Diphenylamine Inhibits Respiration of Green Bell Peppers

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ABSTRACT. The mechanism by which diphenylamine (DPA) controls superficial scald in apples and reduces chilling injury in green bell peppers [*Capsicum annuum* L. var. *annuum* (Grossum Group)] has been assumed to be related to its antioxidant activity. In the present study, DPA inhibited the respiratory activity of green bell pepper fruit as well as oxygen uptake by the mitochondria isolated from them. When the alternative oxidase was inhibited with *n*-propyl gallate or disulfiram during state 4 respiration, DPA did not further inhibit O₂ uptake. Treating green bell peppers with DPA before storage did not alter the induction and abundance of the alternative oxidase protein in mitochondria which was maximally induced in peppers stored at 4 °C. Whether added before or after the uncoupler, 2,4-dinitrophenol, DPA negated the enhanced O₂ uptake associated with uncoupling of electron transfer in isolated mitochondria. These observations indicate that DPA inhibits the flow of electrons through the cytochrome path, probably somewhere in the cytochrome *bc*₁ complex. Although the secondary amine function of DPA makes it a powerful antioxidant, the effectiveness of DPA in reducing chilling injury in green bell peppers and superficial scald in apples [*Malus sylvestris* (L.) Mill. var. *domestica* (Borkh.) Mansf.] also may be due, in part, to its inhibition of respiration.

Diphenylamine (DPA), used commercially in the United States to control low-temperature-induced superficial scald in apples [*Malus sylvestris* (L.) Mill. var. *domestica* (Borkh.) Mansf.] during storage (Smock, 1961), was recently reported to reduce chilling injury in cold-stored green bell pepper [*Capsicum annuum* L. var. *annuum* (Grossum Group)] fruit (Purvis, 2002). The mechanism by which DPA reduces chilling injury in bell pepper and superficial scald in apple is not known, but the prevalent hypothesis for the reduction of superficial scald in apples is that DPA inhibits the oxidation of α -farnesene to conjugated trienes, which are thought to be toxic to fruit tissues, possibly through their initiation of free-radical-mediated chain reactions and/or decomposition to harmful volatiles (Whitaker, 2000). Diphenylamine, however, also reduced α -farnesene synthesis, ethylene production and respiration in apples (Lurie, et al., 1989; Whitaker, 2000), and altered chlorophyll fluorescence parameters in cold stored green bell peppers (Purvis, 2002), but had no effect on chlorophyll fluorescence parameters in apples stored in refrigerated air (Mir et al., 1998).

Diphenylamine was first tested as a potential inhibitor of apple scald (Smock, 1955, 1961) based on a report that it inhibited cytochrome oxidase activity in mitochondria isolated from rabbit liver (Abood and Gerard, 1953). Cytochrome oxidase is the terminal oxidase in the electron transport chain of both animal and plant mitochondria. Diphenylamine was found to only marginally inhibit cytochrome oxidase activity in mitochondrial particles of apple fruit but it severely inhibited the oxidation of succinate (Yatsu, 1960). However, succinate dehydrogenase and NADH diaphorase (dehydrogenase) activities of sweetpotato (*Ipomoea batatas* L.) mitochondria were not inhibited by DPA (Baker, 1963). Since the reduction of the cytochromes was delayed, but no reduced-oxidized pairs were observed, Baker (1963) suggested that DPA inhibits somewhere between the dehydrogenases and the first components of the electron transport chain. The dehydrogenases reduce a pool of ubiquinone that is in large molar excess to other components of the respiratory chain in the mitochondrial inner membrane (von Jagow and Link, 1986). Oxidation of ubiquinol (reduced ubiquinone) by

the cytochrome *bc*₁ complex, the first component in the cytochrome path that terminates with cyanide-sensitive cytochrome oxidase, develops a proton gradient across the inner membrane that is dissipated with the synthesis of ATP (Tyler, 1992). Thus, electron flow through the cytochrome path is tightly coupled to the synthesis of ATP. In addition to the cyanide-sensitive cytochrome oxidase, plant mitochondria have a cyanide-insensitive alternative oxidase that oxidizes ubiquinol directly without developing a proton gradient across the inner membrane (McDonald et al., 2002).

Baker (1963) reported that DPA inhibited the oxidation of succinate by turnip (*Brassica rapa* L.) mitochondria 80% to 90% and by sweetpotato mitochondria 50%, with the remaining 50% being completely inhibited by antimycin A, an inhibitor of the cytochrome *bc*₁ complex. O₂ uptake by turnip mitochondria is almost completely inhibited by cyanide, whereas O₂ uptake by sweetpotato mitochondria is moderately resistant to cyanide, i.e., they have relatively high alternative oxidase activity (Baker, 1961; Baker and Lieberman, 1962). Thus, DPA may differentially inhibit the cytochrome and alternative paths of electron transport in plant mitochondria.

Since the studies by Baker (1963), components of respiratory electron transport in plant mitochondria have been more fully characterized (McDonald et al., 2002). Although the alternative oxidase protein is constitutively present in most higher plant mitochondria, its abundance increases in certain conditions, such as chilling stress. O₂ uptake by green bell pepper mitochondria, for example, becomes almost totally insensitive to cyanide when the peppers are exposed to 4 °C for 4 to 7 d (Purvis, 2001). The alternative oxidase is activated when the sulphydryl groups on the enzyme are fully reduced (Umbach and Siedow, 1993) and pyruvate, or some other α -keto acid, is present (Millar et al., 1993). The objective of the study reported here was to determine if DPA inhibited respiration of green bell peppers and O₂ uptake by mitochondria isolated from them. Compounds which differentially inhibit the cytochrome and alternative paths of electron transport in mitochondria were used to determine the site in the respiratory electron transport chain inhibited by DPA.

Materials and Methods

PLANT MATERIAL. ‘Camelot’ green bell peppers were harvested

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from the field of a local grower and from the University of Georgia horticulture farm in Tift County, Ga. Peppers of unknown variety were also purchased at a local supermarket for isolating mitochondria when freshly harvested peppers were not available.

TREATMENTS AND STORAGE CONDITIONS. Peppers harvested from the field were brought to the laboratory, rinsed with tap water, sorted, and randomized into groups for treatments. One group was treated with DPA by injecting 2.0 mL of a DPA solution (11.8 mM DPA in 5% DMSO and 0.05% Tween 20) into the internal cavity of the fruit through the stylar scar with a syringe and needle, followed by dipping each pepper for 2 min in the DPA solution (Purvis, 2002). Another group was treated similarly with DMSO and Tween 20 without DPA and served as controls. The peppers were allowed to air-dry before being placed in storage. No fungicides were used. The peppers were stored in open trays in dark storage rooms at 1, 4, or 15 °C and 70% to 80% relative humidity for 5 to 7 d.

RESPIRATORY MEASUREMENTS. Respiratory measurements were made on duplicate samples of six peppers from each treatment before and after storage. The six peppers from each treatment were weighed and sealed in 10-L glass jars at 20 °C in the dark. O₂ and CO₂ concentrations in the headspace gases were determined at hourly intervals with an Oxymax Respirometer (Columbus Instruments International Corp., Columbus, Ohio). The respirometer was equipped with the open-flow high-metabolic rate option, which allowed the system to automatically refresh the headspace in the jars with room air after each sampling.

MITOCHONDRIA ISOLATION. Mitochondria from pepper pericarp were isolated and purified on a Percoll gradient by a modification of the procedure previously described by Purvis (1997). The isolation buffer contained 400 mM sucrose, 34 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS), 30 mM Tris[hydroxymethyl]aminoethane (Tris), 5 mM EDTA, 6 mM cysteine, 2 mg·mL⁻¹ bovine serum albumin (BSA; fatty acid free), and 1.0% PVP (w/v) at pH 7.5. The pericarp tissue with the cuticle intact was homogenized (1 g tissue/mL of buffer) in a Waring blender with five 1 s bursts on low speed. The resulting homogenate was squeezed through two layers of cheesecloth and centrifuged in a centrifuge (Sorvall Superspeed RC2-B; DuPont Medical Products, Wilmington, Del.) in a GSA rotor at 2100 g_n for 10 min. The pellet was discarded and the supernatant was centrifuged again at 16,000 g_n for 10 min. The supernatant was discarded and the pellet was suspended with an artist's brush in 1 mL of wash buffer containing 250 mM sucrose, 10 mM MOPS, 5.9 mM Tris and 25 μg·mL⁻¹ BSA, pH 7.2, then brought to 14 mL with wash buffer and centrifuged (SM-24 rotor) at 1600 g_n for 10 min. The supernatant was decanted into clean 16-mL tubes and centrifuged at 11,100 g_n for 10 min. The supernatant was discarded. The loose green material on top of the pellet was removed by carefully adding 2 mL wash buffer, swirling gently and decanting. The mitochondrial pellet was then suspended with an artist's brush in 1 mL wash buffer and layered onto a 2-step discontinuous Percoll gradient. Gradients for the 16-mL tubes consisted of 12 mL 23% Percoll in wash buffer underlaid with 2 mL 45% Percoll in wash buffer. The gradients were centrifuged at 26,000 g_n for 20 min. The band (≈1 cm) of mitochondria at the 23% to 45% Percoll interface was collected with a 2-mL syringe or a Pasteur pipette, diluted with 12 mL wash buffer and centrifuged at 11,100 g_n for 20 min. The resulting supernatant was removed with a Pasteur pipette to reduce agitation of the loose pellet. The pellet was resuspended in 12 mL wash buffer and centrifuged at 11,100 g_n for 10 min. The supernatant was discarded and the pellet was suspended to the desired volume (1.5 to 2.5 mg protein/mL) in respiration buffer (below).

O₂ UPTAKE MEASUREMENTS. O₂ uptake was measured with a Clark O₂ electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio) calibrated with deionized water saturated with air (240 nmol O₂/mL) at 25 °C as described previously (Purvis et al., 1995). Reactions were conducted with 200 or 250 μg mitochondrial protein in a total volume of 3.0 mL of respiration buffer (250 mM sucrose, 10 mM MOPS, 5.9 mM Tris, 2 mM MgCl₂, 2 mM KH₂PO₄, 2 mM KCl and 500 μg·mL⁻¹ BSA, pH 7.4) and substrates and inhibitors. Substrate concentrations were 10 mM succinate, 1 mM NADH, 250 μM ATP and 100 μM ADP or 400 μM ADP to keep mitochondria in state 3. Inhibitor concentrations were 1 mM KCN, 10 μM antimycin A, 10 μM myxothiazol, 25 to 300 μM diphenylamine (DPA), 50 μM disulfiram, 100 μM *n*-propyl gallate (*n*PG) and 200 μM 2,4-dinitrophenol (DNP). Two millimolar pyruvate was used to activate the alternative oxidase.

PROTEIN DETERMINATION. Protein was determined according to Bradford (1976), using BSA as standard.

SDS-PAGE AND IMMUNOBLOTTING. Mitochondrial membrane proteins (40 μg) were solubilized by diluting mitochondria 1:2 with SDS reducing buffer containing 62.5 mM Tris-HCl (pH 6.8), 25% (v/v) glycerol, 0.02% (w/v) SDS, 0.0001% (w/v) bromophenol blue, and 0.5% (v/v) 2-mercaptoethanol and heated at 95 °C for 4 min. Electrophoresis was performed using the system of Laemmli (1970). Reduced mitochondrial protein sample (20 μL) was loaded onto a 12% (w/v) polyacrylamide gel and electrophoresis was performed at 120 V for 140 min. The resolved proteins were transferred to a polyvinylidene difluoride (PVDF) membrane at 90 mA for 800 min with 25 mM Tris (pH 8.3), 192 mM glycine, and 20% (v/v) methanol used as the transfer buffer. The PVDF membrane was washed three times with PBS-Tween [10 mM Na₂H₂PO₄ (pH 7.2), 150 mM NaCl, 0.3% (v/v) Tween 20] for 5 min each. The membrane was then blocked in PBS-Tween with 5% (w/v) nonfat dry milk for 30 min to reduce binding of antibodies to nonspecific proteins followed by probing for 2 h with antibodies developed against the alternative oxidase of *Sauromatum guttatum* (generously provided by T.E. Elthon). After three 5-min washes with PBS-Tween, the membrane was incubated for 1 h with an anti-mouse IgG-alkaline phosphatase conjugate diluted 1:1000 in PBS-Tween. The membrane was washed twice with PBS-Tween for 5 min each and an additional 5 min with 100 mM Tris (pH 9.5), 100 mM NaCl, and 5 mM MgCl₂. The membrane was immersed in 10 mL fresh pH 9.5 MgCl₂ buffer, to which 3.3 mg nitroblue tetrazolium and 1.7 mg 5-bromo-4-chloro-3-indoyle-phosphate had been added to visualize the protein. When the desired color intensity was achieved, the membrane was washed for 10 min in 20 mL PBS-Tween with the addition of 1 mL water saturated with EDTA.

STATISTICAL ANALYSES. Analysis of variance and Duncan's multiple range or least square means tests were performed on all data using the SAS statistical package (SAS Institute, Cary, N.C.).

Table 1. Differences of least squares means from comparisons of O₂ uptake rates by bell pepper mitochondria with various substrates, treatments, storage temperatures, and respiratory states.

Effect	<i>t</i>	<i>P</i> > <i>t</i>
Succinate vs NADH	-8.37	<0.0001
+DPA vs. -DPA	-2.00	0.0483
15 vs. 1 °C	-1.17	0.2428
15 vs. 4 °C	-7.43	<0.0001
1 vs. 4 °C	-6.25	<0.0001
Substrate state vs. state 3	-20.80	<0.0001
Substrate state vs. state 4	1.14	0.2579
State 3 vs. state 4	21.94	<0.0001

Fig. 1. Rates of substrate, state 3 (St 3) and state 4 (St 4) O_2 uptake by mitochondria isolated from freshly harvested green bell peppers and peppers treated with 11.8 mM DPA or untreated and stored for 5 to 7 d at 1, 4, or 15 °C. Substrate was 10 mM succinate (Succ). Means and SE of four isolations.

Fig. 2. Rates of substrate, state 3 (St 3) and state 4 (St 4) O_2 uptake by mitochondria isolated from freshly harvested green bell peppers and peppers treated with 11.8 mM DPA or untreated and stored for 5 to 7 d at 1, 4, or 15 °C. Substrate was 1 mM NADH. Means and SE of four isolations.

Results

Green bell peppers treated with DPA had lower rates of O_2 uptake and CO_2 production than untreated peppers (data not shown) as has been reported for apples (Lurie et al., 1989; Whitaker, 2000). Treating peppers with DPA before storage also reduced the rates of O_2 uptake during succinate and NADH oxidation by mitochondria isolated from the peppers after storage (Table 1; Figs. 1 and 2). For bell peppers stored at 4 °C, compared to those stored at 1 or 15 °C, isolated mitochondria had increased rates of state 3 (high ADP:ATP) and state 4 (low ADP:ATP) O_2 uptake (Table 1) when either succinate (Fig. 1) or NADH (Fig. 2) was oxidized. O_2 uptake rates were lower with NADH than with succinate.

Antimycin A (10 μM) and myxothiazol (10 μM), inhibitors of the ubiquinone reducing and ubiquinol oxidizing centers in the cytochrome bc_1 complex, respectively, inhibited state 4 succinate (Fig. 3) and NADH (Fig. 4) oxidation less in mitochondria isolated from stored peppers than from freshly harvested peppers. In general, treating peppers with DPA before storage did not alter the sensitivity of their mitochondria to inhibitors of the cytochrome bc_1 complex (Figs. 3 and 4). However, myxothiazol inhibited state

Fig. 3. Percent inhibition by 10 μM antimycin A (Anti A) or 10 μM myxothiazol (Myxo) of state 4 O_2 uptake by mitochondria isolated from freshly harvested green bell peppers and peppers treated with 11.8 mM DPA or untreated and stored for 5 to 7 d at 1, 4, or 15 °C. Substrate was 10 mM succinate. Means and SE of four isolations.

Fig. 4. Percent inhibition by 10 μM antimycin A (Anti A) or 10 μM myxothiazol (Myxo) of state 4 O_2 uptake by mitochondria isolated from freshly harvested green bell peppers and peppers treated with 11.8 mM DPA or untreated and stored for 5 to 7 d at 1, 4, or 15 °C. Substrate was 1 mM NADH. Means and SE of four isolations.

4 succinate oxidation less in mitochondria isolated from peppers treated with DPA and stored at 1 °C than from untreated peppers (Fig. 3). Inhibition of NADH oxidation by antimycin A was less in peppers stored at 4 °C compared to those stored at 15 or 1 °C (Fig. 4).

Storing bell peppers at 4 °C, but not at 1 °C, increased the abundance of mitochondrial protein reacting with *S. guttatum* alternative oxidase antibodies (Fig. 5). Treating peppers with DPA before storage did not alter the pattern or abundance of mitochondrial protein that reacted with *S. guttatum* alternative oxidase antibodies compared to controls without DPA.

Inhibition of state 3 O_2 uptake by mitochondria oxidizing succinate was nearly linear with DPA concentrations up to 200 μM , in the absence or presence of inhibitors of the cytochrome and alternative paths (Fig. 6). Since the upper limit of solubility of DPA in aqueous solutions is $\approx 270 \mu M$, the increased inhibition when the upper limit of solubility was exceeded may be attributed to partitioning of DPA in the lipids of the mitochondrial membranes (Baker, 1963). When the cytochrome bc_1 complex was inhibited with myxothiazol or antimycin A, or when alternative oxidase activity was inhibited with 100 μM nPG or 50 μM disulfiram, inhibition of

Fig. 5. Western blot of mitochondrial proteins isolated from green bell peppers and probed with *Sauromatum guttatum* alternative oxidase antibody. Peppers were freshly harvested and stored, untreated or treated with 11.8 mM DPA, for 6 d at 1, 4, or 15 °C.

Fig. 6. Effect of DPA concentration on the inhibition of state 3 (St 3) O_2 uptake in the presence and absence of 10 μM antimycin A (AA), 10 μM myxothiazol (MX), 100 μM *n*-propyl gallate (nPG) or 50 μM disulfiram (DS) by mitochondria isolated from green bell peppers that had been stored at 4 °C for 5 to 7 d to induce the alternative oxidase. Substrate was 10 mM succinate. Pyruvate (2 mM) was included to activate the alternative oxidase. Mean of three isolations.

Fig. 7. Effect of DPA concentration on the inhibition of state 4 (St 4) O_2 uptake in the presence and absence of 10 μM antimycin A (AA), 10 μM myxothiazol (MX), 100 μM *n*-propyl gallate (nPG) or 50 μM disulfiram (DS) by mitochondria isolated from green bell peppers that had been stored at 4 °C for 5 to 7 d to induce the alternative oxidase. Substrate was 10 mM succinate. Pyruvate (2 mM) was included to activate the alternative oxidase. Mean of three isolations.

state 3 O_2 uptake by DPA was linearly related to the concentration of DPA up to 200 μM (Fig. 6). Concentrations of DPA of 100 μM or less slightly stimulated state 4 O_2 uptake by mitochondria oxidizing succinate, whereas concentrations of DPA greater than 100 μM linearly inhibited state 4 O_2 uptake (Fig. 7). In the presence of inhibitors of the cytochrome path, inhibition of state 4 O_2 uptake was also nearly linear with DPA concentrations up to 200 μM . In contrast, state 4 O_2 uptake in the presence of 100 μM nPG or 50 μM disulfiram, inhibitors of the alternative oxidase, was not inhibited further by DPA concentrations up to 200 μM . The residual O_2 uptake, i.e., at 300 μM DPA, was completely inhibited by a combination of inhibitors of both the cytochrome bc_1 complex and the alternative oxidase (data not shown).

Typical traces of O_2 uptake by green bell pepper mitochondria oxidizing succinate are depicted in Figs. 8 and 9. In both traces, state 3 respiration was first established with 100 μM ADP and the mitochondria were allowed to return to state 4 (after ≈ 2 min) before the addition of respiratory inhibitors. One hundred micromolar nPG was then used to inhibit the alternative oxidase and divert all of the respiratory electrons to the cytochrome path which produces a proton gradient across the mitochondrial inner membrane. The addition of 200 μM DPA did not further inhibit or stimulate O_2 uptake by the mitochondria (Fig. 8; see also Fig. 7). The subsequent addition of 200 μM of the uncoupler, DNP, also did not enhance O_2 uptake. In the absence of DPA, 200 μM DNP enhanced O_2 uptake (Fig. 9). However, the subsequent addition of 200 μM DPA apparently blocked electron flow through the cytochrome bc_1 complex, thus preventing the formation of a proton gradient and O_2 uptake. Myxothiazol inhibited the residual O_2 uptake (Figs. 8 and 9). Thus, DPA negated the uncoupling of oxidative phosphorylation by DNP during state 4 O_2 uptake by green bell pepper mitochondria whose alternative oxidase activity was inhibited with nPG, whether the DNP was added before or after the addition of DPA (Figs. 8 and 9).

Discussion

In the present study, DPA reduced both O_2 uptake and CO_2 evolution by whole green bell peppers and O_2 uptake by mitochondria isolated from the pericarp tissue of the fruit. Although treatment with DPA reduced the respiratory capacity of green bell pepper mitochondria ($P < 0.05$), in general no differences in sensitivity to respiratory inhibitors were observed between mitochondria isolated from DPA-treated and untreated green bell peppers. Because DPA is volatile, it may dissipate from fruit tissues rapidly and become diluted in the storage room atmosphere. Diphenylamine is also readily transformed to glycosyl conjugates of several hydroxylated DPA metabolites in fruit tissues even in cold storage conditions (Kim-Kang et al., 1998), but it is not known if these metabolites are active in DPA-mediated reactions. In contrast to treating green bell peppers with DPA, storing them at 4 °C increased the abundance of alternative oxidase protein in their mitochondria compared to storage at 15 or 1 °C, as has been observed previously (Purvis, 2001).

The slight stimulation of state 4 O_2 uptake by green bell pepper mitochondria with concentrations of DPA less than 100 μM in the absence of other respiratory inhibitors could be due to either uncoupling by low concentrations of DPA or increased electron flow through the alternative oxidase if the cytochrome path is partially blocked by DPA. Masubuchi et al. (1999) reported that concentrations of DPA of 100 μM or less caused up to a 2-fold stimulation of state 4 O_2 uptake in rat liver mitochondria, whereas all concentrations of DPA inhibited state 3 O_2 uptake in a concentration-dependent manner. Mammalian mitochondria generally are more tightly

Fig. 8. Clark O_2 electrode traces of O_2 uptake (nmol O_2 consumed/mg protein/min) by mitochondria isolated from green bell peppers that had been stored at 4 °C for 5 to 7 d to induce the alternative oxidase. Additions as indicated were: 10 mM succinate (Succ.), 100 μ M ADP, 100 μ M *n*-propyl gallate (*n*-PG), 200 μ M diphenylamine (DPA), 200 μ M 2,4-dinitrophenol (DNP), and 10 μ M myxothiazol (Myxo).

Fig. 9. Clark O_2 electrode traces of O_2 uptake (nmol O_2 consumed/mg protein/min) by mitochondria isolated from green bell peppers that had been stored at 4 °C for 5 to 7 d to induce the alternative oxidase. Additions as indicated were: 10 mM succinate (Succ.), 100 μ M ADP, 100 μ M *n*-propyl gallate (*n*-PG), 200 μ M 2,4-dinitrophenol (DNP), 200 μ M diphenylamine (DPA), and 10 μ M myxothiazol (Myxo).

coupled than plant mitochondria and, unlike plant mitochondria, do not have a nonenergy conserving alternative oxidase, although mitochondria in certain mammalian tissues, i.e., brown adipose tissue, do contain an uncoupling protein that diverts energy from ATP synthesis to thermogenesis (Nisoli et al., 2003). Since the inhibition of oxidative phosphorylation by DPA in apple mitochondria (Yatsu, 1960) and turnip and sweetpotato mitochondria (Baker, 1963) was not reversed with DNP, DPA was regarded as an uncoupler, rather than an inhibitor, of energy transfer (Baker, 1963). We observed, however, that DPA negated the uncoupling of green bell pepper mitochondria by DNP whether it was supplied before or after the DNP. Thus, in green bell pepper mitochondria DPA appeared to interfere with electron transfer through the cytochrome *bc*₁ complex and prevent the establishment of a proton gradient across the mitochondrial inner membrane and the synthesis of ATP. Furthermore, when electron transfer was restricted during state 4 respiration in green bell pepper mitochondria, DPA did not further inhibit O_2 uptake in the presence of alternative oxidase inhibitors. The residual O_2 uptake, however, was inhibited with myxothiazol, an inhibitor of the cytochrome *bc*₁ complex in the cytochrome path. Altogether, these observations indicate that DPA inhibits electron flow through the cytochrome path, rather than the alternative oxidase, probably somewhere in the cytochrome *bc*₁ complex, but at a site different from myxothiazol. The initial observation of Baker (1963) that antimycin inhibited the residual O_2 uptake by sweetpotato mitochondria when DPA was present suggesting that DPA may be a selective inhibitor of an alternative path was subsequently ruled out since turnip mitochondria do not exhibit antimycin A insensitivity. However, had Baker (1963) supplied pyruvate to the DPA and antimycin A-inhibited sweetpotato mitochondria, O_2 uptake may have been restored since it has been shown that pyruvate or another α -keto acid is required to activate the alternative oxidase (Millar et al., 1993).

In the study reported here, compounds that differentially inhibit the cytochrome and alternative paths were used in conjunction with DPA in an attempt to determine where in the electron transport chain DPA inhibits. Myxothiazol and antimycin A, inhibitors of the ubiquinol oxidizing and the ubiquinone reducing centers in the cytochrome *bc*₁ complex, respectively (von Jagow and Link, 1986), were used to inhibit respiratory electron transport through the cytochrome path. Disulfiram, which inactivates the alternative oxidase by promoting the formation of mixed disulfides with one or more of the sulfhydryl groups on the enzyme (Grover and Laties, 1981), and *n*PG, which apparently competes with ubiquinol as a reductant for the alternative oxidase (Siedow and Bickett, 1981), were used to inhibit respiratory electron transport through the alternative path. The ubiquinol oxidizing center of the cytochrome *bc*₁ complex catalyzes single electron transfers from ubiquinol to the iron-sulfur (Fe-S) protein and cytochrome *b*, producing the one electron-reduced ubisemiquinone intermediate (Tyler, 1992). Sharp et al. (1999a) reported that sub-millimolar concentrations of DPA bind at the ubiquinol oxidizing center of the cytochrome *bc*₁ complex of *Rhodobacter capsulatus* chromatophores and affect the ubiquinol oxidizing center function without disrupting the interaction of ubiquinol with the Fe-S protein. The ubiquinol oxidizing center binds two ubiquinol molecules simultaneously at two distinct sites, one with a high affinity for ubiquinol, and the other with a low affinity for ubiquinol (Ding et al., 1995; Sharp et al., 1999a). The ubiquinol at the high affinity site acts as a catalytic cofactor whereas the ubiquinol in the low affinity site serves as substrate (Sharp et al., 1999b). Excess DPA displaces ubiquinol from the low affinity substrate site, but not from the high affinity

catalytic cofactor site (Sharp et al., 1999a, 1999b). Thus, differences in the binding affinities of ubiquinol and DPA at the low affinity site could account for the differences observed in DPA inhibition of electron transfer and O₂ uptake by mitochondria isolated from various species, i.e., green bell pepper, turnip and sweetpotato. This concept is supported by the observation that the cytochromes (cytochrome *b*, *c*, and *a*) downstream from the ubiquinol oxidizing center in cyanide-sensitive turnip mitochondria remained oxidized in the presence of DPA whereas those in cyanide-resistant sweetpotato mitochondria were reduced, but much more slowly than in the absence of DPA (Baker, 1963). Thus, DPA may compete with ubiquinol at the substrate site of the cytochrome *bc*₁ complex but not serve as a reductant for the alternative oxidase. In contrast, myxothiazol, which also inhibits at the ubiquinol oxidizing center, binds at a site different from the catalytic and substrate ubiquinol-binding sites (von Jagow and Link, 1986), and therefore probably does not compete with either ubiquinol or DPA.

Reduction of superficial scald in apples and chilling injury in green bell peppers by DPA could be related to the inhibition of respiratory electron transport by DPA. Reactive oxygen species are thought to be involved in the manifestation of several stress-induced injuries, including chilling injury (Scandalios, 1993). Ubisemiquinone, the transient intermediate in the ubiquinol oxidizing and the ubiquinone reducing centers in the cytochrome *bc*₁ complex, and the one-electron flavoprotein components of the dehydrogenases in mitochondria can reduce molecular oxygen to superoxide, the precursor of other more detrimental reactive oxygen species (Rich and Bonner, 1978). Inhibiting electron transfer through the cytochrome *bc*₁ complex with DPA would enhance the flow of respiratory electrons through the nonenergy conserving alternative oxidase, if present and activated, thereby eliminating the transient ubisemiquinone and one of the potential sites for the production of reactive oxygen species. An active alternative oxidase would also enhance the flow of respiratory electrons through the flavoprotein components of the various dehydrogenases during state 4 respiration. Indeed, recent evidence indicates that less superoxide is produced by plant mitochondria when electrons are transferred from ubiquinol to O₂ via the alternative oxidase (Maxwell, et al., 1999; Parsons et al., 1999; Purvis, 1997; Purvis and Shewfelt, 1993; Purvis et al., 1995). In preliminary studies, we found that DPA inhibited superoxide production by green bell pepper mitochondria in a concentration-dependent manner (data not shown).

Although its secondary amine function makes DPA a powerful antioxidant (Sugihara et al., 1993), we have shown that DPA also inhibits respiratory electron transport in plant mitochondria. The effectiveness of DPA in reducing superficial scald in apples and chilling injury in green bell peppers therefore may be due, in part, to a reduction in the flow of electrons through the cytochrome path. Involvement of respiratory activity in the development of superficial scald in apples and chilling injury of sensitive plant tissues has been suggested in previous reports (Kim-Kang et al., 1998; Lyons and Breidenbach, 1990).

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