphate buffer (A), with CO bound to heme a\(_3\) (B), in the presence of N\(_2\)O (C), followed by CO (D), in D\(_2\)O buffer (E), and in D\(_2\)O buffer with CO bound to heme a\(_3\) (F). Scans were taken at 20 °C. The major visible (Soret) absorbance at 440 nm is due to heme a\(_3\). Binding of CO shifts absorbance to a lower wavelength, where it combines with the absorbances of cytochromes b and c; therefore the "shoulders" at 440 nm disappears upon CO binding, and at the same time the peak near 430 nm gains intensity (see arrow). Since the major contributor to the alpha peak at 600 nm is heme a, the enzyme component that accepts electrons but does not bind substrate, the spectrum in this region is not changed with CO binding.

Fig. 2: Respiratory activities of bean-seed mitochondrial particles in the presence of increasing concentrations of CO. Solid lines represent activity in the presence of CO; dashed lines represent recovery after a 30-min air purge. Error bars represent range in measurement of at least triplicate assays on two or more preparations. The control value (100% activity) for O\(_2\) was 244 mmols min\(^{-1}\) kg\(^{-1}\), protein basis.

The rate of exchange was rapid, within the time of the assay (3 min), as testing H-particles in D-containing buffer resulted in 44% of control activity. Recovery within the assay time, determined by testing D-particles in
Table 3: Effects of D2O on bean-seed mitochondrial respiration. Values are expressed as percent of control activity*. Letters indicate mean separations at the 5% level.

<table>
<thead>
<tr>
<th></th>
<th>H-buffer</th>
<th>D-buffer</th>
</tr>
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<tbody>
<tr>
<td>H-mitochondria</td>
<td>100.0 A</td>
<td>44.2 C</td>
</tr>
<tr>
<td>D-mitochondria</td>
<td>75.8 B</td>
<td>41.3 C</td>
</tr>
</tbody>
</table>

* Control activity (100%): O2: 440.8 ± 46.1 mmols min⁻¹ kg⁻¹, protein basis, calculated from four replicate assays on each of three mitochondrial preparations.

H-buffer, was not complete, and averaged 76% of control activity.

CcO Binding and Activity

Visible-Soret spectra of bean CcO contained maxima at 414 and 600 nm (oxidized), and 436 and 602 nm (reduced); addition of CO shifted the Soret peak to 428 nm (Fig. 3 a — c), consistent with ligand binding at heme a₃. Spectra of our bean enzyme preparation showed no effects of N₂O or D₂O on the heme a sites (Fig. 3 d - g).

Table 4: Effector molecules and bean-CcO activity.

<table>
<thead>
<tr>
<th>Activity (O₂: mmol min⁻¹ kg⁻¹, protein basis)</th>
<th>mean ± S.D.*</th>
<th>% of control</th>
<th>% recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>air</td>
<td>924±12</td>
<td>100</td>
<td>—</td>
</tr>
<tr>
<td>80% CO/20% O₂</td>
<td>175±4</td>
<td>19</td>
<td>77</td>
</tr>
<tr>
<td>80% N₂O/20% O₂</td>
<td>592±9</td>
<td>64</td>
<td>100</td>
</tr>
<tr>
<td>D₂O</td>
<td>434±31</td>
<td>47</td>
<td>not determined</td>
</tr>
</tbody>
</table>

* n = 3 for each treatment.

The activity of the bean CcO preparation was reduced to 19% of the control in the presence of 80% CO/20% O₂ (Table 4). The CO-treated enzyme recovered to 77% of control activity after a 30-min air purge. Exposure to 80% N₂O/20% O₂ reduced activity to 64% of control that was partially reversed to 79% of control values after a 30-min air purge (Table 4). The enzyme preparation was labile to the conditions of gas exposure, and lost activity even under air; therefore, recovery was based on controls exposed to a comparable air purge. Dioxygen utilization by D-exchanged enzyme in D₂O assay buffer was 47% of the control value. Recovery of D-exchanged enzyme in H-containing buffer was not determined due to limited amount of enzyme, but is expected to be comparable to mitochondrial recovery.

Fig. 3: Visible-Soret absorbance spectra of bean CcO (protein: 1 g L⁻¹) oxidized (A), reduced (B), reduced with bound CO (C), reduced in the presence of N₂O (D) followed by CO (E), reduced in D₂O buffer (F) with bound CO (G).
Discussion

The effector molecules did not decrease total bean-seed germination, thus it is reasonable to assume that mitochondria and CcO were allowed to assemble and function during imbibition. However, we observed reduced root growth in seedlings during the first stages of germination. Early studies on CO effects at concentrations of 10 mL L\(^{-1}\) on more than 100 plant species indicated growth reduction that was variable in terms of sensitivity and expression of effects, with potential damage to mature plants occurring as leaf injury or other physiological abnormality causing lower yield, quality, or growth (Zimmerman et al., 1933). Winter-rye seeds have been shown to germinate in an atmosphere of 100% CO, with green coleoptiles 0.5–3 cm high after 3 to 4 days (Siegel et al., 1962). Plants therefore might contain an inherent ability to utilize CO, possibly by oxygenation to CO\(_2\) via CcO.

The potential of plants to metabolize and therefore remove atmospheric CO has been considered by others (Bidwell and Fraser, 1972; Chappelle and Krall, 1961), but with limited information regarding the actual mechanism involved. Bean leaves were shown to incorporate CO into incorporation and also to convert CO to CO\(_2\) (Bidwell and Fraser, 1972). Experiments with cell-free extracts of spinach and barley leaves revealed light- and O\(_2\)-dependent incorporation of radiolabeled CO with CO\(_2\); as a major product (Chappelle and Krall, 1961). Although bovine heart CcO has been shown to possess catalytic oxygenation activity of CO to CO\(_2\) (Young and Caughey, 1986), this aspect has not been studied in enzyme preparations from plant tissues.

We have observed nonlinear bean mitochondrial respiration rates only with CO, similar to the two distinguishable steady states of dioxygen utilization observed in isolated pigeon heart mitochondria in the presence of CO (Chance et al., 1970). Unlike their animal counterparts, however, plant mitochondria seem to have a readily reversible response to CO binding. This undoubtedly reflects inherent differences between plant and animal CcO. Denis and Richaud (1982) studied mechanistic aspects of CcO in potato-tuber mitochondria compared to beef heart mitochondria and found differences in active-site environment, including CO recombination steps after flash photolysis.

Genetically different sensitivities of CcO to CO have been described in tall and dwarf wheat cultivars (Naik and Nicholas, 1986). Recently, CcO in C\(_3\) plant leaves has been shown to be more sensitive to CO binding compared with C\(_4\) counterparts (Naik et al., 1992). Ready reversibility to CO binding was demonstrated in leaves from spinach, wheat, pearl millet, and sorghum (Naik et al., 1991). Further study of this less sensitive and more readily reversed CO response might elucidate a CO-oxygenase capability in plant CcO.

CcO seemed to be most sensitive to N\(_2\)O compared with mitochondrial (Sowa et al., 1987) or seedling respiration and growth. The activity of our enzyme preparation was reduced 34% by 30-min exposure to 80% N\(_2\)O, with partial recovery after a comparable air purge. These results are similar to effects observed on bovine heart CcO, which document 40% activity reduction after 1 h exposure to 100% N\(_2\)O, and complete recovery after a 15-min purge of N\(_2\); infrared spectra showed N\(_2\)O occupation of four sites of different polarities within the enzyme, with similar diversity in environment, but the sites did not include the ligand pocket (Einarsdóttir and Caughey, 1988).

The N\(_2\)O inhibition of bovine CcO activity was observed in air-saturated buffer. We, too, observed effects of N\(_2\)O to be evident under conditions in which the partial pressure of N\(_2\)O was greatly reduced in the assay: whole seedling respiration was inhibited by prior exposure to N\(_2\)O and subsequent testing in air-saturated water. The respiration assay took 20 min, indicating that both the N\(_2\)O and CO remained inside the seedling during this time, which is considerably longer than the time required for enzyme activity measurement. Air purges, then, seemed to reverse the binding more efficiently than exchange through aqueous media. Sensitivity of the bean CcO to gas purges in general make it difficult to evaluate the completeness of the recovery of enzyme activity.

CcO activity is also inhibited by D\(_2\)O (Einarsdóttir et al., 1988; Laser and Slater, 1960; Shibata and Watanabe, 1949). The CcO-catalyzed reaction, the reduction of dioxygen to water, involves electron transfer and protonation and is accompanied by proton pumping. Electron transfer and protonation sites in the respiratory chain are known targets of D\(_2\)O, with inhibition averaging 40–60% (Wilms et al., 1981). Specifically, D\(_2\)O was shown to inhibit the activity of bovine heart CcO about 40% (Einarsdóttir et al., 1988). D\(_2\)O might easily influence catalysis at a rate-limiting proton donation step, possibly by small changes in protein structure (changes in hydration/hydrogen bonding) that do not affect ligand binding yet slow activity, with reversibility upon replacement in H\(_2\)O.

Our experiments showed a definite reduction/delay of growth in germinating bean seeds in the presence of D\(_2\)O. The explanations provided in the literature for observed biological effects of D\(_2\)O, i.e. changes in hydration, especially protein structure, kinetic differences due to a heavier iso- tope, changes in respiration, etc., all seem reasonable and likely contributors to the effects we observed. In seeds, hydration is a very important process in terms of initiating metabolism during the early stages of germination, especially in the assembly and function of mitochondria and cytochrome C oxidase.

Our results provide insight into the importance of respiratory activity to seedling vigor, especially CcO with its unique ability to exist in a preformed state that is activated by water. As loss of vigor is the first sign of seed deterioration (Roos, 1986), perhaps this vigor loss is somehow associated with decline in CcO function. More severe denaturation of CcO could result in release of partially reduced oxygen species as the binding pocket "loosens" as shown by infrared spectroscopy of enzyme at pH and temperature extremes (Einarsdóttir et al., 1988). These oxyradicals then could further contribute to seed deterioration. We are continuing our study of CcO as it occurs in seeds, especially comparisons of oxygen binding sites in seeds of varied viability/vigor to gain biochemical insight to the preservation of our valuable plant genetic resources.
References


